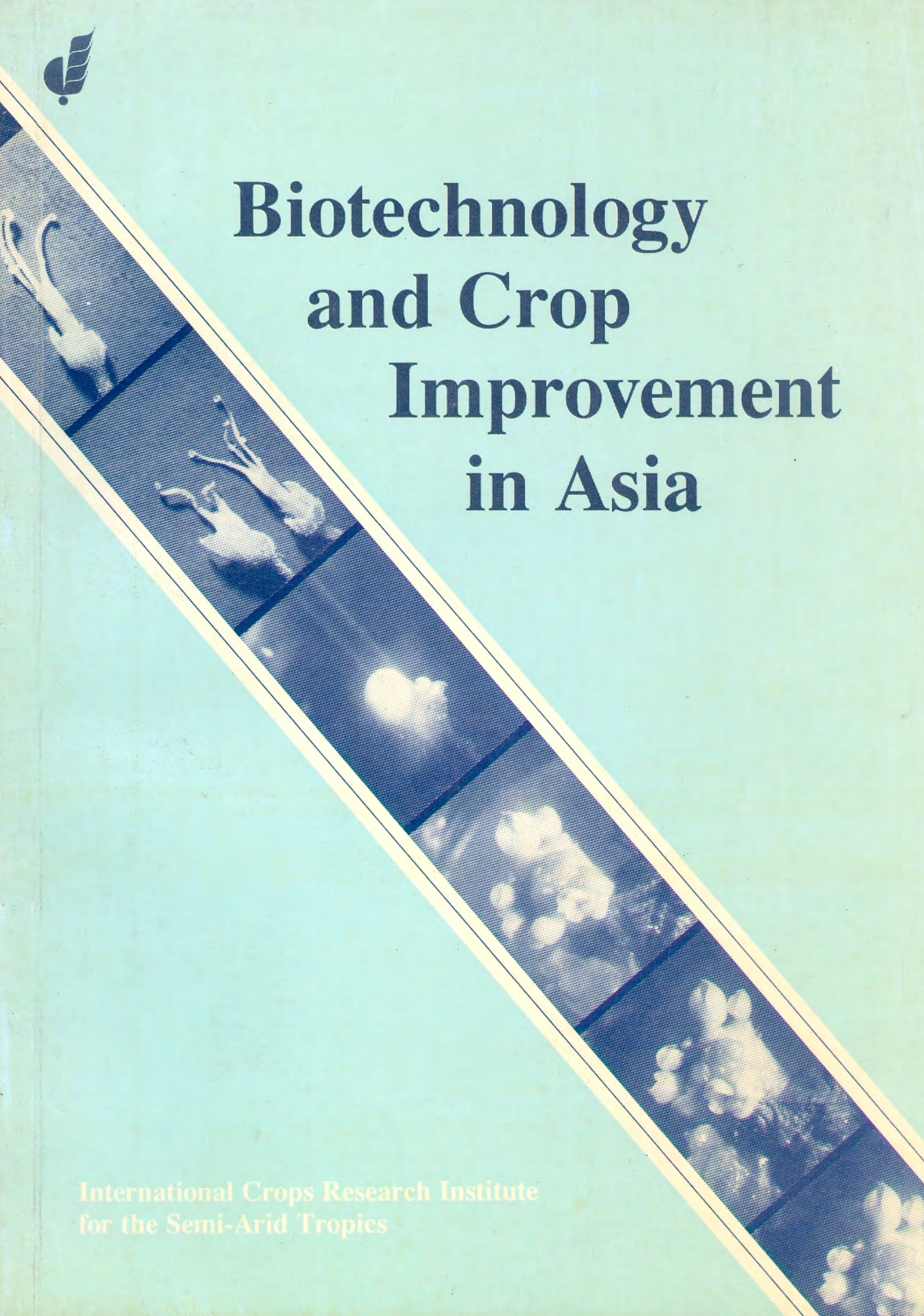
Biotechnology and Crop Improvement in Asia



International Crops Research Institute
for the Semi-Arid Tropics

Abstract

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This book results from a workshop held at ICRISAT 3-7 Dec 1990 sponsored by the Asian Development Bank (ADB). The participants were representatives of Asian countries, and scientific leaders in the various fields of biotechnology.

The introductory chapter outlines the role of the ADB in strengthening biotechnology research in Asia; this is followed by a presentation of the Indian biotechnology program and the problems that are being addressed.

A chapter on Industry and Public Sector Cooperation highlights the advantages and disadvantages of private companies becoming involved in biotechnology research.

The country representatives presented papers on the status of biotechnology in their country, and these chapters give an indication of the wide range of achievement in the area. Participants also outlined those crops and areas of crop improvement to which biotechnology could be applied.

The remaining chapters are reviews of the major disciplines in biotechnology, and reports by Asian scientists and others on research relevant to Asian agriculture. These reviews cover cell and tissue culture, with emphasis on legumes, cereals, and haploids. There are four reviews on transformation, covering Agrobacterium-mediated transformation, physical methods of gene transfer, the use of viruses as vectors, and gene action in transformed plants. The section on genome characterization and diagnostics covers the genomes of plants—nuclear, mitochondrial, and plastid—and of viruses, and the relevance of genome research to plant improvement, through the use of markers—restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR), and random amplified polymorphic DNA (RAPD).

The recommendations of the workshop give a useful insight into how a representative cross section view the future development of biotechnology. The book contains a glossary and index.

Résumé

La biotechnologie et l'amélioration des cultures en Asie. Ce livre est le résultat d'un atelier qui a été tenu à l'ICRISAT du 3 au 7 décembre 1990 et parrainé par la Banque asiatique de développement (ADB). Les participants étaient des représentants des pays asiatiques ainsi que les spécialistes dans divers domaines de la biotechnologie.

Le chapitre préliminaire esquisse le rôle de l'ADB dans le renforcement des travaux de recherche biotechnologique en Asie, suivi d'une présentation du programme indien sur la biotechnologie et les problèmes abordés.

Un chapitre sur la coopération entre l'industrie et le secteur public souligne les avantages et les inconvénients de la participation des sociétés privés à la recherche biotechnologique.

L'importance des réalisations dans ce domaine est mise en évidence dans les chapitres comportant les communications présentées par les représentants des divers pays sur le statut de la biotechnologie dans leur pays. Les participants ont également défini les cultures et les aspects d'amélioration de cultures auxquels la biotechnologie peut être appliquée.

Les autres chapitres constituent des revues des disciplines majeures de la biotechnologie, et des rapports par les chercheurs asiatiques et autres sur la recherche pertinente à l'agriculture en Asie. Ces revues traitent de la culture des cellules et des tissus, avec l'accent sur les légumineuses, les céréales et les haploïdes. Il existe quatre revues sur la transformation, comportant la transformation par l'Agrobacterium, méthodes physiques de transfert de gènes, l'emploi des virus comme vecteurs, et l'action des gènes dans les plantes transformées. La section sur la caractérisation et le diagnostic génomiques couvre les génomes des plantes—nucléaires, mitochondriales, et plastides—ainsi que des virus, et la pertinence des travaux de recherche sur les génomes à l'amélioration des plantes, par l'intermédiaire des marqueurs—RFLP, PCR, RAPD.

Enfin, les recommandations de l'atelier donnent un aperçu utile de la manière par laquelle un groupe représentatif envisage le développement futur de la biotechnologie. L'ouvrage comporte un glossaire et un index.

Resumen

La biotechnología y la mejora de cosechas en Asia. Este libro es el resultado de un seminario celebrado en ICRISAT del 3 al 7 de diciembre de 1990, patrocinado por el Asian Development Bank (ADB). Concurrieron representantes de países asiáticos y líderes científicos en distintos campos de la biotecnología.

El capítulo introductorio subraya el papel del ADB en el fortalecimiento de la investigación biotecnológica en Asia; le sigue una presentación del programa biotecnológico de la India y los problemas a los que está siendo aplicado.

Un capítulo sobre Industria y Cooperación del Sector Público señala las ventajas e inconvenientes de las empresas privadas que se ven envueltas en la investigación biotecnológica.

Los representantes de los países presentaron papeles sobre la situación de la biotecnología en su país, y estos capítulos dan una indicación de la gran diversidad de avances en dicho campo. Los participantes tambien hablaron de las cosechas y ámbitos de la mejora de cosechas a las que se podía aplicar la biotecnología.

El resto de los capítulos consisten en reseñas de disciplinas principales dentro de la biotecnología, e informes realizados por científicos asiáticos y otros sobre investigación significativa para la agricultura asiática. Estas reseñas tratan sobre el cultivo celular y tisular, con especial referencia a las legumbres, cereales y haploides. Hay cuatro reseñas sobre transformación, que tratan la transformación mediada por agrobacterium, métodos físicos de transferencia genética, el uso de virus como vectores, y la acción genética en las plantas transformadas. La sección sobre caracterización y diagnóstico de los genomas habla de los genomas de plantas—nucleares, mitocondriales y plástidos—y de virus, y la importancia de la investigación del genoma para la mejora de plantas, mediante el uso de marcadores—RFLP, PCR, RAPD.

Las conclusiones del seminario nos ofrecen una útil aclaración de cómo ve una sección mixta representativa el futuro desarrollo de la biotecnología. El libro consta de glosario e índice.

Biotechnology and Crop Improvement in Asia

Edited by
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Part III

Foreword

In January 1987 a biotechnology workshop was held at ICRISAT Center to identify institutional constraints we may face in moving into sophisticated biotechnological research.

Three important recommendations resulted from these discussions. First, the institute should refrain from doing basic research in molecular biology. Instead, ICRISAT should use proven biotechnological methods to enhance the effectiveness of its traditional crop improvement procedures. Where basic research is needed, it should be done as cooperative projects with experts in mentor institutes.

Second, to make full use of these new technologies, it will be essential for ICRISAT to upgrade its existing research facilities, develop new facilities where needed, and increase its expertise in cellular and molecular biology. This required funds, and we are thankful to the Asian Development Bank for their support. Funds from the bank allowed ICRISAT to remodel existing facilities, construct new facilities, and purchase the needed equipment to do research in virology and cell biology. The bank also made available funds for postdoctoral research fellowships. These fellows brought needed expertise in cereal virology and in cell biology to the institute. The Asian Development Bank is also sponsoring this workshop as part of an effort to coordinate biotechnological research on tropical crop improvement in Asia.

Third, ICRISAT should carefully consider its priorities for use of biotechnological techniques, and structure its research facility to cater to these needs.

These were excellent recommendations.

The facility that we established comprises two service units, one for electron microscopy and another for experiments involving the use of radioisotopes, and research laboratories for virology, legumes cell biology, and cereal cell biology.

Research in the Legumes Cell Biology Unit concentrates on transferring genes from wild species of *Arachis* to the cultivated groundnut. Substantial progress has been made in transferring resistance to rust, late leafspot, and the aphid-borne component of groundnut rosette disease from wild diploids in section *Arachis* to breeding populations. This unit is also doing research on improving methods for efficient regeneration of functional plants from groundnut, pigeonpea, and chickpea explants for eventual use in genetic transformation of these legumes.

In the Virology Unit research concentrates on the identification and characterization of viruses that infect ICRISAT mandate legumes. It cooperates with scientists in cereal pathology on studies of sorghum virus diseases. New techniques to screen for resistance to virus diseases were developed, allowing breeders to make rapid progress in breeding for resistance to bud necrosis disease, peanut mottle virus, peanut clump virus, peanut stripe virus, and peanut chlorotic mild mottle virus.

The Cereal Cell Biology Unit cooperates with scientists at the Institute for Plant Science Research in the United Kingdom in constructing molecular genetic maps of pearl millet, and with scientists at Milan University in Italy in constructing RFLP maps of sorghum. These molecular markers are being associated with traits such as resistance to insect pests and adaptation to heat and drought stress, and will eventually be used to manipulate these complex genetic traits in breeding populations.

The unit is also studying the effects of downy mildew infection on enhancing regeneration of functional plants from immature pearl millet spikelets. It is now possible to regenerate functional plants from a range of genotypes. This is the first step in using recombinant DNA technology to transfer resistance to the parasitic weed *Striga* from sorghum to pearl millet.

The need for research cooperation with private industry or with universities in developed countries has changed the scientific and legal environment in which ICRISAT operates, particularly in respect to biotechnology and plant breeding. Products of research have become commodities with commercial value. ICRISAT recently explored the effects on research management in an expanding domain of intellectual property rights. Three of the conclusions from this workshop may be of interest to participants in this meeting.

First, biotechnological research on staple food crops of the tropics is not likely to attract private research resources. Research on these crops does not promise quick or sufficient financial returns to make them attractive to private industry. This will make it necessary for the International Agricultural Research Centers and National Research Programs in the tropics to develop expertise in the field of biotechnology. Another research area where private companies are not likely to compete with Centers and National programs is in improving crop adaptation to unfavorable agricultural environments.

Second, the importance of germplasm held in trust by International Agricultural Research Centers and by National Programs will increase. It is therefore essential that a mechanism be established to assure ready availability of such germplasm to all potential users, without the Centers losing control over the further use of germplasm or products of research.

Third, private and public sector research organizations in developed countries increasingly require collaborating institutes to enter into contracts providing them with control over the disposition of intellectual property resulting from such collaborative research. This suggests that International Agricultural Centers and National Research Programs can no longer rely on goodwill alone to safeguard the intellectual property resulting from such research. This is a development that has to be kept in mind when discussing possible research networks among National Programs and with ICRISAT, or when considering cooperation with research institutions in developed countries.

J.M.J. de Wet
Director
Cereals Program

Preface

The Asian Development Bank has sponsored two workshops at ICRISAT to stimulate the development of biotechnology in Asia. The first was held 12-15 Jan 1987, and the proceedings were published as Biotechnology in Tropical Crop Improvement. The second was held 3-7 Dec 1990. The participants were representatives of Asian countries, and scientific leaders in the various fields of biotechnology.

The objectives of the workshop were

- To review the status of biotechnological research in crop improvement.
- To evaluate the status of such research in the Asian region.
- To discuss the role of the private sector, the National Agricultural Research Systems, and International Agricultural Research Centers.
- To discuss cooperative research and development; biosafety responsibilities; status of biotechnology in developed countries; and socioeconomic issues affecting the application of biotechnology in developing countries.
- To develop research cooperation between national programs in Asia, advanced laboratories, and ICRISAT.
- To set up a training program for Asian national programs.

The country representatives presented papers on the status of biotechnology in their country, and these give an indication of the wide range of achievement in the area, from countries that are starting to develop biotechnology programs, to those that have established laboratories. Participants also outlined those areas of crop improvement to which biotechnology could be applied.

The remaining papers covered a range of scientific disciplines within biotechnology, and the interactions of public and private sector undertakings. The poster presentations appear as short papers.

Emphasis was given to collaboration, and many of the participants are actively engaged in collaborative projects, or involved in research on problems related to Asian agriculture.

The participants made a number of recommendations (Appendix II). These focused on a rapid deployment of biotechnology in the region to overcome some of the constraints to crop improvement.

This publication aims:

- To give an indication of the status of biotechnology and crop improvement in the Asian region.
- To present a cross section of biotechnologies relevant to crop improvement.
- To help scientists and others involved in biotechnology to develop applied research programs.
- To foster collaboration between scientists worldwide to develop joint programs in research and training.

J.P. Moss

Part I

Potentials and Challenges of Biotechnology in Asia

The Stakeholders

The Country Perspectives

The Stakeholders

The Role of ADB in Strengthening Biotechnology Research in Asia

D. Nangju[1] and M.S. Rao[2]

Abstract

Since its establishment in 1966, the Asian Development Bank has accorded high priority for agricultural development in the Asia-Pacific region through its lending and technical assistance operations. As a part of these activities, the Bank has provided support to both international and national research institutions for the development of appropriate technologies for improving the well-being of small farmers. During the past 22 years, the Bank has provided substantial loans and technical assistance grants in support of agricultural research in its developing member countries. During the same period, the Bank has given a total of U.S.$ 21.7 million in grants to 16 international agricultural research centers including the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

In April 1989, the Bank approved technical assistance amounting to U.S.$ 1.25 million to ICRISAT for the establishment of a Plant Biotechnology Research and Training Unit. This has been the largest single grant provided by the Bank to any international agricultural research center. In approving the technical assistance, the Bank wanted ICRISAT to maintain not only its leading role in crop improvement but also its role as an agricultural research center of excellence. It is hoped that the biotechnology unit will help accelerate the development of appropriate technologies and high-yielding varieties that are well suited to the harsh environments of the semi-arid tropics and the mandate crops of ICRISAT. Simultaneously, it would provide training to national scientists both from within and outside Asia and foster collaboration of these with ICRISAT and with each other.

Introduction

On behalf of the Asian Development Bank (ADB), I welcome all the participants in this International Workshop on Biotechnology and Crop Improvement in Asia. As you know, this workshop has been organized by ICRISAT as part of the activities under the technical assistance financed by ADB. The technical assistance grant of U.S.$ 1.25 million for the establishment of a Plant Biotechnology Research and Training Unit was approved by the Bank in April 1989 with the main objective of accelerating crop improvement using biotechnology so that high-yielding and quality seeds of ICRISAT's mandate crops can be made available to the developing countries to help solve their food and feed problems.

The workshop has been organized to discuss several important issues.

- The issues related to biotechnology including the current status of biotechnology in developed and developing countries and future opportunities for its application to tropical food crops
- The role of the private sector, the National Agricultural Research Systems (NARS), and International Agricultural Research Centers (IARCs)
- Cooperative research and development, biosafety responsibilities, and socioeconomic issues affecting the application of biotechnology in developing countries

At the conclusion of the workshop, we expect that the participants would reach a consensus to establish a regional biotechnology research network with

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Nangju, D., and Rao, M.S. 1992. The role of ADB in strengthening biotechnology research in Asia. Pages 3–7 *in* Biotechnology and crop improvement in Asia (Moss, J.P., ed.). Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

ICRISAT's biotechnology unit playing a pivotal role in its development.

The Bank's Role in Agricultural Development

Since its establishment in 1966, ADB has always accorded high priority to agricultural development in the Asia-Pacific Region. This is due to the fact that the agricultural sector is the most dominant sector. The Region has over 70% of the world's farming families but has only 25% of the world's arable land; and, nearly two-thirds of the world's poor and hungry are in the Asia-Pacific Region. Over the past 24 years, the Bank has undertaken four major studies of the Region's agricultural sector in order to assess development constraints, identify investment opportunities, and formulate strategies. During 1968-1989, agriculture and agroindustry have remained the most important sector of Bank operations, accounting for about one-third of its cumulative lending of about U.S.$ 28.6 billion, and about one-half of its grant-financed technical assistance. Bank lending has supported the Region's growth in agricultural production, rural employment, and farm incomes. In recent years, the focus of the bank's lending has been shifting increasingly to agricultural diversification, poverty alleviation, facilitating greater participation of women in developmental efforts, and environmental protection.

In the initial years of its operation, the Bank has been fortunate to assist its developing member countries to exploit the new green revolution technologies that were evolved in the 1970s. Thus, both the Bank and its developing member countries capitalized upon the technologies generated as a result of the support for research provided mainly by the Rockefeller and Ford Foundations. At the same time the Bank had begun, albeit in a small way, to support agricultural research in the IARCs and NARS; this support has increased over the years. Details of ADB support for international agricultural research are given in Table 1. As of September 1990, ADB has provided total grants of U.S.$ 22.9 million for 56 activities undertaken by 16 IARCs, including four CGIAR centers and four non-CGIAR centers. About 50% of the total support (U.S.$ 11.1 million) was given to the four CGIAR centers, of which 88% was to ICRISAT and IRRI.

Between 1976 and 1990, the Bank approved nine technical assistance grants to ICRISAT for a total amount of U.S.$ 4.4 million (Table 2). A recent assessment of the impact of ADB's technical assistance grants to ICRISAT shows that the ADB assistance has facilitated the development of a number of technologies that can significantly improve yields of grain legumes, and has improved the research facilities at ICRISAT Center. It has also promoted the intensive participation of the staff of the NARS in the workshops, seminars, and training programs of ICRISAT in order to further strengthen their capacity to conduct research.

The ADB's policy in agricultural research is described in a document approved by the Board of Directors in 1983. The policy stated that the Bank will continue its support to both international and national research institutes. However, special attention will be paid to the development of appropriate technologies for less favorable environments (i.e., rainfed farm-

Table 1. Bank assistance to International Agricultural Research Centers.

Centers	No. of Projects	Total Grant (U.S.$ millions)
CGIAR Centers		
IRRI	12	5.410
ICRISAT	9	4.445
IFPRI	2	.748
ISNAR	2	.555
Sub Total	24	11.158
Non-CGIAR Centers		
AVRDC	8	4.065
ICLARM	2	.825
IIMI	10	3.030
IBSRAM	3	.902
Sub Total	23	8.822
Other Centers		
IJO	2	.950
CABI	1	.130
CIRDAP	1	.180
EPA	1	.250
ICIMOD	1	.540
INFOFISH	1	.334
OIE	1	.150
NACA	1	.380
Sub Total	9	2.914
Total	56	22.894

Table 2. Description of Bank Technical Assistance Grants to ICRISAT (1975-1990).

Title of Grant	TA No	Date Approved	Duration (Yrs)	Amount (U.S.$ thousands)	Expected output
Research on Animal-Drawn Equipment and Allied Implements	5036	17 Jun 76	1	325	Animal-drawn implements for more efficient land preparation; Machinery shop and laboratory
Establishment of a Genetic Resource Laboratory	5087	09 Nov 81	1	450	Improved ICRISAT facilities
Strengthening Chickpea Research in Pakistan in collaboration with ICRISAT Phase I	5118	26 Dec 84	2	300	Chickpea varieties resistant to *Ascochyta* blight; Improved NARS in Pakistan
Strengthening Chickpea Research in Pakistan in collaboration with ICRISAT Phase II	882	09 Jun 87	2	350	High-yielding legume varieties; Improved NARS
Strengthening Grain Legume Research in South Asia	5268	04 Dec 87	2	350	High yielding legume varieties; Improved NARS
Pigeonpea Varietal Adaptation and Production Studies in Sri Lanka in collaboration with ICRISAT	1139	16 Mar 89	2	230	High yielding pigeonpea varieties; Increased pigeonpea area in Sri Lanka
Establishment of a Plant Biotechnology Research and Training Unit	5331	25 Apr 89	2	1 250	Improved ICRISAT facilities; Trained biotech researchers
Strengthening Grain Legumes Research in Asia	5393	10 Jul 90	3	590	Higher yields and production of grain legumes; Improved NARS
Strengthening Genetic Resources Unit	5405	07 Sep 90	3	600	Additional collected germplasm; Increased utilization of germplasm in crop improvement; Improved safety features of the gene bank
Total			4	445	

ing); for crops other than wheat and rice; and to the integration of crop, livestock, and forestry sectors.

The Bank's Role in Biotechnology Research

The discovery of the structure of deoxyribonucleic acid (DNA), the fundamental genetic material, by Crick and Watson in 1953 has enabled scientists to apply it in biochemistry and cell biology for plant and animal improvement. This application is now known as biotechnology or genetic engineering. Biotechnology has been cited as a fast-growing area of science where new opportunities are constantly being created for curing diseases, prolonging lives, and improving crop and animal production around the world. In developed countries, biotechnology has been successfully utilized to manufacture wonder drugs by the drug industry, to develop superior animals that can give tastier meat, fine wools, or high milk production, and to produce horticultural crops that are high yielding and resistant to diseases.

Although most biotechnology research is currently being carried out in laboratories in developed countries, several of the Bank's Developing Member Countries (DMCs), notably India, the People's Republic of China, Indonesia, Malaysia, the Philippines,

Singapore, and Thailand, have already acquired a national capability in biotechnology for development. ADB believes that biotechnology has opened up future possibilities for the development of the semi-arid and rainfed areas of the Asia-Pacific Region.

The population expansion in the developing countries is at present one of the most serious problems facing humankind. This is due to the fact that the green revolution, which occurred in the 1970s, has increased food production and farm incomes only in the irrigated areas in the past 20 years, while annual population increases in the semi-arid and rainfed areas outpaced annual increases in food production by about two percentage points. The rainfed areas, including the semi-arid tropics, account for more than 65% of the total cultivated area in the Asia-Pacific Region. These areas are characterized by inadequate and uncertain rainfall, large areas of infertile and fragile soils, high prevalence of diseases and pests, and widespread poverty. Although conventional plant breeding techniques have made considerable progress in the development of improved varieties for the rainfed areas, they have not been able to keep pace with the increasing demand for food in DMCs. Therefore, many international agricultural research centers, including ICRISAT, decided to introduce biotechnology to speed up the crop improvement process, since there are good possibilities that advances and quantum yield increases that have been achieved in the developed countries with the use of biotechnology could be duplicated in the developing countries. Scientists are generally optimistic about the potential of biotechnology in increasing food production.

In view of the above considerations, in April 1989 ADB accepted ICRISAT's proposal for the establishment of a plant biotechnology research and training unit by approving a grant of U.S.$ 1.25 million. This was the largest grant given by the Bank to any IARC during the past 22 years. ADB believes that the IARCs like ICRISAT should maintain not only their leading role in crop improvement but also as agricultural research centers of excellence. At present, several DMCs can provide only a modest level of budget support for adaptive agricultural research and are unlikely to be able to invest in basic biotechnological research in the near future. The technical assistance to ICRISAT for the establishment of a plant biotechnology research and training unit is viewed as an investment in the future growth of food production in the DMCs and in fostering the biotechnology capacity in these countries. It would also open future possibilities for Bank financing for the development of the semi-arid and rainfed areas of the Region.

The technical assistance is aimed at accelerating the crop improvement process and the availability of high yielding and quality seeds to DMCs for those crops for which ICRISAT has a mandate. The scope of the technical assistance includes the establishment of a biotechnology unit at ICRISAT through rearrangement and modification of the existing facilities, provision of equipment, and fellowships and training of scientists from the Bank's member countries, and a workshop on biotechnology.

Although the technical assistance will be implemented for two years (from April 1989 to April 1991), ICRISAT has agreed to provide thereafter the necessary funds from its core budget to operate and maintain the biotechnology unit and research program to ensure that the technical assistance objective would be achieved as envisaged.

Biotechnology and Third World Concerns

The Centre for Research on Sustainable Agricultural and Rural Development (CERSARD) based in Madras, India, has identified a number of issues related to biotechnology which are of major concern to the Third World. These issues include the following:

- Since in developed countries microorganisms and the whole plant resulting from biotechnological research can be patented, how will resource-poor farmers in developing countries have access to the fruits of biotechnological research covered by intellectual property rights?
- Will the products of biotechnology research contain built-in seeds of social discrimination? A criticism voiced against green revolution technologies in the 1970s has been that they had helped to make rich farmers richer and did not help the resource-poor farmers much. How can we design mutually reinforcing packages of technology, services, and public policies which can ensure that all rural people—rich and poor, landowners or landless labor families—derive economic and social benefits from new technologies?
- Will priorities in biotechnology research be solely market-driven or will they also take into consideration the larger interests and the long-term well-being of humankind, whether rich or poor?
- A major concern relates to the safety aspects of genetic engineering research. Will tests be done in the Third World that are not permitted in the "industrialized countries"?

- Will crop varieties with multiple resistance to pests contain toxins that will ultimately affect the health of the human beings or animals that consume them. What kinds of safety evaluation procedures are needed for food ingredients produced by micro-organisms, single chemicals and simple procedures, and whole foods and other complex mixtures?

These are some of the issues that will be discussed in an international conference to be held by CERSARD in early 1991. I hope that those attending this workshop will also have the opportunity to discuss the socioeconomic issues affecting the application of biotechnology in developing countries.

Conclusion

In conclusion, we would like to assure you that the Bank will continue to support the international and national agricultural research centers to develop appropriate technologies for small farmers. We look forward to fruitful deliberations at this workshop. We hope that we would be able to obtain feedback from ICRISAT and the participants of the workshop regarding the Bank's future activities in agricultural research.

Agricultural Research Under Conditions of Biotic and Abiotic Stress and Scarce Resources

S. Ramachandran[1]

Abstract

Based on technological breakthroughs and developments, it has now been generally accepted that biotechnology has its greatest impact on agriculture and health. Food production is a natural target for the new technology.

The prospect of being able to transform plants in directed ways opens up new possibilities for breeders. Biotechnology has made possible the manipulation of cell organella, thereby increasing the capability of the cell to be able to produce the desired trait. With the advent of new technologies it is now possible to transfer genes from one species to another, regardless of how unrelated they are. Further, contributions of new technologies are not only improving yield potential, but also increasing resistance to pests and disease, breeding for resistance to crop hazards, such as water stress, and storm, and to adverse soil conditions such as salinity, toxicity, etc.

A great deal of genetic variability is expressed in plants regenerated via tissue culture. A large number of mutants have been selected from tissue culture and a few of these have been characterized as stable genetic variants at the whole plant level. Disease resistance, herbicide tolerance, stress tolerance (drought, salt) are some of the most sought after mutant traits.

Transformation studies have offered the possibility of specific introduction of traits of economic importance without causing drastic changes in the genetic background of the recipient genome. It is now possible to isolate specific genes and introduce them into plants. Transgenic plants thus produced have provided a means to understand the mechanism of gene expression such as tissue-specific expression of light-regulated genes, seed storage proteins, heat shock proteins, herbicide tolerance genes, and insect-resistance genes. A number of such genes have been transferred into higher plants from a wide range of plant and bacterial sources.

The fundamental basis of agricultural biotechnology are two important techniques: tissue culture and recombinant DNA. The most direct way to use biotechnology to improve crop agriculture is to genetically engineer plants. Recently, techniques like RFLP and PCR have become the most enabling technologies for crop improvement. Plant tissue culture has become an important tool for research and the application of these technologies is rapidly increasing in plant breeding and crop management.

The new capabilities to manipulate the genetic material present tremendous potential and find use in many novel experiments and applications. These developments have generated a sense of concern among scientists working in biological areas and others to find ways to safely do research in the field, how this should be carried out, and to also find possible means to regulate the work involving pathogenic microorganisms and virulent genes. With the safety consideration in view, the Government of India has evolved the Recombinant DNA Safety Guidelines. These guidelines cover areas of research involving: (1) genetically engineered organisms; (2) genetic transformation of green plants and animals; (3) rDNA technology in vaccine development; and (4) large-scale

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Ramachandran, S. 1992. Agricultural research under conditions of biotic and abiotic stress and scarce resources. Pages 9–15 *in* Biotechnology and crop improvement in Asia (Moss, J.P., ed.). Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

production and deliberate/accidental release of organisms, plants, animals, and products derived by rDNA technology.

The application of biotechnology requires a highly developed educational system, an adequate infrastructure, and sustained R&D support. In collaboration with other departments and institutions, DBT has evolved a model system of teaching/training in biotechnology at the postgraduate/postdoctoral level. DBT has also created a network of infrastructural facilities for building up a strong R&D base.

Several biotechnological processes are particularly advantageous for developing countries. Efforts should be made to involve untapped talent in the universities and other R&D systems in a meaningful tie-up. International and regional cooperation can play an important role in putting biotechnological developments into proper perspective in order to ensure the transfer of the most relevant technologies to developing countries.

Introduction

In the light of the developments in modern biology over the past three or four decades, it could be stated that modern biology has come to the fore among the various scientific revolutions witnessed in recent times. Biotechnology provides the various tools and techniques needed to alter the functioning of living systems at the basic genetic level and helps to develop products and processes of economic and commercial importance in the fields of agriculture, animal husbandry, human health, and environment. The techniques and tools include various recombinant DNA technologies: tissue culture, cell fusion, cell regeneration, and production of transgenic plants; diagnostics for detecting diseases and discriminating between plant varieties; and compression of production cycles using techniques such as Restriction Fragment Length Polymorphism (RFLP) and Polymerase Chain Reaction (PCR), as well as the development and use of symbiotic and helpful microbial and other living organisms for pest and weed control and as biofertilizers.

Past Progress

The progress achieved in the area of plant sciences with reference to new biotechnologies has exceeded our expectations even during the past 10 years. The techniques based on vector-based transformation, direction introduction of DNA, and somatic and germ line cell fusion are becoming readily usable in the laboratory. Various methods of foreign DNA introduction, integration, and expression along with techniques of tissue culture, cell fusion, protoplast regeneration, somatic embryogenesis, and embryo rescue are enabling scientists to introduce into plants new genetic traits of scientific and economic importance. Recent progress in the area of RFLP and PCR has greatly facilitated the fairly simple and straightforward detection of transferred genetic material and its further segregation into daughter progenies, thus leading to significant compression of the breeding cycle in evolving new economically important varieties. The first generation applications of genetic engineering to crop agriculture are targeted at issues that are currently being addressed by traditional breeding, i.e., improved breeding efficiency and enhanced capacity for survival under adverse environmental conditions. Genetic engineering methods complement plant breeding methods by increasing the diversity of genes available for incorporation into crops and shortening the time required for production of new varieties and hybrids.

Agroclimatic Conditions in Asia

The various agroclimatic conditions prevailing in Asia represent the entire spectrum seen across the globe. All these regions are also densely populated and are, therefore, heavily dependent on local agriculture and food production. Large areas of land experience a variety of abiotic and biotic stresses. Some of the major stresses, against which the agricultural scientist is called upon for crop improvement, are salinity, drought, water stagnation, excessively high and low temperature, and degraded and impoverished soils. In addition, a variety of pests, diseases, and weeds also reduce crop yield considerably. Post-harvest losses due to insects, microorganisms, and poor storage facilities are staggeringly high. Generally a majority of the farms are small (less than 2 ha) or marginal (less than 0.75 ha). Most of the farmers are poor and indebted and can ill-afford to give the

small farms even minimum inputs of water, fertilizers, and pesticides. In addition, most of the farm labor is done using animal energy which in turn competes for water, land, and biomass. The challenge for agricultural scientists within the next decade is to solve these very problems, both through biotechnology and conventional techniques. It is here that the new tools of biotechnology offer much scope and promise for introducing biotic and abiotic tolerance/resistance, higher productivity, better nutritional quality, diagnostic and plant variety identification, mass propagation of elite lines, and development and introduction of low-cost and environmentally friendly methods of biological pest control and biofertilizers.

The Indian Agroclimatic Scene

The situation existing in India in many ways represents that existing in other developing countries. There is high population growth with most of the cultivable land and sources of irrigation already being exploited. India has used most of its fertile land for its green revolution, which took agricultural productivity from about 50-60 million t of food grain per year to the 172 million t that was harvested last year. The Indian population has already crossed the 800 million level and at the present growth rate, is expected to touch the 1000 million mark by the year 2000. This situation necessitates the production of approximately 250 million t of food grains by the year 2000, an actual (incremental) increase of 7-8 million t each year between 1990 and 2000. This rate of increase, which is much more than that achieved in the first green revolution, would primarily have to be achieved with land and resources far less endowed than those used for the first green revolution. The strategy therefore must be to produce much more from resource-rich land, to introduce improved and new varieties of crops for the marginal lands, and to stretch the use of land that is in the degraded arid and semi-arid zones, to introduce production of non-crop food growing as an integrated farming strategy, and to reduce postharvest losses and introduce new postharvest techniques so that more of the harvested food reaches the consumer, both in terms of quantity and quality.

Other Scientific Approaches

In addition to biotechnology, there are a number of other new technologies that would also have to be suitably introduced to assist farming in Asian countries. These include a variety of water conservation, harvesting, and utilization techniques appropriate to different regions and extensive mapping of ground water resources and their judicious utilization and replenishment. Equally important is the extensive use of organic and biological fertilizers appropriate to the crop and climate; development of integrated pest management practices that reduce dependence on chemical pesticides and lead to a more favorable ecological balance; use of space imaging for crop and soil fertility monitoring, surface- and ground-water estimation, and better land use; use of computer- and satellite-aided medium- and long-range weather forecasting for advising the farmers suitably on farming practices appropriate to weather conditions; and use of various techniques for tapping nonconventional energy sources that would enable decentralized utilization of nonfossil fuel based energy for farming and for meeting societal needs especially in rural communities.

Specific Crop Priorities

Some of the major irrigated and dryland crops that need continuous attention for improving yield, disease, and pest resistance and adaptation to environmental stresses are discussed below.

Rice

Seventy percent of the cultivated area in India is rainfed and 58% of the rice is grown under such conditions. Out of these 23 million ha of rainfed rice, about 10 million ha is drought prone and 13 million ha is lowland and flood prone. Rice production rose from 20.6 million t in 1950-51 to over 72 million t in 1989-90, mainly because dwarf rice varieties cover about 25 million ha. The main problems of rice have been identified for biotechnological approaches.

- Introduction and amplification of resistance to grassy stunt virus and sheath blight by genome screening and somaclonal variation
- Wide hybridization with special reference to diversification of cytoplasm and use of alien genes for biotic and abiotic resistance development
- Protoplast culture and in vitro selection
- Development of RFLP techniques

Wheat

Considerable areas of wheat growing tracts are drought prone and afflicted with saline soils. The areas identified for biotechnological application in wheat are RFLP mapping for rust resistance, wide hybridization, and resistance to rust, kernel bunt, and foliar blight.

Sorghum

Most of the 16 million ha is rainfed. Although proper fertilizer application and pest and disease control would increase yield significantly, development of pest- and disease-resistant hybrids and varieties is a priority.

Pearl Millet

Most of the 13 million ha under this crop is prone to severe drought.

Pulses

Pulses assume high importance in a country where they serve as a main source of protein. Chickpea (*Cicer arietinum*) and pigeonpea (*Cajanus cajan*) are major pulses that account for 45% of the area and 60% of the production.

Chickpea is the most frequently grown pulse and the biotechnological-based approaches are resistance to pod borer (*Helicoverpa armigera*) through biotechnological introduction of genes, resistance to chickpea blight (*Ascochyta rabiei*) and chickpea wilt (*Fusarium oxysporum* f.sp *ciceri*) by wide hybridization, and embryo rescue.

Oilseeds

Oilseeds are grown on over 26.6 million ha. In recent years, production has gone up to about 15-16 million t. The most important oilseed crops are groundnut, mustard, soybean, sunflower, sesamum, and safflower.

Brassicas

The biotechnological approaches are these.

- Breeding for stress resistance
  1) against *Alternaria* through in vitro screening
  2) for salt tolerance using somaclonal variations
  3) for aphid resistance from protease inhibitors
- Building linkage maps for exploiting hybrid vigor through RFLP mapping
- Creating alloplasmic lines, gametosomatic hybrids, and protoplast fusion

Cotton

Cotton is another important crop in which yield has been raised from 95 to 200 kg ha^{-1}. Hybrid cotton has already been introduced. The biotechnological approach is to introduce bollworm (*Helicoverpa*) resistance through *Bacillus thuringiensis* gene introduction.

Breeding for Stress Resistance/Tolerance

Stress Resistance

There is a wide range of stresses to which plants are subjected, and various techniques that can be used to produce resistant plants are in vitro techniques, cell fusion and protoplast regeneration, and rDNA techniques. These can be used to obtain disease resistance, herbicide resistance, and salinity tolerance. Other approaches are the use of the *Bacillus thuringiensis* gene and chitinase inhibitor for insect pest resistance and viral coat protein genes for resistance to viruses. Probes, inhibitors, botanicals, and antifeedants all have a part to play in stress resistance breeding.

Salinity problems in agriculture are primarily found in the wasteland and semi-arid regions of the world, which comprise more than 25% of the earth's surface. The salt concentration in the soil after evapotranspiration may become four to ten times that of the salt concentration in irrigation water. A level of 800 ppm salt is generally recognized as the threshold of severe agricultural and nonagricultural damage. In many crops, resistance to salinity is greater during seed germination than at emergence and early growth and later stages of development. The role of the plant breeder has become increasingly important since modern agriculture needs plants fit for the available environment.

Traditionally the development of new crops has depended on the efforts of the plant breeder. The identification and isolation of salt-tolerant germplasm in related species of crop plants is a basic requirement

in a breeding program for salt tolerance. The recurrent selection technique can also be applied for salt tolerance selection. The major drawbacks in such breeding programs are the time required for effective selection and the difficulty in maintaining the background genotypes for maximum gene expression.

Besides conventional breeding, tissue culture techniques are now being widely used for developing salt-tolerant varieties. Cell or protoplast cultures can be used; from these, spontaneous or induced variants with increased tolerance can be isolated. Techniques of gene isolation and transfer can be used to directly move genes for increased tolerance from one cultivar, species, or genus, to another.

Of the abovementioned techniques, the technique of gene transfer is the one receiving maximum research attention today because very few genes regulating salt tolerance have been identified, mapped, and sequenced. Genetic variability of salt tolerance has been characterized for certain agronomic crops such as barley. It is possible to introgress the salt tolerance gene from wild into cultivated gene pools. High salt tolerance has been characterized in a wild relative of tomato, *Lycopersicon cheesmanii*. Hybrids between this species and tomato were synthesized and advanced tomato lines exhibiting salt tolerance have been successfully developed.

However, salt-resistant variants can clearly be isolated in plant cell cultures and the resistance mechanism observed in them is similar to those known to operate in intact plants. The polygenic nature of salt resistance might be expected to limit the frequency of occurrence of such a genetic change. There is, however, greater likelihood of eventual success in the field if the salt stress employed for selection reflects the ionic composition of the soil salts in the area for which the crop is intended.

Drought Tolerance

During its life cycle, a green plant is exposed to a variety of stresses and is particularly vulnerable to desiccation injury and overheating hazards. Breeding crop varieties for adaptation to locations characterized by physical environmental stress involves combining a good yield potential in the absence of stress, an appropriate phenology to provide maximum escape from stress, and selected traits that provide tolerance to the major stress in the particular environment.

The stress tolerance mechanism at the molecular level is more amenable to biotechnological manipulation than the mechanism at whole plant or crop level. The potential for molecular biology to contribute to stress tolerance is dependent on the level of the plant organization at which the trait exists. The ability of any plant to tolerate stress—drought for example—results from the cumulative effects of component physiological and biochemical functions. Manipulation of such a trait via genetic engineering will require an understanding and an ability to manipulate the genetic determinants of the components. RFLP mapping can help in identifying the important genetic components and in following their inheritance in breeding programs. If root structure and function are important determinants of drought tolerance, the addition of Ri plasmid genes, which alter root phenotype, might be beneficial. Scientists are trying hard to generate the knowledge base that will make possible the genetic engineering of complex traits like drought tolerance.

Biotechniques with Priority for Adoption

RFLP analysis of complex traits can supply us with these types of information and hence may furnish a means of identifying those endogenous species that would impact complex traits most significantly. Major quantitative trait loci (QTL) can be localized along the chromosome through correlation of the genotype of multiple, mapped RFLP loci with portions of phenotypic variance.

The impact of each individual locus on the total plant phenotype can also be ascertained through determining the amount of variance accounted for by it. At present, genes are identified as candidates likely to effect multigenic traits by either physiological or biochemical analysis of pathways that would logically seek to be involved in the trait of interest. The RFLP strategy is perceived as an alternative approach on the basis of genetic analysis and dissection of the whole plant phenotype.

Recently Polymerase Chain Reaction (PCR) has become one of the most enabling technologies. It helps in rapidly amplifying small amounts of DNA. Its most unique feature is its use of a thermostable DNA polymerase, the Taq polymerase. This enables one to perform multiple rounds of DNA synthesis with repeated heating and cooling cycles without having to add fresh enzyme each time.

Protoplast fusion has a great deal of promise for crop improvement, but the successful agricultural application of somatic hybridization is dependent on overcoming several limitations. For successful practi-

cal application of this technology, it is essential that there be efficient plant regeneration from protoplasts. Fusion of protoplasts is possible, but production of somatic hybrid plants offers problems. Somatic hybrids must be capable of sexual reproduction. It may be necessary to use back fusion or embryo culture to produce gene combinations that are sufficiently stable to permit incorporation into a breeding program. In order to transfer useful genes from a wild species into a cultivated crop, it is necessary to achieve intergenomic recombination or chromosome substitution between two sets of chromosomes. It may, therefore, be necessary to manipulate the chromosome number before releasing new varieties. Protoplast fusion results in unique mixtures of genetic information that may facilitate transfer of nuclear and cytoplasmic genetic information between species. Novel somatic hybrids may also be valuable as bridges in the development of distant gene combinations. Hybridization of cytoplasmic transfer using protoplast fusion has also been explored as a possible method to transfer genes between species in one step.

It has now been well established that potentially useful variation is derived from somaclonal variation and supports the earlier contention that somaclonal variation can be used as a breeding tool for genetic improvement of crops. Several research applications are likely to emerge through somaclonal variation in the near future. Additional research in the efficiency of somaclonal vs induced variation is needed in order to make an objective judgement about methods of producing useful variation.

Program in India

The Department of Biotechnology (DBT) has launched a network of programs consisting of 15 projects focusing on prioritized areas and feasible biotechnological approaches on four major crops.

- Rapeseed and mustard – breeding for *Alternaria*, salinity, and aphid resistance and RFLP mapping and protoplast fusion
- Rice – resistance to grassy stunt virus and sheath blight
- Chickpea – resistance to pod borer through Bt gene transfer, resistance to chickpea blight, and chickpea wilt
- Wheat – RFLP for rust resistance and molecular basis for kernel bunt and foliar blight

DBT has established three Centers for Plant Molecular Biology (CPMB) through strengthening existing plant molecular biology groups in the country. These CPMBs have the responsibility for both research and training in plant molecular biology and are linked to the existing universities and institutions. It is intended to have developments under two components in these centers: infrastructure strengthening for conducting basic research in plant molecular biology; and initiating 3-4 highly focused crop-based research programs on molecular aspects related to plant tissue culture, RFLP mapping, chloroplast and mitochondrial DNA research, molecular biology of mitochondria, and the basis of cytoplasmic male sterility and transformation and regeneration systems; and training in the area of crop agriculture-based plant molecular biology. The three centers are Jawaharlal Nehru University, Tamil Nadu Agricultural University, and Mahatma Gandhi Krishi University.

Role of International R&D Centers

The CGIAR had the vision more than 2 decades ago of establishing a global network of specialized international agricultural research centers in various locations. These international centers were based on specific crops and animal husbandry. Crops were selected on the basis of regional and other socioeconomic and agroclimatic needs. The International Rice Research Institute was established in the Philippines and ICRISAT for semi-arid crops was established at Hyderabad, India. The preeminent success achieved by the Consultative Group on International Agricultural Research system—in terms of evolving a large number of new and improved varieties that are related to the specific needs of mostly developing countries, and a number of related farm practices—has been a consistent encouragement to efforts to establish other international R&D centers and network activities. The two relevant examples that could be quoted here are the establishment of the International Centre for Genetic Engineering and Biotechnology (ICGEB) at Trieste and New Delhi and the International Co-operative Programmes on Rice Improvement under the Rockefeller Program.

Briefly the international R&D centers have these distinct advantages.

- They became a sound basis for south-south and north-south cooperation.
- They have played exceedingly important roles in training scientists, technologists, and extension workers from the developing countries in areas most relevant to their own country and regional needs.
- These centers are primarily involved in working on

problems under the present conditions in which these problems occur (agroclimatic and socio-economic).

- Most of these centers have been located in developing countries.
- These centers provide an excellent means of germplasm evaluation and exchange between the various research groups.
- The centers significantly improve the scientific competence of the developing countries and, therefore, the level of confidence in developing countries for tackling their regional problems.
- They provide a sense of participation and contribution to the developing countries.
- They demystify modern technology to the third world and in the process reduce unjustified fears and risk in the application of modern technology and its products.
- They have shown that multilateral cooperation-based research centers are an extremely cost-effective method of introducing high technology for solving regional problems in developing countries.
- International R&D centers located in developing countries have shown that their locations and the state of problems being worked on by them need not in any way reduce the quality and merit of their scientific output when compared to any other international center.

Industry and Public Sector Cooperation in Biotechnology for Crop Improvement

J.I. Cohen and J.A. Chambers[1]

Abstract

Concurrent with the need to improve global agricultural production are challenges requiring continued scientific innovation to enhance environmental acceptability of agricultural products. To meet these challenges, new technologies, including biotechnology, are becoming part of the scientific foundation comprising modern-day agriculture. While biotechnology offers new potentials, its impact on agriculture may be limited to those countries best able to access and exploit the new technologies.

An emerging priority of international agricultural research is the integration of biotechnology with crop improvement programs targeted for the developing world. Among the challenges posed by this integration is the increasing trend towards privatization of biotechnology in developed countries, and its impact on the future of development assistance efforts in international agricultural research. This paper outlines this trend and its potential effects on donors, the CGIAR system, and national programs, and the challenges and opportunities offered by establishing collaborative partnerships with the private sector.

Introduction

This century has witnessed a critical transition in agriculture. This transition is from a system that has been historically dependent upon the use of natural resources to one that has relied on science and technology to increase agricultural yields (Ruttan 1990). This newer system has provided the foundation for the development of high-yielding cultivars that have increased agricultural production.

It is widely recognized that increased yield per ha or increased production efficiency have been, and will continue to be, critical for agricultural competitiveness in the next decade. However, the trend towards knowledge-intensive agriculture leaves less-developed countries (LDCs) at a distinct disadvantage when competing with developed countries. This is largely due to the presence of well-established public and private technological infrastructure in developed countries, which makes them more adept in the acceptance and application of biotechnological innovations in agriculture (Parton 1990).

In the past, development efforts have partially addressed this disadvantage through support to the International Agricultural Research Centers (IARCs). Donor investment in agricultural research at the centers has been rewarded. Contributions of the IARCs in agricultural research are credited with significant increases in food production achieved during the green revolution of the 1960s. Increases in yield have, in turn, translated into increased income generation for many developing country farmers, thus accomplishing some key goals of development assistance efforts in agriculture.

Yet, even today, despite the transition to science-based agriculture and the substantial achievements of the IARCs, food self-reliance continues to be an elusive goal for many developing nations, and worldwide agricultural production continues to suffer substantial losses approaching 20-40% due to pests, weeds, and

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diseases (Walgate 1990). Moreover, worldwide concern for a fragile natural resource base continues to mount in the face of increasing population pressures, decreased availability of prime lands for cultivation, and obvious environmental demise. Thus, concurrent with needs for greater production efficiency are new challenges requiring continued scientific innovation to enhance environmental acceptability of agriculture.

In an attempt to meet these challenges, new technologies, including biotechnology, are becoming an integral part of the scientific foundation that comprises modern-day agriculture. Continuing investment in this technology derives from the perceived advantages offered over more traditional, science-based techniques that are inadequate to meet present-day challenges for sustainable increases in agricultural production.

The tools of biotechnology—when provided to plant breeders—present many opportunities for increased efficiency and reliability in crop production while ensuring increased profitability and environmental compatibility (Schneiderman 1990). Despite the potential offered through the application of biotechnology, impacts on developing country agriculture will be limited since applications and access will be oriented to those countries best able to exploit the technology. Thus, an emerging goal for international agricultural research is the integration of biotechnology with conventional crop improvement programs when these alone have not resolved specific constraints on enhanced productivity (Cohen et al. 1988).

Each of these facts defines an essential role for donor organizations and the CGIAR system in the acquisition and integration of biotechnology into international agricultural research. While biotechnology offers potential benefits for international agricultural research, its application in this arena also poses a series of interesting challenges. Among these is the increasing trend towards privatization of the technology in developed countries and its impact on the future of development-assistance efforts in international agricultural research. As such, the aim of this paper will be to outline this trend, discuss its potential effects on donors, the CGIAR system, and national programs, and to present the challenges and opportunities offered by the establishment of new partnerships with the private sector.

Defining the Need for New Partnerships

A report on maintaining U.S. competitiveness in agricultural biotechnology provides documentation for the widely recognized trend towards increased private sector participation in biotechnology research in the United States. As demonstrated in Table 1, private sector investments in agricultural biotechnology as recently as 1987—although significantly lower than biomedical investments—nonetheless approximately doubled research support by the public sector in this area (National Research Council 1987). The United States is not alone in this trend as other industrialized countries, such as the United Kingdom, France, West Germany, Japan, and the Netherlands, have recognized the potential for national economic benefit and are instituting changes in policy geared towards the development of a competitive biotechnology industry (Olson 1986).

Several factors have contributed to the exponential growth of the biotechnology industry in developed countries. The perception that both public advocacy for the technology and consumer demand will increase has contributed to private sector involvement. This increase will come about as the economic, environmental, and social benefits of resultant products—

Table 1. Funding levels for biotechnology and agriculturally related biotechnology research by selected sources.

Source	Amount ($ U.S. millions)
Agriculturally-related biotechnology	
U.S. Department of Agriculture	
Agricultural Research Service	24.5
Cooperative State Research Service	
Competitive grants	30.0
Hatch Act and special grants	18.4
State agricultural experiment stations[1]	
State	16.2
Industry	5.4
Private Industry[2]	150.0
All other biotechnology[3]	
Environmental Protection Agency	1.5
Food and Drug Administration	2.6
National Institutes of Health	1,849.5
National Science Foundation	81.6

1. Nonfederal support; fiscal year 1984 data (National Association of State Universities and Land-Grant Colleges, 1985).
2. Estimation based on data from the Agricultural Research Institute (1985).
3. Fiscal year 1985 (General Accounting Office, 1986). Funding by non-USDA federal agencies may include some agriculturally related biotechnology research.

Source: National Research Council (1987).

developed through initial investments in biotechnology—become more evident and accessible (Fry 1990). The capacity of the new technological tools to shorten the historical lag time between basic biological discoveries and product development has additionally motivated the trend towards privatization (National Research Council 1987). Finally, the growth of venture capital firms, most apparent in the United States and largely limited to other developed countries, has contributed significantly to the development of the biotechnology industry through support for small, entrepreneurial, academic-like companies (Bull et al. 1982).

Accordingly, in the United States the institutional structure of agricultural research has undergone significant change (Barker 1990). Although publicly-supported institutions, such as the land-grant universities, are attempting to respond to this institutional shift in support and location for biotechnology research through increases in staff and programs, they are less able to effect greater unilateral participation due to lack of budgetary control (Barker 1990) and overall decreased public support for basic agricultural research (National Research Council 1987). This changing institutional base—when coupled with the obvious commercial potential of the technology—has been a catalyst for greater collaboration between public institutions and the biotechnology industry (Fig. 1).

Several legislative rulings in the United States, which underscore the responsiveness of U.S. patent policy to technological innovation, have also contributed to an evolving partnership between the public and private sectors. This partnership has particular relevance for future IARC and LDC activities in biotechnology with publicly-supported U.S. institutions. The 1930 Plant Patent Act (PPA) and the 1970 Plant Variety Protection Act (PVPA) permitted the protection of most asexually produced plants and sexually produced varieties, respectively (Jondle 1989). The 1970 ruling, in particular, has dramatically affected U.S. private sector participation. For example, the number of private sector soybean breeding programs has increased from 5% to 30% after the PVPA ruling (CSSA 1989). Similarly, private investment in plant research in the U.S. from 1970 to 1980 has increased with no accompanying increase in the market cost of products (Barton 1982).

The 1980 landmark Supreme Court decision in Diamond vs. Charkrabarty, which granted a patent on

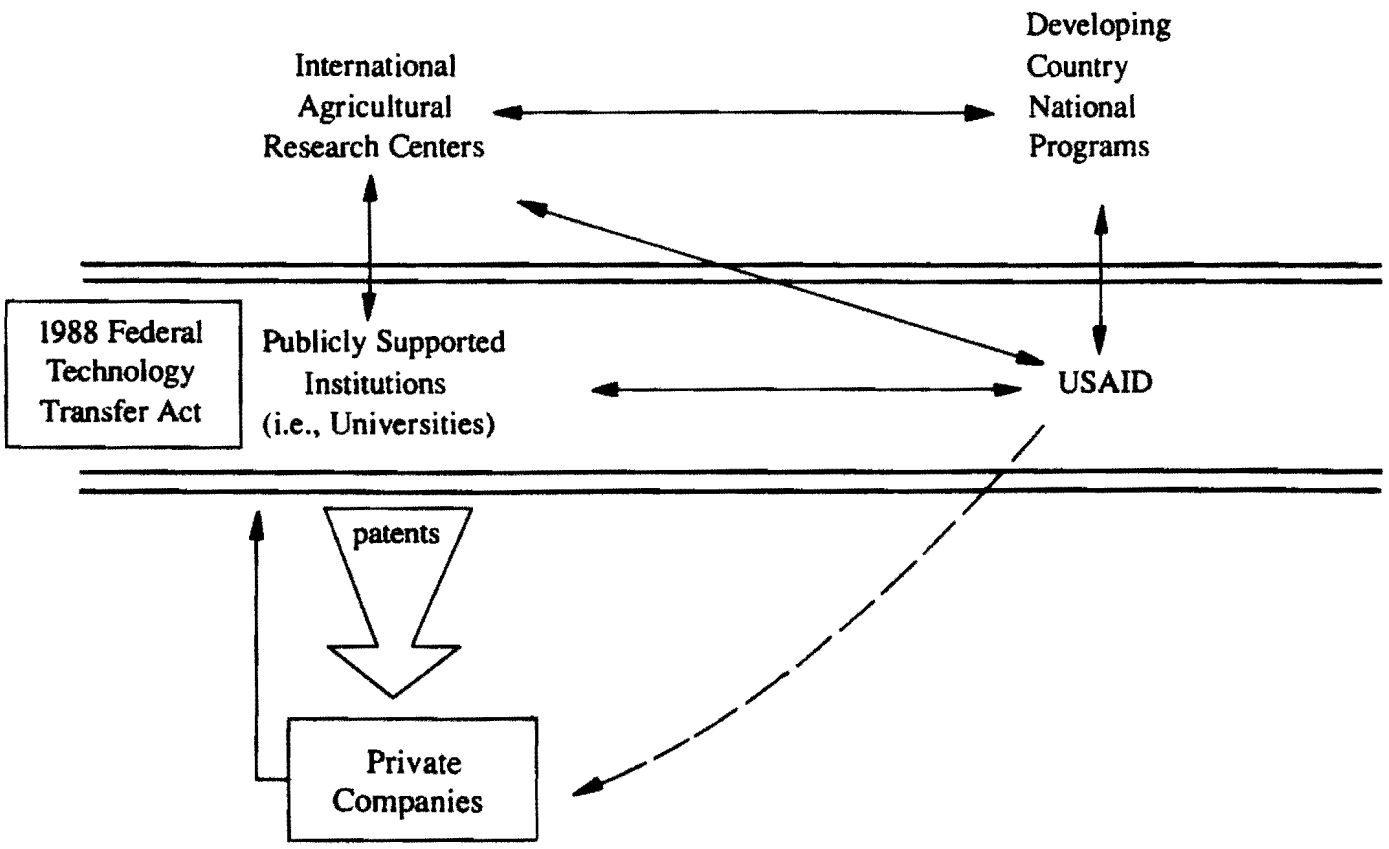

Figure 1. A model for collaboration between IARCs, public institutions, and the biotechnology industry.

a genetically engineered microorganism and, in so doing, ruled in favor of the protection of animate nature whose existence resulted from human intervention, has directly favored the generalized growth of the biotechnology industry in the United States (Beier and Straus 1985). Agricultural biotechnology has specifically benefitted from the 1985 U.S. Patent Office reversal of the Ex Parte Hibberd decision, which relied heavily on Diamond vs. Charkrabarty and which granted a utility patent for plants, thus permitting the protection of a single trait in a particular plant (CSSA 1989).

Finally, agricultural research initiatives at land-grant universities and other publicly-supported institutions have been directly affected by the 1986 Federal Technology Transfer Law. This legislation responded to the perception that research and development results generated in federal laboratories were not being readily adopted by the private sector for the effective production of new products for consumers and farmers. This act authorized government-supported agencies and institutions to enter into coopera tive research agreements with U.S. private companies (Tallent 1989). It was developed to bridge the gap between the fundamental research undertaken by public institutions and the more downstream, applied research being done by the private sector. The Act strengthens past efforts by the U.S. government in this area by providing the ability to grant an exclusive license to one company to put into practice an invention arising from Federal funds (Tallent 1989) and has transformed publicly-supported institutions into more desirable industrial partners (National Research Council 1987). As a result, collaboration between public and private research groups in the U.S. has markedly increased and is now influencing the current trend towards privatization of biotechnology research.

Historically, the IARCs and national programs in the LDCs have relied on public sector institutions in developed countries for advances in basic research which then may be adapted for application. What does the trend towards privatization in biotechnology and the evolving liaison between the private sector and traditional IARC partners mean for development goals in international agricultural research? Donor, IARC, and LDC access to biotechnology through public institutions—once a timely and responsive way of conducting agricultural research geared towards developing country problems and constraints—is becoming increasingly limited. Increased private sector participation in biotechnology, the more comprehensive protection of intellectual property throughout the entire agricultural community in developed countries, and the desire to achieve more rapid benefits from technological innovations may ultimately deny IARCs and LDCs access to beneficial technologies.

This point is illustrated by an informal survey recently conducted by the Office of Agriculture of the United States Agency for International Development (USAID) for the International Potato Center (CIP). CIP has been actively involved, through several collaborations with public sector institutions in numerous developed countries, in applying biotechnology to improve potato breeding programs and to overcome constraints affecting potato production throughout the developing world (Sawyer 1989). Continued success in a variety of additional biotechnology initiatives will necessarily depend on CIP's continued ability to access valuable techniques and genetic constructs useful in the generation of improved and novel germplasm. Accordingly, CIP requested USAID's assistance in the identification of genes and constructs located within the U.S. public sector that might be available to CIP for the generation and worldwide distribution of improved potato germplasm.

Although several universities were willing to collaborate with CIP, a number of prominent U.S. universities recognizing the commercial value of obtaining patents on their genes and constructs, were unable to collaborate with CIP. Many had previously enjoyed an open collaboration with the IARCs for the exchange of materials. The reasons most often cited for their inability to cooperate were the remunerative potential of licensing agreements with private firms, patent processes which were still in development for certain materials, and a perceived incompatibility in the IARCs' global distribution mandate with the IARCs' ability to protect university patents. Some of these universities, such as Cornell University's Biotechnology Program, have already established an integral relationship with several private companies that is based on the university's ability to provide patented inventions. Perhaps anticipating this response, and to explore alternative partnerships, CIP also requested USAID assistance in the identification of outside legal expertise to aid in the acquisition of novel technologies and materials from the private sector. This expertise was provided through external patent attorneys to assist CIP in negotiating licensing agreements.

Options Available for the International Agricultural Research Centers

The IARCs and donors must re-examine their support for collaborative partnerships that seek to acquire and develop new technologies (Cohen 1990). Some of these options have been examined regarding the integration, development, and application of biotechnology for the benefit of client countries in the developing world (Plucknett et al. 1990). Roles for each center will vary according to the crop and objectives established, but resident expertise at the centers will be critical to ensure success. This expertise will generally not be used to develop new basic knowledge in support of biotechnology, but rather to apply these scientific advances towards the needs of developing country agriculture (TAC 1988).

Once center expertise has been established, centers will need to consider technology acquisition, development, and dissemination (Plucknett et al. 1990). One option for acquisition will be to continue to work exclusively with the public sector, primarily with universities in developed countries. However, as outlined above, this relationship may become more difficult to maintain as more universities file patent applications with hopes of receiving financial returns for their research investments.

In the light of changing relationships with traditional partners, a second option would be to expand interaction with the private sector. This would be consistent with the centers' charge to adapt the tools of biotechnology and to consider new methods for acquiring technologies. Since evidence indicates that commerce is a new incentive driving many applications of genetic engineering, the Consultative Group on International Agricultural Research (CGIAR) system has the responsibility to encourage private sector involvement in international research by challenging the private sector to extend its proprietary technologies to the needs of developing countries. Without efforts by the CGIAR and donors, private firms will work where markets exist, where products can be protected, and where they can establish their own in-country expertise. Such a strategy is reasonable from a profit perspective, but will overlook potential benefits gained from working with the public sector or CGIAR institutions.

The desirability of expanding the range of collaborative partnerships available to the CGIAR centers, and hence their host country clients, has been increasingly documented (Sawyer 1989). If the CGIAR centers, donors, and national programs are to explore these new relationships with the private sector, new ways of "doing business" will be required, and a number of issues must be considered that are critical to the establishment of successful working relationships with the private sector. The identification and appreciation of the relative strengths of the IARCs and the commercial sector is of paramount importance in this process and will need to be defined as an initial step in collaborations involving these diverse partners.

For example, collaboration with IARCs could potentially provide companies with valuable, perhaps otherwise unavailable, information regarding the stability of proprietary genetic constructs in various crops and in numerous breeding programs. Such field evaluation would be useful for determining the potential value of proprietary constructs in more commercial backgrounds. IARCS, in turn, could benefit from the private sector's ability to translate valuable technologies into commercially marketable products. In certain instances, the contribution of IARCs in the production of a commercially viable product may realize additional income for the centers through sales to commercial producers, which in turn could be applied to research on a variety of noncommercial crops.

Equally important is the issue of legal and contractual expertise. The IARCs are currently at a disadvantage in their inability to negotiate binding legal agreements regarding proprietary technologies. Most commercial research firms possess "in-house" corporate attorneys or have access to such expertise through retainers. In the past, such expertise has not been required by the CGIAR system and, accordingly, has not yet been introduced.

In addition, numerous questions regarding the image projected to donors and developing country clients may arise as the CGIAR centers enter into contractual relations with private sector firms. Some of these issues involve the CGIAR system's pride in being able to distribute research results and improved germplasm without restriction. As in the initial relationships established between U.S. universities and private biotechnology companies (Olson 1986), the centers' scientific integrity must be maintained for the mutual benefit of both partners. IARC participation in these ventures must not be misconstrued as a means to facilitate unfair profits for the private sector at the expense of client country interests. Such concerns are real and demonstrate the need to employ suitable legal counsel so that the needs of CGIAR centers, host country clients, donors, and the private sector are kept in balance. The realization that each party stands to

benefit should be the driving factor for achieving agreement. Carefully drafted initial agreements will begin to alter the preliminary misconceptions of partners that lack previous experience with one another.

The equitable distribution of germplasm derived from CGIAR research programs, which can occur through the international trial system, special requests to the international centers, and through germplasm announcements, is also an issue of paramount importance. This system of distribution must be re-examined in the context of protecting intellectual property. Commercial partners will require assurance that a system of accountability can be developed for the release of modified germplasm. Thus, when distribution policies are discussed with a commercial partner, agreement should be reached regarding a center's responsibility and liability for the use of proprietary genetic information. As such, in order to continue unrestricted distribution of germplasm, and to ensure that proprietary sources of germplasm or technologies have not been inappropriately handled, it may be necessary for the IARCs to publish the origin and characteristics of each release to preclude unauthorized claims to the material (Duvick 1989).

In many cases, it may be possible for the centers to license technologies or genetic materials to national programs from which additional income may be gained. If free, unrestricted distribution of results occurs, centers must be sure that they have not inadvertently violated terms of the initial agreement.

Many of these considerations will be based upon the particular crop being modified and the system that is used to distribute new germplasm. For example, many of the CGIAR commodity crops are distributed through the public sector or parastatal seed companies. This type of distribution does not present ready access for commercial investment or return on investment. However, if proprietary germplasm, modified through new technologies, has a distinct market advantage, commercial development may become a reality.

Such may be the case with cassava. Commercial production and sales of cassava have begun to increase recently and are being treated differently from local production or that resulting from international agricultural research programs. A recently developed agreement between USAID and Monsanto Corporation, which will be discussed in detail below, could present African scientists with an opportunity to commercially produce the transgenic stocks to be developed within this agreement. While this first collaboration involves the donation of the technology and the gene by Monsanto Corporation, it will provide greater incentive and perhaps a more useful means of delivery of products to the end user to facilitate the development of meaningful business arrangements within Africa for other projects.

It may be difficult to enforce violations of proprietary agreements which result from unauthorized use of germplasm or technologies and litigation may not always be possible or warranted. Nonetheless, corporate partners will differentiate between those institutions which make good partners and those which present greater risk. This is an important point, for once a reputation as a bad partner has developed, opportunities for further collaboration will decrease as other commercial firms become aware of the problem. Thus, those who violate legal agreements may gain in the short term, but stand to suffer later as new material is developed and no longer released to a particular center or national program.

Finally, it must be recognized that certain centers within the CGIAR system will hold patents for their own inventions, whether developed independently or in collaboration. Patents can be essential to control production of inventions, ensure appropriate distribution and pricing, and prevent unauthorized use. Thus, there are numerous benefits that may come from the protection of intellectual property within the CGIAR system. Often, privatization is regarded negatively and is seen as a barrier to access of technologies by perpetuating technological dependence and imposing non-equitable conditions for acquisition (Sasson 1990). However, the positive advantages mentioned above, gained through protection of intellectual property rights, should be more fully explored and established within the CGIAR system.

A question which has been explored on only the most superficial level is this: Can a CGIAR center sign an exclusive agreement with a private source for either technologies or genetic material? However, with appropriate legal counsel provided for the center and national partners, it is one that deserves greater attention.

Hypothetically, genetically engineered constructs for insect resistance protected by proprietary patents of interest to a center may be obtained through negotiation with a private company. Its useful application in the mandate crops, however, may require that a center expend its resources to successfully adapt the genes involved, to achieve expression in germplasm of agronomic importance for a particular region or client country, and to demonstrate a safe and efficacious history of field testing.

For initial agreements after successful testing and expression, it is recommended that limited distribu-

tion of new germplasm be made in accordance with the ability to offer intellectual property protection though plant breeders' rights or patents.

After successful testing and expression, it is recommended that limited distribution of the new germplasm be made only as far as it can be protected by plant breeders' rights or patent protection. This is important because it demonstrates that the CGIAR system is willing to protect the investment made by the private sector, thus becoming better business partners for industry. It is also important in that cooperation with the private sector on proprietary protection is also becoming more important to donor agencies.

Limited distribution may also involve some financial remuneration to both the CGIAR system and the private industry involved. Such agreements must consider the liabilities involved and seek mutual protection through legal documents that make clear the consequences of illegally appropriating genetic material or new technologies.

These initial activities, which could be assisted by donor participation and outside legal expertise, when conducted in a manner which protects the private sector's investment, will set valuable precedents for the improvement of global agriculture through biotechnology and for the establishment of additional partnerships with the private sector.

ICRISAT Crops

As an example, the commodity crops in ICRISAT's mandate—sorghum, pearl millet, chickpea, pigeonpea, and groundnut—may be considered in relation to potential collaboration with the private sector. These crops are critically important to farmers in the semi-arid tropics. Some of these crops, especially hybrid sorghum, have caught the attention of the private sector and are being produced commercially in India, Thailand, parts of Africa, and to a large extent in Latin America. As shown in Table 2, special opportunities may exist here of interest to commercial partners that do not exist for other crops or that may be additionally limited by the technology being considered. Germplasm enhancement, or pre-breeding, in regard to the legume programs and wide hybridization, offer points of interest for all concerned with expanding the genetic diversity of food crops.

Table 2. ICRISAT mandate crops, breeding objects, and likelihood of applying biotechnology towards their resolution.

Crop and breeding priorities	Application of Biotechnology: Scientific opportunity	Application of Biotechnology: Commercial interest
Sorghum		
A. Stress tolerance	Long term	No
1. Drought	Yes	Possible
2. Photoperiod	Yes	Yes
3. Disease/Insect	Yes	Yes
B. Hybrid cultivars	Yes	Yes
C. Utilization		
1. Value added traits	Yes	Yes
Pearl Millet		
A. Stress tolerance		
1. Drought	Long term	No[1,2]
2. Seedling emergence	No	No[1,2]
3. Disease/Insect	Yes	Yes
B. Hybrid cultivars	Yes	Yes
Chickpea		
A. Stress tolerance		
1. Drought	Long term	No[1,2]
2. Low temperature	No	No[1,2]
3. Early growth vigor	No	No[1,2]
4. Disease/Insect	Yes	Yes
B. Hybrid cultivars	Yes	Yes
Pigeonpea		
A. Stress tolerance		
1. Drought	No	No
2. Temperature effects	No	No
3. Disease/Insect	Yes	Yes
B. Plant nutrition		
1. Nodulation and N-fixing ability	Long term	No
C. Utilization		
1. Value-added traits	Yes	Yes
D. Hybrid cultivars	Yes	No
Groundnut		
A. Stress tolerance		
1. Photoperiod response	No	
2. Drought	No	
3. Disease/Insect	Yes	Yes
4. Nematodes	Yes	Yes
B. Wide cross program	Yes	No
C. Oil quality	Yes	Yes

1. No immediate short-term interest due to complexity of problem.
2. Less commercial crop interest.

The Role of Donors in the Evolution of New Partnerships

The burden of reaching such agreements should not be placed upon the CGIAR centers alone. One opportunity to offset concerns that may arise regarding initiatives with the private sector is to involve a donor agency, preferably one with the ability to tap national and commercial interests and legal expertise. Donor intervention may also be useful in the identification of potential partners, and in the establishment of better linkages for biotechnology research and development between the centers, their industrial partners, and national programs. Funding to support these links could come largely from donor agencies in developed countries (Barker 1990). Donors have a vested interest in providing this support. Without such linkages and in view of current institutional trends, the disparity between developed and developing countries for technology acquisition will continue to widen with negative results on worldwide agricultural productivity.

Donor activities could also include the provision or identification of legal retainers (as in the case of CIP), money to support research, co-development of research initiatives, biosafety and regulatory protocols, and the management of genetic material. In this manner, the centers would be less exposed to perceived conflicts of interest. Such relations would also benefit donors because they would link and coordinate development assistance objectives and interests with those of the CGIAR system.

USAID's Activities with the Private Sector

Due to similar constraints being imposed on the centers with regard to technology acquisition, donors are also beginning to extend their collaborative efforts in research to include the private sector. This is becoming increasingly necessary with regard to donor initiatives in biotechnology. USAID efforts in this regard include activities located in the central offices and in client country missions. Several of these current and pending initiatives in biotechnology involving private sector relationships are described below.

Cooperative Agreement with Monsanto Corporation

As previously described, the ability to patent biological technologies has stimulated private investment in agricultural research. Accordingly, Monsanto Corporation has invested heavily in transformation technologies and has achieved demonstrated results in their field-tested applications. Abilities in these areas—far ahead of those in the public sector—were first brought to the attention of USAID and the international community during USAID's biotechnology conference (Delanny 1989). These abilities were coupled with an interest in developing country-oriented application by Dr Ernest Jaworski and were discussed with USAID's Office of Agriculture.

Following extensive negotiations, a grant agreement was signed between USAID and Monsanto to use proprietary vector expression technologies, coupled with coat protein virus technologies developed at Washington University, for the production of virus-resistant root and tuber material for Africa. It will involve the placement of two senior African scientists for a three-year period at Monsanto's laboratory in St Louis, Missouri.

Advantages of such an agreement are presented in Table 3. To reach this agreement, direct negotiations between USAID's Office of General Counsel and Monsanto's corporate attorneys were required. These discussions were necessary to certify USAID's authorization to grant directly to a major commercial entity. This may also be an issue for collaborative projects involving CGIAR centers. In the case with Monsanto, Agency technical officers worked directly with their counterparts in USAID's contractual and legal offices to ensure that the grant was properly understood. In the end, USAID issued a letter to Monsanto attorneys that indicated USAID's ability to award such a grant.

Once this was established, appropriate rights had to be retained by USAID to assure timely access to those technologies that were developed under the agreement. The grant also delineated exactly which proprietary technologies were involved and under what conditions they were to be made available. Accordingly, Monsanto has agreed "... to grant a royalty-free license to use in Africa any proprietary technology (including disarmed Agrobacterium strains, intermediate vectors, selectable markers, promoters, other expression and transformation technology and coat protein based virus tolerance technology) developed by African scientists embodied within any cassava, yam or sweet potato germplasm developed in this program" (USAID 1990).

It is important to realize that Monsanto is assuming a degree of risk in this agreement. Understanding this risk and that faced by other potential commercial partners is the first step in arriving at a work

Table 3. Advantages derived from the USAID and Monsanto Corporation grant regarding the transformation of economically-important root and tuber crops.

Secures specific rights for specific proprietary technologies as used in accordance with conditions of the grant.
Places senior African scientists within commercial laboratory settings to become familiar with commercial orientation, concerns, and decisions.
Familiarizes African scientists with testing protocols and regulatory compliance that commercial firms undertake to bring recombinant products to market.
Establishes the relation between proprietary technologies of Monsanto and those developed at Washington University.
Successful production of transformed germplasm will provide all parties with additional data regarding performance of genetic constructs, which may be useful in future research efforts.
Establishes precedence for donor grants to commercial agricultural research entities and gains acceptance for such opportunities within the commercial legal establishment.
Establishes a multi-directional relationship between a national program, private sector, donor, and IARC for agricultural biotechnology research and product development.

able agreement. Clearly, the risk posed here involves the unauthorized use or dissemination of these proprietary technologies beyond the objectives of the grant with USAID. This illustrates the complexities which may arise in IARC negotiations with the private sector and the unique nature each agreement will present to the collaborating parties involved.

Although commercial profits from cassava will not be realized by Monsanto through this agreement, demonstrated use of Monsanto's technologies will give credibility to the appropriate use of biotechnology for crop improvement. In addition, by involving a partnership with USAID, Monsanto has gained the benefit of the biotechnology and biosafety expertise available for international testing and use. The grant also involves the International Institute for Tropical Agriculture to ensure that an international center has been brought directly into this type of agreement.

Plant Biotechnology for Developing Countries: Recommendations of a National Research Council Panel

At the request of the Office of Agriculture, the National Research Council (NRC) convened a group of experts for discussions concerning priorities in plant biotechnology research that could benefit agriculture in developing countries in the near future (3-5 years). The objective was to identify areas of biotechnology that, in the panel's view, were sufficiently advanced for transfer to USAID client countries through collaborative initiatives with U.S. scientists.

The panel found a distinct need for a technical assistance program that encourages and assists developing country researchers in the acquisition of technologies focused on critical problem areas of local importance. A number of priority areas were identified in the meeting with specific recommendations to be developed upon consultation with sources of expertise most familiar with local problems, needs, and opportunities (National Research Council 1990). Institutional priorities identified included three areas: **biosafety**—USAID should assist developing countries to implement and monitor appropriate biosafety regulations; **intellectual property**—USAID should participate in the development of policies to promote international cooperation in intellectual property rights; and **human resource development and networking**—USAID should enhance biotechnology capabilities through doctoral and postdoctoral fellowships and nondegree training for plant biotechnologists.

This report highlights many of these topics and recognizes that development assistance agencies must accept the challenges of biotechnology if they are to play a relevant role in its future. Progress will not be possible without equal attention devoted to the institutional dimensions as well as to the technical aspects of biotechnology.

On the basis of recommendations contained within this report, the Office of Agriculture is designing a

new project in plant biotechnology that will include research in the technical areas described by the NRC report. Emphasis will also be placed on the management of biotechnology in developing countries. In this regard, provisions for commercial development of products, training, establishment of a network, regulatory review, and intellectual property protection of research inventions are being considered within the context of this project. Integration both with other USAID projects in biotechnology and with increasingly important natural resource management initiatives will also be established within this project. Participation of the private sector or U.S. universities in either constraint-oriented research or commercialization efforts, targeted to the enhanced agricultural production in less developed countries, will be commissioned through a request for proposals. Awards will be made on the basis of technical merit and competitive bidding.

Commercial Efforts in Developing Recombinant Vaccines

Research sponsored by the United States federal sector can be geared in a direction that explores the commercial potential of the research yet simultaneously serves the needs of the developing world. Such an example stems from a 5-year cooperative agreement initiated between the Office of Agriculture, and the University of Florida, Gainesville. This project, titled, "Improved Animal Vaccines through Biotechnology, Phase II: Anaplasmosis and Babesiosis," has utilized legal expertise at the University to file patent applications on much of the research, which has been eventually published in peer-reviewed journals. This project has also employed the services of a commercial consulting company, Ernst and Young, to develop business plans along lines consistent with the intent of the cooperative agreement.

By proceeding in such a fashion, the project offers a new way of doing business to potential corporate partners. This strategy has worked well as judged by the interest expressed by several potential commercial partners in this project. These have included site visits and discussions regarding the production of recombinant vaccines and diagnostic probes for the various regions of the globe in which USAID works. Ultimately, the senior project director, in conjunction with USAID, will have to select the best commercial partner. Exclusive rights or license will be assigned to this firm for the production and distribution of these products in the developing world. Similar interest exists for their use in the United States and of course this would be the subject of other negotiations.

An integral part of this project involves an external Technical Advisory Group (TAG). This body serves to provide nonproject associated advice, review, and guidance related to obstacles, goals, and objectives confronted by project scientists. Such an approach would seem appropriate for IARC-sponsored research. A TAG could include members of the commercial research community as well as legal representation.

Commercial Focus of USAID/Mission Projects in Biotechnology

The USAID missions have become increasingly aware of the potential benefits of research in biotechnology for improving agricultural production in client countries. As such, USAID missions presently support a wide range of activities in biotechnology related to developing country agriculture. Several missions have recently instituted programs designed to stimulate the growth of technology-based industry. To date, these have often included research and development efforts in biotechnology.

For example, in India, USAID/New Delhi and the Indian government have sponsored the Program for Advancement of Commercial Technology (PACT), which provides financial support for jointly submitted proposals between U.S. and Indian companies for the "development and commercialization of innovative technical products and processes to the benefit of both economies" (Jones 1989). Although not specifically planned as such, many projects currently sponsored under this program have a biotechnology component. Those related to agriculture include an agreement between Ecogen Inc (USA) and Gujarat State Fertilizers Company (India) for control of certain pests in cotton, tobacco, groundnut, and pulses using *Bacillus thuringiensis*-based bioinsecticides, and an agreement between DNA Plant Technology Corporation (USA) and Southern Petrochemical Industrial Science Foundation (India) for the development of improved varieties of coffee and roses by tissue culture.

Similarly, the Science and Technology for Development project has been established in Thailand through the provision of three-way support between USAID, the Royal Thai Government, and private sector interests. The project is designed to expand the contribution of science and technology to the devel-

opment process through the establishment of more effective linkages between industry, the public sector research community, and government policy initiatives in Thailand. Nearly two-thirds of the project's total resources are devoted to problem-solving research, development, and engineering in three priority technical areas of concentration. Biotechnology has been identified as one of these three critical areas.

Biosafety and the International Agricultural Research Centers

Maximum coordination will be required to assure that requisite parameters are understood and established to test for both efficacy and safety of recombinant products. Gaining such information will require direct communication between regulators, principal scientists, and donor agencies. Without establishing such communication links, research projects run the risk of "spinning" the request-review-approval-permit cycle multiple times until appropriate questions have been answered (Fig. 2). This will be costly, because each time a request is submitted costs are entailed for the subsequent testing and documentation (Cohen and Chambers 1991).

Once contained testing is warranted, consideration must be given to each host-country's ability to monitor the test, its degree of containment, contingency plans if problems occur, termination procedures, and responsibility for supervision. Costs associated with such experimental modifications will need to be estimated within research proposals so that more realistic funding levels can be determined. Private sector biotechnology firms have the greatest experience to date on these issues, and their expertise is currently being only minimally used by both the CGIAR system and donors.

Many environmental and safety parameters will vary globally. While some of these may be obvious, special attention will need to be paid to ensure that an appropriate assessment of such differences is obtained well in advance of host-country testing. It would be of great value for regulatory agencies in the industrialized countries to make their personnel available for site inspections and recommendations regarding each proposed test. Private sector involvement will share the burden associated with these tests

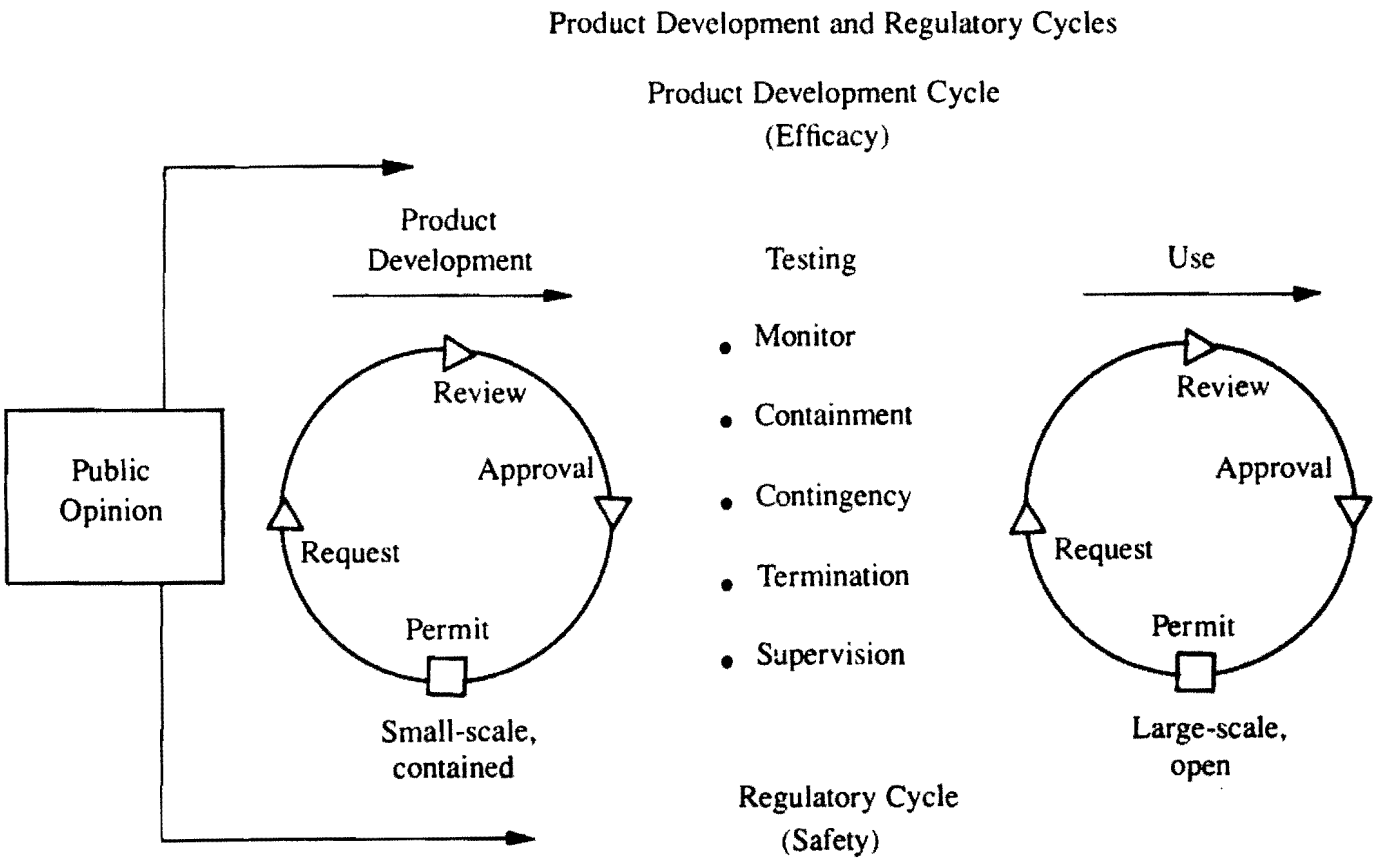

Figure 2. Gaining information is necessary to avoid wasting time when developing new products and meeting regulations.

and form an important collaborative approach to ensure that safety considerations are followed.

Conclusions

Biotechnology, when integrated with traditional crop improvement programs, enables a more efficient, environmentally compatible and, ultimately, cost-effective utilization of resources for improved agricultural productivity. The technology is highly appropriate for developing countries, where present technical inadequacies are often magnified by increasing population pressure, intensive agricultural practices that in turn have contributed to the degradation of natural resources, and the poor quality of many soils. Presently, most biotechnology research has been conducted in developed countries with practical applications limited to those problems and scenarios unique to commercial agriculture. In these countries, changes in patent law, public funding for research, and the relationship between industry and publicly-supported institutions are contributing to increased privatization of biotechnology research for the development of proprietary technologies and products. This may ultimately limit the impact of biotechnology on agricultural research supported by donors and conducted by the IARCs and national programs in developing countries. The international agricultural research community must develop equitable, collaborative relationships with the private sector for the acquisition of beneficial techniques and materials that may be applied to international agriculture. Intellectual property agreements based on a mutual understanding of goals, perceived risks, and possible benefits will be a necessary component of such collaborations. Donor initiatives, such as those recently planned or undertaken by USAID, are beginning to address the issue of private sector collaboration in biotechnology. As such, donors may be in the position to provide assistance to IARCs and national programs in this regard. Initial agreements potentially offer exemplary mechanisms and strategies for the development of continuing, mutually beneficial partnerships for the international agricultural research community.

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Biotechnology at the International Board for Plant Genetic Resources

L.A. Withers[1]

The International Board for Plant Genetic Resources (IBPGR) undertakes biotechnology research within its In Vitro Conservation, Seed Conservation, Pathology and Quarantine, and Genetic Diversity programs. Collaborators in this research include other CGIAR Centers, universities, National Program institutes, and regional institutes.

CGIAR Centers

ICRISAT (India)
CIAT (Colombia)
IITA (Nigeria)
CIMMYT (Mexico)
ILCA (Ethiopia)

National and Regional Institutes

Agriculture Canada (Vancouver, Canada)
CATIE (Costa Rica)
BARI (Bangladesh)
IRHO (France)
IVIA (Valencia, Spain),
John Innes Institute (UK)
National Centre for Research in Biotechnology (Bogor, Indonesia)
ORSTOM (France)
Research Institute for Estate Crops (Bogor, Indonesia)
United States Department of Agriculture (Georgia, USA)
Volcani Centre (Israel)

Universities

Australia: Queensland
China: Zhongzhan
Israel: Jerusalem
Turkey: Istanbul
Canada: London, Saskatchewan
Malaysia: Universiti Pertanian Malaysia
Mexico: UNAM
Netherlands: Wageningen
UK: Nottingham, John Innes Institute
USA: Clemson, Cornell, UC Davis, Florida, Georgia, Kansas State, Ohio State, Utah State

In vitro techniques are being developed to solve problems in the genetic conservation of certain crops, primarily those that are clonally propagated, e.g., cassava, aroids, and *Musa* (banana and plantain), and those that produce recalcitrant seeds, e.g., coconut, mango, and jackfruit. Conventionally, these crops are conserved in field genebanks that are costly to maintain and in which the germplasm is exposed to risks of loss through disease, weather damage, and neglect.

Although storage technologies are the basis of the in vitro approach to genetic conservation, they form part of a system that commences with germplasm collection and flows through disease indexing and eradication, propagation and storage, to distribution and utilization. This system parallels the field genebank and potentially offers both "active" storage by slow growth at a reduced temperature or in the presence of retardants, and "base" storage by cryopreservation in liquid nitrogen.

Problems are often encountered in collecting germplasm. Orthodox seeds may be scarce, in poor condition, or immature. Recalcitrant seeds and vegetative tissues such as budwood, corms, or suckers can deteriorate in transit. Collected material is often bulky or heavy, and therefore costly to transport. These practical and biological problems may be solved by using simplified in vitro inoculation techniques in the field. IBPGR is developing and testing techniques for coconut, temperate fruits, citrus, and forage grasses. A training course on in vitro collecting was held at CATIE during 1990.

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Withers, L.A. 1992. Biotechnology at the International Board for Plant Genetic Resources. Pages 31–32 *in* Biotechnology and crop improvement in Asia (Moss, J.P., ed.). Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

IBPGR supports research on both slow growth and cryopreservation, with most attention being given currently to the development of cryopreservation techniques for cassava, coconut, and *Musa*. Molecular techniques are also being applied to conserved cultures of maize and potato to determine the extent of any risk to genetic integrity in vitro. To date, no evidence has been presented to suggest that cryopreservation is unsafe in this respect. However, RFLP studies of the stored seed of soybean and maize have shown deterioration that is manifested by DNA breaks.

IBPGR and CIAT have been collaborating to develop and test management procedures for cassava in slow growth storage. The Pilot In Vitro Active Genebank Project, now in its fourth year, will produce recommendations applicable to other cassava collections and to the conservation of other crops. Within the project, isozyme analysis is being used to characterize cassava genotypes and to monitor the stability of stored cultures. No evidence of instability has been found but in another project involving *Allium* spp, certain slow growth conditions have been found to lead to isozyme instability.

In association with the IBPGR-CIAT Pilot In Vitro Active Genebank, molecular genetic techniques involving RFLP and analysis of short, highly-repetitive DNA sequences are being used to investigate the cassava genome. In other projects, RFLP analysis is also being used to obtain new information on the amount and distribution of genetic diversity, on species relations and on crop plant evolution. Current studies include the analysis of isozyme and cpDNA variation in *Vigna* spp, the investigation of cytoplasmic DNA variation in cultivated and wild *Musa* and the use of RFLP markers to determine optimum conservation procedures in wild *Phaseolus*.

The development of rapid indexing methods for diseases that create bottleneck problems in the safe and efficient movement of germplasm is an area in which biotechnology can make a great contribution. For example, banana bunchy top is a major disease of banana and plantain and causes serious losses in Asia. The lack of a reliable indexing method prevents the safe movement of germplasm from its center of origin to Latin America where the disease is absent. In research to develop a nucleic acid hybridization test for the banana bunchy top virus (BBTV), a probe has been produced that reliably detects all isolates of BBTV tested so far. As well as specific indexing projects, IBPGR has a major interest in the development of broad spectrum tests for viruses. Research is also supported on virus eradication by in vitro thermotherapy and micrografting, and on virus indexing of in vitro plantlets.

IBPGR recognizes the value of biotechnology in solving problems in plant genetic resources work and takes a pragmatic approach to its application alongside more conventional technologies. Molecular investigations of the genome will supplement rather than replace conventional techniques of characterization and evaluation. Secure, comprehensive, efficient, and accessible conservation of crop genepools will best be achieved by the integrated use of different technologies. The balance between seed storage, the field genebank, in situ conservation, and storage of in vitro cultures, pollen, and perhaps DNA, will be different in each case and, moreover, may alter with time as new technologies develop to higher levels of reliability and as breeders' needs change.

Application of Biotechnology in Crop Improvement at the International Center for Agricultural Research in Dry Areas

ICARDA[1]

Improving farming in the dry rainfed areas of West Asia and North Africa is the main objective of ICARDA. Over the past few years, biotechnology has been gradually incorporated in research and training programs. Current research covers all ICARDA mandated crops (winter cereals and cool season food legumes), and collaboration exists with a large number of advanced institutions engaged in biotechnology research.

One activity is the development of efficient techniques of cereal haploid plant production. Two different methods have been developed, anther culture and interspecific crosses followed by embryo rescue, and have become integrated into cereal breeding programs. Immunodiagnostic techniques are an important component in viral and microbiological research at ICARDA. ELISA kits are being prepared for several viruses. Fluorescent antibody and ELISA techniques also allow investigations of critical areas of *Rhizobium* behavior in soil and *Rhizobium*-plant interactions. In order to use the gene pools of wild relatives, attempts are being made to establish an ovule-embryo rescue technique to cross the cultivated species *Lens culinaris* with *Lens nigricans*. A DNA fingerprinting technique has been established for genotype identification and genetic purity testing. Preliminary work has been done in order to explore the use of RFLPs in marker-assisted breeding.

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ICARDA (International Center for Agricultural Research in Dry Areas). 1992. Application of biotechnology in crop improvement at the International Center for Agricultural Research in Dry Areas. Page 33 *in* Biotechnology and crop improvement in Asia (Moss, J.P., ed.). Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

The Country Perspectives

Proposed Priority Areas of Plant Biotechnology Research and Development in China

Shi-Rong Jia[1]

Abstract

Taking into account the constraints existing in Chinese agriculture, the priorities of plant biotechnology research and development are proposed. Particular emphases are given to rice improvement; the genetic engineering of viral, bacterial, and insect resistance in wheat, potato, and cotton; genetic transformation; and the diagnosis of plant pathogens.

Introduction

For several decades, traditional breeding has made a significant contribution to crop improvement. During the last 40 years in China, the cultivars of major crops used in commercial agriculture have changed two or three times. A noticeable example was the dwarf rice varieties developed in the 1950s and extensively used in production thereafter, but which were replaced in the 1970s by hybrid rice. These now occupy 15 million ha, about half of the total area of rice production. Although the development of new varieties has tremendously increased the yield of some crops—for example, in 1989 the total grain output in China had risen to 0.4 billion t (Editorial Committee of China Agriculture Yearbook 1989)—traditional breeding has also met with some problems. The narrow genetic background within a given species, the difficulties of sexual hybridization between remote species, and the long process to break down the linkages between desirable and undesirable genes are some of the constraints faced by breeders.

Biotechnology is a newly emerging, highly rewarding technology with large potential applications in crop improvement, since the gene(s) with agronomic value, isolated from remote or exotic organisms or even artificially synthesized, can be manipulated in vitro and transferred into cultivars. However, it is important to keep in mind, historically, that a new technology should always be related to or be combined with existing technologies. Their relationship is a grafting or a complementing process rather than a replacement. The initial objective of a biotechnology project is to identify the agricultural problems that need to be solved and that may benefit from the application of new technologies. The critical issue is to determine what the new technologies of cellular and molecular biology will allow us to do that cannot be done by conventional breeding programs. This paper discusses the infrastructure for biotechnology in China, the input of biotechnology into rice, and the constraints in other crops to which biotechnology is being applied.

Infrastructure for Biotechnology in China

In accordance with the needs of agricultural development in China and the progress and potential application of biotechnology in agriculture, the Chinese government has put great emphasis on research and development (R&D) in plant biotechnology.

To promote R&D of plant biotechnology in China, key laboratories have been set up for different fields of study. These include the Biotechnology Research Center (BRC), Chinese Academy of Agricultural Sciences (CAAS), Beijing; the Laboratory of Plant Genetic Engineering, Beijing University; the Laboratory of Agricultural Biotechnology, Beijing Agriculture

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University; the Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology; the Laboratory of Molecular Biology, Shanghai Institute of Biochemistry; and others. In 1986, the CAAS established BRC, which specializes in developing technologies for molecular and cell biology and monoclonal antibodies. In a total of 36 CAAS-affiliated research institutes, there are more than 20 units involved in biotechnology research in both plant and animal sciences. Furthermore, similar laboratories have been set up in most of the provincial and municipal academies of agricultural sciences and the agricultural colleges. A new agrobiotechnology research system is emerging in China.

In the Sixth (1981-85) and Seventh (1986-90) Five-year Plans, the agrobiotechnology R&D program was one of the major programs in biotechnology. In addition, the government in 1986 initiated a long-term (1986-2000) National High Technology Development Project (NHTDP), in which priority was given to biotechnology, especially agrobiotechnology. The national policy of biotechnology was published in a Bluebook for Chinese Science and Technology by the Chinese National Committee of Science and Technology (1990). The Ministry of Agriculture has also taken appropriate measures to strengthen R&D in agrobiotechnology, including the identification of priority areas, some of which are presented in this paper. These include the setting up of a leading committee and an advisory board for biotechnology to coordinate all the R&D activities in agricultural research institutes and universities, the exchange of academic information, the training of personnel, the participation in international cooperation, and the establishment of an information network.

Rice Improvement

Regarding the order of economic importance of the grain crops, rice has been given high priority by the government NHTDP. The cultivated area of rice occupies 32 million ha, of which 15 million ha are hybrid rice. In the NHTDP, priority has been given to hybrid rice breeding through which a 20% yield increase is anticipated by the year 2000.

Hybrid Rice

The strategies of this project are to use photoperiod-sensitive genic male sterile lines to simplify the procedures for making hybrids—since in this system there is no need for using maintainer lines—and to make hybrids between *japonica* and *indica* rice to increase heterosis. To approach such a goal, genes controlling both the photoperiod-sensitive genic male sterility and broad compatibility are needed. Molecular biologists are now cooperating with rice breeders to attempt to identify and clone these genes.

Anther Culture

Anther culture and haploid breeding have proved to be one of the powerful tools for developing new varieties. Superior homozygous diploid lines could be obtained quickly by selection of regenerated plants for agronomic characters after chromosome doubling of haploid plants derived from anther culture of F_1 or F_2 plants. The characters of these lines were found to be relatively stable and breeding programs could be shortened considerably through rapid achievement of homozygosis. By a rough estimate, there were about 1000 institutions involved in anther and tissue culture in China in the late 1970s (Lou and Tang 1985). Since then, many new superior rice and wheat varieties with stress tolerance and high yields have been obtained by this technique. Of these the rice cultivars Zhonghua Nos 8, 9, 10, and 11, developed by the Institute of Crop Breeding and Cultivation, CAAS (M.-F. Li, personal communication, 1990), and the wheat cultivars Jinghua Nos 1, 3, and 5, bred by Beijing Academy of Agricultural Sciences (D.-F. Hu, personal communication, 1990) have been certified and released to the farmers. The total planting area of new varieties developed by anther culture technique in recent years has reached 0.65 million ha, out of which rice varieties account for 0.35 million ha. Therefore, haploid breeding will continue to be on the priority list for plant biotechnology development in China.

Restriction Fragment Length Polymorphism (RFLP) in Rice

RFLPs as molecular markers have a broad prospective application in gene mapping and crop breeding. With RFLP maps, one can even conduct a breeding program without having identified the gene of interest or understanding its biochemical mechanism. This is carried out by analyzing the results of a genetic cross and following how the trait is inherited with respect to the distribution of RFLP markers in offspring. The

selection of one particular trait can be done more precisely without environmental influences, thus it can substantially speed up the success rate of a breeding program. Another powerful advantage of RFLP maps is that they make it possible to conduct breeding programs for traits controlled by multiple genes. In China, the study of rice RFLP and its application to rice breeding are being conducted. The emphasis of this study is to identify the RFLP markers responsible for hybrid vigor and resistance to rice blast (L.- W. Zhu, Institute of Genetics, Academia Sinica, personal communication, 1990). The study of RFLP in wheat and potato is also being considered as a priority.

Virus Resistance

Since it was proved that it is possible to obtain virus resistance by introducing a virus coat protein gene into tobacco, tomato, and potato cultivars (Powell-Abel et al. 1986; Tumer et al. 1987; Cuozzo et al. 1988; Lawson et al. 1990), the same approach is being applied to rice, wheat, and potato breeding in China. Chinese scientists have now obtained tobacco transgenic lines resistant to both tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) that are now under field testing (K.Q. Mang, Institute of Microbiology, Academia Sinica, personal communication, 1990). The future priorities with respect to this field are as follows.

Barley Yellow Dwarf Virus (BYDV)

BYDV is one of the most epidemic and serious viruses and results in great losses in wheat production. Regular outbreaks of BYDV in the 14 northern provinces cause serious damage, reaching levels that can cause yield losses as high as 500 000 t in the three provinces (Shanxi, Gansu, and Ningxia) alone, as happened in 1970.

At present, no wheat cultivars resistant to BYDV are available. It is known that some wheat relatives possess BYDV-resistance gene(s), and progress has been made in a program to introduce the BYDV resistant gene(s) from wild species into cultivars through remote hybridization followed by embryo rescue and anther culture (Xin et al. in press). In another program, the coat protein gene of BYDV has been cloned, prior to introduction into wheat cultivars, to obtain transgenic plants resistant to BYDV. With either of the above approaches, we expect to provide wheat cultivars resistant to BYDV in 5 years.

Potato Viruses

China has the second largest area of potato cultivation in the world, with 2.7 million ha. However, the average yield is low. One of the main reasons for this is the severe degeneration of cultivars caused by virus infection, i.e., Potato Virus X (PVX), Potato Virus Y (PVY), and Potato Leaf-roll Virus (PLRV). At present, control is by using virus-free seed tubers produced by meristem culture; these are grown on about 10% of the area at present and have reduced the yield losses. However, it is necessary to renovate the seed tubers after 2 or 3 years because of virus reinfection. Therefore, the coat protein genes of PVX and PVY have been isolated and will be introduced into Chinese potato cultivars. The transgenic potato clones are expected to be field-evaluated in 5 years.

Bacterial Resistance

Bacterial wilt caused by *Pseudomonas solanacearum* is a worldwide disease that results in serious crop damage. In the southern and central part of China where temperature and humidity are relatively high, bacterial wilt causes dramatic yield losses in groundnut, potato, and tomato production. At present, there are no resistant potato and tomato cultivars nor is resistant germplasm available; this has hindered the progress of conventional breeding. The Cell Biology Laboratory, BRC, Beijing, in collaboration with Fudan University, Shanghai Plant Physiology Institute, the CAAS Institute of Vegetable and Ornamental Crops, Beijing, and the International Potato Center (CIP), are currently investigating the feasibility of genetic engineering of bacterial wilt resistance, in which an artificially synthesized antibacterial polypeptide gene, Cecropin B, has been introduced into Chinese potato cultivars through *Agrobacterium*-mediated gene transfer. To date, we have obtained 245 individual transgenic plants. Some of them were randomly chosen for Polymerase Chain Reaction and dot-blot analysis, and it was evident that the foreign gene was integrated into the potato genome. The transgenic plants have been micro-propagated and are being tested for bacterial wilt resistance by means of soil inoculation under greenhouse conditions.

Since bacterial wilt is also a serious disease in groundnut production, genetic engineering of bacteria

wilt resistance has a high priority in China. The first step is to establish an efficient transformation system for this species either by *Agrobacterium*-mediated gene transfer or by other physical means of direct gene transfer. The possibilities of achieving this through collaborative research are being explored.

Insect Resistance

It has been known for decades that *Bacillus thuringiensis* (Bt) is an effective biopesticide. Proteinase inhibitors have also been shown to be insecticidal agents. Recombinant DNA and tissue culture technologies now enable us to isolate and transfer into cultivars the Bt delta endotoxin gene and the proteinase inhibitor genes that will confer insect resistance.

In recent years Chinese scientists have isolated and cloned Bt genes from different strains of *B. thuringiensis* (Y.-L. Fan, BRC, CAAS, personal communication, 1990) and the proteinase inhibitor genes from different crops (Z.-W. Qi, Shanghai Institute of Biochemistry, Academia Sinica, personal communication, 1990). It is anticipated that delivering these genes into plants would help to control the lepidopteran larvae in rice, cotton, corn, vegetable, and woody species. So far, the Bt gene has been introduced into rice protoplasts that regenerated transgenic plants (Yang et al. 1988). The Bt gene has also been delivered into cotton by pollen tube pathways. The transgenic rice and cotton plants were confirmed by Southern hybridization (Y.-L. Fan, BRC, CAAS, personal communication, 1990). Since the commercial cotton cultivars lack insect resistance and the cotton boll worm (*Heliothis armigera*) and cotton pink boll worm (*Pecpinothoera gossypiella*) cause great damage, and particularly since cotton lint is not an edible part of the plant, it is reasonable to consider that the genetic engineering of insect resistance in cotton would be the right choice.

Genetic Transformation

The major present limitation to the genetic engineering of many crop species is the lack of regeneration and transformation systems, especially in the cereals. Although more than 40 plant species can be genetically engineered to date, the frequency of transformation in some species is still relatively low. While *Agrobacterium*-mediated gene transfer has host-range specificity, other direct gene transfer methods require sophisticated equipment. Furthermore, the transformation and regeneration of crops are highly genotype-specific. Until advances in cell biology allow culturing, selection, and regeneration of any genotype and until genetic transformation technology can be utilized on any genotype in plant breeding programs, biotechnological advances will not move directly from the laboratory to the market in the most elite genotypes available. It is, therefore, most important to work out an efficient plant regeneration and transformation system for each major crop; in particular, the system should be efficiently suited for different genotypes (Jia 1990).

In the practical sense, it is important to point out that genetic engineering should also obey the two rules of plant breeding, i.e., the creation of variation and the selection of resulting variants. In addition, since the mechanism of foreign gene integration is not well understood, the gene is randomly inserted in different chromosomal loci which results in position effects, and the individual transgenic plants differ greatly in the level of gene expression; therefore, the more transgenic plants obtained, the more chance for selecting individuals with good agronomic characters plus the gene of interest being integrated. This is another reason why there should be more effort to establish highly efficient transformation systems, which will be applied to different economically important Chinese crops (Jia 1990).

Meanwhile, a wider range of useful genes, such as stress-tolerance genes, need to be identified and cloned to expand the range of application of recombinant DNA technologies in plant breeding.

Diagnosis of Plant Pathogens

Monoclonal or polyclonal antibodies and nucleic acid probes have been proven to be powerful tools for more rapid and accurate diagnosis of plant diseases that can be used for quarantine service, selection of pathogen-free planting materials, breeding for disease resistance, and the detection and monitoring of pathogens in epidemiological studies to design more effective control measures. Monoclonal antibodies with high sensitivity and specificity—used particularly in virus detection—are a new immunological assay for detection of antigens at very low levels in plant materials.

In recent years Chinese scientists have developed monoclonal antibodies against different strains of PVX (Xiao et al. 1989), PVY (Guo et al. 1990), TMV, Soybean Mosaic Virus (SMV), *Pseudomonas so-*

lanacearum (Wang et al. 1989), and MLOs (mycoplasma-like organisms, e.g., the MLO causing mulberry dwarf disease), some of which are already being used. In order to meet the needs for diagnostic kits of monoclonal antibodies in the domestic market, a National Pilot Workshop is being constructed in the Biotechnology Research Center, CAAS, which will come into use in 1991. The BRC is currently developing and producing monoclonal antibody kits for diagnosis of PVX, PVY, PLRV, Sweet Potato Feathery Mottle Virus (SPFMV), and Sweet Potato Latent Virus (SPLV). This technology could be shared with other Asian countries by technology transfer or exporting diagnostic kits.

At the present time, China has an active program to produce polyclonal antibodies that can also be extremely useful. Their broader spectrum can sometimes be more useful than the highly specific monoclonal antibodies. Nucleic acid probes, on the other hand, can be used for detection of all classes of plant pathogens. But their wide practical use will be limited until stable, sensitive probe markers can be developed, and not, as at present, dependent on the use of radioactive labels. The potential of these for identification of potato viruses is being explored in China.

Future Prospects for Biotechnology in China

The extent of the commercial application of plant biotechnology is the important mark for measuring the vitality of this newly emerging technology. China as a developing country always emphasizes that research achievements should be transformed into productivity. Chinese farmers are benefiting from research achievements, for example, cultivars of wheat and rice produced through haploid breeding and virus-free potato tubers from tissue culture.

China is rich in animal, plant, and microorganism resources, as well as having many scientists with good experience in plant cell and tissue culture. This is a favorable condition for the development of biotechnology. In addition, China has a broad market at home with a reasonably priced labor force and low product costs. The unfavorable conditions are a shortage of funds, lack of quick access to the international market, and lack of management staff who have rich experience in foreign trade. The most urgent problem to be solved in many laboratories is to get more international financial support in order to have direct supplies of instruments or chemicals from other countries. Also needed are people to be trained for Master or Ph.D. degrees and the opportunity to participate in international symposia.

Carrying out economic reform along with opening to the outside world is a fundamental long-range national policy. We hope to strengthen cooperation with international institutions and companies under the conditions of equality and mutual benefit by way of technical collaboration, joint investment, and sharing the results and profits in order to speed up the development of plant biotechnology.

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Biotechnological Studies in Japan: Relevance to Tropical Agriculture

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Abstract

Among the biotechnological studies carried out in Japan, several may have an impact on agricultural development in tropical countries.

One major area of practical application of biotechnology involves development of methods to diagnose or assay plant diseases or to identify plant genes and genomes. Remarkable progress has been made in the molecular diagnosis of plant diseases caused by viruses including rice tungro disease. The same approach is being attempted for the diseases caused by viroids or mycoplasma-like organisms. Genes and genomes of crop plants have been analyzed at the molecular level. A set of probes to analyze the linkage of genes in rice is now available. Genes for key functions such as lipoxigenase genes in soybean and male sterility-controlling mitochondrial genes in rice have been analyzed. Information has accumulated about other genes such as those for seed storage proteins and for pathogenesis-related proteins.

For propagation, shoot tip cloning techniques developed in Haplopappus gracilis *is being tested for wider application.*

Transformation of plants is now possible through various methods. Use of tomato mosaic virus coat protein genes to protect tomato plants has proved to be effective. Several other attempts to introduce novel characters into plants are currently in progress.

In the studies on protoplasts, interspecific cell fusion in Citrus *spp and trials of asymmetric cell fusion in carrot and other plants have led to interesting results.*

Progress has also been made in the manipulation of ploidy and reproductive processes of crop plants. Induction of haploid plants in wheat and barley through pollination with corn pollen grains followed by 2,4-D treatment and embryo rescue (ICARDA-TARC cooperation) resulted in a breakthrough in the breeding procedures for these crop plants. A thermosensitive male-sterile gene induced by gamma-irradiation in rice could be widely utilized in heterosis breeding of rice.

Introduction

Biotechnology as a modern methodology for research on biological sciences offers a wide potential for application to the promotion of human welfare. Through the growing awareness and support of the public, biotechnology is making steady progress in Japan. Among such fields of research, several may have an impact on agricultural development in tropical countries.

Diagnosis

One major area of practical application of biotechnology involves diagnostic or assay devices. Enzyme-Linked Immunosorbent Assay (ELISA) using monoclonal antibodies has been used widely not only for the diagnosis of diseases but also for the assay of biologically active components. With the development of gene structure analysis, nucleic acid hybridization-based assay using cloned DNA or synthetic

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oligonucleotides as probes has become a practical tool for indexing various biological agents at the gene level.

Disease Diagnosis

Modern technology for indexing plant pathogens is required especially in the following areas: (1) assay of pathogens for the international exchange of germplasm or for pathogen-free propagation of crop plants; (2) quantitative assay of pathogens for the screening of disease resistance in breeding schemes especially where synergism of the pathogens or long latency disturbs the evaluation of symptoms; and (3) distinction of virus strains.

For diagnosis relying on nucleic acid hybridization, information on the structure of the genome and genes of plant pathogens is essential. Accumulated information on the genome structure of viruses and viroids is of great use. Gemini viruses, which are mostly causal agents of tropical plant diseases, have been extensively studied by Ikegami's group and the full-length nucleotide sequence of some viruses has been elucidated (Ikegami et al. 1989; Morinaga et al. 1990). The molecular structure of pathogens of rice such as rice dwarf virus (Nakashima et al. 1990), rice stripe virus (Kakutani et al. 1990), and rice tungro bacilliform virus have been analyzed to serve as a basis for the development of DNA probe techniques. Information on the viroid genomes made available by the Hokkaido University group (Sano et al. 1988) should also be useful to index tropical pathogens.

Tungro disease of rice caused by the synergy of two types of viruses, rice tungro spherical virus and rice tungro bacilliform virus (Omura et al. 1983), poses a serious threat to rice culture in the Asian tropics. Since two types of pathogens are involved, they have to be indexed separately in breeding schemes for the development of resistance or in the analysis of resistance genes in rice. Use of ELISA in breeding programs has been attempted in a collaborative research project carried out in Malaysia by the Malaysian Agricultural Research and Development Institute (MARDI) and the Tropical Agricultural Research Center (TARC). A similar project directed at a broader rice growing area has been started at the International Rice Research Institute (IRRI) in close collaboration with Japanese breeding and pathological research teams.

Sweet potato, which is an important carbohydrate source in Japan as well as in many other Asian countries, harbors several viruses that have not yet been characterized. TARC is engaged in a collaborative study with the International Potato Center (CIP) on the characterization of viruses newly found in Central and South America in order to enable modern diagnostic measures.

Some destructive outbreaks of diseases caused by mycoplasma-like organisms (MLOs) have been recognized in Southeast Asia, including sugarcane white leaf in northeast Thailand and sesame phyllody in Myanmar. To address these problems, a DNA-probe technique for indexing MLOs is being developed by a TARC/NARC (National Agricultural Research Council) research group. A probe that effectively indexes rice yellow dwarf disease was developed (Nakashima et al. in press).

Identification of Plant Genome and Genes

For the efficient management of highly heterogeneous germplasm of vegetatively propagated crop plants, a phylogenetic approach to the relatedness among cultivars and accessions of wild relatives using Restriction Fragment Length Polymorphism (RFLP) and other biotechnological means can be adopted. In the selection of breeding lines in cross-breeding of any crops for characteristics related to gene-gene or gene-environment interaction, it is also desirable to be able to identify useful genes biochemically. A project to characterize rice genomes by RFLP is underway in a network with the National Institute of Agrobiological Resources (NIAR) and other national and private institutes (Yano et al. 1990; Kawase et al. 1990). A set of probes to analyze linkages of genes is now available. Identification of rice genomes in in situ hybridization has also been attempted (Iijima et al. in press).

Characterization of genes for specific functions is also under investigation in soybean, rice, sweet potato, and other crops. Glycinin, a group of seed storage proteins of soybean, has been subjected to extensive studies (Hirano and Watanabe 1990). Lipoxygenase, a key enzyme for generation of n-hexanal (Matoba et al. 1985) and peroxidation of lipids in soybean in relation to grain quality and storability, has been studied using a biotechnological approach combined with induced mutation (Kitamura 1990). A mutant lacking all three lipoxygenase isozymes in the grains was induced (Hajika et al. 1990). The genes for the isozymes have been isolated and are now being characterized.

In rice, a biotechnological approach to the gene structure has made remarkable progress. The achievements include the analysis of full length chlo-

roplast genome (Hiratsuka et al. 1989) and of genes for glutelin seed storage proteins, including the upstream region regulating the expression (Takaiwa and Oono 1990). Recently, a mitochondrial gene controlling male sterility has been identified through DNA analysis (Kodowaki et al. 1990).

Genetic studies on sweet potato using a biotechnological approach are able to unravel the gene structure. Formerly gene analysis in this crop plant was considered to be difficult due to the complex cross incompatibility system as well as the presence of hexaploidy. Cloning of the genes in the prokaryotic system and analysis of their structure were able to circumvent the barrier. Some of the genes for incompatibility (Kowyama et al. 1989, 1990), as well as some for root storage proteins (Hattori et al. 1989), have been isolated and partially characterized.

Propagation

Plant breeding, dissemination of recommended varieties, and seed/seedling propagation are closely linked activities. National, prefectural, and private institutes are involved in these processes in various ways depending upon the kinds of crop plants. With increased demand for high technology for the identification of cultivars for registration under the Seed and Seedling Law and for the testing of pathogen-free seed/seedling stocks, the National Center for Seed and Seedlings, which has been reorganized, is promoting technical services in using biotechnological procedures especially in vegetable and ornamental crops.

Research to develop mass-propagation techniques using tissue culture is actively conducted in the private sector as well as in public institutes. The shoot tip cloning technique developed by Tanaka (1983) in *Haplopappus gracilis* is now being applied to a wide range of crop plants. Additionally, techniques of somatic embryogenesis have made steady progress (Galiba and Yamoda 1988; Komatsuda and Ohyama 1988). In vitro propagation of pineapple for minimizing somaclonal variation is a research objective pursued at the Okinawa Branch, TARC.

Transformation in Plants

Various methods are currently available for the introduction of exotic genes in plant cells. Transformation using the *Agrobacterium* Ti-plasmid system is widely used in dicotyledonous plants. Electroporation and other methods using protoplasts have been developed in rice, since, as in the case of other monocotyledonous plants, the Ti-plasmid system can hardly be applied.

Isolation and characterization of the genes to be introduced are crucial steps in the transformation. The utilization of virus coat protein genes for the protection of tomato from infection with tomato mosaic virus is an example of this type of work (Motoyoshi 1990). Analysis of the gene structures is being carried out also for some other viruses in order to use the information on the nucleotide sequence of these viruses and their satellite components for the cross-protection of crop plants. Successful attempts in cross-protection through inoculation with attenuated strains of cucumber green mottle mosaic virus as well as tomato mosaic virus (Motoyoshi 1988) suggest that this approach is effective.

An herbicide resistance gene of bacterial origin was introduced into rice callus cells by electroporation by Toki et al. (1990). Induced expression of a foreign gene in transgenic plants was demonstrated in lettuce (Enomoto et al. 1990) in which the promoter region of the pathogenesis-related protein gene (Ohshima et al. 1990) of tobacco was introduced.

Protoplast Fusion

Somatic hybridization through protoplast fusion is being currently attempted to produce market-quality carnation and other ornamental plants (Nakano and Mii 1990). On the other hand, some new strategies are under investigation. In *Citrus*, the selection advantage of amphidiploids on hormone-free media for regeneration has been utilized for the development of interspecific somatic hybrids between navel orange (*C. sinensis*) and grapefruit (*C. paradisi*) (Ohgawara et al. 1989). Asymmetric cell fusion, where a normal cell line and another with nuclear genes inactivated are mated to induce the unilateral transfer of extrachromosomal genes, has been attempted in carrot for male sterility (Tanno-Suenaga et al. 1988) and other crop plants.

Manipulation of Reproductive Processes and Ploidy

Following the pioneering research in the 1970s on anther culture in tobacco and rice, the use of doubled haploids in breeding has been attempted in various crop plants. Recently, an interesting strategy for the

production of haploid plants has been devised in wheat and barley (Inagaki and Tahir 1990). In the course of collaborative studies between the International Center for Agricultural Research in the Dry Areas (ICARDA) and TARC, it was discovered that *Triticum aestivum* and *Hordeum vulgare* set seeds at a significant rate after pollination with corn pollen grains and treatment with 2-4-D. Plants grown by embryo rescue were found to be haploids. Rates of haploid plants obtained were 9.5% for wheat and 7.8% for barley.

As another example of recent progress in the manipulation of reproductive processes, a mutation for thermosensitive male sterility was induced in rice (Kato et al. 1990). Chromosomal male sterility expressed only at a higher temperature offers advantages over cytoplasmic male sterility in that the male sterile line can be maintained and it can be grown for grain production without an elaborate restorer gene system and the male-sterile gene can be easily introduced in desired genotypes. Having elucidated the behavior of the mutant gene in *japonica* rice, the NARC research group plans to collaborate with IRRI to explore the usefulness of the gene in tropical *indica* rice.

Biochemical Markers for Selection

In vitro selection of cultured cells or tissues forms another useful area of biotechnology in crop improvement. In fact, selection of callus cells for tolerance to pathotoxin and other in vitro stresses, which has been attempted since the early days of tissue culture in the mid-1960s, is being actively pursued. A few substantial achievements have been reported. Many such attempts, however, ended up with inconclusive results and were not reported since even in "successful" cases, some of the products were rather unstable. Difficulties in such an approach may be attributable to the following causes.

- Dedifferentiated plant cells cultured in vitro do not express normal phenotypes except for a few special traits such as immunity to viruses (different from hypersensitive types of resistance) and production of some secondary metabolites including anthocyanins.
- "Pathotoxins" used in in vitro selection are often crude preparations containing various secondary metabolites of microbes with non-specific toxicity to plant cells.
- Epigenetic variations often encountered in the cells cultured under stresses disturb the selection.
- Among the difficulties mentioned above, the problem relating to impure toxin preparations should be avoided by using pathotoxins purified and characterized biochemically and phytopathologically. The concept of host-specific toxins proposed by Nishimura (1983) and further expanded by Kohmoto and co-workers (Otani et al. 1990; Kohmoto and Otani, in press) should be referred to in such an attempt.

Perspective for Future Development

Biotechnology to benefit human welfare is one of the areas of modern science that needs to be strengthened by further investment of resources, and international cooperation should play a crucial role. Collaboration among institutions in developing and developed countries, as well as international agriculture research centers, should be strengthened to promote the progress of technologies for worldwide use. Concerted efforts may be focused, at least in the near future, on the following targets for the development of tropical agriculture.

- Application of available biotechnological tools in solving urgent problems should be sought. Diagnostic use of biotechnology in germplasm management, and breeding for disease resistance will be the prime target.
- Analysis of genetic and biochemical mechanisms underlying biological processes using biotechnological means should be promoted. Factors determining grain quality as well as tolerance to various stresses will be elucidated in terms of the expression of the genes concerned. Achievements will be utilized in the screening and selection of genotypes with desirable traits in cross-breeding.

Breeding of new plants through manipulation of genes may be attempted along with the analysis of the function of the genes concerned. It should be noted that meaningful achievements in breeding through such innovative approaches can be attained only based on sound analytical studies. International and interdisciplinary cooperation will be of crucial importance in this statement.

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Plant Biotechnology in Malaysia: Current Status and Future Prospects

Hassan Mat Daud[1]

Abstract

The main constraints to increasing crop production in Malaysia include low yields, disease and pest problems, seasonality of fruiting, long juvenility period, the perishable nature of fruits, and adaptation of crops to marginal lands. During the past few years, plant biotechnology programs were initiated in four major public research institutions, four major universities, and research agencies in the private sector, aimed toward solving some of these problems. Current research includes generation and application of Restriction Fragment Length Polymorphism (RFLP) clones and other molecular markers in plant breeding and selection, cloning and manipulation of genes conferring resistance to pests and diseases, and transformation of plants with cloned genes controlling traits of interest in regeneration of transformed plant materials. However, the number of research projects and their progress was limited by the number of existing personnel, support staff, and facilities. Thus, only priority problems such as viral diseases of crops, molecular markers for cloning, and breeding and selecting superior rubber, oil palm, and local fruits were given attention.

Intensive efforts under the 6th Malaysian Plan (1991-1995) are channeled to upgrade existing laboratory facilities, train and recruit more personnel and support staff, widen the research scope, and increase international, as well as national, involvement and cooperation among institutions in order to achieve rapid progress of the projects.

Introduction

Crop production in Malaysia is an important component of the nation's economy. The country is one of the world leaders if not the leading producer of rubber, palm oil, and cocoa. Beside these three crops, rice, fruits, coconuts, vegetables, and field crops are grown to meet internal requirements and as minor export commodities. The crop production sector is facing a number of constraints such as a labor-intensive requirement for the growing and harvesting of crops, disease and pest infestation, the seasonality of fruiting of some local fruits and the perishable nature of fruits, and the lack of high-yielding clones or varieties that suit diverse cropping environments.

The discovery and development elsewhere of recombinant DNA technology that provides man with a powerful tool to change the way whole organisms function has prompted Malaysia to embark heavily in developing biotechnology research and development (R&D) to improve agricultural productivity. The foundation for R&D was laid in the disciplines that were emphasized in the late 1980s. It is anticipated that continuing rapid expansion and development will take place in the 1990s, since even greater emphasis and support for biotechnology were given by the government—primarily for plant biotechnology—under the 6th Malaysian Plan (1991-1995). This paper reports the current status of research and achievements in plant biotechnology and briefly identifies some potential contributions that may be anticipated in the next few years.

Institutions and Organizations

There are four major public research institutes that deal with plant biotechnology R&D. Two of the insti-

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tutes, the Rubber Research Institute of Malaysia (RRIM) and the Palm Oil Research Institute of Malaysia (PORIM), undertake research on rubber and oil palm (and their products). The other two institutes are the Malaysian Agricultural Research and Development Institute (MARDI), which deals with rice, cocoa, vegetables, field crops, and ornamentals, and the Forest Research Institute of Malaysia (FRIM), which specializes in forest tree research. Administratively, RRIM, PORIM, and FRIM are placed under the Ministry of Primary Industries. MARDI, on the other hand, is under the Ministry of Agriculture. In addition to the major research institutes, a number of plant biotechnology research programs are carried out in four universities under the Ministry of Education. These are the University of Malaya, the National University of Malaysia, the Science University of Malaysia, and the Agriculture University of Malaysia. In addition to support received from the respective ministries that govern the institute or university, the Ministry of Science, Technology, and Environment also provides support and funding for biotechnology R&D. The National Biotechnology Committee helps to coordinate projects that receive funding through the Ministry.

Malaysian R&D in Plant Biotechnology

Each institute and university department currently has a strong plant biotechnology program designed toward increasing agricultural productivity in the country. The Biotechnology Centre at MARDI was established on 1 March 1990 with the primary objective of conducting R&D in selected areas of plant, animal, and environmental biotechnology. Since this paper deals with plant biotechnology, only research in the area of molecular and cellular biology and in vitro technology are reported.

Tissue culture research was initiated long before the Biotechnology Centre was created. Numerous crops such as banana, cassava, abaca, orchid, pineapple, muskmelon, strawberry, potato, sweet potato, African violet, and fragrant-flowered species have been successfully propagated through tissue culture. A significant breakthrough in tissue culture research was reported when a method for in vitro propagation of cocoa was proved successful (Mohd. Tamin 1990).

In the area of molecular and cellular biology, projects on rice biotechnology were undertaken with additional support from the Rockefeller Foundation. The projects have two main thrusts: the tagging of the Tungro virus-resistant gene with the RFLP probes, and plant regeneration from *indica* protoplasts. In the first project, F_2 plants of the crosses involving Tungro-resistant varieties MR 81, Katarithoug, Basmati, and Puteh Perak with the susceptible variety TN1 are screened for resistance. The resistant plants are then probed with several RFLPs obtained from S.D. Tanksley. Initial results showed that some probes may be useful in tagging the Tungro virus-resistant gene in rice. Further evaluation on the F_2 and F_3 populations are currently in progress to ascertain the usefulness of the probes. Fingerprints for locally released varieties and advanced breeding lines of rice have been developed using 2 RFLPs. The varieties involved were Sekembang, Setanjung, Makmur, Sri Malaysia I, Bahagia, Kadaria, MR 81, Mahsuri, MR 84, MR 52, Pulut Siding, Pulut Malaysia I, Manik, and Murni.

In the second project, protocols of Hodges (1989) and Cocking and Finch (1989) were used on some local varieties of rice. Callus was recovered from protoplasts of these plant materials, but no viable plant has yet been obtained. Experiments to optimize the conditions for efficient regeneration of plants from local *indica* protoplasts are in progress.

In another project, routine applications of *Agrobacterium* transformation (using reporter genes) have been developed in tobacco. Other work that has been initiated recently includes RFLPs of durian (Abu Bakar and Hamat 1990) and mangosteen and physical mapping of molecular markers such as RFLPs, species-specific, and rDNA probes. MARDI is also hosting the National Gene Bank Centre whose aim is collecting, screening, preserving, and distributing pure cultures of biological materials and genes.

The Biotechnology, Biochemistry, Molecular, and Cell Biology Division of RRIM, on the other hand, undertakes a number of tissue culture and molecular biology studies for applications into the rubber industry. Tissue culture techniques are well established as a means of regenerating complete plants from single cells or tissues. In rubber, in vitro propagation techniques involving anther culture have successfully been used to develop a whole plant. The parent material was selected from high yielding trees having other important characteristics such as disease and wind resistance. Other areas of in vitro techniques that are being studied include cell and protoplast culture, embryo rescue, and cryopreservation.

In the field of molecular biology, researchers at RRIM are actively developing RFLP clones to be used in genetic variation studies, in developing fingerprints for clones, and as molecular markers in breeding and selection of rubber. RFLP probes were

developed from genomic, cDNA, and ctDNA libraries. Using these probes, it was demonstrated for the first time by RFLP techniques that genetic diversity exists in the 1981 International Rubber Research and Development Board (IRRDB) *Hevea* germplasm collection from Brazil (Low and Chow 1990). Work is in progress to expand the collection of RFLP clones screened from the three libraries. In another experiment, the large subunit of D-ribulose-1-5-bisphosphate carboxylase gene, *rbcL*, of rubber has been cloned and partially sequenced (Cheong and Koh 1990). The data revealed homologies in the corresponding sequence of tobacco (92%), rice (84%), and liverwort (84%). Work is in progress to obtain the full sequence data of the cloned gene.

At PORIM, the Division of Plant Science and Biotechnology Section has developed methods for the clonal propagation of oil palm. However, a problem of flower and fruit abnormality of the tissue-cultured plants has been encountered. Current research is focused on understanding and overcoming this problem. The approaches taken thus far involve determining possible causes such as somaclonal variation, mutation, and over-production of certain growth substances or hormones, and the possibility of detecting the phenomenon at the seedling stage by isozyme profiles, quantification of cytokinin, and the use of RFLP clones. Probes for RFLP analysis in two species of oil palm (*Elaeis guineensis* and *E. oliefera*) have been generated (Cheah et al. 1990). Seven clones have detected polymorphism within the *E. guineensis*. With the existing data, it can be concluded that DNA polymorphisms in oil palm may not be as extensive as that in other plants such as maize. Work has been initiated to screen the single- or low-copy number DNA sequence of the genomic DNA for RFLP probes.

The four local universities are also active in plant biotechnology research. Highlights of their research include clonal propagation of various crop plants through tissue culture, RFLP technology, gene cloning, and nucleotide sequencing.

In general, in vitro research in Malaysia has been well established for a wide range of crops. This achievement has been facilitated and stimulated by the growth of commercial farms and plantations that require mass propagation of superior clones of planting materials. This has encouraged the accompanying growth of industries that produce these kinds of materials through in vitro techniques. Despite plant biotechnology being a relatively new area of research in Malaysia, intense research activities have taken place in all research institutions. The activities include RFLP technology, gene tagging and cloning, sequencing, transformation, mapping of molecular markers, and library construction. The nature of the research projects undertaken reflects a long-term commitment of the institutions to plant biotechnology as a major contributor toward increasing agricultural productivity in Malaysia.

Research Facilities and Personnel

Almost all laboratories in the research institutions and universities mentioned above are equipped with facilities and instruments that enable basic and some specialized molecular biology techniques to be carried out. Researchers are also sensitive to the development of new and better instruments such as pulse field gel electrophoresis, protein sequencer, and polymerase chain reaction, as well as the new protocols and techniques in the field of molecular biology and DNA recombinant processes.

In terms of research personnel, almost all laboratories lack a sufficient number of researchers and support staff to rapidly carry out existing projects. A survey done by a consultant showed that personnel should be doubled. It is envisaged that with greater support and emphasis given by the government under the 6th Malaysian Plan (1991-1995) a greater number of personnel will be trained and hired.

More interaction with researchers outside the country is also needed in order to facilitate rapid growth in plant biotechnology in Malaysia. This can be achieved through exchange of information and materials such as probes, cloned genes, or even transformed materials between local and international researchers. This is particularly important to the Malaysian researchers since, except for rice, not many are working on the same plant species.

Conclusions

Current developments in plant biotechnology in Malaysia are encouraging. The various applications of molecular biology and in vitro technology described above reflect the thrust and confidence in using such tools to improve agricultural productivity. With greater emphasis expected in the next five years, the future of plant biotechnology in Malaysia is even brighter.

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Biotechnology Research in Myanmar Agriculture

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Abstract

In Myanmar, 60 crop species are being cultivated for food for people, feed for animals, and raw materials for factories. Among these important crops, rice occupies 50% of the total cultivated area. Sesame, groundnut, and sunflower are the major oil crops. Strategic plans for increasing crop production are crop intensification and use of improved varieties and technologies.

Biotechnology research in Myanmar concentrates on crop improvement and screening for stress tolerance, using anther culture for rice breeding, and tissue culture for other crops (groundnut, potato, broccoli).

In vitro screening for salt tolerance of rice varieties and tissue culture of groundnut and sesame for selection for drought tolerance is carried out at the Agricultural Research Institute.

Future research priorities will be: anther culture of important crop species (rice, maize, wheat); meristem culture; rapid multiplication of selected fruit trees, ornamental and medicinal plants; mutant selection for stress tolerance, pest and disease resistance in in vitro culture; embryo culture (intra- and interspecific hybridization); and protoplast fusion.

Introduction

In Myanmar 60 crop species are being cultivated for food for people, feed for animals, and raw materials for factories. Of the 60 crop species, 22 are considered more important for local consumption and export. Among these important crops, rice occupies 50% of the total cultivated area. Sesame, groundnut, and sunflower are major oil crops for producing edible oil, although production is not yet sufficient for local consumption. The area under these crops will possibly be increased from 20-30% of the total cultivated area in order to meet the requirements of increasing population. Food legume crops are also important for local consumption and for export.

In Myanmar, we are using three strategic plans for increased crop production. They are: area expansion, increasing yield per unit area, and crop intensification. There is a potential area of approximately 10 million ha, which includes fallow lands and virgin soils in different regions, and the Ministry of Agriculture and Forests is now stressing the exploitation of these areas. In order to increase yield per unit area, the Myanmar Agriculture Service has been implementing high yielding programs for rice in 82 townships, wheat in 13 townships, maize in 9 townships, oil crops in 22 townships, fiber crops in 12 townships, and food legumes in 15 townships. Use of improved varieties and technologies and high inputs is the significant feature for increasing productivity. Consequently, yield per unit area of each crop has approximately doubled. For example, rice yield has increased from 1.5 t ha^{-1} to 3 t ha^{-1} and, similarly, maize yield has increased from 0.7-1.0 t ha^{-1} to 1.73 t ha^{-1}. There are also considerable yield increases for the other crops involved in high-yielding programs. Crop intensification is being promoted through rice-based or non-rice-based double- and triple-cropping systems, in which intercropping and relay cropping are included. Apart from these approaches, adoption of biotechnology in crop improvement, improved fertilizers, and plant protection are new strategies for crop production at low cost.

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Current Biotechnology Research in Myanmar

Crop Improvement

Anther culture is being used in rice breeding, and tissue culture for other crops (groundnut, potato, broccoli). Conventional methods of rice improvement require at least 6 to 10 generations before varietal evaluation is conducted over a 2 to 3-year period. In the Plant Physiology Division of the Agricultural Research Institute (ARI), 20 F_1 crosses of rice have been tested for callus induction and plant regeneration efficiency. The range of callus induction frequency was 2 to 29%, with an average of 4%, and green plant regeneration was 3 to 17% with an average of 10%. Anther culture derivatives from these crosses were planted in the field for further selection and, in the second generation, promising lines with required characters were observed.

Attempts at anther culture of maize to generate homozygous inbreds has not yet been successful, except for callus formation.

Screening for Stress Tolerance

In a program of in vitro screening for salt tolerance of rice varieties, plantlets of three varieties, Shwewahtun, Shwethwehtun, and Kyawzeya, were regenerated from salt-tolerant calli and the seeds of these plants were sown for evaluation. Further testing for tolerance will be extensively carried out in salinity-prone areas.

In Myanmar, there is a drought-prone area of approximately 3 million ha, where groundnut, sesame, cereals, and food legumes are largely grown for local consumption and export. Of these dryland crops, groundnut and sesame are of vital importance; these crops always suffer from drought, which results in low yields, or even total crop failure for sesame. The Plant Physiology Division of ARI initiated tissue culture of groundnut and sesame for selection for drought tolerance.

Tissue Culture and Micropropagation

Five varieties each of groundnut and sesame are being studied for callusing ability of four seedling explants on four different media. Differentiation of plants from callus is under study.

Another area is maintenance of hybrid vigor in broccoli, which degenerates if the plants are raised from seeds of the first generation. So lateral buds of broccoli were cultured on Murashige and Skoog medium, and the plantlets derived were maintained in test tubes; these grew vigorously. In the 1991 cold season the plants will be transplanted into the field.

Extensive production of orchids is undertaken by the Extension Division of Myanmar Agriculture Service (MAS).

Conclusions

It is realized that the application of biotechnology is important in crop improvement (especially to generate new varieties within a shorter period), as well as in fertilization and crop protection.

Research programs of plant cell and tissue culture and control of pests and diseases will be strengthened in the following areas.

- Anther culture of important crop species (rice, maize, wheat)
- Meristem culture
- Rapid multiplication of selected fruit trees, ornamental, and medicinal plants
- Mutant selection in in vitro culture (selection for stress tolerance, pest and disease resistance)
- Embryo culture (intra- and interspecific hybridization)
- Protoplast fusion

Current Status and Future Prospects of Biotechnology Research in Pakistan

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Abstract

Drought, excess salinity, acidity, and diseases are stresses that limit crop production on more than 50% of arable lands. The irrigated lands are subjected to the hazards of salinity and waterlogging resulting in reduced production potential. A considerable portion of the cultivated lands are rainfed with limited productivity.

Conventional plant breeding is being employed to develop varieties resistant to stress but progress has been slow. The transfer of salt tolerance from alien tolerant species is being attempted in wheat and rice. Gene transfer is restricted in interspecific and intergeneric crosses and cannot be utilized through standard breeding procedures.

There are areas where immediate impact is expected through the use of fast-developing biotechnological approaches such as: micropropagation of particular plant genotypes for multiplication and disease elimination; in vitro screening and subsequent plant regeneration to produce variants with increased stress tolerance; induction of new genetic variability; elimination of viruses from plants; preservation of germplasm; production of haploids, and overcoming the reproductive barriers in noncompatible species and genera for the transfer of alien genetic material.

This chapter outlines the institutions in Pakistan that are tackling these problems, the priorities that have been set, and recommendations for future research.

Introduction

Drought, excess salinity, acidity, and diseases are stresses that limit crop production on more than 50% of arable lands. However, irrigated lands are subject to the hazards of salinity and waterlogging resulting in reduced production potential. In Pakistan, about 40 000 ha y^{-1} are lost due to salinization (Jones and Gorham 1986). A survey showed that the salt-affected area was about 8.9 million ha in the year 1989 (Table 1)

A considerable portion of the cultivated lands are rainfed with limited productivity. In Pakistan, more than five million ha lie in arid and semi-arid regions without irrigation facilities. This constitutes about 25% of the total cultivated area in the country (Table 2).

Of irrigated areas, marginal lands make up a considerable proportion. These are characterized as having a low nutrient profile, low-lying patches with poor drainage, and heavy clay or sandy soils. This causes low crop productivity. Crop productivity can be increased in stressed environments by developing plant types that do well under specific stress conditions.

Conventional plant breeding is being employed to develop varieties resistant to stress, but progress has been slow (Akbar 1986; Srivastava and Jana 1984). The transfer of salt tolerance from alien tolerant species is being attempted in wheat and rice (Dvorak and Ross 1986; Akbar 1986). There is great need to ex-

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Table 1. Area of waterlogged and saline soils (millions of ha) in Pakistan during 1975.

Province	Waterlogged area			Salt affected area			
	0-150 cm	150-300 cm	Total	Moderate saline[1]	Severe saline[2]	Saline alkali	Total
Punjab	1.18	2.64	3.84	0.41	1.21	2.10	3.72
Sind	1.26	1.87	3.13	3.23	1.87	-	5.10
NWFP	-	-	0.04	-	-	-	0.04
Baluchistan	-	-	0.04	-	-	-	0.04
Total	2.44	4.51	7.01	3.64	3.08	2.10	8.90
% area affected	17	32	50	26	22	15	64

1. Moderately saline: EC < 15 mmohs
2. Severely saline: EC > 15 mmohs

Source: Agricultural Statistics of Pakistan, 1988-89.

Table 2. Irrigated and rainfed areas (millions of ha) in provinces of Pakistan during 1988-89.

	Mode of Irrigation					Total cultivated area	Percent share of total area
	Canal	Tube-well	Others	Total	Rainfed		
Punjab	7.87	3.68	0.27	11.82	2.50	14.32	17.46
Sindh	2.31	0.12	0.06	2.49	1.52	4.01	37.91
NWFP	0.70	0.05	0.09	0.84	1.11	1.95	56.92
Baluchistan	0.35	0.12	0.06	0.53	0.09	0.62	9.62
Total	11.23	3.97	0.48	15.68	5.22	20.90	24.98

Source: Agricultural Statistics of Pakistan, 1988-89.

ploit all genetic variability that can be used in breeding for stressful environments. Gene transfer is restricted in interspecific and intergeneric crosses and cannot be utilized through standard breeding procedures. Use of nonconventional techniques would be instrumental in the exploitation of gene sources and the speeding up and supplementing of the application of conventional plant breeding methods.

Fast-developing biotechnological techniques and their impact on the biochemical industry are sufficient evidence for their application and potential to bring about breakthroughs in agriculture. There are areas in which immediate impact is expected through the use of these technologies, such as micropropagation of particular plant genotypes for multiplication and disease elimination, i.e., to shorten the time required to produce improved cultivars; in vitro screening and subsequent plant regeneration to produce variants with increased stress tolerance; induction of new genetic variability; elimination of viruses from plants; preservation of germplasm; production of haploids; and overcoming the reproductive barriers between noncompatible species and genera for the transfer of alien genetic material.

Biotechnology Resources and Facilities

In Pakistan, the important implications of plant biotechnology were realized in the mid-1970s. Projects and proposals were prepared and forwarded to different government agencies for setting up the tissue culture and biotechnology laboratories. Pioneering work was started in 1976 in the Department of Botany, University of Peshawar. Thereafter, many tissue culture laboratories were established in the early 1980s.

At present, a total of 14 laboratories are engaged in using different biotechnological approaches for crop improvement. More than 82 scientists are actively engaged in research work. Of these scientists, 22 have Ph.D. degrees, 3 hold M.Phil. degrees and 45 have M.Sc. degrees (Table 3). A summary describing the location of laboratories and their area of interest, is given below.

Botany Department, Peshawar University, Peshawar

This is one of the oldest tissue culture laboratories in Pakistan. It was established in 1976 and has worked on a number of crops, the main crops being poppy, ginger, saffron, cardamom, potato, eucalyptus, groundnut, and *Juniperus* spp. Most of the efforts were concentrated on callus induction and plant regeneration. A scheme has now been approved for a period of 4 years in which targeted end users are the pharmaceutical industry, foresters, and the department of agriculture.

Pakistan Atomic Energy Commission (PAEC)

Three tissue culture laboratories are working in PAEC. One is located at the Nuclear Institute for Ag-

Table 3. List of biotechnology laboratories with personnel classified according to their academic qualification.

Institute	Ph.D.	M.Phil.	M.Sc.	B.Sc.	Others	Total
Botany Dept, Peshawar University, Peshawar	1	1	1	-	-	3
Pakistan Atomic Energy Commission[1]	5	1	9	-	2	17
Pakistan Council of Science and Industrial Research, Lahore	2	-	4	2	-	8
Ayub Agricultural Research Institute, Faisalabad	1	-	7	-	1	9
University of Agriculture, Faisalabad	3	-	8	-	-	11
Centre for Advanced Molecular Biology, Lahore	2	-	9	-	-	11
Botany Department, Punjab University, Lahore	3	-	-	-	4	7
National Nematological Research Centre, Karachi	1	-	2	-	-	3
Tissue Culture Laboratory, Rawalpindi	-	-	2	-	-	2
Central Cotton Research Institute, Multan	-	-	2	-	-	2
Agricultural Department, NWFPAU, Peshawar	1	-	-	-	1	2
Pakistan Forest Institute, Peshawar	-	-	-	-	1	1
Gomal University, D.I. Khan	-	-	-	-	1	1
National Agricultural Research Centre, Islamabad	3	1	1	-	-	5
Total	22	3	45	2	10	82

1. These include NWAB, NIFA, and AERL figures.

riculture and Biology (NIAB), Faisalabad; the second at the Atomic Energy Agricultural Research Centre (AERC), Tandojam; and the third at the Nuclear Institute for Food and Agriculture (NIFA), Peshawar. Specifics on each are given in detail.

Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. This laboratory was established in 1979 with tissue culture studies on mango. Later, *Citrus*, kallar grass, and chickpea were added. The research thrusts are more toward applied aspects with long-term objectives to solve problems in the areas of plant breeding, physiology, biochemistry, and pathology. Presently, a project is underway for isolation of protoplasts in Basmati-370 rice and a salt-tolerant kallar grass, to transfer salt tolerance by protoplast fusion.

Nuclear Institute for Food and Agriculture (NIFA), Peshawar. The tissue culture laboratory was started at NIFA in 1985-86. The laboratory is fully established with all the basic equipment and facilities, and is now planning to initiate a collaborative research scheme with the International Atomic Energy Agency (IAEA). Presently, the scientists are engaged in the production of haploids in *Brassica* for further utilization in breeding programs, elimination of virus from potato and soybean seed, induction of virus resistance in potato, establishment of potato protoplast culture, and the study of somaclonal variation generated through chemical mutation.

Atomic Energy Agricultural Research Centre, (AERC), Tandojam. There are two tissue culture laboratories at AERC. One is located in the Plant Genetics Division, which was established in 1976 with sugarcane as the major crop. The second, located in the Plant Physiology Division, was established in 1982 and focuses on in vitro and in vivo studies on the mode of salt tolerance/salt sensitivity in rice and wheat.

Pakistan Council of Scientific and Industrial Research (PCSIR)

The PCSIR is the oldest research organization in Pakistan dealing with research that directly relates to industry. Due to the growing importance of tissue culture and related biotechnologies for agro- and bio-based industry, the PCSIR established two tissue culture laboratories, one at PCSIR Laboratories, Lahore, and other at PCSIR Laboratories, Peshawar. In both laboratories emphasis is given to medicinal plants, particularly for the isolation of their metabolites. Work is also being done on the regeneration of superior quality pistachio and screening for disease resistance in *Brassica*.

Ayub Agricultural Research Institute (AARI), Faisalabad

There are two tissue culture laboratories at AARI, Faisalabad. One is located in the Virology Section and the second is in the Directorate of Horticulture. Recently, the Agricultural Biotechnology Research Institute has also been established at AARI, Faisalabad. Research is mostly focused on the eradication of virus through meristem tip culture in potato. Some work has also been initiated on cereals, including wheat, barley, and maize, and some horticultural crops.

University of Agriculture, Faisalabad

This is one of the biggest agricultural universities in Pakistan. Courses regarding tissue culture and biotechnology are taught in the Departments of Botany, Crop Physiology, and Horticulture. Tissue culture laboratories exist in the Departments of Botany and Horticulture. Besides the courses offered in tissue culture, research is being carried out on some cereals and horticultural crops, i.e., guava, kinnow, strawberry, fig, apricot, pear, plum, apple, carnation, chrysanthemum, roses, and bougainvillea. Work is mostly concentrated on developing virus-free plants, cloning plants true to type, and also creating genetic variability for different traits.

Centre for Advanced Molecular Biology (CAMB), Lahore

The CAMB is located temporarily within the premises of the Department of Botany, University of Punjab, Lahore. Preliminary work is in progress on cloning vectors among naturally occurring plasmids in Phyto-oncogenic bacteria using chickpea as a host. The work will be extended to some other dicotyledons and monocotyledons for cloning superior genotypes, for stresses such as salt and drought, and also for disease resistance.

Botany Department, Punjab University, Lahore

The tissue culture program was started in October 1987, in the morphogenesis laboratory, Department of Botany, Punjab University, Lahore. Some work is being undertaken on sugarcane and chickpea. The work has academic, as well as applied value, and the end users will be scientists and farmers. Some basic studies are done on salt tolerance in *Brassica* with emphasis on the isolation and characterization of DNA and RNA in salt-stressed calli. Studies on understanding the relationship between cell culture and gene expression at biochemical and molecular levels have also been done.

National Nematological Research Centre (NNRC), University of Karachi

The center is located at the University of Karachi and is engaged in research on nematodes. Tissue culture techniques are being used for aseptic root cultures in tomato and corn. Studies are being undertaken for the induction of nematode resistance in tomato cultivars using in vitro technologies.

Tissue Culture Laboratory for Horticultural Plants, Rawalpindi

This recently established laboratory, sponsored by the government of Punjab, has initiated work on the propagation of apples, date palm, and some ornamental species.

Central Cotton Research Institute (CCRI), Multan

Cotton is a very important cash crop in Pakistan. Recently, work has been initiated on utilization of tissue culture and other biotechnological techniques for the production of better yielding, disease- and stress-tolerant cotton cultivars.

Agricultural Department, Northwest Frontier Province Agricultural University (NWFPAU), Peshawar

The main research work of the Agricultural Department of NWFPAU is located at the Agricultural Research Institute, Tarnab, Peshawar. There are some other monocrop and multicrop research stations working under this department. One, located at Abbotabad and working on potato, has all the capabilities to start tissue culture. Two scientists have been trained in the tissue culture laboratory, NARC, to initiate work on the production of virus-free seed potato.

Pakistan Forest Institute, Peshawar

A tissue culture laboratory was established in this institute in 1984. This laboratory will primarily be working on the propagation of forest trees.

Gomal University, D.I. Khan

The tissue culture laboratory is attached to the Department of Botany. Presently, the emphasis is only on the academic side and later will be extended to some basic research in different crop species.

National Agricultural Research Centre (NARC), and Pakistan Agricultural Research Council (PARC), Islamabad

The credit to push tissue culture forward as a frontline area of research in the agricultural sector goes to PARC, Islamabad. In 1980, the importance of tissue culture was fully recognized and, in 1981, an independent section for research in plant tissue culture was established for production of virus-free seed potato through meristem tip culture. Funding was approved for a period of 3 years from the non-development expenditure. This was approved as a permanent program of the council and subsequently, date palm, rice, wheat, and tomato were incorporated in the mandate. Future thrusts will include several important research areas.

- Undertake long-term basic research in plant tissue culture.
- Micropropagate selected horticultural and vegetable crops.
- Isolate variants in crop plants, particularly cereals.
- Use in vitro selection to develop variants resistant to environmental stresses.
- Train scientists of provincial research institutions and universities in basic tissue culture techniques.

Priorities

Technical and personnel resources for genetic engineering of plants are still painfully limited. Substantial improvements in experimental biotechnological techniques are also needed to understand the plant systems. Modern equipment and instrumentation are necessary to strengthen the existing laboratories to take advantage of experimental opportunities.

Available human resources are very low, but the number of trained personnel in this field is rising fast. Very recently, the Ministry of Science and Technology launched a program for human resources development in selected fields of biotechnology in foreign universities. Proper arrangements should be made to teach molecular biologists, geneticists, tissue culturists, and biotechnologists at graduate and postgraduate levels. These young trained people with multidisciplinary approaches would be able to define problems and devise appropriate strategies to work within local systems and environments.

New institutes of international caliber in biotechnology and genetic engineering should be established and be equipped with sophisticated equipment and para-scientific facilities staffed by scientists of international repute and devotion. Biotechnology is characterized by its internationalism and information intensiveness and first-rate institutes having trained leadership devoted to achieving meaningful results.

Collaboration between national academic institutions and research laboratories, as well as with advanced foreign research institutes, is essential for efficient use of available methodologies. There need to be links between organizations dealing with plant biotechnology both on a national and an international basis. Access to specialized training and graduate education is critical.

Biotechnology is currently used mostly for the isolation of stress-tolerant variants in higher plants. Recent developments made in wheat, rice, potato, *Brassica*, cotton, legumes, and trees have far-reaching implications in international agriculture. It is also envisaged that the next agricultural revolution will primarily be based on in vitro technology, specifically for the production of stress-tolerant crop plants.

Conclusions and Recommendations

Intervention at Academic Institutes

At present, no academic courses are offered either at undergraduate or graduate levels; therefore, courses covering most aspects of biotechnology should be introduced in the universities. Moreover, high priority should be given to strengthening biological sciences in agricultural universities with emphasis on molecular biology and biotechnology as separate disciplines.

Since there are rapid enhancements and changes in the field of biotechnology on the global level, those engaged in teaching should get training and refresher courses in tissue culture and genetic engineering.

Improvement and Coordination at the National and International Levels

It has been realized that there is no coordination among the research scientists working in different disciplines of biotechnology in different laboratories. This creates the possibility of overlapping and duplication in their research activities. Collaboration between national academic institutions and research laboratories, as well as with foreign research institutes, is essential for efficient use of available methodologies. There is a need to develop links between organizations dealing with plant biotechnology both on national and international levels.

It is further suggested that a National Coordination Cell headed by a coordinator be established. This national coordinator would be responsible for building linkages between research laboratories in the country as well as worldwide. In addition, a Biotechnology Tissue Culture Society should be established. It would provide a forum for biotechnology scientists to meet regularly and, in turn, improve coordination.

Collection and Dissemination of Information

The national coordinator should also be responsible for the preparation of an annual review report highlighting the achievements of all the biotechnology laboratories in the country. The National Coordination Cell should be responsible for publishing a biotechnology journal and a newsletter. The coordinator should also be responsible for holding periodic symposia, seminars, and project evaluation meetings.

Training of Scientists

Training at all levels is one of the efficient media in technology development and improvement, as well as in technology transfer. Therefore, emphasis should also be given to short- and long-term training for young, enthusiastic scientists.

Opportunities for postdoctoral fellowships and participation in international symposia should be created for updating scientists in their respective fields of specialization.

Promotion of Research Facilities

The existing working units in the country are suffering due to shortages of funds, equipment, and skilled manpower. Therefore, sufficient funds for the support of existing tissue culture centers should be allocated and modern equipment and instrumentation to strengthen and expand the scope of laboratories should be provided.

Issues for Research

Since there are a lot of stressful environments that prevail on a large scale in the country, there is a definite need to develop cultivars that can do well under each of them. Important examples are salinity, acidity, drought, diseases, and low fertility situations.

Most of the countries in Asia have the common problem of fungal diseases, environmental stresses, and insect pests. Therefore, emphasis should be focused on the potential of biotechnology in controlling these hazards. In this regard the potential of *Bacillus thuringiensis* (Bt) pesticidal genes through transgenic plant technology to achieve insect resistance in major crops has sufficient justification for exploration. The identification of *Brassica* genes for useful traits such as aphid and drought resistance and salt tolerance through the use of restriction fragment length polymorphism (RFLP) is an interesting area for future collaborative research in developing countries.

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Current Status and Future Prospects of Biotechnology in the Philippines

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Abstract

Increased production of improved seeds and planting materials, effective microbial inoculants and compost biofertilizers, and microbial pest- and disease-control agents are needed to strengthen the agricultural production system in the Philippines. Biotechnology tools such as tissue culture and recombinant DNA when used to complement conventional breeding techniques could accelerate the progress. Present research is focused more on the use of tissue culture for rapid propagation of planting materials. More basic studies are needed on selected molecular genetic techniques. An operational pool of personnel and facilities has been established in a few research centers but the other regional laboratories also need to be developed. More financial support is needed. Training on repair and maintenance of equipment and facilities is required. Cooperation with advanced research centers abroad should be strengthened.

Introduction

There is a growing consensus among planners, researchers, and extension workers in the Philippines for the development of an equitable, efficient, and ecologically sustainable agricultural system to achieve competitive production targets and to increase the real income of the small farmer (BAR 1989).

More and more scientists, nevertheless, are becoming aware that Philippine agriculture is beset with gargantuan problems, the foremost of which is social inequity, which cannot be solved through new technology (SAC 1990). However, as social and political scientists and legislators should actively spearhead work on these problem areas, science and technology workers likewise cannot remain complacent and should vigorously uphold advancement of science and technology for extensive countrywide utilization. Science and technology improvement would always be an indispensable component in enhancing agricultural production and income.

A basic strategy in improving agricultural production and income is to strengthen the production system. The availability and efficient use of planting materials and farm inputs should be enhanced. Biotechnology is an indispensable tool in this strategy. Biotechnology in combination with conventional breeding can make full use of its potential to increase the supply of improved planting materials and make available biological farm inputs such as inoculants and compost biofertilizers, and pest- and disease-control agents. The general aim of this paper is to discuss the status and prospects of biotechnology in the Philippines. Details will be presented on: crop production constraints for biotechnology applications, local biotechnology resources, future needs and priorities, existing priority areas and needs, and proposed areas for collaborative research.

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Crop Production Constraints for Biotechnology Applications

The recurring major problems always mentioned by the different agricultural sectors during the Bureau of Agricultural Research (BAR) consultations revolved around several issues.

- Insufficient supply of seeds and planting materials.
- Lack of disease-resistant and stress-tolerant seeds of crop cultivars.
- Unavailability of low-cost but effective farm inputs such as biofertilizers and biological control agents.

These problems are precise target areas for biotechnology.

As adopted in the national research and extension agenda (BAR 1989), the priority crops for research and development are, of course, the cereals, which include rice (*Oryza sativa*) and corn (*Zea mays*). The priority fruit crops identified include banana (*Musa sapientum*), citrus (*Citrus mitis*), mango (*Mangifera indica*), rambutan (*Nephelium lappaceum*), guava (*Psidium guajava*), jackfruit (*Artocarpus hetephyllus*), cashew (*Anacardium occidentale*), grapes (*Vitis vinifera*), and guyabano (*Annona muricata*). Coffee (*Coffea arabica*), cacao (*Theobroma cacao*), coconut (*Cocos nucifera*), and sugarcane (*Saccharum officinarum*) are the priority plantation crops. The priority legumes are mung bean (*Vigna radiata*), groundnut (*Arachis hypogaea*), soybean (*Glycine max*), and cowpea (*Vigna unguiculata*), while the important vegetable crops include tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena*), snapbeans (*Phaseolus vulgaris*), pole sitao (*Vigna sesquipedalis*), squash (*Cucurbita maxima*), cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), pechay (*B. napus* var. *chinensis*), and pepper (*Piper nigrum*). Cassava (*Manihot esculenta*), and potato (*Solanum tuberosum*) are the priority root crops. Most of the on-going biotechnology research studies as well as those being planned are focused on these priority crops.

In rice, some of the major constraints to production are pests and diseases and stresses such as drought and flooding. Insect damage alone can bring losses of 10-30% in total yield. At the International Rice Research Institute (IRRI), wide hybridization as a tool of biotechnology has been utilized to transfer a gene for resistance to grassy stunt virus from *Oryza nivara*, a wild rice from India, to IRRI breeding lines. It is providing good resistance in numerous lines used in Asia and has saved farmers hundreds of millions of dollars in crop loss and pesticide costs. Hybrids with possible resistance to blast and bacterial blight have been produced and are now being tested (Toenniessen 1990).

Pests are also a major problem in corn. Preliminary studies on anther culture in corn are being done at the Institute of Plant Breeding (IPB), UP at Los Banos. Results may later be used to generate resistant cultivars for improved pest management (Villareal et al. 1989).

An increased supply of improved planting materials is needed in fruit crop production. Protocols for rapid propagation using embryo culture, meristem culture, and organ culture have been variously developed for banana, pommelo, calamansi, sweet orange and rambutan (Villareal et al. 1989).

Pests and diseases are also common problems in plantation crops, legumes, and vegetable and root crops. Wide hybridization is being used in mung bean, sugarcane, and other crops to produce resistant cultivars. Mutation by chemicals and irradiation is used to induce variability in bulb crops, legumes, banana, and ornamentals.

Lack of fertilizer inputs is definitely a major constraint. Successful fertilizer supplementation with biofertilizers could mean added income to the farming sector. Studies on microbial strain evaluation and inoculant production, and genetic improvement of strains for improved biological nitrogen fixation, are being conducted at the National Institutes of Biotechnology and Applied Microbiology (BIOTECH). Studies on mass production of compost biofertilizers are also being conducted in the same institute, as well as fermentation studies for mass inoculant production and improvement of product formulations (BIOTECH 1990).

Biotechnology Resources: Facilities and Personnel

The bulk of biotechnological research in the country is being conducted by a number of government and private agencies and one international agency. These include the University of the Philippines at Los Banos (UPLB), the University of the Philippines at Diliman (UPD), the Department of Agriculture (DA), the Visayas State College of Agriculture (VISCA), the International Rice Research Institute (IRRI), and others.

A few of the government laboratories have a minimum amount of equipment, support facilities, and trained personnel to conduct biotechnology research compared to those at UPLB and UPD. A number of

regional laboratories such as those at VISCA, Central Lugon State University (CLSU), and Mariano Marcos State University (MMSU) would need considerable support to build up their facilities.

Many of the private centers have equipment and facilities geared mainly for rapid propagation of planting materials for selected crops (Barba and Patena 1989). They normally hire experienced researchers for particular activities. Some of them conduct contract research with preferred government agencies.

A number of government research units have a pool of senior researchers with training in biotechnology. BIOTECH has eight full-time Ph.D. researchers with training in various biotechnological activities, as well as eight part-time staff with Ph.D.s and training. IPB has a biotechnology core staff of six with Ph.D.s, two with M.S., and one B.S. CLSU has one staff person with a Ph.D. and two with biotechnology training. Other agencies have one to two staff with Ph.D.s on biotechnology-related activities.

IRRI has a strong pool of biotechnology facilities and well-trained staff. There is an on-going formalization of research and training cooperation arrangements between UPLB and IRRI. This should pave the way for more effective sharing of information and resources for targeted goals.

Future Needs and Priorities: Facilities Development and Personnel Training

In a few relatively strong local biotechnology laboratories, additional equipment and facilities are badly needed to conduct more basic studies. Cold storage units, generators, refrigerated centrifuges, air conditioning units, screenhouses and other equipment such as laminar flow hoods are insufficient. One of the basic constraints is that all these items are imported and their repair and maintenance are always a problem. Those laboratories which received good equipment 5 to 10 years ago are now faced with the difficulty of how to repair or replace them.

Almost all required chemicals are imported as well and funding support for supplies and materials is always needed. More government and private assistance is needed to develop local support industries to ease the problems around chemicals and equipment. Possible sourcing of these items through cooperation and sharing with international research agencies may be explored. International donor agencies have to be tapped for possible funding.

Opportunities for personnel training should be enhanced. Training will need to be a continuing activity considering rapid biotechnological advances. Research cooperation with advanced laboratories in other countries should be pursued. There is a great need to train more personnel on biochemical, cellular, and molecular aspects of biotechnology.

A foremost basic need also is to train technicians and engineers on the maintenance, repair, and manufacture, if possible, of the basic equipment used in biotechnology research.

Priority Areas and Needs for Research and Development

The priority areas for research and development are those pertaining to tissue culture, genetic improvement, germplasm conservation, recombinant DNA technology, and fermentation. Some proposed specific studies under these priority areas are presented in Table 1.

An underlying need in pursuing these research areas is to undertake basic studies on development of protocols for the various techniques involved. This process could be accelerated through collaborative research with other advanced laboratories abroad coupled with on-the-job training of personnel. Other techniques relevant to these basic studies could be learned through sharing publications and other forms of communication. Grants from foreign cooperating agencies will be needed to obtain the much-needed equipment, chemicals, and other supplies.

Priority Areas for Collaborative Research

The proposed areas for collaborative research are shown in Table 2. These areas are planned to support the on-going activities.

Conclusions

It is clear among local scientists that biotechnology is an indispensable tool to strengthen the agricultural production system. Biotechnology used to complement conventional breeding techniques could accelerate the production of improved seeds and planting materials and the development of improved microbial strains for use as inoculants, biofertilizers, and pest-

Table 1. Priority areas for research and development.

Priority Area	Nature of Studies
Tissue culture	Applications for rapid multiplication of priority crops such as bamboo, coconut, selected forest tree species, potato, pineapple, and others
Genetic improvement	Development and routine application of protoplast fusion, hybrid embryo rescue, and inter-specific and wide hybridization
	Production of homozygous lines through anther culture
	Use of chemical mutagens and irradiation for somaclonal variation
Genetic stability and maintenance of germplasm	Use of isozymes and RFLP's to establish genetic grouping of lines and populations to monitor genetic segregation and recombination
	Development of in vitro techniques for short- and long-term storage
Recombinant DNA technology	Protoplast fusion
	Gene transfer for selected crops
	Gene transfer in nitrogen-fixing bacteria for strain improvement
	Gene transfer in *Bacillus thuringiensis* for strain improvement
Fermentation	Mass production of inoculants
	Improvement of product formulations

Table 2. Priority areas for collaborative research.

Areas	Studies
Cell culture systems and induced mutagenesis	In vitro cell selection
	Protoplast fusion
	In vitro mutant selection for stress tolerance
Utilization of isozymes, protein and restriction length polymorphism	Application for gene mapping of important qualitative and quantitative traits in rice and corn
	Assessment of genetic stability in in vitro culture
	Fingerprinting
	Hybridity assessment
	Wide hybridization
	Diversity assessment
Recombinant DNA technology	Virus and viroid identification
	Transfer of useful genes to target crops
	Gene transfer for improvement of selected microbial strains

control agents. But this growing realization is apparently not yet shared by national planners and policymakers. Scientists will have to step up lobbying efforts with government officials and foreign donors for increased funding support.

A few government agencies have equipment, facilities and trained personnel to undertake biotechnology research; but they are currently hampered by problems on repair and maintenance and lack of chemicals and supplies. Both increased funding support and continuing training of personnel are needed. Regional laboratories have to be strengthened.

Present plant breeding efforts using biotechnology are focused more on rapid propagation of planting materials. The move to undertake basic studies to develop protocols for biotechnological techniques has to be accelerated. The development of microbial strains as biofertilizers using biotechnology is important.

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Current Status and Future Prospects of Biotechnology in Sri Lanka

P. Ganashan[1]

Abstract

There are 24 well-defined agroecological zones in Sri Lanka, in which a wide range of crops are grown, and which necessitate a range of crop varieties adapted to the different zones. Rice is the staple crop, and emphasis has been given to increasing yield and resistance to biotic and abiotic stresses. Techniques that have been used are clonal propagation and production of pathogen-free plants, and in vitro conservation. Future emphasis needs to be placed on widening the genetic base, breeding for tolerance to environmental stress, for pest and disease resistance and for herbicide resistance. Improved food quality and better storage characteristics are also important.

Introduction

The diverse climatic conditions that prevail in Sri Lanka permit the cultivation of a wide range of crops from the typical tropical and sub-tropical to temperate species. Twenty-four well-defined agroecological regions have been identified; these have necessitated the development of a range of crop varieties for specific agroecological needs. To cater to the needs of these diverse agroecological conditions, agricultural research has been decentralized into nine Regional Agricultural Research Centers (RARCs) that are supported by different Agricultural Research Stations (ARSs) and Adaptive Research Centers (ARCs) in the respective regions. Crops on which research is being actively pursued are rice, coarse grain cereals, grain legumes, roots and tubers, condiments, oil seeds, fruit crops, vegetables, and fiber crops.

Rice being the staple crop, special emphasis has been given to that crop over the last three decades. As a result, a number of high-yielding varieties have been developed—mainly by conventional breeding methods—which have resistance to major diseases and insect pests. Today more than 95% of the island's rice is grown with these varieties. They have a yield potential of 5–10 t ha^{-1} and are adaptable to a range of rice-growing ecosystems. However, there is a yield gap between the potential yield and the current national average yield of 3.6 t ha^{-1}. To attain self sufficiency, the national average yields must be increased from the present 3.6 to 5 t ha^{-1}. Since no further increase in rice-growing lands is possible in the future, concentrated efforts have to be made to improve rice production in the low-potential lands in the country, and for this new breeding strategies have to be developed. Most of the newly bred varieties do not possess resistance to drought, submergence, cold, salinity, or adverse soil conditions (iron toxicity, zinc deficiency, or acid sulfate). Therefore, to increase production and to stabilize yield, varieties with suitable plant characteristics have to be developed.

Considering the other food crops, which include chillies, onions, grain legumes, coarse grain cereals, oil seeds, roots and tubers, and vegetable crops, high yielding varieties with better response to inputs and greater productivity have been developed by conventional breeding. However, breeding for resistance to biotic and abiotic stresses has to be accomplished in some crops in order to stabilize yields. Food quality is also important. Growth duration is an important factor in rainfed grain legumes, coarse grain cereals, and oil seeds where the varieties developed should match the rainy period, with a goal toward rainwater satisfying the crops' water requirements.

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Plant breeding programs in Sri Lanka were also influenced by the adoption of techniques to improve the genetic makeup of crop plants. Induced mutation studies using physical and chemical mutagens have been undertaken since the late 1960s. These studies resulted in the production of rice mutant H4 dwarf (formerly called MI-273 m), which is a recommended variety (Ganashan 1971). A high-protein pasture grass mutant was also produced by induced mutations.

Development of Tissue Culture Activities

Rapid Clonal Propagation

The tissue culture technique as an initial biotechnological activity was initiated in 1976 by the Department of Agriculture for the rapid clonal propagation of orchid and anthurium for the cut-flower industry. This tissue culture activity is being continued at the Royal Botanical Gardens for commercial purposes. About 170 indigenous orchids of the *Dendrobium* and *Vanda* species are found in Sri Lanka.

Pathogen-free Plant Production

In 1984 a Tissue Culture Laboratory was established at the Central Agricultural Research Institute (CARI) with the assistance of FAO for the pathogen-free micropropagation of fruit crops such as pineapple, citrus, banana, passion fruit, and papaya. Since 1985 mass in vitro propagation of these crops was undertaken to raise homogenous plants for adaptability studies. In addition, micropropagation of other crops, i.e., rambuttan, strawberry, and ginger were also undertaken. Mini-tuber production and in vitro conservation of potato varieties were also done.

Mass micropropagation of potato using meristem culture of disease-free plants has been undertaken at RARC, Bandaraviela, since 1987. Stem cuttings obtained from plants developed through meristem culture are planted in sterilized soils kept in net houses to produce tuberlets that are free of all soil-borne diseases and viruses. These tuberlets are categorized as basic seed and planted to produce foundation and certified seed. As an alternate technique, the disease-free stem cuttings are planted directly in the field, especially in higher elevations; this produces high quality tubers.

In Vitro Conservation and Other Biotechnological Activities

The Plant Genetic Resources Centre (PGRC), which was established with the assistance of the Japanese International Cooperation Agency (JICA), initiated activities in 1988. It has facilities to undertake biotechnological research. In vitro conservation to maintain the germplasm resources of root and tuber crops, i.e., sweet potato, cassava, potato, and *Dioscorea alata* are done at this center. Callus culture and plant regeneration studies in rice, embryo culture in groundnut, chillies, and rice, and electrophoretic studies in root and tuber crops are being undertaken.

Potential Contributions of Biotechnology to Crop Improvement

Although advances in crop improvement have been made by using conventional plant breeding methods, there are limitations for further improvement. Some of the major constraints are given here along with the biotechnological methods which could be used to enhance the efficiency of traditional crop improvement methods, or replace conventional methods that have failed.

Rice

Rapid adoption of new varieties for cultivation has resulted in narrowing genetic diversity in the field. The varieties currently in use have genes predominantly from Cina and Latisail, both contributing from 18.8 to 50.0% nuclear gene sources (Javier et al. 1989). All the new improved semidwarf varieties in use have their dwarfing gene from Dee-geo-woo-gen. Major genes for gall-midge resistance were obtained from OB 677/678 and used in six popular rice varieties under cultivation. Five years after release, the gall-midge resistance in these varieties and the donor OB 677/678 broke down. For brown plant-hopper resistance, PTB-33 was the gene source; this also now shows a degree of vulnerability. Instances of breakdown of the resistance to blast disease have also been reported. The narrow genetic base in the cultivated varieties has generated the risk of genetic vulnerability towards pests, diseases, and other hazards.

Breeding for tolerance to such environmental stresses as salt, drought, and adverse soil conditions also faces several difficulties. Plant biotechnologies

offer plant breeders a mechanism for speeding up the development of varieties resistant to environmental stresses. Developing anther culture techniques and performing plant regeneration from callus offer efficient methods for the production of haploids. Callus induction and plant regeneration in *indica* rice is significantly lower than in *japonica* varieties. However, appreciable anther callus production of some Sri Lankan Bg varieties were reported by Pathinayake and Johnson (1989). Anther culture has the potential of compressing breeding cycles, increasing selection efficiency, providing for early expression of recessive genes, and exposing gametoclonal variants.

Potato

Potato is cultivated predominantly in the upcountry intermediate and wet zones (7000 ha) during the *maha* (wet) and *yala* (dry) seasons. However, a sizable extent (500 ha) is cultivated in some parts of the low-country dry zone during the *maha* season, during which the minimum temperature goes to 22°C. At these temperatures tuberization takes place in some varieties, although the minimum temperature requirement for tuberization is considered to be below 20°C. Since some varieties tuberize at 22°C in the dry zone, further improvement in developing types that could tuberize under both *maha* and *yala* seasons could be explored. Varieties that tuberize above 22°C are not available elsewhere. Current potato production in Sri Lanka is threatened with severe attack by cyst nematode in the upcountry intermediate and wet zones, and this has led to greatly reduced cultivation. In vitro mutagenesis in potato protoplasts and subsequent regeneration appears to be easier (Jones et al. 1984), and this technique should be explored to develop varieties adaptable to the dry zone so that potato cultivation could be increased.

Pigeonpea

The remarkable adaptability of pigeonpea varieties in the environmental continuum—ranging from the drought-prone dry zone to the more stable environments in the intermediate zones has been observed. Pod borer (*Helicoverpa* sp) is a serious problem and to overcome this, wild relatives—*Atylosia* and *Rhynchosia* species—should be taken for hybrid embryo production, or even somatic hybridization could be tried. Wild relatives of pigeonpea are found growing under natural conditions in Sri Lanka.

Groundnut

There is an urgent need to have a very short-duration and drought-resistant groundnut variety for cultivation in the dry zone. To develop these varieties, biotechnological approaches should be tried.

Sorghum

Sorghum was a traditional dry zone crop and several landraces were available. Of these, Thambagala was seen to be more adaptable under dry zone conditions. During the 1960s, the Department of Agriculture undertook a project for the development of sorghum as a substitute for rice in which they developed a short-statured sorghum. Its grain appeared to resemble the popular round grain samba type rices of Sri Lanka, but its cooking quality was not acceptable and it was not preferred. With biotechnological approaches, attempts could be made to develop sorghum types with the food quality of rice grains. It is possible that the prolamin content of the sorghum grain has to be reduced to the level of that of rice grain to gain consumer acceptability.

Citrus

The genetic diversity of sweet orange (*Citrus sinensis*), linae (*Citrus aurantifolia*), and other citrus species has been rapidly depleted over the past 30 to 40 years due to drought, virus (*Tristeza*), and fungal diseases. A program to develop orange cultivation has been undertaken in the 1980s. The popular Bihele Sweet Orange was found to be compatible as a graft on wood apple stock and it comes to early bearing, but declines after 5 years. Although it produces more fruit, the external appearance and rind thickness of the fruit are unacceptable. Production of somatic hybrids of Bibele Sweet Orange with other species should be tried. Successful somatic hybrids of citrus have been produced by Kobayashi and Ohgawara (1988).

Conclusion

The following goals for crop improvement using biotechnological approaches should be taken up either

locally or in collaboration with international institutes.

- Develop plant varieties that are tolerant to high salinity or flooding.
- Improve water-use efficiency of plants and develop drought-resistant varieties.
- Improve the resistance of plants to diseases and pests.
- Develop herbicide-resistant crop plants.
- Modify the amino-acid composition of storage proteins in cereals and legumes to improve nutritional value.
- Improve the composition and storage life of fruits and vegetables.

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Current Status and Future Prospects of Biotechnology in Thailand

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Abstract

Tissue culture, for regenerating plantlets resistant to stress, pests, or diseases, is very popular among plant breeders. However, it is rare that a new successful or exciting and useful line or variety is derived from it. Protoplast fusion and virus gene transfer are being tried in some laboratories for developing new crop varieties.

Fermentation for obtaining useful products from microorganisms is another field of research that is rather popular in Thailand; biological control and fertilization are also considered exciting subjects. However, there is rather small development on an industrial or commercial scale from the mentioned research work.

Current Situation

Many research areas related to biotechnology that have been explored in Thailand in recent years, such as fermentation, decomposition, biofertilizers, and products of microorganisms, are very popular. Mushroom growing as food and as a health food may be a useful application of microbiology. Biological control is also another application, but it is still in the experimental stage. Culture and inoculation of *Rhizobium* for nitrogen fixation in legumes is also a useful application that is being used in farming.

For crop improvement, rapid propagation by tissue culture in orchid and potato is very popular. In addition, techniques for production of disease or virus-free planting materials of citrus, sugarcane, strawberry, and papaya have been developed by some scientists. Somaclonal variation has been induced in sugarcane and rice for variety improvement, but improved varieties have not yet been produced.

Biotechnology has become popular among scientists in Thailand, especially those in academic institutes. Scientists or plant breeders aim at variety improvement through genetic engineering or DNA manipulation to transfer DNA responsible for a useful trait to a particular plant variety. Aims are to improve nutritive food value or resistance to diseases or stress. Unfortunately, this is not easy work; in addition, plant regeneration from excised plants, cells, or callus is very difficult in certain species and much work continues on this problem. Conventional breeding techniques remain the major process of crop improvement and it is hard to predict how much input biotechnology will have in the future.

Potential Research Projects

Some of the research projects (Yuthavong and Bhumiratana 1989) that are related to crop improvement are as follows:

- Selection and development of virus-resistant potato by using protoplast culture and fusion technique.
- Production of disease-free potato seed for commercial scale.
- Application of tissue culture techniques for improvement of steroid and alkaloid yield from *Solanum* and *Duboisia* spp.
- In vitro propagation and screening of indigenous *Costus lacerus* for high diosgenin production.
- Development of a new variety of rice for saline and acid soil through tissue culture.

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- Development of methodologies for cowpea tissue culture.
- Technological development of flowering and ornamental plant production.
- Development of biotechnology for cultivation of shiitake mushroom.
- Application of tissue culture for the propagation and development of papaya tolerant to papaya ringspot virus.
- Improvement and multiplication of pineapple by aseptic techniques for the canning industry.
- Use of biotechnological techniques for selection and breeding of high-yielding rubber clones.
- Technological development of good oil palm for Thai growers through tissue culture.
- Development and application of tissue culture methods for rapid multiplication and improvement of coconut and arecanut.
- In vitro conservation and propagation of the economic species of rattans.
- In vitro conservation and germplasm exchange of bamboo.
- Development of tissue culture of teak (*Tectona grandis* Linn. F.).
- Application of vegetative propagation to improve timber yield of red gum (*Eucalyptus camaldulensis* cv. Dehah).

Potential Contributions of Biotechnology to Improved Crop Production

Pests, diseases, and stresses are the main constraints to production in most crops. A crop variety effectively resistant to stress, diseases, and pests, especially insects, but maintaining a good yield and quality, is a distant goal. In the conventional breeding method, transfer of resistant genes from related species of crops has been attempted; but no viable embryo has been obtained in certain wide crosses and embryo rescue by tissue culture has been tried in some crops. Genetic engineering techniques will be useful, but we may not be ready to enter the field due to many problems and constraints, especially the lack of well-trained or sophisticated scientists and assistants, as well as the necessary equipment. Most scientists who have some training in biotechnology still need to gain knowledge and experience for practical application to crop improvement.

Biotechnology Resources: Facilities and Personnel

Colleges or faculties of science in some universities have personnel and facilities for DNA manipulation. The group at the Faculty of Science in Mahidol University may be the most advanced. However, research work on biotechnology is also carried on at other universities, e.g., Chulalongkorn University, Kasetsart University, Srinakarintharawirot (Prasanmit), and Prince Sonkla Nakarin University. Most scientists have received financial support from Japan, as well as USA organizations, and the National Center for Genetic Engineering and Biotechnology, Office of the Permanent Secretary, Ministry of Science, Technology, and Energy, and from other sources. Within the Department of Agriculture, Ministry of Agriculture and Cooperatives, there is a rather small amount of research work on biofertilizer in the Division of Soil; fermentation by microorganisms and tissue culture in the Division of Plant Pathology and Microbiology; tissue culture or in vitro screening for stress resistance in certain crops in the Division of Botany and Weed; tissue culture techniques related to plant propagation and variety improvement in Horticulture, Rice, Rubber, and Field Crops Research Institutes; and biological control in the Division of Entomology and Zoology. In conclusion, biotechnology especially in crop improvement is rather weak in terms of facilities necessary for the work and well-trained personnel in this advanced branch of science and related important fields, as well as in molecular biology or DNA manipulation to handle the work and the sophisticated equipment.

Future Needs and Priorities for Facilities Development and Personnel Training

Efficient gene transfer and screening techniques are necessary, especially in breeding for resistance to insects and diseases caused by fungus, bacteria, or virus. For gene transfer, efficient tissue culture facilities and well-trained personnel are needed for embryo rescue, regeneration, and transformation. Facilities for detecting the gene or related DNA and protein are also needed. Occurrence of disease is not always uniform or regular and this causes a problem in developing a true and efficient resistant variety. Therefore, efficient and practical facilities for disease screening are needed in crop improvement.

In certain crops, regeneration of plants from callus, cells, or fused protoplasts is still a problem, although many plant breeders, especially in academic institutes, have tried to induce in vitro somaclonal variation and selection for stress and disease resistance in addition to using conventional breeding methods

Priority Areas and Needs for Research and Development

First priority should be given to breeding for resistance to pests and diseases, especially virus diseases in the economically important crops, e.g., rice, cotton, rubber, sugarcane, legumes, and sesame, as well as some horticultural crops. This goal needs cooperation between breeder or geneticist, biochemist, plant pathologist, and entomologist. However, this seems to be hopeless at present, since it is very difficult to get such cooperation, as well as the equipment and facilities necessary for research and development. Even though tissue culture is popular among plant breeders, regeneration and embryogenesis seem to be difficult to achieve. Many scientists are attempting to develop somaclonal variation and make selection in vitro as well as in vivo. Some scientists are also working on protoplast culture and fusion to transfer traits governed by chromosomal as well as cytoplasmic genes.

Areas for Collaborative Research

The Rockefeller Foundation and Cornell University have supported Thai scientists in biotechnology and genetic engineering research in rice variety improvement, especially in breeding for disease resistance and improved quality. Many breeders and scientists are also partly financially and technically supported by the United States and Japanese governments or organizations, e.g., the United States Agency for International Development, the Science and Technology Development Board, the Japan Society for Promotion of Science, and the Thai National Center for Genetic Engineering and Biotechnology in the Ministry of Science, Technology, and Energy. Additionally, many internationally supported projects are also involved, to certain degrees, in tissue culture or in vitro techniques for crop improvement, e.g., soybean, mung bean, cowpea, rubber, potato, oil palm, corn, and papaya.

Conclusions

The more sophisticated biotechnology for use in crop improvement, especially that which involves genetic engineering, is rather new and sometimes inapplicable to general crop breeding in a developing country, and it seems to be difficult to develop research in an efficient and proper way. Scientists who have been trained in this area still need collaborative or cooperative manpower assistance in their research areas, especially in DNA manipulation. Collaboration could be done according to the crop commodity; this should give an opportunity for those who have a common interest to work together. However, because of bureaucracy and the promotion system, it is rather difficult to get people in different disciplines to work together. Outside scientists from research institutes such as ICRISAT should play an important role. Most present collaboration under foreign or international support has been done by visits and short training. This might not be enough for practical research work aimed at variety improvement by biotechnology and genetic engineering.

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Some Contributions and Future Research Problems of Biotechnology in Vietnam

Ho Huu Nhi[1]

Abstract

Little biotechnological work has been conducted in Vietnam, but in 1980 the National Biotechnological Research Program was begun. Since then biotechnology has made some progress and now contributes to the development of agriculture and the food industry in Vietnam and has facilitated the future development of modern biotechnology.

With its available resources, the National Biotechnological Research Program outlines major aspects of biotechnological development and identifies priority areas in order to overcome problems in crop production.

Introduction

As in other developing countries, biotechnological research in Vietnam is scattered and insufficiently financed. In 1980, a biotechnological research network was set up. The biological and agricultural researchers within the network began to work on a common program. Their goal was to successfully apply the achievements gained in the world of biotechnology to improve production practices and contribute to overcoming food problems and raising the living standards of the Vietnamese people.

The government biotechnological network has centralized all the scientific research staffs in the laboratories of six research institutes and four institutes of higher education throughout the country. They include in the North: the Vietnam Institute of Agricultural Sciences, the Institute of Biology, the Institute for Agricultural Genetics, the Veterinary Institute, the Hanoi Polytechnical Institute, and the Hanoi Agricultural College; and in the South: the Research Center for Applied Biology, the Ho Chi Minh Polytechnical Institute, the Ho Chi Minh University, and the Thu Duc Pedagogical College.

In-country Technological Research

In spite of poor, insufficient facilities and lack of scientific information, several contributions have been made to agricultural development through biotechnological efforts in Vietnam.

First, in the area of collection and conservation of plant genetic resources, Vietnam has conserved a diverse germplasm of crop plants, including 67 collections of different plant species. Of these, the most important are the collections of tropical, industrial, and fruit plants. These include: *Nephiliums, Achras sapota, Citrus, Ananas, Litchi chinensis, Musa, Magifera indica, Dyera* sp, rubber, coffee, pepper, and *Theobroma cacao.*

An agricultural crops genebank has been established at the Vietnam Institute of Agricultural Sciences (INSI). Besides storing plant seeds in the cold rooms, the Institute has been storing and conserving the collections of plants vegetatively propagated by in vitro techniques. About 200 samples of varieties and lines of sweet potato, cassava, potato, banana, pineapple, taro, and sugarcane have been conserved in

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this manner. The minimal growth storage method is used for those cultivars by adding 3% mannitol to the culture medium and maintaining a low incubation temperature (16-20°C). This germplasm can be maintained from 6 to more than 8 months. Collections of yam, coconut, and onion are being transferred from the field to be conserved in vitro. The samples for in vitro conservation are heat-treated and cultured by meristem-tip culture techniques, therefore they are almost free of diseases.

Cryopreservation has begun to be used for pineapple and banana, but so far the results are not satisfactory. Sweet potato and banana propagated by tissue culture are available for distribution in vitro.

Rice and Corn

Plant breeding using introduced and local crop germplasm to select new varieties for production has made considerable achievements, especially for the main food crops, rice and corn. Before the green revolution in Vietnam, poor local varieties of rice were grown. These were characterized as tall and had a low yield (1.0-1.2 t ha^{-1}). With developments in world biology and agriculture and through international cooperation and scientific exchange, Vietnam has accumulated a diverse collection of initial genetic materials with various characteristics, i.e., disease and pest resistance, a wide range of adaptations to diverse ecological conditions, and high yielding potential. Based on the conventional methods of breeding (single cross, three-way cross, and backcross), Vietnamese plant breeders have successfully transferred valuable characteristics, e.g., semidwarf and high yield, from the introduced lines to local genetic resources. Thanks to the contributions of conventional biotechnology, between 1985 and 1990, the number of rice varieties in production has increased greatly. The following rice varieties are widely cultivated.

Varieties with tolerance to acid-sulfate and alkaline soil. These varieties, 314, 424, and V15, have tolerance to high concentrations of aluminum (60 ppm) and iron toxicity (200 ppm) (Chuong 1990). They are semidwarf and have a high yield of 4.0 t ha^{-1}. Their tolerance is controlled by the genes derived from the local varieties "Sai Duong" and "Chiem Three Leaves".

Varieties with cold tolerance. Variety NN 75-3 is widely cultivated in the North. It has cold-tolerant genes taken from a Romanian variety.

Varieties with disease and pest resistance and wide adaptability. CR 203 and IR 1561 are the most important and cover 60-70% of the rice cultivation area.

Varieties for intensive cultivation and very high yield (up to 12 t ha^{-1}). These are NN 8, Xuan No. 2, and DT-1.

With such diversity in genotype and phenotype, the rice varieties mentioned above have increased the average yield up to 2.5 times and rice production in Vietnam has doubled (Song et al. 1984). Beginning in 1989, we produced enough rice for local consumption and also exported more than 1 million t each year.

Production of corn hybrid seeds has not been taken up in Vietnam, but by a combination of breeding and periodical ear-to-row selection or biparental progeny selection, about 30 new corn varieties have been released for production. These are short duration varieties (75-80 days) with a yield of 2 t ha^{-1}, for example, Santa Rose and Nakula (Mien 1985).

Other Crops

Crop improvement is also conducted for other plants, i.e., sweet potato, soybean, and groundnut, but the results are unsatisfactory compared to rice and corn.

Beside the conventional hybridization methods used since 1980, tissue and cell culture are being used for crop plant variety improvement, but the usable results are limited. This technique is mainly focused on the culture of haploid plants, recovery and rapid multiplication of some vegetatively propagated plants with low natural multiplication rates, and establishment and separation of vegetative mutations.

Anther culture was first applied in rice breeding. Many haploid plantlets were successfully generated from anther cultures of more than 20 *indica* varieties and lines, mostly local varieties (Uyen 1985). Ten to twelve percent of anther cultures formed plantlets. To obtain doubled haploids, haploid seedlings were treated with colchicine. In comparison with the original varieties, these doubled haploids had earlier maturity and were highly homogeneous. Furthermore, haploid shoots were multiplied in liquid medium to produce a suspension of single microshoots that could be placed on petridishes for selecting salt-tolerant rice lines. The selected lines could grow on medium supplemented on 10 g l^{-1} NaCl (Dung and Uyen 1986).

Pollen culture and haploid plant generation of tobacco were also successfully used for plant breeding. The doubled haploid tobacco plants were allowed to self-pollinate through many generations. The F_4 individuals appeared to be morphologically uniform. It is clear that the generation of homogeneous lines from haploid plants can shorten plant breeding time (Binh 1983).

In vitro recovery and rapid multiplication were applied to vegetatively propagated crops with low natural multiplication rates. Because of hot, humid conditions and many diseases and pests, the potatoes in Vietnam degenerate very quickly and thus the average yield is about 10-12 t ha^{-1}. To overcome the constraints causing plant degeneration, Nhi et al. (1990) tried to develop a method for recovery of local varieties and rapid multiplication of newly introduced potato varieties. Recovered varieties had a low coefficient of virus infection (5-7%) and high yield (20-22 t ha^{-1}) against 30-50% and 10-12 t ha^{-1} for non-recovered varieties.

By the system of seed multiplication, in vitro techniques, and post in vitro culture in greenhouses or nurseries, the annual multiplication rate of potato has been increased from 3-4 times to more than 2000 times. This work is also conducted in several laboratories in the North and in the Dalat Research Center for Applied Biology in the central highland. It ensures a continuous supply of good seed tubers for potato production in Vietnam.

Agave, a fiber-producing plant, is cultivated in drought-prone regions. Its natural multiplication coefficient is so low (4-5 times), that it cannot meet plantlet production requirements for growers. The Biological Research Institute has developed in vitro propagation techniques in which shoot tips were used as explant material. Shoots or multiple shoots were formed on MS medium supplemented with 0.2 mg l^{-1} BA. The propagation rate obtained at the Institute is more than 1000 times (Muoi and Binh 1990).

On MS medium supplemented with IBA and BA, many multiple shoots are generated. The generated plantlets have preserved their characteristics during in vitro multiplication. Using this process, the sugarcane line F 105, which has a high yielding potential, can be introduced rapidly into sugarcane production in Southern Vietnam. (Minh and Lien 1986).

The Applicable Results of Microbiology

Some results in nitrogen fixation research have been of great significance. Many local microbial strains are being field tested. The Microbiology Department of the Vietnam Institute of Agriculture Sciences has proposed a scheme for microbial Nitrazin production. Their microbial inoculants are being effectively applied to some legumes, such as soybean and groundnut, with an increase in yield of up to 45% (Viet and Cuong 1986). Isolation, strain selection, and production of blue-green algae—a nitrogen fixer for rice—were also being researched.

In Vietnam, the need for gibberellic acid (GA) increases from day to day. GA stimulates the growth and increases the yield of tea, vegetables, and tomatoes. GA also controls flowering of coffee and many other plants, which increases their economic value. To meet the production requirements in our country, research on a GA production technology scheme has been conducted. To date, we can produce 50-100 g of GA per day. Its quality is high and its price is lower than that of imported GA (Uyen 1990).

The use of *Aspergillus* for molasses fermentation to produce citric acid gives good results. Every year about 500 t of molasses is being fermented giving large amounts of citric acid. This meets the consumption requirement at a lower price than imported citric acid. (Uyen 1990).

Since 1986, mushroom production has become more and more popular. The mushroom-growing technique has been accepted by many farmers' families. The most widespread mushroom species in Vietnam are *Pleuritis ostrealus*, *Agaricus bisporus*, and *Volvaniella volvaces*. Their average yield is 12-15 kg m^{-2} (Kiet and Lien 1986). Mushroom growing gives many benefits. For example, it makes the most of agricultural byproducts like straw, hulls, and bagasse and creates an additional source of employment for farmers. Mushrooms are a valuable product for export to Japan and Italy, as well. Seventy t of mushrooms are exported each year (Nha 1987).

The Main Constraints to Agricultural Production

According to government statistical data from 1989, the cultivated area in Vietnam is about 8 million ha. Of these, acid-sulfate and alkaline soils occupy about 2 million ha and drought-prone soil 1.8 million ha. Thus far, soil improvement efforts have not produced satisfactory results. Most crops grown in these soils have low yield (1.0-1.2 t ha^{-1} for rice, 0.6-0.8 t ha^{-1} for groundnut), even though rice and groundnut have good adaptability. There is still much noncultivated soil in this category.

Like other tropical countries, Vietnam has climatic conditions favorable for plant growth. At the same time, these conditions encourage development of pests and diseases, especially viruses. These diseases make many plants, like root and tuber crops, degenerate quickly and cause yield losses up to 30-40%. The most important virus diseases in Vietnam are:

- Potato viruses X, Y, A, M, and S
- Bean yellow and bean common mosaic viruses
- Citrus tristeza and mycoplasma-like diseases
- Rice grassy stunt, rice transitory yellowing, and rice dwarf viruses
- Maize stripe and maize dwarf viruses

An FAO expert reports that each 100 kg of rice biomass contains 50 kg of rice straw and 10 kg of rice hulls (Barreveld 1989). Each 100 kg of sugarcane biomass contains 18 kg of bagasse and 3 kg of molasses. By that, every year Vietnam has about 20 million t of bagasse and molasses. These are a very big source of lignocellulose from which Vietnamese industry has not manufactured products of economic value.

Every year Vietnamese farmers use about 3 million t of nitrogen fertilizer, of which one-third is produced in the country; the rest is imported. The lack of nitrogen fertilizers could be partly compensated for by preparations of nitrogen-fixing microorganisms. We have taken the first steps in this field and our results are very promising, but a small amount of these biofertilizers is produced and applied to only a few crops.

Conclusions

Future Needs of Biotechnology

The objectives of biotechnology in Vietnam for the future include application of biotechnology achievements to conserve and effectively use the biological resources, and the utilization of biotechnology to solve real problems, such as employment and improvement of living conditions for Vietnamese people. Simultaneously, it is necessary to establish the material base for modern biotechnology. To realize this, we will direct our biotechnology research toward the following.

- Isolation, culture, and storage of microbial strains that are active in nitrogen fixation for legumes and non-legumes (biological nitrogen fixation).
- Collection and storage of genetic resources of crop plants to overcome genetic erosion in nature and to supply valuable materials for plant breeding.
- Crop improvement in order to raise crop yield, especially in difficult to cultivate areas such as drought-prone, acid-sulfate, and alkaline soils.
- Recovery of degenerated crops, especially root and tuber crops and fruit trees, and rapid seed multiplication of these crops and trees.
- Development of biological conversion for rural food fermentation, biofuels from agricultural crops and residues, mushroom production, plant biomass conversion, and animal vaccines.
- Molecular biology and genetic engineering, in general.

The Top Priority Problems in Biotechnology Research

- Isolation, screening, and testing the local nitrogen-fixing organism collection of rhizobial bacteria with legumes and blue-green algae with rice.
- Industrialized production of microbial fertilizer (and its subsequent application in production and contributions), to overcome nitrogen-fertilizer deficiency.
- Use of tissue and cell culture techniques for conservation and storage of vegetatively-propagated crops, recalcitrant seeds, and also for crop improvement and breeding.
- Fermentation of cereals and legumes to improve their nutritive value and conversion of agricultural waste into animal feed or other useful products.

Material Base and Training for Biotechnology

Many research institutes and institutes of higher education take part in the biotechnology research program. They include six laboratories for cell and tissue culture, seven laboratories for microbiological research, and seven laboratories for biochemistry and molecular biology research.

Some of these are well equipped thanks to the help of United Nations organizations and of other countries. The rest, in general, have poor facilities; their equipment is old and chemicals are in short supply.

To develop biotechnology in the coming years, Vietnam will promote research and training on the following:

- Food biochemistry
- Microbial nitrogen fixation
- Plant breeding using in vitro techniques
- Disease testing techniques, especially virus testing for in vitro plants
- Genetic engineering

We hope that with our great efforts and the kind-hearted help of the international scientific community we will successfully realize the goals of our biotechnology research and development program in the coming years.

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Part II

The Scientific Disciplines

Cell and Tissue Culture

Transformation

Genome Characterization and Diagnostics

Cell and Tissue Culture

Cell and Tissue Culture in Cereals

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Abstract

Cereals provide three-quarters of world energy needs and more than one-half of protein needs, and are important as cattle feed. By the year 2000 it is estimated that food production should be doubled to feed the increasing population. Most uncultivated land has limited potential for cropping and a production increase must come from the improvement of the genetic potential of cereals. Recent advancements in cell and tissue culture techniques in cereals have opened up a wide scope for creating novel genetic variation that could be best used in further improvement programs. However, the initiation of organogenesis in vitro is a complex morphogenetic phenomenon, wherein both extrinsic and intrinsic factors play a role. Several factors that are believed to play a direct or indirect role in regulating organogenesis in vitro are reviewed. Studies over the past several years have indicated that regulatory mechanisms operate at various levels, i.e., donor genotype and explant selection, medium components, exogenous hormone level, physiological status, cytological changes, and biochemical level. Manipulation of medium, culture environment, and selection of responding genotypes have resulted in great progress in regeneration of cereals. However, we are still far from understanding how organogenesis is regulated. In the final analysis, this answer may come from the area of molecular biology, an area that is presently under intensive study.

Introduction

By 2000, it is estimated that the world's population will exceed 6×10^{10} people. This will necessitate doubled food production within the next ten years. Production increase must come mainly from higher crop yields.

Genetic variability in the cereals has not been exhausted. With recent advancement in in vitro culture, new and sophisticated techniques are becoming available for crop improvement. They include cell and tissue culture, cell fusion and genetic transformation.

The earliest report on in vitro culture in cereals dates back to 1964 by Furuhashi and Yatazawa. Nishi et al. (1968) were the first to succeed in regenerating a whole plant from rice calli and were also the first to succeed with monocotyledon species. In the same year, Niizeki and Oono produced haploid rice seedlings from pollen grains. In this chapter, the recently published results on various aspects of regeneration in cereals—rice, sorghum, pearl millet, finger millet, wheat, barley, oats, rye, and triticale—are reviewed and progress made in our laboratory is presented.

Anther and Pollen Culture

Anther culture remains the most extensively investigated aspect in cereal tissue culture due to the possibility of obtaining haploid cells, tissues, and complete plants that have only a single dose of genetic information. Most of the reports on organogenesis in vitro fall into the category of manipulation. In these, successful organ formation is achieved through selection of suitable explant, proper choice of the medium, and control of the physical environment.

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Selection of the Explant

The choice of the explant is of paramount importance in achieving success in regeneration.

Several factors such as the genotype of the donor plant, physiological and ontogenetic age, the tissue source, size of explant, physical environment, and the overall quality of the donor plant from which explants are to be obtained are found to be involved.

Genotype. It is well known that induction frequency and regeneration ability of the pollen callus or embryoids vary greatly with the genotypes of anther-donor plants. Several researchers like Niizeki and Oono (1968), Chu (1982), Rush et al. (1982), Ding et al. (1983), Zapata (1985), Hu (1985), Boyadzhiev and Fam Viet Kong (1989), Guiderdoni et al. (1986), Mikami and Kinoshita (1988), and Quimio and Zapata (1990) have observed genotype differences for culturability in rice. Similarly, Foroughi-Wehr and Zeller (1990) in wheat, Powell (1988) and Knudsen et al. (1989) in barley, and Barlov and Beckert (1989) in maize have reported similar results.

Genotypic variation is mostly seen in the extent of response rather than in the existence of responsive and nonresponsive genotypes. In general, *japonica* rice genotypes responded better than *indica* (Chu 1982; Gun 1982; Zapata 1985; Manimekalai and Sree Rangasamy 1983 and 1987; Yang 1988; Narasimman et al. 1990; Quimio and Zapata 1990), and F_1 hybrids perform better than their inbred parents (Cornejo-Martin and Primo-Millo 1981; Chen 1986(a); Manimekalai and Sree Rangasamy 1987). It was suggested that the pollen callus induction frequency is controlled by genes of the diploid heterozygous anther wall tissue and not mainly by genes of the hemizygous microspores (Junwen 1986). Genotypic variation to the extent of green plant or albino plant regeneration is also reported in rice (Wu et al. 1987; Datta et al. 1990) and in wheat (Agache et al. 1989). In sorghum, anther culture studies are very limited. Zenkteler (1980) reported haploid albino and green plantlets from *S. halepanse*. Rose et al. (1986) obtained albino plantlets from sorghum anthers.

Rice *japonica* races seem to be good combiners for callus induction as compared to *indica* (Miah et al. 1985; Quimio and Zapata 1990). Studies on the genetics of anther culturability revealed that this trait was heritable, was controlled by multiple genes (Miah et al. 1985), had independent inheritance (Davoyan 1987), and had no maternal influence (Bullowck et al. 1982; Ouyang et al. 1983; Foroughi-Wehr and Friedt 1984; Quimio and Zapata 1990). On the contrary, Mathias and Fukui (1986) and Sagi and Barnabas (1989) observed the effect of nuclear-cytoplasmic interaction and/or nuclear effect on plant regeneration capacity. However, in some cytoplasmic substitution lines, the cytoplasm can significantly affect tissue culturability (Mathias and Fukui 1986; Mathias et al. 1986; Szakacs et al. 1988; Sagi and Barnabas 1989). Studies in wheat chromosome substitution lines revealed that the 7A and 1B chromosomes had considerable effect on callus induction and 3A and 2D had a definite influence on green-plant regeneration (Szakacs et al. 1988). Agache et al. (1989) observed that the genes on the CS1D chromosome and the 5BL chromosome arm increased embryo frequency. Genes involved in regeneration were located on the 1RS chromosome arm and a gene increasing albino frequency was located on the Chinese Spring 5B chromosome. The best responding winter wheat cultivar "Florida" was characterized by the presence of the 1B/1R wheat-rye translocation chromosome (Foroughi-Wehr and Zeller 1990).

Ontogenetic age and physical condition of the donor plant. The physiological state of the donor plant at the time of explant collection is known to affect response in a number of plants (Foroughi-Wehr and Mix, 1979). Anthers from flower buds arising early in the onset of flowering are better than from the buds arising later in the season (Narayanaswamy and Chandy 1971; Sunderland 1971). In rice, panicle-to-panicle differences in callus induction have been reported. Although during the incubation of anthers, light has not been found to have a crucial effect on callus induction, the light condition under which the parent plant is grown appears to have a considerable effect. Plants grown under natural light during the normal season have been found to be better than those grown under artificial light during off-seasons (Pelletier 1972 cited in Pandey (1973)). In rice, temperature and sunshine during flowering affected anther response markedly (Hu et al. 1978). The indica rice, Basmati-370, gave a better response when flowering when the average maximum-minimum temperature range was 34.2-23.3°C, (Raina et al. 1987). In wheat, no significant differences were observed between the three environments studied, i.e., 15°C-16/8 h light/dark, 20°C-16/8 h light/dark, and 20°C-12/12 h light/dark). However, better response was observed at 20°C with 16/8 h light/dark conditions (Jones and Petolino 1987). Significant genotype × environment interaction was observed for embryo formation. Zhong and Liang (1981) observed that the pollen callus yield in barley was correlated with the thick-

ness of anther tapetum, gradually decreasing with the decrease of tapetum thickness. The growth condition of the donor plant was complicated, yet the most important conditions might be temperature, light, and nutrient supply at the stage of anther formation. For the chosen explants, significant differences were observed among the individual wheat plants (Carman et al. 1987).

Donor plants—culture treatments. Large improvements in embryoid formation could be made by improving environmental conditions (Bjarnstad et al. 1989). The effect of the age of the parent plants, the stage of flowering, and the position of the bud in the inflorescence indicate that the content of growth hormones in the growing tissues of the young bud may be relevant in this connection. Thus, hormones–IAA, NAA, or gibberellic acid, alone or in combinations–sprayed on the parent plant about 1 week before using the bud, may be beneficial for induction of pollen haploidy (Pandey 1973). Wang and Chen (1980) reported that the pollen callus yields of winter wheat were significantly increased when the seeds were first germinated and vernalized artificially and then sown in the field in spring just before the ice in the soil began to melt, instead of sowing the seeds normally in autumn. Picard et al. (1987) observed that spraying with a chemical hybridization agent (Fenridazon-potassium) to the flower buds undergoing meiosis resulted in a 20-fold increase in embryo production.

Pre- and postinoculation physical environmental conditions. The pre- and post-inoculation treatments are given as a means of neutralizing the more powerful male potency normally present in the young microspores of the majority of flowering plants. During the cold-shock treatment, additional divisions of the nongametophytic type are known to occur and these facilitate the induction process in the dedifferentiating culture medium (Nitsch 1974). In graminaceous crops, it has been reported that cold-shock pretreatment of young spikes was effective for anther culture of *Oryza sativa* (Zhou and Cheng 1982), *Hordeum vulgare* (Huang and Sunderland 1982), *Secale cereale* (Sun et al. 1978), and triticale (Sun et al. 1980). Among the *indica* and *japonica* rice genotypes, the cold shock pretreatment requirements varied (Zhou et al. 1983). They also pointed out that when cold treatment duration exceeded a certain limit, the induction frequency decreased markedly. They also observed that cold treatment not only significantly increased anther response but also enhanced green plant production. In rice, different temperatures and incubation timings were found to give better results: 6°C for 5 d (Chaleff et al. 1975); 10°C for 4-8 d (Hu et al. 1978); 5°C or 10°C for 7 or 10 d or 13°C for 10 or 14 d (Genovesi and Magill 1979); 2-4°C for 48 h (Cornejo-Martin and Primo-Millo 1981); 7°C for 3 d (Chaleff and Stolarz 1982); 9-10°C for 20 d (Zhou et al. 1983); 8°C for 4–8 d (Zapata et al. 1983 and 1985); 10°C for 10 d (Manimekalai and Sree Rangasamy 1987); 10°C for 11 d (Gupta and Borthaker 1987); 4°C and/or 10°C for 10-18 d (Datta et al. 1990); and 6-8°C for 8 d (Quimio and Zapata 1990).

However, in wheat, the effects of cold pretreatment have been unsatisfactory. Indeed some preliminary experiments showed that cold pretreatment was beneficial to wheat anther culture (Pan et al. 1975). However, repeated experiments showed that the effect was unstable. Liang et al. (1982) and Muller et al. (1989) were unable to observe beneficial effects of cold pretreatment on wheat anther culture. However, Huang (1987) reported that pretreatment at 30°C for 8 d gave 40% more microspore callus and green plants. At present in wheat anther culture, this pretreatment may be adopted as a measure to store young spikes to obtain a continuous supply of anthers and decrease the workload and pressure during inoculation. In barley, 4°C for 21 d gave the highest response (Powell 1988). In maize, 15°C for 4 d doubled the number of embryo-like structures (Pescitelli et al. 1990). Heat treatment rather than cold pretreatment is also known to be stimulatory in rice: 40°C for 15 min (Rush et al. 1982); 35°C for 15 min or 5 min prior to 10°C for 7 d (Zapata and Torrizo 1986).

Besides temperature, centrifigation of rice panicles at 2000 rpm for 10 min (Zhu and Wang 1982), and gamma irradiation of anthers prior to culture (Sun et al. 1978; Yin et al. 1984; Zapata et al. 1986) have been found to increase the callusing efficiency by more than 28 times.

Developmental stage of explant. In rice, anthers inoculated at the mid- to late-uninucleate pollen stage have been found to be the most suitable for culture. Oono (1975), Chen and Lin (1976), Chen (1977), and Cornejo-Martin and Primo-Millo (1981) conducted detailed studies. They concluded that anthers at the tetrad stage do not respond at all, and early uninucleate pollen may respond poorly. Mid- to late-uninucleate pollen responds the best. Anther response falls sharply after the first pollen mitosis. Anthers in early stages of nuclear division respond more readily than those in the later stages. Calli from late-uninucleate pollen tends to show less regeneration potential and produce more albinos. Staging of microspores in iron

album-hematoxylin improved the rate of callus induction (Gupta and Borthakar 1987).

In wheat also the developmental stage of the pollen at the time of culturing is very important. He and Ouyang (1984) found that in some genotypes haploid callus and subsequently haploid green plantlets could be obtained from microspores at various stages, from early meiosis to binucleate pollen. The highest peaks of callus induction and plantlet regeneration were observed from mid- or late-uninucleate microspores. Xu et al. (1981) found that the pollen at mid-uninucleate stage was optimum for in vitro anther culture in barley.

Culture media for induction. Niizeki and Oono (1968) used Blaydes (1966) medium in their first report of successful rice anther culture. Until 1975 subsequent researchers used the same formulation or attempted only slight changes. Oono (1975) examined several other media and recommended Murashige and Skoog (1962) medium (MS) as the most suitable medium for anther culture. Yang (1975) was the first one to improve the basic ingredients of wheat anther culture medium. He reduced the concentration of four major inorganic salts of MS medium to half their original strength but increased the concentration of KH_2PO_4 to 275 mg l^{-1} with biotin at 0.5 mg l^{-1} (Maiji 1 medium). The success of this medium was much higher than the original MS medium in wheat anther culture. Almost at the same time, Chu et al. (1975) reported a medium, N6, for rice anther culture. It was found to be suitable for induction of callus from wheat pollen. The effects of Maiji 1 and N6 medium were similar. Later, however, N6 medium was much more widely adopted for wheat anther culture. Recently, C1 medium (Chen and Wang 1979), C17 medium (Wang and Chen 1983), and D medium (Zhang & Meng 1982) were proposed for anther culture. In general, the induction frequencies of pollen calli by these media were significantly higher, but the green plantlet regeneration ability of these calli was lower than N6.

Rice anthers respond to many basic media such as Miller medium (Chen et al. 1974; Wang et al. 1974), modified MS medium (Chen 1977), LS and modified LS media (Chaleff and Stolarz 1981), and modified White's medium (Tsai and Lin 1977). So far, N6 medium (Chu et al. 1975) has been widely adopted for *japonica* rice. These authors pointed out that the growth and differentiation of rice pollen callus are influenced by major salts, especially by NH_4 salt. An NH_4^+-ion concentration lower than 7 mM is beneficial. Best results were obtained when the concentrations of $(NH_4)_2SO_4$ and KNO_3 were 3.5 mM and 28 mM, respectively. For *indica* rice, a concentration of 3.5 mM NH_4^+-ion was optimum for callus induction. Increasing KH_2PO_4 to 800 mg l^{-1} and iron salts to 2-fold strength of MS medium was found to be beneficial. Subsequently, He5 basic medium was developed (Huang et al. 1978) for *indica* rice. SK3 medium has been developed for *japonica-indica* hybrids. Increasing Fe-EDTA, $MgSO_4$, and KH_2PO_4 in Miller medium favored both callus induction and subsequent regeneration. By combining the inorganic salts of N6 and organic ingredients of MS, the induction effects were far better than that of modified MS medium in *japonica* and *japonica-indica* hybrids. Two new media, L-8 and 'General', have been formulated in China and N6-Y1 (Chung and Sohn 1986) in Korea. N6-Y1 is essentially the same as N6, but its nitrogen source consists of 28 mM KNO_3, 1.75 mM $(NH_4)_2SO_4$, and 1.75 mM L-glutamine, instead of the 28 mM KNO_3 and 3.5 mM $(NH_4)_2SO_4$ of N6. Chen et al. (1986) observed that more rice anthers formed callus when either 0.1 mM Na_2Fe-EDTA or 0.1 mM NaFe-EDTA was added to the culture medium. However, increasing the iron concentration to 0.2 mM led to toxicity. Manimekalai and Sree Rangasamy (1988) reported that the response of rice anthers was better on modified N6 medium. Raina et al (1989) formulated two media, SK-1 and MSN, for efficient anther culture of some *indica* rice hybrids. MSN medium is a blend of MS and N6 medium. The nitrogen source in SK1 is mainly NO_3^-, but vitamins, amino acids, sucrose, and hormones were similar for both the media. Boyadzhiev et al. (1986) and Boyadzhiev and Fam Viet Kong (1989) also reported that N6 was better for callus induction in rice while MS was better for obtaining regenerants. Rout et al. (1989) reported that potato-2 medium was superior to N6 in terms of increased anther response, early callus induction, multiple calli formation, and overall green plant regeneration.

Culture medium for differentiation. Many reports point out that media with a relatively high content of inorganic salts are more suitable for the differentiation of callus. The differentiation frequencies of rice pollen callus on different media were as follows:

Modified N6 > RM > MS ½MS > Miller > Nitsch

Addition of various concentrations of Na_2Fe-EDTA, NaFe-EDTA, or a combination of the two improved plantlet production in rice (Chen et al. 1986). Use of modified MS medium containing reduced nitrogen

(glutamine and casein hydrolysate) and 10% (W/V) Ficoll might favor microspore embryogenesis and green-plant regeneration in rice (Datta et al. 1990). In wheat, a medium based on twice the MS inorganic salt concentration gave a significantly better response, and inclusion of MS vitamins at normal strength appeared to be essential for optimum response (Carman et al. 1987a). Shimada and Otani (1989) reported that potato medium produced more pollen embryoids than N6 medium. Potato aqueous extract which was derived from potato tuber with rind had a more stable effect than the one without rind.

Concentration of carbon sources. Sucrose is added to the medium as a carbon source and to adjust osmotic pressure. Various concentrations of sugar ranging from 1.5 to 12% were tested. In general, a low concentration (3%) favored androgenic initiation and callus induction, while a higher concentration (8%) inhibited callus formation. The differentiation frequency of green plantlets was higher at 6% than at 3% sucrose (Clapham 1973). Chen (1978) reported that 6% and 9% sucrose enhanced both callus formation and subsequent regeneration, but most plantlets that differentiated from callus with media containing 9% sucrose were albinos. Chaleff and Stolarz (1981) suggested that the influence of sugar was due to the effect on the osmotic pressure of the medium, which was more important than its utilization as a carbon source. Liang et al. (1980) tested the penetrability of late-uninucleate microspores of rice in both 0.24 M and 0.12 M sucrose solutions and found that the penetrability of the grains in 0.12 M solution was one and a half times higher than that in 0.24 M. In rice, a higher sucrose concentration (6-12%) induced anther callusing (Kim and Raghavan 1988) as compared to a lower (2%) sucrose level (Mercy and Zapata 1986). In wheat, several studies have revealed almost the same conclusion that 9% and 10% sucrose concentrations (Junwen 1986) favoured callusing frequency as well as regeneration ability. In barley, Sorvari and Scheider (1987) observed that starch medium together with melibiose had revolutionary effects in producing a high number of embryoids and green plantlets and also drastically reduced the number of albinos. Finnie et al. (1989) observed that the overall green plantlet differentiation was higher in maltose-based media as compared to one based on a malt extract.

Exogenous Hormones

Callus induction. A wide variety of growth-stimulating hormones have been tried, singly and in numerous combinations. A combination of auxins (IAA, NAA, 2,4-D), cytokinins—kinetin (Kin), zeatin, and 6-benzylaminopurine (BAP)—and complex growth substances—coconut water (CW), yeast extract (YE), and casein hydrosolate (CH)—have been used for rice and wheat anther culture. However, in rice, the responses from these combinations was lower than 2,4-D (2 mg l^{-1}) alone (Raina 1977). He also observed that addition of zeatin (0.01 mg l^{-1}) or YE (2 mg l^{-1}) enhanced the response slightly. NAA (2 mg l^{-1}) increased callusing frequency as compared to 2 mg l^{-1} 2,4-D. However, plantlet regeneration was lower in NAA-induced pollen calli. Chou et al. (1978) tested 22 chemicals and found that 2,4,5-T and TCP (picloram) were effective in callus induction. Cornejo-Martin and Primo-Millo (1981) found that 2,4-D (2 mg l^{-1}) favored callus induction in some genotypes while NAA (2 mg l^{-1}) favored callus induction in others.

Of late, most researchers now employ several hormones as "combined initiation factors". In *japonica* rice, Yang et al. (1980) used a combination of 4 mg l^{-1} NAA, 1 mg l^{-1} 2,4-D, and 1-3 mg l^{-1} Kin. For *indica* rice, they used 2 mg l^{-1} 2,4-D + 2 mg l^{-1} NAA + 3 mg l^{-1} Kin. However, a combination keeping 2,4-D at 1 mg l^{-1} or lower and using Kin at 1-3 mg l^{-1} has been found especially suitable for subsequent regeneration (Chen et al. 1986). Rout et al. (1989) reported that NAA was significantly superior to 2,4-D in terms of callus induction.

In wheat, 2,4-D (1-3 mg l^{-1}) was found suitable for anther calli induction. Besides the higher sucrose concentration, older pollen developmental stage (Research Group 301 1977) and the genotype of anther donor also affected filament callus formation (Shimada and Makino 1975).

Regeneration. In rice, all pollen calli do not regenerate, and shoot differentiation from 50% of the calli is considered very good. Best regeneration response is observed when calli are transferred after 10-12 days of their emergence (about 2 mm size). Shoot induction could be achieved by transferring 2,4-D-induced pollen calli to IAA plus Kin medium (Niizeki and Oono 1968) or to a hormone-free medium (Wang et al. 1974). The relative ratio of IAA, NAA, and Kin also played a role in differentiation. It was found that IAA favored root elongation while NAA benefitted the sturdy growth of root. So, a combination of Kin, NAA, and IAA was generally used to induce differentiation for higher green-plant regeneration (Wakasa 1982). Chaleff and Stolarz (1982) obtained a high rate of regeneration using modified MS medium (R3) +

NAA (2 mg l^{-1}) + Kin (0.3 mg l^{-1}). Organic supplements such as YE (250 mg l^{-1}) or CH (250-500 mg l^{-1}) or hydrolytic nucleic acid (20-200 mg l^{-1}) in the induction medium improved the callus quality and subsequently the regeneration ability (Yang et al. 1980). Addition of CH or CW in regeneration medium also improved the regeneration ability. Zhou et al. (1983) pointed out that alanine, folic acid, and coenzyme A enhance the callus formation of *indica* rice. Zapata et al. (1983) using B5 medium reported about 40% regeneration efficiency. Chung and Sohn (1986) noted that calli from induction medium containing NAA, Kin, and abscisic acid (ABA) showed better regeneration ability than 2,4-D-induced calli. Chung (1987) noted that addition of ABA prolonged the regeneration potential. Rout et al. (1989) also reported that calli formed in the presence of NAA and Kin were the earliest to differentiate as compared to calli induced on 2,4-D- and Kin-containing medium.

In wheat anther culture, the sucrose level and especially the auxin level in the regeneration medium should be greatly lowered (Zhuang and Jia 1983). In general, sucrose (3%), IAA (0.2-2 mg l^{-1}), and Kin (0.2-2 mg l^{-1}) were found to be optimum (Ouyang et al. 1973). Increasing thiamine to 1 mg l^{-1} was also found to be advantageous (Junwen 1986). Thus, it was seen that the effects of these hormones were complicated. The influence of these hormones on culture not only depended upon their concentration and mutual ratio, but were also related to the concentration of iron salts, sugar, and other components in the medium.

Physical Environment

The relevant factors in the physical environment are many. These include the physical form of the medium, i.e., whether solid or liquid, pH, humidity, light, and temperature. These factors can have a profound effect on organogenesis. The light factor involves a combination of several components such as light intensity, daily light period, and quality. Although the light requirement for photosynthetic activity is minimal, it may be required for certain photo morphogenic events. In triticale, Bernard (1980) reported a 2.25-fold increase in embryoid induction in darkness, as compared with light incubation at 3000 lux. In wheat, Bjarnstad et al. (1989) reported that high-intensity light during the incubation strongly suppressed induction but stimulated regeneration of green plants in recalcitrant genotypes. Weak diffuse light did not inhibit induction. However, the positive effect on regeneration was maintained. Raina et al. (1987) maintained an incubation temperature of 23 +1°C and 2000 lux light intensity for regeneration in rice and Datta et al. (1990) maintained a photoperiod of 16 h light, at 4000 lux light intensity and at 25°C. However, Cornejo-Martin and Primo-Millo (1981) and Manimekalai and Sree Rangasamy (1987) reported that dark incubation gave a better response.

The position of the anther at plating also influences anther callusing in rice (Mercy and Zapata 1987) and in barley (Powell et al. 1988). Jones and Petolino (1988) observed that liquid regeneration medium produced twice as many embryos when compared to agar-solidified medium. Addition of Ficoll to liquid medium further increased the response. They also observed that addition of activated charcoal resulted in increased response in wheat anther culture.

Albinism

Albinism is a serious problem in cereal anther culture (Nitzshe and Wenzel 1977). Many factors have been found to affect the degree of albinism. The frequency of albino plants seems to be genotype-dependent (Yang 1988). Wang et al. (1978) observed the presence of proplastids in leaf cells of albinos and Sun et al. (1978) reported the absence of ribosomes and fraction I protein. No definite relationship could be established between the albinos and media components (Chu 1982). However, Feng and Ouyang (1989) reported that a higher concentration of KNO_3 (35 mM) in CI medium increased the green plant to albino ratio in wheat anther culture. In rice, use of high sucrose concentration (9 to 12%) (Woo and Chen 1982; Mercy and Zapata 1986), high level of 2,4-D or NAA (10 mg l^{-1}) (Chen 1983), higher pretreatment temperatures of panicles at 35°C for 3-5 days (Qu and Chen 1983), higher incubation temperature of more than 25°C (Song et al. 1978), and the physical environment of the donor plant (Huang et al. 1983) seem to increase the frequency of albinos. On the other hand, low pretreatment temperature (10°C), 6 times higher strength of iron salts in N6 medium (Guo 1983) seem to increase the frequency of green plants. Day and Ellis (1984) reported that plastids in most of the albino plants have deletions in their genomes up to 80%. During the development into binucleate pollen, the number of ribosomes in the proplastids decreases and completely disappears in the binucleate pollen. Chen et al. (1988) observed variation in the plastids of albino plants. These include amoeboid plastids with different shapes and structures, amyloplasts with numerous starch grains and prolamella-like bodies and

membrane dilution in grains, and plastoglobuli varied in shape and size. These seem to explain the occurrence of albinos in anther culture.

Isolated Pollen Culture

This technique offers many opportunities for production of haploids for breeding and in studies relating to androgenesis, mutagenesis, genetic manipulation, and transformation. The success rate was very low in rice (Chen et al. 1980) and in maize (Coumans et al. 1989). Cold pretreatment at 10°C for 10 to 15 days was found to be helpful (Chen 1983). Preculture was found to be important for most of the pollen to undergo division and subsequent regeneration (Cornejo-Martin and Primo-Millo 1981; Chen 1986a).

Float Anther Culture

Float anther culture was first successfully attempted in *japonica* rice by Chen et al. (1979). Zapata et al. (1983) reported high frequency anther callusing in rice by float culture. However, the regeneration efficiency of calli was lower than that obtained from semi-solid medium (Chen 1983). Recently, Chen (1986a) observed that liquid medium containing 20% potato-extract improved callus induction as well as green-plant regeneration. Datta et al. (1990) regenerated plants through microspore embryogenesis from *indica* and *japonica* varieties. The response was higher in *japonica* varieties. Through secondary embryogenesis, direct embryo germination and more frequent regeneration with multiple tillers have been obtained.

Direct Pollen Plants

In rice anther culture, there have been problems of regeneration and instability associated with the callus phase. Ouyang et al. (1983) succeeded in inducing direct plants from rice pollen, but the response was very low. Several hormone combinations involving NAA, Kin, and/or BAP were tested (Raina 1989) and direct plantlet formation was occasionally observed. Ling et al. (1984) and Song and Li (1986) claimed direct pollen plantlet production. However, in this case both pollen calli formation and subsequent regeneration occurred in the same media. For the first time in our laboratory, direct androgenesis (embryogenesis directly from the pollen grains) was observed in some of the hybrid combinations (e.g., IR 50 × Ponni) when cultured in modified N6 media supplemented with low concentrations of BAP (Manimekalai and Sree Rangasamy 1987). However, reproducibility was poor.

Ovary Culture

In cereals, San Noeum (1976, 1979) was the first to succeed in inducing haploid barley by means of ovary culture. Subsequently, haploid plantlets were raised from cultured ovaries of wheat (Wu and Chen 1982) and rice (Asselin de Beauville 1980; Zhou and Yang 1980; Wu and Chen 1982) barley (Huang et al. 1982) and corn (Ao et al. 1982). Embryological studies traced the origin of proembryo and calli from the embryo sac (Zhou and Yang 1981). Further investigation by Yang et al. (1984) revealed that gynogenic embryoids originated mainly from the synergids. The induction frequency in ovary culture (1.5-12% in japonica) was usually lower than that of induced pollen grains. Most of the ovary-culture-derived plants were haploids (Zhou and Yang 1981). The optimal stage for inoculation ranged from uninucleate to tetra-nucleate embryo sacs, corresponding to late uninucleate to early binucleate pollen stage. Best results were obtained when an unhusked flower with intact pistil and glumes attached to a piece of receptacle was cultured as a unit (Chang et al. 1986). For callus induction, 2-methyl-4-chlorophenoxyacetic acid (MCPA) at a concentration of between 0.125 and 0.5 mg l^{-1} was found optimum. He and Yang (1987) reported that picloram promoted embryoid differentiation in rice ovary culture. With improvement in the culture technique, ovary culture in unfertilized rice appears to be a potential tool for haploid and dihaploid production for utilization in crop improvement (Zhou et al. 1986).

Somatic Embryogenesis and Regeneration

Shoot and plantlet regeneration has been achieved from different somatic tissues in rice (Le et al. 1989; Rush 1987; Gupta et al. 1989; Mikami and Kinoshita 1988), in wheat (Purnhauser et al. 1987; Bapat et al. 1988; Redway et al. 1990; Quareshi et al. 1989), in barley (Abarbanell and Breiman 1989; Breiman et al. 1987; Coppens and Devitte 1990), and in maize (Duncan et al. 1985; Swedland and Locy 1988; Pareddy and Petolino 1990).

Abe and Futsuhara (1986) examined 60 rice cultivars, and reported that only a few of the *indica* and

japonica varieties, and *japonica* × *indica* hybrids showed shoot regeneration potential. Strong genotypic effect on the capacity and frequency of plant regeneration was also observed in wheat (Carman et al. 1987; Bapat et al. 1988), and in maize (Close and Ludesmon 1987). A considerably reduced level of 2,4-D, or substitution by weak auxins such as IAA or NAA, favored shoot formation in rice (Le et al. 1989; Rush 1987; Jun 1987; Ling and Yoshida 1987; Gupta et al. 1989), in wheat (Quareshi et al. 1989), and in maize (Swedland and Locy 1988; Pareddy and Petolino 1990). The frequency of shoot, bud, and plantlet differentiation could be further increased in the Kin-supplemented medium (Ling et al. 1984). Ling and Yoshida (1987) reported that 2,4-D (1 mg l^{-1}) and Kin (1 mg l^{-1}) are necessary for somatic embryogenesis and a lower concentration (0.2 mg l^{-1}) is needed for explant development. Addition of 0.136 mg l^{-1} ABA in subculture medium maintained embryogenic calli. Mutagenic treatment with diethylsulphate and N-methyl-N.nitrosourea reduced regeneration frequency (Maddumage et al. 1989). Addition of tryptophan (50 or 100 mg l^{-1}) or dicamba (1 mg l^{-1}) induced a high frequency of green-plant regeneration. Songstad et al. (1988) suggested the use of ethylene inhibitors (norbornadiene and silver nitrate) to increase plant regeneration. Carman et al. (1987a and b) observed that 2,4-D (3.62 μM) or dicamba (9.05 μM) is required to induce and maintain embryogensis. Kin at 4.65 μM together with 2,4-D or dicamba increased the embryoid formation significantly. Addition of either 6-furfuryl-aminopurine or CW increased precocious germination of both embryo explants and embryoids. Ling and Yoshida (1987) reported that 6% sucrose was required for embryogenesis. However, Gupta et al. (1989) regenerated plants on a hormone-free N6 medium. Redway et al. (1990) observed that "aged callus" supplemented with 2,4-D, CH, and glutamine upon further subculture, formed friable embryonic calli. Chu et al. (1987) regenerated plants from 1½ year-old callus culture via somatic embryogenesis. Ozawa and Komamine (1989) established a system for high-frequency embryogenesis from long-term cell suspension culture of rice. Sticklen et al. (1989) reported the possibility of converting non-embryogenic calli to embryogenic calli by media conditioning.

The progress in cell and tissue culture research in sorghum has been presented in detail in the reviews of Smith and Bhaskaran (1986) and Kresovich et al. (1987). In sorghum very young tissues close to a meristematic state—immature panicle, immature embryo, and seedling—have proven responsive in culture. Along with the above explants, in our laboratory we found that tissues like stem pith, young leaves, nodal plate sections, anthers, and ovaries are also responsive. Successful callus formation from immature embryos has been reported by Gamborg et al. (1977) and Brar et al. (1979). Kresovich et al. (1986) and Smith and Bhaskaran (1986) reported callus initiation from young seedlings. Callus has also been initiated from unemerged, immature inflorescences (Brettel et al. 1980; Boyes and Vasil 1984; George and Eapen 1988; Eapen and George 1990) and immature leaves (Wernicke and Brettel 1980; Wernicke et al. 1982).

An efficient and repeatable, high-frequency regeneration system is necessary not only to study the changes occurring during in vitro culture but also for genetic manipulation in crop improvement programs. However, so far no medium has been found suitable for regeneration of all varieties. Gamborg et al. (1977) observed morphogenesis and plant regeneration from immature embryos (10-15 days post pollination) of sorghum hybrid 4004A. Brar et al. (1979) reported somatic embryogenesis from immature embryos of sorghum hybrids GPR.148, NK-300, and 4004A. Ma et al. (1987) highlighted the responsiveness of 9-to-12-day-old embryos. Kresovich et al. (1987) reported that the overall embryogenesis across genotypes ranged from 26 to 69%. The frequency of response for embryos from individual panicles within a genotype was as high as 95%. Kresovich et al. (1987) observed that a high degree of phenotypic plasticity existed for in vitro responsiveness. Ma et al. (1987) reported that the ability to differentiate plants from callus was heritable and controlled by two gene pairs and acted as a dominant trait. Regeneration from mature embryos was reported by Thomas et al. (1977) and El'Konin et al. (1986).

Brettell et al. (1980) obtained somatic embryogenesis from cultured immature inflorescences of several sorghum cultivars and hybrids including CK 60, Regular Hegari, Plainsman, WAC 692, CS22DR, FS 302, and G83F. The inflorescence 10 to 20 mm in size, yielded the greatest frequency of embryogenic cultures compared to those 10 to 50 mm. However, it was not consistent across genotypes (Brettell et al. 1980). Successful results of somatic embryogenesis from young inflorescence was reported by Eapen and George (1990), Lusandi and Lupolto (1990), and Cai et al. (1990). Wernicke and Brettell (1980) and Wernicke et al. (1982) reported somatic embryogenesis from immature leaves of G522DR.

In sorghum, Kresovich et al. (1987) established biochemical differences discriminating embryogenic

from nonembryogenic calli, that can be utilized as a rapid screening tool for responsive cell types. They reported differences in esterase isozyme patterns between embryogenic and nonembryogenic calli of RTx430 and Regular Hegari. In our laboratory, studies on peroxidases and isoesterases indicated the change in the banding pattern during dedifferentiation and redifferentiation in the three genotypes studied (CO 26, CO 27, and CSH 5). Both peroxidase and esterase markers for embryogenic calli were obtained in all these genotypes (Kumaravadivelu 1990). Maheswaran and Sree Rangasamy (1988) reported that esterase isozymes could be used as markers in organogenesis.

Bajaj and Dhanju (1981) and Haydu and Vasil (1981) induced embryogenic callus from leaf fragments of *P. purpureum* and obtained viable plants that were cultivated in the glasshouse and in fields. Bui-Dang and Pernes (1987) have obtained a large number of plants of the hybrid Massue × Ligai by microspore culture. The results of in vitro responses in *Pennisetum* are listed in Table 1.

Finger Millet (*Eleusine coracana* (L.) Gaertn.)

In finger millet, callus has been induced from the first leaf when 0.5-0.6 cm in length. The in vitro responses of varities HPB 7-6 and ROH 2 were studied. The medium suitable for leaf callus was B5 supplemented with NAA (3×10^{-6} M). No organogenesis was obtained (Nandini and Mohan Ram 1980). Callus was also produced from mesocotyl segments and coleoptile cut ends but showed no differentiation. Mohanty et al. (1985) successfully initiated callus from leaf base segments of 3 to 4 day-old seedlings. Callus was also derived from mesocotyl segments on MS and B5 with 2,4-D (2.0 mg l^{-1}) and casein hydrolysate (500 mg l^{-1}). Regeneration was obtained from MS supplemented with 2,4-D (0.25 mg l^{-1}). Enlarged apical domes were developed (Wakizuka and Yamaguchi 1987). Callus was obtained on MS supplemented with 2,4-D (3.0 mg l^{-1}) and Kin (0.1 mg l^{-1}) with casamino acids (500 mg l^{-1}) and 5.0% coconut water. Regeneration was obtained from MS + 2,4-D (0.1 mg l^{-1}) + Kin (0.2-0.5 mg l^{-1}). Ramesh and Farook (1988) developed methods for callus culture from leaf explants and obtained regeneration. Prasanna et al. (1990) reported induction of embryoids and plantlet formation. Eapen and George (1989) reported somatic embryogenesis and plantlet formation from seed callus.

Somaclonal Variation and its Significance

Due to in vitro culture processes, abnormalities have been detected in plants regenerated from undifferentiated callus. There has been considerable interest in the assorted stable genetic changes induced in these plants. These heritable variations from somatic tissue have been termed somaclonal variation (Larkin and Scrowcroft 1981). The variation from cell cultures can be grouped into gametoclonal and somaclonal variation.

In anther, pollen, and ovary culture, different types of chromosomal changes in in vitro regenerated plants have been reported. The ploidy status of pollen plants of *indica* rice was different from that of *japonica* rice (Ying 1986). The frequency of haploids and dihaploids was almost the same and, in some cases, 50-60% were dihaploids (Oono 1978). However, the frequency of other polyploids ranged from 5-10%. Chu and Zhang (1985) obtained 11.4% aneuploids; 6.7% were primary trisomic (2n+1), 0.7% were double trisomic (2n+1+1), 1.7% were tetrasomic (2n+2), 1.3% were monosomic (2n-1) and 1%, were nullisomic (2n-2).

In addition to chromosomal variation, much genetic variability in pollen plants has been reported. The rate of gene mutation was higher in haploids. The recurrent variations observed in in vitro cultures were earliness of growth, dwarfness, and changes in fertility (Chen 1986b). Some morphological aberrations such as dwarfness, twisty raches branches, and very low fertility also occurred in pollen plants derived from F_1 hybrids (Ying 1986); and these traits were heritable in the A_2 also. Phenotypic variation among the plants regenerated from anthers has been reported in rice (Ying 1986) and other cereals.

In somatic cell culture, the regenerants also showed phenotypic variation. However, the rate of variation is less than in anther/pollen culture. Several cell lines resistant to salt have been identified (Wong et al. 1986; Abrigo et al. 1985). Somaclonal variation for several biometrical traits has been reported in rice (Raina et al. 1989). Rush (1987) observed that the rate of somaclonal variation was cultivar-dependent. Jun (1987) observed that the rice somaclones outyielded their parent by 12%. Le et al. (1989) observed significant variation for rice grain length, weight, and qualities such as gel consistency, amylose content, gelatinization, cooking temperature, and chalkiness of endosperm. Jacquemin and Dubois (1988) observed variation in yield and protein content in wheat. Ling et al. (1985) screened somaclones for *Helminthosporium oryzae* toxin and identified one resistant variant.

Table 1. Results of in vitro culture response in *Pennisetum*.

Species	Explant	Medium	Culture	Growth	Reference
P. typhoides	Mesocotyl 5 days old	MS+2,4-D (10 mg l^{-1}) + CW (15%) + IAA (0.2 mg l^{-1}).	25+2°C diffuse light	Plants	Rangan (1976)
P. purpureum	Young inflorescence	MS+2,4-D (5 mg l^{-1})	2,4-D free medium	Plants	Bajaj & Dhanju (1981)
P. glaucum	Immature embryo	MS+2,4-D (2.5 mg l^{-1}) +Thiamine (5 mg l^{-1})	27°C diffuse light 16 h	Scutellar callus	Vasil & Vasil (1981a,b)
	Callus	MS+2,4-D (2.5 mg l^{-1}) + CS (5%)	27°C in dark	Embryoid suspension	
	Embryoid	MS+IAA (0.4 mg l^{-1}) adenine (80 mg l^{-1}) + Zeatin (1 mg l^{-1})	27°C diffuse light 16 h	Plants	
P. purpureum	Leaf Young inflorescence	MS+2,4-D (0.5 mg l^{-1}) + BAP (0.5 mg l^{-1}) NAA (0.1 mg l^{-1})+ CM (0.5%)		Callus Embryoids	Haydu & Vasil (1981)
Somatic embryogenesis					
P. glaucum	Immature embryo	LS+2,4-D (11.3 mg l^{-1}) + CW (5%)		Somatic embryos & plants	Vasil & Vasil (1980a)
P. glaucum	Immature inflorescence	MS+2,4-D (2.5 mg l^{-1}) + CW (5%)		Somatic embryos & plants	Vasil & Vasil (1981a)
P. glaucum × *P. purpureum*	Immature inflorescence	MS+ABA (0.4 mg l^{-1})		Plants	Vasil & Vasil (1981b)
P. purpureum	Leaf	MS+2,4-D (2.3 mg l^{-1}) NAA+(5-4 mg l^{-1})+BA (2.3 mg l^{-1})+CS (5%)		Somatic embryos & plants	Haydu & Vasil (1981)
Protoplast culture					
P. glaucum	Immature embryo protoplast cell suspension	LS+2,4-D (2.5 mg l^{-1}) CW (5%)	27°C in dark	Callus	Vasil & Vasil (1980b)
P. glaucum	Hypocotyl 3 days old protoplast cell suspension	LS+2,4-D (0.25 mg l^{-1}) + NAA (7 mg l^{-1}) + Kinetin (0.01 mg l^{-1})	27°C in dark	Callus	Vasil & Vasil (1979)
	Young inflorescence protoplast cell suspension	MS+2,4-D (2.5 mg l^{-1}) liquid medium		Plants	
Disease Resistance					
P. glaucum	Seed callus	MS+2,4-D (5 mg l^{-1})	Callus grown on culture filtrate of ergot	Differentiated callus growth	Bajaj et al. (1980)
HB 3	Diseased immature	MS+IAA (3.5 ppm)	Callus growth	Few plantlets	Bhagyalakshmi et al (1984)
P. glaucum	Peroxidase isozyme pattern			Isoperoxidase imparting resistance	Chahal et al. (1988)
Salt tolerance					
P. glaucum	Young inflorescence	MS+2,4-D (2.5 - 5 mg l^{-1})	Callus grown on medium containing NaCl 0.1-1%	Salt tolerant cell lines. *P. purpureum* more tolerant than *P. glaucum*	Bajaj & Gupta (1986)

In sorghum, somaclonal variation was reported for leaf morphology by Gamborg et al. (1977) and Ma et al. (1987). Plants derived from callus culture initiated from immature embryos exhibited heritable variation in height, waxy midrib, and male and female sterility. In Chinese sorghum genotype C401-1, somaclonal variation for seed number, chlorophyll content, shoot weight, plant height, days to flowering, and yield was reported by Bhaskaran et al. (1987).

MacKinnan et al. (1986) evolved selection methods for derivation of NaCl-tolerant plants of Regular Hegari, Keller, and Rio. Many plants were obtained from selections on semi-solid rather than liquid medium. Bhaskaran et al. (1986) observed differences between selected and non-selected progeny with reference to enhanced tolerance to 0.1 to 0.5% NaCl. Plants tolerant to acid soil, $AlCl_3$, NaCl, and drought were reported by scientists at Colorado State University (TCCP 1987) and by Duncan and Widholm (1989).

Protoplast Culture

Of late, protoplast isolation, culture and regeneration is being taken up in an intensive manner. This technique is very useful for transferring cytoplasmic male sterility, for obtaining hybrid vigor through mitchondrial recombination, and for genetic transformation/engineering in plants. Although limited success in plant regeneration from protoplast culture of cereals has been obtained in the past decade (Vasil and Vasil 1984), in the past 3 years progress has been achieved in plant regeneration from protoplasts of rice (Abdullah et al. 1986). In *indica* rice Kyozuka et al. (1988) could not develop suspension cultures while Dayuan et al. (1989) developed suspension cultures from which protoplasts were isolated, but further cell division could not be induced. Various reports of success on plant regeneration from rice protoplasts have come from several countries (Raina 1989). The various reports on protoplast culture in cereals are listed in Table 2.

Research reports on sorghum protoplast culture are very limited. Brar et al. (1979) reported the establishment of sorghum protoplast culture. Karunarathne and Scott (1981) obtained protoplasts from leaf tissues. Protoplast fusion studies were conducted between rice and sorghum (Bajaj 1983) and sorghum and corn (Brar et al. 1980). Murthy and Cocking (1988) isolated protoplasts of sorghum, but no regeneration was obtained. Wei and Xu (1990) isolated protoplasts from embryogenic suspension cultures derived from immature inflorescences of two cultivars of *Sorghum vulgare*. The cells divided after 4-5 days. Later procalli formed, followed by whole plant regeneration. Sixty plants were obtained along with only two albinos. The plants were grown in soil to maturity and produced seed. Transient gene expression in sorghum protoplasts was reported by Qu-Lee et al. (1986). In pearl millet, Vasil and Vasil (1981a) obtained protoplasts from embryogenic cell suspension cultures, that differentiated into callus and regenerated. Regeneration was also obtained from cultures of young spikes and immature embryos of *P. typhoides* × *P. purpureum* hybrids by somatic embryogenesis. Lorz et al. (1981) also regenerated plants from protoplasts obtained from embryogenic suspensions.

Table 2. Reports of successful culture and regeneration in cereals.

Crop	Author	Source of protoplasts[1]
Rice	Fujimura et al. 1985	CS
	Abdullah et al. 1986	CS
	Coulibaly & Demarly 1986	C
	Toriyama et al. 1986	CS
	Yamada et al. 1986	CS
	Kyozuka et al. 1987	CS
	Finch et al. 1989	CS
	Hodges et al. 1989	CS
	Nayak and Sen 1989	CS
	Tan et al. 1989	CS
	Zu et al. 1989	CS
	Wang et al. 1990	C
Maize	Ludwig et al. 1985	C
	Hodges et al. 1986	CS
	Imbrie-Millingan et al. 1987	C
	Rhodes et al. 1988	C
	Sun et al. 1989	CS
Wheat	Maddock 1987	CS
	Hayashi and Shimamoto 1988	C
	Adachi et al. 1989	PP
Barley	Luhrs and Lorz 1988	C
Sorghum	Brar et al. 1980	PP
Triticale	Stolarz et al. 1986	C
Rye	Dalton 1987	C

1. C = callus; CS = cell suspension; PP = plant part.

Progress at Tamil Nadu Agricultural University (TNAU)

Anther Culture

Our studies with rice anther culture have been concerned not only with the *indica* parents but also with *japonica* lines, *indica* × *indica* hybrids and *indica* × *japonica* or *japonica* × *indica* derivatives. The various factors influencing anther culture have been investigated. The results in our laboratory confirm the influence of genotype on anther culturability. In general, the response of the various genotypes is in the following order: *japonica indica* × *japonica* or *japonica* × *indica indica* × *indica indica*. The callus induction frequency varied among the *indicas* (11-30%), *japonicas* (22-26%), *indica* × *indica* (8-17%), *indica* × *japonica* (17-21%), and *japonica* × *indica* (20-23%). The regeneration efficiency also varied among *indicas* (80-98%), *indica* × *indica* (79-83%), *japonica* × *indica* (75-83%), and *indica* × *japonica* (62-89%). The frequency of albinos produced also varied with genotype (Manimekalai and Sree Rangasamy 1987; Narasimman et al. 1990) Plate I. The effect of six media—N6, modified N6, MS, modified MS, B5, and potato extract medium—on callus induction was tried. The results suggested that modified N6 medium gave the highest response (28%), followed by N6 medium (20%). Dark incubation was found to give better response (18.6%) than light incubation (9.6%) (Manimekalai and Sree Rangasamy 1987). Cold pretreatment at 10°C for 10-12 days gave better response (Manimekalai and Sree Rangasamy 1987; Narasimman, 1989). Sucrose at 6% was found optimum for callus induction and green plant regeneration (Narasimman 1989). Among the hormones, 2,4-D (1.5-2.0 mg^{-1}) and Kin (0.5 mg^{-1}) have induced higher frequency of embryogenic calli. IAA (1.0 mg l^{-1}), BAP (1.0 mg l^{-1}), and YE (500 mg l^{-1}) were found essential for regeneration. Embryoids were formed in MS medium supplemented with coconut milk and thiamine. A higher level of cytokinin without auxins promoted faster and quicker multiplication of embryoids. IAA and NAA were found to favor shoot and root development from the embryoids (Manimekalai and Sree Rangasamy 1987 and 1988). Androgenesis was observed in some of the hybrid combinations (e.g., IR 50 × ARC 6650) when cultured in modified N6 medium with a low level of BAP (Manimekalai and Sree Rangasamy 1988). Among the regenerants, variations in chromosome number from haploid to tetraploid were observed (Manimekalai and Sree Rangasamy 1987). However, in some genotypes dihaploids were predominant while in others, haploids predominated. Trisomic plants were observed in very low frequency (1.0%) (Manimekalai and Sree Rangasamy 1981, 1987, and 1988). The dihaploids were stable and homozygous as revealed by progeny tests in the field (Manimekalai and Sree Rangasamy 1987). Significant variation was observed in quantitative traits such as plant height, tiller number, panicle length, grains per panicle, and grain weight per plant (Sree Rangasamy et al. 1987). The anther-derived lines from Ponni were found to be useful as an alternative genetic source for short stature in rice breeding (Sree Rangasamy et al. 1988) (Plate 2). Three anther-derived cultures from F_2s of Vaigai × CO 40 (developed in a TNAU-IARI collaboration) were semidwarf, medium duration, and photoperiod insensitive. Culture 433-A-R1 is resistant to blast and rice tungrovirus (RTV); 433-A-R5 is resistant to blast and moderately resistant to RTV and bacterial leaf blight (BLB); and 433-A-R6 is resistant to blast and moderately resistant to RTV, BLB, and brown spot (Sree Rangasamy et al. 1989) (Plate 3).

In vitro culturability of sorghum anthers was studied by Gnanam (1987). She reported that no visible callus or embryogenic activity was observed up to 45 days after inoculation of uninucleate microscpores in N6 medium with 2,4-D at diferent levels.

Anther response to callusing, embryoid induction, and regeneration of plantlets was studied in sorghum hybrid CSH 5 and fodder variety CO 27 (Kumaravadivelu and Sree Rangasamy 1989). Successful callus induction (16%) was obtained in N6 medium with 2,4-D at 2-3 mg l^{-1}. Incubation of anthers at an elevated temperature of 30°C for 30 days triggered callus induction and splitting of anther lobes. Haploid and dihaploid plants were regenerated and transferred to the glasshouse.

In the second generation of the dihaploid lines, variability within lines was nonsignificant indicating the immediate fixation of alleles. The homozygous banding pattern for the esterases in the dihaploid lines of the hybrid CSH 5 indicated the possibility of identifying dihaploid lines in the plantlet stage. High-yielding lines were advanced for further testing.

Somatic Cell Culture

Maheswaran and Sree Rangasamy (1989) reported genotypic variation for callus induction from mature seeds as well as for regeneration. Among the five genotypes (*O. spontanea*, *O. glaberimma*, and *O. sativa*

cultivars IR 50, CO 43, and IR 1522), the regeneration efficiency ranged from 0 (CO 43) to 28.5% (*O. spontanea*). A concentration of 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kin was found to give better callus induction (59.7%) and subsequent regeneration (40.9%) in MS medium supplemented with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kin.

Krishna Raj (1989) and Narayanan and Sree Rangasamy (1990a and b) stressed the seed calli of IR 20 and IR 50 in vitro at 3000, 6000, and 9000 ppm of NaCl for 5 to 6 cycles and regenerated plants from the resistant calli. We also succeeded in selection of regenerant progenies, from calli stressed in salinized medium, that survived screening at the R_1 seedling stage at 6000 ppm NaCl.

Among the different explants of sorghum — young leaves, immature inflorescences and immature embryos — callus induction varied from 50 to 55% for young leaves of 45-day old sorghum plants, 80 to 85% callus induction from immature inflorescence and immature embryos, and 20 to 25% from anthers (Gnanam 1987; Veera Ragavan 1988; Kumaravadivelu and Sree Rangasamy 1989).

Callus induction depends on a reliable protocol to overcome the exudation of phenols in culture. Gnanam (1987) reported that cold pretreatment of explants for 3 days followed by 1% ascorbic acid treatment for 15 min and dark incubation for 4 days arrested the exudation of tannins that interfered with callus induction and plant regeneration. Presoaking explants for 1 to 1½ h in liquid MS medium was found to leach out the tannins and increase frequency of callus induction in grain sorghum variety CO 26 and hybrids CSH 5 and COH 3 (Kumaravadivelu 1990). Addition of 0.2 to 0.4 g l^{-1} activated charcoal or doubling the quantity of Fe-EDTA and thiamine also increased the callus induction frequency (Kumaravadivelu 1990). MS medium modified with B5 vitamins and double the concentration of Fe-EDTA and thiamine was found to give the best response (Gnanam 1987; Kumaravadivelu 1990). The phytohormones, particularly auxins, play a major role in callus induction when added along with very low concentrations of cytokinins. 2,4-D at 2 to 2.5 mg l^{-1}, with 0.3 to 0.5 mg l^{-1} of BAP increased the frequency of callus induction in all the explants (Gnanam 1987; Veera Ragavan 1988; Kumaravadivelu 1990).

Regeneration and establishment of plants was obtained from immature inflorescence (75-80%), young leaf (50-70%), and immature embryo (60%) (Gnanam 1987; Veera Ragavan 1988; and Kumaravadivelu 1990). Morphogenesis and regeneration of plantlets took 100 to 120 days.

Pearl Millet

The in vitro response of different explants—seed, immature embryo, and inflorescence—of 11 genotypes was studied on a range of media. Callus induction frequency ranged from 40 to 80%, regeneration from 20 to 50%. The best callus-induction medium was N6 supplemented 2,4-D (2.0 to 2.5 mg l^{-1}) and Kin (0.5 mg l^{-1}). The best regeneration medium was MS supplemented with BAP (1 mg l^{-1}) and NAA (0.5 mg l^{-1}) (Table 3, Plate 6). Addition of adenine increased embryoid differentiation and regeneration frequency. Three per cent sucrose was most suited for callus induction, and 1.5% sucrose for regeneration. Genotype and explant differences were observed for callusing and regeneration (Table 4). Cold treatment of the inflorescences at 10°C for 1 to 7 days did not improve regeneration frequency.

Studies on the in vitro response of seeds, leaves, inflorescences, and roots of four finger millet varieties, CO 9, CO 11, CO 12, and CO 13—revealed that

Table 3. Culture media used at TNAU for different explants in pearl millet.

	Media composition	
Explant	Callus induction	Regeneration
Seed	MS+2,4-D (2.5 mg l^{-1})	MS+BAP (2-2.5 mg l^{-1})
Immature embryo	MS+2,4-D (2-2.5 mg l^{-1})	MS+Kn (1-2 mg l^{-1}) + IAA (0.5 to 1 mg l^{-1})
Inflorescence	N6+2,4-D (2.5-5 mg l^{-1}) +Kn (0.5 to 1 mg l^{-1})	MS+BAP (0.5 to 1 mg l^{-1}) +NAA (0.1 to 0.5 mg l^{-1})

Table 4. Regeneration efficiency in pearl millet.

Genotype	Explants					
	Seed		Immature embryo		Inflorescence	
	CI[1]%	Regn[2]%	CI%	Regn%	CI%	Regn%
UCH 11	60	40	65	39	80	60
PT 3075	59	38	60	40	75	55
PT 3095	55	35	53	38	74	50
PT 1921	50	30	50	35	65	48
PT 1890	48	30	45	28	60	40
ICMS 7703	48	40	45	28	60	40
CO 7	45	30	43	25	55	39
732 A	40	25	40	25	50	34
732B	43	27	43	25	55	35
81A	40	23	40	20	50	25
81B	45	25	40	23	55	30

1. CI - Callus induction
2. Regn - Regeneration

callus browning could be reduced by addition of casein hydroloysate. Callus induction occurred on MS with 2.0-2.5 mg l^{-1} 2,4-D and 0.4-1.0 mg l^{-1} Kin. Supplementation of MS medium with 1.5 to 2.0 mg l^{-1} of BAP and 1.0 mg l^{-1} of IAA favored regeneration (Table 5). Sucrose at 3% was suitable for callus induction and regeneration. More than 100 embryoids were recovered from the callus. Roots were induced on ½ MS basal medium. CO 11 responded well for callus induction and regeneration (Kalamini et al. 1991).

Pearl Millet Napier Grass Hybrid (*P. glaucum* × *P. purpureum*)

The triploid interspecific pearl millet Napier grass hybrid is now an agriculturally established and commercially grown, multicut, perennial green fodder crop that is sterile. Owing to the high sterility, it is vegetatively propagated, and so variability obtained can be easily preserved. Shirly (1985) investigated pith, leaf, and young inflorescence for their morphogenetic potential. It was found that the unemerged inflorescence was more totipotent than other explants studied. MS medium was found to be the most suitable. Supplemental 2,4-D was effective for callus induction, NAA for rooting, and BAP for shooting. Of 720 regenerated plants, 542 were from inflorescence. Distinct morphological variations were observed for leaf width, midrib color, waviness, and color of panicle. Cytological studies indicated 7 II + 1 I in a few regenerants, which is due to selective elimination of B genome. The study on quantitative traits such as plant height, number of tillers, number of leaves, leaf size, internode number, and stem thickness of regenerated plants revealed a high degree of variation.

Among the 11 genotypes, UCH 11 responded very well for callus induction and regeneration, followed by genotypes PT 3075, PT 3095, and PT 1921. Field survival of the regenerants was 50%. Among the 3 explants tried, the immature inflorescence was found to be the best for a high frequency of callus and regeneration. Cold treatment of the inflorescence at 10°C for 1 to 7 days did not improve the regeneration frequency.

Somatic embryoid differentiation in the inflorescence explants is such that 200-300 embryoids could be recovered from each callus mass. Apart from this indirect somatic embryogenesis, 25% of the inflorescences directly differentiated into somatic embryoids without passing through the callus phase. These embryoids also regenerated into plantlets. If dormancy could be induced in somatic embryos, the possibility arises that they could be incorporated into artificial seeds either by coating or encapsulation. These artificial units could then be handled like normal seeds and stored, shipped, and planted. Because of their innate properties as embryos, somatic embryos may prove useful for long-term storage such as in germplasm banks. The result of in vitro response in *Pennisetum* is reviewed in Table 1.

Table 5. In vitro response in finger millet.

Variety	Explant	Best CI[1]	Best RM[2]	CI		RM	
				Range	Mean	Range %	Mean %
CO 9	Seeds	MS+2.5 2,4-D + 0.5 kin MS+3.0 2,4-D + 0.4 kin	-	0 - 34	10.3	-	-
	Leaf	MS+2.0 2,4-D	-	0 - 27	2.8	-	-
	Inflorescence	MS+2.5 2,4-D	-	0 - 49	5.7	-	-
CO 11	Seeds	MS+2.0 2,4-D + 0.5 kin	MS+2.0 mg l^{-1} BAP + 1.- mg l^{-1} IAA	0 - 38	16.0	0 - 48	28.1
	Leaf	MS+2.0 2,4-D +0.5 kin	MS+2.0 mg l^{-1} BAP +1.0 mg l^{-1} IAA	0 - 55	5.6	0 - 34	19.5
	Inflorescence	MS+2.0 2,4-D +0.5 kin	MS+2.0 mg l^{-1} BAP +1.0 mg l^{-1} IAA	0 - 84	15.2	0 - 46	24.8
CO 12	Seeds	MS+2.0 2,4-D +1.0 kin	-	0 - 72	11.3	-	-
	Leaf	MS+2.0 2,4-D +1.0 kin	-	0 - 27	3.2	-	-
	Inflorescence	MS+2.0 2,4-D	-	0 - 42	7.4	-	-
CO 13	Seed	MS+2.0 2,4-D +0.5 kin	-	0 - 79	13.5	-	-
	Leaf	MS+2.0 2,4-D +1.0 kin	-	0 - 26	4.4	-	-
	Inflorescence	MS+2.0 2,4-D +0.5 kin	-	0 - 45	7.1	-	-

1. CI = callus-induction medium.
2. RM = regeneration medium.

Protoplast Isolation, Culture, and Regeneration in Rice

At TNAU, 3-week-old mature seed embryogenic callus was used to initiate cell suspensions. Subculturing once a week was vital to maintain cell viability and growth. One full growth cycle took 12 days. Protoplasts were successfully isolated on the 4th day of subculture when cells were in an exponential growth phase. Enzymes used were 1% Cellulase LS + 0.1% Macerozyme R-10, digested for 3 h on a 50 rpm shaker followed by 1 h stationary digestion. The protoplast yields were 1.05×10^6 ml^{-1}. Ninety-eight percent of the suspension cells were viable and 92% of the protoplasts isolated from them were viable (Reena, 1990).

Protoplasts were cultured by the feeder layer technique. Feeder cells used were Norin 6, Norin 8, IR 50 and Paspalum scorbiculatum. Protoplast division was first seen on the 5th day of culture and microcalli were seen within a month. Several techniques enhanced protoplast division; these were: exposure to a shock at 45°C for 5 min before culturing, presence of feeder cells, reduction in osmotic pressure of the medium, culturing in 0.8% agarose solidified medium, reducing ammonia and ferric ions, and addition of bovine serum albumin (BSA). The maximum plating efficiency achieved was 7%. Microcalli were nurtured in proliferation media for 15 days and were then transferred to regeneration media and plants were recovered (Plate 11).

Protoplast Culture in Finger Millet

Seeds of CO 11 were used for callus induction and protoplast isolation. Among the various media tried

for suspension culture of seed calli, AA and MS media with 2.0 mg l^{-1} 2,4-D was found to be most suitable. Good suspensions were established in 5 to 6 months. Protoplasts were isolated from callus, suspension cultures, and leaves. High protoplast yields were obtained from callus when treated with 2% Cellulase RS and 0.5% Macerozyme R10 on a shaker for 4 h. Stationary incubation for 6 h was adequate to get a maximum number of protoplasts when these were treated with 4% cellulase RS and 1% macerozyme R10. Protoplasts isolated from suspension cultures of cultivar CO 11 and plated on a feeder layer in protoplast-culture medium (KPR medium) gave rise to micro-colonies. Colonies transferred to MS with 2,4-D (2.5 mg l^{-1}) + Kin (0.5 mg l^{-1}) showed slow growth.

Conclusions

A number of potential techniques have been developed and some are becoming economically viable and of proven benefit. Embryo-culture techniques are now available for rescuing hybrid embryos from otherwise unsuccessful crosses. In the case of recalcitrant hybrid embryos, it is advisable to try indirect regeneration through callus cultures. The anther-culture technique is useful in breeding new rice varieties and several anther-bred varieties have been grown on a large scale in China. However, the anther-culture technique still suffers some drawbacks. Research efforts are directed at:

- enhancing the anther response and green-plant regeneration from calli;
- developing a stable and repeatable protocol for direct pollen plant induction through pollen embryogenesis; and
- reducing the occurrence of albinos.

Advances in plant molecular biology have generated renewed interest in the isolated pollen culture technique. Efforts are directed toward improving the efficiency of pollen culture.

Considerable progress has been achieved in somatic callus initiation and subsequent regeneration. Techniques are now available for regeneration from long-term cultures. Several in vitro mutants (somaclonal variants) have been identified for different biotic and abiotic stresses. Micropropagation techniques are available for rapid multiplication of elite genotypes, male-sterile lines, F_1 hybrids, and endangered species. Of late, protoplast technology has offered opportunities for genetic manipulation, gene cloning, transformation, and gene expression. Plant regeneration from protoplasts is being taken up in several laboratories in an intensive manner. Improved protoplast techniques will help to study gene cloning, transformation, and expression.

Initiation of organogenesis in vitro is a complex morphogenetic phenomenon in which extrinsic and intrinsic factors play a role. Studies over the past years have indicated that regulatory mechanisms operate at various levels, e.g., explant selection, medium components, exogenous hormone level, physiological status, cytological changes, and at the biochemical levels, but manipulation of medium or cultural environment and proper selection of material has resulted in great progress in the regeneration of cereals. We are still far from an understanding of how the organogenetic process is regulated. There are perhaps two major reasons for this slow progress. One probable reason has been the lack of truly suitable experimental systems for such studies; and the second reason has been the lack of synchrony in the process of organ formation in these systems (Thorpe and Bondi 1981). Without a much better understanding of the biochemical, physiological, physical, and structural basis of organogenesis, many recalcitrant species will remain as such, but biochemical and molecular approaches will give us an answer that can enhance the progress of in vitro modifications of cereals.

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Field Performance of Somaclones and Androclones in Sorghum Hybrid CSH 5

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We have standardized the protocols for high frequency callus induction and regeneration of the sorghum hybrid CSH 5 from young leaves, immature inflorescences and anthers. We have compared the progenies of the regenerants from leaves and inflorescences of somaclones (SC_2) and anther culture derived plants, androclones (A_2), with F_2. In the present study on sorghum hybrid CSH 5, 300 plants from 10 families each from F_2, SC_2, and A_2 generations were analyzed for the frequency distribution, extent of variability, h^2, and GA for seven traits. The range and variation for the biometrical traits in SC_2 was wider than in F_2. In A_2, the distribution was still wider than in SC_2. In F_2 generation, the plants were more frequently centered and occupied higher values of the traits. A similar trend was noted in SC_2 also. In SC_2, the wider range of variation than F_2 was due to the addition of in vitro culture induced variations. In A_2, the recessive genes also expressed directly.

The number of plants that can be selected in A_2 for high values of the economic traits was high compared to F_2 and SC_2 as is evident from the frequency distribution in the high values. The present study indicated (1) the possibility of in vitro generation of useful or economic variations for biometric traits (2) expression for the recessive genes in the immediate generation enables the basic genetic research besides selection (3) widest range of variation in both the direction in A_2 generation helps the breeder to select more efficiently for families with shorter duration and high LAI, DMP, HI, and grain yield, and (4) since the alleles are fixed in the A_2 itself, it quickens the process of crop improvement in Sorghum.

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Tissue Culture and Regeneration in Legumes

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Abstract

Legumes are one of the most significant groups of agriculturally important plants, and have consequently been the subject of widespread efforts to improve desirable traits through in vitro manipulations. Much effort has been expended to develop and optimize efficient regeneration systems in order to facilitate development of a variety of technologies. Despite the widely reported in vitro recalcitrance of legumes, at least 75 species from 25 genera have undergone de novo regeneration to date, and limited contributions to crop improvement have been realized. Successful regeneration has been accomplished in large part by species-specific determination of parameters critical to regeneration, such as explant source, genotype, and media constituents. In recent years, attention has focused on the development of regeneration systems amenable to gene transfer technology. Organogenic systems have been successfully employed in transformation of some legumes. More recently, indirect embryogenesis and direct secondary embryogenesis have shown great promise as prolific regeneration systems amenable to gene-transfer for those legumes which do not regenerate via organogenesis. The induction of embryogenesis in these systems generally requires the application of exogenous auxin. Initially, limitations in efficiency were a result of minimal conversion, as manifested by the inability of somatic embryos to develop, germinate, and progress to seedling growth. Improvement in the conversion ability of somatic embryos has been achieved for some species by limiting exposure to inducing auxins and including a desiccation period in the embryo maturation process. Presently, sufficient knowledge exists to design and optimize de novo regeneration systems for most legumes

Introduction[2]

Legumes are rivaled only by the grasses in terms of diversity and economic importance, and are grown throughout the world as sources of food, feed, oil, forage, fuel, wood, and even fiber. Legumes are also used as ornamentals, as green manure, and as ground covers to help control erosion. Not surprisingly, many species within this family have been the subject of efforts towards nonconventional plant improvement using cell-culture techniques. These efforts have resulted in a large array of in vitro protocols for legumes.

Legumes exhibit a diversity of responses when cultured in vitro. Depending on several factors, regeneration occurs via organogenesis and/or embryogenesis, either directly from explanted tissue or indirectly after an intervening callus phase. While several species are limited to either organogenesis or embryogenesis, others regenerate via both. Methodologies leading to the diversity of in vitro responses are a relatively new accomplishment. With few exceptions, legumes were commonly described as recalcitrant species with regard to tissue culture—a comment especially true of the large-seeded legumes. Today, there are reports of regeneration from

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2. Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid; 2,4-DB = 2,4-Dichlorophenoxybutyric acid; 2,4,5-T = 2,4,5-Trichlorophenoxyacetic acid; 2 i-P = 2-Isopentyladenine = 6(γ,γ-Dimethylallylamino)-purine; ABA = (±)-2-*cis*,4-*trans*-Abscisic acid; BA = N^6-Benzyladenine = 5-Benzylaminopurine; GA_3 = Gibberellic acid; IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; KT = Kinetin = N^6-furfuryladenine = 6-furfurylaminopurine; NAA = α-Naphthaleneacetic acid = Naphthylacetic acid; pCPA = p-Chlorophenoxyacetic acid; PIC = Picloram (Tordon) = 4-Amino-3,5,6-trichloropicolinic acid; Z = Zeatin = 6-[4-Hydroxy-3-methyl but-2-enylamino]purine

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at least 75 species from 25 genera in the Leguminoseae (Table 1). With the exceptions of East Indian walnut, *Albizia lebbeck* (L.) Benth., *A. richardiana* King, red bud, *Cercis canadensis* L., and sensitive plant, *Mimosa pudica* L., all reports of regeneration are confined to the Papilionoideae, the legume subfamily of greatest economic importance.

The first legumes regenerated from culture, such as *Crotalaria* (Ramawat et al. 1977), *Cicer* (Mukhopadhyay and Bhojwani 1978), and *Indigo* (Bharal and Rashid 1979), were those that could be induced to undergo organogenesis from various vegetative explants. Other early attempts achieved regeneration from legumes that formed somatic embryos after a callus phase. Notable in this group were alfalfa, *Medicago sativa* L. (Saunders and Bingham 1972; Saunders and Bingham 1975; Bingham et al. 1975), and red clover, *Trifolium pratense* L. (Phillips and Collins 1979b; Beach and Smith 1979).

Several important achievements led to the development of efficient regeneration systems for large-seeded legumes that have not regenerated from callus or via de novo organogenesis, such as soybean, *Glycine max* (L.) Merr. These developments centered on the selection of appropriate genotypes and the use of relatively high auxin concentrations for induction of somatic embryogenesis from immature zygotic embryos. Other advances have resulted in simple protocols for embryo maturation and conversion into plants. Application of these principles, which will be discussed at length in the section on somatic embryogenesis, may serve as guidelines for experimental approaches leading to regeneration of other legumes.

This review focuses on the main points of whole plant regeneration and crop improvement. Our discussion distinguishes between organogenic and embryogenic systems. However, this distinction is not always easy, particularly when regenerating tissues are sufficiently abnormal as to defy classification (Bingham et al. 1988). In addition to the problem of precisely describing and categorizing regeneration systems, overlapping and conflicting terminology have often been used. For clarity and simplicity in this review, the word "regeneration" will refer only to de novo regeneration, that is, formation of shoots (organogenesis) or embryos (somatic embryogenesis) in the absence of preexisting meristems. We will use the terms shoot proliferation and micropropagation to describe shoot formation from apical meristems, axillary buds, or cotyledonary nodes, even though the use of the term organogenesis to describe this phenomenon is, unfortunately, common in the literature. Finally, the term germination will refer to enlargement and development of roots from somatic embryos. Since germination need not lead to further development, the term conversion is used to describe the recovery of a plant from a somatic embryo.

Organogenesis

Definition

Organogenesis is the process by which a cell, or group of cells, differentiates to form organs. Organogenesis refers equally to the formation of roots or shoots, but since recovery of plants is usually the objective, the formation of shoots is of greater interest. Organogenesis is commonly induced by manipulations of exogenous phytohormone levels and occurs either directly from explanted tissue or from callus. Often, what is referred to as direct organogenesis is, in fact, shoot proliferation or micropropagation from preexisting meristems instead of de novo formation of a meristem. Even in callus-mediated organogenesis, organ-forming capacity limited to "primary" callus indicates the potential existence of meristems embedded in the original explant.

The distinction between de novo organogenesis and shoot proliferation from preexisting meristems is not trivial. Aside from the distinction that de novo organogenesis requires a redetermination of existing genetic programs expressed within a cell, the application of these two processes differs. Micropropagation of meristems is more easily achieved whenever attempted than is the induction of de novo organogenesis. However, *Agrobacterium*-mediated transformation of preexisting meristems is unsuccessful or very inefficient, which is perhaps due to the low number of attachment sites for *Agrobacterium* in the cell walls of meristematic cells (Matthysse and Gurlitz 1982). Nevertheless, micropropagation techniques are amenable to microprojectile-mediated transformation, as evidenced by recent work in soybean (McCabe et al. 1988; Christou et al. 1989, 1990). Microprojectile-mediated transformation of preexisting meristems followed by micropropagation has several advantages over de novo organogenesis: it can be used in species where de novo organogenesis does not occur, it is less subject to somaclonal variation, and less time is required than when regeneration from callus is necessary (Christou 1990). In general, the same media used to induce organogenesis are effective for micropropagation. A list of legumes that have been propagated from meristems, including shoot

Table 1. Explant source and type of regeneration observed for various species of legumes from which whole plants have been obtained.

Species	Type of regeneration[1]	Explant[2]	Reference
Albizia lebbeck (L.) Benth	Org.	H, R	Gharyal and Maheshwari 1981; Tomar and Gupta 1988
	Emb.	H	Gharyal and Maheshwari 1981; Tomar and Gupta 1988
A. richardiana King	Emb. Org.	H	Tomar and Gupta 1988
Arachis chacoense Krap. et Greg.	Org.	L	Pittman et al. 1983
A. hypogaea L.	Org.	A, C, E, H, L, ZE	Mroginski and Fernandez 1980; Mroginski et al. 1981; Bhatia et al. 1985; Atreya et al. 1984; Illingworth 1968, 1974; Martin and Rabéchault 1976; Narasimhulu and Reddy 1985; Pittman et al. 1983; Reddy and Narasimhulu 1989; Rugman and Cocking 1985; McKently et al. 1990
	Emb.	A, C, CN, S, ZE	Bajaj 1983b; Bajaj et al. 1980; Ozias-Akins 1989; Sellars et al. 1989
A. paraguariensis Chod. et Hassl.	Emb.	A, ZE	Sellars et al. 1989; Still et al. 1987
A. pintoi Krap. et Greg.	Org.	L	Burtnik and Mroginski 1985
A. villosa Benth.	Org.	A	Bajaj et al. 1981b
	Emb.	A	Bajaj 1983b; Bajaj et al. 1981b
A. villosulicarpa Hoehne	Org.	L	Pittman et al. 1983, 1984
Cajanus cajan (L.) Millsp.	Org.		Kumar et al. 1983
Cercis canadensis L.	Emb.	ZE	Geneve and Kester 1990
Coronilla varia L.	Emb.	Ca, L, R	Duskova et al. 1990; Arcioni and Mariotti 1982
Crotalaria juncea L.	Org.	A, CP, L, S	Mohan Ram et al. 1982; Ramawat et al. 1977; Ramanuja Rao et al. 1982
	Emb.	CP	Ramanuja Rao et al. 1982
	?	A	Mohan Ram et al. 1982
Dalbergia sisso Roxb.	Org.	R	Mukhopadhyay and Mohan Ram 1981
Glycine argyrea Tind.	Org.	C, CP, L, P	Hammatt et al. 1989

Continued

Table 1. *Continued*

Species	Type of regeneration[1]	Explant[2]	Reference
G. canescens F.S. Herm.	Org.	C, CP, H, HP, L, P, R	Myers et al. 1989; Newell and Luu 1985; Kameya and Widholm 1981; Hammatt et al. 1987b
	Emb.	C	Grant 1984
G. clandestina Wendl.	Org.	C, CP, H, L	Myers et al. 1989; Hammatt et al. 1987c; Hymowitz et al. 1986
G. falcata Benth.	Org.	C, L, P	Hammatt et al. 1987a
G. latrobeana (Labill.) Benth.	Org.	C, L, P	Hammatt et al. 1987a
G. max (L.) Merr.	Org.	CP	Wei 1988
	Emb.	C, EA H. I. O.	Christianson et al. 1983; Lazzeri et al. 1985; Li et al. 1985; Ranch et al. 1985; Ghazi et al. 1986; Barwale et al. 1986; Tétu et al. 1987; Lazzeri et al. 1987a; Hammatt and Davey 1987; Parrott et al. 1988; Hartweck et al. 1988; Lazzeri et al. 1988; Finer 1988; Finer and Nagasawa 1988; Komatsuda and Ohyama 1988; Shoemaker and Hammond 1988; Xin-Hua et al. 1989; Kien et al. 1989; Buchheim et al. 1989; Parrott et al. 1989; Komatsuda and Ko 1990
G. tomentella Hayata	Org.	H	Kameya and Widholm 1981
G. tomentosa Benth. (=*G. tomentella*)	Org.	H	Kameya and Widholm 1981
Indigofera enneaphylla L.	Org.	C, H	Bharal and Rashid 1979
Lathyrus sativus L.	Emb.	ST	Gharyal and Maheshwari 1983
Lens culinaris Medik.	Org.	E, L	Polanco et al. 1988; Williams and McHughen 1986
	Emb.	EA	Saxena and King 1987
Lotononis bainesii Bak.	Org.	C,L	Wier et al. 1988
Lotus corniculatus L.	Org.	I	Swanson and Tomes 1980a
	Emb.	L	Arcioni and Mariotti 1982
	?	LP	Ahuja et al. 1983b; Webb et al. 1987a
Lupinus angustifolius L.	Org.	H	Sator 1990
L. polyphyllus Lindl.	Org.	A, H	Sator 1990
Medicago arborea L.	Org.	R	Mariotti et al. 1984

Continued

Table 1. *Continued*

Species	Type of regeneration[1]	Explant[2]	Reference
M. borealis L.	Org.	P	Kuchuk et al. 1990
M. coerulea Less.	Emb.	LP	Arcioni et al. 1982
M. difalcata Sinsk. (=*M. sativa* spp. *difalcata*)	Emb.	LP	Gilmour et al. 1987
M. falcata Arcengeli (=*M. sativa* spp. *falcata*)	Emb.	LP	Gilmour et al. 1987
M. glutinosa M.B. (=*M. sativa* spp. *glutinosa*)	Emb.	LP	Gilmour et al. 1987; Arcioni et al. 1982
M. hemicycla Grossh. (=*M. sativa* spp. *hemicycla*)	Emb.	LP	Gilmour et al. 1987
M. media Pers.	Emb.	R, H	Nagarajan and Walton 1987
M. sativa L.	Org.	L, LP, O, P, S	Bianchi et al. 1988; Walker et al. 1979; Dos Santos et al. 1980; Skotnicki 1986
	Emb.	C, H, L, LP, O, P, S, SC, ST	Hartman et al. 1984; Reisch and Bingham 1980; Yu et al. 1990; Bianchi et al. 1988; Walker and Sato 1981; Meijer and Brown 1987a; Senaratna et al. 1989; Fujii et al. 1989; Meijer and Simmonds 1988; Meijer 1989; Xu et al. 1982; Strickland et al. 1987; Kao and Michayluk 1981; Latunde-Dada and Lucas 1988; Meijer and Brown 1988; Dos Santos et al. 1980; Kao and Michayluk 1980; Johnson et al. 1981; Novák and Konecná 1982; Stuart and Strickland 1984b; Arcioni et al. 1982; Lupotto 1986; Meijer and Brown 1987b; Strickland et al. 1987; Bianchi et al. 1988; Mitten et al. 1984; Seitz Kris and Bingham 1988
	?	A, CaP H, I, O P	Bingham and McCoy 1977; Stavarek et al. 1980; Lu et al. 1982; Bingham and McCoy 1986
M. truncatula Gaertn.	Emb.	L	Nolan et al. 1989
M. varia Mart. (=*M. sativa* spp. *varia*)	Emb.	LP	Gilmour et al. 1987
Onobrychis viciifolia Scop.	Org.	H, L	Arcioni and Mariotti 1982; Hamill et al. 1986
	?	LP	Ahuja et al. 1983a
Phaseolus acutifolius Gray	Emb.	L	Kumar et al. 1988b
Psophocarpus tetragonolobus C.D.C.	Org.	C, E, EP, I, L, P	Wilson et al. 1985; Gregory et al. 1980; Kao and Michayluk 1980; Gill 1990
Pisum sativum L.	Org.	L	Mroginski and Kartha 1981a; Rubluo et al. 1984

Continued

Table 1. *Continued*

Species	Type of regeneration[1]	Explant[2]	Reference
	Emb.	LP, ST, ZE	Kysely et al. 1987; Lehminger-Mertens and Jacobsen 1989; Kysely and Jacobsen 1990
Robinia pseud-acacia L.	Emb.	ZE	Merkle and Wiecko 1989
Sesbania rostrata Brem. et Oberm.	Org.	C, H, ZE	Vlachova et al. 1987
Stylosanthes guianensis (Aubl.) Sw.	Org.	H, L, LP, R	Godwin et al. 1987; Gregory et al. 1980; Meijer and Broughton 1981; Mroginski and Kartha 1981b; Szabados and Roca 1986
S. hamata (L.) Taub.	Org.	Ra	Scowcroft and Adamson 1976
S. humilis H.B.K.	Org.	C, L, R	Manners and Way 1989; Manners 1988
Trifolium africanum Ser.	Org.	C	Webb et al. 1987b
T. arvense L.	Emb.	H	Bhojwani et al. 1984
T. alexandrinum L.	Org.	A, H	Barakat 1990; Mokhtarzadeh and Constantin 1978
T. alpestre L.	Emb.	C, H, R	Yamada and Higuchi 1990
T. amabile Humb., Bonpl. et Kunth	Emb.	C, H, R	Yamada and Higuchi 1990
T. apertum Bobrov	Emb.	C, H, R	Yamada and Higuchi 1990
T. batmanicum Katzn.	Org.	C	Webb et al. 1987b
T. caucasicum Tausch	Emb.	C, H, R	Yamada and Higuchi 1990
T. cherleri L.	Emb.	C, H, R	Yamada and Higuchi 1990
T. heldreichanum Hausskn.	Emb.	C, H, R	Yamada and Higuchi 1990
T. incarnatum L.	Org.	H	Beach and Smith 1979
	Emb.	H	Pederson 1986
T. kotulae	Org.	C	Webb et al. 1987b
T. laevigatum Poiret	Org.	C	Webb et al. 1987b
T. masiense Gillett	Org.	C	Webb et al. 1987b

Continued

Table 1. *Continued*

Species	Type of regeneration[1]	Explant[2]	Reference
T. medium L.	Emb.	P	Choo 1988
	Org.	P	Choo 1988
T. michelianum Savi	Org.	C	Webb et al. 1987b
T. montanum L.	Emb.	C, H, R	Yamada and Higuchi 1990
T. nanum Torr.	Org.	C, H, N	Webb et al. 1987b
T. nigrescens Viv.	Org.	H	Webb et al. 1987b
T. pratense L.	Org.	E, H, R, S	Beach and Smith 1979; Phillips and Collins 1979b
	Emb.	A, Ca, H, P, SC, ZE	Keyes et al. 1980; Collins and Phillips 1982; Maheswaran and Williams 1986b; Phillips and Collins 1980; Bhojwani et al. 1984; McGee et al. 1989
	?	H, O, P	Broda 1984; MacLean and Nowak 1989
T. radiosum Wahlenb.	Org.	C, H	Webb et al. 1987b
T. repens L.	Org.	C, Ca, CaP, H, LP, P, R	Gresshoff 1980; Yamada 1989; Webb et al. 1987b; Ahuja et al. 1983a; White 1984; Oswald et al. 1977
	Emb.	H, L, ZE	Maheswaran and Williams 1986a; Bhojwani et al. 1984
	?	C, H, L, LP, St	Bond and Webb 1989; Webb et al. 1987a; Pelletier and Pelletier 1971; Bhojwani et al. 1984
T. resupinatum L.	Org.	C	Webb et al. 1987b
	Emb.	ZE	Maheswaran and Williams 1986b
T. rubens L.	Emb.	H	Parrott and Collins 1983
T. subterraneum L.	Org.	C, H	Webb et al. 1987b
	Emb.	ZE	Maheswaran and Williams 1986b
T. tridentatum Lindl.	Org.	C, H	Webb et al. 1987b
T. uniflorum L.	Org.	C, H	Webb et al. 1987b
T. vesiculosum Savi	Emb.	H	Pederson 1986
Vicia faba L.	Emb.	ZE	Taha and Francis 1990
V. narbonensis L.	Emb.	ST	Pickardt et al. 1989

Continued

Table 1. *Continued*

Species	Type of regeneration[1]	Explant[2]	Reference
Vigna acontifolia (Jacq.) Marechal	Org.	P	Krishnamurthy et al. 1984
V. acontifolia	Emb.	L, LP	Shekhawat and Galston 1983; Kumar et al. 1988a
V. glabrescens × *V. radiata* (L.) Wilczek	Emb.	ZE	Chen et al. 1990
	Org.	ZE	Chen et al. 1990
V. radiata (L.) Wilczek	Org.	C	Mathews 1988; Mathews et al. 1986; Mathews 1987; Mathews and Rao 1984

1. Org. = organogenesis, Emb. = embryogenesis, ? = unknown
2. A = anther, C = cotyledon, CN = cotyledonary node, CP = cotyledon protoplast, Ca = callus, CaP = callus protoplast, E = epicotyl, EA = embryonic axis, H = hypocotyl, HP = hypocotyl protoplast, I = internode, L = leaf, LP = leaf protoplast, O = ovary, P = petiole, R = root, Ra = radicle, S = stem, SC = suspension culture, ST = shoot tip, St = stolon, ZE = zygotic embryo

tips, cotyledonary nodes, and axillary buds is provided in Table 2.

Beach and Smith (1979) defined a three-step culture system for successful regeneration of red and crimson clover, *Trifolium incarnatum* L., as follows: (1) callus was initiated on B5 basal medium with 11 μM NAA and 10 μM each of 2,4-D and KT. (2) For shoot development, callus was transferred to a medium with a lower auxin to cytokinin ratio, achieved by removal of the 2,4-D, and use of 15 μM adenine as the cytokinin. (3) Roots were induced on a rooting medium containing 1.1 μM NAA. These three steps, callus initiation, shoot differentiation, and root differentiation, are common to indirect organogenic systems. The main factors that appear to control organogenesis in legumes are explant type and age, auxin/cytokinin concentrations and combinations, and genetic effects among and within species.

Explant. Organogenesis in legumes has been reported from several tissue types, including cotyledons, leaves, petioles, hypocotyls, stems, roots, and anthers. As the information in Table 1 reveals, leaves have been used most frequently in organogenic systems. In most cases where use of different explants has been reported, only leaf explants have consistently given rise to shoots. Nevertheless, there appear to be some limitations to the use of leaf tissues. Shoots derived from leaf explants of indigo, *Indigofera enneaphylla* L., died, while shoots from cotyledon and hypocotyl explants developed into plants (Bharal and Rashid 1979). Both the age of the leaf and the growth regulators used can affect the success of regeneration (Rubluo et al. 1984), with younger leaves responding to a wider range of growth regulators. In addition, genotype is important. Genotypes within *Glycine canescens* F.J. Herm., *G. falcata* Benth., *G. latrobeana* (Meissn.) Benth., *G. tomentella* Hayata (Hammatt et al. 1987a), and *G. argyrea* Tind. (Hammatt et al. 1989) differ in their regeneration capacity from leaf tissues. Finally, species differences are also evident. Pittman et al. (1983) cultured leaflets from several *Arachis* species, but only achieved regeneration in the *Arachis* and *Extranervosae* sections of the genus.

The use of cotyledon tissues tends to be successful whenever attempted, but not to the extent to which leaves are successful. Cases in which organogenesis from cotyledon explants was not obtained include *G. tomentella* and *G. canescens* (Kameya and Widholm 1981), and 59 of 72 *Trifolium* species evaluated (Webb et al. 1987b). Atreya et al. (1984) observed a gradient in regeneration potential, such that proximal regions of cotyledons gave rise to shoot regeneration. In this study, de novo shoot formation was confirmed since the proximal cotyledon regions were distinct from the node. Organogenesis from cotyledons has been reported in the absence of growth regulators. For example, peanut plants were regenerated by placing whole or segmented cotyledons on moist filter paper in the absence of nutrient media and hormones (Bhatia et al. 1985; Illingworth 1968), although the

Table 2. Reports of successful micropropagation of various leguminous species.

Species	Explant[1]	Reference
Acacia koa Gray	RS, ST	Skolmen 1986
Arachis hypogaea L.	CN, EA, ST	Narasimhulu and Reddy 1985; Atreya et al. 1984; Bajaj et al. 1981a; Kartha et al. 1981; Braverman 1975; Russo and Varnell 1978; Mhatre et al. 1985; Oelck et al. 1982; Oelck and Schieder 1983
Cicer arietinum L.	CN, ST	Bajaj 1983a; Kartha et al. 1981; Bajaj and Dhanu 1979
Glycine max (L.) Mill.	AB, CN, E, EA, ST	Wright et al. 1986; Freytag et al. 1989; Graybosch et al. 1987; Cheng et al. 1980; Kartha et al. 1981; Braverman 1975; Oelck and Schieder 1983
Lathyrus sativus L.	ST	Gharyal and Maheshwari 1983
Lens culinaris Medik.	E, SN, ST	Polanco et al. 1988; Williams and McHughen 1986
L. esculentum Moench	ST	Bajaj and Dhanu 1979
Medicago sativa L.	ST	Cheyne and Dale 1980
Melilotus officinalis Willd.	SN	Oelck and Schieder 1983
Mimosa pudica L.	CN, ST	Gharyal and Maheshwari 1982
Phaseolus areus Roxb.	ST	Bajaj and Dhanu 1979
P. coccineus L.	ST	Ruiz et al. 1986
P. mungo L.	ST	Bajaj and Dhanu 1979
P. vulgaris L.	ST	Kartha et al. 1981; Ruiz et al. 1986
Pisum sativum L.	ST	Bajaj and Dhanu 1979
Robinia pseudacacia L.	SN, ST	Chalupa 1983; Barghchi 1987
Trifolium alpestre L.	ST	Parrott and Collins 1983
T. amabile Humb., Bonpl. et Kunth	CN, ST	Webb et al. 1987b
T. armenium Willd.	CN, ST	Webb et al. 1987b
T. arvense L.	CN, ST	Webb et al. 1987b
T. batmanicum Katzn.	CN, ST	Webb et al. 1987b
T. cherleri L.	CN, ST	Webb et al. 1987b
T. curvisepalum	CN, ST	Webb et al. 1987b

Continued

Table 2. *Continued*

Species	Explant[1]	Reference
T. heldreichianum Hausskn.	CN, ST	Webb et al. 1987b
T. hirtum All.	CN, ST	Webb et al. 1987b
T. hybridum L.	CN, ST	Webb et al. 1987b
T. incarnatum L.	ST	Parrott and Collins 1983
T. kotulae	CN, ST	Webb et al. 1987b
T. laereigatum Poiret	CN, ST	Webb et al. 1987b
T. lagopus Pourr. et Willd.	CN, ST	Webb et al. 1987b
T. ligusticum Balbis et Loisel.	CN, ST	Webb et al. 1987b
T. masaiense Gillett	CN, ST	Webb et al. 1987b
T. medium L.	CN, ST	Webb et al. 1987b; Parrott and Collins 1983
T. michelianum Savi	CN, ST	Webb et al. 1987b
T. montanum L.	CN, ST	Webb et al. 1987b
T. nanum Torr.	CN, ST	Webb et al. 1987b
T. nigrescens Viv.	CN, ST	Webb et al. 1987b
T. parviflorum F.E. Ehrh.	CN, ST	Webb et al. 1987b
T. pauciflorum D'Urv.	CN, ST	Webb et al. 1987b
T. phleoides Pourret et. Willd.	CN, ST	Webb et al. 1987b
T. pilulare Boiss.	CN, ST	Webb et al. 1987b
T. pratense L.	E, FH, SN, ST	Skucinska and Miszke 1980; Broda 1984; Phillips and Collins 1979a, 1979b; Cheyne and Dale 1980; Parrott and Collins 1983; Collins and Phillips 1982; Webb et al. 1987b; Campbell and Tomes 1984; Oelck and Schieder 1983
T. radiosum Wahlenb.	CN, ST	Webb et al. 1987b
T. repens L.	CN, SN, ST	Parrott and Collins 1983; Cheyne and Dale 1980; Barnett et al. 1975; Bhojwani 1981; Webb et al. 1987b
T. resupinatum L.	CN, SN, ST	Webb et al. 1987b; Oelck and Schieder 1983
T. rubens L.	ST	Parrott and Collins 1983
T. scabrum L.	CN, ST	Webb et al. 1987b
T. stoloniferum Muhl et. Eaton	ST	Singha et al. 1988

Continued

Table 2. *Continued*

Species	Explant[1]	Reference
T. striatum L.	CN, ST	Webb et al. 1987b
T. subterraneum L.	CN, ST	Parrott and Collins 1983; Webb et al. 1987b
T. tomentosum L.	CN, ST	Webb et al. 1987b
T. tridentatum Lindl.	CN, ST	Webb et al. 1987b
T. uniflorum L.	CN, ST	Webb et al. 1987b
Vicia faba L.	ST	Busse 1986
V. unguiculata (L.) Walp.	ST	Kartha et al. 1981

1. AB = axillary bud, CN = cotyledonary node, E = epicotyl, EA = embryonic axis, FH = flower head, RS = root sprouts, SN = shoot node, ST = shoot tip

addition of BA increased the frequency of regeneration (Bhatia et al. 1985). Likewise, in mung bean, *Vigna radiata* (L.) Wilczek, direct shoot formation occurred on MS basal medium without hormones from cotyledon explants with the embryonic axes removed (Mathews 1987).

As with cotyledons, the use of hypocotyl explants has generally been successful, except for the sensitive plant (Gharyal and Maheshwari 1982), and 68 of 72 *Trifolium* species (Webb et al. 1987b). Various factors appear to affect regeneration from hypocotyls. High frequency of direct organogenesis from hypocotyl segments was reported in *G. canescens* (Kameya and Widholm 1981). The frequency of organogenesis was dependent on seedling age and length and position of the hypocotyl explant. When sections about 5 mm in length were placed on medium containing NAA and BA, the acropetal end of the explant produced numerous shoots.

Regeneration from root explants has been sporadic at best. Successful regeneration has been reported in Indian teakwood, *Dalbergia sissoo* Roxb. (Mukhopadhyay and Mohan Ram 1981), Brazilian lucerne, *Stylosanthes guyanensis* (Aubl.) Sw. (Meijer and Broughton 1981), East Indian walnut, *Albizia lebbeck* (Gharyal and Maheshwari 1981), and *Medicago arborea* L. (Mariotti et al. 1984). Alternatively, regeneration from root explants has been obtained in only one of 72 *Trifolium* species tested, and has been unsuccessful in *G. tomentella* and *G. canescens* (Kameya and Widholm 1981), sensitive plant, *Mimosa pudica* L. (Kameya and Widholm 1981), and groundnut, *Arachis hypogaea* L. (Narasimhulu and Reddy 1985).

Auxins and cytokinins. The use of the cytokinin, BA, has been widespread for the induction of organogenesis in legumes and most studies to date have used BA exclusively. A few studies suggest that BA can be the more effective cytokinin, but comprehensive studies comparing the effectiveness of BA to that of other cytokinins are lacking in legumes. Regeneration frequencies of Brazilian lucerne were higher with the use of BA than KT (Meijer and Broughton 1981), although these two cytokinins were not compared at the same auxin level, making it difficult to draw inferences on their relative effectiveness. Kinetin was equal (Narasimhulu and Reddy 1985) or superior (Martin and Rabéchault 1976) to BA for organogenesis in groundnut, and Z was equal to BA in birdsfoot trefoil, *Lotus corniculatus* L. (Ahuja et al. 1983b). The use of BA was more effective than both KT and 2,i-P in the regeneration of berseem (Egyptian) clover, *Trifolium alexandrinum* L. (Mokhtarzadeh and Constantin 1978), and pea, *Pisum sativum* L. (Mroginski and Kartha 1981a). In this last species, both BA and KT induced regeneration, although with differing effectiveness, while Z and 2,i-P did not (Rubluo et al. 1984). In the case of indigo, BA effectively induced organogenesis, but KT and Z did not (Bharal and Rashid 1979).

Similar observations apply to the use of auxins during organogenesis, although no single auxin ap-

pears to be used most often. In the most complete evaluations carried out in legume tissue culture, the effectiveness of different auxins depended on the maturity of the explant tissue which consisted of pea leaves (Rubluo et al. 1984). The youngest leaves responded to NAA, IAA, and IBA, while leaves of intermediate maturity responded to NAA and IAA. The most mature leaves responded only to NAA, and no leaves responded to either 2,4-D or PIC. The use of NAA was superior to IAA for regeneration of berseem clover (Mokhtarzadeh and Constantin 1978), and superior to 2,4-D for the regeneration of Brazilian lucerne (Meijer and Broughton 1981) and *Glycine canescens* (Newell and Luu 1985). This last report was in conflict with another report on the same species (Hammatt et al. 1987b), in which only IBA, and not NAA, IAA, or 2,4-D, induced organogenesis. Similar conflicting results have been reported for winged bean, *Psophocarpus tetragonolobus* (L.) DC., in which NAA was ineffective (Gregory et al. 1980), or superior to IAA (Wilson et al. 1985). In other reports in which auxins have been compared with each other, 2,4-D and NAA were unable to substitute for IAA for the regeneration of indigo (Bharal and Rashid 1979). In alfalfa, 2,4-D was more effective than KT in a Schenk and Hildebrandt basal medium, while KT was more effective in a Linsmaier and Skoog medium (Stavarek et al. 1980). For regeneration of Indian teakwood, NAA induction produced more shoots than IAA induction, but shoots induced with IAA were more normal (Mukhopadhyay and Mohan Ram 1981). In the sensitive plant, the use of 2,4-D or NAA gave more primary shoots, but IBA was more effective in the multiplication of primary shoots (Gharyal and Maheshwari 1982).

In addition to the type of auxins and cytokinins used, the ratio of these growth regulators is important. The conventional use of high auxin-cytokinin ratios for root-induction, low ratios for shoot-induction, and intermediate ratios for dedifferentiation is applicable to legumes. Various auxin to cytokinin ratios have been used effectively to induce organogenesis. At one end of the spectrum, cytokinin alone has been used to induce organogenesis. Concentrations of 0.05 mg l^{-1} NAA and 0.5 mg l^{-1} BA have been effective for shoot induction in both *Medicago arborea* (Mariotti et al. 1984) and in various clover species (Webb et al. 1987b). Much lower auxin:cytokinin ratios have been used for other species, with perhaps a 1:10 auxin:cytokinin ratio being successfully used the most often.

In conclusion, several factors must be considered for the selection of appropriate growth regulators in organogenic systems. These include species, genotype, explant, and even the composition of the basal medium. The use of balanced factorial designs in future experiments should help to further define the relative importance of these factors. Critical studies evaluating the relative efficiencies of various cytokinins are especially needed.

Genotype and species limitations. Explants show a pronounced inter- and intraspecific diversity of organogenic response, which renders the selection of genotype among the most important factors in establishing successful in vitro regeneration protocols. Some examples have already been discussed. In another example, callus and organogenesis from cotyledon explants were observed in various accessions of *G. canescens*. Accession G1171 was the most responsive of all the accessions investigated, undergoing organogenesis in over 70% of cultures, while other accessions underwent organogenesis at low frequency. Hypocotyl sections of *G. canescens* and *G. tomentella* underwent organogenesis. In contrast, it was not possible to induce organogenesis from hypocotyls, roots, or cotyledons of *G. soja* Sieb. and Zucc., *G. falcata* Benth., *G. tabacina* (Labill.) Benth., *G. latifolia* (Benth.) Newell and Hymowitz, and soybean (Kameya and Widholm 1981).

Cultivar differences were also observed for initiation of organogenesis from groundnut cotyledons (Bhatia et al. 1985). Reddy and Narasimhulu (1985) noticed genotypic differences in seedling explant culture in groundnut. While it was possible to regenerate plants from seedling explant calli of ICG 4367 and TMV 2, the cultivars TG-196 and US-48 did not respond under similar conditions. Similarly, shoot-bud regeneration from immature leaves was obtained in only 5 of 10 groundnut cultivars (Mroginski et al. 1981). Culture of seedling leaves from all groundnut cultivars tested produced callus, but the frequency of organogenesis was highly cultivar dependent (Johnson 1981; Pittman et al. 1983).

Organogenesis and gene-transfer systems. In general, de novo organogenic regeneration systems have been amenable to the standard leaf-disc method of *Agrobacterium*-mediated transformation (Horsch et al. 1985) or modifications thereof. The legumes transformed by this method so far include birdsfoot trefoil (Jensen et al. 1986), lotononis, *Lotononis bainesii* Baker (Wier et al. 1988), Townsend stylo, *Stylosanthes humilis* H.B.K. (Manners 1988; Manners and Way 1989), and pea (Puonti-Kaerlas et al. 1989).

Somatic Embryogenesis

General Considerations

Somatic embryogenesis is a process whereby a cell or group of cells from somatic tissues form an embryo. In general, development of somatic embryos parallels that of zygotic embryos. As with organogenesis, somatic embryogenesis in legumes is not inherently different from somatic embryogenesis in other plants and the same fundamental principles appear to apply in all cases. Depending on the species, somatic embryogenesis can occur indirectly from an intervening callus phase, or directly from explanted tissues. Indirect somatic embryogenesis is best characterized by alfalfa, while direct embryogenesis from explants is characterized by soybean. It is the latter that has led to the successful development of tissue culture and regeneration systems for recalcitrant species.

Indirect Systems

Two genera of small-seeded legumes that have received considerable attention are *Trifolium* (the clovers) and *Medicago* (alfalfa, lucerne). In fact, both genera have been considered at various times to be model systems for somatic embryogenesis among legumes, but indirect embryogenesis has not become common in legumes. Both *Medicago* and *Trifolium* tissue culture in general, and somatic embryogenesis in particular, have been recently reviewed elsewhere (Bingham et al. 1988; Williams et al. 1990), so only the salient points are considered here. Clovers are considered first, since multiple modes of regeneration are found within this genus.

Somatic embryogenesis of clover. The clovers have perhaps been the subject of the greatest number of reports on tissue culture and regeneration in legumes and provide examples of both direct and indirect embryogenesis. The greatest amount of work has been done on white clover, *Trifolium repens* L., a species that is amenable to the greatest diversity of regeneration techniques used for a leguminous species. Sporadic recovery of shoots from callus cultures of white clover has occurred with diverse protocols based on the use of various combinations of auxins and cytokinins (Pelletier and Pelletier 1971; Oswald et al. 1977; Gresshoff 1980; Ahuja et al. 1983a; Bond and Webb 1989; Webb et al. 1987b). While the mode of regeneration in white clover has been reported to be somatic embryogenesis from callus tissues (Bhojwani et al. 1984), the mode of regeneration is not always clear in other cases. A recent report of regeneration in white clover clearly is direct organogenesis (Webb et al. 1987b). In all cases, genotype is important, with efficient regeneration limited to a few genotypes with high regeneration capacity. Once identified, these genotypes have been used for regeneration studies from cell or protoplast culture (Bhojwani et al. 1984; White 1984; Webb et al. 1987a; Yamada 1989).

Pederson (1986) was able to obtain somatic embryos in culture from callus tissues of white clover, but these failed to develop. Nevertheless, indirect somatic embryogenesis from callus induced on auxin-cytokinin combinations leading to the recovery of plants has occurred in several species of the genus *Trifolium*, including red clover (Phillips and Collins 1980; Bhojwani et al. 1984; McGee et al. 1989), *T. rubens* L. (Parrott and Collins 1983; Cui et al. 1988; McGee et al. 1989), rabbitfoot clover, *T. arvense* L. (Bhojwani et al. 1984), crimson clover, and arrowleaf clover, *T. vesiculosum* Savi (Pederson 1986).

The first reports of direct somatic embryogenesis in white clover (and other clovers) resulted from exposure of very immature zygotic embryos to BA at 0.2-2.0 mg l^{-1} without the presence of an auxin in the medium (Maheswaran and Williams 1984, 1985, 1986a). Most recently, somatic embryos have been obtained directly from immature cotyledons of white clover upon exposure to high levels of 2,4-D (Parrott, unpublished results). Given that a large number of zygotic embryos respond to either auxin or cytokinin, and that each zygotic embryo represents a different genotype, one can infer that a larger number of clover genotypes have the ability to regenerate via direct embryogenesis than have the ability to regenerate via indirect embryogenesis. Thus, white clover can regenerate via organogenesis or somatic embryogenesis, and the latter can be stimulated by either auxins or cytokinins, and be either direct or indirect.

Somatic embryogenesis in alfalfa. Alfalfa was first regenerated from callus tissues in 1972 (Saunders and Bingham 1972). Regeneration has occurred from callus induced from virtually any part of the plant (Bingham et al. 1988). Regeneration in alfalfa was initially improved by the realization that the induction and the development of somatic embryos were separate processes. Induction was accomplished by the presence of 2,4-D in the medium, while development occurred upon removal of auxin from the medium (Walker et al. 1979). The basic callus induction with auxin-cytokinin combinations followed by transfer of tissues to hormone-free medium has become standard

in alfalfa tissue culture, and somatic embryogenic protocols in general.

Different genotypes of alfalfa differ in the amount of callus they form prior to the initiation of somatic embryos (Meijer and Brown 1987a). The presence of at least 12.5 mM NH_4^+ in the medium has also been found to be required for somatic embryogenesis to occur (Walker and Sato 1981). Recently, desiccated somatic embryos of alfalfa have been proposed for use as artificial seeds. Senaratna et al. (1989) found that treatment of somatic embryos with ABA prior to desiccation improved desiccation tolerance and conversion of desiccated somatic embryos sown directly to soil. Embryos were more responsive following a slow desiccation treatment than a fast treatment. Stress pretreatments prior to desiccation tended to improve desiccation tolerance, but reduced successful conversion of embryos into plants. Of treatments that included low temperature, high temperature, and water and nutrient shock, only heat shock was comparable to ABA treatment in improving desiccation tolerance without negative effects on conversion. Desiccated alfalfa somatic embryos were stored for 8 months without any loss in viability.

Effect of genotype in indirect systems. Genotypic influence on embryogenic capacity has been documented in virtually all studies that have addressed the subject. For example, calli from different plants of crown vetch, *Coronilla varia* L., and birdsfoot trefoil, Lotus corniculatus L.—two outcrossing and self-incompatible species—exhibited different embryogenic capacity (Arcioni and Mariotti 1982). Genotype was also considered as the most important factor in establishing successful in vitro cultures of alfalfa and clover (Williams et al. 1990; Arcioni et al. 1990). In alfalfa, the creeping rooted cultivars Rambler, Roamer, and Drylander are better regenerators as a group than noncreeping cultivars. The cultivar Rangelander, from which essentially all genotypes regenerate (Atanassov and Brown 1984) is the best regenerating cultivar currently known in alfalfa. Mitten et al. (1984) studied regeneration capacity of 35 tetraploid alfalfa cultivars representing all the nine germplasm sources of U.S. alfalfa. Calli derived from hypocotyls from 20 genotypes of each cultivar were tested for regeneration. Ladak, Norseman, Turkistan, and Nomad were clearly superior sources of regenerating genotypes while the remaining cultivars possessed a much lower frequency of regenerating genotypes. It has been possible to breed genotypes of clover (Mezentsev et al. 1982) and alfalfa (Bingham et al. 1975) characterized by a high capacity for regeneration. After two cycles of selection in alfalfa, regeneration frequency was increased from an initial 12% to 67% (Bingham et al. 1975). This alfalfa germplasm, known as Regen-S, has facilitated a broad range of studies, including: (1) physiological aspects of somatic embryogenesis (Walker et al. 1979; Walker and Sato 1981; Stuart and Strickland 1984a; Stuart and Strickland 1984b); (2) in vitro selection and subsequent recovery of plants resistant to the toxin produced by *Fusarium oxysporum* f. sp. *medicaginensis* (Weimer) Snyd. and Hans.; (3) recovery of plants regenerated from protoplasts (Johnson et al. 1981; Dijak et al. 1986); and (4) transformation of alfalfa plants (D'Halluin et al. 1990). Diploid alfalfa has also been bred for regeneration capacity and is now available (Ray and Bingham 1989).

The genetic control of regeneration has been best studied in alfalfa. Two dominant genes, *Rn1* and *Rn2*, were originally found necessary for a high level of regeneration to occur (Reisch and Bingham 1980). Since then, two dominant genes were also found to be necessary for regeneration in other populations of alfalfa, and were named *Rn3* and *Rn4* (Wan et al. 1988) and *Rna* and *Rnb* (Hernández-Fernández and Christie 1989). These gene pairs, although given different names, have not been tested for allelism, so the possibility that they are the same genes in all populations has not been eliminated. In contrast to alfalfa, the regeneration capacity of red clover has been attributed to a recessive gene, *rg*, while the ability to form callus prior to the formation of somatic embryos is due to a single dominant gene, *C* (Broda 1984).

Direct Systems

The following discussion details the factors important for soybean somatic embryogenesis in those species in which embryos do not originate from callus, and reviews aspects of somatic embryogenesis in other leguminous species using soybean as a model.

Somatic embryogenesis in soybean. Soybean is the most extensively grown and economically important legume in the world, and has been the subject of widespread efforts to develop tissue culture and regeneration systems for nonconventional crop improvement. Soybean is offered here as a model system for other recalcitrant legumes for several reasons. First, several regeneration protocols are available that provide options for future research. Second, current regeneration protocols are remarkably simple and efficient. Embryos induced on basal medium with

auxin subsequently require no exogenous growth regulators for continued differentiation, maturation, germination, and plant establishment (Parrott et al. 1988; Buchheim et al. 1989). Third, soybean somatic embryogeny mirrors the developmental events of zygotic embryogeny. For example, maturation and desiccation clearly enhance conversion of both zygotic and somatic soybean embryos (Hammatt and Davey 1987; Parrott et al. 1988; Buchheim et al. 1989; Rosenberg and Rinne 1986; Rosenberg and Rinne 1988). Finally, the development of successful embryogenic systems for soybean represents a historical progression from extreme recalcitrance to efficient in vitro regeneration of normal plants. In early studies, the recalcitrance of soybean to in vitro manipulation was widely reported, and at one time the crop was referred to as "the archetype of material recalcitrant to the induction of differentiation" (Mroginski and Kartha 1984). From this perspective, successful efforts to overcome recalcitrance in soybean may prove useful when applied to other legumes. Already, the development of regeneration systems for other legumes has been based on aspects of soybean regeneration. A recently described system for somatic embryogenesis in groundnut was based entirely on a protocol developed for soybean (Sellars et al. 1989). This review will not cover the various reports of organogenesis in soybean, as these most often hinge upon the presence of a meristem in the explant tissue. As such, these are best considered cases of micropropagation, not de novo organogenesis.

Development of embryogenic systems in soybean. The first evidence of culture-induced differentiation that could be interpreted as somatic embryogenesis in soybean was reported by Beversdorf and Bingham (1977). These authors described the development of "growth centers", "embryo-like structures", and "calli with leafy protrusions". No plantlets were obtained from any of these structures. Subsequently, Phillips and Collins (1981) developed a reproducible system for the generation of numerous globular and heart-stage embryos from suspension culture, but recovered no plants. Several genotypes of both soybean and *G. soja* responded differently in the number of somatic embryos produced per ml of culture medium. Cultivar Essex was the most prolific of five commercial cultivars, with a frequency of 0.2 embryos ml^{-1} while PI 81762 was the highest of three *G. soja* genotypes at 3 embryos ml^{-1}. Embryogenesis occurred at the highest frequency with 2,4-D as the inducing auxin, while other auxins (NAA, IAA, IBA, and PIC) were inferior (Phillips and Collins 1981). Gamborg et al. (1983) generated somatic embryos in suspension cultures from hypocotyl-derived calli. Again, 2,4-D was effective for the production of embryos, but embryos were also induced with PIC. The auxins NAA, IBA, and IAA were again ineffective. Consistent with the findings of Phillips and Collins (1981), cv Essex was the most prolific of seven soybean genotypes tested, and PI 81762 was the most regenerable of three genotypes of *G. soja*. No embryos were detected in cultures of *G. canescens*, *G. clandestina* Wendl., *G. falcata*, *G. tabacina* (Labill.) Benth., and *G. tomentella*.

Christianson et al. (1983) reported the first incidence of plant recovery via somatic embryogenesis. Immature embryonic axes (1-2 mm) were routinely subcultured on medium with 5 mg l^{-1} 2,4-D. By replacing the nitrogen salts of MS medium with ammonium citrate, one piece of tissue with numerous embryos was produced. These embryos were either maintained by serial subculture in liquid N-amended medium supplemented with 2,4-D, or were induced to develop shoots by transfer to a medium containing 0.005 mg l^{-1} IBA and 0.2 mg l^{-1} BA. Since root formation in these cultures was extremely rare, shoots were transferred to a standard root-inducing medium (0.1 mg l^{-1} IAA) for the recovery of plantlets. Lippmann and Lippmann (1984) next reported the induction of somatic embryos from immature zygotic embryos. Embryos arose directly from explants of immature cotyledons cultured on medium supplemented with any of several auxins (NAA, IBA, 2,4,5-T, IAA, 2,4-D, pCPA). Consistent with earlier studies, strong auxins (2,4-D and pCPA in this case) were the most effective for inducing embryogenesis. The addition of BA, Z, or KT at 0.5 or 1.0 μM to the auxin-supplemented induction medium strongly inhibited embryogenesis. The developmental stage of zygotic embryos was an important parameter affecting the frequency of induction of embryogenic tissue and embryos. Cotyledons 4-5 mm in length provided maximum production of somatic embryos. Embryos failed to develop further when subcultured onto induction medium. Transfer to a medium containing 0.5 μM Z resulted in occasional root and shoot formation from the same embryo, but no plants were recovered. In retrospect, these studies (Christianson et al. 1983; Lipmann and Lipmann 1984) established critical factors that were to become important for reproducible and efficient somatic embryogenesis. Virtually all subsequent reports have described auxin-induced embryogenesis from a specific developmental stage of immature embryos. A concurrent, independent study

described a similar protocol for *G. canescens* (Grant 1984).

Li et al. (1985) described a novel protocol for soybean somatic embryogenesis. Pods were immersed in liquid nitrogen for 20 minutes, then thawed at 60°C for 20 minutes. Immature embryos were excised from unbroken pods and cultured on a medium supplemented with 1-2 mg l^{-1} 2,4-D. A suspension was derived from the resulting callus and maintained on 0.5 mg l^{-1} 2,4-D and 5% coconut milk. Hanging drop cultures of single cells divided to form colonies containing small somatic embryos that were then subjected to various growth-regulator treatments for further development. "Differentiated calli" containing globular and heart-shaped embryos were transferred to liquid regeneration medium supplemented with 0.5 mg l^{-1} 2,4-D and 5% coconut milk. Mature embryos and plantlets were detected in liquid culture after 6 months.

Lazzeri et al. (1985) induced somatic embryos from immature zygotic embryos, either whole or with the embryonic axis removed, on medium containing 5 mg l^{-1} 2,4-D or 10 mg l^{-1} NAA. Seeds of 3-6 mm in length provided the most responsive embryos. A diverse range of genotypes were responsive to the protocol, including 30 cultivars and 10 *G. soja* genotypes. Embryogenic cultures could be maintained by periodic subculture to fresh induction medium. The auxin 2,4-D was superior for maintaining the embryogenic capacity of subcultured tissue and for producing the highest frequencies of somatic embryos. However, somatic embryos induced with 2,4-D were more abnormal than those induced by NAA. Development and regeneration of somatic embryos was accomplished by transfer to a medium supplemented with 0.15 mg l^{-1} NAA and 0.033 mg l^{-1} each of BAP, KT, and Z. Embryos were subcultured on this medium for 20-30 days until outgrowth of the shoot apex was observed. Embryos not induced to germinate from this treatment were transferred for 5-15 days to a similar medium but with 0.05 mg l^{-1} NAA and 0.33 mg l^{-1} each of BAP, KT, and Z. Plantlets with primary leaves were transferred to half-strength MS medium with 0.0005 mg l^{-1} IBA. Rooted plants were transferred to sterilized soil and covered for acclimatization.

Ranch et al. (1985) described a similar regeneration system with substantial and important differences. Again, embryogenic tissue was derived from whole, immature zygotic embryos or immature cotyledons in the presence of an inducing auxin, and embryogenic tissue was maintained and increased by subculture onto the induction medium. Embryos 0.5 to 4 mm in length were more responsive than those 4-8 mm long, while embryos of less than 0.5 mm length did not survive the culture protocol. Consistent with the previous findings, morphologically abnormal embryos were produced upon induction with concentrations of 2,4-D below 45.2 μM. Increasing the concentration of 2,4-D reduced the growth rate of embryogenic tissue, but resulted in the most normal and developmentally responsive globular-stage somatic embryos. Maturation was accomplished by transferring somatic embryos to a medium containing 6 μM IBA and 1 or 10 μM ABA. For conversion, mature somatic embryos were transferred to 0.6 μM IBA + 0.3 μM GA_3. Embryos exhibiting development of the apical region were then transferred to a medium containing 0.6 μM IBA for establishment of plantlets, and plants were then grown to maturity in the greenhouse. Considerable variation in response (percentage of explants with organized structures) among genotypes was observed (the lowest was 12.5% of explants with organized structures and the highest was 67.1%) yet "no detrimental genotypic effect upon initiation, proliferation, and plant regeneration from immature embryo-derived organized callus cultures of soybean" was observed.

Consistent with these findings, Barwale et al. (1986) reported regeneration from 54 soybean genotypes with only minor differences in percent regeneration. As with earlier studies, 2,4-D was superior for the production of embryogenic cultures, but somatic embryos were abnormal from cultures induced from 20 μM 2,4-D and were produced at low frequencies on 45 μM and 67 μM 2,4-D. Raising the level of thiamine-HCl and nicotinic acid in the basal medium increased the percentage of embryogenesis. Embryogenic cultures were maintained and serially propagated on 21.4 μM NAA in the dark with no loss of embryogenic capacity over a period of 12 months. For "germination", torpedo-stage somatic embryos were transferred to either MS basal medium + 1.7 μM BAP + 0.2 μM IBA or MS basal medium + 9.8 μM IBA + 5.0 nM BAP + 5 μM GA_3 and then to MS basal medium without growth regulators. For efficient plant recovery, rooted plantlets were grown in liquid Hoagland's before transplantation to soil in the greenhouse. All regenerated plants and their progeny were fertile.

Although no genotypic differences in percent regeneration were reported in the previous study, a concurrent study (Kerns et al. 1986) reported that genotypes with a high capacity for cotyledonary shoot proliferation were also found to have a high capacity for somatic embryo development. Somatic embryos

were produced from cotyledon or hypocotyl-derived callus in liquid suspension cultures containing 0.4 mg l^{-1} 2,4-D as the sole growth regulator. Cultures were evaluated for average number of somatic embryos per two culture vessels, resemblance to normal embryos, and relative numbers of additional "clusters of proliferating embryos". These three characteristics were positively correlated, i.e., flasks with the highest number of embryos also had the most normal embryos and the highest number of embryonic clumps. As with earlier systems that utilized callus-derived suspension cultures (Beversdorf and Bingham 1977; Phillips and Collins 1981), somatic embryo development was incomplete and no plant regeneration occurred.

All reports subsequent to that of Kerns et al. (1986) have described auxin-induced somatic embryogenesis from immature zygotic embryos. Ghazi et al. (1986) induced the most responsive somatic embryos from immature cotyledons 6 mm in length in the presence of 10 mg l^{-1} 2,4-D and 0.264 mg l^{-1} ABA. Hammatt and Davey (1987) found heart-stage embryos to be more responsive than globular or cotyledonary-stage immature embryos. In this study, genotypes differed in percentage of explants producing somatic embryos [Fiskeby (38%) AP 120 (18%) ACCO 101 (9%)]. To improve germination rates, somatic embryos were cultured for a total period of 8 weeks, then desiccated in empty petridishes for 1-4 weeks until somatic embryos had shrunk to 40-50% of original volume. While only 5% of all nondesiccated embryos germinated upon transfer to fresh medium, 35% of Mitchell and 72% of AP 120 embryos germinated after the desiccation treatment. Germinated embryos also developed viable apical meristems. Other reports have substantiated the improvement of conversion rates after maturation and desiccation of soybean somatic embryos. Parrott et al. (1988) found that conversion was substantially improved when somatic embryos were matured for 1 month on hormone-free medium and then desiccated for 1 week. Buchheim et al. (1989) expanded on these studies by desiccating somatic embryos in atmospheres with defined relative humidities. A 4-day desiccation period in a relative humidity of 75% was superior to either 40% or 93% relative humidity for embryo viability and conversion.

Embryo morphology has been an important indicator of maturation, germination, and conversion ability. Lazzeri et al. (1987a) found that embryos of normal morphology were most likely to germinate, regardless of the growth regulator content of the germination medium. Likewise, Ranch and Palmer (1987) reported that morphologically normal embryos were the most developmentally responsive, regardless of the induction or propagation medium. The influence of morphology on conversion was systematically evaluated by Buchheim et al. (1989). Somatic embryos were classified in nine morphological categories and evaluated for conversion frequency and rate. Normal somatic embryos converted at higher frequencies than any of several abnormal embryo types.

Several recent reports have characterized and improved upon auxin-stimulated somatic embryogenesis from immature cotyledons. The first report describing the origin and mode of formation of somatic embryos was that of Hartweck et al. (1988). Using the system of Lazzeri et al. (1985), it was found that 95% of somatic embryos were formed on cotyledons with the abaxial surface oriented on the culture medium. Somatic embryos were observed to arise directly from a "compact green rim" on the perimeter of the explant. A friable callus also formed along the periphery of the explant, but no differentiation from this tissue was observed. This study also reported an auxin-orientation interaction for frequency and normality of somatic embryogenesis. The highest number of embryos were induced with 2,4-D on explants with either their abaxial or adaxial surface in contact with the medium. The greatest number of normal embryos arose from either NAA-induced explants with the abaxial surface on the medium, or from adaxially-oriented explants induced with 2,4-D. Embryos arising from the margin of the cotyledon were more normal than those arising from the center. NAA-induced embryos were more likely to arise from single or small cell groups than embryos induced with 2,4-D. A sucrose-auxin interaction also has been observed (Lazzeri et al. 1988), such that normalcy of embryos increased as sucrose levels decreased and auxin levels increased. Finally, there appears to be an inverse correlation between the length of induction on an auxin and the normalcy of the resulting somatic embryos (Parrott et al. 1988).

Prolific suspension cultures have been derived from globular-stage somatic embryos induced on 2,4-D (Finer and Nagasawa 1988). Secondary embryos proliferate from terminal or apical areas of primary somatic embryos (Finer 1988). This mode of proliferation is markedly different from the secondary proliferation described by Ranch et al. (1985), who observed secondary embryos arising from the base of primary somatic embryos in a rosette-like fashion. The use of microprojectile acceleration techniques in conjunction with repetitively embryogenic suspension

cultures is currently the most promising system for the transformation of soybean (McMullen and Finer 1990). This system may prove useful for legumes in which embryogenic suspensions can be obtained.

Komatsuda and Ohyama (1988) detected differences in somatic embryogenesis and conversion ability among a number of Japanese genotypes. In another evaluation of genotypic effects on regeneration, Parrott et al. (1989) found all genotypes evaluated were embryogenic, but genotypes with 'Manchu' or 'A.K. Harrow' in their pedigree regenerated at higher frequencies. A cross between Shiro (a very low regenerator) and Manchu (a high regenerator) resulted in F_1 progeny with intermediate regeneration capacity. Recently, 290 genotypes of soybean and 5 genotypes of *G. max* subsp *gracilis* Smartt were evaluated for regeneration capacity. An accession from South Africa, cv Brownie, was found superior to all other genotypes (Komatsuda and Ko 1990).

The current soybean regeneration protocols are summarized in Figure 1. Several factors important for the induction, maturation, and conversion of normal somatic embryos are clear from the body of literature on soybean tissue culture. Explant source is of utmost importance as immature embryo explants are the only tissue from which regenerated plants have been recovered via direct somatic embryogenesis. Orientation of immature cotyledons is also critical as explants with the abaxial surface oriented on the culture medium produce considerably greater numbers of more normal embryos than explants with the adaxial surface on the culture medium (Hartweck et al. 1988). Although no genotype is completely recalcitrant to somatic embryogenesis, genotypes differ by 200x in embryogenic capacity, and also differ substantially in conversion ability. With the most recent and efficient protocols, growth regulators are required only to induce embryogenesis (Parrott et al. 1988; Buchheim et al. 1989). The greatest number of embryos are produced from explants induced for a short time by 2,4-D. In early studies embryos induced with 2,4-D were generally abnormal, presumably due to carryover effects. The use of activated charcoal in the maturation medium has apparently reduced auxin carryover, increasing the recovery of normal somatic embryos (Buchheim et al. 1989). For optimal conversion frequencies and rates, soybean somatic embryos require a maturation period that is approximately equivalent to maturation times required by zygotic embryos. Another similarity to zygotic embryos is the fact that desiccation markedly enhances germination and conversion rates (Hammat and Davey 1987; Parrott et al. 1988).

General Considerations for Direct Embryogenic Systems

Explant age. Immature embryos have been utilized for the induction of somatic embryogenesis from a number of legumes (Table 1). The developmental age of the explant has proven important for most species. In red bud, *Cercis canadensis* L., the highest numbers of somatic embryos were produced from zygotic embryo explants collected from pods that were 75 and 82 days postanthesis (Geneve and Kester 1990). In black locust, *Robinia pseudoacacia* L., somatic embryos were initiated from a single culture collected and cultured 4 weeks after anthesis. As only one explant regenerated, this response cannot be unequivocally attributed to explant age. However, immature seed collected 1-2 weeks after anthesis exhibited no growth and those collected 8-10 weeks after anthesis simply germinated. In each case, the response would likely preclude embryogenesis (Merkle and Wiecko 1990). With groundnut, the age of the explant appears less important since somatic embryogenesis has been reported from developmental stages ranging from late-heart-stage zygotic embryos (Sellars et al. 1989) to nearly mature cotyledons (Ozias-Akins 1989). Embryonic axes ranging in size from 3 to 6 mm have also proven responsive to the production of somatic embryos (Hazra et al. 1989).

Auxin. Auxin type, concentration, and exposure time have also proven important for the initiation of somatic embryogenesis in legumes other than soybean. As with soybean, 2,4-D has proven superior to NAA for initiating greater numbers of embryos per explant. In one experiment, zygotic embryo explants of red bud were the most embryogenic when induced with 2,4-D, rather than NAA (Geneve and Kester 1990). Normal embryo morphology for this study was not correlated with auxin type (NAA or 2,4-D), and in contrast to soybean, explants cultured on low levels of 2,4-D (5 μM) produced the greatest numbers of normal embryos. The optimum exposure time for somatic embryo formation in this study was 20 days.

Groundnut appears to be responsive to a variety of synthetic auxins. Sellars et al. (1989) reported the highest frequency of somatic embryos resulted when PIC was used in the inducing medium, although lower frequencies were observed with either NAA or 2,4-D. Another study (Ozias-Akins 1989) reported successful initiation of somatic embryogenesis using PIC, but little success when NAA was used. Hazra et al. (1989)

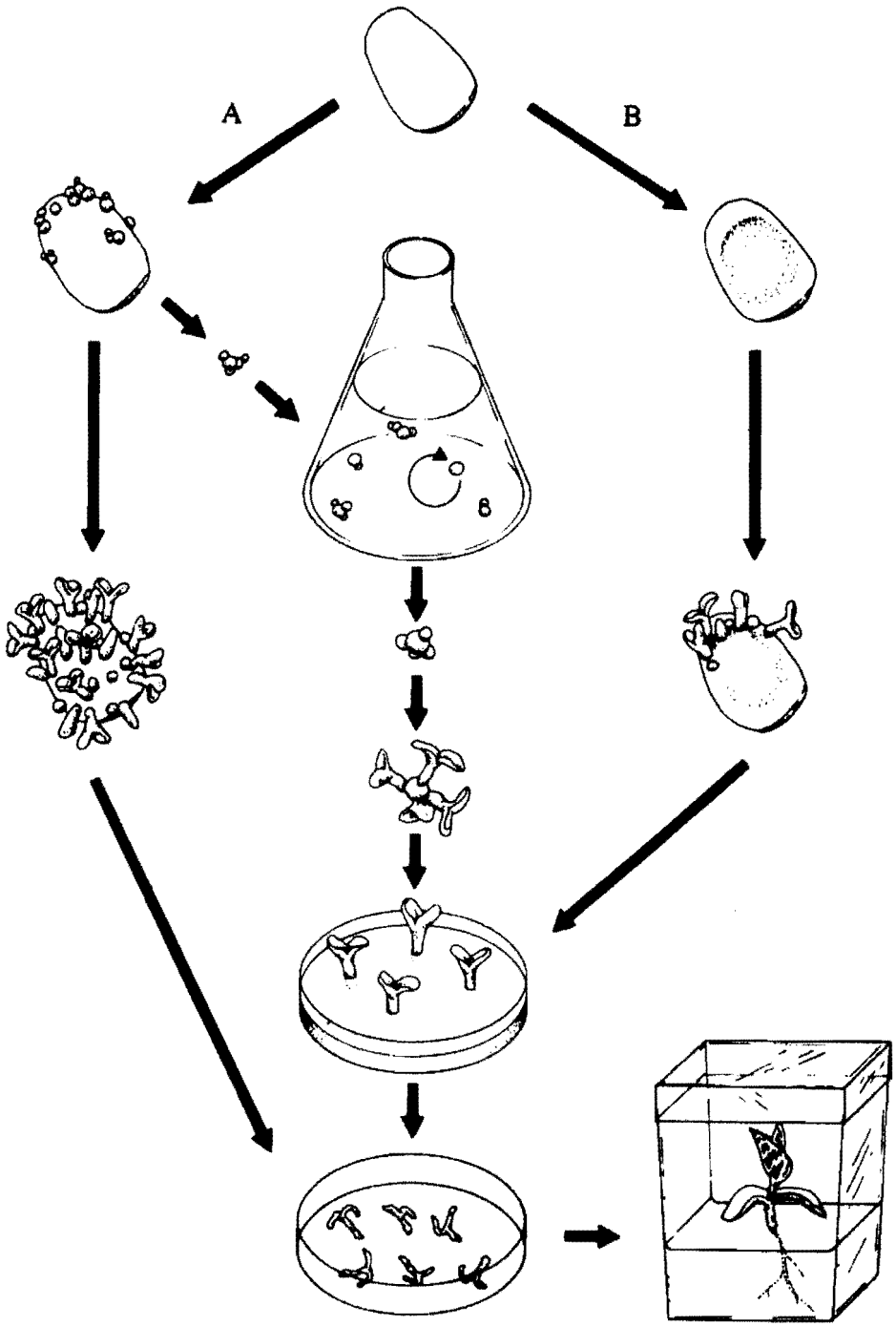

Figure 1. Generalized scheme for auxin-stimulated somatic embryogenesis from immature cotyledons of soybean. (A) Induction with high levels (>20 mg l^{-1}) of 2,4-D for 30-60 d results in proliferation of primary and secondary somatic embryos. These may be serially propagated in suspension culture containing lower levels of 2,4-D (5 mg l^{-1}) or transferred to hormone-free solid medium (MSO) containing activated charcoal for development and maturation. After a maturation period equivalent to that necessary for their zygotic counterparts, somatic embryos are removed to a dry vessel for partial desiccation, then transferred back to hormone-free medium for germination and seedling growth. (B) Induction with 10 mg l^{-1} of NAA for 10 d results in formation of embryogenic tissue around the distal periphery of the explant. Transfer of induced explants to MSO allows development of somatic embryos. Further maturation is achieved by isolation of embryos and transfer to fresh MSO. Separation of embryos from embryogenic clumps can also be used for maturation of somatic embryos from suspension as depicted in (A). Desiccation promotes germination and seedling growth from any of these protocols. [Protocols modified from Buchheim et al. (1989); Parrott et al. (1988); Finer and Nagasawa (1988)].

found 2,4-D to be superior to NAA for inducing somatic embryogenesis from embryonic axes.

Cytokinin. As mentioned previously, very young zygotes exposed to a cytokinin form somatic embryos, an ability lost to older embryos. Cotyledonary-stage embryos, however, respond to auxins. These results can probably be explained in terms of the concept PEDCs (Pre Embryogenically Determined Cells) of Sharp et al. (1982). Very young zygotic embryos consist of PEDCs, and only a mitotic stimulus is required for cells within the embryo to form additional embryos. On the other hand, slightly older embryos have lost some of their PEDC property, presumably due to termination of embryogenic developmental programs within the cells and their replacement by other developmental programs. Such cells must be exposed to an auxin to restore their embryogenic potential. While the presence of a cytokinin in the growth medium is required to obtain vigorous callus growth, the rationale for inclusion of a cytokinin if the objective is to obtain somatic embryos from immature cotyledons is not evident. Use of an auxin is all that is necessary. In those cases where both auxins and cytokinins were used, the presence of the cytokinin either had no effect or had a detrimental effect.

Role of Tissue Culture in Legume Improvement

In the following section we will draw examples from the literature to highlight some of the areas where tissue culture has proven useful to legume improvement. These areas include long-term storage of germplasm, virus elimination, somaclonal variation, and somatic cell selection. Much of the work to date has involved alfalfa and clover since these were among the first legumes to be manipulated in tissue culture. However, similar approaches may prove useful in other legumes in which tissue culture techniques have been developed. An emerging area of importance is the use of tissue culture in genetic transformation. Since this area will be covered elsewhere in these proceedings, we will not include it here.

Germplasm Preservation

While most cultivated legumes are seed propagated and therefore amenable to long-term storage, some wild species are perennial or reproduce poorly from seed and require clonal propagation for germplasm maintenance and long-term storage (Mroginski and Kartha 1984). Cheyne and Dale (1980) were able to store shoot-tip cultures of red clover, white clover, and alfalfa for 15 to 18 months without subculture and recover viable plants from more than 70% of their cultures. Their storage regimes were either 2-4°C with an 8-hour photoperiod, or 4-6°C under complete darkness. Also, Bhojwani (1981) stored shoot cultures of white clover for 10 months without subculture at 5°C in darkness. This treatment did not affect the potential for rapid multiplication of the cultures. Following cryopreservation for 20 months in liquid nitrogen (–196°C), meristem cultures of groundnut and chickpea, *Cicer arietinum* L., exhibited 42% and 57% survival (Nagarajan and Walton 1987). Examples of other long-term storage regimes can be found in Bajaj (1990).

Virus Elimination and Evaluation

When very small regions of rapidly growing meristems are isolated and cultured, resulting plants are often free of viruses present in the original plant material. Virus elimination is particularly important in the production of clover seed. Most clover cultivars are synthetics, resulting from open pollination of several source genotypes. The progenitor genotypes are maintained through clonal propagation and are thus susceptible to continued infection and spread of viruses. The end result is a reduction in seed production (Williams et al. 1990). Barnett et al. (1975) used a combination of cold treatment (10°C for 13-15 wks) and meristem-tip culture of white clover to successfully eliminate viruses from plants. Also, Phillips and Collins (1979a) found that two-thirds of the meristem-derived plants from red clover were virus free. In the latter study, virus elimination was enhanced by using smaller apical meristems to initiate cultures. Shoot-tip or meristem culture offers both clonal propagation and virus elimination to seed producers. The production of aseptic, virus-free plants in vitro expedites the international transfer of germplasm since the aseptic cultures are usually easily passed through quarantine. Techniques for meristem culture have been developed for several other legume genera (Mroginski and Kartha 1984) and should prove useful for virus elimination from these species as well.

Jones et al. (1981) investigated the ability of cell cultures of several clover genotypes to support virus replication in vitro. They found the in vitro response closely approximated the response of the genotypes

when inoculated in situ, indicating that selection for virus resistance is possible using tissue-culture techniques. This finding is important since it would allow pathologists to evaluate viral resistance in vitro and thus reduce or eliminate the risk of accidental viral release into previously uninfested regions.

Somaclonal Variation

Plant breeders are continually searching for new genetic variability that is potentially useful in cultivar improvement. A portion of plants regenerated by tissue culture often exhibits phenotypic variation atypical of the original phenotype. Such variation, termed somaclonal variation (Larkin and Scowcroft 1981), may be heritable, i.e., genetically stable and passed on to the next generation. Alternatively, the variation may be epigenetic and disappear following sexual reproduction. The heritable variation is potentially useful to plant breeders.

Somaclonal variation in legumes has been studied most in alfalfa (Bingham and McCoy 1986) and, to a lesser extent, in soybean (Barwale and Widholm 1990). In alfalfa, the most common type of somaclonal variation derived from tissue culture involves changes in chromosome number. Saunders and Bingham (1972) observed octoploid and albino plants among the first alfalfa regenerants, and numerous other studies have also indicated chromosomal changes ranging from spontaneous doubling of chromosome number to aneuploidy and translocations (Binarová and Dolezel 1988; Taha and Francis 1990; Nagarajan and Walton 1987; Reisch and Bingham 1981; Groose and Bingham 1984; Johnson et al. 1984). Among these studies, no difference in number of variants was observed in plants regenerated from suspension or callus culture (Groose and Bingham 1984; Shahin et al. 1986; Binarová and Dolezel 1988). The work of Nagarajan and Walton (1987) indicates that frequency of aneuploidy in alfalfa tissue cultures may be genotype dependent. The number of aneuploids observed during development of Regen-S, a line bred for high regeneration capacity (Bingham et al. 1975), was probably minimized since selection for vigor and fertility were performed after each regeneration cycle. In a study specifically designed to evaluate the frequency of somaclonal variation, Groose and Bingham (1984) constructed two tetraploid lines of alfalfa that were heterozygous for four simply inherited traits. One culture resulted in 116 regenerated plants of which 21% were variant for one or more of the characters. Sixty percent of variant regenerants exhibited a change in chromosome number. However, they observed that variation induced by tissue culture did not result in major rearrangements in either the alfalfa chloroplast or mitochondrial genomes.

In another study with alfalfa (Reish et al. 1981), cell suspensions of a diploid cultivar, HG2, were mutagenized with methane sulfonic acid ethylester (EMS) and selected for resistance to the toxic effect of ethionine (an analog of methionine). Two types of variants were observed in the regenerated plants: those that were resistant to ethionine, and those that exhibited phenotypic variation in morphology, fertility, and herbage yield. Few variants were observed among plants regenerated from mutagenized cultures that were not treated with ethionine, leading the authors to suggest that the mutagenic effects of ethionine may have been responsible for the appearance of the additional variants (Reisch and Bingham 1981). One variant, a spontaneous tetraploid (NS1) arising from a control culture, yielded threefold more herbage dry matter than diploid or tetraploid controls. A subsequent experiment (Pfeiffer and Bingham 1984) confirmed these results and indicated significant improvement in fertility of NS1. However, the yield increase realized in NS1 was not equal to that achieved through selection during inbreeding of HG2, the diploid progenitor or NS1 (Pfeiffer and Bingham 1984).

Barwale and Widholm (1990) described three types of variants that arose after cotyledonary-node culture of soybean: traits exhibiting simple inheritance, traits with stable inheritance but aberrant segregation ratios, and epigenetic traits. Among the variants observed were chlorophyll deficiency, partial or complete sterility, numerous abnormalities in leaf morphology and number, twin seeds, changes in developmental growth habit, and multiple shoots. Although not as common as in alfalfa, changes in ploidy were also observed among soybean regenerates (Barwale and Widholm 1990). Variations in quantitative traits have been observed as well. Greybosch and coworkers (1987) reported decreased yield (2 families) and increases in height (3 families) among 89 families derived from plants regenerated from cotyledonary nodes of soybean. Changes in maturity date and increases in oil content among regenerates have also been reported (Barwale and Widholm 1990).

Somatic Cell Selection

Somatic cell selection, or in vitro selection, is a process by which variant cells or cell lines are selected in vitro for the ability to grow in the presence of selec-

tive agents, such as fungal filtrates, herbicides, or high salinity, which inhibit the growth of nonvariant cells. For the technique to be successful, spontaneous or induced mutations must be present in the cell cultures, and the resistance reaction must be active at the cellular level (Bingham et al. 1988). This procedure has the advantage—over selection imposed following plant regeneration—of increased efficiency of screening for rare variants among large numbers of cells in culture.

There are three major areas where somatic cell selection has been practiced with legumes: selection for resistance or tolerance to fungal filtrates, selection for tolerance to phytotoxic levels of herbicides, and selection for salinity tolerance.

In vitro selection for resistance to several fungal diseases in alfalfa has been attempted. Selection for resistance to fusarium wilt was achieved by adding culture filtrates from the fungus into the plant-culture medium (Hartman et al. 1984). Resistance was observed in regenerated plants under greenhouse conditions, and these plants gave rise to resistant cell cultures. However, there are no reports of progeny evaluated for resistance. In the previous study, selection for resistance among long-term cultures resulted in plants with elevated ploidy (hexaploids and octoploids), but selection from short-term cultures yielded tetraploid plants that were also resistant. Other studies have also indicated success in selecting for *Fusarium* resistance (Binarová et al. 1990; Arcioni et al. 1987). While the resistance obtained in these studies has been stable through asexual propagation, reports are lacking as to whether the resistance is heritable. Attempts to select for resistance to *Verticillium* wilt in alfalfa were unsuccessful (Latunde-Dada and Lucas 1988; Yu et al. 1990). When selecting for resistance to *Verticillium*, toxic levels of culture filtrate apparently selected for cells that were incapable of regeneration. When selection was carried out with levels of the filtrate that allow regeneration, there was little evidence of increased resistance to the fungus among regenerated plants (Latunde-Dada and Lucas 1988). Mezentsev et al. (1982) found a direct correlation between resistance in red clover at the seedling and cellular level to *Sclerotinia trifoliorum* using culture medium. Selection for resistant cell lines was successfully achieved in irradiated cultures, but resistance in regenerated plants was not tested.

Several attempts have been made to select for increased tolerance to phytotoxic levels of phenoxy analog herbicides (2,4-D, 2,4,5-T, and 2,4-DB) in clover. Oswald et al. (1977) reported increased tolerance of cell lines to all three analogs following a 5-day exposure of suspension cultures to phytotoxic levels of the herbicides. Selecting for tolerance to 2,4-DB alone also conferred tolerance to the other two herbicides. Tolerance was maintained through subsequent cell generations and the report indicated that plants were regenerated from selected cells although response to the herbicides at the whole plant level in these regenerates was not discussed. In another study (Taylor et al. 1989), in vitro and field responses to 2,4-D of eight diverse clover populations were correlated ($r=0.77$) and increased tolerance to 2,4-D was selected in several callus lines, but plants could not be regenerated from the callus. In birdsfoot trefoil a positive correlation was found between whole plant and callus tolerance to 2,4-D (Swanson and Tomes 1980b) and selection for 2,4-D-tolerant cell lines was successful (Swanson and Tomes 1980a). However, the level of tolerance achieved through in vitro selection did not equal that exhibited in a cultivar previously selected for field resistance to 2,4-D (Swanson and Tomes 1980b).

Selection for salinity tolerance has been largely unsuccessful in alfalfa. In two separate studies (McCoy 1987; Smith and McComb 1983), there was no correlation between in vitro and whole plant response. One study (Smith and McComb 1981), however, identified a cell line with increased NaCl tolerance. This cell line originated from a cultivar previously selected to perform well in tissue culture (Bingham et al. 1975).

Miscellaneous Uses

Correlations have been noted between whole-plant and callus tolerance to stress factors, such as salinity, in legumes. Such a correlation for tolerance to salinity and acidity has also been identified in alfalfa (Mezentsev et al. 1982). Therefore, callus cultures have been proposed as sensitive assays for evaluation of whole plant tolerance (Smith and McComb 1981). Recently, Parrott and Bouton (1990) have exploited this relationship and used callus assays to identify alfalfa plants most tolerant to toxic levels of aluminum in acidified media; and Stephens et al. (1990) were able to detect differences in iron efficiency between soybean cultivars by using a callus assay. Finally, genotypic differences in the capacity for tannin production are evident in tissue culture (Lees 1986), raising the possibility that programs breeding for low tannin content can use callus assays to identify plants with low tannin contents to use as parents in the breeding program.

Conclusions

General conclusions about legume tissue culture, modified from those of Bingham et al. (1988), appear to be generally applicable, whether regeneration is via organogenesis or embryogenesis.

- Some species of legumes are amenable to regeneration by both organogenesis and embryogenesis.
- Regeneration is genotype-specific.
- Most genera or species which have been seriously addressed contain genotypes capable of regeneration via one mode or another.
- Media combinations will probably need to be optimized for each species.
- Explant type and age can affect regeneration capacity.
- Genotypes or populations with a high regeneration capacity can be developed through conventional breeding.

Tissue-culture systems for legumes have been and continue to be developed to meet the challenging demands of plant improvement and genetic engineering. Various options for the design of regeneration systems are currently available. The desirability of the mode of regeneration is contingent on specific goals. The mode may be irrelevant if one simply needs to recover plants from protoplasts or following in vitro selection. Simple shoot proliferation is all that is necessary for virus elimination or germplasm conservation. Organogenic regeneration systems have traditionally been more effective for the purposes of genetic transformation, but transformation techniques have now been adapted to exploit the unique features of somatic embryogenesis. This is important since de novo regeneration occurs exclusively via somatic embyrogenesis in several species.

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In Vitro Culture Techniques for Forage Genetic Resources

J. Hanson and T.J. Ruredzo[1]

Introduction

Inadequate nutrition in livestock, resulting from feed shortages and the poor quality of available feed throughout the year is the major constraint to increased livestock production in sub-Saharan Africa. Forage crops play an important role as animal feed resources and germplasm is being tested to select adapted forages for further development and use by small-holder farmers in Africa. A large pool of germplasm, which can be used for evaluation and selection, is essential for development of improved forages for animal feeds.

Many native forages show considerable potential for further development and incorporation into small-scale farming systems for livestock production. Forage grasses of the genera *Pennisetum, Cognition, Digitaria, Panicum, Chloris, Cenchrus, Setaria*, and *Brachiaria*, which form the major components of native African pastures, are a major feed resource. Indigenous tree legumes such as *Sesbania, Erythrina, Faidherbia*, and *Acacia*, together with introduced genera such as *Leucaena*, have considerable forage potential in the African environment.

Although conventional management of forage germplasm relies on seeds, some important species of forage grasses rarely produce seeds and other slow growing trees may be outcrossing or take several years to produce seeds, thus inhibiting rapid dissemination of selected germplasm. Plant in vitro culture techniques have provided solutions and alternative approaches to overcoming constraints in the management of these genetic resources.

An important constraint to the rapid development of these forage species for utilization is the long time they take to reach maturity and produce seeds or, in the case of grasses, inadequate production of seeds for collection, multiplication, dissemination, and utilization. In vitro culture techniques have a potential use for those tropical forage legumes and grasses that cannot be conveniently handled by conventional methods for genetic resources management.

Use of In Vitro Culture for Germplasm Management

Over the last decade in vitro plant culture has proved invaluable to crop plant improvement, providing solutions and alternative approaches to overcoming constraints in management of genetic resources. In vitro techniques have been used for germplasm collection (IBPGR 1984; Withers 1987), disease elimination (Kartha 1981, 1986), micropropagation (Hussey 1983), and germplasm dissemination and conservation (Withers 1980). In vitro techniques have also been used for the creation of variability through somaclonal variation (Larkin and Scowcroft 1981; Scowcroft 1984), in vitro pollination, fertilization, embryo culture, somatic hybridization (Melchers 1982; Melchers et al. 1978), and genetic transformation (Wullems et al. 1982). Haploid plants which are invaluable for plant breeding can be produced from in vitro anther and pollen culture (Sunderland and Cocking 1978; Sunderland 1979). Forage species have received much less attention but investigations in in vitro methods for management of forestry species (Hartney and Kabay 1985; Bonga and Durzan 1982) and on forage grasses at the Welsh Plant Breeding Station (Dale 1978, 1980) and at the International Livestock Centre for Africa (ILCA) (Ruredzo and Hanson 1990) have shown that these techniques are equally useful in management of forage genetic resources and plant improvement.

Many of the early concerns about the stability and genetic integrity of plant in vitro cultures have now been resolved. Plants that are recovered from the non-adventitious growth of shoot tips, axillary buds, meristems, embryos, and gametes have been found to be genetically stable (Scowcroft 1984). Plants that are

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directly regenerated from nonaxillary regions of the plant are also stable, although their genotype will depend on the mother tissue (D'Amato 1975). Indirect regeneration through a callus stage is believed to release somaclonal variation giving rise to genetic variants from the same tissue, which can include enhanced desirable characteristics (Larkin and Scowcroft 1981). Plants that are recovered from these therefore need to be assessed for genetic stability and enhanced desirable characters.

In Vitro Research at ILCA

Work on in vitro culture technology has been in progress at ILCA since 1986 when the International Board for Plant Genetic Resources (IBPGR) and ILCA agreed to collaborate in a 2-year project to develop minimal facility methods for in vitro collection and in vitro slow growth methods for the conservation of *Cynodon aethiopicus*, *C. dactylon*, and *Digitaria decumbens*. A suitable basal medium for grass species was identified for the growth of these species in vitro and suitable exogenous growth substances (auxins, cytokinins, and gibberrellins) and carbon source for normal rates of growth in vitro were determined. Cultures of the forage grasses *Cynodon* and *Digitaria* were successfully initiated, multiplied and rooted, and slow growth conservation techniques using low temperature were developed for these species (Ruredzo and Hanson 1990). It is now possible to maintain cultures at 15°C for up to 18 months without subculturing and to recover them into normal growth conditions. A minimal facility method for the transfer of these species to soil was developed and a minimal facility method for the collection of *C. dactylon* using locally available sterilizing agents, antibiotics, and fungicides as supplements to the media to control contamination was also developed and tested.

In 1989 the work was expanded to multipurpose tree legumes in a project supported by the International Development Research Centre (IDRC) to develop in vitro culture methodology for rapid multiplication. *Sesbania sesban*, *leucaena leucocephala*, and *Erythrina brucei* were successfully initiated in vitro from embryo-derived cotyledons, hypocotyls, and embryo axes of *Sesbania sesban* (Ruredzo and Hanson 1990) and from embryo-derived explants in *Faidherbia albida* and *Acacia tortilis*. Nonadventitious regeneration was successfully achieved from shoot tips in *Erythrina brucei*, *Faidherbia albida*, and *Acacia tortilis*.

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Plant Regeneration in Chickpea

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Micropropagation is one of the most widely used tissue culture techniques for rapid asexual in vitro propagation. Such techniques for grain legume crops have been rare but are of recent interest. Chickpea (*Cicer arietinum* L.) is a major food legume in many countries and is one of the most important pulse crops in the world. A technique was standardized to propagate chickpea and its wild relatives. A medium was developed to induce multiple shoots from excised shoot tips of 15-day-old aseptically raised seedlings and 30-day-old greenhouse-grown plants. Shoot-tip explants (2-8 cm long) of six chickpea genotypes (L 550, K 850, ICCC 32 (ICCV 6), ICC 12237, ICCC 42, and C 235) and four wild *Cicer* spp (*C. bijugum* No. 201, *C. cuneatum* SL 157, *C. judaicum* No. 185, and *C. pinnatifidum* No. 188) were cultured under two light regimes. Multiple shoots were produced from shoot tip explants of all the genotypes tested on medium containing 2.0 mg l^{-1} 6-Benzyl Aminopurine (BA) and 0.5 mg l^{-1} Indole Acetic Acid (IAA). All the chickpea genotypes except ICCC 32 and ICC 12237 produced a greater number of shoots under 16 h than 24 h daylength. Explants of *C. cuneatum* SL 157 and *C. judaicum* No. 185 also responded better under 16 h than 24 h daylength. *Cicer bijugum* and *C. pinnatifidum* were not tested under 16 h daylength but did produce shoots under 24 h daylength.

Shoots produced were excised and subcultured individually on fresh medium at 6-week intervals to ensure a high rate of multiplication. One shoot-tip explant of the genotype K 850 produced a total of 134 shoots after three transfers. The shoots were excised and transferred individually to a rooting medium supplemented with Indole-3 Butyric Acid (IBA) or Napthylene Acetic Acid (NAA). The IBA-supplemented medium induced normal roots, while NAA-supplemented medium induced short, thick roots. The plantlets were transferred to polythene bags or small plastic pots containing sterile sand and then drenched with an antimicrobial solution (Benlate® + Thiram® + Agrimycin® mixture) to reduce plant mortality. Finally, the plantlets were established in a sand + Vertisol (3:1) medium in pots and maintained in the greenhouse. Normal pods were produced from such micropropagated plants.

Attempts were also made to regenerate plants from callus. Callus was induced from a range of explants (leaflets, mature cotyledons, immature cotyledons, immature embryos, hypocotyls, epicotyls, shoot tips, and stems) of *C. arietinum* genotypes and wild *Cicer* spp. Callusing response varied with explant, media, light, and temperature. Medium containing 2,4-D, alone, or in combination with other hormones, induced callus with embryogenic structures that resembled embryogenic initials. Further differentiation into embryo-like structures did not occur. However, some of the calli produced roots.

Medium supplemented with BA and IAA induced callus from the cut end of stem explants in contact with the medium. Very few calli produced shoot buds when maintained on the same medium. The callus was transferred to medium without hormones for shoot elongation. The regenerated shoots produced roots on medium supplemented with IBA. This technique will be standardized to improve the frequency of regeneration.

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Tissue and Organ Culture and Regeneration in *Arachis hypogaea* and its Wild Relatives

Nalini Mallikarjuna[1], D.C. Sastri[1], J.P. Moss[1], Amar Kumar[2], and W. Powell[2]

The ability to generate plantlets from in vitro cultures of organs and tissues offers opportunities for in vitro genetic manipulation besides several commercial applications. A range of tissues and organs from *Arachis hypogaea* and some of its wild relatives were cultured on different media. Concentrations, type, and combinations of cytokinins and auxins used in the media elicited different responses. Preliminary results of regeneration were published in 1981 by Sastri et al. Caulogenic callus was always obtained when the medium was supplemented with a combination of NAA and BAP for culture of a range of seedling explants—root discs, shoot meristems, immature leaflets and epicotyl, and cotyledons from mature seeds. All these explants produced shoot-forming calli from *A. hypogaea, A. pusilla, A villosulicarpa,* and *A. correntina* on MS semisolid medium with NAA (0.01-10.0 mg l^{-1}) and BAP (0.1-25.0 mg l^{-1}).

The only exceptions, however, were roots of *A. hypogaea* that did not respond similarly and no organogenesis was observed. Amongst the four *Arachis hypogaea* L. cultivars, JL-24, TMV-2, Robut 33-1, and MK-374, used for cotyledon cultures, JL-24 was superior with respect to the number of cotyledons giving rise to shoot buds. Cotyledons from mature seeds had a greater propensity to form shoot buds and shoots at the nodal end directly, or through a small callus phase. Cotyledons formed shoots and shoot buds on medium with Kinetin also. Amongst the different combinations of NAA and BAP, NAA at 2 mg l^{-1} and BAP at 25 mg l^{-1} induced the maximum number of shoot buds from the cotyledons. All the buds obtained on the above hormone combination did not appear normal. Hence, to recover the maximum number of shoots from those buds, they were either dissected into individual shoot buds and cultured on NAA 0.1 mg l^{-1} and BAP 1.0 mg l^{-1} or the buds were directly transferred to the induction medium, but with a larger culture vessel. It was observed that the second method supported a greater number of buds to develop into shoots. The shoots thus formed have been successfully transferred to soil in high frequency.

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Regeneration in *Arachis hypogaea*

J.P. Moss, M.J.T. Reena, N.R.G. Dutt, and D.C. Sastri[1]

De-embryonated cotyledons have been widely used for in vitro regeneration because of their easy availability and ease of handling. A large number of shoots can be regenerated from a single cotyledon within a short time thus making them good candidates for use in genetic engineering. Although cotyledons placed on moist filter paper will produce shoots, responses vary greatly when cotyledons are placed on different media. The cotyledon is a storage organ that acts as the source of nutrition for the germinating seedling. In this study, de-embryonated cotyledons were placed on a range of media to initiate shoots and/or roots and transferred to basal medium after different periods.

The following phytohormones, a) NAA (Napthylene acetic acid) 1 mg L^{-1} and BAP (Benzylaminopurine) 1 mg L^{-1}, and b) Kinetin 4 mg L^{-1}, were applied, in three formulations: (1) distilled water, (2) with sucrose and agar, and (3) with MS major and minor salts, vitamins, sucrose, and agar. The cultures were maintained at 25°C under high light intensity (2000-2500 lux), and on 16 and 8 h light/dark cycle. After 0.6, 3, 6, 10, and 13 days of culture, the cotyledons were transferred from the different media to MS (Murashige and Skoog) basal medium.

The duration of exposure and the composition of the media had a marked effect on the response of the cotyledons. Number of explants forming roots and shoots were greatest when cotyledons were cultured in distilled water with hormones; the presence of sucrose and agar or MS salts, sucrose, and agar decreased the percentage of responding cotyledons (Fig. 1).

Pretreatment with hormones in distilled water produced up to 39 shoots per cotyledon. The numbers of shoots and roots produced continued to increase with pretreatment time when NAA + BAP were used, but there was an optimum pretreatment time when Kinetin was used (Table 1).

These experiments indicate that cotyledons are a good source of shoots for in vitro manipulation, and that their own nutrient storage is sufficient for initiation of shoots in large numbers. Addition of salts or sucrose decreased the number of explants initiating shoots and roots, and also the number of shoots and roots produced per cotyledon.

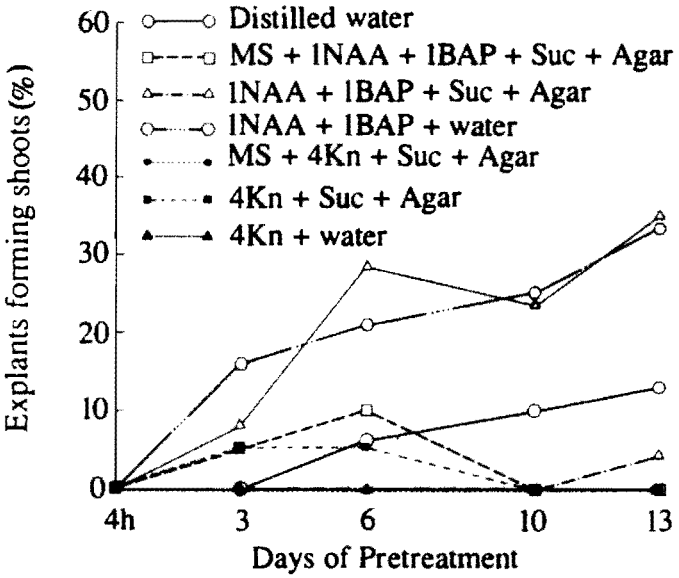

Figure 1. Effect of media constituents and hormones and days of pretreatment on cotyledon response.

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Table 1. Mean response of whole de-embryonated cotyledons to different media and days of pretreatment.

Medium	Days of pretreatment	Number of		Length of	
		Roots	Shoots	Roots	Shoots
Distilled Water	0	0.09	0.00	0.01	0.00
	3	0.23	0.64	0.48	0.01
	6	0.00	0.30	0.00	0.02
	10	0.24	0.90	0.23	0.01
	13	0.43	1.26	0.56	0.04
MS+1NAA+1BAP+3%Suc+0.7%Agar	0	0.17	0.00	0.20	0.00
	3	0.09	0.96	0.05	0.01
	6	0.38	2.24	0.05	0.02
	10	0.00	0.00	0.00	0.00
	13	0.71	1.00	0.80	0.17
1NAA+1BAP+3%Suc+0.7%Agar	0	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00
	6	0.00	0.00	0.00	0.00
	10	0.17	0.00	0.05	0.00
	13	0.43	0.43	0.05	0.00
1NAA+1BAP in water	0	0.00	0.00	0.00	0.00
	3	0.83	8.22	0.39	0.08
	6	1.73	10.36	1.03	0.35
	10	1.38	29.96	0.80	0.39
	13	2.96	39.00	0.82	0.26
MS 4Kn+3%Suc+0.7%Agar	0	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00
	6	0.00	0.00	0.00	0.00
	10	0.00	0.00	0.00	0.00
	13	0.00	0.00	0.00	0.00
4Kn+3%Suc+0.7%Agar	0	0.00	0.00	0.00	0.00
	3	0.00	0.87	0.00	0.01
	6	0.10	4.05	0.01	0.02
	10	0.04	1.57	0.04	0.00
	13	0.70	3.00	0.50	0.08
MS 4Kn + Water	0	0.00	0.00	0.00	0.00
	3	0.00	0.57	0.00	0.01
	6	1.27	14.86	0.13	0.08
	10	0.65	8.15	0.05	0.04
	13	0.26	6.04	0.06	0.07

Effects of Physical Factors on Regeneration in *Arachis*

J.P. Moss, N.R.G. Dutt, and M.J.T. Reena[1]

Regeneration in *Arachis* has been reported, both in the cultivated peanut and its wild relatives, but the wild species regenerate more freely than the cultivated species.

Plant regeneration is influenced and controlled by different chemical, physical, and genotypic factors. To further understand the factors influencing regeneration, de-embryonated cotyledons from two genotypes were cultured on MS media with 1 mg l^{-1} NAA and 1 mg l^{-1} BAP (M1) or 2 mg l^{-1} NAA and 0.5 mg l^{-1} BAP (M2), at two different temperatures—25°C and 30°C—with different light regimes—dark and at low and high light intensities with a 16/8 h light/dark cycle—and with two orientations—either with the abaxial or the adaxial surface in contact with the medium.

Analysis of variance indicated significant effects of hormone level, temperature, light intensity, and orientation on production of roots and shoots and on callus formation, and also significant interactions between these factors.

Differences in temperature affected production of shoots and root growth and callus production. Lower temperature (25°C) induced more shoots than higher temperature (30°C); however, higher temperature encouraged root elongation and stimulated more callus formation than the lower temperature. More explants formed callus at nodal end at low temperature than at a higher temperature.

Light levels significantly affected shoot production and growth, root numbers, and callus production. High light intensity initiated more shoots and greater shoot growth and callus induction than lower light intensities. Incubation in dark drastically reduced the shoot numbers, but did not affect the root formation, although the roots that formed grew longer.

Explant orientation influenced root numbers and growth and shoot growth.

Table 1. Significant factors and interactions affecting response of cotyledons cultured in vitro.

	Dependent variable				
	Root	Shoot	Root	Shoot	
Source of variation	Formation		Formation		Callus Production
Media (M)	**	**	**	**	
Temperature (T)		*	**		**
Light Intensity (L)		**	**	**	**
Genotypes (G)	*		**	**	*
Orientation (O)	*		**	**	
M × T		**		**	**
M × L		**		*	*
T × L	**	**	*	**	
M × O		*		*	
G × O	**	**		**	*
M × T × L		**		*	**
M × T × G	**				
M × T × O	*				
T × L × G		**	**		
T × G × O	*				
L × G × O		*			
M × T × L × G				*	

* $P < 0.05$, ** $P < 0.01$

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Moss, J.P., Dutt, N.R.G., and Reena, M.J.T. 1992. Effects of physical factors on regeneration in *Arachis*. Pages 159–160 *in* Biotechnology and crop improvement in Asia (Moss, J.P., ed.). Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

When Robut 33-1 cotyledons were adaxially placed on medium 1 at 30°C, an increase in light intensity had little effect on the number of explants forming both roots and shoots, but had a dramatic effect (approximately six-fold increase) when abaxially placed (Fig. 1). On medium 2 at 30°C, response at low light intensity showed a similar dramatic increase over the response in the dark for both orientations, which further increased at high light intensity for abaxially orientated cotyledons, but decreased for adaxial orientation. However, responses for the other genotype, JL 24, did not follow this pattern.

There were significant interactions of media with temperature and different light intensities with respect to shoot and callus formation and shoot growth (Table 1). Higher-order interactions were also significant for certain combinations of factors. Notably among them, media, temperature, and intensity significantly influenced shoot numbers, length, and callus induction. Light intensity, cultivars, and explant orientation together affected only shoot formation.

These results indicate that changes in physical conditions can greatly influence response, and the nature and magnitude of the responses differ for different genotypes and media. These factors contribute to the difficulty that has been experienced in regeneration of *Arachis*.

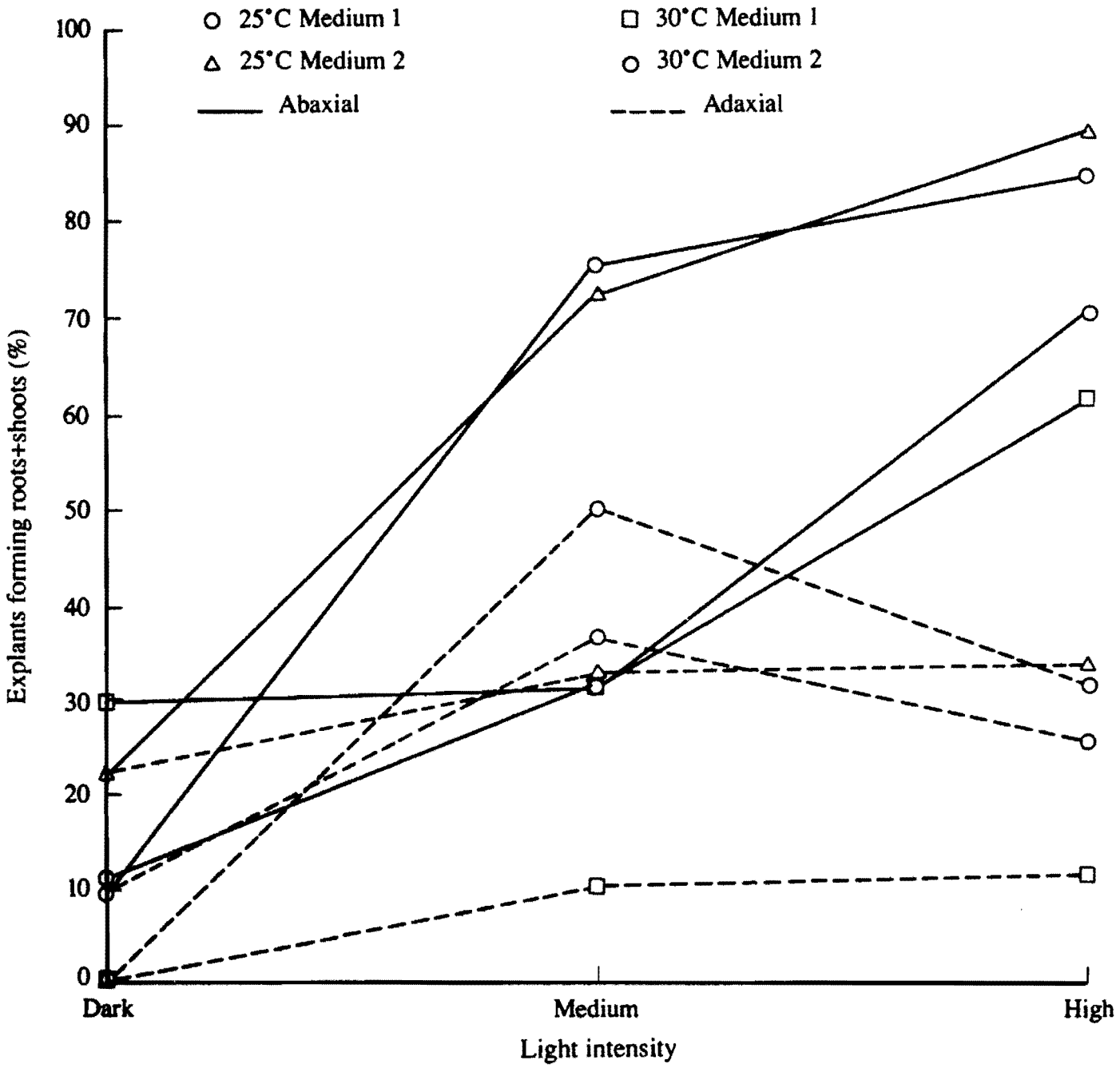

Figure 1. Percentage of Robut 33-1 cotyledons that formed both roots and shoots when cultured under different conditions.

Somatic Embryogenesis from Mesophyll Protoplasts of Wild and Cultivated Species of Pigeonpea

V.V. Ramana Rao, S.N. Chary, and J.K. Bhalla[1]

Pigeonpea, *Cajanus cajan*, has wild relatives that are of potential value, through hybridization, in genetic improvement of the cultivated pigeonpea. However, limited success has been achieved using conventional techniques since some of the wild species are incompatible. Somatic hybridization offers new options that could complement conventional breeding approaches.

In the present program, attempts have been made to isolate, culture, and fuse the mesophyll protoplasts of pigeonpea cultivars, e.g., UPAS 120 and Pant A2 and their wild relatives *Atylosia platycarpa*, *Rhynchosia aurea*, and *Rhynchosia visicidia*. The protoplasts were isolated from the leaves of axenical shoot cultures using an enzyme mixture consisting of 1% cellulase + 0.5% pectinase. The yield of protoplasts was greater from both cultivars than from the wild species. Freshly isolated protoplasts of all species showed maximum viability and in such cells cytoplasmic streaming was clearly seen. Differences in cell size were observed between the cultivated and wild species.

The purified protoplasts were cultured at a density of 2×10^4 ml^{-1} on modified B5, MS, and Blaydes liquid media, with 5% sucrose (0.15M) and 4.6% Mannitol, 1 mg l^{-1} 2,4-D, 0.4 mg l^{-1} BAP, 1.0 mg l^{-1} NAA, and 2 mg l^{-1} glycine, at pH 5.8. After 1 week, a fresh medium devoid of phytohormones was added to the cultures to lower the osmoticum. Cultures were incubated in the dark for the first 2 weeks and thereafter under continuous cool light.

Cell division was observed after 6 days of culture and subsequent development of small colonies was noticed on modified B5 media, which proved to be the most efficient among the media tested. Two species, *Atylosia platycarpa* and *Rhynchosia visicidia*, as well as the cultivar UPAS 120, gave good response.

The growth rate of the cells was fast and they turned into clumps or protoclones after 45 days. After 60 days, the cell clumps showed large numbers of globular structures. The cells constituting the globular structures were isodiametric and compactly arranged with granular cytoplasm, suggestive of being embryogenic cells. Embryoids were observed on the surface of the irregularly shaped cell clumps. Embryoids were attached to the clumps, and some such clumps also formed root-like structures. Both embryoids and root structures developed by division of the cells within the cell clumps. The protoclones developed into typical callus.

The fusion of protoplasts of cultivars and wild species was carried out using polyethylene glycol, and the fusion products were viable for several weeks. The fused products were dispensed on to liquid B5 medium, and the growth of cells has been observed.

Although protoclones were obtained successfully from mesophyll protoplasts of the cultivar UPAS-120 as well as *Atylosia platycarpa* and *Rhynochosia visicidia*, they did not differentiate into plantlets.

Experiments are in progress to identify the heterokaryons and standardize the cultural conditions for callus initiation and further differentiation.

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Tissue Culture and Wide Crosses in Pigeonpea

M.J.T. Reena, P.T.C. Nambiar, J.P. Moss, D.C. Sastri, and M. Mohiuddin[1]

In vitro regeneration of plants from different tissue explants has been achieved in a large number of species. The availability of an in vitro regeneration technique is a prerequisite for the application of most of the biotechnological techniques. Tissue and organ culture in pigeonpea have been attempted and regeneration reported earlier (Mehta and Mohan Ram 1980; Kumar et al. 1984 and 1985). For plant propagation or transformation high-frequency regenerability and reproducibility are important. Among the different explants like immature leaflets, root discs, hypocotyl, and epicotyl segments, cotyledons were chosen for culture because of their high frequency of regeneration.

Cotyledons from mature seeds and seedlings from two cultivars of *Cajanus cajan*, ICPL 87 and ICPL 4, were cultured on Murashige and Skoog's (MS) medium with 3% sucrose, 0.7% agar, and supplemented with different combinations of Napthalene acetic acid (NAA) and Benzyl-aminopurine (BAP) (Table 1). All the cotyledons cultured from mature seeds and 1-day-old seedlings revealed nodular green compact calli on the media supplemented with 1 mg l^{-1} each of NAA and BAP and also with 5 mg l^{-1} BAP and 1 mg l^{-1} NAA. Transfer of this callus to MS + 0.5 mg l^{-1} BAP with or without 0.1 mg l^{-1} NAA triggered the formation of a large number of shoot buds. Best elongation of these buds was seen on MS with GA (Gibberellic acid) 1 mg l^{-1}. Elongated shoot buds were transferred to the rooting media, which were comprised of half strength MS with 0.1 mg l^{-1} NAA or MS with 2 mg l^{-1} NAA and 1 mg l^{-1} Indolbutyric acid (IBA). Root formation was observed only in five shoots on MS medium with 0.1 mg l^{-1} NAA.

Table 1. Percentage of explants forming roots/shoots from mature cotyledons of *Cajanus cajan* cv ICPL 87.

Hormones (mg l^{-1})	% of explants forming roots[1]	% of explants forming shoots
1.0	10	0
1.0 NAA	8	0
5.0 NAA	7	0
1.0 BAP	0	7
1.0 BAP + 1 NAA	0	20
5.0 BAP	0	14
5.0 BAP + 1.0 NAA	0	20

1. 20 cotyledons were cultured on each medium.
NAA - Naphthaleneacetic acid
BAP - Benzylaminopurine

Wide Crosses in Pigeonpea

Hybrid embryos obtained from the interspecific cross between *Cajanus platycarpus* × *Cajanus cajan* were cultured on MS with 0.6 mg l^{-1} BAP (Table 2). Embryos that matured and formed both roots and shoots were transfered to soil. These did not survive long. Some of the embryos callused in culture and then formed shoot buds that did not elongate beyond 0.5 to 1 cm in length. Hybrid callus has been transferred to different combinations of NAA and BAP for further induction and growth of shoots.

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Table 2. Response of hybrid embryos of *Cajanus platycarpus* × *Cajanus cajan* cultured on MS + 0.6 mg l^{-1} BAP.

Cross between accession of *Cajanus platycarpus* ( ) and genotype of *Cajanus cajan* ( )	No. of pollinations done	No. of pods formed	No. of embryos cultured	No. of embryos responding	No. of embryos forming multiple shoot buds from callus	No. of embryos forming both root and shoot
ICPW 65 × ICPL 85030	45	3	6	6	6	0
ICPW 66 × ICPL 85030	43	8	11	10	4	2[1]
ICPW 69 × ICPL 85030	32	7	5	0	0	0
ICPW 70 × ICPL 85030	18	4	7	3	3	0
ICPW 72 × ICPL 85030	18	4	5	1	1	0
ICPW 66 × ICPL 88014	41	15	18	6	4	4[1]
ICPW 69 × ICPL 88014	39	11	9	2	0	2[1]
ICPW 70 × ICPL 88014	46	19	7	0	0	0
ICPW 72 × ICPL 88014	8	1	1	1	0	1[1]

1. Plants transferred to soil but did not survive long.

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Wide Hybridization and Crop Improvement in the Genus *Arachis*

D.C. Sastri[1], A.K. Singh[1], J.P. Moss[1], Nalini Mallikarjuna[1], and X. Zhang[2]

Considerable progress has been made at ICRISAT in genetic improvement of *Arachis hypogaea* using wild relatives with resistances to late leaf spot and rust, and which are easily crossable with the cultivated species.

Hormone-aided pollination followed by in vitro culture of immature ovules and embryos has been developed for production of hybrids from intersectional crosses and also, for example, between sections *Arachis* and *Rhizomatosae* and between sections *Arachis* and *Erectoides*, hitherto reported incompatible by conventional methods.

A large number of hybrid plants from embryo culture derived calli obtained from culture of hybrid embryos (*A. hypogaea* × *A.* sp 276233 (*Rhizomatosae*)) have been transferred to soil but only one has produced flowers. Flowers are mostly sterile but backcrosses with *A. hypogaea* have formed a few pods containing immature seeds. Embryos from these have been cultured in vitro and plantlets obtained.

Meiotic analysis of flower buds of this hybrid plant revealed a considerable variation in the pairing behavior. Bivalents per p.m.c. ranged from 11 to 16 and univalents ranged from 1 to 11. Trivalents and quadrivalents were common. Anaphases I and II contained laggards and bridges.

Another hybrid, *A. duranensis* and *A.* sp 276233, has good vegetative growth and has been established in soil.

This technique of hybridization has been successfully extended to reciprocal crosses between diploid species of section *Arachis* and those of section *Erectoides*. A large number of hybrids have been produced and transferred to soil. Many have formed flowers. Progress has been made in the analysis of these hybrids as well as in their use for introduction of resistances to early leaf spot and rosette virus.

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Tissue Culture Studies in Chickpea

J. Divakar Rao and G.M. Reddy[1]

Chickpea (*Cicer arietinum* L.) is an important pulse crop, grown mainly for its protein content. Establishment of callus cultures and subsequent whole plant regeneration is essential for efficient utilization of these in vitro techniques. The present investigation was aimed at regenerating whole plants directly from explants as well as callus cultures of hypocotyl explants. Somatic embryogenesis could be initiated in epicotyl and cotyledon callus cultures and its histological development was followed through.

Multiple shoots were induced directly from the different seedling explants—hypocotyl, epicotyl, cotyledons, and shoot apices—cultured on different types of media with different hormonal concentrations. Multiple shoot formation from hypocotyls was significantly higher compared to other explants. In contrast, all the wild species of *Cicer* gave higher shoot formation as compared to cultivated species. Micropropagation and production of somaclonal variants in chickpea and its wild relatives will be very useful for chickpea crop improvement. Efficient multiple shoot formation, which could be induced in a number of explants, is visualized to be useful for genetic transformation using Ti plasmids.

A combination of BAP and NAA in B5 medium was found to be more effective in inducing callus from both cultivated and wild species of *Cicer*. Callus induced on the above medium was transferred to the regeneration medium (Blaydes medium) with BAP. Differentiation of shoots was achieved with varying frequencies from hypocotyl-derived callus cultures of JG-62, *C. reticulatum*, and *C. bijugum*. These shoots were rooted in Gamborg's basal medium containing IBA or NAA and transferred successfully to the pots.

Somatic embryogenesis and subsequent shoot development was achieved in cotyledon-derived cultures of *C. arietinum*. Callus was induced on Gamborg's basal medium supplemented with 3% sucrose and different concentrations of 2,4-D, but 2 mg l^{-1} was found to be optimum for the production of embryogenic callus. The embryogenic callus was yellow, smooth, and nodular in appearance. These nodular structures developed into globular and heart-shaped embryoids on the surface of the callus. Somatic embryos transferred were found to have a tendency either to dedifferentiate or to form root-like structures upon transfer to B5 basal media (without hormones). Somatic embryos transferred to B5 media supplemented with BAP, however, produced shoots. Anatomical studies of embryogenic calli derived from cotyledons revealed the presence of pro-embryonic masses and early stages of globular and heart-shaped embryoids. These results would be discussed in the light of recent and relevant literature.

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Production and Use of Haploids in Crop Improvement

G. Wenzel, A. Graner, F. Fadel, J. Zitzlsperger, and B. Foroughi-Wehr[1]

Abstract

Haploid production is possible in principle either via partheno- or androgenesis. As androgenesis is more efficient in most plant species tested, the chapter will concentrate on haploid induction from microspores. The regeneration of haploids from microspores has greatly increased due to three main factors: selection of genotypes with high regeneration capacity, improvement of anther-donor-plant growth conditions, and optimization of in vitro culture conditions, particularly the change to liquid media. To avoid gametoclonal variation, considerable efforts have been made to obtain regeneration via embryogenesis. This was successful in rape seed, barley, wheat, and potato. Due to these improvements the cost of production of one green doubled haploid line in barley dropped from about U.S.$ 100 000 in 1973 to less than U.S.$ 2 in 1990. Today, in such successful crops the question is no longer how to produce haploids but how to incorporate them most efficiently into the normal breeding procedures. For inbreeding barley, one or more haploid steps are recommended, depending on the genetic nature of the starting material (F_1 hybrids or selfed progenies in later generations) as well as on the breeding aims (combination of mono-, or polygenically transferred traits). It will be demonstrated that for breeding programs that include wild types or unrelated genotypes, recurrent selection alternating with haploid steps is the most economic procedure for combining quantitatively-inherited characteristics. Such approaches are applied in a winter barley breeding program, with the central aim of combining the qualitatively-inherited character of resistance to barley yellow mosaic virus (BaYMV) with quantitatively-inherited complexes, particularly yield. Two haploid steps are possible in the tetraploid potato. Along with classical combination breeding and protoplast fusion, new hybrid clones have been produced, combining the complete genome of the haploids. Besides looking at the direct application of haploidization procedures in breeding programs, the use of haploids in restriction fragment length polymorphism (RFLP) mapping of the barley genome will be demonstrated as a more indirect use of haploids.

Introduction

Some tissue culture methods have become applicable in crop improvement. The use of haploids is one that does not demand very sophisticated laboratory equipment and, therefore, may be of immediate use in developing countries. Techniques for most of the subtropical and tropical plants are not yet adopted. This review concentrates on cereals and potatoes, where these techniques have been proved effective. Other crops will be mentioned whenever solid data are available; for further information see reviews of Nietzsche and Wenzel (1977), Dunwell (1985), or Foroughi-Wehr and Wenzel (1991).

A plant breeder who is dependent on genetic variability seeks to circumvent the diploidy of our higher plants, at least in several steps of a breeding program. Of course, the final product must be diploid again, resulting in doubled haploid lines (DH). The principal ideas about how to make use of the simpler haploid genomes are more than 50 years old; and very few new concepts have been added since Blakeslee, Belling, Farnham, and Bergner listed them in 1922. The main advantage of using haploids is the rapid and

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complete homozygosity of the offspring, because phenotype selection for quantitative characters, and particularly for qualitatively inherited characters, is much easier and therefore, breeding is more efficient.

It is no longer in doubt that the gametes are the most appropriate material for the production of haploids. Two principal methods exist, either androgenesis, in which the male gamete develops into the haploid, or parthenogenesis, when the female gives rise to the haploid.

Artificial Induction of Haploids

Spontaneous haploid formation occurs at a low rate and procedures for increasing haploid production were developed. A breakthrough in 1964 was the successful induction of haploids from young anthers cultured in vitro (Guha and Maheshwari 1966). This microspore androgenesis occurred only under artificial in vitro conditions. The same was true for the parthenogenetic method of haploid induction. Here, techniques such as embryo culture or the more sophisticated isolation of unfertilized egg cells and their regeneration in vitro had to be made reproducible.

Since the first successes in in vitro regeneration of haploids, considerable research effort has been directed toward improving the technique for economically important plants such as the cereals. Today, the methodology has reached a stage where private laboratories consider it economically viable to adopt this technique at their own economic risk.

Parthenogenesis

It has been possible to induce parthenogenesis by in vitro culture of unpollinated ovaries and ovules (reviewed by Yang and Zhou 1982). Attempts have been made to culture unfertilized ovules or ovaries in different plant species but growth of most of them stopped at the callus stage; only some crop species developed into haploid green plants (Table 1). As in all tissue culture procedures a different regeneration response was seen depending on the genotype.

The practical application of parthenogenesis is rare because of the low frequency of haploid induction in most of the species except potato. Only in plant species where anther culture has been unsuccessful as in sugar beet (Speckmann et al. 1986) or onion (Muren 1989) are isolated unpollinated ovules or ovaries still of practical interest.

Table 1. Work on parthenogenetic haploid production of important crop plants.

Species	Author(s)	Year of publication
Hordeum vulgare	San Noeum	1976
Triticum aestivum	Zhu and Wu	1979
Nicotiana tabacum	Zhu and Wu	1979
Oryza sativa	Asselin de Beauville	1980
Zea mays	Ao et al.	1982
Citrus sinensis	Kochba and Spiegel-Roy	1982
Beta vulgaris	Hosemanns and Bossoutrot	1983
Hevea brasiliensis	Chen et al.	1988
Allium cepa	Muren	1989

In some interspecific crosses, the phenomenon of chromosome elimination during early embryo development has been reported. Due to a mechanism not yet understood, the chromosomes of one parent were partially or completely lost during the early divisions of the young hybrid embryo. In nature such a loss of chromosomes, normally coupled with failure of endosperm development, leads to failure of seed formation. If all chromosomes of one parent are lost and if embryo rescue in vitro is successful, the procedure results in haploid plants of one, normally the female parent. For barley this system was studied by Kasha and Kao (1970) in crosses of *Hordeum vulgare* × *H. bulbosum*. They demonstrated that the method of crossing diploid barley with diploid *H. bulbosum* could be used to produce high frequencies of barley haploids. Apart from crosses with *H. bulbosum*, haploids of barley have been reported from crosses with rye (Fedak 1977) or with *Psathyrostachys fragilis*, as well as from crosses with other wild barley species (Jorgensen and von Bothmer 1988).

The bulbosum system in barley has been practiced for nearly 20 years. The first doubled haploid variety of barley 'Mingo' was licensed for sale in Canada in 1979 (Ho and Jones 1980). During the past decade, the method has been remarkably improved by selecting more compatible *H. bulbosum* genotypes and by optimizing different culture factors. Field studies of DH lines obtained by the bulbosum method were evaluated for agronomic characters. It was found that there was no significant difference between single-seed descent lines and DH lines as far as grain yield, heading date, and plant height were concerned (Choo

1988). However, since it is possible to make use of the greater potential of the androgenetic system for haploid production, the bulbosum method is of decreasing value for practical plant breeding.

In potato the cross between *Solanum tuberosum* (2n=4x=48) and *S. phureja* (2n=2x=24) leads to parthenogenetic haploids of *S. tuberosum* (2n=2x=24). The reason for the haploid formation is that the second microspore mitosis is blocked in special *S. phureja* genotypes, resulting in only one sperm nucleus that can either fertilize the embryo sac nucleus or the egg cell. In the first case, the egg cell remains unfertilized giving rise to a parthenogenetic haploid seed. From successful pollinations, up to 40% haploid seeds can be detected. Today, 2x potatoes can be produced so efficiently that most potato breeders make use of them in their breeding programs. Since the return from the 2x to the 4x level is not only possible via mitotic doubling, but also via protoplast fusion, the practical strategy is to combine characteristics first by classical combination breeding at the 2x level and then to combine the heterozygotes via fusion, to reach the 4x level. In the vegetatively propagated potato, such heterozygous fusion products can be propagated immediately and may even already be a new cultivar.

Within the legumes most success has been reported for *Medicago sativa* where Bingham and Dunbier (1974) produced maternal haploids of alfalfa cultivars routinely by interploid cross methods (4x × 2x). These haploids are used for transfer of marker genes from the tetraploid to the diploid level. Further experiments are in progress to test whether breeding *Medicago sativa* at the diploid level might be of commercial value. At present, this approach does not look as successful as in potato, but it has become possible to combine wild species with cultivated forms at the diploid level. This was of particular interest for the incorporation of genes for resistances. This technique would also be of value in interploidy crosses making use of embryo rescue such as those for red clover (Phillips et al. 1982) and for *Arachis* (Stalker and Moss 1987).

Androgenetic Haploid Production

The most successful method of inducing DH lines today is microspore androgenesis. Pollen in an early developmental stage can be induced in vitro to form a plantlet. Figure 1 gives an example of haploid research in Germany, and shows how the methods developed during the last 20 years have progressed to commercial utilization. Success in microspore culture is predominantly dependent on the genotype of the anther donor material. In all plant species examined, there are drastic differences between the anther response in different genotypes under a set of culture conditions. Culture conditions could probably be optimized for each genotype, as proposed by Dunwell (1981). We believe, however, that it is less costly to broaden the genetic bases for tissue culture ability by selection and combination breeding.

In barley and potato, high-responding genotypes were crossed with agronomically important varieties. The F_1 generation showed an intermediate reaction between both parents, making it feasible that a high response in anther culture is heritable. In winter barley, the cultivar 'Igri' produced the highest percentage of green structures (Foroughi-Wehr and Friedt 1984). Similar results were reported for rye (Wenzel et al. 1977; Wenzel and Foroughi-Wehr 1990), potato (Wenzel 1980; Uhrig and Salamini 1987), and rapeseed (Siebel and Pauls 1989).

Even if the donor-plant genotype is excellent, the response of microspores in culture is dependent on the growth conditions of the donor parent. Dunwell and Perry (1973) first pointed out the influence of the photoperiod and light regime on the androgenetic response in anther culture of tobacco. In *Brassica napus*, pollen embryogenesis was improved by preconditioning the donor plants at a low temperature (Keller and Stringam 1978). In cereals, the influence of the growing conditions of the donor material is known in wheat, barley, rice, and rye. The cultivar 'Igri' regenerates no green plants at all if the growth conditions of the donor plants are not suitable. In most plant species, field-grown material during the natural season was inferior to greenhouse-grown material.

Anthers harvested at the early or mid-uninucleate stage of the microspore are most responsive in vitro. As soon as starch deposition has begun, no sporophytic development and, subsequently, no macroscopic structure formation occurs. The size of the flower buds that contain anthers in the right stage depends, to some extent, on their position in the inflorescence, on the genotype, and on the plant age.

After surface sterilization of the buds or ears, the anthers are removed aseptically and planted on a liquid or solid culture medium. Certain pretreatments of the inflorescences can have a positive effect on the development of the microspore. The most effective technique used in anther culture, especially for the cereals, has been cold pretreatment. Periods of from 72 hours to 4 weeks at 4-10°C are recommended. On

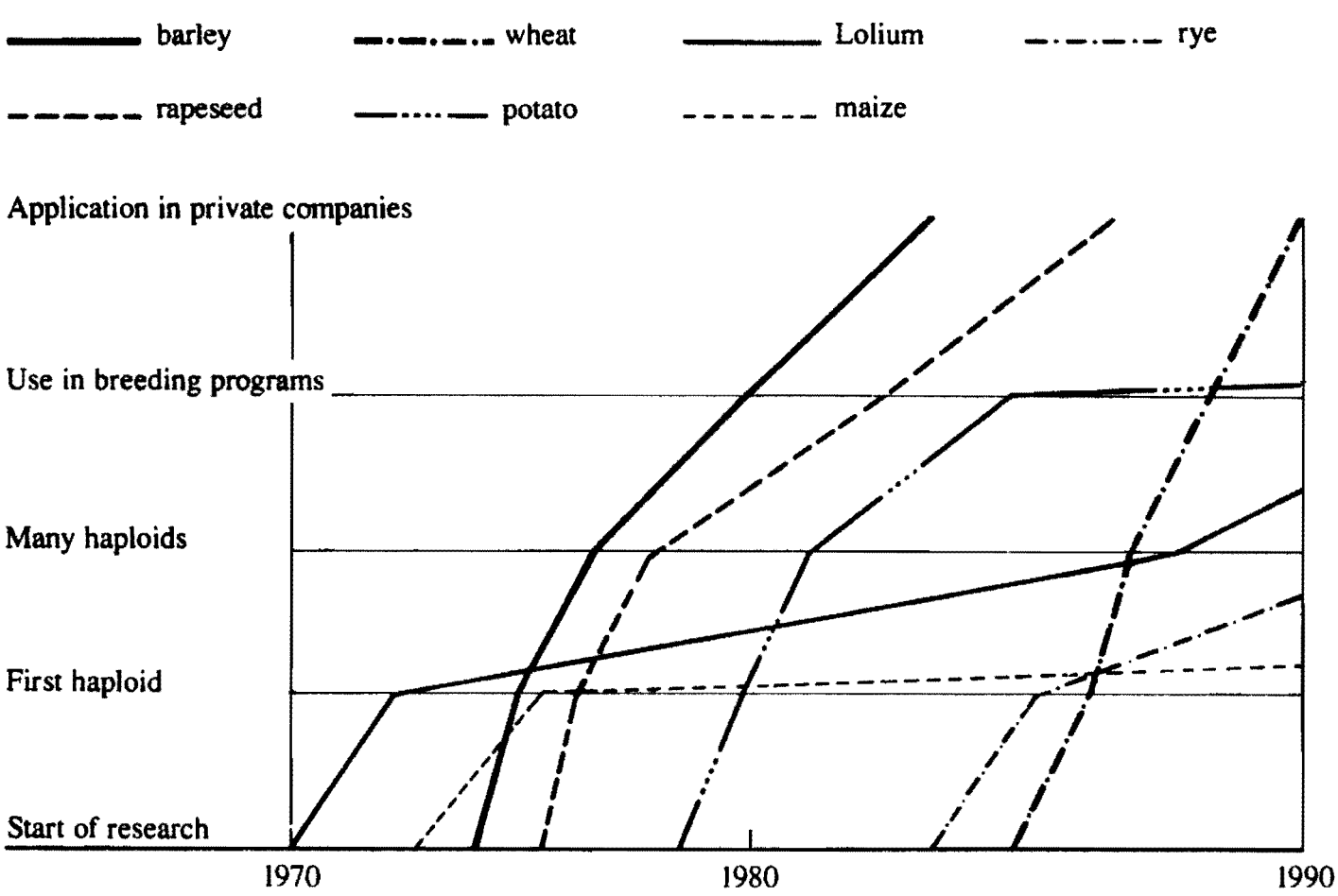

Figure 1. Progress in the use of DH lines in Germany.

the other hand, several investigations showed a high frequency of callus or embryo formation for good-responding genotypes—such as the cultivar 'Igri' in barley—without any pretreatment (Powell 1988). In wheat a negative effect of low temperature on the anther response has also been reported (Marsolais et al. 1984), and if the donor plants were grown under optimal conditions, the promoting effect of pretreatment was minimized and the whole procedure could be omitted.

The available literature does not allow recommendation of any anther culture medium for general applicability. The basal media commonly used have been mainly N6 medium (Chu 1978) or that of Murashige and Skoog (1962). In China, a potato extract medium on which wheat, especially, gave a high androgenetic response was developed and further improved by Chuang et al. (1978). Quite recently, a major research effort was made for haploid production in barley by replacing the agar with barley starch (Sorvari 1986). We were able to verify these results for wheat where wheat starch gave a better response than agar. Kao (1981) reported a considerable increase in the frequency of microspore regeneration by adding Ficoll to liquid medium. Ficoll prevented small structures such as callus or embryos from sinking into the medium, and thus prevented them being killed by anaerobiosis. Liquid media have been preferred for pollen embryo induction. A carbon source is obligatory for microspore embryogenesis, and in most culture media, sucrose has been recommended. Since Raquin (1983) found that maltose improves embryo induction and development in anther culture of petunia, more attention is being paid to this sugar. In potato anther culture, sucrose was found to stimulate embryo induction more than maltose (Batty and Dunwell 1989; Zitzlsperger, personal communication), but plant production was significantly increased in induction medium containing maltose. In wheat (Fadel and Wenzel 1990), and barley (Hunter 1987), maltose in the induction medium increased the rate of regeneration. We were able to regenerate a high frequency of green plants in barley in liquid Ficoll medium containing barley starch and maltose (Kuhlmann and

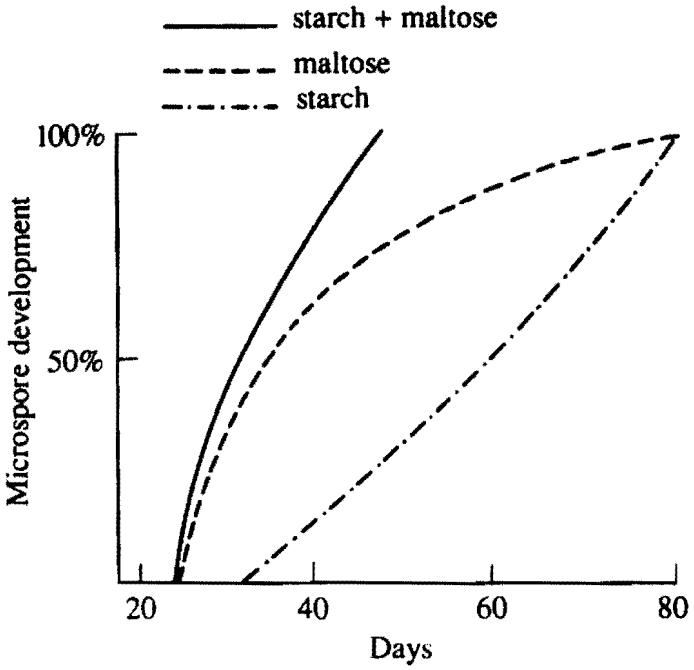

Figure 2. Development of embryos and calli of barley on the basal medium of Chu (1978) with the addition of 80 mg starch per petridish, 20 g l^{-1} maltose, or both (Kuhlmann 1991).

Foroughi-Wehr 1989), but we obtained comparable results in a maltose-free medium; maltose, however, speeds up the development (Fig. 2).

The effects of incubation conditions such as light (quantity, quality, and duration) and temperature have been investigated in a number of studies. Usually the anthers were cultured in the dark at temperatures between 20°C and 30°C. For field-grown material, the optimal culture temperature was generally 2°C higher than for greenhouse-grown material. The orientation of the anthers on the medium with regard to the loculi had either a positive effect on response in Datura (Sopory and Maheshwari 1976), in barley (Hunter 1985; Shannon et al. 1985), and in maize (Tsay et al. 1986).

To date, the culture of isolated microspores has been possible in many plants, such as potato (Uhrig 1985), rapeseed (Lichter 1982), barley (Sunderland and Xu 1982), wheat (Datta and Wenzel 1987), rice (Chen et al. 1980), and maize (Coumans et al. 1989; Pescitelli et al. 1989). The microspores were either isolated mechanically from the anther or shed initially into liquid culture media. To improve the efficiency of isolated microspore culture, it proved to be beneficial to condition the liquid culture medium by addition of anthers or ovules (Kohler and Wenzel 1985). Siebel and Pauls (1989) compared anther culture and microspore culture in *Brassica napus* and demonstrated a much higher efficiency in embryo production in microspore culture. There were no differences between the two populations with respect to agronomic characters in the field. Isolated microspore cultures offer a more effective system of regenerating a random sample from the microspore population than does anther culture. This is particularly important when the desired trait is linked with low plasticity. In addition, these cultures are a good source for in vitro selection and gene transfer (Kuhlmann 1991).

Plants developed from microspores or ovaries were either haploid, diploid, or tetraploid; a few were aneuploid or mixoploid. In barley, potato, rapeseed, and rye, the majority (70%) of the plants that developed were diploid, whereas in maize (Nitsch et al. 1982) and in wheat, most of the regenerants remained haploid. Various factors influence the ploidy level of the regenerated plants. In *Brassica napus*, this character seems to be under genetic control (Chuong et al. 1988) and the ratio of diploids rose with increasing duration of the culture period (Siebel and Pauls 1989). The same was true for potatoes, where the monoploid (x=n=12) formation was dependent on the genotype; it ranged from 0 to 20%. Epigenetic factors such as the pollen stage at the time of inoculation, the hormones in the media, or the culture conditions that influence androgenesis may also influence the ploidy level of the plants.

Haploid plants have been doubled by colchicine, as described by Jensen (1975). This has been done without problems in most plant species. In potato and maize, the efficiency of inducing doubled haploid plants has been very low. But Wan et al. (1989) used colchicine treatment of an embryogenic haploid maize callus initiated from anther culture and produced fertile doubled haploid inbred lines at high frequency. In rice and barley, the induction of doubled haploids has been improved to the extent that colchicine treatment of the remaining haploids can be omitted. Seed set without colchicine treatment could be found in more than 50% of haploid barley plants under field conditions (Table 2). The reasons why there are unreduced gametes are not known.

Haploids in Plant Breeding

Since Wark (1977) obtained improved doubled haploid tobacco lines via anther culture, this technique has worked in practice in potato, rapeseed, and the cereals. Of particular interest in barley is the incorporation of resistance to the soil-borne barley yellow mosaic virus (BaYMV). The use of a haploid step in

Table 2. The ploidy level and the seed set of DH-progenies from different F_1 crosses of winter barley.

	Ploidy level					
	2n=x=7		2n=2x=14		2n=4x=28	
F_1 crosses	No. seed set	No. sterile	No. seed set	No. sterile	No. seed set	No. sterile
1	11	3	14	1	1	0
2	22	10	88	1	9	2
3	16	6	15	1	2	0
4	45	15	207	4	17	2
5	67	35	164	0	26	0
6	10	10	44	1	9	0
Total	171	79	532	8	64	4

the breeding program offers a rapid approach to incorporating BaYMV resistance in commercial cultivars (Foroughi-Wehr and Friedt 1984). In wheat (De Buyser et al. 1987) and rice (Huang et al. 1988), new cultivars were developed via this technique. It is generally accepted today that a stepwise reduction in the ploidy level of potato offers the advantage of a simpler inheritance and also a better chance to combine qualitatively inherited characters (Wenzel et al. 1979). Data now available demonstrate that field resistance to potato virus Y (PVY) and potato leafroll virus (PLRV) was maintained and expressed at all ploidy levels over a 5-year period, which means that both monogenically and polygenically inherited resistances were maintained during the successive haploidization process. When a genome has passed the monohaploid level, a homozygous additive character can be transferred efficiently to the next generation via classical crossing. Similar results have been obtained for polygenic nematode resistance (Uhrig and Wenzel 1987).

In *Brassica napus*, quality characteristics were emphasized in breeding techniques with haploids (Keller 1984; Siebel and Pauls 1989). Vigor of microspores was positively correlated with high glucosinolate content in the androgenetic regenerants. More than 70% of androgenetic plantlets tested had a much higher glucosinolate content than did the original material (Hoffmann et al. 1982). Since the aim of breeding was for low glucosinolate content, spontaneous selection for this character worked against the program. Development of microspores with a high level of glucosinolates—being chemically quite similar to phytohormones—may have been favored. Very low or very high glucosinolate levels are diverse qualities needed either for human consumption or for industry. A simple screening for extremes would increase breeding flexibility at an early stage.

Since regeneration is no longer a main obstacle in using DHs, other questions are gaining importance. Snape and Simpson (1981) already discussed the genetic effects when starting from different filial generations. The question is: can DHs be used more economically in an F_1 generation, a preselected F_2 or F_3, or should the haploid step be combined with other breeding techniques? It could be demonstrated that one haploid step followed by selection in the greenhouse and in the field during the first androgenetic (A_1) and two subsequent selfed generations (A_2, A_3) is the most efficient procedure, if characters from related varieties are to be combined. Haploids can also increase the efficiency for the most complex breeding problem: the transfer of quantitatively inherited traits from distant genotypes to cultivars. For such complex breeding programs, a combination of recurrent selection that guarantees variability, followed by haploid selection that guarantees secure selection, has been developed. This breeding technique is called recurrent selection alternating with haploid steps, and is shown in Figure 3. The degree of relationship of the two parents determines the number of backcrosses needed. The necessary offspring size of the DH population for quantitative characters depends on the degree of linkage and on the number of genes involved. The main advantage of using this method is to speed up the breeding process and to make selection of quantitatively inherited traits more effective and reliable (Foroughi-Wehr and Wenzel 1990).

A rather recent application of DH lines is their use in genome mapping. In most cases, there is no immediate need to always locate the gene directly responsible for a character expressed at the phenotypic level.

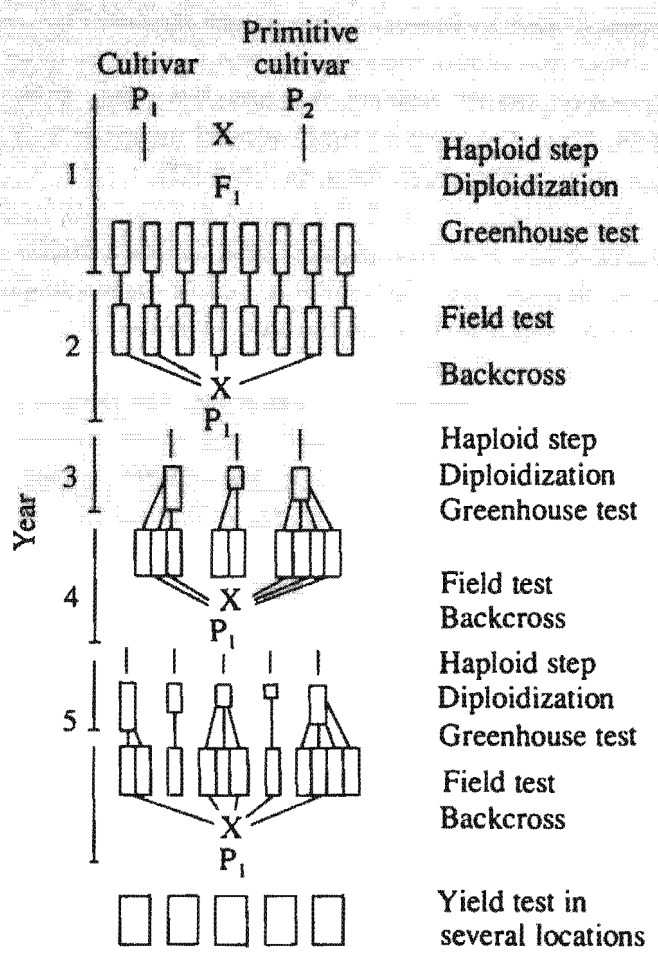

Figure 3. Breeding scheme for the introduction of mono- or polygenically inherited traits from distantly related genotypes: recurrent selection alternating with haploid steps.

It would be sufficient for applied breeding strategies to detect characteristic restriction fragment length polymorphisms (RFLP) correlated to phenotypes. For such correlations, segregating plant populations are a necessary prerequisite. In a population of DH lines the identification of markers is much more secure, as most intermediate phenotypic expressions due to heterozygosity are excluded. A gene will segregate in a 1:1 ratio for both the molecular marker and the phenotype at the plant level (Graner et al. 1990). Figure 4 demonstrates such a 1:1 segregation between DH lines from the F_1 hybrid 'Igri' × 'Franka'. This is of particular importance when polygenically inherited characters need to be mapped for qualitative trait loci (QTL) analysis.

Conclusions

The use of haploids is a common step in modern breeding programs. It is not relevant whether these

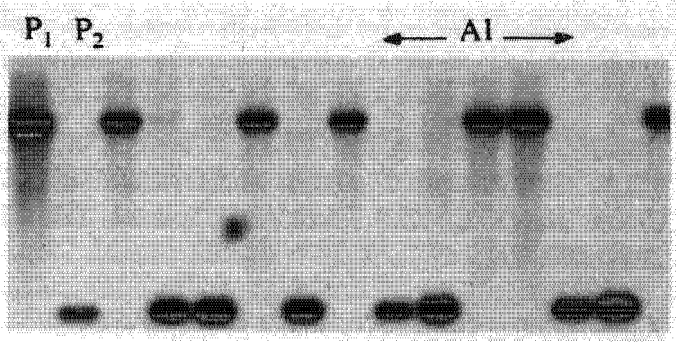

Figure 4. A 1:1 segregation of a single copy marker in a DH progeny of barley. The example shows part of a DH line progeny from the F_1 hybrid 'Igri' x 'Franka' after cutting with BamHI and probing with MWG6H514.

haploids are produced partheno- or androgenetically. In a number of cases, haploid production has been improved so much that the numbers of DH lines necessary for applied work—normally around 100 per donor genotype—can be produced, and it is economically feasible to use haploidy. Using DH lines, the production of the donor hybrids takes 1 year and costs U.S.$ 200 000, production of 2000 DH lines takes 6 months and costs U.S.$ 20 000, and subsequent yield tests take 6 years and cost U.S.$ 1 200 000.

Compared to the costs of the classical procedure this saves about U.S.$ 500 000. Though this is a very rough calculation it shows the potential of a successful haploid step in modern breeding programs. This is particularly true for most cereals and for the Brassicaceae. In crops where there are still difficulties, e.g., legumes, a concentrated input might solve the problem.

In those crops where the technical problems of haploid induction are solved, the question of their incorporation into a breeding scheme needs further investigation. For aims such as rapid incorporation of a monogenic trait, the time gain is interesting, but much more important are strategies that allow the combination of quantitatively inherited characters. Here, haploids have their biggest potential because they allow a very secure selection of polygenic traits in a very early generation. That an A_1 is equivalent to an F_2 is probably the most far-reaching advantage of DH lines, and this is true for crop improvement all over the world.

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Transformation

Transgenic Plants: *Agrobacterium*-mediated Transformation and its Application in Plant Molecular Biology Research and Biotechnology

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Abstract

For several years it has been possible to stably transform plants with cloned genes of interest to agriculture and industry. This ability to obtain transgenic plants offers tremendous opportunities both for studying fundamental processes in plant molecular biology and for engineering agronomically useful traits into crop plants.

The most widely used method for the genetic transformation of plants is based on the interaction between the plant pathogenic soil bacterium Agrobacterium tumefaciens *and wounded plant cells. A part of the bacterial tumor-inducing (Ti) plasmid, the T-DNA, is transferred to the plant cell and integrated into its nuclear genome. Expression of the T-DNA-encoded genes induces the transformed plant cell to form tumorous "crown gall" tissues. However, none of these T-DNA genes are required for T-DNA transfer or integration and they can be replaced by any other DNA sequence, and the* Agrobacterium *transformation system modified into a vector for the introduction of any desired gene sequence.*

Many plants can be routinely transformed with Agrobacterium, *and, where possible,* Agrobacterium*-mediated gene transfer is the transformation method of choice, because it allows stable integration of a well-defined DNA segment in one or a few copies. However, many monocotyledonous plants, including the cereals, remain recalcitrant to this type of DNA transfer. For these plants, alternative transformation methods have been developed. With some of these techniques, fertile transgenic rice and maize plants have been obtained.*

Plant transformation contributes enormously to progress in plant molecular biology research. By introducing reporter genes fused to plant regulatory sequences, many diverse processes can be elucidated. Overexpression or reduction of expression by the use of antisense RNA yields valuable information on the function of the encoded gene product whereas T-DNA or transposon insertion mutagenesis can be used to identify new genes.

On the other hand, Agrobacterium*-mediated gene transfer has revolutionized plant biotechnology. Several economically important traits have been introduced into crop plants.*

Introduction

The last decade has witnessed rapid progress in gene-transfer technologies and a diverse range of plant species can now be stably transformed with foreign genes.

The most widely used method for the genetic transformation of plants is based on the conjugation-like DNA transfer from the plant pathogenic soil bacterium *Agrobacterium tumefaciens* to wounded plant cells. This natural *Agrobacterium* transformation system has been modified into a vector for the introduction of any desired gene sequence in the nuclear genome of plants. Many plants can be routinely transformed with *Agrobacterium*, including model plants such as *Arabidopsis*, petunia, or tobacco, and impor-

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tant crop plants such as potato, sugar beet, cotton, alfalfa, and oilseed rape. Whenever possible, *Agrobacterium*-mediated gene transfer is the transformation method of choice, because it allows stable integration of a well-defined DNA segment in one or a few copies. However, many monocotyledonous plants, including the cereals, remain recalcitrant to this type of DNA transfer. For these plants, alternative transformation methods have been developed, including microinjection, direct DNA transformation of plant protoplasts using polyethylene glycol or electroporation, and particle-gun bombardment. With some of these techniques, fertile transgenic rice and maize plants have been obtained.

Plant transformation contributes enormously to progress in plant molecular biology research: it allowed the development of techniques for unraveling the organization of plant cells at the molecular level. By introducing reporter genes fused to potential regulatory sequences, transcriptional and post-transcriptional control mechanisms of gene expression are elucidated. Overexpression of a gene or reduction of expression by antisense RNA is used to gather information on the function of the encoded gene product and T-DNA or transposon insertion mutagenesis are valuable tools for the identification of new genes.

On the other hand, *Agrobacterium*-mediated gene transfer has revolutionized plant biotechnology, because it allows the introduction into crop plants of specific characteristics that are of interest to agriculture and industry. Insect, virus, and herbicide resistance, genetically engineered male sterility, and the ability to produce large amounts of biologically active peptides in transgenic plants are among the successful applications of genetic transformation in plant biotechnology.

Stable Transformation of Plants with *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a plant-pathogenic soil bacterium causing tumorous "crown galls" on infected plants. The basis of this disease is the transfer of a segment of bacterial DNA to the nuclear genome of the infected plant cells. The transferred DNA (T-DNA) is part of the large tumor-inducing (Ti) plasmid, present in virulent *Agrobacterium* strains. The T-DNA genes are equipped with the appropriate signals for transcription and translation in the plant cell and are thus efficiently expressed. Two kinds of enzymes are encoded by the T-DNA genes: those forming an alternative pathway for auxin and cytokinin production, and those involved in the synthesis of specific metabolites called opines, e.g., octopine and nopaline. The first set of enzymes induces the transformed plant cell to form the tumorous crown gall tissue, which is capable of in vitro growth on hormone-free medium. The opines that these tumors synthesize by means of the second set of enzymes can be specifically used by the inciting agrobacteria as a carbon and nitrogen source. *Agrobacterium tumefaciens* can thus be considered a parasite that obtains useful and specific metabolites by genetically engineering its host plants. A review on the molecular genetics of crown gall can be found in Gheysen et al. (1985).

Elements Required for T-DNA Transfer

The genetic elements encoded by *Agrobacterium* and essential for T-DNA transfer are the T-DNA border sequences, the chromosomal virulence genes, and the virulence (*vir*) genes present on the Ti plasmid, outside the T-DNA.

The 25-base pair (bp) direct repeats flanking the T-DNA (the T-DNA borders) (Yadav et al. 1982; Zambryski et al. 1982) are the only part of the T-DNA important for transfer. The right border repeat is an essential, *cis*-acting element for transfer, whereas the left border repeat is thought to merely signal where the transfer of DNA normally ends.

The attachment of *Agrobacterium* to the plant cell during the infection process is mediated by the chromosomal virulence genes, *chvA*, *chvB*, and *pscA* (Douglas et al. 1985; Thomashow et al. 1987), which are constitutively expressed. Subsequent steps in the T-DNA transfer process require the proteins encoded by the *vir* region, which comprises six complementation groups (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*). *Agrobacterium tumefaciens* infects only wounded, actively dividing plant cells. These cells excrete wound-specific compounds, such as acetosyringone and α-hydroxy-acetosyringone. These phenolic compounds act both as chemo-attractants for *Agrobacterium* (Ashby et al. 1987) and inducers of the *vir* genes (Stachel et al. 1985). Both processes are proposed to be mediated by the gene products of *virA* and *virG* (Stachel and Zambryski 1986a; Shaw et al. 1988). The constitutively expressed VirA protein acts as a chemo-receptor sensing the presence of molecules such as acetosyringone and transmits this information to the VirG protein, possibly by a phos-

phorylation mechanism (Jin et al. 1990). VirG transcriptionally activates the *virB*, *virC*, *virD*, *virE*, and *virG* loci. A number of sugars act synergistically with phenolic compounds to enhance *vir* gene expression. This induction pathway- requires the gene products of *chvE* and *virA* (Ankenbauer and Nester 1990; Cangelosi et al. 1990; Shimoda et al. 1990).

The lower DNA strand of both border repeats are then nicked by the *virD* gene products (Yanofsky et al. 1986; Stachel et al. 1987) and a linear, single-stranded DNA (the T-strand), corresponding to the bottom strand of the T-region, is released, probably by strand displacement DNA synthesis (Stachel et al. 1986). The T-strand, which is probably the intermediate transferred to the plant cell, is at the 5' end covalently attached to the VirD2 protein (Herrera-Estrella et al. 1988; Young and Nester 1988) and possibly covered by the single-stranded DNA-binding protein VirE2 (Christie et al. 1988; Citovsky et al. 1988; Das 1988). These proteins probably protect the T-strand from nucleolytic attack during the transfer. VirD2 might additionally function as a plant nucleus-targeting protein (Herrera-Estrella et al. 1990). Finally, several *virB* gene products are associated with the membrane or localized in the cell envelope (Thompson et al. 1988; Ward et al. 1988, 1990; Engström et al. 1987), suggesting they might form a transmembrane structure through which a T-DNA/protein complex is transported to the plant cell.

In view of the many analogies between T-DNA mobilization to the plant cell and bacterial conjugation, it has been proposed that T-DNA transfer is an adaptation of the conjugation mechanism (Stachel and Zambryski 1986b).

Transformation of Plants with Ti-plasmid-based Vector Systems

The development of plant transformation systems using *Agrobacterium tumefaciens* is based on the fact that, apart from the border repeats, none of the T-DNA sequences is required for transfer and integration. This means that the T-DNA genes can be replaced by any other DNA of interest, which can thus be transferred to the plant genome. Also, as a consequence of the removal of the plant hormone biosynthetic T-DNA genes, the transformed plant cells do not proliferate into tumorous tissues, but can regenerate into normal plants.

The first developed vector systems were of the cointegrate type and made use of *Agrobacterium* strains with nononcogenic Ti plasmids, e.g., vector pGV3850 (Zambryski et al. 1983). pGV3850 is a Ti plasmid derivative that still carries the *vir* genes and the T-DNA borders, but from which most of the T-DNA genes have been removed and replaced by pBR322 sequences. The genes to be transferred to plant cells are cloned in a pBR322 derivative that can then be mobilized to *Agrobacterium* and cointegrated into the T-DNA of pGV3850.

The knowledge that the T-DNA and the *vir* region do not have to reside on the same plasmid allowed the development of binary vector systems (An et al. 1985; Klee et al. 1985; Van den Elzen et al. 1985a; Simoens et al. 1986; Deblaere et al. 1987). The binary vectors contain a broad host range origin that allows them to replicate in both *Escherichia coli* and *Agrobacterium*, and appropriately positioned T-DNA border sequences between which the DNA of interest can be cloned. These vectors are introduced in *Agrobacterium* strains containing a helper Ti plasmid from which the complete T-DNA region, including the border repeats, has been removed. The helper plasmid provides the *vir* functions in *trans*.

To be able to select those cells that are transformed, a chimeric gene, consisting of a bacterial antibiotic-resistance gene or a herbicide-resistance gene fused to plant promoter and 3'-processing signals, is incorporated in the T-DNA of the transformation vectors. The most common selectable marker genes are the neomycin phosphotransferase II (*nptII*) gene (Herrera-Estrella et al. 1983; Bevan et al. 1983), the hygromycin phosphotransferase (*hpt*) gene (Van den Elzen et al. 1985b; Waldron et al. 1985), and phosphinothricin acetyl transferase (*bar*) gene (De Block et al. 1987; Thompson et al. 1987). These genes can also be used as quantifiable markers, since enzymatic assays for measuring their gene products have been described (Reiss et al. 1984; De Block et al. 1987; Datta et al. 1990).

The transformation procedure itself involves the cocultivation of wounded plant cells with the agrobacteria. The wounded plant material can either be single-cell protoplasts (Márton et al. 1979; De Block et al. 1984) or explants derived from leaves, roots, stems, hypocotyls, cotyledons, tubers, etc. (first described for leaf discs by Horsch et al. 1985). After the cocultivation step, the transformed plant cells are allowed to proliferate on selective medium and transgenic plants are regenerated through organogenesis or somatic embryogenesis, usually with an intervening callus phase. The procedure using explants is generally the preferred way of obtaining transformed plants. However, the protoplast cocultivation method has the advantage that very large numbers of transfor-

mants can be obtained, allowing selection for infrequent events. With this method, sequences with plant-promoter activity could be selected from a pool of random DNA fragments (Herman et al. 1986). Furthermore, it allowed the determination of the frequencies of aberrant T-DNA insertions (Herman et al. 1990) and of mutations occurring in the T-DNA during transformation (unpublished results) and the relative importance of the left and right T-DNA borders for the transfer process (Herman et al. 1990).

Table 1 gives an overview of the species for which *Agrobacterium*-mediated transformation and regeneration to transgenic plants has been demonstrated. This list includes several important crop species and model plants for molecular and genetic studies. In some of these reports the described transformation protocols are largely genotype independent, whereas in others the procedure is specific for a certain variety or cultivar. Although monocotyledonous plants are generally considered insensitive to *Agrobacterium* infection, *Agrobacterium*-transformed *Asparagus* plants have been obtained (Bytebier et al. 1987), as well as transformed callus or tumor tissue in the monocotyledonous crop plants *Dioscorea* (*yam*) and rice (Schäfer et al. 1987; Raineri et al. 1989).

For plant species refractory to *Agrobacterium* transformation, methods for delivery of free DNA are available (see Weissinger, this publication). These include polyethylene glycol treatment or electroporation of protoplasts (Potrykus et al. 1985; Fromm et al. 1986), microinjection (Neuhaus et al. 1987), and microprojectile bombardment (Sanford 1988). Fertile transgenic rice and maize plants have been recovered with these methods (Shimamoto et al. 1989; Datta et al. 1990; Gordon-Kamm et al. 1990).

Integration and Stability of the T-DNA in the Plant Genome

If Ti-plasmid-based vectors are to be used to obtain transgenic plants for scientific or commercial purposes, then knowledge on how and where the T-DNA integrates in the plant genome and on the stability and inheritance of the T-DNA are of great importance. Contrary to the detailed information on how a transferrable T-DNA intermediate is generated in *Agrobacterium*, much less is known about the integration of the T-DNA in the plant chromosomes.

The chromosomal localizations of the T-DNA integration sites seem to be randomly distributed (Ambros et al. 1986; Chyi et al. 1986; Wallroth et al. 1986). Tagging experiments with T-DNAs containing a promoterless selectable marker at the right border (Koncz et al. 1989; Herman et al. 1990) indicate a preferential insertion in transcriptionally-active plant DNA, witnessed by expression of the marker gene. The T-DNA usually integrates in one or a few copies per transformed plant. It is still unclear what governs the number of T-DNA insertions.

The integrated DNA is usually an exact copy of the T-DNA present in the bacterium. The right end of the integrated T-DNA corresponds most often exactly with the nick in the right border repeat, whereas the left end point can vary slightly more (Gheysen et al. in press). Apart from normal T-DNA copies, truncated insertions missing one, or both, of the T-DNA ends have been observed. These truncation events are independent of the nature and the length of the T-DNA sequence and are only observed when the right border is present in *cis* (Herman et al. 1990). Furthermore, sequence analysis of the T-DNA:plant junctions has shown that the T-DNA breakpoints are randomly distributed and do not show homology to one another or to the border sequences (Gheysen et al. 1990). Therefore, truncated T-DNA insertions are probably generated by breakage during the transfer or integration process after the formation of a normal T-DNA intermediate in the bacterium.

Sequence analysis of plant target sites before and after T-DNA insertion has shown that the integration process is accompanied by rearrangements of the plant DNA such as small deletions, insertions of "filler" sequences, and large duplications of target DNA (Gheysen et al. 1987, in press). These observations led to a model for the T-DNA integration process analogous to illegitimate recombination in animal cells and involving host functions such as DNA nicking, exonuclease activity, and DNA repair synthesis (Gheysen et al. in press). The genes introduced via the T-DNA appear to be stably maintained and inherited in a Mendelian fashion through several sexual generations (Budar et al. 1986; Chyi et al. 1986; Müller et al. 1987), which is a very important property if T-DNA transformation is used for crop improvement.

Transgenic Plants in Plant Molecular Biology Research

One of the most important consequences of the gene-transfer technology is the development of completely

Table 1. Species for which transgenic plants have been obtained with the *Agrobacterium tumefaciens* vector system.

Species	Reference
Apium graveolens (celery)	Catlin et al. 1988
Arabidopsis thaliana	Lloyd et al. 1986; Valvekens et al. 1988
Asparagus officinalis	Bytebier et al. 1987
Beta vulgaris (sugar beet)	K. D'Halluin, personal communication
Brassica napus (oilseed rape)	De Block et al. 1989; Moloney et al. 1989; Thomzik and Hain 1990
Brassica oleracea	De Block et al. 1989
Cucumis melo (muskmelon)	Fang and Grumet 1990
Cucumis sativus (cucumber)	Chee 1990
Fragaria × *ananassa* (strawberry)	Nehra et al. 1990
Glycine max (soybean)	Hinchee et al. 1988
Gossypium hirsutum (cotton)	Umbeck et al. 1987
Helianthus annuus (sunflower)	Everett et al. 1987
Juglans regia (walnut)	McGranahan et al. 1988, 1990
Lactuca sativa (lettuce)	Michelmore et al. 1987
Linum usitatissimum (flax)	Basiran et al. 1987; Jordan and McHughen 1988
Lycopersicon esculentum (tomato)	Horsch et al. 1985; McCormick et al. 1986
Malus pumila (apple)	James et al. 1989
Medicago sativa (alfalfa)	Shahin et al. 1986; D'Halluin et al. 1990
Medicago varia (alfalfa)	Deak et al. 1986; Chabaud et al. 1988
Nicotiana plumbaginifolia	Horsch et al. 1984
Nicotiana tabacum (tobacco)	De Block et al. 1984; Horsch et al. 1985
Petunia	Horsch et al. 1985
Pisum sativum (pea)	De Kathen and Jacobsen 1990; Puonti-Kaerlas et al. 1990
Populus alba × *P. tremula* (aspen)	De Block 1990
Populus alba × *grandidentata*(poplar)	Fillatti *et al.* 1987b
Populus trichocarpa × *P. deltoides* (poplar)	De Block 1990
Solanun melongena (eggplant)	Filippone and Lurquin 1989
Solanum tuberosum (potato)	De Block 1988; Sherman and Bevan 1988; Stiekema et al. 1988
Vitis rupestris	Mullins et al. 1990

new tools to study the organization of plant cells at the molecular level. Firstly, introduction in plants of reporter genes fused to potential regulatory sequences made it possible to analyze all aspects of gene expression: transcription and the influence of enhancers and silencers, transcript processing, translation initiation and termination and secretion, and targeting of proteins to subcellular locations. The same techniques demonstrated the intricate regulation of gene expression by internal stimuli, such as hormones and devel-

opmental patterns, and external stimuli, such as light, adverse environmental conditions, and pathogen attack. Apart from the previously mentioned marker genes (*nptII*, *hpt*, and *bar*), some other useful reporter genes are the *E. coli* β-glucuronidase (*gus*) and β-galactosidase (*lacZ*) genes (Jefferson et al. 1987; Teeri et al. 1989) and the firefly luciferase gene (Ow et al. 1986). Transformation with chimeric *gus* and *lacZ* genes, in combination with histochemical techniques, is particularly useful to study organ-, tissue-, and even cell-specific gene expression.

To gain insight into the function of a particular protein, the levels at which that protein is present can be modulated in transgenic plants. In this respect, two complementary strategies can be envisaged:

- Overproduction of a protein by placing its coding sequence under the control of a strong constitutive or inducible promoter.
- Reduction of expression by using antisense constructs (Lichtenstein 1988) or possibly ribozymes (Cotten 1990).

Finally, the introduction into plants of insertional mutagens is becoming a very useful tool for the isolation of new plant genes. The advantage of this technique is that the mutated gene is molecularly tagged by the insertion that allows the gene to be cloned. The T-DNA itself can serve as the insertional mutagen (Feldmann et al. 1989; Koncz et al. 1990; Yanofsky et al. 1990; Van Lijsebettens et al. in press). Alternatively, transposon tagging can be used. However, useful transposon systems have not yet been identified in genetically well-characterized plant species such as *Arabidopsis*. Therefore, maize and snapdragon transposon systems have been introduced in heterologous hosts by *Agrobacterium*-mediated gene transfer (Balcells et al. 1991). Some examples of the applications of these techniques in molecular biology research are detailed below.

A Test System for the Molecular Analysis of Mutants Induced by Tissue Culture and Mutagenic Treatment

Genetic variation is known to occur in many plant species during tissue culture and plant regeneration (Scowcroft 1985) and has been termed somaclonal variation (Larkin and Scowcroft 1981). In many cases it has been shown that the regenerated variants are really somaclonal mutants, i.e., the altered phenotype is transmitted to the progeny. On the one hand, somaclonal variation is considered a useful additional source of genetic diversity, which can be exploited for crop improvement (Larkin and Scowcroft 1981; Chaleff 1983; Evans and Sharp 1986; Evans 1989). On the other hand, it is an unwanted phenomenon in cases where clonal uniformity is desired, e.g., the in vitro micropropagation of commercial cultivars, the conservation of germplasm through tissue culture, and the genetic transformation of plants that, for most methods, requires a tissue culture step. Many mechanisms have been implicated as causes of somaclonal variation; transposition, DNA amplification, variations in chromosome number, translocations, deletions, and point mutations are known to occur.

Somaclonal variation has mostly been studied at the morphological, biochemical, karyological, and genetical level. Molecular studies are scarce and little information is available on the frequency and the type of changes that occur at the level of the DNA sequence. We tried to approach these questions by regeneration and selection of protoplasts derived from transgenic tobacco plants. The transgenic plants were obtained by *Agrobacterium*-mediated transformation with constructs containing inactivated *nptII* genes. Reactivation of these genes requires specific DNA alterations such as a point mutation or a small deletion. Mutants with a reactivated *nptII* gene are selected on kanamycin-containing medium and analyzed at the molecular level by Southern blotting and/or sequence analysis of DNA amplified by polymerase chain reaction (PCR). One of the constructs used in this study, pGSFR164, contains a dicistronic transcriptional unit consisting of a plant promoter followed by the *bar* gene and the *nptII* gene; both genes are separated by an intercistronic region of 10 base pairs (Angenon et al. 1989). The *nptII* gene in this construct, which is the downstream gene of the dicistronic unit, is translated with a very low efficiency and hence plants transformed with this construct are sensitive to kanamycin levels higher than 25 mg l^{-1}. One way to reactivate this *nptII* gene is by a deletion creating a +1 frameshift in the 3' end of the *bar* coding sequence. Such a frameshift allows ribosomes initiating at the *bar* start codon to skip the *bar* stop codon, and to continue translation in the *nptII* reading frame.

Protoplasts were prepared from several independent transgenic tobacco plants containing one copy of the pGSFR164 T-DNA and were cultured in the presence of 500 mg l^{-1} kanamycin. Mutants in which the *nptII* gene was reactivated could be scored 8 weeks after selection as small kanamycin-resistant calli. These mutants were recovered with a frequency of

about two in 10^6 plant cells. Plants regenerated from these calli were kanamycin-resistant, contained high levels of NPTII enzymatic activity, and, in the cases analyzed, transmitted the kanamycin resistance phenotype in a Mendelian fashion to the progeny. For seven plants, the DNA region in which the mutation was assumed to be located was amplified by PCR and sequenced. This showed that the mutants contain small deletions in the 3' end of the *bar* gene, ranging from 1 to 34 bp and all causing a +1 frameshift (Angenon, G., Jacobs, A., Bruyns, A., and Depicker, A., unpublished results). The deletions that we can select for with this system have to be located within a 220-bp region and have to cause a +1 frameshift. If we extrapolate these data to the whole tobacco genome (3.2×10^9 bp) and to the three reading frames, we calculate that every regenerated plant should contain about 90 small deletions. Since we apply the selection after the cells have divided 2 to 3 times, we only measure mutations arising in the initial stages of the regeneration process. Somaclonal variation is known to increase with the length of time in culture. Therefore, our data represent a minimum estimate for the mutations that occur.

In a similar way, but with other constructs, we will analyze the type and frequency of other mutations, e.g., base pair substitutions and larger deletions. This type of analysis should lead to a better insight into the mutations induced by tissue culture and the factors influencing this process. It could also be used to study the frequency and the type of mutations induced by chemical mutagens, UV, and γ irradiation.

The Role of Superoxide Dismutases in the Protection Against Oxidative Stress in Plants

Reduced oxygen species such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide, and the hydroxyl radical (OHø) are by-products of oxidative metabolism. Their deleterious effects cause oxidative stress in all aerobic organisms. The hydroxyl radical, which is the most reactive species known in chemistry, is especially harmful for living cells because it causes DNA mutations and damage to membranes and other cell constituents. Hydroxyl radicals can be formed in the Haber-Weiss reaction from the superoxide radical and hydrogen peroxide, which are by themselves not very harmful:

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^- + O_2 + OH\text{ø}$$

Aerobic organisms possess a whole range of defense mechanisms to protect them against reactive oxygen species, such as antioxidants, superoxide dismutases (SOD), catalases, and peroxidases. Clearly, this defense strategy aims to control superoxide radicals and hydrogen peroxide, rather than their highly toxic reaction product, the hydroxyl radical. Catalases and peroxidases remove hydrogen peroxide whereas SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide and oxygen. SOD thus controls the concentration of the two Haber-Weiss reaction substrates and probably plays a central role in the defense against oxidative stress.

Three types of SOD can be distinguished, according to their metal cofactor: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe) SODs. In general, higher plants contain a mitochondrial MnSOD, a cytosolic Cu/ZnSOD, and a Cu/ZnSOD and/or a FeSOD localized in the chloroplasts (for a review, see Bowler et al. in press). The SOD isoforms are all encoded by nuclear genes.

In plants, many stress conditions appear to exert their damaging effects through the production of reactive oxygen species. Examples of such conditions are photoinhibition, exposure to herbicides, such as paraquat, and to air pollutants, such as ozone and SO_2, recovery from anoxia, and infection by certain pathogens. A higher tolerance to these stress factors is often correlated with increased levels of SOD (Bowler et al. in press). In general, it appears that oxyradicals generated in a particular cell compartment lead to elevated SOD levels in that compartment. For instance, in *Nicotiana plumbaginifolia*, oxidative stress in the mitochondria results in induction of the mitochondrial MnSOD, whereas if such a stress affects the chloroplasts, the chloroplastic FeSOD is induced (Bowler et al. 1989; Tsang et al. 1991).

To further unravel the function of SOD and its possible protective role towards stress conditions, transgenic plants with increased SOD levels were analyzed. The *N. plumbaginifolia* MnSOD was overproduced in tobacco plants and targeted to either the mitochondria or the chloroplasts (Bowler et al. submitted). Incubation of leaf discs from the transgenic plants with paraquat was used to measure superoxide damage. The amount of engineered SOD appeared to be higher in older leaves than in younger leaves. This allowed study of the effect of different levels of SOD overproduction. High-level overproduction in a particular cell compartment (chloroplasts or mitochondria) conferred strong protection against damage generated by superoxide radicals in that compartment. On the other hand, low-level overproduction had no or sometimes even deleterious effects. Probably high levels of SOD cause a total depletion of one

of the substrates of the Haber-Weiss reaction (O_2), thus precluding hydroxyl radical formation. A low increase of SOD might only partially remove superoxide, thereby disturbing the normal balance between superoxide and hydrogen peroxide and causing damage if the hydrogen peroxide is not adequately removed (Bowler et al. submitted). In another study (Tepperman and Dunsmuir 1990), a Cu/ZnSOD was overexpressed and targeted to chloroplasts of tobacco and tomato plants. Apparently, there was no difference in superoxide sensitivity between the transgenic and the control plants. A possible explanation for the different results with different forms of SOD might be that Cu/ZnSOD and FeSOD are inactivated by hydrogen peroxide, whereas MnSOD is not.

The effects of MnSOD overproduction at the level of the whole plant and towards other stress factors, such as chilling, air pollutants, and high light intensities, remain to be studied. However, the results obtained so far demonstrate that SOD overproduction is a promising strategy to engineer stress resistance in plants. Furthermore, it has become clear that the type of SOD that is produced, the level of production, the subcellular localization of SODs, and the interaction with other detoxifying mechanisms are all important factors in protection against oxidative stress.

Transgenic Plants Expressing Agronomically Useful Traits

Classical plant breeding programs have greatly improved the yield, quality, and disease resistance of crop plants. The ability to genetically transform plants now adds a new dimension to breeding because it eliminates the barriers of sexual reproduction and greatly extends the pool of genes that are available for crop improvement. Indeed, once the gene encoding a particular trait has been isolated, this gene—be it of plant, animal, or microbial origin—can be stably inserted into the plant genome. Furthermore, recombinant DNA techniques allow the existing genetic material to be modified or combined, thus creating new diversity that is not available in nature. Another advantage of the genetic engineering approach is that a strategy that has proven to be useful in one plant species is readily applicable to all the crops for which transformation methods are available. Since phenotypes encoded by multiple genes are both difficult to analyze and to introduce in transgenic plants, the achievements of genetic engineering to date involve single-gene traits. However, the domains in which the gene transfer technology is being applied are diverse and include crop protection, qualitative improvement of crops, production of male-sterile plants, and synthesis in plants of high-value biological compounds. Some examples of the progress in these fields will be described in more detail.

Insect-resistant Transgenic Plants

Bacilllus thuringiensis (Bt) is a gram-positive soil bacterium which, during sporulation, produces proteinaceous crystalline inclusions with insecticidal activity. The insecticidal crystal proteins (ICPs or Cry proteins) of different Bt strains exhibit a striking difference in their insecticidal spectra and are not toxic to organisms other than insects. The ICPs are classified according to their structural similarities and insecticidal spectra. Four major classes have been identified: *Lepidoptera*-specific (CryI), *Lepidoptera*- and *Diptera*-specific (CryII), *Coleoptera*-specific (CryIII), and *Diptera*-specific (CryIV) proteins (Höfte and Whiteley 1989). Bt crystalline inclusions dissolve in the midgut of insect larvae and release one or more ICPs. The ICPs are usually protoxins, proteolytically converted into smaller toxic polypeptides. The active toxins bind to the midgut epithelial cells of susceptible insects, leading to disruption of the cell membrane. As a consequence, the epithelial cells lyse, and the insects stop feeding and eventually die.

Spore preparations of Bt have been used for many years as a biological insecticide. However, the properties of the Bt toxins, i.e., their proteinaceous nature and their proven safety and efficiency, make them ideal for introduction into plants. Consequently, transgenic plants expressing *ICP* genes were one of the first successful applications of plant transformation technology for crop improvement. Modified *ICP* genes under the control of plant promoter and 3'-processing signals were introduced into tobacco and tomato plants via *Agrobacterium*-mediated transformation (Vaeck et al. 1987; Fischhoff et al. 1987; Barton et al. 1987). The transgenic plants expressed the ICP, were toxic to larvae of *Manduca sexta* (the tobacco horn worm), and were protected against feeding damage by the larvae. Subsequent greenhouse and field trials with tobacco, tomato, potato, and cotton plants expressing ICPs demonstrated that the transgenic plants were protected against a range of lepidopteran insect pests (Delannay et al. 1989; Perlak et al. 1990; Plant Genetic Systems N.V. unpublished results). These results demonstrate the feasibility of

producing transgenic crop plants protected from insect damage as a commercially interesting and environmentally safe alternative to chemical insecticides.

Concerns about the development of insect resistance against Bt toxins have stimulated research into the precise mode of action of ICPs. For different ICPs, specific high-affinity binding-sites (receptors) have been identified on brush border membranes in the midgut of susceptible insects. Furthermore, it has been shown that ICPs that are toxic to a certain insect species bind saturably and with high affinity to midgut receptors of that species, whereas nonsusceptible insects do not possess specific ICP-binding sites (Hofmann et al. 1988; Van Rie et al. 1989, 1990a). This correlation between toxicity and binding suggests that differences in the insecticidal spectrum of ICPs are determined by the ICP receptors of the midgut epithelium. Since the midgut can contain different receptors, a particular insect species can be sensitive to different types of ICPs.

Van Rie et al. (1990b) studied the mechanism of resistance of a *Plodia interpunctella* strain to an ICP. *P. interpunctella* is normally sensitive to ICPs of the CryIA(b) and CryIC types. A CryIA(b)-resistant strain (R-strain) was selected in the laboratory by rearing the insects on a Bt-treated diet. Resistance is correlated with a 50-fold reduction in affinity of the membrane receptor for the CryIA(b) protein. However, the R-strain is not resistant to the CryIC toxin. On the contrary, sensitivity to CryIC is increased. Apparently, CryIC is recognized by a different receptor, the concentration of which is increased in the R-strain. This result can be rationalized if one assumes that the ICP receptors have an important function in the insect midgut. An altered CryIA(b) receptor with reduced ICP affinity could also have lost its normal physiological function. This could be compensated for by an increase in the concentration of the CryIC receptor. These studies also suggest that ICPs binding to different receptors could be used in combination or sequentially as a strategy to prevent or to delay insect resistance.

Herbicide-resistant Transgenic Plants

Several newly developed herbicides combine some very interesting properties, i.e., low toxicity to men and animals, low persistence in the environment, and a high activity implying that they can be used efficiently at low doses. Since the enzymes that these herbicides inhibit are common to all plants, weeds as well as crop plants, they are total herbicides that cannot be sprayed for postemergence purposes. To be able to use these broad spectrum herbicides selectively for crop protection, herbicide resistance has been engineered in plants. Two strategies to obtain herbicide-resistant plants can be followed:

- Decreasing the sensitivity of the plant to the herbicide by overproduction of the target enzyme or by expression of a modified target that is insensitive to the herbicide but retains its enzymatic activity.
- Providing to the plant an herbicide detoxifying pathway.

Examples of the first approach are engineered resistance to glyphosate and sulfonylurea compounds. Glyphosate is an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the biosynthetic pathway of aromatic amino acids. Sulfonylurea herbicides inhibit acetolactate synthase (ALS), an enzyme involved in the biosynthesis of branched-chain amino acids. The introduction of mutant *EPSPS* genes, encoding enzymes less sensitive to glyphosase (Fillatti et al. 1987a; Hinchee et al. 1988; Comai et al. 1985) or the overproduction of a plant EPSPS (Shah et al. 1986) conferred tolerance to glyphosate in the transgenic plants. Similarly, transgenic tobacco plants expressing mutant *ALS* genes were found to be tolerant to sulfonylurea compounds (Haughn et al. 1988; Lee et al. 1988).

High levels of resistance towards several herbicides were obtained with the second approach, i.e., engineering of a novel detoxification pathway in plants. Herbicide-detoxifying enzymes have been identified in several plant species and in microorganisms and are thus potential candidates for introduction in crop plants.

The herbicide glufosinate or l-phosphinothricin (PPT) is an analogue of glutamate and as such a potent inhibitor of glutamine synthetase (GS). Inhibition of GS leads to the rapid accumulation of ammonia and eventually to death of the plant cells. Bialaphos, produced by *Streptomyces hygroscopicus*, is a tripeptide consisting of PPT and two l-alanine residues. Bialaphos is converted into PPT by peptidases. PPT and bialaphos are commercialized by Hoechst as Basta® and by Meiji Seika Ltd. as Herbiace®. A PPT-resistance gene, *bar*, was isolated from *S. hygroscopicus* (Murakami et al. 1986) and was shown to encode the enzyme phosphinothricin acetyl transferase (PAT), which specifically converts PPT to an acetylated, nonherbicidal form (Thompson et al. 1987).

The *bar* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced

into tobacco, tomato, potato, oilseed rape, alfalfa, sugar beet, aspen, and poplar plants via the *Agrobacterium* transformation system (De Block et al. 1987; Botterman and Leemans 1988; De Block et al. 1989; De Block 1990; D'Halluin et al. 1990). In all cases, the *bar* gene conferred resistance on greenhouse-grown transgenic plants to doses of glufosinate and bialaphos that are much higher than normally applied in agriculture. Also in field tests with transgenic tobacco, potato, and alfalfa plants, complete resistance was observed (De Greef et al. 1989; D'Halluin et al. 1990). Furthermore, growth of herbicide-treated transgenic plants and nontransformed, nontreated control plants was undistinguishable (De Greef et al. 1989). These results convincingly demonstrate that the broad-spectrum herbicide glufosinate can be used selectively for postemergence applications on transgenic crop plants.

Bacterial genes encoding bromoxynil and 2, 4-dichlorophenoxyacetic acid (2,4-D) detoxifying enzymes were likewise engineered in tobacco plants and conferred high levels of herbicide resistance (Stalker et al. 1988; Lyon et al. 1989; Streber and Willmitzer 1989).

Engineering of 2S Seed Storage Proteins

Seeds are a very important source of proteins for human and animal nutrition. However, in most seed-storage proteins several essential amino acids are underrepresented. Many breeding programs consequently focus on improving the balance of essential amino acids in those proteins. Alternatively, this problem can be tackled with genetic engineering approaches, e.g., by expressing in a heterologous host naturally occurring or in vitro modified genes encoding seed storage proteins rich in a limiting amino acid. Both these approaches have been worked out with an important class of seed storage proteins of dicotyledonous plants, i.e., the 2S albumins.

The 2S albumins are synthesized as large precursor molecules that undergo extensive posttranslational processing. A signal peptide, an additional amino-terminal fragment, an internal fragment, and some carboxy-terminal residues are removed. The two subunits resulting from this cleavage process are linked by disulfide bridges. 2S albumins are encoded by multigene families. The *Arabidopsis thaliana* family, for example, contains four tandemly clustered members (Krebbers et al. 1988). One of the *Arabidopsis* genes (*at2S1*) was introduced into tobacco plants. The *Arabidopsis* 2S albumin was correctly processed in the seeds of the transgenic tobacco plants and accumulated in the protein bodies of the endosperm and the embryo (De Clercq et al. 1990a). This demonstrates that 2S albumins can be correctly processed and targeted, when expressed in a heterologous host.

Chimeric genes encoding a Brazil nut methionine-rich 2S albumin fused to different seed storage protein 5' sequences, were expressed in tobacco, *Brassica napus*, and *Arabidopsis* plants (Altenbach et al. 1989; Guerche et al. 1990; De Clercq et al. 1990b). The Brazil nut 2S albumins were reported to be correctly processed and targeted in the transgenic plants. In one case (Altenbach et al. 1989), a significant increase in the methionine content of the seeds was obtained. The *Arabidopsis at2S1* gene was also modified in vitro to encode a novel protein with a high methionine content. The alterations were made in a region that is highly variable among different 2S albumins in both length and amino acid composition. This variable region was replaced with different oligonucleotides containing mainly methionine codons. The modified genes were introduced into tobacco, *Brassica napus*, and *Arabidopsis* plants and directed the synthesis of new, methionine-rich 2S albumins (De Clercq et al. 1990b). This shows that 2S albumins can tolerate extensive modifications in their primary sequence. On the other hand, the expression levels of the modified genes should be further optimized to get a large increase in the seed methionine content. It will be interesting to investigate whether other limiting amino acids, or combinations of amino acids, can likewise be engineered in these seed storage proteins. 2S albumin genes have also been modified for totally different purposes. Vandekerckhove et al. (1989) showed that it is possible to produce large amounts of biologically active peptides as part of a chimeric seed storage protein. To demonstrate this, the variable region of the *Arabidopsis at2S1* gene was replaced by a sequence encoding the neuropeptide Leu-enkephalin, flanked by tryptic cleavage sites. The modified gene was introduced into *Arabidopsis* and *Brassica napus* using *Agrobacterium*-mediated transformation. The 2S albumins were isolated from seeds of the transgenic plants and the Leu-enkephalin peptide was released by digestion with trypsin and with carboxypeptidase B to remove an extra C-terminal lysine residue. The peptide recovery per gram of seed was up to 200 nmol for *Arabidopsis* and up to 50 nmol for *Brassica napus* (Vandekerckhove et al. 1989). Advantages of this system include the high stability of the seed storage proteins and thus also of the inserted peptide, and the easy purification of the 2S albumins, even on a large scale.

In conclusion, it has been demonstrated that engineered 2S albumins can be used for the improvement of the nutritional value of seeds and also for "molecular farming", i.e., the production of high-value biological molecules in plants. A discussion of the potential of producing foreign proteins and peptides in plants can be found in Krebbers et al. (in press).

Engineering Nuclear Male Sterility in Plants

Hybrid seeds have become very important in agriculture for increasing crop productivity. Indeed, in many crop species, F_1 hybrid plants perform superiorly, a phenomenon called hybrid vigor or heterosis. However, male-sterile lines for the production of hybrid seeds are not available for most crop plants and the alternative, mechanical removal of anthers, is often impractical, impossible, or very expensive. Recently, Mariani et al. (1990) reported the transformation of plants with a gene preventing pollen development and thus inducing male sterility. The introduced chimeric gene consists of a promoter, directing exclusive expression in the tapetum cells of anthers, linked to the coding region of a cytotoxic ribonuclease.

The tapetum is a cell layer that surrounds the pollen-sac and is essential for pollen development. From a set of anther-specific tobacco cDNA clones, two were found to hybridize exclusively with mRNA in this tapetum layer (Goldberg 1988). The gene corresponding to one of these cDNAs was isolated from a tobacco genomic library (Seurinck et al. 1990). This gene, called *TA29*, is regulated primarily at the transcriptional level (Koltunow et al. 1990). The 5'-flanking sequence of the *TA29* gene was fused to two different ribonuclease genes, an *Aspergillus oryzae* gene encoding RNase T1 and a *Bacillus amyloliquefaciens* gene encoding the ribonuclease barnase (Mariani et al. 1990). The chimeric genes were introduced into tobacco and oilseed rape plants. The majority of the transgenic plants were male sterile, but were identical to untransformed control plants in all other aspects, including the ability to produce viable seed when cross-fertilized. Expression of the *TA29*-RNase genes leads to selective destruction of the tapetum cells and prohibits pollen development. In oilseed rape, both RNases seem to work equally well and one copy of either the *TA29*-RNase T1 or *TA29*-barnase gene is sufficient to induce male sterility. In tobacco, at least four copies of the *TA29*-RNase T1 gene are required to produce male-sterile plants, but for the *TA29*-barnase gene, one copy is sufficient. For crops in which the fruit is not the harvested product, the male-sterile plants can be crossed with any pollinator line. However, if fruit or seed is the desired plant product, full fertility must be restored. This could be achieved by incorporating in the pollinator plants antisense constructs to the RNase gene or by introducing the gene for barstar, which is a specific inhibitor of barnase. The availability of a dominant nuclear male-sterility gene is a very significant breakthrough for the production of hybrid seed, since this technology is potentially applicable to many different crop species.

Conclusions

Any natural or in vitro modified gene can be introduced directly into plants with the newly developed gene-transfer methods. This creates obvious advantages for increasing the yield and improving the quality of crop plants and for fundamental plant biology research. The use of transgenic plants will further increase our knowledge of the molecular fine structure of plant cells, of developmental processes, and of the interactions of plants with both their environment and with other, beneficial or harmful, living organisms.

Many of these new insights will also find applications in the genetic engineering of crop plants. Indeed, more complex traits can be incorporated in plants as the genes controlling them are identified. Furthermore, a detailed knowledge of gene expression is required for the introduction of new characteristics, as shown in the described examples. The precise regulation of gene expression, sometimes at the cellular or even subcellular level, can determine the success of genetic manipulations.

Agronomically useful genes have now been introduced into some of the world's major crop plants; however, for several other important crops, transgenic plants have not yet been obtained and continued efforts will be needed to develop efficient transformation protocols for these plant species and varieties. The benefits of gene-transfer technology for agriculture are demonstrated by the successful field trials with transgenic crop plants resistant to insects, viruses, and herbicides, and the commercialization of these plants which is foreseen for the near future. Although until now plant genetic engineering has mainly focused on the introduction of agronomically important genes, several examples demonstrate the potential to obtain products from transgenic plants with pharmaceutical or industrial applications. Plants have always been a major source of ingredients and

raw materials for the production of pharmaceuticals, special chemicals, and bulk products. Clearly, genetic engineering offers the opportunity to enhance the production levels of these natural products and to broaden the spectrum of molecules that can be produced in plants.

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Transformation of *Lotus* Species Using an Ri Plasmid Vector

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Lotus species are forage legumes produced for pastures in both temperate and tropical regions. Transformation of *Lotus* and other forage legumes would enable the incorporation of useful genes for the genetic improvement of this crop, as well as providing a system for molecular study of symbiotic nitrogen fixation. The ability of transformed roots induced by Ri plasmids of *Agrobacterium rhizogenes* to regenerate into whole plants and to produce detectable opines (Tepfer 1984; Ooms et al. 1984) has generated increased interest in their use as vector systems.

Transformation studies involving *Lotus corniculatus* have been reported using *A. tumefaciens* (Webb 1986; Armstead and Webb 1987) and *A. rhizogenes* (Petit et al. 1986; Petit et al. 1987; Stougaard et al. 1987). This report describes the transformation of two other important species within the genus, *L. tenius* Waldst et Kit and *L. pedunculatus* Cav., using a genetically engineered strain LBA 9402 (pRi 1855 - p Bin 19) which confers resistance to kanamycin.

Materials and Methods

Stem explants from 3-week-old glasshouse-grown seedlings were surface-sterilized in 7.5% v/v 'Domestos' for 20 min followed by six washes in sterile water. Stems were cut into 3 cm lengths and the apical ends inoculated with 20 µl of an exponential culture of *Agrobacterium rhizogenes* LBA 9402 (pRi 1855 - p Rin 19) carrying the neomycin phosphotranferase II (NPT II gene) (Hamill et al. 1987). Bacteria were cultured in yeast mannitol broth (YMB) liquid medium (Ooms et al. 1985) containing 50 µg ml^{-1} of kanamycin sulphate (25°, dark, 100 rev. min^{-1}). Inoculated stem explants were placed base down into hormone-free solidified agar (0.8% w/v, Sigma) Murashige and Skoog (1962) medium (MSO) and maintained under continuous fluorescent illumination (100 lux, 25°C). The explants were transferred after 5 days to MSO agar medium containing 500 µg ml^{-1} cefotaxime (Calforan; Roussel Laboratories, Wembley, UK). Individual transformed roots produced at the inoculation sites were excised and cultured on MSO agar medium containing 250 µg ml^{-1} cefotaxime. Roots were subcultured every 4 weeks, the cefotaxime level being reduced, and subsequently roots were maintained in antibiotic-free MSO agar medium.

For assay of kanamycin resistance, roots were transferred to MSO agar medium containing 50, 100, 150, 200, 250, and 300 µg ml^{-1} kanamycin. Transformed roots were analyzed by paper electrophoresis for the synthesis of opines (Morgan et al. 1987), NPT II activity was assayed as described (Reiss et al. 1984). Segments of transformed roots were placed on media containing various combinations of growth regulators for regeneration. Alternatively, root segments were placed on different callusing media. Calli that proliferated were either transferred to regeneration media or used for establishment of cell suspension cultures.

Results and Discussion

Roots developed on stem explants of *L. tenuis* within 9-23 days after inoculation with *A. rhizogenes* LBA 9402 (pRi 1855 - p Rin 19) and within 10-30 days on *L. pedunculatus* stem explants. Roots emerged directly from the inoculation sites and in some cases localized swellings preceded root emergence. Such roots had poorly developed root hairs while the organs were still attached to the explants. The rooting

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response, based on the percentage of explants producing roots and the mean number of roots produced per explant, was high in both species. 98.3% of *L. tenuis* stem explants and 91.0% of *L. pedunculatus* stem explants inoculated with the Agrobacterium strain produced roots. The mean number of roots produced per explant for *L. tenuis* and *L. pedunculatus* were 20 and 22, respectively. Transformed roots exhibited a characteristic lack of geotropism; they were mostly plagiotropic and some displayed negative geotropism. Phenotypic characteristics of transformed roots were found to be plant species-specific following excision and transfer of roots to culture. Transformed roots of *L. pedunculatus* were yellowish-white, long, less hairy, and with profuse lateral branching. *L. tenuis* transformed roots were creamy-yellow, fairly thick, and very hairy, but with poor lateral branching.

Comparatively, transformed roots of *L. pedunculatus* showed a higher level of kanamycin resistance than transformed roots of *L. tenuis*. In *L. pedunculatus*, of the root clones assessed 45% were resistant to 150 ml^{-1} and 30% were resistant to 200 μg ml^{-1} of kanamycin; 15% tolerated 100 μg ml^{-1} kanamycin, while 10% survived 250 μg ml^{-1} of the antibiotic. In *L. tenuis*, 45% of the root clones assessed were capable of resisting kanamycin at 100 μg ml^{-1}. Shoot regeneration from transformed roots of *L. tenuis* occurred spontaneously at low frequency (approx. 20%) on MSO agar medium containing 50 and 100 μg ml^{-1}. Further regeneration was stimulated when calli established on MS agar medium with 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 2.0 mg l^{-1} -napthaleneacetic acid (NAA), 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 100 μg ml^{-1} kanamycin sulphate were transferred to B5 (Gamborg et al. 1968) agar medium with 0.5-1.0 mg l^{-1} BAP and 50 μg ml^{-1} kanamycin. Roots were produced in abundance on transfer of the shoots to B5 agar medium with 0.05 mg l^{-1} BAP. Transformed roots and callus of *L. pedunculatus* failed to produce shoots.

Paper electrophoresis of crude extracts showed transformed roots and calli of *L. tenuis* and *L. pedunculatus* and transformed regenerants of *L. tenuis* to synthesize silver nitrate positive compounds that migrated to similar positions as agropine and mannopine + mannopinic acid present in the standard mixture. The synthesis of opines confirmed the expression of Ri plasmid T-DNA genes. Further confirmation of the transformed nature of the regenerants and calli was demonstrated by the detection of NPT II activity in the tissues.

The ability of all root clones transformed by LBA 9402 (pRi 1855 - p Bin 19) to resist kanamycin implied a successful, cotransfer of the binary vector (pRin 19-carrying NPT II gene) and the coresistant Ri plasmid (pRi 1855). The differential levels of kanamycin resistance observed in transformed root clones of *L. tenuis* and *L. pedunculatus* has also been reported in roots and regenerated plants of *Lycopersicon* spp (Morgan et al. 1987), and *Nicotiana* and *Solanum* spp (Davey et al. 1987). Factors that may have influenced the level of kanamycin resistance include the TL-DNA copy number inserts, plant DNA and T-DNA sequences flanking the NPT II coding region or the strength of the nopaline synthase gene promoter (Morgan et al. 1987). Although high frequency of spontaneous regeneration has been reported in *A. rhizogenes* transformed roots of *L. corniculatus* (Petit et al. 1987; T.K. Ghose, personal communication) this was a less frequent event in transformed roots of *L. tenuis* and absent in *L. pedunculatus*. A more likely reason for the low spontaneous regeneration could be the presence of a high level of endogenous auxins in the roots resulting in an auxin to cytokinin ratio that was unfavorable for shoot formation.

Cell-suspension cultures were established from the transformed root-derived calli of *L. tenuis* and *L. pedunculatus*. Kanamycin resistance of protoplasts isolated from the cell suspensions would be a useful dominant marker in somatic hybridization programs within the genus *Lotus*.

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Preliminary Experiments on Transformation of Tissues and Organs of *Arachis* Species

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In vitro regeneration in a crop plant is a prerequisite for any attempt to achieve genetic transformation. In legume tissue culture, cotyledons have been established as explants for high-frequency regeneration in many species and techniques have been developed at ICRISAT to use cotyledons, leaflets, and hypocotyls for high frequency of *Arachis* spp regeneration. These techniques were used at the Scottish Crops Research Institute (SCRI) for infection of explants by virulent and avirulent strains of *Agrobacterium tumefaciens.*

Virulent strains of *Agrobacterium tumefaciens* C-58 and A-281 were used for infecting the first internodes (between the cotyledonary node and the first leaf) of 8–12 days old aseptically raised seedlings of four cultivars of *Arachis hypogaea.* Three weeks after inoculation, tumors were observed on all the four cultivars at the points of infection. These tumors continued to grow when excised and cultured on hormone-free MS medium. Tumor tissues were also observed on the cotyledons of JL-24 which had been infected with the above two virulent strains of *Agrobacterium.*

An avirulent strain of *A. tumefaciens* A-281 carrying cointegrate vector PGV 3580 (PKu-2) was used to transform *Arachis hypogaea* cv JL-24 (cotyledons. and leaflet segments) and *A. pusilla* (hypocotyls and leaflet segments). The above plasmid has kanamycin and hygromycin resistance genes. Green, semigreen, and white shoots were obtained on the kanamycin selection medium.

Green buds were obtained from JL-24 leaflet segments and *A. pusilla* leaflet and hypocotyl segments. The shoots from JL-24 cotyledons have been transferred to the rooting medium. The shoots/buds from the kanamycin selection medium will be assayed for NPT II genes.

To test the sensitivity of cotyledons to kanamycin and hygromycin, JL-24 cotyledons were cultured on a medium with 100, 250, 350, and 450 mg l^{-1} concentrations of kanamycin and 10 and 20 mg l^{-1} of hygromycin. Kanamycin at 250 mg l^{-1} supported the formation of tiny buds from the cotyledons that did not grow beyond the bud stage. Cotyledons bleached after 15-20 days of culture on media with hygromycin at 20 mg l^{-1} and no regeneration was observed. This suggests that a hygromycin resistance gene may be better than a kanamycin resistance marker gene for transformation of *Arachis.*

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Viruses as Extra-chromosomal Gene Vectors For Higher Plants

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Abstract

Viruses have been extremely important as gene vectors for bacteria and animals and have played an important role in the molecular genetics of these organisms. However, virus-based vectors for plants are in an early exploratory state of development. With the use of reverse genetics, two plus-strand RNA viruses have been investigated as vectors for amplification and expression of foreign genes in plants. Tobacco mosaic virus and brome mosaic virus have been used as coat protein replacement vectors for the high-level expression of bacterial chloramphenicol acetyltransferase. In addition, representatives of both the single-stranded DNA (geminiviruses) and double-stranded DNA viruses (caulimoviruses) have been developed as gene-transfer agents. These experiments as well as the regulatory regions of viruses that are useful for plant genetic engineering will be reviewed.

Introduction

Gene cloning and expression vectors have been derived from a wide variety of bacterial, animal, and plant viruses, especially those that replicate via DNA intermediates. These viruses are easy to restructure by recombinant-DNA methods. However, it is now feasible to manipulate viruses that replicate only through RNA intermediates with the recent availability of transcription systems for making artificial RNA transcripts from complementary DNA (cDNA) copies. Consequently, the choice of virus to use as the genetic vehicle has grown to include the large number of positive-strand RNA viruses of eukaryotes.

The plant viruses also offer an interesting variety of gene-regulatory elements that are useful in genetic engineering. The strong constitutive 35S promoter of cauliflower mosaic virus (CaMV) has played an important role in transformation technology of plants in recent years, for example, and is a prime example of the potential usefulness that plant virus elements have for manipulation.

Gene vectors of a nonintegrating nature that replicate independently of the cellular genetic apparatus are desirable for plants for a number of reasons. Perhaps the foremost justification is to develop vectors that replicate to a high copy number so that new functions or gene products can be produced at much higher levels than are now possible with Ti plasmid vectors. Viral genomes as autonomously replicating elements (replicons) can be used to amplify any covalently attached chimeric genes.

Manipulating with suitable gene-regulatory regions can insure that the amplified sequences are expressed in plant cells.

Another justification for developing virus-based gene vectors would be the possibility of eliminating the need to regenerate plants via tissue culture in order to introduce foreign genes into plants. With Ti plasmid-*Agrobacterium* systems, the regeneration of plants following transformation as leaf discs or single cells is the most time-consuming step. With virus vectors, it is possible to transmit the vector with foreign sequences directly into intact plants. From a few infection foci following mechanical inoculation, the gene vector will replicate and thence move cell-to-cell until it and foreign genes have spread throughout the plant. In most cases this would occur rapidly, within a

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few days following inoculation. There would be no requirement for the regeneration of plants following the transformation event.

The third factor that favors the development of virus-based vectors is that we know a great deal about the biology of these agents. The genetic constitution and regulatory sequences of several plant viruses have been well defined. Moreover, the genomes of several are available as infectious clones of known nucleotide sequences. Hence, these are ideal subjects for genetic reconstruction as gene vectors.

So far as we know none of the genomes of plant viruses become integrated into plant chromosomes except perhaps as very rare events. Hence, in general, genes transferred via viruses would not be transmitted through seed. This is the greatest disadvantage of most autonomous replicons. However, many of our important crops, including food crops, are vegetatively propagated and these vectors would persist through successive generations in these plants. Consequently, outside the laboratory, virus-based gene vectors would be most useful in vegetatively propagated crops.

Foreign Gene Expression With RNA Plant Viruses

At least two positive-strand RNA viruses have been investigated as autonomously replicating vectors. One of these, brome mosaic virus (BMV), infects monocotyledonous plants and the other, tobacco mosaic virus (TMV), infects dicotyledonous plants. Both have been successfully engineered as episomes to amplify and express foreign genes in plants. In addition, both viruses have a variety of regulatory elements useful in foreign gene expression in plants (reviewed recently by Ahlquist 1990).

At present, only RNA viruses that have genomes of single-stranded messenger-sense RNA (positive sense) have been engineered as vectors. This is a limitation imposed by the need to restructure the viral genomes as double-stranded DNA copies. The required manipulations in vitro of the viral genomes are possible only as cloned cDNA copies, restructured via recombinant-DNA techniques.

The cDNA copies of some positive-sense RNA viruses are infectious when directly applied to the host organism. This is true for several bacterial and animal viruses and also applies to the cDNA clones of plant viroids. However, the cDNA clones of the positive-sense RNA viruses of plants have not been directly infectious. Only a single exception has been reported (Dore and Pinck 1988). In all other cases, it has been necessary to make positive-sense RNA transcripts of cDNA clones in order to initiate infections with viral genome components.

Tobacco Mosaic Virus (TMV) as a Gene Vector

The genome of TMV consists of one positive-sense RNA molecule of 6395 nucleotides with four translational open reading regions. Two of the latter, which encode proteins (126K protein and 183K protein) located at the 5' end of the genome (i.e., they are 5' proximal), are translated from full-length genomic RNA. These 5' proximal genes function in replication of viral RNA. Two other viral proteins (a 30K movement protein and the 17.5K coat protein) are translated from subgenomic RNAs. Messenger RNA (mRNA) synthesis of the latter is believed to be regulated by internal promoters on minus-sense viral RNA similar to that established for BMV RNA (Miller et al. 1985).

Two laboratories have developed complete cDNA clones of the TMV genome from which expression of infectious TMV RNA can be obtained from transcripts produced in vitro. Both laboratories have investigated the vector potential of TMV. In one case the viral coat protein (CP) gene was replaced with a bacterial chloramphenicol acetyltransferase (CAT) gene. The foreign gene was expressed in virus-infected plants (Takamatsu et al. 1987). However, the most successful example of foreign gene expression with TMV has been to insert CAT as a separate cistron between the 30K and CP genes (Dawson et al. 1989). This was done in such a way as to preserve the subgenomic promoter upstream of the CP gene. Expression of the downstream CP gene was obtained by creating a new subgenomic mRNA with the CP gene at its 5' end. These investigators showed that a third subgenomic RNA occurred in plants infected with the hybrid virus (Dawson et al. 1989). This chimeric virus replicated very efficiently, encapsidated well, and moved cell-to-cell in a normal manner when inoculated to intact plants of *Nicotiana tabacum*. The CAT gene was expressed efficiently. Unfortunately, after a time in systemically infected plants, the CAT gene was spontaneously deleted to regenerate the wild type TMV genome (Dawson et al. 1989).

Brome Mosaic Virus (BMV) as a Gene Vector

The second example of an RNA plant virus engineered to express foreign genes is BMV, a virus with

a genome split into three separate RNAs. These are RNA-1 of 3.2 kb, RNA-2 of 2.9 kb, and RNA-3 of 2.1 kb. The two largest RNAs are required for trans-acting RNA replication factors. RNA-3 encodes two proteins. One of these, near the 5' end encodes a 32K protein probably involved in cell-to-cell movement. A cistron toward the 3' end of RNA-3 encodes the 20K viral coat protein (CP). Neither gene is required for replication of viral RNA in protoplasts. The mRNA for CP is a subgenomic RNA of 0.9 kb. A viral complementary DNA expression system was used for restructuring and making the appropriate RNA transcripts (for a review of this system see Ahlquist et al. 1987). When the CP gene was replaced with CAT and artificial transcripts of the modified RNA-3 inoculated to protoplasts of barley in the presence of RNA-1 and RNA-2, all three viral RNAs replicated. The CAT gene was amplified along with the rest of the viral genome and was efficiently expressed in single cells (French et al. 1986). Foreign gene expression in this case was not tested on intact plants.

The sequences controlling expression of BMV subgenomic promoters have been well defined (Marsh et al 1987; French and Ahlquist 1988). Subgenomic RNA is initiated on BMV RNA-3 at a specific site on the negative strand template (Miller et al. 1985). The subgenomic promoter can be moved as a small cassette (120 bp) to a variety of new sites in RNA-3 to direct the production of new subgenomic RNAs. This allows considerable versatility for foreign gene expression of internal cistrons via new subgenomic mRNA production (French and Ahlquist 1988).

DNA Viruses as Gene Vectors

Clones of these viruses are directly infectious to whole plants. Hence it is a simple matter to genetically manipulate these viruses. Several of the regulatory regions of DNA viruses that are involved in their replication and gene expression have been identified. Some of these elements, such as the 35S promoter of CaMV, have been widely used in plant transformation technology. The use of geminiviruses and caulimoviruses as plant gene-transfer agents has been reviewed recently (Gronenborn and Matzeit 1989; Shepherd 1989).

Geminiviruses

These viruses have genomes of small circular single-stranded DNA (ssDNA). Replication is probably through action of DNA-dependent DNA polymerases. They are probably the only group of plant viruses that have no RNA intermediates in the replication cycle. Hence these viruses probably have a lower error rate during replication than other plant viruses.

Some members of the group have genomes consisting of two different circular molecules of approximately equal size; others have genomes of only a single circular molecule of ssDNA. As the viruses undergo replication in nuclei of infected cells, double-stranded DNA intermediates occur that serve as templates for synthesis of single-stranded forms found in virions as well as viral mRNA. In cloned form, the DNAs of tomato golden mosaic virus (TGMV), cassava latent virus (CLV), and wheat dwarf virus (WDV) have been tested as gene vectors.

TGMV is an example of a geminivirus that has a split genome of two small ssDNAs. One portion, DNA-A, of 2588 nucleotides, encodes proteins required for DNA replication. This portion of the genome can replicate in single cells in the absence of DNA-B. DNA-B of 2508 nucleotides, depends on DNA-A for functions required for its replication. DNA-B codes for functions for cell-to-cell transport. The only homologous region between the A and B portions of the TGMV genome is a 230 bp region that probably contains an origin for DNA replication. Both portions of the genome are transcribed bidirectionally to produce viral mRNA.

The TGMV genome has been tested as a gene vector by replacing the coat protein gene encoded by an open reading frame of DNA-A with foreign sequences. The coat protein is not required for viral DNA replication or cell-to-cell movement leading to systemic infection.

Although cloned TGMV DNA is infectious and can be mechanically transmitted to its hosts, a more efficient method for producing systemic infections is to use *Agrobacterium*-mediated inoculation. This has been termed agroinfection (Grimsley et al. 1986). In using this method with DNAs of the geminiviruses, head-to-tail dimers of viral DNA are cloned into Ti plasmid vectors. These are then used by agroinfection to transform host plants with the TGMV genome. In transgenic plants containing dimers of TGMV DNA-A integrated into the chromosome, the viral genome excises, probably through a process of homologous recombination. After excision, it replicates as a monomeric DNA-A. When the coat protein gene is replaced with foreign sequences, the latter is replicated along with the rest of the viral genome.

In initial experiments, a neomycin phosphotransferase gene (NPT II) of bacterial origin was used to replace the coat protein gene in a redundant end clone of TGMV DNA-A (Hayes et al. 1988). The redundant genome was cloned into a binary Ti plasmid vector. The NPT II gene (0.79 kbp) is about the same size as the coat protein gene of 0.74 kbp. The viral sequences were then transformed into *Nicotiana tabacum* tissue via agroinfection. When plants were regenerated, each cell of selected plants had a partial dimer (redundant end clone 1.6 times the length of a DNA-A monomer) of the TGMV genome from which the viral genome excised and replicated. In these plants a functional NPT was produced and enzyme activity corresponded to the copy number of the double-stranded forms of the viral genome (Hayes et al. 1988).

In a second type of experiment with the redundant end DNA-A clone of TGMV, *Agrobacterium*-mediated infections were made by injecting the bacterium into the stems of tobacco plants transgenic for a dimer of TGMV DNA-B. An active and wholly complete virus infection ensued in this case. When DNA-A excised and replicated in the stems, DNA-B was also released and replicated, leading to cell-to-cell movement and systemic infection of plants with DNA-A containing the foreign gene. A very high level of NPT II enzymatic activity and a high gene copy number of the amplified genome were found (Hayes et al. 1988).

In similar more extensive investigations of the vector potential of TGMV genome, a second larger gene, bacterial β-glucuronidase (GUS) of 1.8 kbp, was substituted for the coat protein gene of DNA-A and found to be expressed (Hayes et al. 1989; Elmer and Rogers 1990). However, in both cases spontaneous deletions occurred in the inserted sequences of viral-derived components in plants transgenic for TGMV DNA-B. These deletions suggested that a strong selective pressure existed for wild type sized genome components (Elmer and Rogers 1990).

Similar experiments demonstrating the vector potential of geminiviruses have been done with CLV (Ward et al 1988) and WDV (Gronenborn and Matzeit 1989). The latter virus has also been used as a replicon to introduce a transposable element into cells of wheat, maize, and rice (Laufs et al. 1990). In other experiments foreign gene substitutions for the capsid gene of maize streak virus clones were capable of replication but not capable of systemic spread in plants (Lazarowitz et al. 1989).

Caulimoviruses

This group of viruses have small isometric virions with a single small loop of double-stranded DNA as their genomic material. Several have been completely sequenced. These viruses have six major conserved genes and replicate by reverse transcription.

All genes except gene II of CaMV are required for replication and systemic movement of the virus. Gene II is involved in insect transmission and is not required for replication or movement. Using the genome of CaMV as an example, the six major translational open reading regions (genes) appear in succession on a full-length RNA transcript of the genome (the 35S transcript). One gene (gene VI) has a separate promoter and transcript (the 19S transcript). This gene functions as a translational activator for the closely spaced genes including gene VI itself, which appears on the full-length transcript (Gowda et al. 1989; Bonneville et al. 1989).

The successive genes on the full-length transcript are probably translated in a coupled manner referred to as a "relay-race" type of translation. When CaMV is used as a gene vector, its single nonessential gene II is replaced by foreign DNA. However, this manipulation must be done in a certain way in order to preserve the coupled nature of the translation process. It is important to construct the junctions of each end of the foreign gene as short as possible to favor the migration of scanning ribosomes (the 40S subunit) directly from the stop codon of one open reading region to the nearby start codon of the next downstream cistron without causing detachment of the ribosome from the mRNA.

The first successful use of CaMV as a gene vector was the work of Brisson et al. (1984) in which a bacterial methotrexate-resistant dihydrofolate reductase gene (*dhfr*) was used to replace gene II of this virus. The DNA of the engineered virus was mechanically inoculated to plants. The virus from the resulting infections moved systemically and caused plants to become resistant to methotrexate sprays, which are ordinarily very toxic to plants.

The constructs of the CaMV genome used by Brisson et al. (1984) left as little nontranslated sequence as possible between the *dhfr* gene and the flanking viral genes (genes I and III). In their experiments, a plasmid with only nine base pairs between the region I stop and *dhfr* start codon was stable during replication and successive transfer between plants. A plasmid with a remnant of region II in front of the *dhfr* gene was not stable. With both plasmids, the junction between the *dhfr* gene and viral gene III was a single

base pair. The protein of the *dhfr* gene was detected with an appropriate antibody and dihydrofolate reductase activity was found to be present. In addition, infected plants were shown to be resistant to methotrexate sprays.

Others (Lefebvre et al. 1987) have used CaMV to express a metallothionein in *Brassica campestris*, again demonstrating the feasibility of using CaMV as a vector to propagate and express foreign genes in plants.

The *dhfr* sequence used by Brisson et al. (1984) is a small gene of only 234 base pairs. Its small size allowed encapsidation of the chimeric viral genome and movement in plants as a virion. To what extent larger genes can be accommodated by CaMV has not been clearly defined. Gronenborn et al. (1989) found that the CaMV genome could be enlarged by about 250 base pairs to produce a stable recombinant virus. However, there are nonessential sequences in the large intergenic region that can be replaced by additional foreign DNA.

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Physical Methods for Plant Gene Transfer

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Abstract

Transformation of plants by Agrobacterium*-mediated DNA transfer is currently the most commonly used method of plant gene transfer. However, a growing body of work demonstrates the utility of procedures for the direct introduction of transforming DNA into cells by purely physical means. Some of these, including liposome fusion, microinjection, chemically-mediated DNA uptake, and electroporation, are generally applicable only to protoplasts, cells from which the cell wall has been removed by enzymatic treatments. Other techniques, such as microprojectile bombardment and silicon carbide fiber-mediated transfer, allow introduction of transforming DNA through intact cell walls. Physical methods are especially useful for the transformation of species such as maize, which are not readily infected by* Agrobacterium*. Most have been used to stably transform one or more plant species, although protoplast methods are limited by difficulty in plant regeneration in some species. Applications, especially of electroporation, direct DNA uptake, and microprojectile bombardment, are increasing as techniques improve.*

Although somewhat more demanding technically, physical methods of gene transfer offer several advantages over biologically-mediated procedures. Because no Agrobacterium *Ti sequences are required, vector constructions can be simple and small. Cotransformation with multiple genes can be carried out easily by delivering mechanical mixtures of two or more plasmids. Direct DNA transfer and electroporation of protoplasts provide rapid and efficient means of testing genetic constructions by transient expression. Microprojectile bombardment has the unique feature of allowing the delivery of DNA into intact tissues, where expression of alien genes can be tested for tissue-specific or elicitor-induced expression in the tissue of interest.*

Introduction

Transformation of plants by *Agrobacterium*-mediated DNA transfer is currently the most commonly used means of accomplishing plant gene transfer. Protocols have been developed for efficient *Agrobacterium*-mediated transformation in a very broad range of species, including a large number of crop plants. However, a growing body of work demonstrates the utility of procedures for the direct introduction of transforming DNA into cells by purely physical means. This paper reviews the current state of technology for physical transfer of DNA to plant cells and tissues, and attempts to provide the reader with sufficient information to make rational decisions about the utility of such procedures for particular applications. While these technologies are extremely useful in pure research applications, the present discussion is directed primarily toward understanding each system with respect to its use in crop germplasm enhancement.

Physical methods of DNA transfer are fundamentally different from *Agrobacterium*-mediated transformation. In *Agrobacterium* transformation, genetic sequences are introduced into modified Ti plasmids, which also carry additional genetic elements required for the DNA transfer process. These plasmids are then reintroduced into defined strains of *Agrobacterium*. DNA transfer occurs as part of a modified pathogenesis process, involving complex and highly evolved interactions between the target plant cell and the bacterium. A tightly defined DNA segment is cut

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from the Ti plasmid molecule, transferred into the recipient cell, and integrated into the plant chromosome, all by active processes, mediated primarily by bacterial products.

Conversely, physical methods for gene transfer do not involve any intermediate vector organism. The mechanisms by which the transforming DNAs are incorporated and integrated into the recipient chromosomes are poorly understood, but appear to be the result of plant cell processes, independent of the mechanism of DNA delivery. Purified plasmid DNA (typically based on *Escherichia coli* plasmids and amplified in *E. coli* cells) is introduced through the cell wall and plasma membrane of the recipient plant cell by one of several techniques.

Once inside the recipient cell, alien DNA apparently can undergo immediate transcription to produce the "transient expression" phenomenon. The transforming DNA also can be integrated into the host chromosome, although virtually nothing is known about the events and mechanisms involved. Indeed, it is unclear in most cases whether the physical process by which the DNA is introduced into the cell also deposits DNA inside the nuclear envelope, or whether DNA enters the nucleus by some undefined cellular process. Importantly, integration of alien sequences appears to occur randomly and can involve any part of an introduced plasmid regardless of its genetic content. Unlike the Ti plasmid, there is no known way to define the portion of a plasmid that will be incorporated into the plant genome.

Because no vector organism is involved, physical gene-transfer techniques are not limited by many of the constraints characteristic of *Agrobacterium*-mediated transformation. Genetic sequences can be incorporated into virtually any plasmid that provides the sequences necessary for replication and selection in bacteria. No other sequences, such as the 'vir' or 'border' sequences of the Ti plasmid, are required. Therefore, construction of plasmids for delivery by physical means is simplified, and plasmids tend to be smaller (see Pietrzak et al. 1986, for example). Also, physical introduction of DNA is not dependent upon any bacterial/host cell interaction, and so is theoretically applicable to any species, regardless of its susceptibility to *Agrobacterium* infection. Further, because no *Agrobacterium* "infection" occurs during DNA transfer by physical procedures, there is no requirement for the removal of *Agrobacterium* cells from the resulting transgenic tissues or plants by antibiotic treatment. This both simplifies and expedites the transformation process. The absence of *Agrobacterium* also eliminates the possibility of accidental release of recombinant bacteria into the environment, reducing the probability of unintentional movement of alien genes to nontarget plants.

Cotransformation, the simultaneous delivery of two or more genes during the transformation process, is greatly facilitated by physical DNA transfer procedures. Typically, different plasmids carrying two or more genes of interest, can simply be mixed and introduced into recipient cells. A portion of the resulting transgenic cell population is likely to carry and express all of the introduced traits. An independent selectable marker gene for each plasmid is not required, since transgenic cell lines can be recovered, e.g., by antibiotic resistance, and cotransformants can be identified within this population by other means.

Physical methods for DNA transfer are especially useful for rapid measurement of gene expression. As described above, DNA introduced into cells appears to function directly as template for transcription immediately after delivery. Expression of gene products is often detectable within a few hours after DNA delivery, and continues at measurable levels for several days (depending upon the gene and the level at which it is expressed). This phenomenon, called "transient expression", is observed in all systems for DNA transfer. However, it probably is most useful for measurement of the expression level of introduced sequences in those procedures, such as direct DNA uptake and microprojectile bombardment, where DNA is delivered in large quantities to many cells simultaneously. The absolute level of gene product tends to be large, because of the cumulative effect of its expression in many cells, and can thus be assayed with minimum error. Secondly, although the level of expression of the introduced sequence may vary markedly between individual cells, measurement of product from large populations of cells allows a useful estimation of the "average" level of expression produced when a particular construct is introduced into a specific cell type or tissue. Such measurements are quite useful, e.g., for comparison of the efficiency of different genetic constructions or of expression of a single construction under different environmental conditions. Development of techniques such as microprojectile bombardment and electroporation of intact tissues now also allows DNA to be delivered into organized tissues, permitting measurement of tissue-specific expression.

The following is a discussion of physical transformation procedures which are currently in common use. For convenience, they have been arranged according to the target tissue or cells with which they are typically used.

DNA Delivery to Organized Structures

A reliable procedure for the introduction of transforming DNA into intact tissues or organs of plants would be extremely useful. Such a system might eliminate the requirement for tissue culture and plant regeneration, the most difficult and disruptive aspects of most gene-transfer systems. It could also drastically reduce the time required for the transformation process.

The natural defensive barriers of the plant normally impede the introduction of transforming DNA into organized plant structures. Most external cells of the plant body are covered by cuticle layers and heavy cell walls, in addition to the plasma membrane, and so are resistant to the entry of alien DNA. Further, utility of the process would likely be limited, since most cells of the plant typically do not participate in gametogenesis. It is therefore unlikely that DNA incorporated into somatic cells would be transmitted to progeny.

These difficulties have been circumvented in several published accounts by targeting DNA delivery to floral structures or pollen (Luo and Wu 1988; Ohta 1986; de la Pena et al. 1987). The rationale of these approaches has been that DNA delivered in close proximity to the ovule or pollen tube might be able to enter these cells under certain conditions because of their thin cell walls. Secondly, DNA incorporated into these cells would be much more likely to be inherited than would DNA introduced into somatic cells.

Another approach that appears to have met with success involves the entry of transforming DNA through the cell walls and cell membranes of dry embryos prior to imbibition (Topfer et al. 1989). While this procedure does not appear to allow targeting the specific cells into which the DNA will be introduced, it might still produce transgenic sectors destined to give rise to floral structures, and so may still have a reasonable probability of transferring the alien DNA to progeny.

DNA Delivery to Pollen

In what appears to be the only published account of successful direct DNA delivery to pollen, Ohta (1986) demonstrated gene complementation in maize. A stock of maize that was homozygous recessive for several genes which produce easily scorable phenotypes, including genes involved in pigment biosynthesis, was used as a source of recipient pollen. DNA was isolated from a donor line that was homozygous for the dominant condition of all of these same marker loci. Pollen from recipient plants was combined with a concentrated solution of donor DNA, and the resulting slurry was placed on silks of recipient plants. The pollen/DNA mixture was transferred either immediately after mixing, or was allowed to stand briefly before application to the silks. In an alternative treatment, DNA from the donor was placed on recipient silks, and then the recipient was self-pollinated in the usual manner. Precautions were taken to prevent unintended fertilization. These treatments therefore should have resulted in self pollination of the recipient line, but, if DNA uptake occurred, hypothetically it could have incorporated genes carried in the donor DNA.

Ears resulting from the treatment in which pollen and DNA were mixed and then transferred directly to silks resulted in over 30 kernels that were described as "putatively transgenic", on the basis of altered endosperm characteristics. No other treatment had an observable effect. From this result, it was concluded that alien DNA had been incorporated and were expressed in the endosperm. However, the experiment yielded no evidence regarding whether the DNA was integrated into recipient chromosomes in the endosperm, or which might be present as an unintegrated fragment.

Kernels with both altered and unaltered endosperm characteristics were further analyzed by progeny testing. Kernels were grown, the resulting plants were pollinated, and ears were scored for the presence of kernels with endosperms expressing the dominant genetic effects of the donor line. The results of this test suggested that alien DNA was also active in embryos, although embryonic "transformations" occurred at low frequency. Importantly, a relatively high proportion of the plants grown from kernels with altered phenotypes manifested various anomalies that were interpreted as evidence of the mutagenic effect of DNA treatment.

This study suggests that gene transfer can be effected by treating pollen with exogenous DNA and then carrying out a relatively ordinary pollination, during which some of the DNA is transferred to the developing endosperm and embryo. The data support the hypothesis that DNA transfer occurs, and that it is in some way mediated by the pollen. The genetic data are difficult to refute, particularly those that suggest that the alterations induced by the transformation process appear to be inherited in the following generation. It is also difficult to attribute these changes solely to the mutagenic effect of DNA treatments,

which the author invokes to explain the anomalous plants observed during analysis. The markers used to indicate transformation would require a recessive-to-dominant shift in the plant genotype, an event that seems unlikely to occur at the rate required to explain the frequent shifts observed.

Unfortunately, because gross DNA preparations were used, rather than defined genetic sequences, it was impossible to test the putative transgenics at the DNA level to determine what sequences were transferred, and, if so, where they were integrated. Also, no detailed segregation analysis was performed. Without such supporting data, the efficacy of the procedure remains in doubt.

DNA Delivery to Florets

There are two reports in the literature at present in which gene transfer was observed following treatment of floral structures with plasmid DNA. The first of these involves a series of experiments, by de la Pena and co-workers (de la Pena et al. 1987), in which plasmid DNA was delivered into the leaf sheath surrounding the developing inflorescence of rye (*Secale cereale* L.) plants by a procedure termed "macroinjection". The DNA was not introduced into cells of the recipient plant directly, but rather was introduced into the space inside the leaf sheath surrounding the inflorescence, using a standard hypodermic needle and syringe. Plants were treated 14 days before meiosis with a plasmid carrying a chimeric neomycin phosphotransferase (*NPT II*) gene. Treated tillers were cross-pollinated with other injected plants. Resulting seeds were screened for resistance to kanamycin. Seven resistant seedlings were isolated from among 3023 seeds from 98 treated plants. Two plants tested positive for the production of the NPT II protein, and both of these were shown to carry apparently integrated copies of DNA sequences that hybridized with a fragment from the transforming DNA. Results were consistent with the presence of more than one integrated copy of the transforming sequence. A replicate of this experiment yielded one transformed (NPT II positive) plant from among 1000 seeds produced on treated plants.

The mechanism by which transforming DNA enters cells in this system is not known. The authors point out that both the male and female gametes are devoid of a callose wall at this stage of development. They present no evidence that supports preferential DNA uptake by either male or female cells, but hypothesize that the male gamete is the more probable recipient because of the prevailing condition of its cell membrane at that stage.

Another procedure for DNA delivery to organized structures, described for the transformation of rice by Luo and Wu (1988), is based on earlier work by Duan and Chen (1985). Rice florets were cut a few hours after pollination had occurred, removing the stigmas, exposing the cut surface of the style. Luo and Wu applied plasmid DNA carrying the *NPT II* gene to the cut style, and covered the inflorescence to prevent desiccation. Seed set was reduced to about 20% of normal levels. Seeds were tested for the presence of transforming DNA by an in situ DNA hybridization procedure that detected the introduced sequence in root tips of about 10 out of 54 seeds tested. Thus, the transformation frequency was estimated at 20%. Transgenics were further analyzed by DNA hybridization (Southern blot). Multiple high molecular weight bands were shown to hybridize with a probe consisting of the NPT II coding sequence. NPT II assays were positive for about 50% of the plants.

The authors hypothesized that when the stigma is removed from the pistil, as the floret was cut following pollination, the pollen tube was left largely intact. Its cut end allowed the entry of DNA, which then moved down the style to the ovule. While this hypothesis is a rational explanation for the movement of DNA into position for incorporation into cells, the hypothesis was not tested in the reported study. Further, no explanation was given regarding the site or mechanism of incorporation of the DNA.

Neither of the two procedures for DNA introduction into floral structures appears to have been successfully repeated in other laboratories and both are viewed with skepticism. These accounts continue to be interesting, however, because of their apparent technical simplicity. In practice, they have been passed over in favor of other techniques which, although more intrusive and technically demanding, may require less understanding of the precise timing of events in the reproductive process, and perform in a more reproducible fashion.

DNA Delivery to Dry Seed-derived Embryos

Another potentially useful procedure involves the uptake of transforming plasmid DNA into dry embryos of wheat by imbibing them in a solution of the DNA. Topfer et al. (1989) reported that when embryos removed from dry seeds were allowed to soak for approximately 30 minutes in solutions containing plasmids with various chimeric NPT II constructions,

transient expression of NPT II could be detected in seedlings. The effect was enhanced by the addition of dimethyl sulfoxide to the imbibition mixture, and was sharply reduced or eliminated by soaking the dry embryos in buffer without DNA, prior to imbibition with the DNA mixture. Transient expression increased dramatically when the chimeric marker gene was carried in a plasmid, derived from Wheat Dwarf Virus, which was capable of autonomous replication in plant cells. DNA uptake followed by transient expression of the introduced sequence was also demonstrated in other crop species including oats, barley, rice, rye, triticale, maize, common bean, pea, and *Vicia faba*.

The uptake of exogenous DNA by seeds or seedlings has been reported previously (Ledoux et al. 1974), but this earlier work failed to gain wide acceptance because it involved transfer of undefined DNA sequences and lacked conclusive evidence that could rule out the possibility of bacterial contamination. Topfer et al. (1989) state that, in addition to the use of defined bacterial marker sequences, two key pieces of evidence support the validity of their claim that the introduced sequences were expressed by cells of the recipient plant. First, when chimeric genes were constructed with an array of promoters from various sources, the levels of expression observed in embryos treated with these constructions were as expected when based on results obtained when these promoters were tested in electroporated protoplasts. This effectively rules out the possibility that the introduced DNA was taken up and expressed by microflora that would be expected to give very different expression levels than would be obtained in plants. Secondly, enhancement of expression by the addition of plant replicon for a geminivirus, and evidence proving that replication occurs as expected in plant cells, also supports the assertion that the introduced DNA is being expressed in cells of the treated embryos.

The mechanism by which macromolecules could traverse the dual barriers of cell wall and plasma membrane in order to reach the interior of embryonic cells is not known. The authors suggest that the wall, which is known to exclude molecules the size of the introduced plasmids, may have been damaged during the removal of embryos from dry seeds. This damage would presumably allow DNA to enter some cells, from which it could disperse through plasmadesmatal connections to other cells. Further, since it was shown that expression can only be achieved when embryos are completely dry, the authors hypothesize that dry membranes have local discontinuities that serve as pores through which DNA can pass. These lesions are thought to undergo spontaneous closure as the membranes hydrate during imbibition. Thus, uptake is dramatically reduced when the membranes are allowed to rehydrate in buffer before DNA is added.

The results of this study are interesting and convincing, though subject to further interpretation. If one presumes that DNA uptake does occur in this system, however, it remains unclear how the process could be applied to achieve stable plant transformation. Heritable transformation would require uptake of DNA by cells that would ultimately give rise to gametes, and it is not obvious how this could be made to occur.

Utility of DNA Transfer to Organized Structures

Techniques for introduction of transforming DNA into intact plant tissues are among the most intriguing and controversial of transformation procedures. The potential utility of the procedures described here is obviously great, but the limited amount of literature in this area suggests that the protocols may be difficult or unreliable. Indeed, some workers are openly skeptical of published results, while others report that they have been unable to replicate experiments. Unless, and until, the mechanisms that underlie the reported results are understood and described, and until others can routinely replicate the results, this controversy seems unlikely to be resolved.

DNA Delivery to Protoplasts

The cell walls of plants are formidable barriers to the introduction of macromolecules, and have been a major impediment to the development of transformation systems. One way in which this difficulty has been overcome has been the use of hydrolytic enzymes to remove the cell wall, thus releasing protoplasts which are bounded only by the plasma membrane. This procedure is typically carried out on cultured cells, or protoplasts released from leaf tissue.

Preparation of Protoplasts

The quality or health of protoplasts used in DNA transfer protocols is probably the single most important phenomenon in determining the overall efficiency of the system, whether applied for transient gene expression or used to produce transgenic plants. Protocols for the release of protoplasts vary widely, depending upon the type of explant or cultured mate-

rials used as the source tissue. Development of these protocols can be a difficult and time consuming process, particularly for those who are unaccustomed to working with protoplasts. There are, however, certain underlying features of the process that are similar among most protocols.

Probably the most common source tissues for the release of protoplasts used in transformation work are callus suspension cultures and leaf mesophyll cells. Suspension culture cells typically can be treated directly with an enzyme mixture that hydrolyzes the cell wall and cell-cell attachments, while leaves usually must be abraded lightly to expose inner cell layers to the enzyme mixture. The content of the enzyme mixture must be determined empirically to achieve optimum release of protoplasts from a particular tissue, although an enzyme mixture can often be used effectively with cells other than those for which it was optimized. These mixtures typically contain cellulases and pectinases, and may contain many other purified or crude enzyme preparations. They also contain buffers, divalent cations, and an osmoticum to protect protoplasts from osmotic shock after their release. For practical purposes, it is suggested that the reader examine the literature of microinjection and/or direct DNA uptake, to obtain an enzyme formulation that has been successfully applied to a source tissue as close as possible in origin and physical characteristics to the contemplated source (Crossway et al. 1986a; Crossway et al. 1986b; Fromm et al. 1985; Potrykus 1989; Potrykus et al. 1985).

It is also important to note that tissues can vary in their physical characteristics and chemical composition, sometimes dramatically. This is especially true when cells are carried in culture for extended periods of time. This variation can sometimes sharply alter the efficiency of protoplast release and is usually not accompanied by any observable change in the appearance or growth rate of the culture to warn that it has occurred.

Microinjection

Microinjection is a process by which macromolecules can be introduced into cells using fine glass micropipets controlled by micromanipulators. In a typical experiment, protoplasts are immobilized either by suction on a special holding pipet, by affixing them to a solid support medium with compounds such as polylysine, or by embedding them in agar or other gel matrix. DNA, in an aqueous suspension, is taken up by the pipet which may also be charged with a small volume of oil to facilitate control of the injection process. The injection pipet is introduced through the plasma membrane and, preferably, introduced into the nuclear envelope. A volume of a few picoliters carrying the transforming DNA is then ejected from the pipet, using a manually operated pump.

Microinjection differs in several ways from other procedures for gene transfer to protoplasts. First, cells must be manipulated individually rather than in the large numbers characteristic of direct DNA uptake procedures. As a consequence, fewer cells can be treated by microinjection than with other protoplast techniques. Electorporation protocols, for example, routinely allow treatment of greater than 1×10^6 protoplasts in a single event, while the most rapid injection systems (for immobilized animal cells) permit treatment of a few thousands of cells per hour.

This apparent deficiency is somewhat mitigated, however, by the high frequency at which transforming sequences microinjected into cells become integrated (Crossway et al. 1986b; Morikawa and Yasuyuki 1985; Reich et al. 1986). In the first report of stable transformation of plants following microinjection, Crossway et al. (1986b) found that 14% of calli derived from protoplasts, which had been treated with plasmid delivered by intranuclear microinjection and grown without selection, carried integrated copies of the introduced sequences. This compares quite favorably with the integration frequency observed in protoplasts treated by direct DNA uptake which seldom exceeds 1% (Negrutiu et al. 1987).

The efficiency of microinjection decreased sharply, however, when DNA was delivered into the cytosol, rather than the nucleus (Crossway et al. 1986b; Morikawa and Yasuyuki 1985). The efficiency of stable transformation decreased to only about 6% when DNA was delivered into the cytoplasm. It is, therefore, imperative to immobilize protoplasts so that the nucleus is accessible during the injection event. For animal cells, this is accomplished easily, since cells tend to adhere to solid substrates and the nucleus is prominently exposed. Plant cells do not adhere to solid supports, however, and must be immobilized for injection in some other way, either by embedding them in a solid matrix (e.g., agar or agarose) or by holding them individually with a special holding pipet. Neither system is ideal, however. Embedding holds cells effectively but permits a random orientation of nuclei, thus complicating intranuclear injection. Holding pipets provide maximum precision, but are laborious and slow to use.

Stable transformants produced through microinjection can often be recovered without selection (Aly

and Owens 1987). Selection is a vital part of most transformation systems because the probability of integration is so low that identification of transgenics by any form of screening would likely be prohibitively expensive and time-consuming. The probability of an introduced gene becoming integrated is sufficiently high following intranuclear injection, however, that screening without antibiotic or herbicide selection is a manageable task. Transgenic cell clusters (colonies) can be screened either by biochemical assay for the introduced gene product, or by DNA analysis for the presence of the introduced sequence. Because DNA analysis is independent of gene expression, this procedure may be useful for isolating transgenics in which the alien gene is present, but is not expressed in culture or does not produce a selectable product. This could circumvent the requirement for cotransformation with a selectable marker capable of expression in isolated cells or calli.

At present, most transformation experiments involve the transfer of only one or, at most, a few foreign genes. In general, too little is known about the specific genes or gene products involved in the development of more complex traits to allow their manipulation at the molecular level. It is hoped, however, that as the methodology of gene isolation and characterization improves, it will become increasingly desirable to be able to insert large DNA molecules, and even engineered chromosomes, into recipient cells. Microinjection is unique among the technologies of gene transfer in its ability to accommodate such large transforming DNA.

Griesbach (1987) has reported the chromosome-mediated transformation of *Petunia hybrida* via microinjection. Chromosomes isolated from *P. alpicola* were injected into *P. hybrida* protoplasts. Recipient protoplasts were evacuolated in order to minimize the possibility of releasing toxic substances into the cytosol during injection and to prevent vacuolar autophagic degradation of the introduced DNA.

Survival rates and transformation frequency were high among protoplasts injected with the alien chromosomes. About 80% of treated cells received one or more chromosomes. Injection reduced cell viability by 42% relative to untreated controls. Chromosome fragments and/or aberrations were observed in about 60% of the treated cells. Small calli, about 1 mm in diameter and containing about 25 cells, could be recovered after 3 weeks. Of 60 such calli tested, 2 had protein alterations that were characteristic of the donor parent, suggesting that transformation had occurred. Plants regenerated from putatively transgenic calli had flavonoid characteristics similar to the donor (the altered protein profiles in these transgenics were known to be related to flavonoid biosynthesis in the donor species).

The introduced genes were also transmitted to progeny. In some, it appears that the gene of interest was no longer present as part of an additional chromosome, but rather was translocated to a site on a native chromosome of the recipient. Many of the transgenic cell lines produced in this manner were not stable, however, and reverted to the recipient phenotype.

This report suggests that microinjection with alien chromosomes can be an efficient means of transferring genes from one species to another. Unfortunately, no DNA data were presented to corroborate the evidence from protein and flavonoid testing. Such evidence, along with more detailed cytological data, would allow a more meaningful analysis of the transformation efficiency attainable with this technique.

Another small, but potentially troublesome, difficulty with the transfer of chromosomes derives directly from the technique's great strength. Because the transfer of a single, highly specific gene could be monitored in a very large proportion of the calli examined following injection, it seems reasonable to hypothesize that transfer of some genetic material occurs even in cells that are not obviously transformed. This suggests that in transgenics produced in this manner, undesirable alien sequences could inadvertently be transferred. This could alter other, more subtle characteristics of crop varieties.

Although Neuhaus et al. (1987) successfully transformed small, multicellular microspore-derived embryoids by microinjection, most systems for microinjection require the use of protoplasts. The difficulty typically encountered in regeneration of whole plants from protoplasts appears to have sharply limited the number of crop species that have been stably transformed by microinjection (Uchimiya et al. 1989). These include tobacco (Crossway et al. 1986b) and *Petunia* (Neuhaus et al. 1987), both considered among the most manageable species in culture. At present, the need to regenerate plants from protoplasts is by far the greatest impediment to the widespread use of microinjection in plant systems.

Another disadvantage is that the equipment required for efficient microinjection is complex and expensive. Apparatus usually consists of: one or more laminar flow hoods; an inverted microscope; pipet-pulling apparatus for manufacturing microinjection needles; a microforge, for further manipulation of needles; holding pipets, etc.; and one or more micromanipulators for holding and positioning cells and

pipets and accomplishing the actual injection. Modern microinjection apparatus is often also equipped with computer-aided positioning mechanisms for precise targeting of cells and subcellular compartments. The cost of purchase and maintenance of this apparatus places it beyond the reach of many workers.

Despite these disadvantages, microinjection can be the most efficient means for DNA transfer, and is the only method currently developed for the delivery of intact chromosomes for the transfer of complex traits. These features will undoubtedly continue to make microinjection an appealing option for some gene-transfer applications.

Liposome Fusion

Liposomes, synthetic single or multilamellar lipid vesicles, have been used successfully as vehicles for delivery of both RNA and plasmid DNA into protoplasts (Deshayes et al. 1985; Fraley 1983; Matthews and Cress 1981; Nagata et al. 1981; Ohgawara et al. 1983; Rouze et al. 1983; Uchimiya and Harada 1981). Lipid vesicles are typically prepared by a reverse phase evaporation technique (Fraley 1983), in which an aqueous solution of plasmid DNA is combined with lipid carried in an organic solvent. The two phases are then mixed in an inert atmosphere by sonication, after which the organic solvent is evaporated under reduced pressure. This process results in the encapsulation of approximately 25-40% of the total DNA. Liposomes are then further purified (to remove unencapsulated plasmid and residual lipid) by sedimentation onto a Ficoll gradient.

Protoplasts are fused with liposomes by incubating them together in a buffered aqueous fusion medium, usually composed of an osmoticum (e.g., mannitol), divalent cations, and an appropriate buffering medium. The protoplast membrane and lipid vesicle are induced to fuse by treatment with polyethylene glycol or other polyalcohol. Cell membrane and lipid vesicle are thought to fuse during this process, but the exact mechanism by which nucleic acid transfer occurs does not appear to have been unequivocally demonstrated (Fraley 1983).

Various conditions affecting the nucleic acid delivery efficiency of liposome fusion systems have been characterized in detail (Deshayes et al. 1985; Fraley 1983; Rouze et al. 1983). Critical factors include vesicle phospholipid composition, cationic concentration of incubation buffer, concentration and type of polyalcohol (e.g., polyethylene glycol or polyvinyl alcohol), nucleic acid concentration (i.e., total DNA or RNA content in the fusion mixture), and incubation period (Deshayes et al. 1985; Fraley 1983). Under optimum conditions, rates of transformation as high as 80% (of treated protoplasts) have been reported.

A particularly useful account is to be found in the report of Deshayes et al. (1985), in which the effect of several of these factors was quantified on the basis of the number of stably transformed calli recovered under various conditions when an NPT II construct was delivered to isolated tobacco mesophyll protoplasts that were subsequently subjected to kanamycin selection. The quantity of liposomes, i.e., the total amount of transforming DNA incubated with a standard number of protoplasts, appeared to have the greatest effect on transformation efficiency. This relationship was also noted by others (Ohgawara et al. 1983). Transformation efficiency was also found to vary greatly between experiments even when identical conditions for DNA transfer were applied. The authors suggest that this may be due to the physiological state of the recipient cells, which could make them more or less competent to be transformed. While this phenomenon is difficult to quantify, it probably affects all gene-transfer systems as well.

The efficiency of liposome-mediated DNA transfer has also been compared with that of *Agrobacterium*-mediated transformation of protoplasts and direct DNA uptake methods (DeBlock et al. 1984; Fraley 1983; Krens et al. 1982). Although less efficient than *Agrobacterium*-mediated transformation, liposome encapsulation provides a significant increase in transformation efficiency, compared with results obtained by transfection with naked DNA. This may reflect an increase in the efficiency of the transfer process, or may be a reflection of the enhanced longevity of nucleic acids sequestered from enzymatic degradation by the encapsulating liposomes (Fraley 1983).

Despite the apparent efficiency of the technique for introduction of transforming DNA into target protoplasts and numerous accounts of transient expression, there are at present only two published accounts of plant transformation and analysis of transgenic progeny in which gene transfer was mediated by liposome fusion (Bellini et al. 1989; Deshayes and Herrera-Estrella 1985; Uchimiya et al. 1989). In the first, stably transformed calli, derived from tobacco mesophyll protoplasts fused with liposomes carrying a chimeric NPT II gene, gave rise to transgenic plants (Deshayes and Harrela-Estrella 1985). Three of these were analyzed in some detail. All of these carried the integrated marker as a single dominant allele. Al-

though results of both genetic and molecular analysis were consistent with the integration of the gene into only a single chromosomal site, in each case examined, the gene was present at the transgenic locus as multiple tandem repeats. Plants were positive upon assay for NPT activity, but NPT level was not measured quantitatively. All three appeared to carry the introduced marker as a single dominant gene in the heterozygous condition. The trait was transmitted to progeny of self pollination in a strict Mendelian fashion in two of the transgenic families, but could have been homozygote-lethal in the third.

In another study (Bellini et al. 1989), 60 independent transformants derived from tobacco mesophyll protoplasts by liposome fusion were analyzed in detail. From 16 X 10^{-6} protoplasts treated, 245 kanamycin-resistant colonies were recovered. One hundred of these were chosen at random and from these 83 plants were regenerated. Aminoglycoside resistance levels varied widely among the plants. Molecular analysis demonstrated the presence of the introduced NPT coding sequence in all plants, although integration patterns were complex. Plants carried an average of two copies of the NPT sequence. NPT level was not correlated with copy number. Eleven of the 83 plants developed totally sterile flowers. Five of the remaining plants failed to express resistance to antibiotic and were dropped from further analysis. Of the remainder, 53 displayed Mendelian inheritance of the introduced gene, while 10 clones showed non-Mendelian transmission. A significant proportion of the transformed plants had morphological abnormalities, but upon genetic analysis, all of these were attributed to somaclonal variation rather than insertion mutagenesis or other effects associated with the introduced gene or its product. It is unclear, however, whether the process of gene transfer, i.e., the liposome fusion process, the introduction of alien DNA, etc., increased the rate of somaclonal variation among transformants above the "background" variation inherent to the protoplast culture procedure.

Liposome fusion with protoplasts is an effective means for delivering transforming DNA or RNA into protoplasts. It has been used successfully to produce transgenic plants in at least one species. Transgenic cells and plants showed integration and expression patterns similar to those observed in other transgenic materials produced by physical methods. Transformation efficiency is high when compared with naked DNA transfer procedures, but is lower than that reported for *Agrobacterium*-mediated transformation of protoplasts. The limited data available suggest that the process produces genetic anomalies at high frequency, although it is unclear that this difficulty is greater for liposome-mediated transformation than for any other protoplast-based procedure.

Although liposome fusion is clearly a viable technique for plant transformation in species in which plants can be regenerated from protoplasts, it does not appear to have gained the popularity accorded other protoplast techniques. It is probable that for most workers the perceived difficulty of producing lipid vesicles outweighs the advantage of slightly enhanced transformation frequencies.

Direct DNA Uptake: Chemical Treatments and Electroporation

The plasma membranes of protoplasts can be made temporarily permeable to DNA by treatment with chemicals, exposure to an intense electric field, electroporation, or both. While these procedures are generally discussed as separate and distinct techniques, they are addressed together here, because the mechanisms involved in trans-membrane movement of DNA are thought to be quite similar.

Protoplasts were shown to incorporate plasmid DNA after treatment with zinc sulphate and poly-L-ornithine as early as 1977 (Lurquin and Kado 1977). Krens et al. (1982) were able to transfer modified Ti-based plasmid DNA into protoplasts by treatment of protoplasts with polyethylene glycol (PEG). Direct DNA uptake was developed into an effective means of transformation, however, by better defining conditions required for DNA uptake and by the development of minimal transformation plasmids that included antibiotic resistance markers, such as NPT, that permitted the direct selection of transgenic cell progenies following DNA transfer (Negruitiu et al. 1987).

Generally, chemically mediated DNA transfer is quite simple, requiring in its least complex form little more than addition of DNA to a buffered osmoticum in which protoplasts are suspended. However, a very large number of factors have been studied in attempts to improve the efficiency of the process. Major parameters were found to include plant species from which protoplasts are derived; state, i.e., linear versus open or covalently closed circular forms, and concentration of transforming DNA; the presence of carrier, i.e., nontransforming, DNA; and the inclusion of electroporation in the treatment (Negruitiu et al. 1987). By proper adjustment of these and other parameters, the efficiency of chemically-mediated DNA

transformation has been increased from about 10^{-6} to nearly 10^{-2} (Shillito et al. 1985).

Comparison of electroporation parameters and results is made difficult by the existence of at least two rather different approaches. In one of these, a pulse of about 100-500 V cm^{-1} is applied to the DNA/cell suspension from a capacitor of 50-2000 mfd (Callis et al. 1987; Fromm et al. 1985; Potter et al. 1984). This results in a pulse length of about 5-10 ms. An alternative electroporation protocol, which has been widely applied as a part of a "hybrid" procedure (with Polyetheylene glycol (PEG) treatment), involves treatment of protoplasts with a pulse of greater voltage (typically greater than 1000 V cm^{-1}) delivered from a relatively small capacitance bank of approximately 10-50 mfd (Shillito et al. 1985). This treatment results in exposure of cells to a pulse length of only about 10-50 μs.

The effects of various parameters of electroporation by the low-voltage procedure have been studied in detail for protoplasts derived from several diverse species and were fully reviewed by Hauptmann et al. (1987). Recovery and subsequent viability of protoplasts was found to be strongly affected by the total charge delivered during electroporation. Viability decreased as voltage and capacitance were increased, while expression of an introduced marker gene, indicative of the efficiency of DNA uptake, increased up to a certain charge value. Above this critical combination of voltage and capacitance, expression declined rapidly because cell death occurred before expression of the introduced gene could be achieved. Electroporation conditions that maximize either transient expression or stable transformation of the target protoplast population must therefore be determined empirically for the species of interest, although it is possible to produce protocols that provide satisfactory results with many different cell types. Importantly, those conditions that maximize transient expression usually are not ideal for recovery of stable transformants (Arthur Weissinger, unpublished results). Although cells may survive long enough to produce a maximum amount of gene product, treatment for maximum transient expression typically causes the population of cells to decline rapidly.

The precise mechanisms by which the cell membrane is made permeable to DNA by chemical or electrical treatments is not well understood. It is hypothesized that both treatments produce similar membrane changes (Hahn-Hagerdal et al. 1986). Polythylene glycol (PEG) is known to reduce the polarity of aqueous media, resulting in the rearrangement of membrane components and simultaneously stabilizing membrane lipid structures. Electric fields are thought to produce local charge anomalies in the membrane. Both processes are thought to result in the formation of pores, which may or may not be reversible, depending upon stringency of the treatment and composition of the membrane. DNA entry can therefore occur for only a limited time following treatment. The duration of this receptive period, in the case of electroporation, can be protracted by holding protoplasts at a slightly reduced temperature following treatment. Generally, electroporation alone may produce slightly less cell damage than PEG treatments, probably because it is possible to control the stringency of electroporation treatment with greater precision.

Little is known about the amount of DNA delivered into protoplasts, and its subcellular location, following electroporation of plant protoplasts (Callis et al. 1987; Jongsma et al. 1987; Riggs and Bates 1986). Bertling et al. (1987), however, have presented an interesting estimation of these phenomena following electroporation of cultured human lymphocytes. They found that DNA uptake was rapid and is a function of the DNA concentration in the electroporation media. Maximum intranuclear incorporation approached 104 copies per nucleus, approaching as much as 8% of the total DNA present in the nuclei of treated cells. Introduced DNA persisted in the nucleus with a half-life of 15-24 hours. Precisely how these estimates might apply to protoplasts is, of course, unknown. It is worth noting, however, that both the relationship between total DNA concentration and uptake efficiency and the approximate half-life of biologically active plasmids in the cell are in keeping with the limited findings available from plant studies.

As described in the discussion of liposome fusion above, direct DNA transfer procedures appear to be affected by the physiological state of recipient cells. An interesting attempt to use knowledge of this phenomenon to enhance performance of transformation systems was reported by Meyer and coworkers. They reported a marked increase in DNA uptake efficiency by synchronously dividing cell lines (Meyer et al. 1985). Uptake was also strongly influenced by the stage of the cell cycle at which DNA delivery occurred. After M-phase, transformation efficiency fell to levels comparable to those in unsynchronized cell lines.

At present, direct DNA uptake procedures have been used to produce transgenic plants of at least 10 species (Uchimiya et al. 1986). Importantly, these include major crops, such as rice (Shimamoto et al. 1989), oilseed rape (Guerche et al. 1987), and maize

(Rhodes et al. 1988), although not all of these reports include the production of progeny by primary transgenics. In general, it appears that direct DNA transfer procedures offer a useful alternative to *Agrobacterium*-mediated transformation in some crops, producing transformation efficiencies that are comparable with, or exceed, those of most other techniques. Protocols are well developed for many species. Perhaps more importantly, the process for optimization of parameters for any specific application is very well defined; and there are numerous examples that can be consulted for guidance during the development of new protocols.

Although recovery of stably transformed germplasm sources is likely to be the primary interest of those who might use direct DNA transfer for crop applications, these techniques have made perhaps their largest long-term contribution to the science of gene transfer by providing a means for monitoring gene function. These techniques are ideally suited for some transient expression studies because of their ability to transfer large amounts of DNA or RNA into a very large population of cells. This often results in high levels of expression of the introduced gene, increasing the accuracy of biochemical assays, and reducing error.

Although direct DNA uptake protocols are highly developed, and the technique has been available nearly as long as that for *Agrobacterium*-mediated transformation, the technique has not contributed transgenic germplasm at a rate equal to that of other procedures. This is largely because the techniques are almost exclusively applicable to protoplasts, and plant regeneration from protoplasts is not a well-established technology. Thus, although it is an excellent means for introducing DNA into cells, direct DNA transfer is not yet an ideal process for producing transgenic plants.

General Considerations Regarding the Use of Protoplasts

An ongoing concern about all methods of plant gene transfer, especially when used to alter some characteristic of an elite crop variety, is the extent to which the transformation process alters the original genotype in unintended ways. Such effects could be the result of either culture-associated phenomena (somaclonal variation) or could result from loss of gene function in the recipient, which is caused by insertion mutagenesis in the locus at which an introduced gene integrates into the plant chromosome. The effects of somaclonal variation could be especially severe with techniques that employ protoplasts, since time in culture and environmental exposure are maximized.

This effect was amply demonstrated in a study by Bellini et al. (1989), in which a substantial number of transgenic families, derived by liposome fusion with tobacco mesophyll protoplasts, were examined in detail. Among other concerns, the workers hypothesized that insertion mutagenesis, occurring as a result of the integration of alien sequences, could give rise to heritable anomalies. Transgenic families varied considerably in levels of transgenic products, and many also differed from wild type controls in characteristics such as fertility and leaf morphology. Genetic analysis of these variant plants revealed that in every instance the physical anomaly of interest failed to segregate with the newly introduced sequence. This was interpreted as strong evidence that the "mutations" resulting from the transformation process were the result of somaclonal variation, not insertion mutagenesis or any other phenomenon associated with integration of the transforming DNA.

From studies of this kind, as well as from a mounting body of anecdotal evidence from those working in this area, it has become increasingly obvious that the process of protoplast isolation, culture, and regeneration gives rise to an array of detectable genetic aberrations—apart from and in addition to other deleterious effects of gene transfer. These results, although supportive data could be extremely difficult to acquire, also suggest the possibility that cryptic mutations may be produced as a result of these manipulations. If such hidden mutations do occur, they could pass on into breeding populations derived from transgenic materials even though families with detectable mutations were discarded. For this reason, the use of protoplast-based techniques for crop improvement should be approached cautiously.

Delivery of DNA to Walled Cells

Delivery of DNA into walled cells was an intriguing but elusive goal during the late 1970s and early 1980s, a period during which rapid progress was made in practically all areas of plant gene transfer. A system for efficient DNA delivery to walled cells would offer numerous advantages. It could circumvent all of the problems associated with the use of protoplasts, including loss of totipotency and perturbation of physiological conditions within the cell, resulting in anomalous gene expression patterns. It would also be faster and less expensive than protoplast-based tech-

niques, since it would eliminate treatments required for removal of the cell wall. Finally, direct delivery of DNA into walled cells could permit treatment of an almost unlimited range of explant tissues. This might allow gene transfer in species that are not easily grown in culture or into tissues from which plant regeneration is more efficient.

Electroporation of Intact Tissues

Until recently, most evidence suggested that transformation of walled cells by electroporation was unlikely because the cell wall forms an impenetrable barrier to the passage of DNA. Attempts to electroporate cells with intact or slightly degraded cell walls had largely met with failure. In the few instances where electroporation of walled cells had produced transient expression of an introduced gene, results were inconclusive because of the possibility that apparent success could be due to the presence of free protoplasts in electroporation mixtures (Morikawa et al. 1986; A. Weissinger, unpublished results; M. Fromm, personal communication).

Electroporation of intact cells could be extremely useful, however, in protocols for production of stably transformed plants, especially for transient expression studies. Measure expression of a genetic construction in protoplasts may not provide accurate models for expression in whole plants because protoplasts are not always physiologically identical to the cells from which they are derived. Introduction of DNA into intact tissues could provide a better model.

Dekeyser et al. (1990) have recently reported transient expression of a chimeric gene delivered into intact and organized tissues of rice leaf bases, and have also applied their technique to other species and explant types. Leaf base explants from 1-week-old rice seedlings were chosen as the initial test tissue for the technique because they contain several cell types, which makes them ideal for studies of tissue-specific expression. Also, they contain meristematic cells capable of development into embryogenic callus, and therefore could produce stably transformed plants. Explants were treated with a plasmid carrying a chimeric NPT II gene. Expression of the introduced construction was maximized by electroporation of tissue in a medium containing 150 mM NaCl, at 375 V/cm, using a 900 μF capacitor as the source of the transforming pulse. These conditions resulted in a pulse duration of about 300 msec. Enzyme levels increased in a linear fashion as DNA concentration in the electroporation buffer increased from 5-100 μg ml^{-1}. Linear and circular DNA conformations gave similar results. Maximum gene expression was achieved at 4 days following treatment, and could be detected as early as 1 day and as late as 8 days. Interestingly, DNA was found to penetrate more than six cell layers, a distance comparable to that achieved by microprojectiles during bombardment (Stomp et al. 1991).

Addition of 0.2 mM spermidine enhanced expression threefold. Spermidine is known to reduce nuclease degradation of DNA, and also has been shown to prevent lysis of oat protoplasts. Thus, spermidine treatment may increase transformation efficiency by protecting transforming DNA and by increasing cell viability.

The mechanism by which DNA enters walled cells is not known. However, the authors put forward three hypotheses to explain their results. First, DNA could be entering only damaged cells. Secondly, the electrical pulse might be able to disrupt both the cell wall and membranes, permitting DNA to pass through both. Finally, DNA might penetrate the cell wall passively during incubation of the tissue in the DNA solution, and the membrane is permeabilized by the electric discharge. The first hypothesis was rejected because expression was not observed only at wound sites. Both of the other hypotheses remain possibilities, but neither was tested during experimentation detailed in this report.

In addition to the original work in rice leaf bases, protocols for electroporation were also found to be effective for leaf bases from maize, wheat, and barley. DNA transfer was also achieved in leaf sheath and lamina tissue from rice. Light regulation of an NPT II coding sequence fused to a light inducible promoter was also observed following electroporation of leaf sheath and lamina explants.

The electroporation protocols for intact tissues outlined in this report could provide an important alternative to microprojectile bombardment for tests involving regulated gene expression, at least in some tissues. The system could also be adapted for production of transgenic plants, although insufficient data are currently available to permit evaluation of this potential.

Microprojectile Bombardment

Microprojectile bombardment, or "biolistic" DNA transfer, is a process by which transforming DNA is associated with microscopic metal (tungsten or gold) particles that are accelerated to high velocity in a

particle acceleration apparatus (particle gun). The particles (microprojectiles) acquire sufficient kinetic energy to allow them to break through intact plant cell walls and plasma membranes. DNA carried on the particles remains biologically active. Once inside the cell, it can be expressed transiently, or can be integrated into the chromosomal DNA to effect stable transformation. Although DNA is introduced into intact cells rather than protoplasts, microprojectile bombardment shares some characteristics with both microinjection and direct DNA uptake procedures. DNA is physically driven into cells, as in microinjection, but large numbers of cells, more characteristic of direct DNA uptake protocols, can be treated simultaneously.

The particle gun apparatus originally designed by Sanford and coworkers (Klein et al. 1988a) is powered by a .22 caliber blank cartridge, and operates in a manner analogous to a shotgun. DNA is precipitated by the addition of calcium chloride and spermidine to a solution containing transforming (plasmid) DNA and tungsten particles—the microcarrier—approximately 1 μm in diameter. DNA appears to coat the particles, although the mechanism involved in this process is not fully understood. A small volume of a slurry of particles and DNA is placed on the leading face of a cylindrical plastic "macroprojectile". This macroprojectile is then placed into an acceleration tube (barrel), and the blank cartridge is loaded behind it. A perforated "stopping plate" made of tough plastic material is held in place at the lower end of the acceleration tube. When the cartridge is fired, the macrocarrier, along with its load of particles, is accelerated to a velocity of approximately 400 m s^{-1}. At the far end of the acceleration tube, the movement of the macroprojectile is halted by the stopping plate. The DNA-coated microprojectiles fly free and pass through the aperture of the stopping plate. They thus acquire sufficient momentum so that they can penetrate the walls of the cells undergoing treatment. The entire process is carried out under a partial vacuum to reduce air resistance, which tends both to slow and to disperse the particles in flight.

The apparatus for particle acceleration has improved rapidly since its introduction. At present there are several different methods by which particles are accelerated, including the use of compressed air to drive a macroprojectile like that in the original device (Iida et al. 1990; Oard et al. 1990), acceleration by the shock wave produced by an electric discharge (McCabe et al. 1988), or by the explosive release of compressed helium (E.I. DuPont de Nemours and Co., Inc., personal communication). Although each of these methods has unique characteristics, the major differences between them appear to relate primarily to ease of use, precision of velocity control, and limitation of tissue damage. The least damaging apparatus at present are those, like the DuPont PDS-1000 (He) (E.I. DuPont de Nemours and Co., Inc., Wilmington, Delaware), which deliver particles from a carrier sheet where they are spread prior to delivery. The carrier sheet is coated with DNA-covered particles that are allowed to dry in place on the sheet prior to delivery. This type of gun avoids the release of high-velocity debris, which is the major source of tissue loss during bombardment. Because velocity can be regulated accurately, treatment parameters can be adjusted and optimized with greater precision than was possible with previous models.

Proof that living cells could tolerate the trauma of bombardment was first obtained when tungsten spheres approximately 4 μm in diameter were introduced into living onion (*Allium cepa*) epidermal tissue. Bombarded cells, even those that were struck by multiple particles, were not ruptured and continued to exhibit cytoplasmic streaming. It was subsequently demonstrated (Klein et al. 1988a) that active nucleic acids could be delivered into cells in this fashion.

Stably transformed plants and their progeny have now been derived by bombardment of both intact explant tissues (McCabe et al. 1988; Tomes et al. 1990) and cultured cells (Finer and McMullen 1990; Gordon-Kamm et al. 1990; Klein et al. 1988b) in several plant species, including some which have proven to be quite difficult to transform by other means. While the conditions for particle preparation and other aspects of the bombardment process were very similar for all species transformed; explant source and the process of recovering transgenic plants has varied widely.

Tobacco was transformed by bombardment of leaf explants, followed by selection of resistant callus colonies proliferated on the explant surface (Tomes et al. 1990). Plants selected from transgenic calli showed no evidence of chimerism, and carried from 1 to more than 20 stably integrated copies of the introduced sequence. Transgenes were inherited primarily in a Mendelian fashion, although some more complex patterns of inheritance were observed. Although no vector organism was used, this process followed a course not unlike leaf-disk protocols used to produce transgenic tobacco plants with *Agrobacterium*.

Transgenic maize plants were recovered by applying a selection with bialophos to embryogenic suspension cultures bombarded with a plasmid carrying the "bar" gene, encoding PAT (phosphinothricine

acetyl transferase) (Gordon-Kamm et al. 1990). Transgenic plants were derived directly from bombarded tissue through embryogenesis. Finer used a virtually identical approach to produce transgenic cotton (Finer and McMullen 1990).

The transformation of maize and cotton provides an important demonstration of the principles involved in recovery of transgenic plants from bombarded embryogenic materials. First, the efficiency of the bombardment process was undoubtedly enhanced by the diffuse, flat target geometry presented when the suspension culture cells were spread over the surface of solidified media. This geometry also facilitates antibiotic or herbicide selection, reducing the probability of recovering plants that appear to be resistant to the selective agent, but which have merely escaped because of their spatial relationship to the selective media. Embryogenesis is also the primary route to regeneration for some taxa, and, it is therefore essential to be able to treat such cultures effectively.

McCabe et al. (1988) transformed soybean (*Glycine max*) by bombarding the apical meristems of immature embryos. Plasmid DNA carrying a chimeric beta glucuronidase (GUS) gene was coated onto gold particles that were accelerated by an electric discharge apparatus. Approximately 2% of the shoots derived from treated meristems through organogenesis displayed chimeric expression of GUS. A small proportion of the primary transformant plants that were carried to the greenhouse continued to express the introduced gene (approximately 1:50-1:360). From these plants, a total of 10 seeds were sampled, of which 3 were positive for GUS expression. A single GUS positive R_1 plant was recovered.

Recovery of transformed plants from bombarded meristematic tissues is potentially a very important technique that could find broad application. Since culture procedures are minimized, it is likely that the procedure would have far less deleterious genetic effect than other, more culture-intensive techniques. Secondly, it could provide a route for transformation in those species for which there are no well-developed culture protocols.

The high probability of obtaining chimeric primary transgenics that could be difficult to use in development of breeding materials is the single greatest limitation to such an approach. Indeed, primary transgenics were chimeric. In later genetic analysis of transformed soybean plants, however, Christou et al. (1989) demonstrated that inheritance of alien genes in the R_1 progeny was in strict accordance with expected Mendelian ratios. This evidence suggests that although the primary transgenics may be chimeric, progeny are stably and homogeneously transformed. Furthermore, plants cotransformed with both NPT and GUS were recovered and also exhibited Mendelian segregation patterns for both transgenes. Concerns about chimerism therefore appear to be unfounded after the first transgenic generation.

The biolistic process has also been used to deliver transforming DNA into plant organelles (Daniell et al. 1990; Svaab et al. 1990). Svaab et al. (1990), in the only published account of plastid transformation in higher plants, bombarded *Nicotiana tabacum* leaves with tungsten particles coated with plasmid DNA carrying a 3.7 kb fragment of plastid DNA containing the coding sequence for the 16S ribosomal RNA. Within this coding sequence, there was also a streptomycin resistance mutation, and a spectinomycin resistance mutation. Transformants were selected on spectinomycin, and could be distinguished from spontaneous mutants by cotransformed streptomycin resistance and the presence of an identifying restriction site. The transgenic plastid traits were transmitted to seed progeny. These seed progeny were uniformly resistant to spectinomycin, but were chimeric for the (unselected) streptomycin resistance trait.

Mitochondrial transformation by microprojectile bombardment continues to be an elusive goal in plants, although Johnston et al. (1988) have reported the transformation of mitochondria in yeast. In that study, yeast mitochondrial transformants were recovered from among a population of yeast cells that were treated simultaneously with genes encoding a selectable nuclear trait, and a mitochondria-specific marker. Mitochondrial transformants were isolated from among yeasts that had been transformed for the nuclear trait. Yeast cells with a respiratory deficiency caused by the presence of a mutant mitochondrial gene were bombarded with 1.0 μm diameter tungsten particles carrying a plasmid with both the nuclear marker, as described above, and the wild-type complement of the mutant mitochondrial gene. Mitochondrial transformants were selected by growing nuclear transformants on media that would support the growth of wild type yeast, but not the deficient mutants. One mitochondrial transgenic has been recovered per 1000 to 2000 nuclear transformants. Integration of the introduced sequence occurred by homologous recombination. The trait was inherited as a dominant gene producing expected segregation ratios in crosses of the mitochondrial transgenics with deficient strains.

It is not clear whether such a transformation procedure will be effective for gene transfer in higher plants. At present, two difficulties appear to limit the

probability of success. First, the mutant stocks and complementary genes available for use in the yeast system are ideal for the purpose and may be without parallel in plants. Secondly, the very low efficiency of mitochondrial transformation relative to nuclear events suggests that a very large number of stable transformants would have to be generated to provide a reasonable probability of recovering mitochondrial transgenics. Production of this number of independent transformants may be near the practical limit of the current technology for particle delivery. Several laboratories are working to achieve mitochondrial transformation, however, and it seems likely that the goal will eventually be achieved.

Microprojectile bombardment may also become a predominant method for use in transient expression studies. It is often desirable to introduce DNA into cells, and then to observe expression soon after introduction (Kartha et al. 1989; Klein et al. 1988b). In many such studies, integration of the introduced gene is not necessary to achieve the objectives of the test, e.g., to determine whether or not a particular genetic construction will function properly in a specific cell type. Transient expression tests of this type have been conducted frequently by using electroporation to introduce DNA into protoplasts. In this situation, longevity of the protoplasts is relatively unimportant, and regeneration capacity is immaterial, so that some of the major limitations associated with the use of protoplasts do not interfere with the utility of the procedure. With the advent of microprojectile bombardment, however, the use of electroporation in some types of transient expression tests is becoming obsolete, and microprojectile bombardment is opening new possibilities for transient expression tests that are impossible with any other method of DNA delivery.

First, bombardment permits introduction into intact cells without removing cell walls. This greatly reduces the time required for DNA delivery. It also permits delivery of DNA into virtually any cell type, even if it is difficult to release protoplasts from the explant of interest. It is also not necessary to treat cells with the enzyme mixtures used to release protoplasts, nor are cells exposed to the trauma of wall removal, either of which could possibly alter expression of the introduced gene.

Microprojectile bombardment also permits the introduction of genetic constructions into organized tissues (Klein et al. 1989; Loopstra et al. 1990; Ludwig et al. 1990; Morikawa et al. 1989). Tissue-specific gene expression can then be observed, if desired, in a fully homologous system. This approach has been applied to test the function of a regulatory gene involved in the production of anthocyanin pigments in maize (Klein et al. 1989; Ludwig et al. 1990). A plasmid carrying a maize coding sequence, *Lc*, a member of the *R* gene family, was precipitated onto tungsten particles, and delivered into various tissues of maize that lacked *Lc*. Test lines were either permissive for expression of the pigment, or else were deficient for a factor other than *Lc*. Aleurone, coleoptile, root, mesocotyl, scutellar node, coleorhiza, and marginal leaf hairs were treated. The introduced gene, driven by a constitutive promoter was expressed, and induced formation of pigment in all tissues except the endosperm, in which pigmentation has not been reported. This response indicated normal tissue-specific function of the gene product and proved the utility of the technique for studies of tissue-specific gene activity.

Tissue-specific expression has also been reported for pollen-specific constructs delivered to maize pollen (Twell et al. 1989), and for light-regulated genes delivered into leaf tissue of barley, rice, and oat (Bruce et al. 1989). Twell et al. (1989) introduced genes with either the CaMV 35S promoter or a pollen-specific promoter from *Lycopersicon* into tobacco pollen. Expression of the marker gene (GUS) was observed in bombarded pollen and leaf tissue in patterns similar to those observed in stably transformed plants carrying these constructions.

Bruce et al. (1989) developed a rapid transient expression assay for light-regulated genes by introducing DNA-coated particles into intact dark-grown maize seedlings. Particles carried a chimeric gene consisting of the 5' untranslated region of a phytochrome gene from oat, fused to a chloramphenicol acetyl transferase (CAT) coding sequence. As hypothesized, the fusion was down-regulated by white light in monocotyledons, but failed to be expressed in three dicotyledons: tobacco, cucumber, and *Arabidopsis thaliana*.

Successful bombardment of wood, however, is perhaps most indicative of the extreme versatility and potential of this technique. Loopstra et al. (1990) delivered a plasmid carrying a chimeric GUS gene into the developing wood tissue of mature *Pinus taeda* stem segments. GUS activity was observed in different cell types, including tracheids, ray parenchyma, and axial parenchyma associated with resin canals. Interestingly, particles were able to deliver DNA into cells that were at least partially lignified. These experiments clearly demonstrate that microprojectile bombardment provides a means of delivering transforming DNA into virtually any cell type, and that it

could be used, therefore, to study tissue-specific gene activity in essentially any tissue type.

Bombardment offers several advantages over protoplast-based systems for transient expression studies. First, DNA can be introduced into explant or cultured cells immediately, without the need for developing protocols for release of protoplasts. Secondly, it is probable that walled cells are a more useful model for response in whole plants. Finally, it is possible to deliver DNA into organized structures, allowing observation of the regulation of a genetic construction in a specific tissue, under a defined set of environmental parameters. This was possible previously only by producing stably transformed plants, a far longer and more involved process than bombardment of an explant.

Biolistic gene transfer has several other advantages over other transformation methods. First, it allows delivery into walled cells from virtually any source. DNA can be delivered into at least superficial cell layers on essentially any explant that can fit into the vacuum chamber of the apparatus. This permits great freedom in choice of explant tissues, allowing use of explants that perform well in culture, for example, rather than explants that may not perform as well, but are more suited for the DNA delivery process. Because intact and/or organized explants can be used, culture effects can be minimized, reducing the probability of undesirable genetic changes occurring as a result of the transformation procedure. The ability to deliver DNA into cells in organized structures facilitates studies of tissue-specific regulation of gene expression.

Although several aspects of the biolistic process are not fully understood, it has been developed empirically into an efficient and reproducible means of gene transfer. Importantly, almost all aspects of the bombardment process can be controlled with precision. This allows optimization of treatment protocol for a specified tissue, which can be applied to that type of tissue easily and reproducibly thereafter. It is equally applicable to virtually any species.

Despite its many favorable attributes, microprojectile bombardment remains imperfect, and may be unsuited for some applications. The apparatus continues to be more expensive than that required for most other methods. This situation may eventually change, but, at present, cost of the apparatus has limited access to the technology. The apparatus, though improving steadily, may still contribute significantly to variability in experimental results. Particle and shock wave damage continue to adversely affect efficiency of DNA delivery and tissue survival rates.

Fiber-mediated DNA Transfer

Fiber-mediated transfer of transforming DNA is a promising new technology for gene transfer to walled plant cells. In the procedure described by Kaeppler et al. (1990), plasmid DNA was combined with a suspension of silicon carbide fibers averaging 0.6 μm in diameter, and 10-80 μm in length, and the suspension was vortexed, presumably bringing DNA and fibers in contact with one another. Intact cells from suspension cultures were then added to the DNA/fiber mixture, and the resulting suspension was vortexed for approximately 60-70 seconds at high speed. The suspension, still carrying fibers, was then transferred to solidified media for incubation.

Following incubation, transient expression of a beta glucuronidase (GUS) marker gene was assayed by the histochemical procedure of Jefferson (1987). With the reported treatment parameters, the authors obtained approximately 140 expressing cells in a treated mass containing about 2.5 X 10^{-6} Black Mexican Sweet (BMS) maize suspension culture cells, or an approximate average frequency of transient expression of 10^{-4} per experiment.

The technique was applied successfully to three different types of suspension culture cells: BMS, a nonregenerable maize cell suspension; an embryogenic maize cell line; and a tobacco suspension culture. The highest efficiency of transiently expressing cells was obtained with the tobacco cell culture, while the lowest occurred in the embryogenic maize cell line.

The mechanism of DNA transfer in this process is unknown at present. Electron microscopic examination of treated cells reveals that a significant proportion of the cells have been penetrated by fibers; and it is assumed that the openings through cell wall and membrane allow the entry of DNA into the cell. It is unclear, however, whether the delivery of DNA involves any active involvement of the fibers, such as binding of DNA to silicon carbide, followed by its release in the cell matrix.

Parameters for DNA transfer with silicon carbide fibers have not been fully optimized, even for the cell lines tested in the original study. It seems likely that efficiency will increase as the mechanisms involved are elucidated, and as additional factors are optimized. While there is no mention of gene transfer to organized structures, as has been reported for microprojectile bombardment, such an application appears to be feasible if methods for treatment of larger explants can be devised.

The procedure, while clearly in the early stages of development, holds significant promise as a relatively simple, efficient, and inexpensive means of delivering transforming DNA into walled cells. For maximum utility, it must be adjusted to increase efficiency and allow treatment of other explant types. There is not yet evidence that the process will produce stable transformants, but there is every reason to believe that if adequate DNA can be delivered to produce observable transient expression, recovery of stable transformants is probable.

Conclusions

The physical methods for DNA delivery described above are rational technologies for the enhancement of crop germplasm resources through production of stably transformed plants. These techniques are especially useful as alternatives to *Agrobacterium*-mediated transformation, particularly in those species which are resistant to *Agrobacterium* infection. They also provide methodology for direct and immediate observation of gene activity through transient expression experiments.

All techniques for physical delivery of DNA into plant cells appear to share certain characteristics. Perhaps most important among these are the phenomena of DNA integration observed following physical transfer of DNA.

Integration of parts of introduced plasmid DNA can involve any part of the plasmid molecule, and there is little evidence that one part of the plasmid can be induced to integrate more often than others. Similarly, the chromosomal site at which introduced sequences integrate is generally unpredictable.

The genes introduced into plants at the present time are almost exclusively dominant or additive in action. Typically, one to many copies of the introduced sequences become integrated. Where many copies are involved, they are often integrated as tandem repeats and do not produce many independent transgenic loci. Integrated copies are found, virtually without exception, in the heterozygous (or, more properly, hemizygous) condition, as determined by segregation analysis. Inheritance of integrated sequences is normally according to strict Mendelian patterns, although non-Mendelian patterns are commonly seen. The mechanisms producing these anomalous ratios are not known. Once a gene has undergone stable integration and exhibits Mendelian inheritance, however, both expression and inheritance of the gene usually remain stable thereafter.

The level at which an introduced gene is expressed is unpredictable. Integration of nonfunctional genes or gene fragments is commonplace. The level of gene expression in stably transformed tissue or plants may or may not be correlated with the number of integrated copies of the gene.

Transient expression follows similar patterns, regardless of the means used to deliver the transforming DNA (Stomp et al. 1991). Expression typically becomes observable within a few hours after DNA delivery. Expression then usually increases to some plateau level, followed by slow decline. Measurable levels of the gene product persist for about 1 week, after which detection becomes impossible. Because of this decline in gene expression over time, it is a common practice to measure gene product levels at a specified interval following treatment.

The mechanism of this reduction in transient expression is not fully understood, but is thought to involve the intracellular degradation of the newly introduced DNA. Ohgawara and coworkers (1983), for example, observed that plasmid DNA introduced into protoplasts by liposome fusion was almost entirely absent following only 1 week in culture. It is possible, however, to recover stably transformed cells from treated cultures well past the period during which transient expression can be observed, and indeed, it is common practice to do so. Therefore, it is obvious that integration of alien sequences must occur prior to the complete loss of the newly introduced DNA. It is possible that the integration process, which may involve DNA repair mechanisms, could actually be facilitated by partial degradation of the plasmid.

Most methods for physical transfer of transforming DNA should be applicable to virtually any plant species with equal effect. The primary limitation is that those systems which are dependent upon the use of protoplasts may be inappropriate for production of transgenic plants, because protocols for their regeneration are unavailable. The difficulty of regeneration and increased rates of mutation associated with protoplasts provide strong arguments against their use for the production of stable transgenics that might find application in crop improvement.

The technologies for physical transfer of DNA to plants continue to undergo rapid evolution. For the worker interested primarily in the development of new sources of genetic diversity through transformation, it is important to be aware of technical innovation, but not to move blindly to the newest technique or to the one for which the highest transformation efficiency is promised. It is especially important to realize that the development of a new transformation

protocol is a major undertaking, and must be viewed within the context of the overall program of germplasm improvement.

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Gene Action in Mutant and Transformed Plants

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Abstract

The successful application of the techniques of "reverse genetics" to improve crop plants requires the availability of a variety of plant genes affecting properties such as nutritional quality, herbicide and disease resistance, stress tolerance, and production of primary and secondary metabolites. In this chapter, we shall first emphasize the role of mutants—not only for their possible agronomic value—but also in providing markers and recipients for gene transfer, as well as a means for identifying important genes. Examples related to nutritional quality improvement by designing crops with enhanced essential amino acid content will be emphasized. In the second part, ways to analyze and control gene expression will be presented since regulation of foreign gene action during development is often desirable for plant breeding purposes. The characterization of regulatory elements involved in transcription control in the case of the alcohol dehydrogenase gene will be specifically described. Data on the stability of expression in progenies of transgenic plants grown in field conditions are becoming available for a few crops. The demonstration of the stability and the long-term safety of genetically modified crops represents an essential step for the future of plant biotechnology.

Introduction

It is important to reassess the central role played by mutant analysis in the genetic dissection of biological structures and functions. The development in the last 20 years of a somatic cell approach with the blossoming of new screening methods for isolating mutants disrupting important functions has allowed a revival in this field. One exemplary case concerns the photorespiratory pathway for which simple and elegant selection procedures have allowed the accumulation of a whole array of defective mutants altering most of the biosynthetic steps (Somerville 1984). The diversity of new types of mutants can be best illustrated by the state of art reached by the model species *Arabidopsis thaliana* (Estelle and Somerville 1986).

The availability of such mutated genes has also led to the development of methods for isolating them by diverse approaches which go from complementation in the corresponding bacterial or yeast mutants to testing homology between a heterologous probe and the corresponding plant gene. Insertional mutagenesis based on transformation with Ti plasmid or with available transposable elements from corn and *Antirrhinum* also represents a promising way to tag genes. The existence of restriction fragment length polymorphism (RFLP) maps—although for a still limited number of species—and recent progress in the preparation and separation of large DNA fragments, combined with cloning tools such as yeast artificial chromosomes that can carry DNA segments up to 500 kb (Burke et al. 1987) represent another approach to isolating individual plant genes known only from their phenotype.

Thus, plants with functions deleted, amplified, or modified have been obtained by mutagenesis-selection procedures. To such classical mutants, we have, at present, to adjoin transgenic plants characterized by new functions added by transformation. The molecular and cellular techniques allowing gene transfer from one organism to another represent powerful tools for changing plants in a directed way. It is quite

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clear that control of foreign gene expression is desirable for crop improvement. In addition, the availability of transgenic plants has allowed investigation of the basic mechanisms of gene regulation. Considerable progress has been accomplished in the identification and characterization of 5' upstream regulatory elements involved in transcriptional control (Schell 1987). The added gene may still include its own promoter and its expression can be evaluated under various environmental and developmental conditions. By using chimeric genes in which the promoter under study is fused to the coding sequence of a reporter gene such as β-glucuronidase (GUS), the expression of that reporter gene can be measured, e.g., by a color reaction, at organ, tissue, and cell level during the development of the plant. Another strategy consists of replacing the original promoter by another one which can be constitutive, or inducible, or developmentally regulated, or tissue and organ specific. Such alterations in the normal expression pattern of a gene may contribute to a better understanding of its role in the plant.

Ways for characterizing the cis-acting regulatory motives in the promoter region use a range of in vitro mutagenesis techniques such as the construction of deletion mutants of the concerned region that are often associated with site-directed mutagenesis which allows identification of key nucleotides in the sequence of interest.

Although less information is available for plants than in eukaryotes, the regulation at transcriptional level presents much analogy with animal systems. The presence and role of the TATA box are clearly demonstrated. The CCAAT box is represented in many plant promoters and modifications introduced by mutagenesis in the -40 to -110 region deeply alter promoter activity (Bruce and Gurley 1987). Moreover, various "upstream" elements involved in the control of gene expression have been identified in plants in relation with light inducibility in the case of rbcS gene (Timko et al. 1985) or the light harvesting chlorophyll a/b binding protein (*lhcp* gene) (Simpson et al. 1986), or environmental factors such as anaerobic conditions for the maize alcohol dehydrogenase (*ADH1*) gene (Ferl and Nick 1987).

The global expression of the added gene in the transgenic plant may be influenced by the copy number and the insertion site, as well as rearrangement of the foreign DNA. Very often, a whole array of variation is observed in the expression of independently obtained transformants which principally carry the same introduced gene. This has been associated with the so-called position effect. As a function of its location, the gene is strongly or weakly expressed or becomes silent.

In transformed plants, most genes are transcribed following the same regulatory pattern as in the donor plant. The endosperm activity of a zein promoter in transgenic tobacco plants was clearly demonstrated (Schernthaner et al. 1988). When the gene coding for phaseolin is introduced into the tobacco genome, it is correctly expressed in the seeds (Sengupta-Gopalan et al. 1985).

The promoter activity of a defined construct can also be rapidly assayed by the technique known as "transient gene expression". This relatively simple transfer technique consists of protoplast incubation with plasmid DNA in the presence of polyethylene glycol and divalent cations (Negrutiu et al. 1987).

In this article we consider two aspects of work with systems well suited for the study of gene action in mutants and transformants: nutritional quality and enzyme induction by environmental stress. Finally, we present some data about the evaluation and behavior of transgenic tobacco plants under field conditions.

Nutritional Quality Improvement: Designing Crops with Enhanced Essential Amino Acid Content

Amino acid imbalance is an important factor in determining the nutritional value of some food and feed grains. Cereals are mostly deficient in lysine, threonine, or tryptophan, while legumes are generally deficient in the sulfur amino acids. Various approaches aiming to improve the amino acid composition of the storage proteins have been considered (Bright and Shewry 1983; Boulter 1988), such as decreasing the proportion of prolamins in the grain or increasing the content of "high-lysine" proteins, or by changing the amino acid composition by replacing, for example, basic residues with lysine, or by adding sequences coding for a high-quality protein (Altenbach and Simpson 1990). Increasing the amount of the desired essential amino acids in the soluble fraction of the plant represents another possible way for reaching higher crop quality.

Selection of Mutants Affecting Aspartate Family Amino Acid Biosynthesis

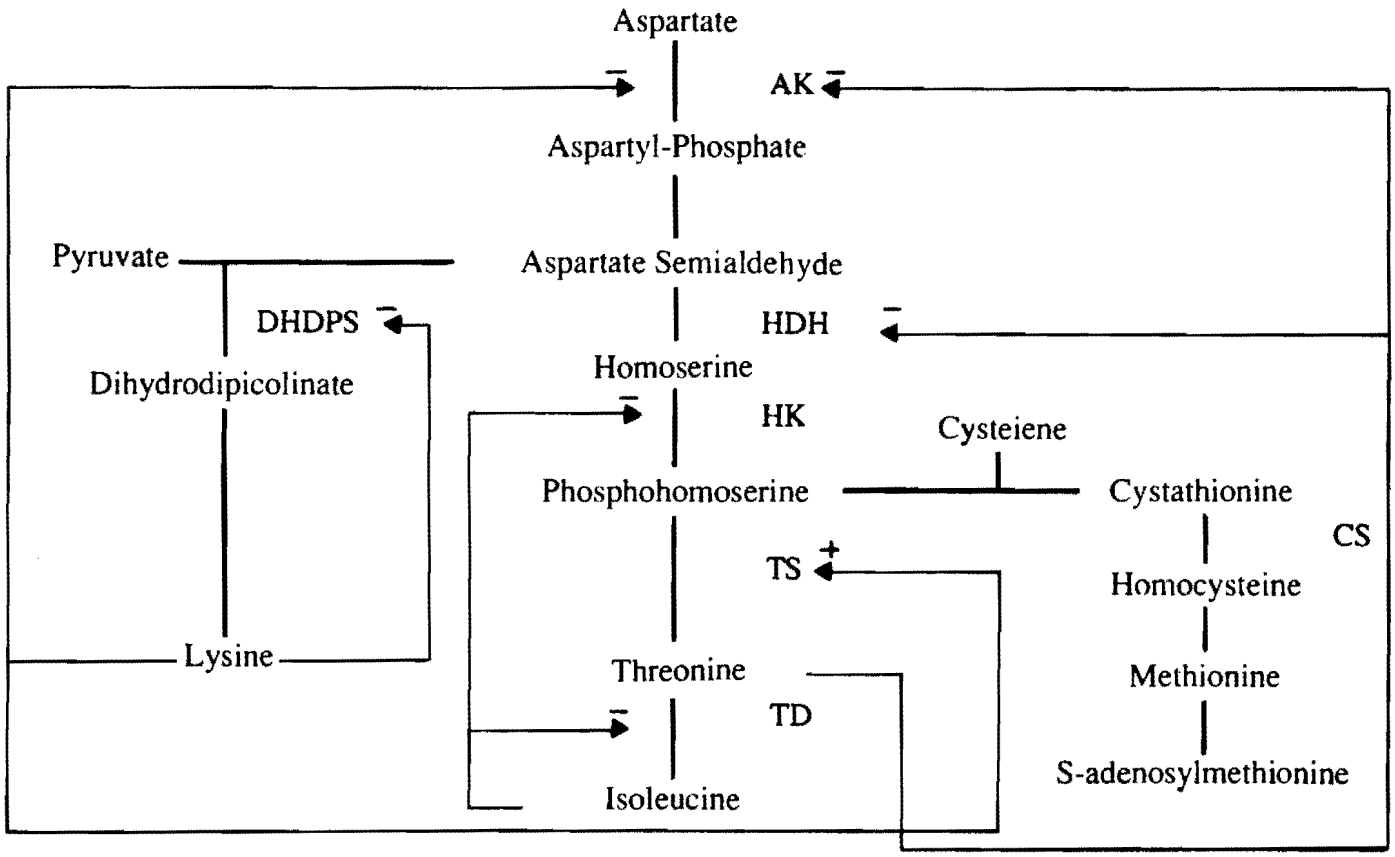
Amino acid biosynthesis is an essential process for plant growth and development, yet little is known

about the genetic regulation of specific enzymatic steps in most of the biosynthetic pathways. The accumulation of free amino acids and their incorporation into proteins affect the nutritional value of crops for nonruminant food or feed and as such can be an important factor in crop breeding.

The nutritionally essential amino acids, lysine, threonine, methionine, and isoleucine all derive from aspartate via a branched biosynthetic pathway. Research has been conducted in the enzymology of the pathway and in the search of mutants with altered regulation, which are characterized by the accumulation of the end-product amino acids. This pathway, therefore, shows a central interest and gives the opportunity to associate a biochemical and genetical analysis with the realization of important progress in the nutritional value of crops.

The major way of regulating this biosynthetic pathway is feedback inhibition at the level of the key enzymes, such as aspartate kinase (AK), the first enzyme of the pathway; dihydrodipicolinate synthase (DHDPS), the first enzyme of the lysine pathway; and homoserine dehydrogenase (HDH), the first enzyme of the branch leading to threonine and methionine (Fig. 1). Generally AK is inhibited by both lysine and threonine (Bryan 1980), while DHDPS is strongly feedback inhibited by lysine and HDH by threonine.

Selection methods for identifying potential mutants characterized by an increased production of lysine and/or threonine were developed for various species (Green and Phillips 1974; Miflin et al. 1983). They are based on the growth inhibition caused by a combination of lysine and threonine present in the culture medium, or by addition of a lysine analog



AK: aspartate kinase
DHDPS: dihydrodipicolinate synthase
HD: homoserine dehydrogenase
TS: threonine synthase
CS: cystathionine synthase
HK: homoserine kinase
TD: threonine deshydratase

Figure 1. Diagram of the biosynthetic pathway of the aspartate family of amino acids with known regulatory interactions (– = inhibition, + = stimulation of enzymatic activity by the indicated compound).

such as S-2-aminoethyl-L-cysteine (AEC). This growth inhibition was considered to be the result of feedback inhibition of the mentioned regulatory enzymes, leading to starvation for methionine or lysine.

Mutants of Aspartate Kinase (AK)

Genetic and biochemical characterization of altered AK activities have been reported in various species and in our laboratory in embryos and seeds of barley (Cattoir-Reynaerts et al. 1981a), sorghum (Piryns et al. 1988), *Arabidopsis* (Cattoir-Reynaerts et al. 1981b), suspension cultures of carrots (Cattoir-Reynaerts et al. 1983), or protoplasts of *Nicotiana sylvestris* (Negrutiu et al. 1984; Jacobs et al. 1990). In most of the mutants, AK showed a decreased sensitivity to lysine inhibition compared to the wild-type enzyme and evidence for the presence of AK isozymes was provided. When available, genetic analysis indicates that the mutants transmitted the resistance to lysine and threonine (LT) as a dominant single gene trait (Bright et al. 1982a).

In barley, the basis for resistance has been found to be an alteration of the feedback inhibition of a lysine sensitive AK, characterized as the AKIII isoenzyme (Bright et al. 1982b). The consequence of the mutation for amino acid accumulation is an increase in free threonine in the plant and in grains. Threonine in the soluble fraction of mature seeds from the mutant was increased from 1.9% to 9.1% of the total threonine content.

By using tissue culture selections, two LT-resistant maize mutants were recovered (Hibberd and Green 1982; Diedrick et al. 1990). Both mutants overproduce free threonine, especially in the kernels, and have altered AK activity. Increases in protein-bound methionine, lysine, and glycine concentrations were also noted.

In *N. sylvestris*, diploid leaf protoplasts were mutagenized and then submitted to selection on medium containing lysine plus threonine. The simultaneous presence of these two amino acids at high concentrations causes maximal feedback inhibition of the AK activity, therefore preventing further synthesis of lysine and then threonine, and more importantly, finally, methionine which is not supplemented in the medium. Thus, growth inhibition caused by methionine starvation can be relieved if its precursors are added to the medium (Green and Phillips 1974). Regeneration of LT-resistant plants allowed genetical analysis to be made. The resistance character was found to be Mendelian and dominant, and heterozygous in the parent mutant named RLT 70. High amounts of free threonine, reaching 70% of the total of the free amino acids, were found in the heterozygous RLT 70, against 4 to 9% in the wild type. The biochemical basis of this resistance is due to a loss of sensitivity of AK to feedback inhibition by lysine.

In order to study and compare inhibition patterns of the AK in RLT 70 versus wild type, a partial purification procedure was developed. After a G25 gel filtration step, the activity of wild type AK was measured, and inhibition in the presence of one or both feedback inhibitors was determined. Between 70 and 80% of the AK activity is lysine sensitive, while the remaining 20 to 30% is sensitive to threonine inhibition. When both amino acids are present, an additive feedback control is observed. In the AK enzyme from homozygote RLT 70, AK activity is completely desensitized to feedback inhibition by lysine (tested up to 50 mM, final concentration). Threonine still inhibits 20 to 30% of the enzyme total activity. Considering the inhibition pattern observed in *N. sylvestris*, by using a gel-filtration fraction, it appeared that at least two AK isoenzymes probably coexisted. Since isoenzymes in several species have already been separated on the basis of their different ion exchange properties, "fast protein liquid chromatography" was used. When the mutant enzyme was analyzed, the larger peak corresponding mainly to the lysine-sensitive AK activity was, as expected, less sensitive to feedback inhibition by lysine.

Thus, a desensitized lysine AK isozyme, in the RLT 70 mutant is thought to be the molecular basis of the resistance to LT growth inhibition and threonine overproduction.

Mutants of Dihydrodipicolinate Synthase (DHDPS)

Selection for modification of the regulatory properties of DHDPS was accomplished by incorporating S-2-aminoethyl-L-cysteine (AEC) a lysine analog in the culture medium. It is not completely clear whether AEC acts mainly as a competitor of lysine for protein synthesis or as an inhibitor of DHDPS. This type of selection applied to crops such as maize, barley, and *Pennisetum* has resulted in the recovery of resistant cell lines, but without lysine overproduction. In barley (Bright et al. 1979) and wheat (Kumpaisal et al. 1988), AEC mutants behave as recessive and are characterized by a decreased AEC uptake.

We have selected two AEC-resistant *N. sylvestris* callus lines by using UV-mutagenized protoplast cul-

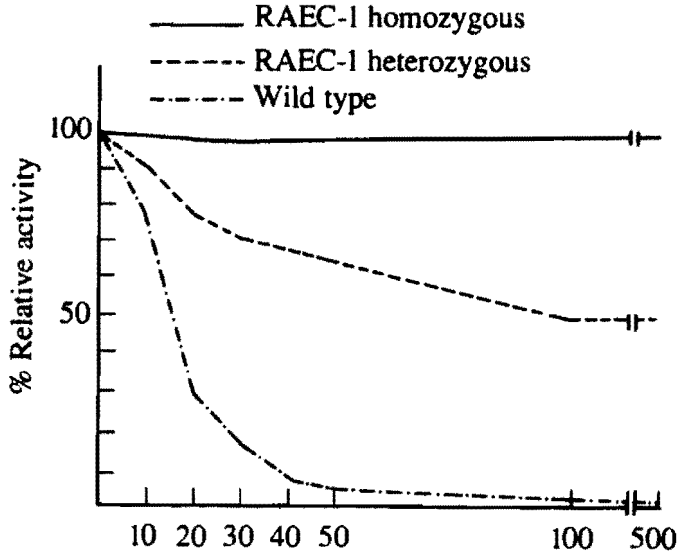


Figure 2. Effect of lysine on the activity of dihydrodipicolinate synthase (DHDPS) in leaf extracts of wild type, heterozygote, and homozygote mutant RAEC-1 (activity expressed as percentage of the total without inhibition).

tures (Negrutiu et al. 1984). Heterozygous AEC-resistant plants regenerated from selected colonies of RAEC-1 exhibited up to a 20-fold increase in free lysine of leaf and root tissues compared with the wild type. Homozygous mutants were obtained by crosses and selecting again on AEC-containing medium. The resistance in the mutant RAEC-1 was related to an altered dihydrodipicolinate synthase with a total insensitivity to feedback inhibition by lysine or AEC (Fig. 2). Study of the lysine-insensitive form of DHDPS did not reveal any other modifications compared to the sensitive form except the inhibitory properties. No activity or sensitivity changes were observed at the AK level. Analysis of the lysine overproduction in the homozygous plant was performed during the life cycle. A clear peak of lysine overproduction—up to 25% in the free amino acid pool—was appearing just before the flowering stem elongation. The AEC resistance trait was inherited as a single dominant gene.

Characterization of the Lysine and Threonine Overproducing Genes

Our approach consisted of increasing the amount of the desired amino acids in the soluble fraction by mutagenesis-selection procedures with the goal to develop plants with modified regulatory mechanisms leading to a higher production of specific amino acids. This mutagenesis-selection procedure led to the obtention of potentially interesting mutants whose genes are presently characterized. However, the selection methods cannot be easily applied for all crops and the overproduction of lysine was only observed in the case of the RAEC-1 mutant of *N. sylvestris*. Thus, a new, complementary strategy based on gene cloning, transfer, and expression in transgenic crops has been developed.

Approaches to Cloning the Plant Aspartokinase Gene

Rafalski and Falco (1988) have isolated a yeast AK clone on the basis of its ability to complement the HOM3 mutation causing threonine and methionine auxotrophy in yeast.

Analysis of the putative homology between the yeast probe and the plant gene was performed through Southern blotting with relaxed hybridization conditions. Hybridization signals have been detected on a Southern blot between the yeast probe and genomic DNA of *Arabidopsis thaliana*. A conserved sequence, deduced from the nucleotide sequence comparison of both bacterial and yeast AK, allowed the design of an oligonucleotide probe which could be homologous to the plant enzyme. Southern blot analysis is presently performed with such an oligonucleotide probe.

Cloning and Characterization of DHDPS Gene from *N. sylvestris* RAEC-1 Mutant

We have started the cloning in *N. sylvestris* and *Arabidopsis thaliana* of DHDPS genes by using different approaches (Ghislain et al. 1987). One was based on the use of the purification of DHDPS that enabled us to obtain a corresponding oligonucleotide probe deduced from a partial amino acid sequence as well as an immunological probe. The *N. sylvestris* enzyme was purified after ammonium sulfate precipitation, heat treatment, anion exchange chromatography, and finally gel-filtration chromatography. After separation on a two-dimensional native-SDS gel, the DHDPS subunit was eluted as a few microgram sample (Ghislain et al. 1990). Amino terminal microsequenc-

ing, performed by J.C. Guillemot (Unité des Protéines, Sanofi-Elf Biorecherche, Toulouse) allowed identification of 10 of the first 11 amino acids. Thus, DNA sequences corresponding to the DHDPS amino acid sequence could be synthesized. Such oligomers can be labeled and used as probes for hybridizations on Northern and Southern blots or used to screen complementary DNA (cDNA) libraries. Three sets of oligonucleotides were synthesized, but they still comprise 96 possible sequences of 17-mer oligonucleotides; that is the reason we decided to first use the immunological approach and then to retest to learn which would be the correct oligomer probe. Purified DHDPS subunits were used to produce polyclonal antibodies from rabbit. The best immunoreacting serum on Western blotting analysis was used to screen a cDNA gene expression library. The EcoRI site of the Lambda gt11 expression vector was used to clone cDNA from leaf poly(A)+RNA fraction of *N. sylvestris* wild type. Among 106 clones screened, several clones appeared positive with the use of alkaline phosphatase anti-rabbit IgG conjugate as a secondary antibody detection system. Sixteen clones remained strongly positive upon purification and are now studied at the DNA sequence level. Another approach developed very recently is based on the maize DHDPS cDNA recently cloned in DNA:DNA hybridization experiments (Frisch et al. unpublished results). Several Southern blots reveal a possible homology between maize, *N. sylvestris*, and *A. thaliana* DHDPS genes.

Genomic clones will be isolated from genomic DNA from *Nicotiana* wild type (for the lysine-sensitive DHDPS form) and from an already available homozygous RAEC-1 mutant (for the lysine-insensitive DHDPS form). Characterization of the DHDPS gene, once wholly sequenced, will be achieved by the identification of the different functional regions: promoter analysis (looking for the conserved CAAT and TATA boxes), site of initiation of transcription (S1 nuclease mapping), coding sequence structure (presence of introns), and 3' termination structure. This analysis of the DHDPS genes, by comparing the nucleotide sequences of the wild-type gene with the mutated gene coding for the lysine-insensitive form, will then designate which nucleotides and amino acid changes are responsible for the feedback deregulation.

Prospects are to construct chimeric genes including the mutated gene leading to feedback insensitivity under the control of its own promoter, along with the transit peptide sequence necessary for appropriate chloroplast targeting of the enzyme associated with a selectable marker gene. The plant promoter can also be replaced by a strong, constitutive promoter such as the 35S promoter of cauliflower mosaic virus (CaMV), or by a tissue-specific promoter such as a seed-specific promoter obtained from cereals.

Such an approach has been realized by using the *dap A* gene of *E. coli* that codes for a DHDPS less sensitive to inhibition by lysine than the plant enzyme. Glassman et al. (1989) have obtained transgenic tobacco plants with high levels of lysine-tolerant bacterial DHDPS and an accumulation of free lysine in leaves, but not in seeds. The expression of the bacterial gene was also accompanied with an aberrant morphology.

These data strongly suggest that a fine control of the expression level of the mutated DHDPS gene is necessary to obtain nutritionally significant elevations of free lysine compatible with a fully normal development.

The *Arabidopsis* Alcohol Dehydrogenase (ADH) System

Properties

The ADH system represents, as illustrated by the work on maize (Freeling and Bennet 1985), an exemplary case for studying gene expression. In *Arabidopsis*, ADH is regulated in a tissue-specific and developmental fashion and moreover by environmental factors such as anaerobic stress, and application of the synthetic auxin 2,4-D. *Arabidopsis* ADH is encoded by a single gene locus that was cloned thanks to the high degree of homology observed between maize and *Arabidopsis* at both DNA, RNA, and protein levels (Chang and Meyerowitz 1986; Dolferus et al. 1990). This raised interest in identifying the cis-acting promoter elements and the trans-acting protein factors that are responsible for the expression features of the *ADH* gene. ADH null mutants can be very conveniently selected in M2 seeds obtained from seeds mutagenized by ethyl methane sulfonate by using allyl alcohol as a suicide substrate for the enzyme (Jacobs et al. 1988). The genes from two mutants were recently cloned and fully sequenced (Dolferus et al. 1990). As expected for ethyl methane sulphonate, both mutants were identified as point mutations and more specifically GC to AT transitions.

Arabidopsis is also amenable to in vitro culture and transformation techniques. We have obtained *Arabidopsis* plants by root transformation using the

method developed by Valvekens et al. (1988), and by direct gene transfer into protoplasts using a modified procedure based on the protocol from Damm and Willmitze (1988).

Characterization of the *Arabidopsis* ADH Promoter

For the identification of cis-acting elements or enhancers, and trans-acting regulatory proteins interacting with these promoter elements, three approaches are generally followed. One consists of comparing the promoter sequence of genes with a related expression pattern. A second method involves the construction of chimeric genes and the establishment of an in vivo expression. For the application of the first method, the most obvious candidates are the maize *ADH*1 and 2 genes, which have been most extensively characterized. Walker et al. (1987a) have mapped the maize ADH1 promoter by deletion mapping. This work revealed a 40 bp region between positions -140 and -99, which was essential for anaerobic inducibility of ADH/CAT chimeric gene constructs, in a maize suspension protoplast transient expression system. Linker scanning mutagenesis showed that this region consists of two subregions (region I, -140 to -124, and region II, -113 to -99), which destroyed anaerobic expression after mutation. These regions, called "anaerobic response elements" (ARE), were shown to be conserved in other maize anaerobically regulated genes (*ADH*2, sucrose synthase, aldolase), as well as in the pea and wheat ADH genes (Walker et al. 1987b). Also in the *Arabidopsis* ADH gene, this element was found to be conserved and consists of a TTGGTTT sequence motive between -150 and -160.

To determine more precisely which regions of the *Arabidopsis* ADH promoter play which roles (tissue specificity, anaerobic induction, or auxin induction), the complete promoter was uncoupled from the rest of the gene by site-specific mutagenesis and used to construct a chimeric gene using the GUS and CAT reporter genes. We also used a Sau3A restriction fragment, leading to a truncated promoter including the presumptive ARE sequences to make a chimeric gene construct.

Such constructs were first used in transient expression assays. Protocol described by Negrutiu et al. (1987) was used to introduce the plasmids into protoplasts derived from *Nicotiana plumbaginifolia* suspension cultures. It was possible to demonstrate ADH-driven reporter gene expression in these protoplast systems (Fig. 3).

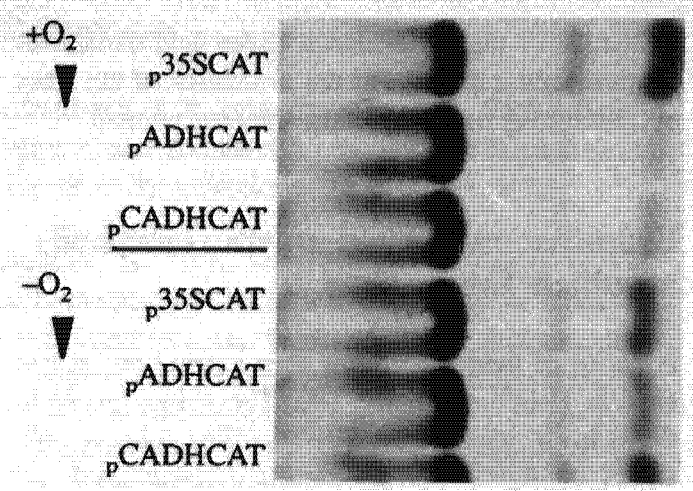


Figure 3. Expression analysis of chimeric genes by transient expression assays. Chimeric genes were constructed using a short Sau3A promoter fragment (ADH: –230 to +30) or the complete promoter (CADH: –962 to +58) or the constitutive 35S promoter from the cauliflower mosaic virus (CaMV) fused to the CAT gene (coding for chloramphenicol acetyl transferase activity). The complete ADH promoter retains its anaerobic inducibility in suspension protoplasts from *Nicotiana plumbaginifolia* while the truncated version is poor in terms of activity.

These chimeric gene constructs were mobilized to binary Ti-vectors for the obtention of transgenic *Arabidopsis* plants through *Agrobacterium*-mediated transformation and also for direct gene transfer experiments. *Arabidopsis* plants transformed with these chimeric genes will not only allow the evaluation of the influence of the 5' deletions on the anaerobic or 2,4-D response of the ADH promoter, but also the determination of the basis for tissue specificity and developmental expression.

Assessment of Transgenic Crop Plants

The development of recombinant DNA technology together with the techniques available nowadays to transfer genes among plants provides us with new opportunities to improve defined traits of our agricultural crops. Plants with new properties such as herbicide resistance, virus and fungal resistance, and insect resistance have been developed by genetic engineering (Fraley et al. 1988). The field testing of engineered crops represents an essential step in the application of plant biotechnology. The information that can be obtained concerns mainly two aspects: the

evaluation of the performance under field conditions of transgenic material and the evaluation of the risks of introducing such plants into the environment.

Examination of Field-grown Genetically Modified Tobacco

A series of field trials have been carried out at two sites in Belgium and the UK. The plants grown in these trials were genetically modified tobacco, obtained by *Agrobacterium tumefaciens*-mediated leaf disc transformation with a construct containing the CaMV35S promoter fused to the GUS gene and the nopaline synthase promoter fused to the neomycin phosphotransferase gene (NPT-II), conferring kanamycin resistance. Nonmodified plants that had been subjected to the same tissue culture procedures but not to transformation were also grown.

The aim of the trials was to examine genetically modified plants in an agricultural context. Two main aspects were examined: the stability and inheritance of the introduced characters and the development of the transformed plants in comparison to the nonmodified plant (Paul et al. 1989).

The experiment includes two transgenic lines that originally express the GUS gene at high and low levels. The plants were grown during the summer of 1989 and examined for variation in growth rate and development. In addition, samples were taken at intervals during plant development to measure the level of GUS activity. Progeny analysis at the level of kanamycin resistance was also performed. The main conclusions obtained until now are briefly outlined below.

Plant Morphology

It was not possible to distinguish between the two transgenic lines and the control line on the basis of any aspect of morphology including plant architecture, leaf shape and color, flower color, or inflorescence structure.

Plant Growth and Development

On the basis of the mean value of the height of the plants measured at regular intervals, it was observed at the beginning that the control plants were significantly shorter than those of the other two lines, but that the subsequent growth rate of the control lines was higher. This resulted in no significant difference between the lines at later measurements.

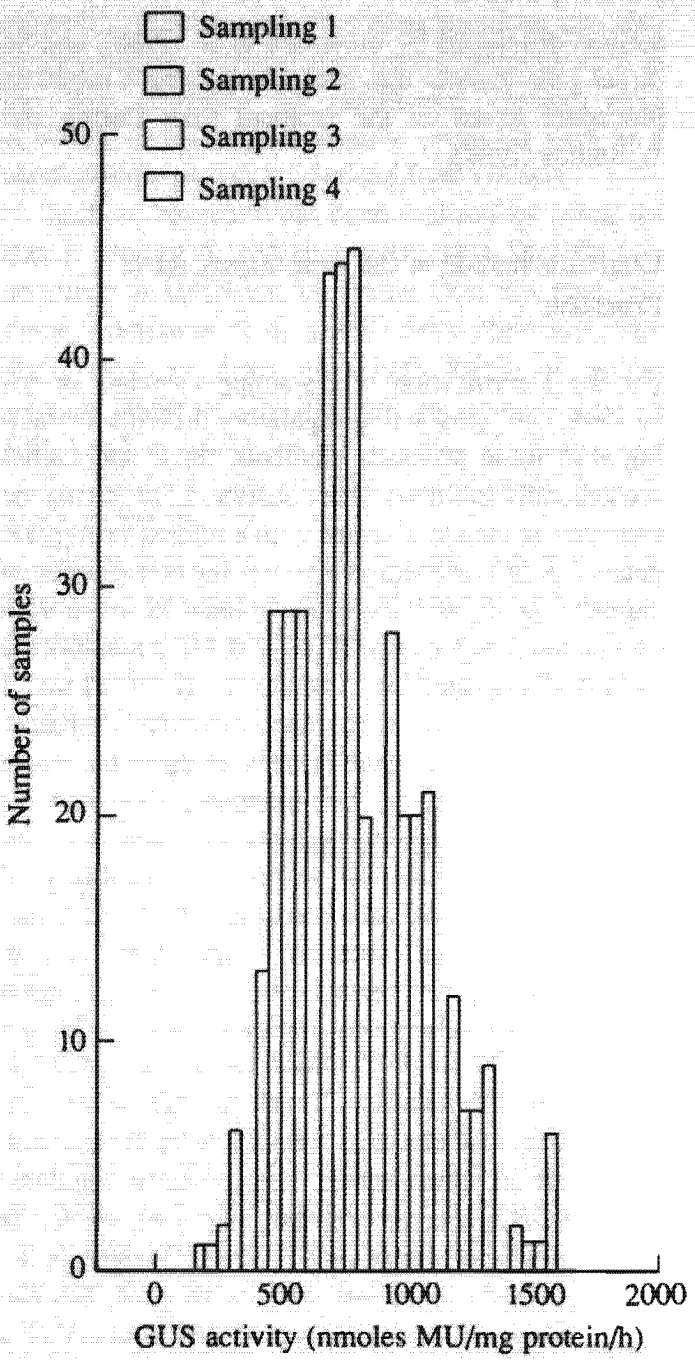


Figure 4. Frequency distribution in GUS activity of 100 samples from the high line tobacco plants taken at four harvests at the VUB site (Belgium).

Capsules produced on the first inflorescences were harvested, counted, and weighed. At both sites, the mean capsule weight was significantly lower in the control line than in the other two lines. In general, the differences could not be related to the level of activity of the introduced characters. It means that the high GUS expressing line did not differ from the control line to a greater extent than the low-expressing line.

Foreign Gene Expression

In the genetically modified lines, no clear trend in GUS activity during development of the plants could be detected and the level in the control line remained at a background level. The marked differences between the high and low expressing lines were maintained (Fig. 4). The molecular basis for the difference in GUS activity level has been associated with a rearrangement within the introduced GUS gene in the low line.

A first series of analyses concerning the behavior of seedlings growing in the presence of kanamycin (tested in at least 100 progenies) allowed observing a high degree of stability in the expression of resistance in the high line. However, "segregation-like" patterns of sensitive plantlets appear to characterize around 10% of the progeny of the low-expressing lines. A series of such sensitive plants have been rescued by transfer on a medium without antibiotics and will be analyzed at a molecular level to determine the possible origin of the lack of such expression as loss of the gene, rearrangement within the gene, or extinction of the expression by methylation.

The actual conclusions of the field tests show that the introduced traits did not affect plant growth or reduce yield and that their expression level was comparable to the one observed originally in the laboratory tests.

Conclusions

Plant improvement through cellular and genetic engineering is becoming a reality. Plant mutants with functions altered in amino acid metabolism have contributed to increasing our understanding of the biochemical genetics of higher plants, and have also proved their utility for changing the amino acid production level. The technology of gene transfer allows adding functions to plants and provides new ways of understanding the regulation of gene expression during development. In terms of nutritional quality improvement, transformation can extend the value of a mutated allele restricted to a defined species or to a wide range of crops. In that case, the properties of mutants and transformants appear as complementary and may lead to practical applications. The future success of transgenic crops depends also on large-scale field testing demonstrating the stability and the long-term safety of engineered plant material.

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Targeting of Proteins to the Nucleus: Development of an In Vitro Expression Vector System

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In recent years there has been considerable progress in understanding the mechanisms of intracellular sorting of newly synthesized proteins. These advances have been widely applied in the targeting of foreign proteins to different compartments of a cell by recombinant-DNA techniques. We have been interested in the mechanism of selective transport of proteins into the nucleus. The nucleus is separated from the cytoplasm by a double-layered nuclear envelope containing nuclear pores that are proteinaceous channels involved in nucleocytoplasmic traffic. The transport of a karyophilic protein into the nucleus has been shown to depend on the presence of a basic nuclear location signal sequence within the mature polypeptide (reviewed by Gerace and Burke 1988). The requirements for specific nuclear transport of a protein were earlier studied in vitro by using highly purified nuclear proteins (Newmeyer et al. 1986; Markland et al. 1987). In order to increase the versatility of such systems, we have developed an assay to study the transport of any nuclear protein for which a cloned gene is available (Parnaik and Kennady 1990). The messenger RNA (mRNA) obtained by P6 RNA polymerase transcription of a specific gene cloned in an SP6 expression vector was translated in a rabbit reticulocyte lysate containing 35S-methionine. Subsequently, mouse liver nuclei were added and nuclear-localized 35S-labelled proteins were analyzed by gel electrophoresis. Transport of nuclear proteins into isolated nuclei was rapid, temperature-dependent and ATP-dependent. A specific nuclear accumulation of >20-fold was observed for the animal cell tumorigenesis-related nuclear proteins, p53 and E1b, and the nuclear enzyme, thymidine kinase, whereas transport of the nonnuclear proteins, dihydrofolate reductase and simian virus 40 small T antigen was negligible.

The ability of nuclear location-signal peptides to target proteins into the nucleus has been extensively studied in animal cells and yeast. Recently, a bacterial peptide has been found to act as a plant nuclear targeting signal (Herrera-Estrella et al. 1990). In this study, the amino-terminal portion of *Agrobacterium* VirD2 protein, which contains the eukaryotic prototype signal sequence, (Arg-Lys-Gly-Arg), was shown to direct a β-galactosidase fusion protein into tobacco nuclei. These studies have provided convincing evidence for the role of VirD2 as a pilot protein in the transfer of T-DNA from *Agrobacterium* into plant cell nuclei, where the T-DNA subsequently integrates into the plant nuclear genome. The exact mechanism of such nuclear transport processes can be conveniently determined in in vitro systems, such as the assay we have described. Our assay also provides a sensitive method to study the requirements for the targeting of newly engineered proteins to the nucleus.

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Genome Characterization and Diagnostics

Organization of Viral Genomes: the Potential of Virus Genes in the Production of Transgenic Virus-resistant Plants

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Abstract

The extent of our knowledge of the nucleotide sequences of plant virus genomes is increasing rapidly and the genome organizations thus revealed are proving to be intriguingly diverse. The strategies include production of independently translatable mRNA, bypass of termination codons by read-through or frameshift to express downstream sequences, and proteolytic cleavage of precursor proteins. Knowledge of the sequences and modes of expression of plant virus genes has facilitated their isolation and subsequent insertion into plant genomes by transformation. Most work has involved attempts to mimic cross-protection by using coat-protein genes, and a measure of resistance to viruses from 11 different taxonomic groups has been achieved in this way. Results with other genes have been mixed but many possibilities remain to be explored.

Introduction

Recent years have seen significant advances in the development of biotechnological methods for modifying agronomically useful cultivars so that they become resistant to virus infection. This technology has become possible because of the availability of complementary DNA (cDNA) copies of appropriate genes and the development of methods for transforming plants by the introduction of 'foreign' genes (reviewed elsewhere in this volume). Before a virus gene can be used in transgenic work, it must be identified and something must be learned about its expression during virus multiplication. Therefore, this chapter will discuss the different ways in which virus genomes are expressed, consider how virus gene sequences might be expected to impart resistance to a plant, and present examples of what has been achieved by exploiting known genomes.

Genome Organization

General Principles

Most plant viruses have single-stranded positive-sense RNA genomes and only these will be discussed. Plant virus genomes are very small; most comprise between 4 and 15 kb of RNA that contain three to eight genes. Plant messenger RNA (mRNA) molecules are monocistronic, that is, each molecule is translated to give a single gene product. However, virus genomes contain fewer RNA species than genes, and plant viruses have evolved a variety of strategies for synthesizing the translation products of the different genes in the polycistronic genomic RNA. The mechanisms for virus RNA genome expression are:

- Division of genome into separate mRNA
- Transcription of subgenomic mRNA
- Readthrough of termination (suppression)
- Bypass of termination (frameshift)
- Bypass of initiation (multiple frames)
- Proteolysis of a polyprotein

Each will be discussed with reference to the four examples shown in Figure 1.

Division of the Genome into Separate mRNA

This is perhaps the simplest strategy. For example, the genome of alfalfa mosaic virus (AlMV) consists of

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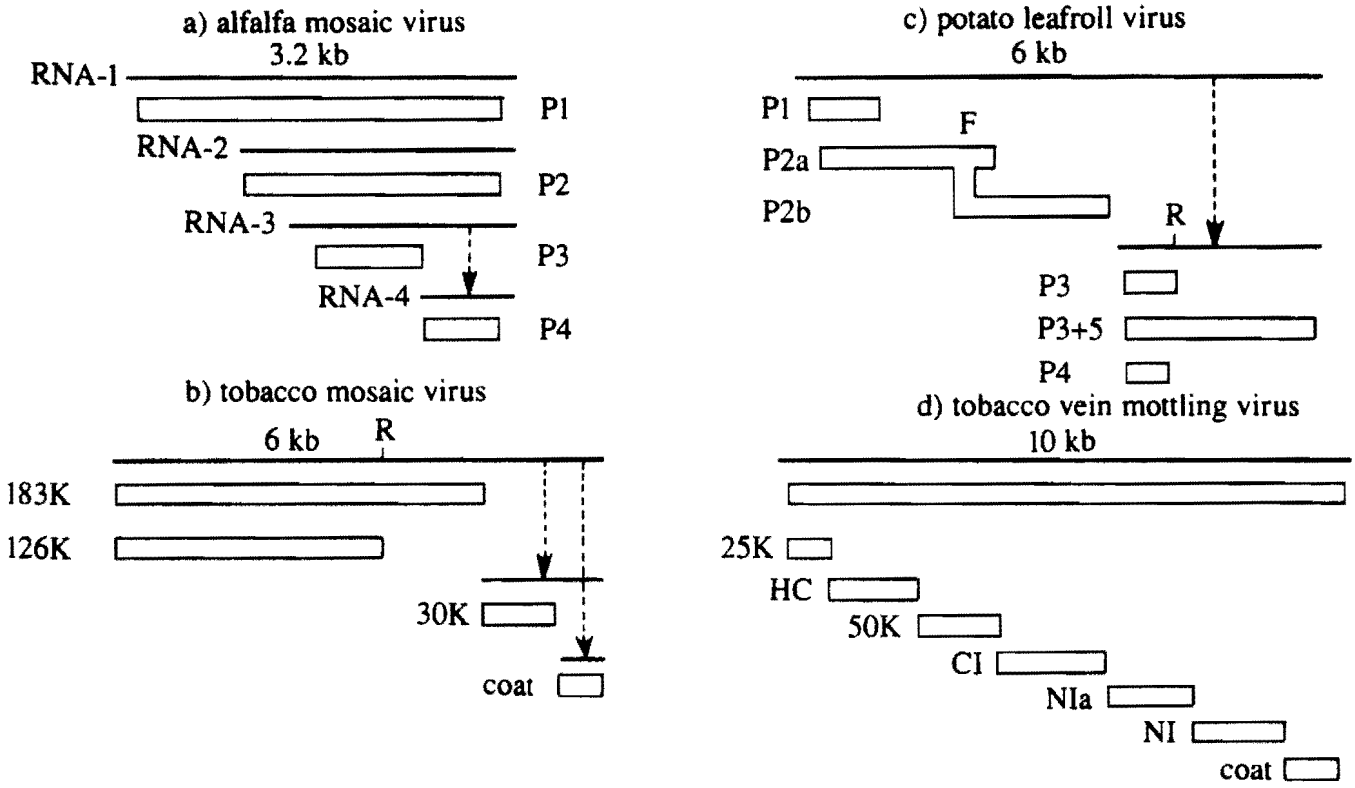

Figure 1. Diagram of the genome expression strategies of four diverse plant viruses: (a) alfalfa mosaic virus, (b) tobacco mosaic virus, (c) potato leafroll virus, and (d) tobacco vein mottling virus. F shows the position of frame-shift readthrough, R of In-frame readthrough. ——— Indicates RNA (to scale), ▭ Encoded proteins (to scale), and ↓ Production of subgenomic RNA.

three positive-sense RNA species (Fig. 1a; Symons 1985). RNA-1 and RNA-2 each encode one polypeptide. However, although the other genome RNA (RNA-3) is translated to give polypeptide P3, it also encodes a fourth gene product, P4. This product is the coat protein and is made by translation of a subgenomic RNA (see below). No positive-sense, single-stranded RNA (ssRNA) virus genome comprises only monocistronic mRNA.

Transcription of Subgenomic mRNA

Subgenomic RNA molecules are produced by partial transcription of a larger, usually genomic, RNA. By this means genes that are downstream of the first encoded protein become the 5'-most open reading frame (ORF) of a mRNA. This strategy is very common among viruses. For example, two genes of tobacco mosaic virus (TMV) (Fig. 1b; Morch and Haenni 1987) and two genes of potato leafroll virus (PLRV) (Fig. 1c; Mayo et al. 1989; Tacke et al. 1990) are expressed in this way. The noncoding parts of the subgenomic mRNA may be important in controlling the expression of these genes.

Readthrough of Termination

Although termination codons specify the end of translation of an mRNA, some suppressor transfer RNA (tRNA) species can recognize termination codons and cause an amino acid to be incorporated into the polypeptide being produced. The result of this 'read through' is that translation then proceeds to the next termination codon. The 183K protein of TMV (Fig. 1b) arises by readthrough of the termination codon of the 126K protein (Morch and Haenni 1987). Likewise, readthrough of the termination codon of the gene for

the coat protein (P3) of PLRV (Fig. 1c) results in synthesis of a larger protein (Bahner et al. 1990). Another way of avoiding a termination codon is by frameshift. This happens when, during translation of RNA from some viruses, a proportion of the ribosomes involved in translation transfer to another reading frame in the RNA. The result is that a termination codon is bypassed. The P2b part of the putative replicase of PLRV is thought to be synthesized in this way by frameshift near the C-terminus of P2a (Fig. 1c; Mayo et al. 1989). Thus, the shorter P2a is analogous to the 126K protein of TMV and the longer P2a + P2b protein is analogous to the 183K protein of TMV.

Bypass of Initiation Codons

The principle of genetic economy is taken further by some viruses. For example, in the PLRV genome (Fig. 1c; Mayo et al. 1989), one piece of nucleotide sequence encodes genes in each of two of the three possible reading frames. Thus ribosomes either initiate near the 5' end and translation yields P1, or they initiate at another, out-of-frame, initiation codon and translation yields protein P2a. The genes for P3 and P4 proteins of PLRV are also in different reading frames, but it is not known if these proteins are translation products of one subgenomic RNA that arises by bypass, or if there are two subgenomic mRNA species.

Proteolysis of a Polyprotein

The genomes of some viruses, for example those of potyviruses (Fig. 1d; Morch and Haenni 1987), contain only one large ORF that encodes a single large protein. This polyprotein contains sequences that have protease activity and this specifically cleaves the polyprotein into functional gene products. Thus, only the sequence encoding the 5'-most gene product has an initiation codon.

Combined Strategies

As is clear from the examples shown in Figure 1, different genes in one virus genome are frequently expressed in more than one way. And there is no theoretical reason why any combination of strategies should not occur. For example, comoviruses have two genomic RNA species, each of which encodes a polyprotein, but in the smaller RNA species, alternative initiation codons are used which result in the production of two sizes of polyprotein (Franssen et al. 1982). The different genome RNA of some other bipartite genome viruses are also expressed in different ways. For example, the larger genomic RNA of beet necrotic yellow vein virus contains a single ORF mRNA (Bouzoubaa et al. 1987) but the smaller genome RNA contains more than one ORF. The 3'ORF (or ORFs) is expressed by translation of a subgenomic mRNA and the 5'ORF is expressed by direct translation to yield either a M_r (Mol wt) 21 000 protein or, by readthrough of the termination codon, a M_r 75 000 protein (Bouzoubaa et al. 1986). These examples show that little can be predicted concerning the genome organization and gene expression strategy of a virus before one knows both the sequence of its genome—or at least that of a close relative—and the identity and nature of the translation products of its RNA.

Satellite RNA and Defective Interfering RNA

Satellite RNA molecules and defective interfering (DI) RNA molecules multiply in association with some viruses, but are not strictly parts of the virus genome (i.e., not part of the minimum infective RNA complement). They have important effects on the functioning of the genomes of some viruses and are relevant to later sections of this article. Satellite RNA molecules are usually small and occur naturally in some virus cultures. They depend on these helper viruses for multiplication, but have no appreciable sequence homology with them (Fritsch and Mayo 1989). Some but not all satellite RNAs are mRNAs.

DI RNAs are also small nonessential RNA molecules that depend on the helper virus for replication and are found in some virus cultures. They differ from satellite RNA in that they comprise parts of the virus genome that normally include the 3' and 5' ends of the genome; they arise from genome RNA by deletion (e.g., Hillman et al. 1987). Satellite RNA or DI RNA molecules may cause a decrease in yield of the helper virus (Fritsch and Mayo 1989; Burgyan et al. 1989; Li et al. 1989; Marsh et al. 1990), presumably by competing with viral RNA for the replicase activity.

Background to Producing Transgenic Resistance

Although natural genes conferring virus resistance have been incorporated into the genomes of some

crop plants by breeding, this is not always possible. One nongenetic control measure that has proved successful in some instances is cross-protection. This occurs when prior inoculation with a mild strain of a virus protects a plant against the effects of subsequent infection by a second, related virus. In effect, this is using many, or even all, the genes of the virus inoculated first against the second virus. Although, simply stated, cross-protection is a complex phenomenon that can occur by different mechanisms (Sherwood 1987), it nevertheless works and has been used for practical control of virus diseases. For example, the effects of citrus tristeza virus infection of citrus crops has been controlled by inoculating young plants with a mild strain (Costa and Muller 1980) and similar measures have been used for the control of tomato mosaic virus (ToMV) in tomato crops (Rast 1972), papaya ringspot virus in papaya (Yeh and Gonsalves 1984; Wang et al. 1987), and zucchini yellow mosaic virus in squash (Lecoq et al. 1989).

A second approach has been to inoculate pepper plants with cucumber mosaic virus (CMV) containing a satellite RNA that ameliorates symptoms of CMV infection so as to protect plants against subsequent infection by CMV strains that induce severe symptoms (Tien et al. 1987). In this system the satellite RNA competes with virus genomic RNA, thereby suppressing its synthesis.

However, with all these approaches, there is a risk that the cross-protecting strain or satellite RNA might mutate into a form that induces a more virulent effect. As little as one nucleotide change can convert a satellite RNA of CMV from one that causes symptom amelioration to one that causes necrosis (Sleat and Palukaitis 1990). Moreover a mild strain of TMV that was used to cross protect tomato crops against infection with ToMV was found to have mutated in the field to a more virulent form (Fletcher and Rowe 1975).

Cross-protection was perhaps the first type of nongenetic control to be thought to be imitable by transforming plants with virus genes (Hamilton 1980). Various viral genes have been proposed as agents to simulate cross-protection or other protective effects. These, and others, are discussed below.

Potential for Transgenic Interference

Table 1 is a simplified description of the stages of the infection cycle of a virus in an infected cell. There is a potential for transgenic interference at each stage and an agent that could cause such interference is listed.

The first stage in a viral infection is the uncoating of virus particles. One of the mechanisms proposed to explain cross-protection was that protein from the protecting virus in some way prevented uncoating of the particles of the challenge virus (Sherwood and Fulton 1982). If this is true, a similar effect might be expected to occur if viral proteins were already present in the inoculated cell. Transforming plant cells with a viral coat protein gene so that they synthesize this protein has proved to be an effective way of protecting plants from infection (see below).

Once uncoated, or partially uncoated, virus RNA must be translated by ribosomes to give the proteins necessary for replication. Translation of mRNA in plant cells can be inhibited by the presence of complementary RNA sequences (antisense RNA) (Ecker and Davis 1986). These are, in effect, genes or parts of genes that have been reversed in the transformation vector so that the RNA transcripts made in the transformed cells have sequences complementary to that of the target mRNA. Anti-sense and target RNA therefore hybridize together. Virus RNA translation also may be susceptible to this sort of inhibition.

Ribozymes (Haseloff and Gerlach 1988) are also RNA molecules that have stretches of sequence com-

Table 1. Possible transgenic interference during a virus infection cycle.

Stage	Action of transgene	Active agent
Uncoating	Compete for RNA	Coat protein
Translation	Block or cut mRNA	Antisense or ribozyme
Transcription	Inhibit enzymes	Antibodies
Replication	Compete for enzymes	Satellite or DI RNA
Assembly	Compete for capsids	Satellite or DI RNA
Dissemination	Interfere with vector acquisition	Mimic of virus or helper component

plementary to the target RNA, and which therefore bind to the target RNA by hybridization. Because the nucleotide sequence in the center of the ribozyme between the regions that bind to target RNA has catalytic activity, the result of hybridization to a ribozyme is a cleavage of the target RNA. Figure 2 shows a ribozyme constructed to cleave the putative polymerase gene of PLRV (Lamb and Hay 1990). The arms hybridize to the target RNA and the central catalytic domain is that described by Haseloff and Gerlach (1988). This ribozyme cleaves its target RNA in vitro but there is no information about its activity in vivo (Lamb and Hay 1990). However, transgenic expression of a ribozyme in mammalian cells has been shown to cause cleavage of human immunodeficiency virus RNA (Sarver et al. 1990).

The next steps in the development of a viral infection are the transcription and replication of virus RNA by replicase enzyme(s). Plants transformed with antibody genes have been shown to produce serologically active antibodies (Hiatt et al. 1989) and therefore expression of antibodies to the virus replicase enzymes might be an effective way to inhibit virus replication.

Replication is probably the stage at which satellite RNA is active in suppressing virus RNA synthesis. Some satellite RNA molecules, in particular small type C or type D satellites (Murant and Mayo 1982), appear to compete with virus RNA for the viral replicase; satellite RNA multiplication therefore diminishes that of the helper virus RNA. DI RNA molecules are similar except that they are derived from the viral genome RNA and can arise spontaneously (Li et al. 1989; Burgyan et al. in press). DI RNA and satellite RNA molecules are encapsidated by the helper virus coat protein and therefore may compete with genomic RNA molecules for coat protein during the particle assembly stage of the infection cycle.

Another virus gene product that could be vulnerable to transgenic interference is any virus-encoded protease used during multiplication to cleave polyproteins into functional gene products. Such proteases could be interfered with directly by the antibody approach described above. A more indirect approach could be exploited to obtain transgenic transformation of the plant with the gene for a relatively innocuous precursor of a toxin in which the potentially toxic parts were separated by a linking stretch of amino acids, such as that for the ricin A and B chains (Halling et al. 1985). If the linker stretch contained an amino acid sequence recognized by the viral protease, the protease then produced after viral infection of the transgenic cell would cleave the harmless precursor and release the toxin which would kill the cell before virus multiplication could occur. The host would thus be transgenically hypersensitive.

The final stage of virus multiplication is spread to other cells. Many viruses are disseminated by being specifically carried by a vector, often an invertebrate. They are then transmitted when released during feeding on another host plant. With some persistent viruses, binding can also be to internal organs of the vector. With some viruses, for example potyviruses, transmission by aphid vectors involves a helper component—a virus-coded protein, which may act by forming a link between the virus particle and the retention site in the vector (Harrison and Murant 1984). Thus, if plants are transformed to express a molecule that mimics the binding of either the virus particle protein or the helper component, this could compete with infective virus and thus dilute the inoculum carried by the vector.

These active agents and their modes of action are all speculative, and some are highly so. Nevertheless, it is apparent that there are many possible ways in which transformed plants can express a transgene derived from, or acting specifically against, a virus in order to inhibit the development of a virus disease in a plant or in a crop. The following section describes some of the successes that have been obtained.

Progress in Achieving Transgenic Resistance

Coat Protein Mediated Effects

Since the first demonstration that expression of TMV coat protein induced resistance to TMV infection in tobacco (Powell-Abel et al. 1986), there has been a rapid rise in the number of reports of coat protein-mediated resistance effective against other viruses (Mayo and Barker 1990). Table 2 lists reports of coat protein-mediated virus resistance and there are studies going on with a number of others. Resistance has been induced against challenge infection by 16 viruses belonging to 11 virus groups. The host most commonly used has been tobacco, probably because it is relatively easy to manipulate in culture and is a host for many viruses. However other, less amenable hosts, such as potato (Hoekama et al. 1988; Hemenway et al. 1988) and sugar beet (Kallerhof et al. 1990) have been investigated. Progress in achieving trans-

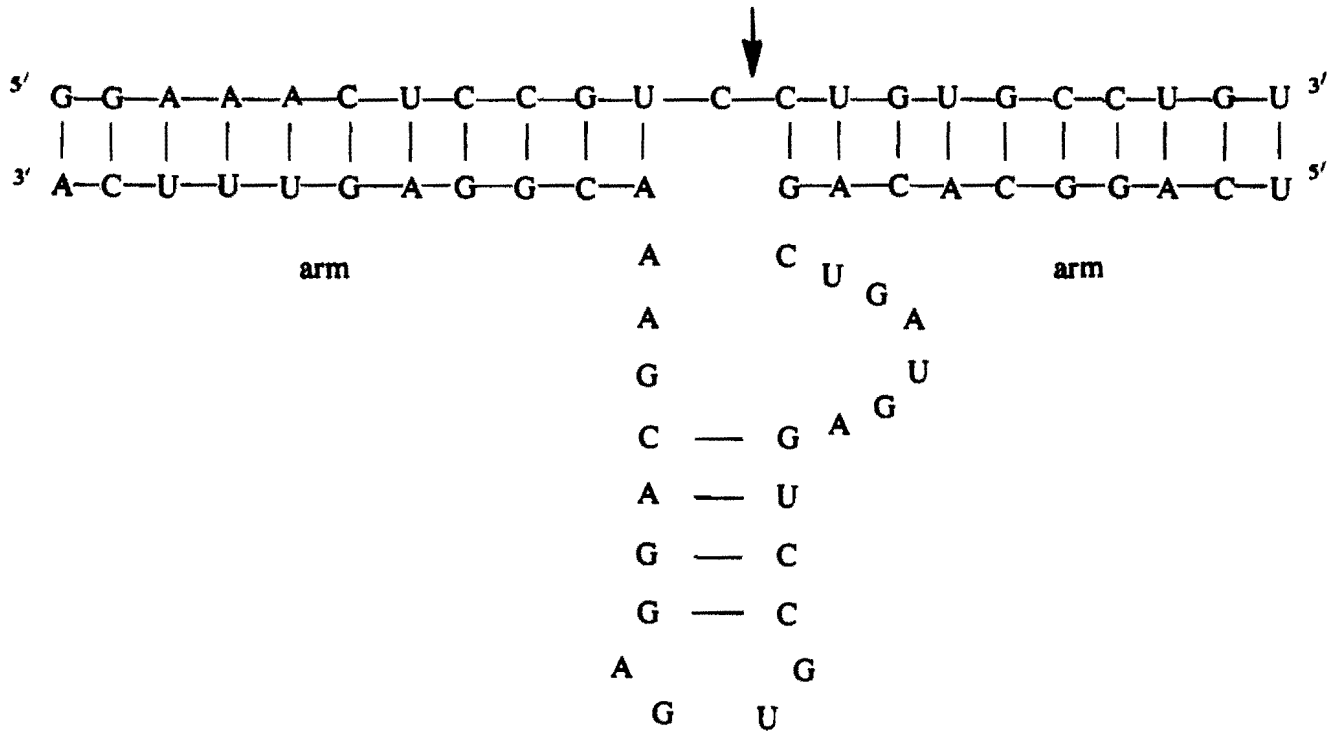

Figure 2. Binding of a ribozyme to part of the putative polymerase gene of potato leafroll virus. The upper sequence is that of PLRV RNA, the lower sequence is that of the ribozyme synthesized in vitro. Arms are the parts of the ribozyme that hybridize to the RNA target. The arrow marks the cleavage site. (Redrawn from data shown by Lamb and Hay 1990).

formation and regeneration seems certain to extend this range of hosts in the next few years.

Coat-protein genes have been taken mainly from viruses that express their coat protein by translation of a subgenomic mRNA (e.g., TMV), but coat protein-encoding sequences have been excised from the genes encoding the polyproteins of potyviruses, e.g., soybean mosaic virus (Stark and Beachy 1989) and tobacco vein mottling virus (Murphy et al. 1990), and linked to an initiation codon prior to the transformation. An alternative strategy is to transform plants with a larger piece of the virus genome that would include the gene for the viral protease as well as that for the coat protein. Expression of the protease sequence should then cleave the polyprotein accurately to release the functional coat protein (Wefels et al. 1990). There appears to be no taxonomic barrier among positive-sense, ssRNA viruses to obtaining virus resistance by transformation with coat-protein genes.

Transformation with some coat-protein genes provides effective resistance against heterologous viruses if the amino acid sequences of the two coat proteins are appreciably similar. Thus, TMV coat protein was effective against ToMV and tobacco mild green mosaic virus, which have coat proteins more than 66% identical to that of TMV (Nelson et al. 1987). In contrast, expression of the coat protein of one strain of tobacco rattle virus (TRV) did not protect plants against infection by another strain of TRV that had a coat protein only 39% identical to the first strain (Van Dun and Bol 1988). However Anderson et al. (1989) have reported weak protection by coat proteins of TMV and AlMV against infection by unrelated viruses.

The effect that transformation of a plant with a viral coat-protein gene has on virus multiplication appears to be that fewer infective centers form in inoculated leaves (Nelson et al. 1987), that systemic spread is slower than in control plants (Nelson et al. 1990), and that less virus accumulates in tissues that do become infected (Cuozzo et al. 1988; Hemenway et al. 1988; Lawson et al. 1990). The degree of protection is inversely dependent on the concentration of the inoculum (Powell-Abel et al. 1986; Hemenway et al. 1988) and, in most cases, seems to depend on the amount of coat protein made in the transgenic cells (Hemenway et al. 1988; Halk et al. 1989). However, this is not always so (e.g., SMV: Stark and Beachy 1989). Indeed, transgenic plants transformed with the coat-protein genes of TRV (Angenent et al. 1990) or PLRV (Kawchuk et al. 1990) have been obtained that are

Table 2. Coat protein-mediated resistance.

Virus group	Resistance induced to	Host	Source of coat protein	Reference
Tobamovirus	TMV	tobacco	TMV	Powell-Abel et al. 1986
	TMV	tomato	TMV	Nelson et al. 1988
	ToMV	tomato	TMV	Nelson et al. 1989
	ToMV	tobacco	TMV	Stark et al. 1990
	TMGMV	tobacco	TMV	Stark et al. 1989
	SHMV	tobacco	TMV	Anderson et al. 1989
Potexvirus	PVX	tobacco	PVX	Hemenway et al. 1988
	PVX	potato	PVX	Hoekama et al. 1988
	PVX	tobacco	TMV	Anderson et al. 1989
	PVX	tobacco	AlMV	Anderson et al. 1989
Cucumovirus	CMV	tobacco	TMV	Anderson et al. 1989
	CMV	tobacco	CMV	Cuozzo et al. 1988
AlMV	AlMV	tobacco	AlMV	Loesch-Fries et al. 1987
	AlMV	alfalfa	AlMV	Halk et al. 1989
	AlMV	tomato	AlMV	Tumer et al. 1987
	AlMV	tobacco	TMV	Anderson et al. 1989
Ilarvirus	TSV	tobacco	TSV	Van Dun et al. 1988a
Tobravirus	TRV	tobacco	TRV	Van Dun et al. 1987
	PEBV	tobacco	TRV	Van Dun and Bol 1988
Potyvirus	PVY	potato	PVY	Lawson et al. 1990
	PVY	tobacco	SMV	Stark and Beachy 1989
	TEV	tobacco	SMV	Stark and Beachy 1989
	TEV	tobacco	PaRSV	Ling et al. 1990
	TEV	tobacco	TVMV	Murphy et al. 1990
	TVMV	tobacco	TVMV	Murphy et al. 1990
Carlavirus	PVS	*N debneyii*	PVS	Mackenzie and Tremaine 1990
Nepovirus	TBRV	tobacco	GCMV	Candresse (pers. comm.)
Furovirus	BNYVV	sugar beet	BNYVV	Kallerhof et al. 1990

Abbreviations: AlMV = alfalfa mosaic virus; BNYVV = beet necrotic yellow vein virus; CMV = cucumber mosaic virus; GCMV = grapevine chrome mosaic virus; PaRSV = papaya ringspot virus; PEBV = pea early browning virus; PVS = potato virus S; PVX = potato virus X; PVY = potato virus Y; SHMV = sunnhemp mosaic virus; SMV = soybean mosaic virus; TBRV = tomato black ring virus; TEV = tobacco etch virus; TMV = tobacco mosaic virus; TMGMV = tobacco mild green mosaic virus; ToMV = tomato mosaic virus; TSV = tobacco streak virus.

resistant to challenge infection even though no virus coat protein could be detected in them.

The mechanism by which coat-protein genes mediate resistance is not clear. At least two distinct mechanisms seem to be involved. Although plants made resistant to inoculation with particles of TMV or AlMV by transformation with their respective coat-protein genes were not resistant to inoculation with virus RNA (Nelson et al. 1987; Loesch-Fries et al. 1987), those expressing potato virus X (PVX) (Hemenway et al. 1988) or potato virus S coat proteins (Mackenzie and Tremaine 1990) were resistant to in-

oculation with either virus particles or RNA. Also, although plants transformed with an AlMV coat-protein gene—one that had been mutated so that RNA accumulated but translation was impossible—were not resistant to challenge inoculation (Van Dun et al. 1988a), transgenic plants in which no TRV or PLRV coat protein could be detected were resistant (see above; Angenent et al. 1990; Kawchuk et al. 1990). A further development in the application of coat-protein gene transformation has been the production of potato plants with field resistance to both PVX and PVY by transforming plants with two different coat-protein genes at the same time (Lawson et al. 1990; Kaniewski et al. 1990).

Satellite-mediated Resistance

Satellite RNA species have been found in association with a number of viruses (Fritsch and Mayo 1989), but only those from CMV and tobacco ringspot virus (TobRV) have been used as sources of resistance. Transformation of tobacco with DNA sequences complementary to satellite RNA variants that ameliorate the symptoms induced by CMV (Baulcombe et al. 1986; Harrison et al. 1987a) or TobRV (Gerlach et al. 1987) resulted in plants that showed milder symptoms and accumulated less virus than control plants after inoculation with the homologous virus.

Plants transformed with CMV satellite cDNA sequences also showed milder symptoms than control plants after inoculation with tomato aspermy virus (which is somewhat related to CMV) but the amounts of virus made by the two types of plant were not greatly different (Harrison et al. 1987a). A secondary effect of infection of satellite-transformed plants was that virus transmitted from the transgenic plants contained satellite RNA and was therefore potentially less pathogenic for nontransgenic hosts than the original satellite-free virus (Harrison et al. 1987b).

Transformation with satellite sequences has been found to give an effective measure of resistance in field trials: CMV infection of transgenic pepper (Tien et al. 1990a), tobacco, or tomato plants (Tien et al. 1990b) resulted in milder disease symptoms and less virus accumulation than infection of control plants.

Results With Antisense RNA

Cuozzo et al. (1988) showed that plants expressing antisense CMV RNA were resistant to infection in that systemic spread was slower and virus accumulation was less than in control plants. However, the effect was only detectable when the inoculum concentration was low; coat protein-mediated protection was effective against CMV inoculum concentrations that overcame the antisense RNA-mediated effect (Cuozzo et al. 1988). Similar results were observed with antisense PVX RNA (Hemenway et al. 1988) and antisense TMV RNA (Powell et al. 1989). In contrast, expression of antisense TRV RNA did not enhance the resistance of plants to TRV infection (Baulcombe et al. 1987; Angenent et al. 1990).

Results With Other Virus Genes

Transformations with genes encoding a variety of nonstructural virus proteins, for example putative replicase proteins from AlMV (Van Dun et al. 1988b), from TRV (Angenent et al. 1990), or the 'transport protein' (30K protein: Fig. 1b) of TMV (Deom et al. 1987), have not yielded virus-resistant plants. However, although Golemboski et al. (1990) found that transformation with sequences encoding the 126K protein of TMV (U1 strain) did not induce resistance, they also found, in contrast, that plants transformed with sequences encoding the 54K C-terminal part of the 183K putative replicase protein (see Fig. 1b) were virus resistant. Moreover, these plants were resistant to inocula containing very high concentrations of virus, sufficient to rapidly overcome resistance mediated by coat-protein expression. Also, unlike plants protected by coat-protein expression, these plants were resistant to infection by RNA inocula. This virtual immunity was much more specific than coat protein-mediated resistance; both the U2 strain of TMV and ToMV readily infected these plants.

Plants transformed with a cDNA copy of the complete genome of a mild strain of TMV were also resistant, not only to challenge inoculation by particles of a genetically very similar but virulent strain of TMV, but also, like those transformed with the 54K gene, to challenge by RNA inocula (Yamaya et al. 1988).

The potential of transformation with genes for certain nonstructural proteins is emphasized by some other recent studies. For example, plants transformed with sequences encoding the cylindrical inclusion protein of TVMV (CI: see Fig. 1d) were more resistant to infection by TVMV than were control plants, but they were also abnormal in appearance; infection with tobacco etch virus induced more severe symptoms in these plants than it did in control plants (Murphy et al. 1990).

Table 3. Field performance of transgenic virus resistance.

Host	Transgene	Yield increase	Reference
Tomato	TMV coat protein	40%	Nelson et al. 1988
Potato	PVX+PVY coat proteins	38%	Kaniewski et al. 1990
Tomato	CMV satellite	14%	Tien Po (pers. comm.)

For definition of virus acronyms, see Table 2.

Prospects and Conclusions

The prospects for being able to confer resistance on agronomically valuable cultivars by transformation with viral genes are very promising. A useful measure of virus resistance has been induced in several plants by transformation with coat-protein genes or satellite-encoding sequences (Table 3). Also, it was found recently that transformation with sequences encoding the nucleoprotein of tomato spotted wilt virus induced virus resistance in tobacco plants (R. Goldbach, personal communication). Other sources of resistance have been tried to a much lesser extent and with less success but many combinations and variations remain to be tested. One further point that should perhaps be made is that although little studied so far, genes encoding antipathetic agents, such as antisense RNA, antibodies or ribozymes, may offer significant promise where there is concern about the release of virus genes into the environment as part of the genome of a crop plant.

Progress to date with transgenic virus resistance has drawn on knowledge of the genome organizations of the target viruses obtained from past, or sometimes very recent, research (e.g., PLRV: Kawchuk et al. 1990). New target viruses may well necessitate study of further genomes. Moreover, the unexpected finding that sequences encoding part of the replicase gene of TMV can induce strong resistance (Golemboski et al. 1990) emphasizes that, even with well-studied viruses like TMV, there are unexplored phenomena available for exploitation in future work.

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Gene Sequences in Viruses, Viroids, and Prokaryotes, and the Development of Diagnostic Tools

R. Goldbach, P. de Haan, and B.J.M. Verduin[1]

Abstract

Gene sequences of plant viral genomes and viroids have provided detailed insight into the genetic properties and interrelationships among pathogens. Plant pathogenic bacteria, however, have been analyzed much less extensively and for only some bacterial avirulence genes have complete sequences been established. With the available sequences, it is possible to develop and apply new diagnostic tools. The use of nonradioactive probes in the detection of nucleic acids has increased and has resulted in sensitivities similar to radioactive methods. With the polymerase chain reaction (PCR) technique—a recent technique by which sequences are enzymatically amplified—viruses have been detected without the use of any specific probe. This technique has immense potential in the detection and identification of pathogens.

Introduction

The current molecular technology, in particular recombinant DNA techniques, has allowed the study of gene composition and nucleotide sequences of plant pathogens. For a growing number of the smallest plant pathogens, i.e., the viroids and viruses, complete nucleotide sequences of their genomes are available. This has enabled the determination of the proteins and the functions encoded by plant viral genomes and the unravelling of genetic relationships between plant and animal viruses. Sequence analysis of viroids has demonstrated that these pathogens do not encode any protein and can be regarded as highly advanced parasites. Data on genome organization and nucleotide sequence for pathogenic bacteria are available for a limited number of cases. The first part of this chapter will discuss the genetic properties of viruses, viroids, and bacteria that infect plants.

The availability of well-defined cloned sequences of viruses, viroids, and bacteria has facilitated the identification and detection of these pathogens based on nucleic acid hybridization, especially utilizing nonradioactive probes. These will be discussed in the second part of this chapter. Another powerful technique for the detection and identification of plant pathogens is based on the polymerase chain reaction (PCR). The advantages and disadvantages of the PCR technique as a diagnostic tool will also be discussed.

Supergrouping of Plant RNA Viruses Based on Genome Sequences

Over 75% of the more than 500 distinct plant viruses possess a single-stranded RNA genome of positive polarity. These viruses have been classified by the International Committee on Taxonomy of Viruses (ICTV) into more than 30 taxonomic groups. They show a wide variation in capsid morphology, and their genomes may be segmented or unsegmented. Some of them have terminal structures such as genome-linked proteins (VPg) or cap structures at the 5'-end, or possess a poly (A) tail or transfer RNA (tRNA) -like structure at the 3'-end. Despite these differences, computer-assisted sequence comparisons of the nonstructural proteins they encode have shown that they are genetically interrelated. The majority of positive-strand RNA viruses have sequence homolo-

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Table 1. Supergroups of positive-strand RNA viruses infecting plants.

Supergroup	Taxonomic group	Common characteristics
Picorna-like	como nepo	5'-VPg, 3'-poly A no subgenomic mRNA potypolyprotein processing set of conserved genes (hel, pro, pol)[1]
Alpha-like	alfalfa mosaic bromo carla clostero cucumo hordei potex tobamo tobra tymo	5'-cap subgenomic mRNA set of conserved genes (mtr, hel, pro) readthrough (most)
Carmo-like	carmo diantho luteo (BYDV) tombus	small genome conserved pol no mtr, no hel
Sobemo-like	luteo (PLRV, BWYV) sobemo	small genome 5'-VPg, no 3'-poly A set of conserved genes (pro, pol) no mtr, no hel

1. Abbreviations: hel = helicase; pro = proteinase; pol = polymerase; mtr = methyl transferase; ( ) indicates putative.

gies with the animal RNA viruses. Thus, these positive-strand RNA viruses can be clustered into four "supergroups" (Goldbach 1986; Goldbach and Wellink 1988; Goldbach et al. 1990) as summarized in Table 1.

The first supergroup encompasses the "picorna-like" plant viruses como-, nepo- and potyviruses, which are related to the animal *Picornaviridae*. The properties these plant viruses share with those of animal picornaviruses are listed below (see also Table 1 and Fig. 1).

- RNAs have a viral protein genome-linked (VPg) at the 5'-end and a poly (A) tail at the 3'-end.
- RNAs are expressed by translation into a so-called "polyprotein" from which the mature proteins are derived by proteolytic cleavages.
- They code for a number of nonstructural proteins exhibiting significant amino acid sequence homology (shaded regions in Fig. 1).
- These conserved proteins have been shown or suggested to be involved in the viral RNA replication process, and are encoded by similarly organized gene sets (Fig. 1).

The conserved replication proteins include a protein with a nucleotide binding (NTB) domain (indicated * in Fig. 1), which may be a helicase (Gorbalenya et al. 1985 and 1989), a proteinase, or a core polymerase (Franssen et al. 1983; Kamer and Argos 1984).

Of the spherical plant viruses, the comoviruses (type member cowpea mosaic virus) appear to be closely related to the picornaviruses. Although comoviruses have a bipartite genome, parts of their genome are colinear with those of poliovirus, one of the best studied picornaviruses (Fig. 1). They may have a common origin, but differences arose due to adaptation of these viruses to either animal or plant host systems. Indeed, comparison with the picornaviruses suggests that the como- (and nepo-) viral RNA genome has become split (a divided genome is often found among plant viruses, but very rarely among animal viruses) and has acquired genes encoding transport proteins (of 48 and 58 kilodaltons) for cell-to-cell transport, via plasmodesmata.

The second supergroup is formed by the so-called "alpha-like" plant viruses, which are all genetically

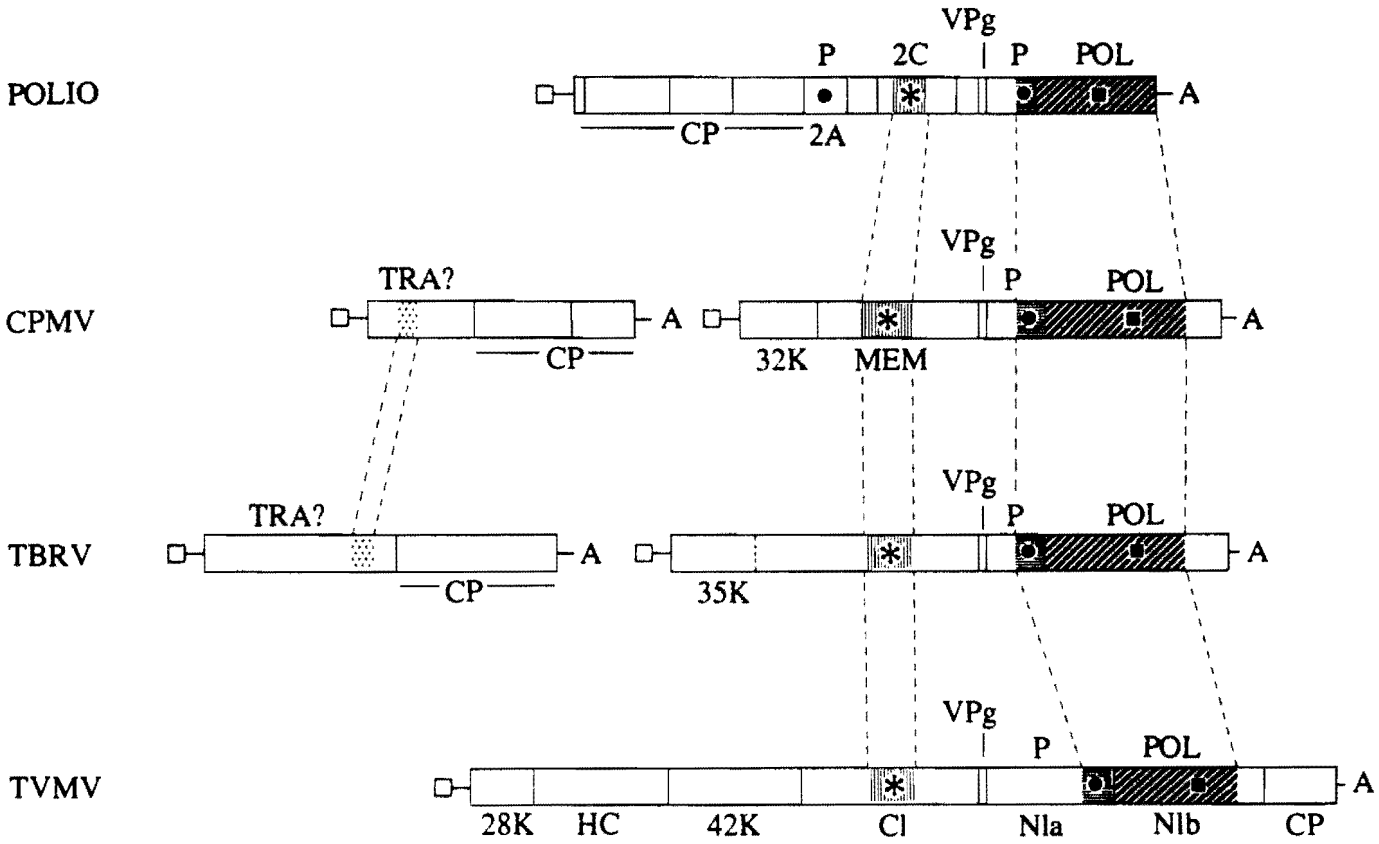

Figure 1. Comparison of the genomes of poliovirus (polio, family *Picornaviridae*) and picorna-like plant viruses: CPMV = cowpea mosaic virus; TBRV = tobacco black ring virus; TVMV = tobacco vein mottling virus. Coding regions in the genomes are indicated as open bars; regions of amino acid sequence homology in the gene products are indicated by similar shading. Other notations: CP = coat protein; TRA = transport protein; P = proteinase; MEM = membrane-binding protein; * = nucleotide binding sequence • = cysteine proteinase ■ = polymerase sequence.

related to the alphaviruses, a genus of the animal virus family *Togaviridae*. In Figure 2 the genomes of a number of alpha-like viruses are compared with Sindbis virus, the best studied member of the alphaviruses. The plant viruses that belong to this supergroup have capped RNA genomes, produce subgenomic mRNAs, and specify proteins exhibiting significant sequence homology to two nonstructural proteins of Sindbis virus. One of the conserved proteins (or protein domains) contains an NTB-domain (* in Fig. 2), which probably represents a helicase, while a second conserved protein or protein domain represents a core polymerase. The alpha-like viruses share, moreover, a third conserved protein (or protein domain), which represents a putative methyl-transferase (Mi et al. 1989), an enzyme that could be involved in capping of the viral genome.

In addition to the two largest supergroups, picorna- and alpha-like plant viruses, there are two smaller supergroups of plant viruses, i.e., the carmo-like and the sobemo-like supergroups (Table 1 and Fig. 3). The carmo-like supergroup comprises small spherical plant viruses like the carmo-, tombus-, tobacco necrosis, and dianthoviruses. They can be distinguished from the alpha-like virus supergroup in that they encode a distinct polymerase, and show partial homology to the polymerases of the animal *Flaviviridae* (Miller and Purcell 1990), and do not possess genes for a putative methyltransferase and helicase (Table 1 and Fig. 3). Since these plant viruses exhibit wide differences from members of the *Flaviviridae*, it may not be appropriate to call these small RNA viruses "flavi-like".

The fourth supergroup are the "sobemo-like" plant viruses that do not exhibit any similarities to known animal viruses. In fact this supergroup shares some picorna-like as well as carmo-like characteristics (Table 1). Sobemo-like plant viruses have a VPg-containing RNA genome and code for a putative proteinase and polymerase like the picorna-like viruses

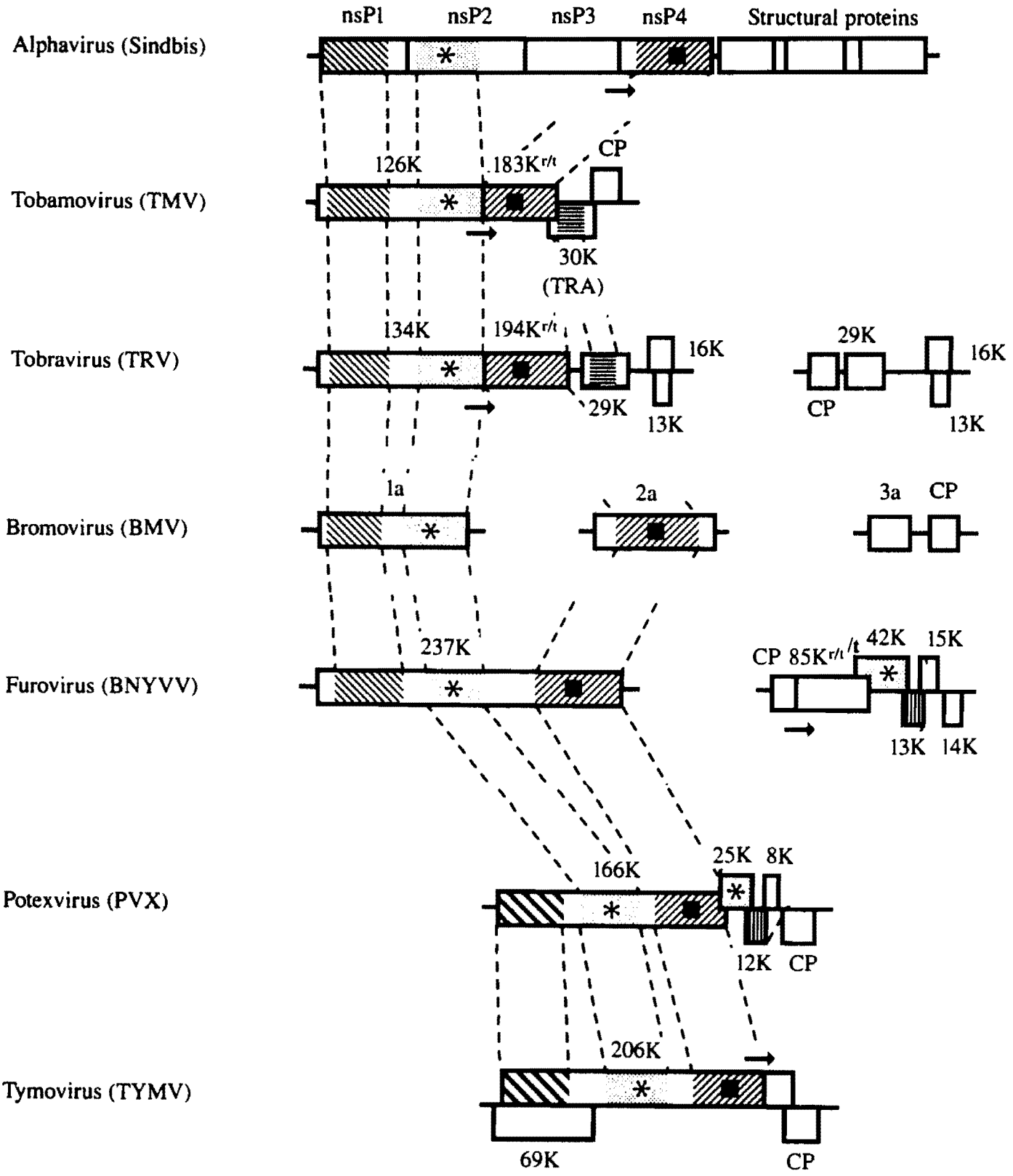

Figure 2. Supergrouping of the alpha-like plant viruses. The genomes of the following viruses are shown: Sindbis virus (genus Alphavirus, family *Togaviridae*); TMV = tobacco mosaic virus; TRV = tobacco rattle virus; BMV = brome mosaic virus; BNYVV = beet necrotic yellow vein virus; PVX = potato virus X; TYMV = turnip yellow mosaic virus. For symbols see Figure 1. Other notations: →▶ = leaky termination codon; r/t = readthrough.

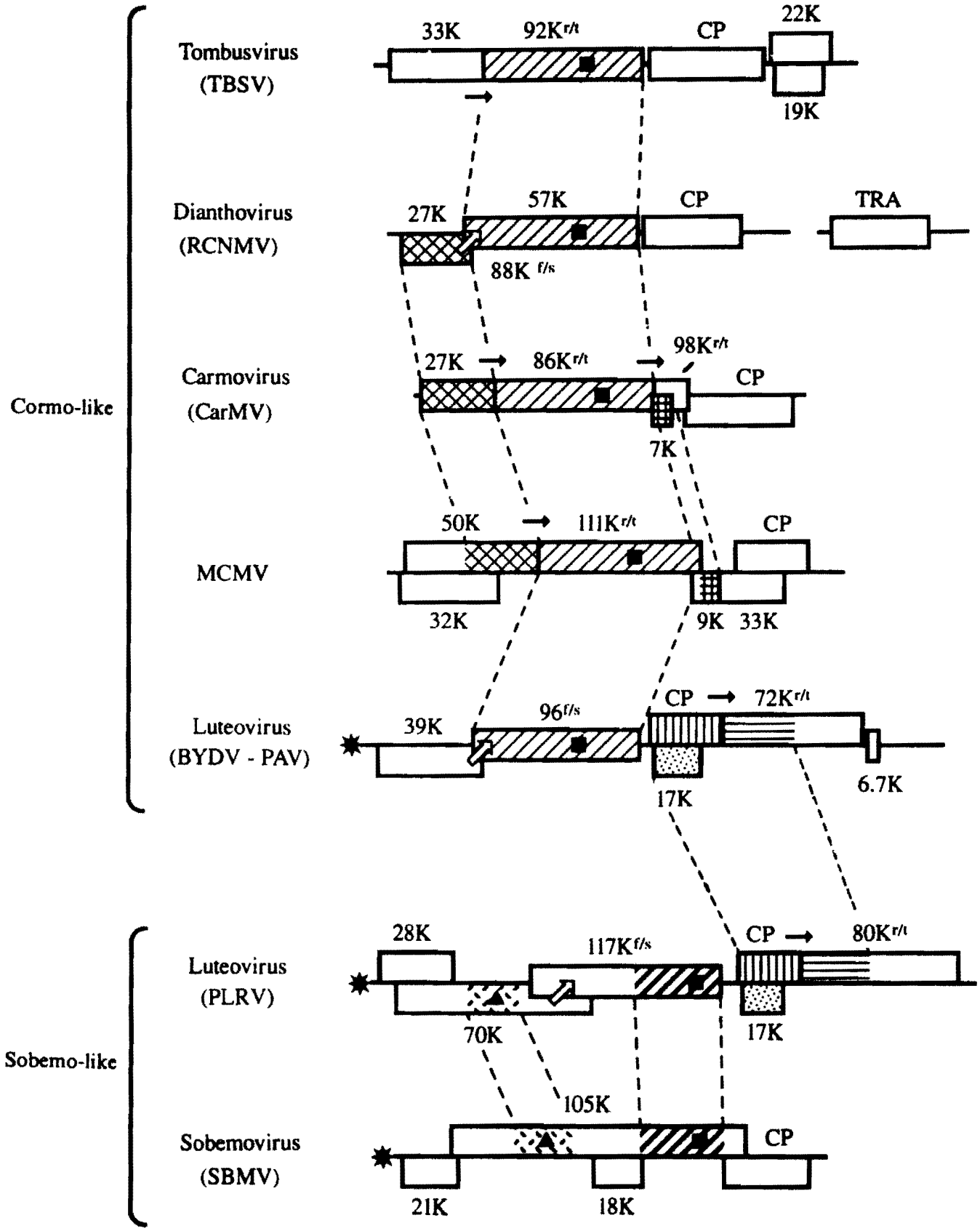

Figure 3. Supergrouping of the small, plant-infecting RNA viruses. Genomes of the following viruses are shown: TBSV = tomato bushy stunt virus; RCNMV = red clover necrotic mottle virus; CarMV = carnation mottle virus; MCMV = maize chlorotic mottle virus (unclassified); BYDV = barley yellow dwarf virus; PLRV = potato leafroll virus; SBMV = southern bean mosaic virus. For symbols see Figure 1. Other notations: ⇗ = ribosomal frame-shift; ✸ = VPg; ▲ = putative serine proteinase domain.

(Table 1 and Fig. 3). Interestingly, some luteoviruses code for a sobemo-like proteinase and polymerase, e.g., potato leafroll virus (PLRV) and beet western yellows virus (BWYV), while some other luteoviruses, such as barley yellow dwarf virus (BYDV), code for a carmo-like polymerase (Fig. 3; Veidt et al. 1988; Van der Wilk et al. 1989). Since the majority of luteoviruses, like sobemoviruses, possess a VPg-containing genome, it is likely that the typical luteoviral genome encodes a sobemo-like polymerase and the atypical luteovirus such as BYDV contains a recombinant RNA that acquired a carmo-like polymerase gene by interviral recombination. As discussed by Goldbach and Wellink (1988) recombination appears to be a major evolutionary mechanism that has led to the current similarities and differences in gene sequences among plant viruses.

Tomato Spotted Wilt Virus, a Bunyavirus Infecting Plants

Compared to the positive-strand RNA viruses, the negative-strand RNA viruses of plants have not received much attention. Until recently the only known negative-strand viruses were rhabdoviruses, which are not known to cause many economically important diseases. Tomato spotted wilt virus (TSWV), which is known to cause many economically important diseases of field crops and horticultural crops, has recently been shown to contain negative-strand RNA. TSWV is unique among plant tissues in that it has a distinct particle morphology and genome structure. It is the only plant virus that is known to be vectored only by thrips (*Thysanoptera*). TSWV particles are spherical, membrane-bound, 80-110 nm in diameter and covered with spikes consisting of two glycoproteins (G1 and G2). The genome consists of three linear RNA molecules of approximately 3000 nucleotides (S-RNA), 5000 nucleotides (M-RNA), and 8500 nucleotides (L-RNA). These genome segments are tightly complexed with nucleocapsid (N) proteins to form circular nucleocapsids (Van der Hurk et al. 1977; De Haan et al. 1989). Based on these morphological properties, TSWV resembles bunyavirus. Recently nucleotide sequence homology was detected between S-RNA and L-RNA of TSWV and bunyavirus.

Like uukuviruses and phleboviruses, two genera of the *Bunyaviridae*, TSWV has an ambisense S-RNA segment, and a fully negative-sense L-RNA segment (Fig. 4; De Haan et al. 1990). Partial sequence determination of the M-RNA has revealed that part of this genome is also of negative polarity (unpublished data). A proposal to classify TSWV as a member of a new genus (denoted Tospovirus) within the family *Bunyaviridae*, has been accepted by the International Committee for Taxonomy of Viruses (ICTV).

The ambisense S-RNA segment (2916 nucleotides) contains two genes, the nucleocapsid (N) protein gene in the viral complementary (vc) strand, and a gene encoding a 52.5 kDa nonstructural protein (denoted NSs) in the viral strand (Fig. 4; De Haan et al. 1990). The 52.5 kDa protein seems to be involved in symptom severity of TSWV infection, since TSWV isolates that produce severe symptoms also produce more NSs protein (organized in filamentous structures in the cytoplasm of infected cells), than isolates that produce mild symptoms (Kormelink et al. 1991).

The N gene has been used in our laboratory to obtain transgenic tobacco plants with high levels of resistance to TSWV (R. Goldbach, personal communication). Hence, this approach holds promise for the control of this very aggressive virus, which has one of the widest host ranges among plant viruses. This conclusion has significance because seldom have genes that confer resistance to TSWV been located in cultivated crop plants.

Viroids: Noncoding Parasitic RNA Molecules

Viroids are the smallest known pathogens that are known to infect only plants. Many of them have been identified. However, many viroids which produce cryptic infection are yet to be identified. Viroids are circular RNA molecules with sizes that range from 240 to 380 nucleotides. With the elucidation of the nucleotide sequence of potato spindle tuber viroid (PSTV) by Sänger and colleagues in 1978 (Gross et al. 1978), the first complete genome sequence of a plant pathogen became available. Since then nucleotide sequences for many viroids have been determined (Table 2). From the sequence data available the following conclusions can be drawn:

- Viroids are naked, circular, single-stranded RNA molecules.
- By internal complementarity they have a double-stranded character (Fig. 5).
- They do not encode any protein.

In spite of their small size and lack of coding capacity, viroids are pathogenic. They can be regarded as the ultimate parasites. By comparing sequences of mild and severe (up to lethal) isolates of

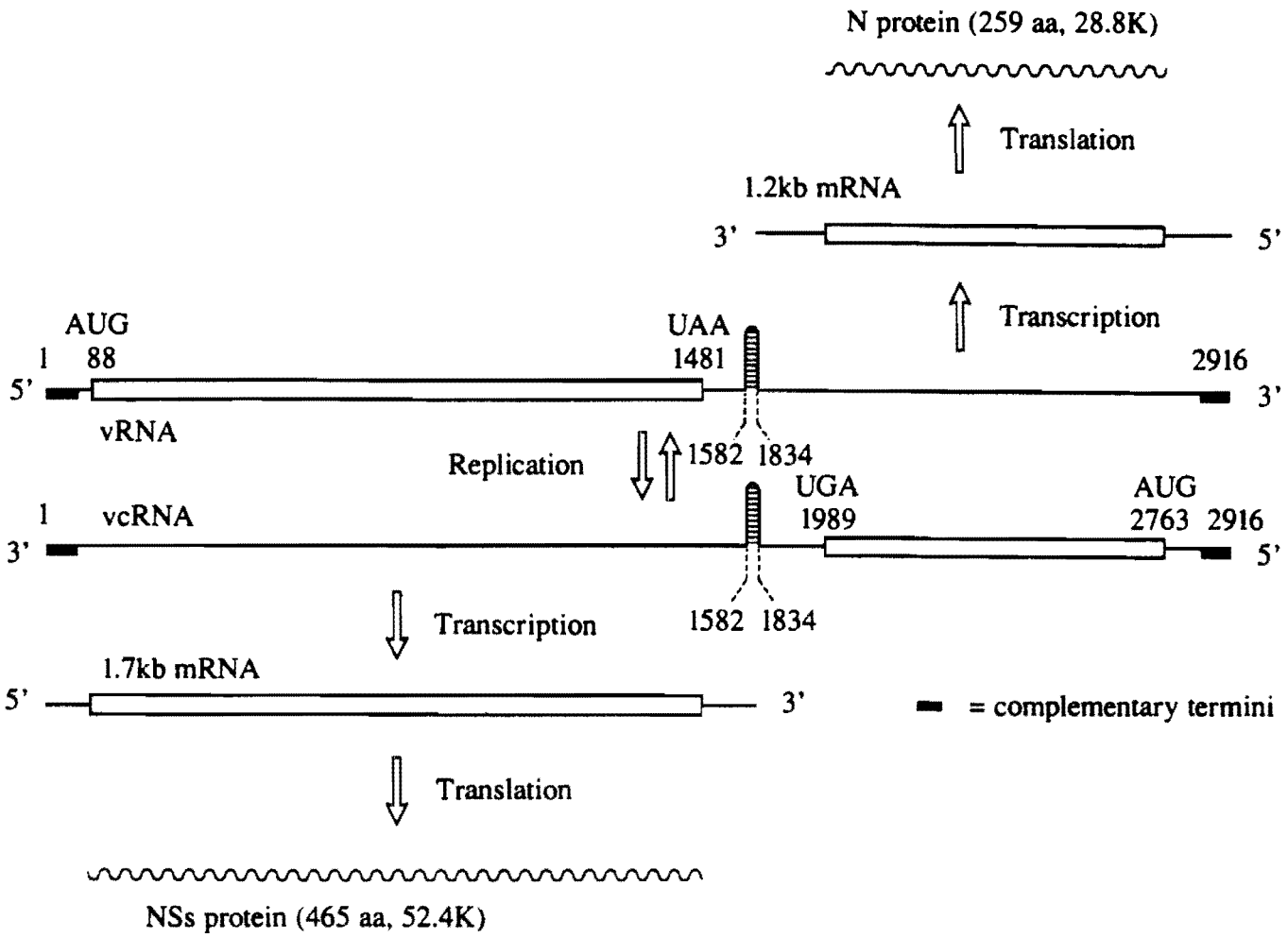

Figure 4. Structure and genetic organization of the ambisense TSWV S-RNA. The S-RNA segment is 2916 nucleotides long, containing one open reading frame in the viral strand (vRNA) and a second open reading frame in the viral complementary (vc) strand. Both open reading frames are expressed from subgenomic mRNAs and specify a non structural protein (NSs) and the nucleocapsid (N) protein, respectively. The intercistronic region contains a long A-U rich hairpin. (Source: De Haan et al. 1990).

PSTV, Sänger and coworkers have tried to unravel the molecular basis for the pathogenicity of viroids (reviewed in Sänger 1987). This has led to the identification of a region (Pathogenicity or P-domain, see Fig. 5) within PSTV that is of great importance for the virulence of the viroid. In spite of the genetic simplicity of viroids, the molecular mechanism of viroid pathogenesis still remains to be elucidated and is still open for speculation.

Sequence analysis of viroids has also revealed that a number of viroids can be regarded as natural recombinants being composed of pieces of up to four distinct viroids (Sänger 1990). Hence, recombination appears to be a very important evolutionary mechanism not only for positive-strand RNA viruses but also for viroids.

Detailed analyses have revealed that the single-stranded viroid RNA has plus polarity and replicates via oligomeric RNA intermediates of both minus and plus polarity, using a rolling circle mechanism (reviewed in Sänger 1987; Tabler and Tsagris 1990). Studies with RNA synthesis inhibitors have shown that DNA-dependent RNA-polymerase II is most likely to be responsible for the synthesis of viroid RNA. The processing of the multimeric replicative intermediates into monomeric viroid molecules is a very intriguing process. While for avocado sunblotch viroid (ASBV) it has been shown that this occurs by autocatalytic self-cleavage, without involvement of host protein or energy (Hutchins et al. 1986), for PSTV and all other viroids tested so far, this cleavage requires host cell factors of still unknown identity. Based on the presence or absence of this self-cleavage capacity and on the nature of the conserved central region (Fig. 5; Koltunow and Rezaian 1989), the viroids have been classified into three distinct groups (Table 2).

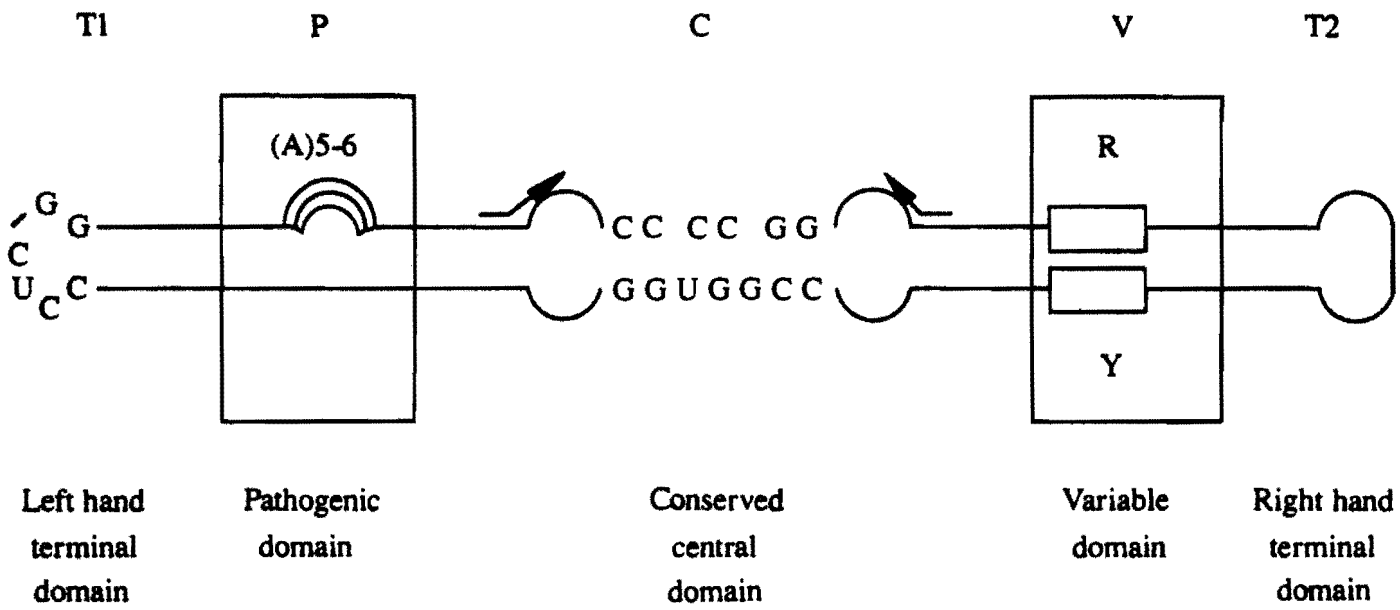

Figure 5. Presence of domains in viroids belonging to the PSTV-group. R and Y represent short oligo-purine and oligo-pyrimidine stretches. (Source: Keese and Symons 1987).

Table 2. Viroids of which complete sequences are known.

Viroids	Abbreviation	No. of nucleotides	Main symptoms
PSTV-group			
Potato spindle viroid	PSTV	359	Stunting
Citrus exocortis viroid	CEV	370,375	Stunting
Tomato planta macho viroid	TPMV	360	Stunting
Chrysanthemum stunt viroid	CSV	354,356	Stunting
Tomato apical stunt viroid	TASV	360	Stunting
Coconut cadang cadang viroid	CCCV	246,247	Chlorosis
Coconut tinangaja viroid	CTiV	254	Chlorosis
Hop stunt viroid	HSV	297	Stunting
Grapevine viroid	HSV-grape	297	Yet undetermined
Hop latent viroid	HLV	256	Latency
Cucumber pale fruit viroid	CPFV	303	Stunting
ASSV-group			
Apple scar skin viroid	ASSV	330	Fruit flecking
Grapevine yellow speckle viroid	GYSV	367	Yellow flecks
Grapevine viroid	GV1B	363	Yet undetermined
ASBV-group			
Avocado sunblotch viroid	ASBV	247	Chlorosis

Source: Sänger (1988) and Koltunow and Rezaian (1989).

Autocatalytic self-cleavage has also been observed during replication of some linear satellite RNAs, e.g., of tobacco ringspot virus, and in particular for circular satellites (referred to as "virusoids"). This self-cleavage occurs at specific secondary structures in the RNA, so-called "hammerhead" structures. Self-cleaving RNA sequences (referred to as "ribozymes") are currently being tested in various laboratories for their potential as antiviral agents (Gerlach et al. 1990).

Table 3. Avirulence genes of bacterial pathogens.

Gene	Bacterium
Chromosomal	
avrA	*P. syringae* pv. *glycinea*
avrB	*P. syringae* pv. *glycinea*
avrAsph1	*P. syringae* pv. *phaseolicola*
avrAspi1	*P. syringae* pv. *pisi*
$avrB_2$	
$avrB_3$	
$avrB_6$	*X. c.* pv. *malvacearum*
$avrB_N$	
$avrB_{In}$	
avrRxv	*X. c.* pv. *vesicatoria*
$avrBs_2$	*X. c.* pv. *vesicatoria*
Plasmidborne	
avrC	*P. s.* pv. *glycinea*
vrBspi2	*P. s.* pv. *pisi*
$avrBs_1$	*X. c.* pv. *vesicatoria*
$avrBs_3$	*X. c.* pv. *vesicatoria*

Source: Vivian (1990).

Genes and Gene Sequences of Plant Pathogenic Bacteria

Compared to viruses and viroids, the plant pathogenic bacteria have been much less intensively studied in terms of isolation and characterization of specific genes and sequences. Two types of genes are of interest and have therefore been studied in most detail. These are the genes that encode the pathogenicity factors, i.e., the proteins and enzymes that make these bacteria parasitic to plants, e.g., pectin lyases and proteinases, and the avirulence (*avr*) genes that interact with dominant resistance (R) genes in the host ("gene-for-gene" interactions, as hypothesized by Flor 1942) to trigger the hypersensitive response (HR) reaction. HR is a localized defense mechanism occurring at or close to the site of inoculation of the pathogen, involving rapid necrosis of plant cells, the accumulation of phytoalexins, and the synthesis of pathogenesis-related (PR) proteins.

While the hypothesis of gene-for-gene interaction (an *avr* gene of the pathogen should match a dominant resistance gene to induce the HR) has been based for many years solely on genetic evidence, direct molecular indications have been obtained for this interaction during the past 5 years. For various plant pathogenic bacteria, *avr* genes have now been cloned and analyzed (Table 3). In particular, the *avr* genes of various pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* have been studied (reviewed in Keen and Staskawicz 1988; Vivian 1990; Keen et al. 1989). Part of these genes are chromosomal while others are plasmidborne. For a number of these genes, DNA sequences are available that may give clues to the mode of action of these bacterial gene products. It is frequently speculated that these products are located at or near the cell surface, thus permitting direct contact with the plant. However, all of the bacterial *avr* gene products determined so far do not exhibit any sequence homology to those in data banks, but certain *avr* genes share significant homology to each other (Vivian 1990).

Development of Diagnostic Tools

Detection of Protein

Enzyme-linked immunosorbent assay (ELISA) is by far the most widely used serological test for the detection of plant pathogens. Several improvements have been made to the classical method developed by Clark and Adams (1977). These include more efficient methods for the isolation and purification of gammaglobulins, of both polyclonal and monoclonal antibodies, and introduction of inexpensive and highly sensitive enzyme/substrate and amplification systems (Stanley et al. 1985). In our laboratory 50 ng of purified potato leafroll virus was detected using enzyme amplification reaction combined with cocktail ELISA (Van den Heuvel and Peters 1989). Polyclonal antibodies made against purified virus preparations are superior compared to monoclonals, because detection of monoclonals is influenced by conformational changes in the pathogen as a result of variable sap conditions.

If amino acid sequences of the surface proteins of pathogens are known, antisera can be developed against linear peptides that are exposed in the protein. These exposed amino acid sequences can be deduced from hydrophilicity plots. If the three-dimensional structure of the surface protein is known, e.g., coat proteins of TMV, SBMV, TCV, TBSV, BPMV, CPMV, surface epitopes can be selected for detection and specific antisera production. Expression of the open reading frame underlying the amino acid sequence of the surface protein of the pathogen, in expression systems, e.g., baculoviruses in insect cells, results in pure antigen that can be used for the induction of antibodies.

The Western blots (combination of ELISA and electrophoresis) allow discrimination of pathogen at the level of the visualized individual proteins. Recently Lin et al. (1990) demonstrated a technique where fresh sections of healthy and infected tissue were blotted on filters. The pathogen was detected in specific tissues with immunological techniques and could be traced with a light microscope on the filter because the precipitate formed a cellular replica of the infected tissue.

Immunoglobulins conjugated to gold have been applied to detect antigen-antibody complexes on filters. They are also currently being used for the detection of pathogens in thin sections of infected leaf tissue. At low magnification with the epi-illumination microscope, a brilliant blue color is seen at the labelled sites while at high magnification in the electron microscope black spheres mark the location of the pathogen (Van Lent and Verduin, 1991).

Detection of Nucleic Acids

Several improvements have been made to the application of complementary DNA or RNA probes to detect pathogens. Mostly ^{32}P-labelled nucleotides were used to label these nucleic acids radioactively, with specific activities up to 10^{10} cpm μg^{-1}, which can detect 1 pg of target sequences. Squash blots have been used successfully with small tissue samples and aphids (Boulton et al. 1984).

Over the past 10 years, several laboratories have reported utilization of nonradioactive probes. At first biotinilated probes were used (Langer-Safer et al. 1982) since they interacted strongly and specifically with avidin and streptavidin, which in turn were coupled to alkaline phosphatase. Streptavidin was preferred because of its more neutral isoelectric point and in causing lower backgrounds. Many other haptens

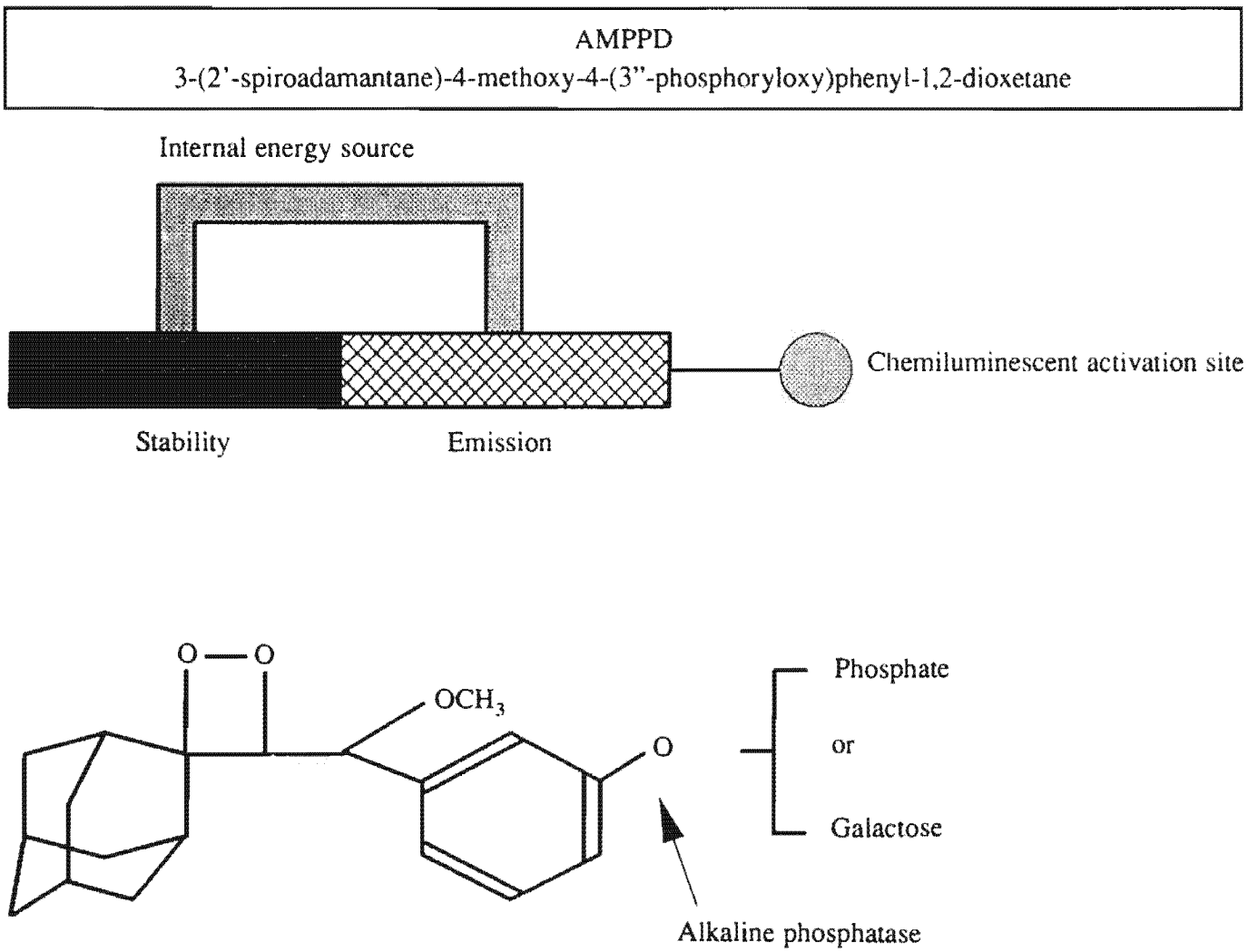

Figure 6. Schematic representation of AMPPD [3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane] and its separate functional building blocks.

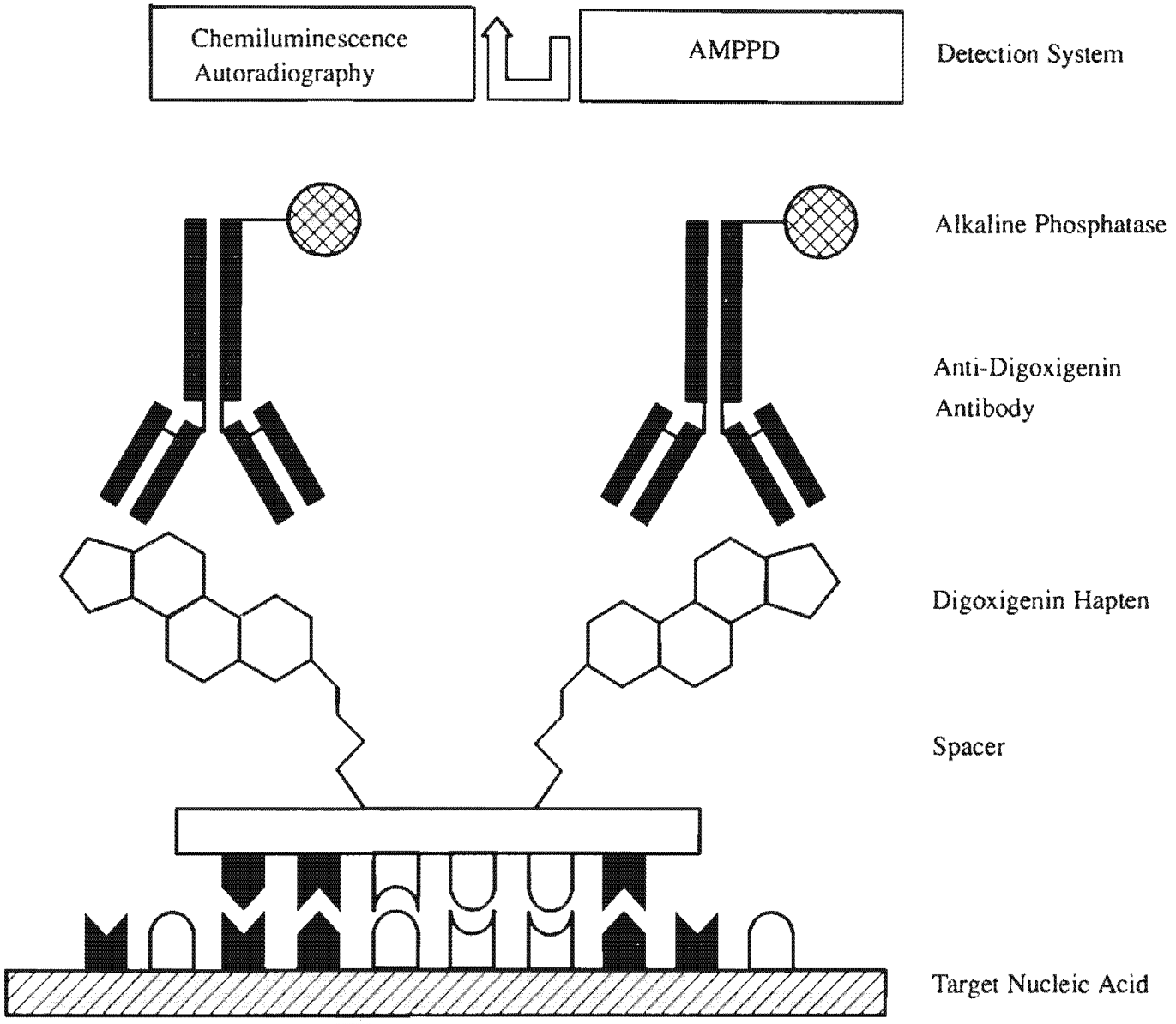

Figure 7. Schematic representation of nonradioactive hybridization and subsequent detection with alkaline phosphatase and AMPPD as chemiluminescent substrate.

such as dinitrophenol (Shoyer and Nakane 1983), 2-acetylaminofluorene (Landegent et al. 1984) and digoxigenin (Boehringer-Mannheim, GmbH., Mannheim, Germany) have been used as substitutes for biotin. Digoxigenin was originally developed for routine detection of viroids but now it is used in many other systems. High-quality digoxigenin-labelled nucleotides, e.g., Dig-dUTP, are commercially available. The detection of the hapten occurs with systems similar to those used for protein detection. The enzyme/substrate combination most commonly used is alkaline phosphatase/nitroblue tetrazolium with dichlorophenol indophenol. The sensitivity of this method is comparable to radioactive labeling, i.e., up to 1 pg of target sequences (Roy et al. 1989).

Computer-aided design and sophisticated synthesis protocols have led to development of a well-defined substrate that meets the requirement of an ideal substrate. AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane) (Bronstein et al. 1990) consists of three different elements (Fig. 6), one to make the compound soluble and stable in water, one that contains the energy source, and one that acts as a fluorophore. This complex is activated by alkaline phosphatase resulting in removal of phosphate. The phosphatase excites many fluorochromes that can emit a constant amount of light for a certain time period. The light emitted by the fluorochromes is detected by autoradiography (Fig. 7). Several exposures can be made on different films. A further advantage is that fluorochrome can be washed away and the alkaline phosphatase can be inactivated, thus permitting probing of target sequences with a different label.

Reprobing is often applied with the restriction fragment length polymorphism (RFLP) method to detect different genes or other molecular markers. This method has been used in detection, classification, and identification of pathovars in fungi and bacteria (Panabières et al. 1989).

In the 1970s, most probes were prepared by nick-translation of double-stranded DNA and, later, primer-directed synthesis of pathogen sequences was achieved by cloning into phage DNA. Often vector sequences were attached to the probe. The development of vectors carrying two different promoters for DNA-dependent RNA polymerases in different directions enclosing a multilinker site enables preparation of RNA transcripts with both polarities and without any vector sequences. Of course the abovementioned nucleotides conjugated to different haptens have also been applied to these transcripts called "riboprobes". The advantages and disadvantages of nonradioactive probes are the following.

Advantages

- Stability of probes
- No radioactivity
- Inexpensive
- Rapid detection

Disadvantages

- Possible stearic hindrance during hybridization, because of hapten presence

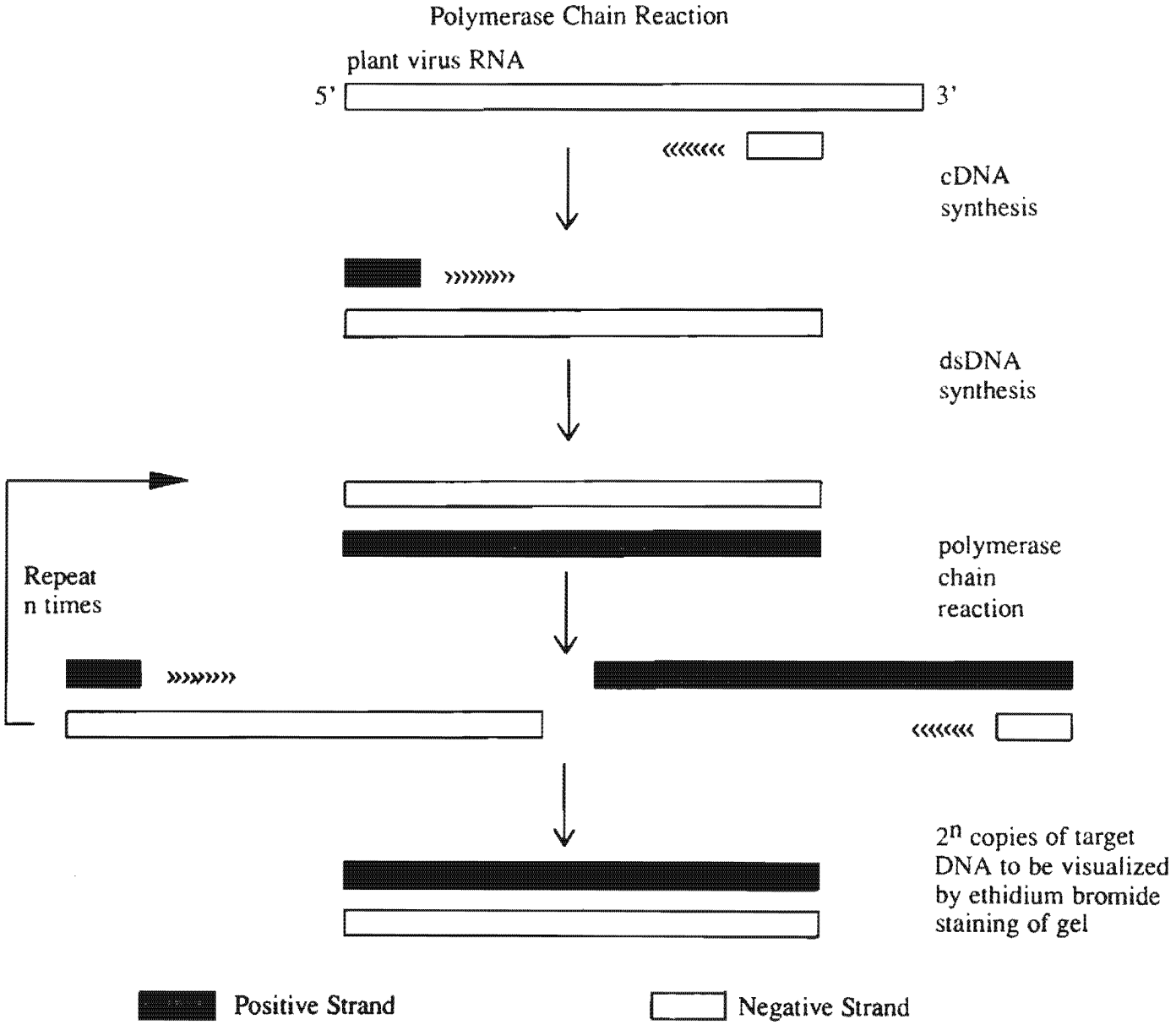

Figure 8. Schematic representation of the amplification of a plant virus single-stranded RNA with the polymerase chain reaction (PCR).

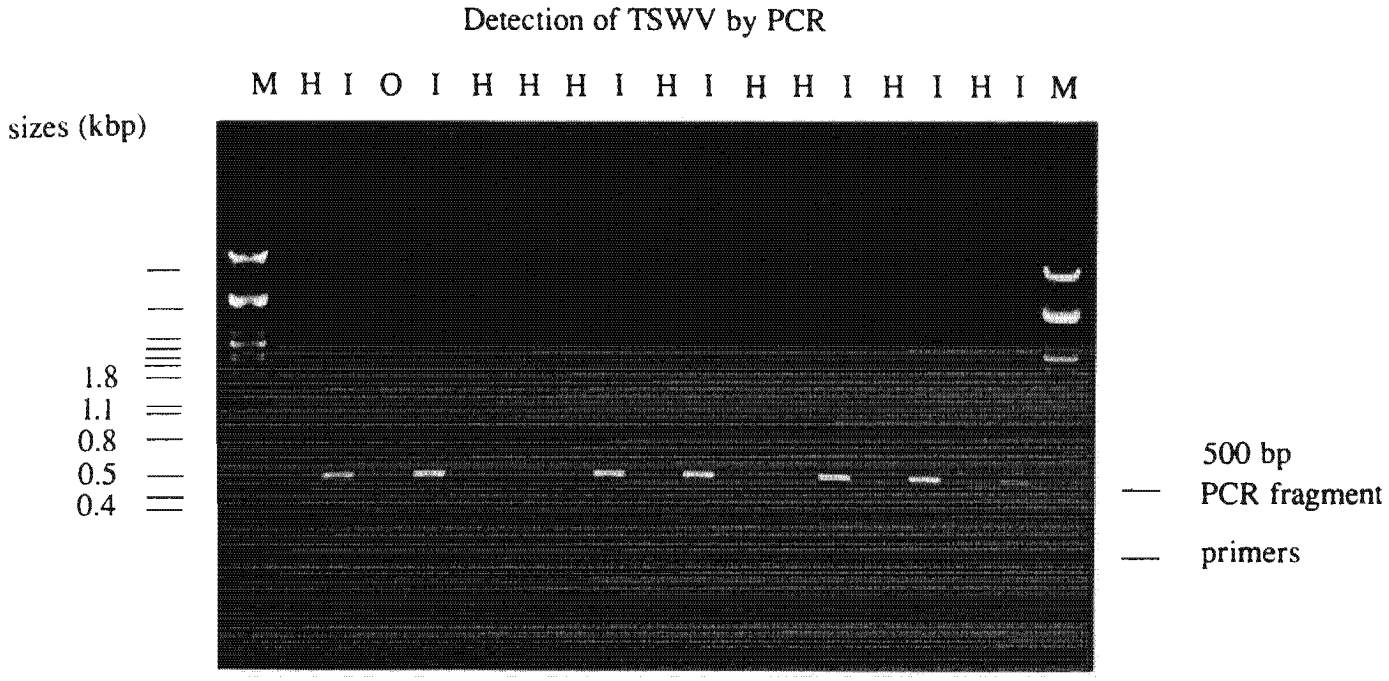

Figure 9. Detection of tomato spotted wilt virus (TSWV) by PCR. The agarose gel shows DNA fragments obtained by reverse transcription and subsequent amplification of leaf sap samples from healthy (H) and TSWV-infected (I) tobacco plants using synthetic oligonucleotides derived from the TSWV S-RNA sequence. M = Marker lane (phage DNA restricted with PStI); O = negative control without leaf sap.

Finally, the most recent and one of the most promising techniques is the polymerase chain reaction (PCR). With this technique nucleic acids can be enzymatically amplified by several cycles of nucleic acid synthesis. PCR requires knowledge of (some) sequences of the nucleic acid to be amplified in order to prepare the oligonucleotide primers. In case of double-stranded DNA the strands are separated at a temperature of around 95°C and after addition of the primers both strands are reannealed at a temperature of around 30°C with their respective primers. The whole mixture, including the four nucleotides and the thermostable Taq-polymerase, is then incubated for several minutes at 50°C to synthesize the full complementary strands. These are denatured again and the whole cycle is repeated (Fig. 8). After 25 cycles, one molecule is amplified to 30 million molecules. In terms of equipment, a simple waterbath where the temperature can be changed within several minutes from 90°C to 30°C and vice versa is sufficient, though sophisticated. Highly sophisticated machines have been constructed that are programmable and can be left overnight to process some 50 different samples.

Possible applications of PCR have been described in recent handbooks (Ehrlich 1989; Innis et al. 1990). For detection of pathogens with known sequences, it is possible to amplify a few molecules from a leaf sample and visualize the product by ethidium bromide staining of the product as shown for TSWV in Figure 9. Examples can also be given of amplified sequences that are hybridized with a specific probe. For example in several picornaviruses there is a region in the genome where the end sequences are highly conserved and where the middle sequences are specific for subgroups like rhinoviruses and enteroviruses. After amplification of several samples with the conserved primers, the product is spotted on filters and hybridized with rhinovirus or enterovirus-specific probes, thus permitting not only detection but also identification of strains or types within the family. A similar approach may be applicable to plant viruses, viroids, and prokaryotes if more genome sequences become available.

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Biotechnology in Plant Virus Research at the National Botanical Research Institute of India

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Recent biotechnological approaches have shown immense potential in various aspects of crop improvement. A wide variety of techniques used for the diagnosis of viruses, methods for eliminating viruses through tissue culture coupled with chemotherapy, and recent techniques of transforming plants with desired genes have not only shown potential in crop improvement, but have also unraveled approaches that can be used to manage virus and viroid incited diseases.

The National Botanical Research Institute (NBRI) is primarily engaged in research on virus diseases of ornamental plants, with special reference to their identification, diagnosis, and management. Viruses affecting *Gladiolus*, *Chrysanthemum*, *Amaryllis*, *Petunia*, *Gerbera*, and Ice plant have been identified on the basis of biological properties, transmission characteristics, and morphology of virus particles, and serology. Viruses that are frequently isolated are bean yellow mosaic virus (BYMV) on *Gladiolus*; chrysanthemum virus-B, tomato aspermy virus (TAV), and a strain of cucumber mosaic virus (CMV) on *Chrysanthemum;* Hippeastrum mosaic virus on *Amaryllis*; petunia mosaic virus on *Petunia;* Dorotheanthus chlorotic spot virus on *Dorotheanthus*; and an unidentified ilarvirus on *Gerbera*. Methods for purification for various viruses have been standardized and polyclonal antisera produced.

Immunodiagnostic techniques such as enzyme linked immunosorbent assay (ELISA), Ouchterlony's double diffusion test, dot immunobinding assay, and immunosorbent electron microscopy for trapping and decoration of particles as well as their labelling with gold particles is being used at NBRI for various purposes. These purposes relate to identification and differentiation among strains, mass detection of virus(es), and detection of contamination of unrelated particles in a purified preparation of a virus.

Double antibody sandwich ELISA (DAS-ELISA) is the method of choice for detecting *Hippeastrum* mosaic virus in *Amaryllis* and in *Hippeastrum* while indirect ELISA is being used for detection of BYMV in *Gladiolus*, and TAV, CMV, and Chrysanthemum virus-B in *Chrysanthemum*.

Tissue culture coupled with chemotherapy is being exploited for elimination of viruses from infected stock. Inclusion of virazole and some dyestuffs in tissue culture medium has given encouraging results. Viruses like potato virus X and Y, eggplant mottled crinkle virus, *Dorotheanthus* yellow spot virus, petunia mosaic virus, and an ilarvirus causing chlorotic stunt in *Gerbera* have been successfully eliminated using virazole. Dyestuffs like ethidium bromide, malachite green, acridine orange, and eosin Y, though effective, were not able to completely eliminate viruses.

Recently, we have initiated work for developing transgenic plants expressing coat-protein genes for management of a virus affecting ornamentals as well as a few other economic plants. A strain of CMV causing lethal necrosis in tobacco has been selected for study. RNA was isolated from purified virus particles. cDNA synthesis was carried out and the complementary ds DNA fragments have been cloned in a plasmid pBSKS. The cDNA library consists of 630 clones of varying sizes.

Five clones were selected and plasmids carrying the inset DNA were digested with XbaI and PstI and separated from the plasmid DNA by electrophoresis on agarose gels. The band was excised and the insert DNA was purified using DEAE paper. The insert DNA thus separated from the gels were labelled by

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nick translation. The labelled probes were used to detect the corresponding viral RNA in Northern blots. Thus, clones representing RNA-1, RNA-2, and RNA-3 have been identified. The RNA-3 clone gave a weak signal with RNA-4 as well, indicating that the clone representing RNA-3 consists of a major portion of RNA-3a and only a minor portion of RNA-3b.

These results have proved that the putative satellite RNA which we earlier observed in preparation of virus particles did not hybridize to any of the clones representing RNA-1, RNA-2, and RNA-3. Additionally this satellite RNA is not infectious in the absence of genomic RNAs that had been worked on earlier by us and reported by other workers as well. It also was earlier observed that approximately 50% of the RNA isolated from purified virus particles constitutes the satellite RNA. Currently, we are studying the role of this RNA in symptom modulation.

Our future work to these clones would embrace several aspects. The primary aim, however, is to raise transgenic tobacco plants expressing the coat protein gene for development of built-in resistance in tobacco against CMV. Subsequent aims include differentiation of CMV strains based on RNA protection assays using clones representing RNA-1, RNA-2, RNA-3, and RNA-4, separately.

Of the six strains of CMV isolated by us from different hosts, five contain satellite RNA. Once we are able to get a representative clone of satellite RNA of the CMV strain causing lethal necrosis in tobacco, we would try to establish its homology/heterogeneity with satellites associated with different strains. Simultaneously, we are attempting to isolate and clone the satellite RNA of the other strains for our future work on the role of satellite RNA in symptom modulation in different host-virus combinations.

Immunological Detection and Epitope Analysis of Plant Pathogens and their Gene Products: Monoclonal Antibodies in Plant Pathology

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Abstract

Various applications of monoclonal antibodies (McAbs) in detection assays, epitope analysis, and characterization of strains/isolates of plant viruses belonging to tobamovirus, ilarvirus, luteovirus, potexvirus, and potyvirus groups are given. In all these cases viral structural proteins were used for McAb production. Examples of monoclonal antibody production for nonstructural viral proteins are also given. McAbs so far have not been widely used for studies on plant pathogenic bacteria. Examples in which McAbs have been produced for bacterial lipopolysaccharides or extracellular endopectate lyases are given. In limited number of cases McAbs have been used for distinguishing bacterial strains. Various prokaryotes for which McAbs are available include spiroplasmas and mycoplasma-like organisms. They facilitated diagnosis and in some cases identification of strains. McAbs are currently not being widely used for the identification of fungal pathogens. Examples of McAb produced for fungi are given.

The full potential of hybridoma technology has been exploited only in the case of plant viruses. The superior discriminatory potential of McAbs, especially for distinguishing strains/biotypes/pathotypes in the case of plant pathogenic bacteria, prokaryotes, and fungi is yet to be tapped.

Introduction

Functions of plant pathologists include diagnosis of plant diseases, detection and identification of disease-causing organisms, and research on biotic pathogens and host-pathogen interactions (Grogan 1981). An ultimate goal in plant pathology is plant health, that is, disease control. The correct diagnosis of any disease is, of course, a prerequisite for its control. The more rapidly and accurately the disease-causing organism can be identified, the sooner proper control measures can be initiated. Common methodologies in disease diagnosis and pathogen detection include symptomatology, microscopy, microbiological techniques, bioassay techniques, and serological techniques.

Plant pathologists have found immunochemical techniques to be extremely useful for the rapid and accurate routine detection of plant pathogens and ultimately the diagnosis of the plant disease (Clark 1981; Hampton et al. 1990). These techniques have also been found useful for the identification and quantitative assay of plant pathogens and for determining the degree of similarity between members of the various groups of plant pathogens (taxonomy), as well as for studying the functional and structural aspects of specific antigens associated with plant pathogens, i.e., structural and nonstructural gene products.

The introduction of hybridoma technology (Kohler and Milstein 1975) has provided methods for the production of homogeneous and biochemically defined immunological reagents (monoclonal antibodies) of identical specificity, which are produced by a single cell line and are directed against a unique epitope of the immunizing antigen. However, monoclonal antibody production has only recently been adapted to the field of plant pathology (Halk and

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DeBoer 1985; Hsu et al. 1984; Sander and Dietzgen 1984; Van Regenmortel 1984a). The purpose of this report is to review the current status on the production, characterization, and use of monoclonal antibodies in plant pathology. Detailed procedures on the production of monoclonal antibodies have been well documented. Readers interested in the various aspects of producing hybridomas are referred to Campbell (1984), Goding (1983), Zola (1987), and Jordan (1990). Attention here will be focused on the utilization of monoclonal antibodies for the detection and identification of plant pathogens, the characterization and analysis of variants and strains, and the study of the antigenic structure of pathogen-related proteins. Additional, alternative, and new avenues in hybridoma production reported in the last few years will also be discussed. For a more detailed consideration of specific pathogen groups or species, the reader should consult reviews by Halk and DeBoer (1985), Hsu et al. (1984), Sander and Dietzgen (1984), Van Regenmortel (1984a, 1984b), and specific research articles.

Some Useful New Avenues in Hybridoma Production

The hybridoma technique has made it possible to develop monoclonal antibodies (McAbs) in almost every situation where polyclonal antibodies are used at present and in several cases where conventionally produced antibodies have been unsatisfactory. Large numbers of hybridomas (high efficiency of fusion of donor spleen cells and myeloma fusion partner) have been developed from fusions using viruses as immunogen, and a large proportion of those hybridomas secreted the desired antibodies specific for the immunogen (high specific efficiency). However, many fusions have had specific efficiencies of close to zero. Realization of the potential of antigen-specific McAbs is often limited by failure to stimulate adequate numbers of antigen-specific B lymphocytes (spleen cells) during the in vitro immunization procedure. This failure can be due to any number of factors. Some of these include: low levels of antigen or 'poorly' immunogenic antigen (resulting in low splenic content of the appropriate cells), an antigen hierarchy response (selective responsiveness to one or a few components of the immunogen preparation; usually the wrong one), or tolerance (antigen-specific unresponsiveness) (Reading 1986). Several techniques have recently been employed to improve the yield of antigen-specific hybridomas. Some of these procedures are described below.

Intrasplenic (direct injection of the spleen in the animal; Spitz 1986) and in vitro (sensitization of cultured spleen cells with antigen; Reading 1986) immunization protocols have been found to increase specific efficiency compared to the standard in vivo immunization. These procedures are extremely useful when very low amounts of antigen are available. The "immunization" schedule is reduced to 4-10 days. However, most of the McAbs obtained are concomitantly of the IgM isotype. An increase in the IgG response can be achieved by using an in vitro immunization as the secondary (or tertiary) immunization (Jordan et al. 1984; Spraganian et al. 1983). Adoptive transfer of spleen cells from immunized animals to X-irradiated syngeneic recipients followed by in vivo antigen boosting has resulted in a 10- to 50-fold increase in the percentage of antigen-specific hybridomas (Spraganian et al. 1983).

Other immunization-related techniques include using more highly immunogenic adjuvant peptides, e.g., N-acetylmuramyl-L-alanyl-D-isoglutamine (Reading 1986), and injecting suspensions of antigen-bearing particles derived from antigen-spotted or blotted nitrocellulose (Abou-Zeid et al. 1987; Knudsen 1985).

Procedures relating to the selection and/or enhancement of antigen-specific B lymphocytes or to the suppression or induced tolerance of nontarget antigen-specific lymphocytes have also been described. Separation of spleenocytes prior to fusion over a Percoll gradient, in order to prevent overgrowth of hybrids by macrophages and fibroblasts and increase fusion efficiency, has led to a higher yield of antigen-specific hybridomas (Van Mourik and Zeizlemaker 1986). Solid-phase immunoadsorption removal of nontarget antigen-specific lymphocytes using anti-nontarget antigen antibodies has also been shown to be a viable protocol for increasing the yield of target-specific hybridomas (George and Converse 1988; Mage et al. 1977). Monoclonal antibody production by receptor-mediated electrically induced cell fusion (Lo et al. 1984) is another form of the B lymphocyte enrichment procedure. Suppression of immune response by cyclophosphamide (Campbell 1984) or induction of tolerance (Hockfield 1987; Hsu et al. 1988, 1990a, 1990b) has also been used. High doses of an identified nontarget antigen presented neonatally can suppress the ability of the immune system to recognize that nontarget antigen later in life when both target and nontarget antigens are present in the immunogen mixture (Golumbeski and Dimond 1986; Hockfield 1987; Hsu et al. 1988, 1990a, 1990b).

The successful production of stable rabbit-mouse hybridomas that secrete complete rabbit Ig monoclonal antibody of defined specificity (Raybould and Takahashi 1988) should enable the production of McAbs to antigens that were not immunogenic in mice.

Monoclonal Antibodies in Plant Virology

Monoclonal antibodies have been produced to numerous plant viruses representing different taxonomic groups since 1981. McAbs have been obtained that react with one or more members of the tobamoviruses (Al Moudallal et al. 1982, 1984; Briand et al. 1982), the potexviruses (Torrance et al. 1986a), the potyviruses (Bahrani et al. 1986; Culver and Sherwood 1988; Dougherty et al. 1985; Gugerli and Fries 1983; Hill et al. 1984; Hsu et al. 1988; Jordan and Hammond, 1991; McLaughlin et al. 1986; Sherwood et al. 1987; Wang et al. 1984; Wisler et al. 1989; Yao et al. 1984), the luteoviruses (Diaco et al. 1986b; Hsu et al. 1984; Hu and Rochow 1988; Martin and State-Smith 1984; Slack et al. 1984; Torrance et al. 1986b), the sobemoviruses (Tremaine et al. 1985a, 1985b), the nepoviruses (Power 1986), the ilarviruses (Aebig et al. 1987; Halk 1984; Halk and Burhop 1985; Halk et al. 1984; Jordan and Aebig 1985; Jordan et al. 1984, 1985a), the phytoreoviruses (Jordan et al. 1985b), the caulimoviruses (Hsu and Lawson 1985), as well as to a number of viruses in other groups (Gracia et al. 1986; Hiruki et al. 1984; Hsu et al. 1990a; Hu et al. 1990; Martin 1984; Parmer 1990; Sherwood et al. 1989; Vela et al. 1986).

Monoclonal antibodies have also been produced to nonstructural viral proteins. For example, McAbs specific to a cloned protein from cucumber mosaic virus were produced by MacKenzie and Tremaine (1988). McAbs specific to potyvirus helper components (Jordan et al. 1990) and potyvirus cytoplasmic inclusion protein (Baker and Purcifull 1990) have also been generated. In addition, McAbs specific to double-stranded RNA have also been produced (Garcia et al. 1986; Lukacs 1990; Power 1987).

In the following sections, we will attempt to illustrate, with specific examples, the application of hybridoma technology to plant virology and how it has provided an expanded battery of both biological and analytical tools for the investigation of plant viruses and virus-host interactions.

Tobamoviruses

The monospecificity of McAbs makes them particularly useful for studying the complex antigenic structure of proteins. One of the first reports in plant pathology using monoclonal antibodies to map the antigenic structure of a protein was given by Al Moudallal et al. (1982) and Van Regenmortel (1984a) with tobacco mosaic virus (TMV). The antigenic structure of TMV was analyzed by measuring the ability of nine McAbs to distinguish between wild type virus and thirteen mutants having single and double amino acid substitutions in the coat protein. The majority of the tested antibodies detected those substitutions that were situated on the outer surface of the virion. In addition, some of them also recognized conformation changes that were induced by substitutions occurring deep within the protein subunit. Each of the nine tested McAbs possessed a unique discrimination pattern with respect to the different substitutions.

In another study with these same nine McAbs (Briand et al. 1982), several serologically indistinguishable (by polyclonal sera) strains of TMV could be differentiated for the first time. Dietzgen and Sander (1982, 1984) produced and identified a virus infectivity-neutralizing McAb that reacted with the carboxy-terminus of TMV coat protein. They proposed a biological function for the carboxy-terminal tetrapeptide based on this data.

Ilarviruses

The ilarviruses, apple mosaic virus (ApMV) and *Prunus* necrotic ringspot virus (NRSV), have been shown to cause serious diseases in a number of crops, including stone and pome fruits, and rose. Halk et al. (1984) produced 14 hybridomas secreting McAbs specific to NRSV, ApMV, tobacco streak virus (TSV) or alfalfa mosaic virus (AMV). Seven of the McAbs recognized six distinct epitopes among the two viruses NRSV and ApMV. These McAbs were able to define 5 serotypes of ApMV and 3 serotypes of NRSV from a collection of 21 ApMV or NRSV strains. In using various immunosorbent and western blot analyzes with these McAbs, it was demonstrated that these seven antibodies possessed seven unique epitopes, two of which are common on other unrelated ilarviruses (Aebig et al. 1987; Jordan and Aebig 1985; Jordan et al. 1985a). Some of these epitopes are present only on intact virions (conformation dependent, external epitopes), whereas other epitopes are present only on disrupted virions (conformation independent, non-surface epitopes). Three of the McAbs have been shown to neutralize infectivity in vitro (Aebig et al. 1987). A modified indirect ELISA using a mixture of three NRSV-specific McAbs as "coating

antibody" and a mixture of two NRSV-specific McAbs as alkaline phosphatase-conjugated "detecting antibody" was shown to be effective in detecting purified virus and virus-infected plants (Halk 1984).

Luteoviruses

Barley yellow dwarf virus (BYDV) is an economically important plant virus worldwide in barley, wheat, and oats. It occurs in many perennial grasses. Several distinct strains have been characterized based upon specificity of aphid transmission. The five North American groups are serologically distinct using polyclonal antisera. Hsu et al. (1984) produced four strain-specific McAbs and three cross-reactive McAbs, illustrating the presence of at least two common epitopes among two separate strain-pairs. They also demonstrated that injection of one of the BYDV-specific McAbs into aphids reduced their ability to transmit virus. Anti-idiotypic polyclonal antibodies were raised against this neutralizing McAb to study epitope specificity of the McAb and to probe salivary gland membranes for virus receptors (Hsu and Rochow 1988).

In another study, Torrance et al. (1986) produced five rat McAbs to a UK isolate of BYDV and used these antibodies to characterize field isolates of BYDV. All of the UK, North American, and Swedish isolates could be placed in one of four groups based on McAb reactivities.

Using 27 McAbs (those above and others) to 4 luteoviruses, D'Arcy et al. (1989) detected strong heterologous reactions with 17 strains of 7 luteoviruses and identified common epitopes among many pairs of luteoviruses.

Potexviruses

A panel of 10 rat McAbs specific to strains of potato virus X (PVX), an economically important virus commonly found in potato-growing areas worldwide, has been produced (Torrance et al. 1986a). One of these McAbs reacted with all 33 PVX isolates tested, whereas another McAb reacted with only 2 of the 33 isolates. Two of the McAbs were shown to be extremely useful in identifying "resistance-breaking" PVX strains from indigenous non-resistance-breaking phenotypes. Polyclonal sera currently used in testing imported germplasm cannot distinguish the two phenotypes.

In further studies with these McAbs, Koenig and Torrance (1986) were able to define at least three antigenic determinants on the protein subunits of the B strain of PVX when native and denatured virus preparations were studied in various serological tests.

Potyviruses

The potyviruses comprise the largest and economically most important group of plant viruses and affect a wide range of crop plants. It is no wonder then that the majority of monoclonal antibodies that to date have been generated to plant viruses have been to members of the potyvirus group (Bahrani et al. 1986; Dougherty et al. 1985; Gugerli and Fries 1983; Hill et al. 1984; Hsu et al. 1988; Jordan and Hammond 1990; McLaughlin et al. 1986; Sherwood et al. 1987; Wang et al. 1984; Yao et al. 1985).

Monoclonal antibodies generated to potato virus Y (PVY) by Gugerli and Fries (1983) reacted with common epitopes of 24 isolates belonging to the tobacco veinal necrosis (N), common (O), and stipple streak (C) strains of PVY. A McAb specific to a common PVY antigenic epitope gave more uniform reactions in ELISA for the detection of PVY viruses than polyclonal antibodies from rabbit serum.

Hill et al. (1984) produced McAbs to lettuce mosaic virus (LMV), soybean mosaic virus (SMV), and to two strains of maize dwarf mosaic virus (MDMV). Seven of the eight McAbs were strain specific. The other, LMV-generated, McAb was very weakly cross reactive with MDMV and SMV when used in radioimmunoassay. Two SMV-specific McAbs that recognize two different epitopes were used in a double monoclonal antibody-based, nonisotopic, biotin-avidin ELISA for the detection of SMV antigen in soybean seeds (Diaco et al. 1985). One of these McAbs was also shown to be useful for the purification of SMV by immunoaffinity chromatography (Diaco et al. 1986a).

Dougherty et al. (1985) prepared 10 McAbs to the capsid protein of tobacco etch virus (TEV). Three of the McAbs were specific to externally located epitopes present only on TEV isolates. The remaining seven McAbs were specific to epitopes not readily accessible on the virion surface of a variety of different potyviruses including TEV, PVY, tobacco vein mottling virus, pepper mottle virus, watermelon mosaic virus II, and MDMV. The latter results suggest that some epitopes common to many potyviruses that reside within the interior of the virions have been conserved during evolution (Dougherty et al. 1985).

Monoclonal antibodies prepared to tulip breaking virus (TBV) (Hsu et al. 1988) that were selected by indirect ELISA when viral antigen was used to coat the plates were not useful in detecting TBV infections in double antibody sandwich ELISA (polyclonal rabbit antibody coated plates). These McAbs were shown, however, in indirect ELISA to be able to differentiate strains of TBV and to react selectively to distinguish at least 10 other potyviruses, including bean yellow mosaic virus (BYMV), iris mild virus (IMMV), and iris severe mosaic (ISMV) viruses (Hammond and Chastagner 1989; Hammond et al. 1985; Hsu et al. 1988).

Using as immunogen a mixture of different potyviruses (including BYMV, ISMV, IMMV, and PVY), 30 potyvirus-specific McAb-secreting cell lines were generated by Jordan and Hammond (1991). All of the McAbs react with at least one BYMV isolate. Fourteen of them recognize epitopes found only on strains of BYMV or BYMV-subgroup isolates, whereas the remaining 16 McAbs react with a BYMV isolate and with at least 1 of the other 43 potyvirus isolates tested. At least 25 different poty-virus coat protein epitopes could be delineated with the McAbs based on antigen specificity using more than 55 potyvirus isolates. As was shown with the TEV McAbs (Dougherty et al. 1985), most of these potyvirus McAbs that were specific to conserved epitopes common to many members of the potyvirus group reacted to antigenic sites not found on the surface of intact virions (Jordan and Hammond, 1991; and Jordan, unpublished data). These McAbs have also been shown to be extremely useful in the detection of various potyviruses in infected plants (Jordan and Hammond 1990) as well as for the detection and characterization of fusion proteins expressed in cloned inserts of the viral coat protein gene (Hammond et al. 1990; and Jordan and Hammond, unpublished).

The above examples illustrate that strain-specific, virus-specific, and possibly even group-specific McAbs have been generated to the potyviruses. Their potential usefulness as serological probes in taxonomic and structure-function studies of potyviral capsid protein, and in routine virus detection, is obvious.

Monoclonal Antibodies in Plant Bacteriology

Plant Pathogenic Bacteria

Although polyclonal antibodies have been widely used for the serological detection, serotyping, and taxonomic classification of bacteria, the application of monoclonal antibodies in plant bacteriology is still in its infancy. Monoclonal antibodies have been made to only a small number of plant pathogenic bacteria. These include *Agrobacterium tumefaciens* (Bishop et al. 1989), *Corynebacterium sepedonicum* (DeBoer and Wieczorek 1984; DeBoer et al. 1988; Magee et al. 1984), *Xanthomonas campestris* (Alvarez et al. 1985, 1986; Norman and Alvarez 1989; Yuen et al. 1986; Civeolo and Hsu, unpublished data), *Erwinia ananas* (Sheng et al. 1986), and *E. amylovora* (Lin et al. 1986). In addition, monoclonal antibodies have been made to lipopolysaccharide of *E. carotovora* subsp *atroseptica* (DeBoer and McNaughton 1987) or extracellular endopectate lyases of *E. carotovora* subsp *carotovora* (Livingston et al. 1986; Klopmeyer and Kelman 1988).

Because monoclonal antibodies are specific to single epitopes, they should be useful in bacterial strain analysis. In fact, monoclonal antibodies produced for *X. campestris* pv. *campestris* have been used for rapid identification of strains and for tracing strains in epidemiological studies of black rot of crucifers (Alvarez et al. 1985; Yuen et al. 1986). Using a panel of monoclonal antibodies, a unique strain of *X. campestris* pv. *citri* causing Mexican citrus bacteriosis was identified (Alvarez et al. 1986). On the other hand, broad spectrum monoclonal antibodies generated to *C. sepedonicum*, which react with all strains of the pathogen, have been shown to be useful probes for disease detection and in seed certification programs (DeBoer and Wieczorek 1984; DeBoer et al. 1988).

Many broad-spectrum monoclonal antibodies, however, when diluted, show different degrees of reactivities to various strains of a pathogen (Sheng et al. 1986; Civerolo and Hsu, unpublished data). Many positive results may be observed when high concentrations of antibodies are used. Fewer positive reactions are recorded when higher dilutions of antibodies are employed in the tests. Such quantitative differences may be useful in differentiating strains of bacterial pathogens.

Enzyme immunoassays (EIA) and immunofluorescence assays are two of the most commonly used methods for identification of bacterial pathogens. Enzyme immunoassays are popular because the techniques are sensitive and the results are visible without aid of specific instruments. Immunofluorescent assays, although not as widely used as EIA for identification of plant pathogenic bacteria, are extremely sensitive methods for localization of pathogens. Application of monoclonal antibodies in immunofluores-

cent assays is a feasible method for identification of pathogens in infected tissues (Lin et al. 1986).

Finally, monoclonal antibodies specific to lipopolysaccharide (DeBoer and McNaughton 1987) or extracellular endopectate lyases (Livingston et al. 1986; Klopmeyer and Kelman 1988) are very useful reagents in studies of host-parasite interaction. They can be high-precision tools for investigating the role of those molecules in pathogenesis. Their usefulness as specific probes is now being investigated (Klopmeyer and Kelman 1988; Maher et al. 1986).

Fastidious Gram-negative Bacteria

Hung et al. (1987) have reported the production of three hybridomas that secrete antibodies specific to strains of the plum leaf scorch bacterium that are responsible for the plant diseases designated as plum leaf scorch, phony peach, Pierce's disease of grapevine, elm leaf scorch, periwinkle wilt, sycamore leaf scorch, and mulberry leaf scorch. One McAb reacted only with the elm leaf scorch bacterium. These antibodies should be useful reagents to begin to understand the biology, epidemiology, and pathology of these organisms.

Spiroplasmas

Spiroplasmas are cell wall-less prokaryotes that are characteristically helical and motile and that have been shown to be important pathogens of plants, insects, and vertebrates. Monoclonal antibodies have been generated that are highly specific to *Spiroplasma citri* (Jordan et al. 1989; Lin and Chen 1985a), to the corn stunt spiroplasma (CSS) *S. kunkelii* (Jordan et al. 1989; Lin and Chen 1985b), or to epitopes present on both spiroplasmas and/or other Group I spiroplasmas (Jordan et al. 1989).

All nine McAbs produced against *S. citri* (Maroc) by Lin and Chen (1985a) were highly specific for 10 of 14 isolates of *S. citri*. None of the McAbs reacted with any of the 27 other spiroplasmas tested. Based on the antigen specificity tests reported, no valid conclusions can be drawn concerning epitope specificity.

In a separate study, Lin and Chen (1985b) produced a panel of 7 McAbs to CSS (1747) that reacted only to 3 strains of CSS and not to any of the 29 other spiroplasmas tested. Again, no statements can be made concerning epitope specificity.

Forty-six McAbs were obtained by Jordan et al. (1989) using a mixture of *S. citri* (R8A2) and CSS (1747, F32, PU8-17) as an immunogen and screening antigen. When tested against 36 strains of spiroplasmas representing group I and groups IV-XI spiroplasmas, 17 McAbs were selected that react only with the strains of *S. citri* tested, and 17 McAbs that react only with *S. kunkelii* isolates. The remaining 12 McAbs react with antigenic sites common in three to eight of the eight group I spiroplasmas, including honeybee spiroplasmas (AS576), Maryland flower spiroplasma (M55), *Cocos* spiroplasmas (N525), and the periwinkle spiroplasma, *S. phoeniceum*. Based on the McAb reactivities to the various spiroplasma strains tested, the 46 McAbs define at least 17 different group I-spiroplasma epitopes. Three of these epitopes are located on nonmembrane-bound protein(s) (Jordan et al. 1989; Konai et al. 1986; Jordan, unpublished).

All of these highly specific serological reagents should be very useful in providing new information on the antigenic relationships among the spiroplasmas, as probes for the detection and identification of spiroplasmas in plants and insects, and as molecular probes in identifying and locating specific spiroplasma proteins.

Mycoplasma-like Organisms

Mycoplasma-like organisms (MLOs) have been implicated in more than 300 yellows-type diseases in plants. Diagnosis of diseases caused by MLOs and identification of the casual agents are not only difficult but also time consuming. Currently, the yellows diseases are only differentiated by host range, symptomatology, and insect-vector relations; none have as yet been cultivated in vitro. Although serology offers several reliable and rapid methods for pathogen detection and disease diagnosis, sufficient pure quantities of MLO have not been obtained for conventional polyclonal antibody production. Monoclonal antibodies, however, have been produced to a limited number of plant pathogenic MLOs including the aster yellows agent (Lin and Chen 1985c), the maize bushy stunt agent (Chen and Jiang 1988), the primula yellows agent (Clark et al. 1989), the peach eastern X-disease agent (Jiang et al. 1989), and an agent that induces tomato big bud disease (Hsu et al. 1990).

Lin and Chen (1985c, 1986) reported the first successful production of McAbs to an MLO agent, the aster yellows (AY) agent, which was achieved by using insect vector salivary glands as the antigen for immunization and for hybridoma screening. In indirect ELISA tests, a selected McAb reacted specifi-

cally with Ay-MLO-infected plants and differentiated the AY agent from other MLO's. In in situ detection by immunofluorescent staining, the McAb bound specifically to the AY-MLO in sieve tubes in infected plants. Similar results were also obtained for the peach eastern X-disease agent (Jiang et al. 1989). Employing an immune tolerance inducing procedure—using mice neonatally injected with nontarget antigens present in immunogen preparations before immunization—20 McAbs specific to the tomato big bud disease MLO agent were produced by Hsu et al. (1990c). The highly discriminatory capacity of these McAbs to differentiate MLOs will be the most important advantage over polyclonal antibodies for MLO disease diagnosis, disease forecasting, and epidemiological studies.

Monoclonal Antibodies in Fungal Plant Pathology

The application of monoclonal antibody technology in the field of fungal plant pathology has, so far, been mostly limited to just a few diseases (Banowetz et al. 1984; Benhamou et al. 1985a, 1985b; Gendloff et al. 1987; Iannelli et al. 1983; Miller et al. 1986; Nameth et al. 1990). Monoclonal antibodies against spores of *Fusarium oxysporum* f. sp *lycopersici* reacted with several *Fusarium* spp (Iannelli et al. 1983). However, based on quantitative differences in specific antibody binding patterns, different *Fusarium* spp, as well as formae speciales of *F. oxysporum*, could be differentiated. Similar types of results were reported by Banowetz et al. (1984) using McAbs directed against teliospores of *Telletia* in immunological comparisons of two species of the wheat bunt fungi. A monoclonal antibody directed against *Pythium aphanidermatum* was shown by Miller et al. (1986) to react with four different species of *Pythium*, all of which have been shown to be involved in Pythium Blight in turfgrass. In a McAb-based diagnostic immunoassay, the *Pythium* spp could be detected in inoculated and naturally infected turfgrass samples and no cross-reactivity was observed with other common turfgrass pathogens. Nameth et al. (1990) developed a McAb against the necrotic ringspot of turfgrass pathogen *Leptosphaeria korrae*. This McAb was shown to be capable of detecting *L. korrae* in culture and in naturally infested bluegrass. A McAb against T-2 toxin, a mycotoxin produced by various plant pathogenic *Fusarium* spp, was produced by Gendloff et al. (1987) and shown to cross react with several T-2 toxin metabolites.

The successful production and use of two monoclonal antibodies specific for a phytotoxic glycopeptide produced by *Ophiostoma ulmi*, the Dutch elm disease pathogen, has been reported by Benhamou et al. (1985a, 1985b). These McAbs were utilized by immunohistochemical and immunocytochemical techniques for the in situ identification of the toxic glycopeptide. The toxin was detected, at the light microscope level, in the fungus by immunofluorescence and immunoperoxidase techniques, and in the infected elm host tissues by the latter technique. At the electron microscope level, the antigenic macromolecule was detected and localized in the fungus and infected elm tissue using monoclonal antibody-protein A-gold complexes. This work demonstrated quite elegantly the use of highly specific antibodies for evaluating qualitatively the distribution of a phytotoxic compound.

Conclusions

The initial intended use of monoclonal antibodies in plant pathology has been for diagnostic purposes. The great potential for McAbs in phytopathological diagnostics is essentially because homogeneous antibody preparations with defined activity and specificity can be produced in large quantities over long periods. Even though the hybridoma technology is a laborious and expensive enterprise compared to standard immunization procedures, in the next few years McAbs will most probably be generated against many plant pathogens, especially those for which there is a demand for large-scale diagnosis. Diagnostic application for plant viruses and for plant pathogenic bacteria and fungi have already been demonstrated, albeit on a limited scale.

Compared to polyclonal antisera, McAbs often possess a superior discriminatory capacity for revealing small differences in the structure of epitopes. McAb use in plant pathology has demonstrated that certain fine details of the antigenic structure, especially conformational aspects, could only be ascertained by using hybridoma technology. Current observations indicate that with McAb probes it should be possible to evaluate qualitatively and quantitatively the antigenic nature of plant pathogenic agents and their gene products, the interactions of these pathogen gene products and toxins with the plant host, especially in discerning the distribution, the site of action, and the cytopathogenic effect of these macromolecules. With the expanded use of McAbs, immunochemistry will continue to be an

even more powerful tool for the detection, assay, differentiation, and topographical and structural analysis of the wide variety of molecules encountered in plant pathology.

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Plant Genomes, Gene Markers, and Linkage Maps

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Abstract

The detection and exploitation of polymorphism in plants and animals represents one of the most significant developments in biology. The tools of molecular biology provide the opportunity to develop large numbers of phenotypically neutral genetic markers in any organism from which DNA can be extracted. The exploitation of both protein and DNA markers in crop improvement are described. Particular attention is given to methods for the identification of desirable recombinant genotypes in segregating populations. Strategies for the creation of linkage maps are considered and the characterization of germplasm at the nuclear and organellar levels is illustrated with reference to crops of particular importance in Asia. The use of near-isogenic lines to rapidly identify genetic markers linked to traits of interest is described and the potential of molecular markers to improve the speed and precision of gene introgression from exotic germplasm into adapted cultivars is emphasized. The role of molecular markers in the manipulation of quantitative traits is discussed and the value of recombinant inbred lines, particularly doubled haploids, in understanding the genetic control of polygenic systems is illustrated in barley. Limitations to the exploitation of marker-based technology are considered and new approaches for detecting polymorphism based on the use of short oligonucleotide primers in conjunction with the polymerase chain reaction is reviewed. The significant developments in plant genome mapping have been dependent on the germplasm. Further advances will demand an integrative, multidisciplinary approach. International networks that provide the conceptual and administrative framework will be an important feature of future genome mapping projects.

Introduction

In most plant breeding programs, the synthesis of new and improved genotypes relies upon the processes of recombination and segregation that occur in the progenies of heterozygous individuals. A major challenge for the plant breeder is to identify the desirable recombinant phenotype among the segregating population. Various scientists have suggested marker-based selection strategies to improve both the speed and precision of plant breeding programs. The theory exploits the fact that a marker locus identifies a chromosomal segment and enables that segment to be monitored in subsequent generations of selfing or crossing. In this situation the marker should be readily scoreable. Reports of the association between seed size and alleles influencing seed color in *Phaseolus* were first reported by Sax (1923). The concept of using morphological markers in plant breeding is therefore not new. Examples include those conferring dwarf stature, inflorescence morphology, and chlorophyll deficiencies. Purple mottled seed color in *P. vulgaris* is tightly linked to a dominant allele controlling resistance to bean common mosaic virus (Temple and Morales 1986).

In a plant breeding context, many morphological markers have undesirable effects on plant phenotype and their use in crop improvement has been limited. Developments in the electrophoretic separation of proteins and in the exploitation of recombinant DNA technology have dramatically increased the number of genetic markers available for use in plant breeding. The greater utility of molecular markers arises from five inherent properties that distinguish them from

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morphological markers (Tanksley 1983). The phenotype of most morphological markers can only be determined at the whole plant level; whereas molecular loci can be assayed at the whole plant, tissue, and cellular levels. Allele frequency tends to be much higher at molecular loci compared with morphological markers. In addition, morphological mutants tend to be associated with undesirable phenotypic effects. Alleles at morphological loci interact in a dominant-recessive manner that limits the identification of heterozygous genotypes. Molecular loci exhibit a codominant mode of inheritance that allows the genotypic identification of individuals in a segregating population. Fewer epistatic or pleiotropic effects are observed with molecular markers than with morphological markers. Hence, a large number of polymorphic markers can be generated and monitored in a single cross.

Inherent in research on genetic transformation is the approval of the regulatory authorities prior to the release of genetically transformed material. Although recombinant DNA technology is used in linkage map creation, the strategies do not include the genetic engineering of plants. Gene mapping research is therefore not constrained by regulations relating to the release of genetically engineered organisms.

The detection and exploitation of polymorphism in plants and animals represents one of the most significant developments in biology and the objectives of this review are: to outline the methods used in detecting polymorphisms in plants; to describe the applications of marker-based technologies in plant breeding; and to highlight limitations to current approaches for the integration of molecular markers with conventional approaches to crop improvement. Molecular markers can be divided into two categories: protein and DNA markers.

Protein Markers

The most widely used protein markers in plant breeding are isozymes. The application of isozymes in plant genetics has been thoroughly reviewed by Tanksley and Orton (1983) and only recent developments, particularly in relation to the selection of disease resistant plants, will be described. Isozymes, or multiple molecular forms of enzymes, are enzymes that share a common substrate but differ in electrophoretic mobility (Markert and Moller 1959). They are revealed when tissue extracts are subjected to electrophoresis in various types of gels and subsequently immersed in solution containing enzyme-specific stains. Electrophoretic separation of complex mixtures of proteins can be accomplished in several types of support media, including starch gel electrophoresis (SGE), polyacrylamide gel electrophoresis (PAGE) and agarose gels, and cellulose acetate membranes (Wendel and Weeden 1990). The two most widely used systems are starch and polyacrylamide electrophoresis. The technique of isoelectric focusing (IEF) is a further protein electrophoretic technique with high resolution (Radola 1980). IEF offers several advantages over conventional continuous systems such as SGE. In the case of IEF, protein separation is carried out in a pH gradient established between two electrodes that are stabilized by carrier ampholytes. Due to their amphoteric properties, proteins migrate to their isoelectric point (pI), the pH at which they possess no net charge. Proteins focus at their pI allowing proteins naturally present at low concentrations to be detected. IEF is thus an electrophoretic technique which gives a high degree of resolution and raises the level of detectable polymorphism. This is illustrated in Figure 1 where zymograms for barley leaf esterase (E.C 3.1.1) are presented for starch gel electrophoresis and IEF.

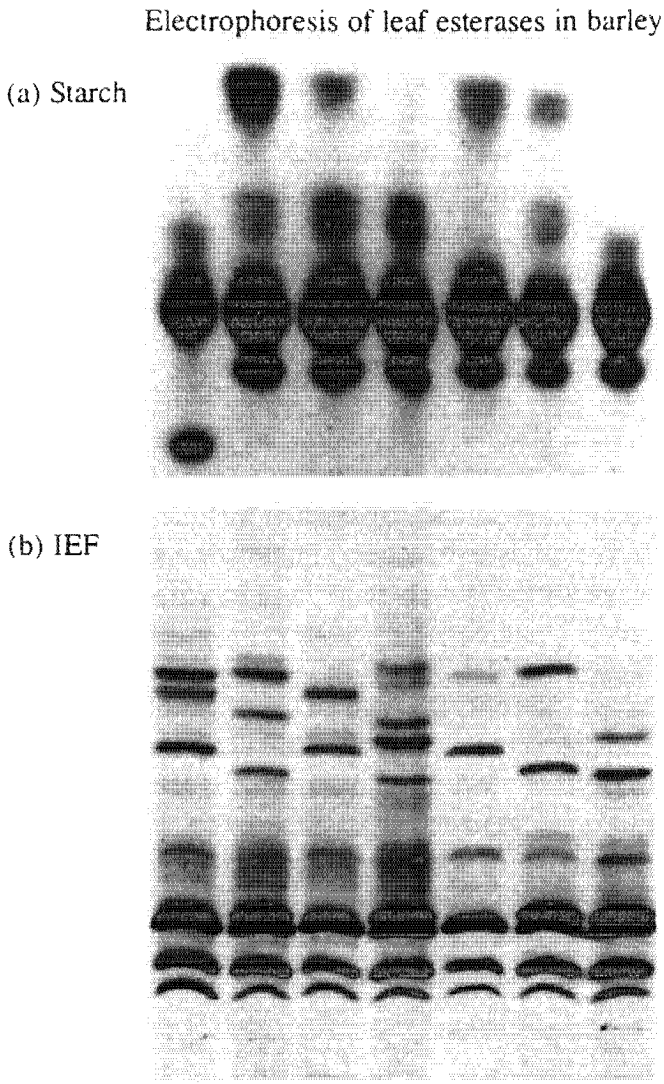

Figure 1. Comparisons of zymograms for barley leaf esterase using starch gel electrophoresis and isoelectric focusing (IEF).

Despite the greater resolving power of PAGE and IEF over SGE, the relative simplicity of starch gel preparation ensures that SGE is still a widely used and effective separation system. In addition, SGE does not involve the use of toxic material whereas the acrylamide used for PAGE and IEF is a neurotoxin. Furthermore, starch gel systems can be easily replicated to allow the simultaneous evaluation of a range of enzyme systems. Thus, where a large number of enzyme systems are being assayed, the efficiency and cost effectiveness of starch may compensate for its lower resolution relative to IEF.

Two of the main uses of isozymes in plant breeding are given below.

Germplasm Characterization

Polymorphic isozyme and storage protein systems have been investigated for use in classification of a wide range of crops including wheat (Cooke 1987), maize (Cardy and Kannenberg 1982), soybean (Cardy and Beversdorf 1984), and barley (Nielson and Johansen 1986). Recently Thompson et al. (1990) have used IEF in conjunction with seven protein and isozyme marker phenotypes to uniquely characterize 27 out of 29 barley varieties examined. The number of phenotypes identified in the spring and winter varieties is given in Table 1 and the zymograms illustrating the protein profiles are presented in Figure 2. The protein profiles at the β-amylase (β-*Amy-1*) and water soluble (*Wsp-2* and *Wsp-3*) loci have been examined in 44 spring and 39 winter barley varieties (Forster et al. 1991). The frequencies of alleles at the three loci were found to be different in winter and spring genotypes. Spring genotypes possessed a wider range of phenotypes than winter cultivars. This is particularly evident in the case of the *Wsp-3b* allele which is very rare in the winter group of barley cultivars. Panda is the only winter cultivar we have tested possessing this allele. Similarly, the majority of winter cultivars possess the β-*Amy-1b* allele. The β-*Amy-1* locus is known to be located on chromosome 4H (Nielson et al. 1983) and segregation analysis indicated that *Wsp-3* and β-*Amy-1* are linked (0.111 ± 0.022) (Forster et al. 1990). Winter and spring barley genotypes can therefore be distinguished on the basis of *Wsp-3* and β-*Amy-1* phenotypes (Fig. 3). Both genes are easily detected on IEF gels and they can be extracted simultaneously from the endosperm in half of the grain, thus the embryo can be retained for future plant regeneration following protein analysis. These characteristics make β-*Amy-1* and *Wsp-3* potentially valuable biochemical markers for spring/winter habit in barley breeding programs.

Isozyme analysis has been extensively applied to rice (*Oryza sativa* L.). Glaszmann (1987, 1988) has used isozymes to classify Asian rice varieties and to examine the relationship between electrophoretic variability and the geographic distribution of rice genotypes. Nonrandom distribution of alleles at 15 isozyme loci was detected and Southeast Asia was identified as the region with the highest genetic diversity in the *japonica* subgroup.

Isozyme variation in old world races of *Sorghum bicolor* was relatively low with an allelic diversity of 2.38 alleles per polymorphic locus (Morden et al. 1989). Based on isozyme information there was no obvious grouping of genotypes related to geographical distribution. Higher levels of isozyme variability were detected in *arundinaceum* spp than in cultivated sorghum (Morden et al. 1990). This observation is consistent with the view that cultivated sorghum exhibited a loss of genetic variability during domestication. Tuwafe et al. (1988) examined the inheritance and geographical distribution of isozyme polymorphism in chickpea (*Cicer arietinum* L.). Six isozyme systems were analyzed and four polymorphic loci were identified. These studies indicated that the greatest genetic diversity was associated with germplasm originating from Middle Eastern, Asian, and East African countries. These regions would therefore appear to be important locations for future germplasm collecting expeditions.

Table 1. Phenotypes identified for spring and winter varieties of barley using eight protein markers.

	Protein Markers							
	β-Amy-1	Est-10	Est-3	α-Amy-1	Wsp-1	Wsp-2	Wsp-3	L. Est
Variety	*Phenotype*							
Spring	ABCD	AB	AB	ABC	AB	ABCD	ABCD	ABCDEFG
Winter	AC	AB	AB	AB	A	ABD	AB	BCDE

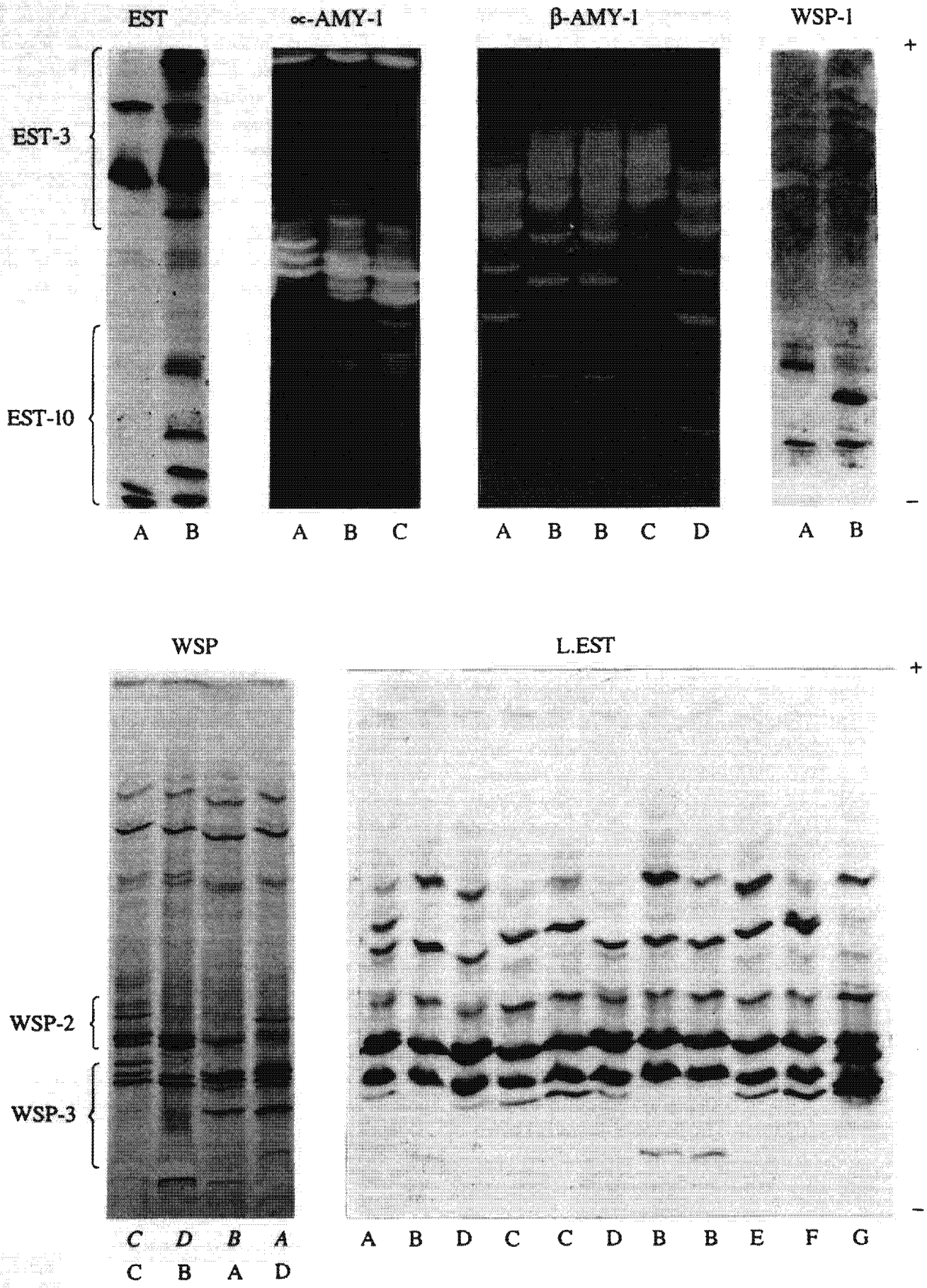

Figure 2. Zymograms of esterase-3 (EST-3), esterase-10 (EST-10), -amylase-1 (*β-Amy-1*), ß-amylase-1 (*β-Amy-1*), water soluble protein-1, -2 and -3 (*Wsp-1, Wsp-2, Wsp-3*), and leaf esterase (L. EST) showing the various phenotypes (A-G) observed in barley.

The identification of suitable sources of disease resistance genes and the incorporation of these genetic traits into commercial cultivars represents a major challenge for plant breeders. Assays for disease resistance necessitate the presence of the pathogen, are often laborious, take several months, can be inaccurate due to variation in disease pressure, and often involve the destructive testing of numerous plants. A biochemical marker closely linked to the gene conferring disease resistance would be a valuable breeding tool. One of the first examples of an isozyme associated with pest resistance was reported by Rick and Fobes (1974). They reported tight linkage between an acid phosphatase locus, *Aps-1* and the locus *Mi* controlling nematode (*Meloidogyne incognita*) resistance in tomato. Detection of this linkage has had a major impact on tomato breeding programs: the codominant *Aps-1* locus is now widely used as a selectable marker for nema-tode resistance in tomato (Medina-Fihlo and Stevens 1980). In this case, the variant *Aps-1* and *Mi* alleles were introgressed from the wild green-fruited tomato species *Lycopersicon peruvianum*.

In the garden pea, an esterase polymorphism can be used to mark *Fw*, the gene conferring resistance to *Fusarium* wilt (Hunt and Barnes 1982) and bean yellow mosaic virus resistance is tightly linked to phosphoglucomutase (Pgm-P) on chromosome 2 (Weeden et al. 1984). More recently, Weeden and Providenti (1988) have proposed the ADH1 isozyme in *Pisum sativum* as a biochemical marker for *En*, the locus controlling resistance to pea enation mosaic virus.

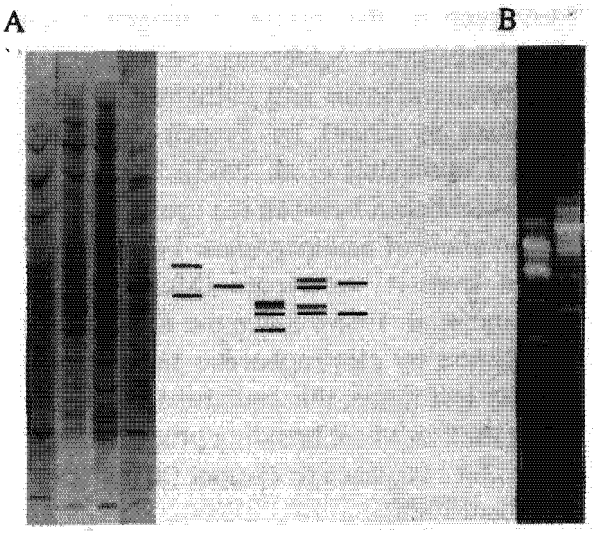

Figure 3a. Polymorphisms of *Wsp-2*, *Wsp-3*, and *ß-Amy-1* in barley.

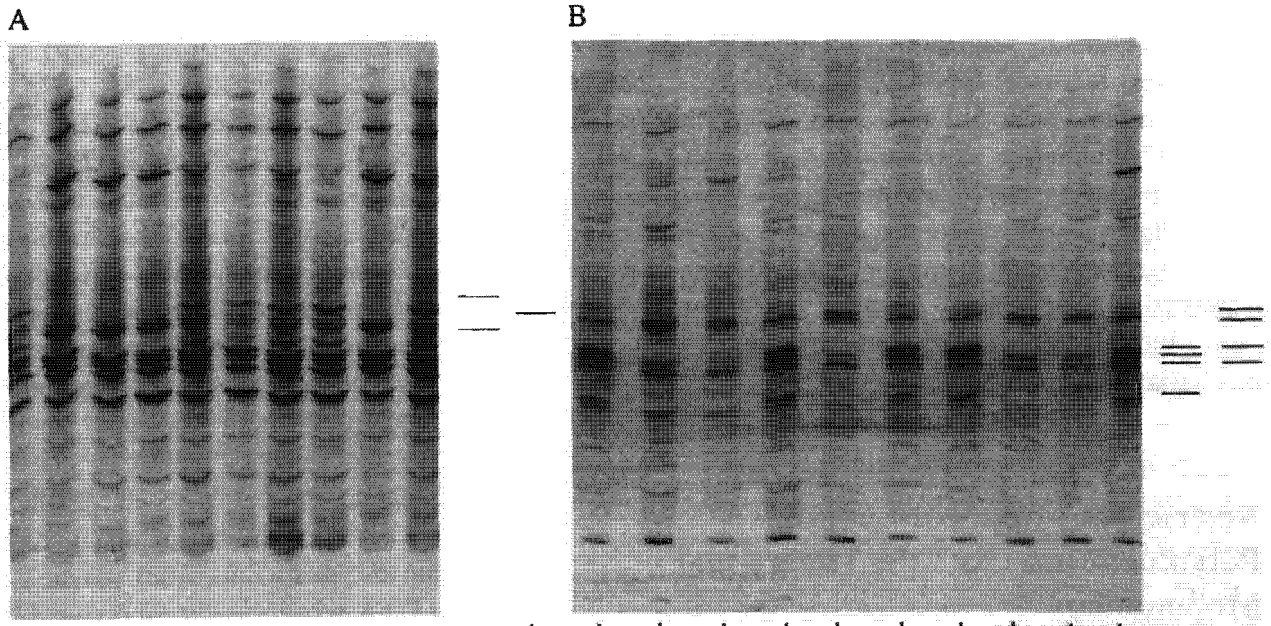

Figure 3b. Segregation of *Wsp-2* and *Wsp-3* genotypes in 10 lines from two doubled haploid populations: (A) 'Dissa' x 'Sabarlis', segregation of *Wsp-2a* versus *Wsp-2b*; (B) TS42/3/5 × 'Apex', segregation of *Wsp-3a* versus *Wsp-3b*.

Resistance to the eyespot pathogen (*Pseudocercosporella herpotrichoides*) has been introgressed from *Aegilops ventricosa* into cultivated hexaploid wheat (*Triticum aestivum*) by French researchers (Maia 1967; Doussinault et al. 1983). Genetic linkage has been established between the eyespot resistance gene and an allele of endopeptidase-1 (*Ep-D1b*) also transmitted from *A. ventricosa* (McMillin et al. 1986; Worland et al. 1988). Since the *Ep-D1b* allele can be established by electrophoretic separations from embryo or leaf tissue, this codominant genetic marker is being used in wheat breeding programs to screen progenies for resistance to eyespot (Summers et al. 1988; Bingham 1986).

Barley yellow mosaic virus (BaYMV) is an important soilborne disease of barley. Resistant barley varieties originating from Japan and China have been identified and segregation analysis indicated that the BaYMV resistance locus is tightly linked to three leaf esterase loci on chromosome 3H (Konishi et al. 1989). The intensity of linkage (1.26 ± 0.622cM) is sufficient to allow the use of leaf esterase isozyme profiles as a means of selecting indirectly for resistance to BaYMV. Bournival et al. (1989) used an interspecific tomato cross to demonstrate that the *Got-2* locus on chromosome 7 is linked to the gene I-3 conferring resistance to *Fusarium oxysporum* f.sp. *lycopersici*. These authors propose that the *Got-2* locus is a suitable marker for the transfer of the race 3 resistance genes into tomato cultivars.

These examples illustrate the principles behind the use of protein markers to identify desirable recombinant genotypes in segregating populations. Furthermore, they highlight the value of biochemical markers as indirect selection tools in plant breeding programs. Compared to DNA markers, isozymes have the further advantage of being relatively inexpensive and a large number of samples can be processed rapidly. Isozymes have also been used to manipulate quantitatively determined characters (Stuber et al. 1987) and this will be discussed further in the section on character dissection.

Restriction Fragment Length Polymorphism (RFLP): Theory and Methodology

Morphological and protein-based marker systems only sample actively expressed regions of the genome. However, the relative ease with which DNA molecules can currently be cloned—allied to the availability of a wide range of restriction endonucleases—has allowed a much greater portion of the plant genome to be assayed for genetic markers. Restriction endonucleases are bacterial enzymes that respond to certain short base sequences in DNA by catalyzing endonucleotic cleavage at a specific point in the sequence, facilitating the creation of a restriction map specific to that enzyme (Botstein et al. 1980). In order to detect polymorphism at the nucleic acid level, genomic DNA from two genetically distinct individuals are digested with a restriction enzyme and the DNA is separated by gel electrophoresis. Following transfer of the DNA to a membrane by Southern blotting, a radioactively labelled DNA clone (probe) is hybridized in solution with the membrane. Polymorphism can result due to differences in the distribution of the restriction sites in the two genotypes and this is illustrated in Figure 4.

The production of probes is crucial for RFLPs and in general single-copy DNA sequences are required for mapping. In most higher plants the nuclear genome consists of a large proportion of repetitive DNA sequences (Flavell 1980). Often these repeats are interspersed with unique single-copy sequences that render the isolation of low-copy DNA sequences more difficult. The proportion of repeated DNA and the extent to which it is interspersed with single-copy DNA is generally a function of the overall DNA content of the organism. Compared with other organisms, plants have a wider range of DNA content and on average have more DNA per nucleus. The large genome size of certain crops makes it difficult to isolate clones consisting entirely of single-copy DNA.

Random complementary DNA (c-DNA) clones have been used extensively for RFLP analysis in plants (Landry and Michelmore 1987). c-DNA clones are derived from gene transcripts and are a good source of single-copy clones (Tanksley and Pichersky 1987). In contrast, the majority of random genomic clones are likely to contain repeated sequences, making them unsuitable for RFLP mapping. Colony hybridization with ^{32}P-labelled total genomic DNA is one method of selecting low-copy genomic clones (Bernatzky and Tanksley 1986a). A strategy for producing genomic libraries enriched for single-copy sequences is based on the use of methylation-sensitive restriction enzymes. Data from maize suggest that repeated DNA sequences are methylated to a greater degree than single-copy DNA, with the result that cloning with methylation-sensitive enzymes such as PstI produces libraries enriched for single copy sequences (Burr et al. 1988). In a comparison of EcoRI and PstI tomato genomic libraries, Miller and Tank-

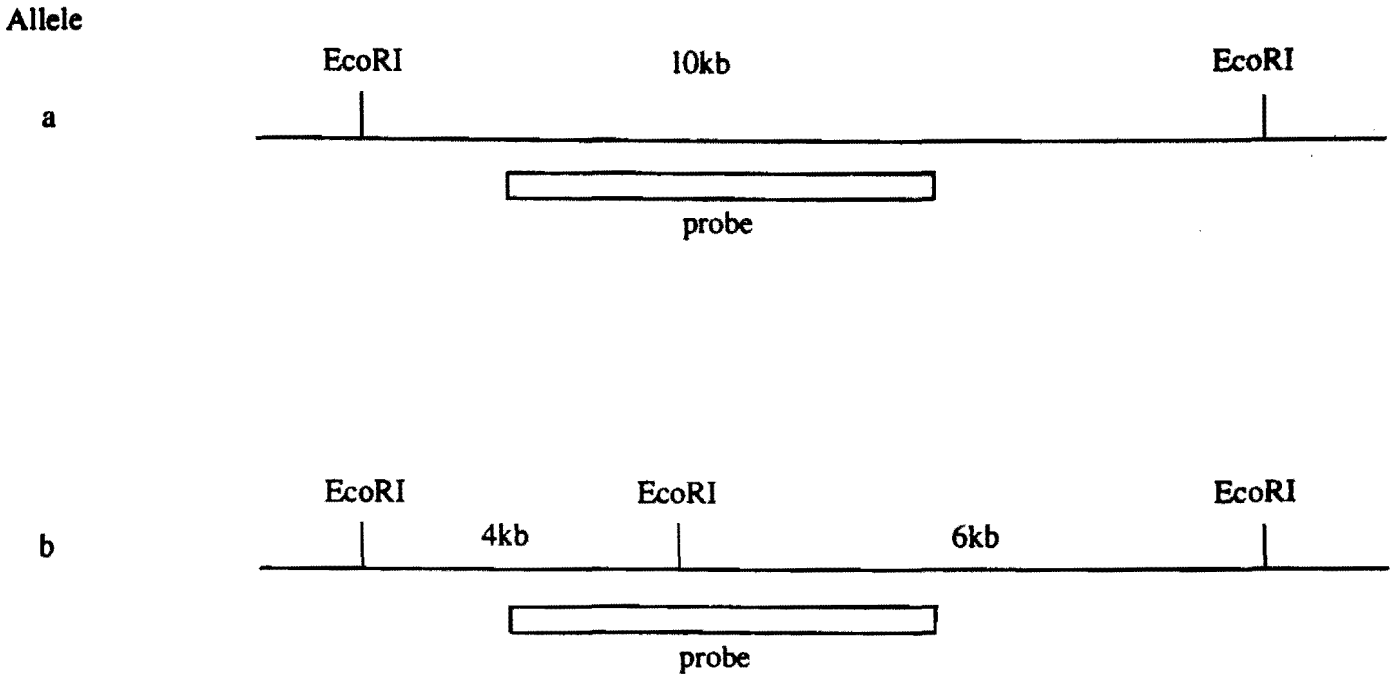

Figure 4a. EcoRI restriction sites of different alleles in which fragment length polymorphism can be detected with a probe hybridizing with the region shown.

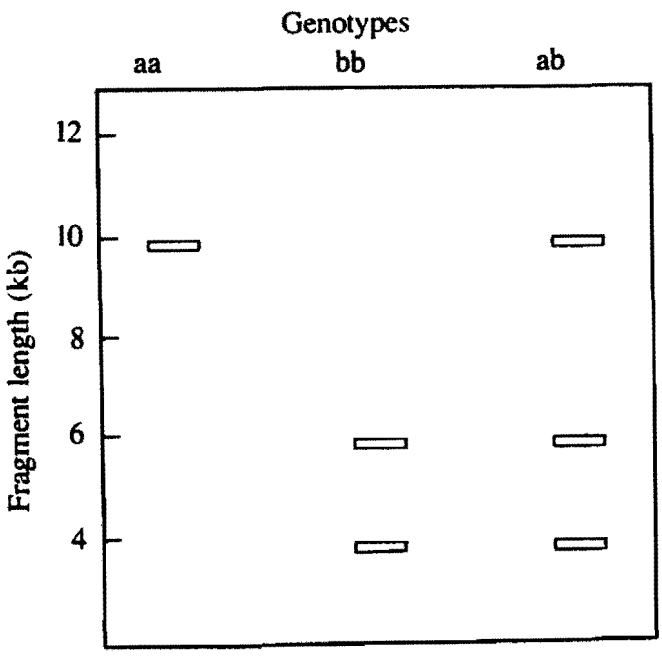

Figure 4b. Autoradiograph of different genotypes when fragments are hybridized with a probe as in Figure 4a.

sley (1990a) have demonstrated that the PstI library contained 92% single-copy clones whereas only 36% of the EcoRI clones were single copy. The efficiency of c-DNA and genomic libraries in detecting polymorphism has been compared in lettuce (Landry et al. 1987). Polymorphism was detected 2.5 times more frequently with c-DNA probes than random genomic probes. Similar results have also been obtained in lentils (Havey and Muehlbauer 1989). Comparisons of c-DNA and genomic probes produced by EcoRI and PstI-digested tomato DNA revealed that c-DNA clones produce 25% more unique restriction patterns than from the PstI library and 50% more clones than from the EcoRI library (Miller and Tanksley 1990b). However, it should be borne in mind that c-DNA clones tend to be shorter than genomic clones and therefore tend to require a longer exposure time with autoradiography (Tanksley et al. 1988).

The methods described have relied exclusively on the use of DNA probes labelled with ^{32}P. The use of radioactive probes in many developing countries is limited by the short half-life of ^{32}P. Alternative non-radioactive probes must meet the criteria of sensitivity and the reuse of filters achieved with radioactive probes. Two nonradioactive detection methods have been employed. Ishii et al. (1990) have published protocols for the detection of rice RFLPs based on the incorporation of digoxigenin-dUTP into probe molecules by random priming and subsequent immunological detection of hybridizing fragments. A chemiluminescence method has been described by Kreike et al. (1990) based on a new substrate AMPPD for the enzyme alkaline phosphatase. The substrate produces light rather than forming colored precipitate on the blot and the detection of the light signal is

carried out with X-ray film. An exposure time of 2 hours is reported to be sufficient for the detection of single copy signals. It is anticipated that with further refinements, such nonradioactive methods for detecting RFLPs will play a vital role in the exploitation of molecular markers in developing countries.

Application of RFLPs

Studies of Genetic Diversity

The maintenance and evaluation of germplasm collections is a major feature of many international agricultural research centers (Williams 1989). Traditionally, genetic resources of crop plants have been characterized using a combination of morphological and agronomic traits. The suitability of using morphological characters to estimate allelic diversity has been questioned by several workers (e.g., Brown 1979; Gottlieb 1977). Molecular and biochemical markers that are not subject to environmental influences provide an opportunity to examine more precisely the genetic relationships between accessions. This can help in the rationalization of existing germplasm collections and allow future collection strategies to be targeted toward specific geographical areas.

Cytoplasmic diversity. The importance of cytoplasmic diversity has been emphasized on several occasions particularly in relation to the monoculture of varieties susceptible to pest attack. The devastation of the U.S. maize crop in 1970-73, all hybrids possessing the T-type cytoplasmic male-sterility factor, illustrates the dangers associated with a narrow genetic base. A similar disaster occurred in India in the early 1970s when pearl millet hybrids (*Pennisetum typhoides*), all possessing a common cytoplasm, were severely damaged by downy mildew disease (Swaminathan 1984). Restriction endonuclease digestion of total cellular DNA followed by Southern transfer and hybridization with chloroplast or mitochondrial-specific probes provides a convenient way of assessing cytoplasmic diversity in crop plants. This approach has been pursued in potato (Waugh et al. in press) and the level of diversity detected in 18 tetraploid potato cultivars and 2 wild *Solanum* species is given in Figure 5. Four cytoplasmic DNA (ctDNA) phenotypes

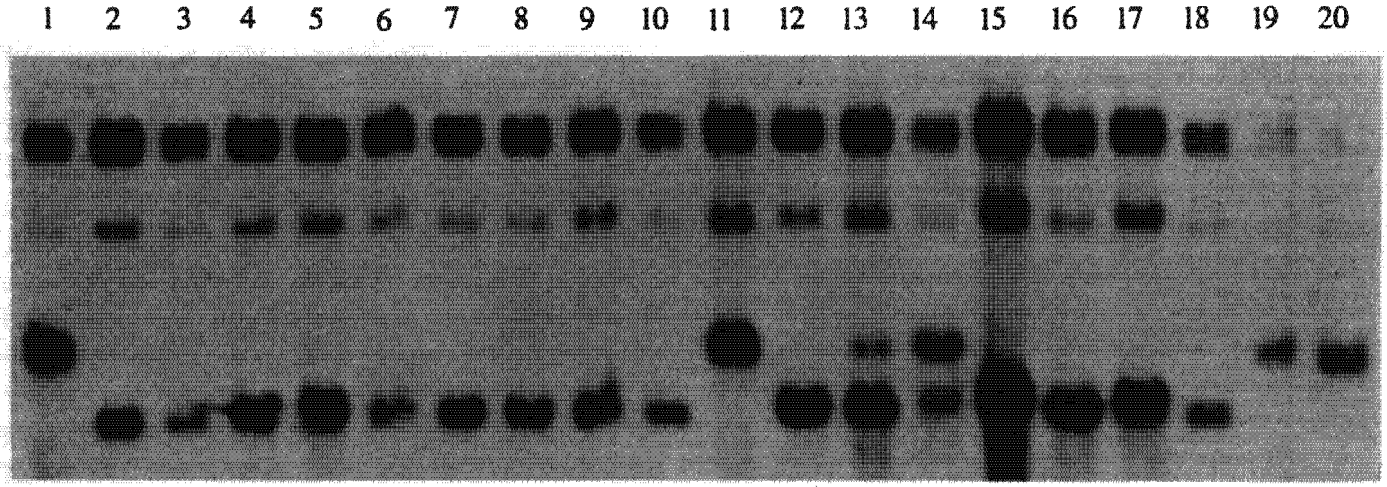

Figure 5. Autoradiogram derived from probing HindIII digested DNA of 20 *Solanum* genotypes with HVC4.

Track
1. Stormont Enterprise*
2. Cara
3. Pentland Crown
4. Foxton
5. Record
6. Pentland Javelin
7. Baillie
8. Golden Wonder
9. Wilja
10. Moira

Track
11. Croft*
12. Morag
13. Estima*
14. Maris Piper*
15. Desiree
16. Fiona
17. Pentland Dell
18. Russet Burbank
19. S. papita*
20. S. stoloniferum*

* indicates variant cytoplasmic types.

can be identified in this figure. The cultivars Croft, Maris Piper, Estima, and Stormont Enterprise differ from the majority of the cultivars examined in terms of their ctDNA restriction patterns. This method allows the detection and characterization of the organellar genomes of potato cultivars without the complication of isolating purified chloroplast DNA. The relatively high (18%) level of chloroplast DNA diversity detected in the European potato cultivars reflects the more broadly-based potato improvement programs, particularly at the Scottish Crop Research Institute (SCRI), that have included the use of wild species and *Andigena* (Neo-tuberosum) populations (Glendinning 1983).

Intraspecific chloroplast DNA variability has been reported in *Zea mays* (Doebly et al. 1987), *Lycopersicon peruvianum* (Palmer and Zamir 1982), *Pisum sativum* (Palmer et al. 1985), and *Hordeum vulgare* (Clegg et al. 1984). In rice, Dally and Second (1990) were able to distinguish between *indica* and *japonica* chloroplast DNA (cpDNA) types. Nine different cpDNA types were identified among 65 *Oryza sativa* cultivars, but in a smaller sample of six *O. glaberima* cultivars the cpDNA was monomorphic. Two studies of pearl millet (*Pennisetum glaucum* L.) by Clegg et al. (1984) and Gepts and Clegg (1990) using both cultivated and wild genotypes of various geographic origins demonstrated monomorphism for cpDNA. These authors propose that a bottleneck induced at some stage in the evolution of pearl millet may have been responsible for the lack of chloroplast DNA diversity. Similar studies with cowpea (*Vigna unguiculata* [L.] Walp) and sorghum (*Sorghum bicolor* L.) that share a common history of evolution in Africa may allow the identification of primary ecogeographic factors that influence cytoplasmic DNA diversity.

The restriction endonuclease digestion of mitochondrial DNA has been particularly useful in the identification of differences between normal and cytoplasmic male sterile (CMS) lines of maize, sorghum, sugar beet, pearl millet, and sunflower. Kemble et al. (1980) demonstrated that Mexican maize lines have greater mitochondrial DNA variation than U.S. maize lines. Mitochondrial DNA probes have also been used to detect variation in CMS and normal maize cytoplasms (Lonsdale et al. 1981). A 10.5 kb soybean mitochondrial DNA fragment has been used as a probe by Grabau et al. (1989) to distinguish between Mandarin (Lincoln) and other soybean cytoplasms.

The hybridization of total cellular DNA to defined chloroplast or mitochondrial sequences provides an effective way to characterize the organellar genomes of crop plants. Chloroplast DNA, in particular, also provides a useful marker for biosystematic studies (Palmer 1987) and can be used to trace the cytoplasmic origin of allopolyploid crops.

Nuclear diversity. Nuclear RFLPs can also be used to provide genetic fingerprints of individual genotypes. Jeffreys et al. (1985) have shown that multiple highly variable regions in humans provide hybridization fragments that are unique to each individual tested. Human mini-satellite clones (33-band 33.15) have been used by Dallas (1988) to fingerprint nine cultivars of *O. sativa* and *O. glaberima*. The M13 repeat probe has been used to detect variation in gymnosperms and angiosperms (Rogstad et al. 1988; Ryskov et al. 1988), to distinguish between *Malus*, *Prunus*, and *Rubus* genotypes (Nybom et al. 1990), and as a basis for paternity analysis in apples (Nybom and Schaal 1990).

In plants, the number of single-copy RFLP markers necessary for fingerprinting of cultivars, protection of breeders' rights, and parentage identification has been calculated (Burr et al. 1983; Soller and Beckman 1983). For example, in an inbreeder where there are two alleles per locus and each allele occurs at a frequency of 0.5 among 20 inbred lines, the probability of distinguishing each of the 20 lines is 0.99 if 20 probes are used. However, in some plants, the level of polymorphism detected is much greater and fewer polymorphic probes are necessary to obtain accurate identification of genotypes (Beckman and Soller 1986; Evola et al. 1986). In potato, two c-DNA clones with one restriction enzyme were sufficient to discriminate between the 27 potato cultivars examined by Powell et al. (1991). This is illustrated in Figure 6 which emphasizes the high level of RFLPs detected in potato. Similar results have been obtained by Gebhardt et al. (1989).

The key factor is the level of variation detected within a species. A number of studies have shown that the level of variation detected as RFLP within a species differs from one species to another (Helentjaris et al. 1985; Bernatzky and Tanksley 1986b; Apuya et al. 1988). The two species that exhibit the two extreme levels of variability are probably maize and tomato. In maize (*Zea mays*), more than 95% of unique sequence clones are capable of detecting polymorphism with up to 13 alleles per locus (Helentjaris 1985). In contrast only 5% of unique sequence clones are capable of detecting RFLPs in *Lycopersicon esculentum*. Furthermore, when polymorphism is detected only

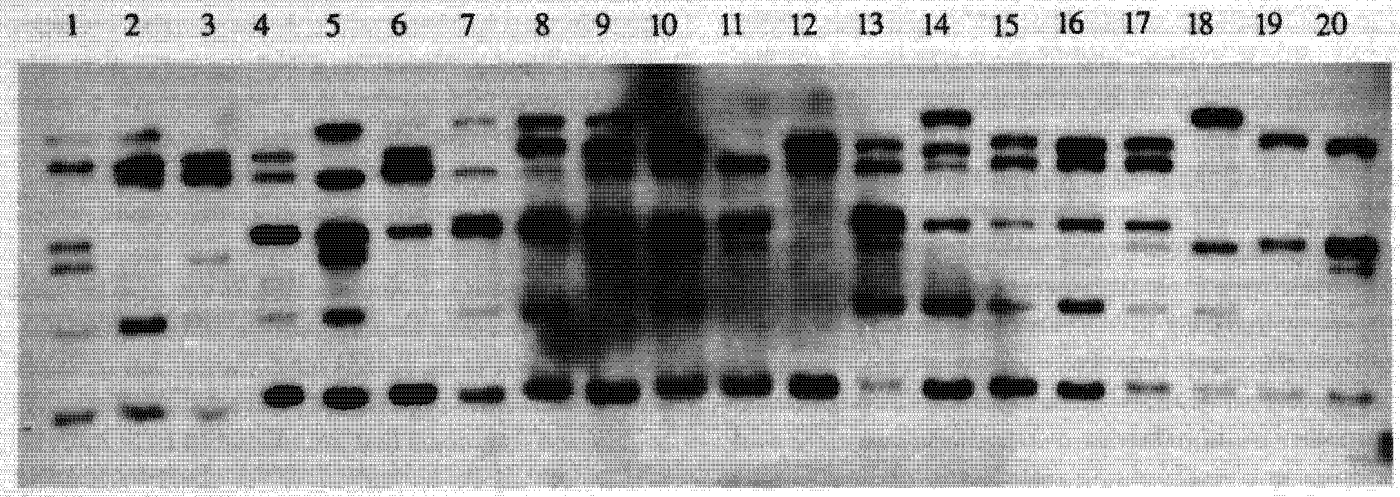

Figure 6. DNA isolated from potato cultivars and wild species was digested with EcoRI and probed with an anonymous potato sprout cDNA clone pSTC34. Lanes 1-20 as in Figure 5.

two alleles are present—one of which predominates in the tomato germplasm.

RFLPs can be due to either a loss of a restriction site or a gain of a new one. This can occur through base changes, deletions, additions, or rearrangements in the DNA sequence homologous to the cloned segment or in the area surrounding the cloned segment. RFLP analysis does not allow the genetic events responsible for these changes to be identified. However, polymorphism revealed by a single restriction enzyme is probably due to a sequence difference within the recognition site of that enzyme. Alternatively, polymorphisms that are revealed by several enzymes may reflect complex rearrangements such as insertions or deletions. In both rice (McCouch 1988) and tomato (Miller and Tanksley 1990a), there is a positive correlation between the size of genomic fragments generated and the amount of polymorphism detected. These results would be expected if polymorphisms are generated by insertions or deletions since the larger the fragment size the more likely that it will encompass an insertion or a deletion. The significant level of RFLPs detected in plants may therefore reflect variation in the frequency of rearrangements whereas the frequency of single nucleotide substitutions would be similar between species. Shattuck-Eidens et al. (1990) have recently used polymerase chain reaction (PCR) amplification and sequencing to compare DNA sequence variation in maize and melon. As would be predicted from RFLP analysis, the sequence variation in maize is much higher than in melon, both in the number of base changes and DNA rearrangements. More significantly, the processes generating and maintaining the neutral point mutations in maize differed for the four genomic regions examined. Transposable element activity may be one of the factors responsible for the high RFLP diversity observed in maize (Schwartz-Sommer et al. 1985).

The development of molecular marker techniques has provided more detailed assessments of genetic diversity. For a number of crop improvement programs, particularly maize, progress is based on the exploitation of heterosis. Pedigree information and the field evaluation of F_1 hybrids has been the main method used to predict and test heterotic combinations. Isozymes have been used to study the relationship between genetic diversity and heterosis (Frei et al. 1986). These studies were limited by the number of markers and the number of alleles per locus. The advent of RFLPs has allowed the relationship between genetic diversity at the DNA level and heterosis to be examined. Extensive studies by Lee et al. (1989) and Godshalk et al. (1990) have indicated that RFLP analysis is of value in allocating maize inbred lines to heterotic groups but no relationship between RFLP-based genetic distance and hybrid performance was detected.

Genetic Maps and Gene Introgression

The main application of RFLP technology is in the development of detailed genetic linkage maps. An attractive feature of RFLP mapping is that it can be accomplished in any plant species that undergoes sexual recombination. Genetic maps are based on meiotic recombination among homologous chromosomes

carrying alternative alleles. To determine linkage relationships among genetic loci, one must generate crosses that segregate for the genes of interest. Since most of the traditional genetic markers affect some aspects of whole plant phenotype, it has been difficult to score more than a few segregating markers simultaneously in a single cross. The generation of classical genetic maps has thus required hundreds of crosses and the analysis of thousands of segregating progeny. Since the level of allelic variation is higher with DNA markers than conventional morphological markers, detailed genetic linkage maps can be created from a single cross.

A key feature in the development of a linkage map is the identification of parents that will segregate for a trait of importance. However, it is difficult to predict the extent of RFLP variability between parents for use in linkage map creation. Since the usefulness of RFLPs is dependent on the degree of polymorphism existing in the organism of interest, the first phase of RFLP mapping often involves screening potential parents for variation at the nucleic acid level. Van de Ven et al. (1990) screened 16 *Vicia faba* accessions for RFLP variability with both four- and six-base pair cutters in conjunction with c-DNA clones. This approach allowed the identification of relatively diverse but hybridizable parents that could be used to create a mapping population. Having identified the parents, DNA extracted from each inbred can be cut with a range of restriction enzymes and sequentially probed with single copy clones. This is illustrated in Figure 7 for three *V. faba* genotypes: Optica, 172, and ABC. Polymorphism is detected between 172 and ABC with the restriction enzymes: BamHI, BglII, EcoRI, HindIII, and XbaI. These probe/enzyme combinations can then be used to monitor the segregation of alleles in the mapping population derived from 172 and ABC as parents. Both the F_2 and backcross generations can be used for linkage map creation, but Tanksley et al. (1988) have emphasized that the F_2 population offers the greatest resolution for a given number of individuals. Recombinant inbred lines offer an attractive al-

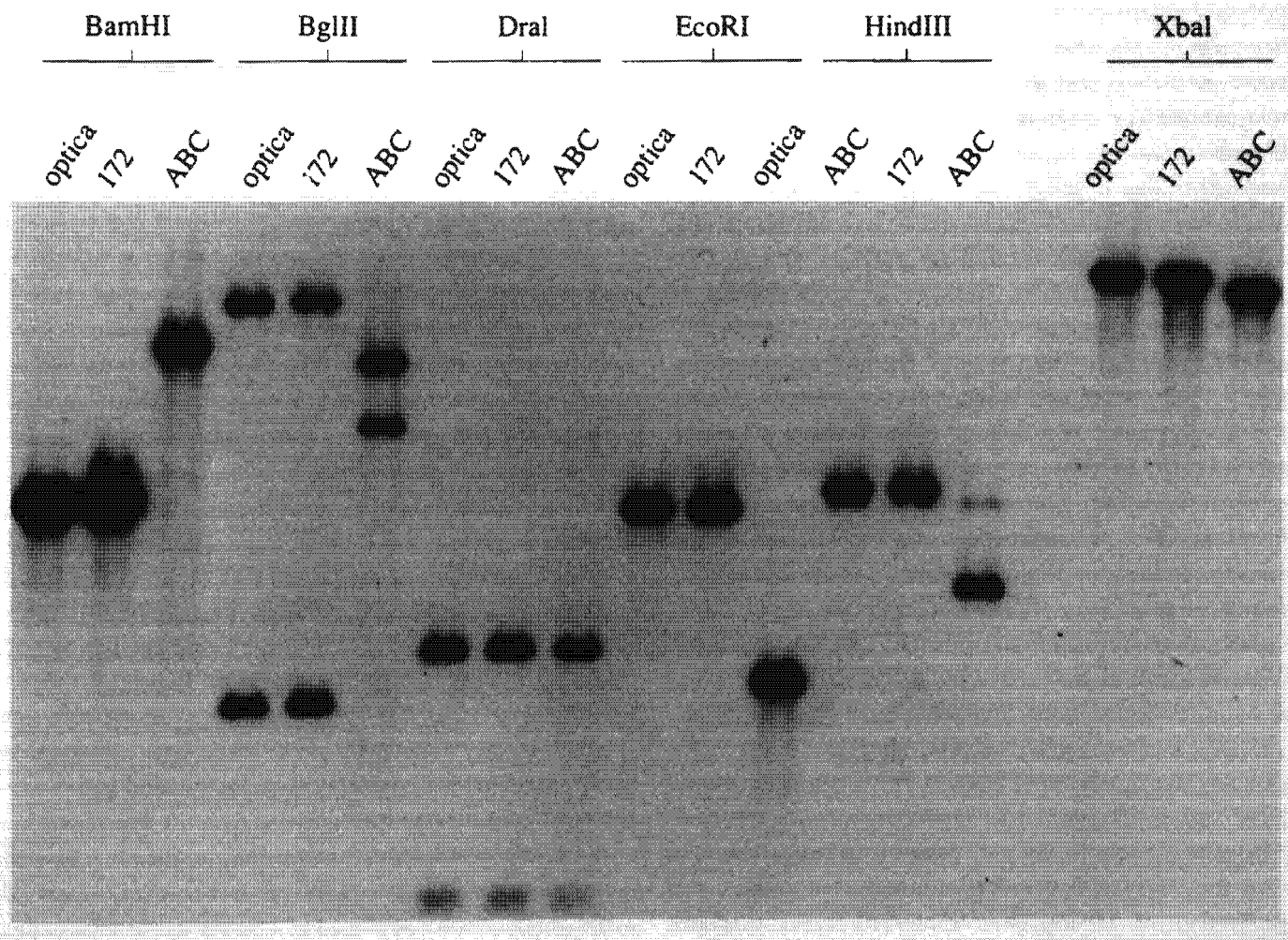

Figure 7. DNA isolated from three *Vicia faba* genotypes (Optica, 172, and ABC) digested with six 3 bp cutting enzymes and probed with cDNA clone 7.14.

ternative to F_2 and backcross generations since they represent fixed, homozygous individuals that can be used indefinitely for gene mapping studies (Burr et al. 1988). Recombinant inbred lines can be created by single-seed descent (Brimm 1966) or doubled haploidy (Bajaj 1990) and are particularly relevant for the mapping of quantitative traits. The availability of aneuploid genetic stocks can facilitate the localization of specific RFLP loci to chromosomes (Carlson 1972; Khush 1973). Helentjaris (1986) used monosomic analysis to map maize RFLP loci to chromosomes and Evola et al. (1986) have proposed that the B-A translocation method can also be used in maize for localization of RFLP loci to chromosomes. Primary trisomics have been used to assign gene markers to chromosomes via gene dosage effects in tomato (Young et al. 1987) and rice (McCouch et al. 1988). The isolation of alien chromosome addition lines in the *Triticeae* (Gale et al. 1988) has allowed RFLP probes to be assigned to chromosomes prior to formal linkage analysis.

Backcross, F_2, and doubled haploid (DH) families have been used to create linkage maps. It is interesting to note that in maize where high levels of DNA polymorphism exist, it is sufficient to make crosses between unrelated inbreds. In the case of rice, a cross between *indica* and *japonica* rice cultivars was used to generate the linkage map (McCouch et al. 1988). However, cultivated tomato is highly monomorphic, and both the creation and exploitation of linkage maps have involved interspecific hybridization between *Lycopersicon esculentum* and *L. chmielewskii* (Tanksley et al. 1989). The use of wild species is relevant for two reasons. First by using diverse parents in creating a linkage map, the probability of detecting polymorphism is increased and this facilitates the development of a linkage map. Second, primitive races and wild species possess a wealth of valuable genetic variation. The segregation of genes controlling resistance to various pests, pathogens, and abiotic stresses in the mapping population provides an opportunity to search for linkage between molecular markers and traits of importance. RFLP markers have been identified that are tightly linked to genes for resistance to tobacco mosaic virus (Young et al. 1988), *Fusarium* wilt, bacterial speck, and root knot nematodes in tomato (Paterson et al. 1988). A dominant resistance gene for *Fusarium oxysporum* f.sp. *lycopersici* race 3 was introgressed from *L. pennellii* into the cultivated tomato (McGrath et al. 1987), and Sarfatti et al. (1989) have identified RFLP markers linked to the resistance gene on chromosome 11 of tomato. Recently, Barone et al. (1990) have identified linkages between RFLP markers and a major dominant locus conferring resistance against the root cyst nematode *Globodera rostochiensis* on chromosome 9 of potato. The resistance gene was transferred from *S. spegazzinii* into one of the diploid *S. tuberosum* parents used for the mapping study. The genetics and breeding of potato have been hampered by the absence of a genetic linkage map. The identification of easily scored markers linked to important disease resistance genes emphasizes the rapid rate of progress that can be achieved following the detailed characterization of a mapping population.

Plant breeders have recognized the potential of exotic germplasm for varietal improvement and notable successes have been achieved, as reviewed by Stalker (1980). However, even when sexual hybrids can be obtained, the introgression of desirable traits into adapted cultivars is a slow and unpredictable process. The most common method of transferring genes from exotic germplasm into adapted varieties is through backcross breeding. The objective is to eliminate the exotic donor germplasm as rapidly as possible, replacing it with the recipient cultivar genome while retaining the gene of interest from the donor. RFLP markers that are tightly linked to traits of interest can be used to select backcross derivatives with the least amount of undesirable donor DNA. In this way RFLP markers can be used to expedite the transfer of small amounts of genetic information into commercial varieties. The introgression of important genes from wild species into cultivated plants is regarded as the most significant contribution of RFLP technology to plant breeding (Tanksley et al. 1989). This feature of RFLP technology is well illustrated by reference to rice (*Oryza sativa* L.) in which an RFLP map already exists (McCouch et al. 1988). The wild *Oryza* species possesses many agronomically-useful traits, such as resistance to pests and diseases and tolerance to biotic stresses. For example, hybrids between *O. sativa* and *O. nivara*, *O. rufipogen*, *O. officinalis*, and *O. longistaminata* have been created and backcross procedures are used to selectively introduce resistance to brown plant hopper, bacterial blight, and tolerance to stagnant flooding (Toenniessen et al. 1988). The identification of marker genes tightly linked to these traits will allow gene introgression to proceed with greater speed and precision. The cultivated groundnut, *Arachis hypogaea* L., is an important food legume that could also benefit from the application of RFLP technology. Many of the wild *Arachis* species are known to possess genes conferring resistance to rust (*Puccinia arachidicola*), leaf spots (*Cercospora arachidicola*), viruses (e.g.,

groundnut rosette virus, peanut mottle virus), and certain insect pests. Considerable effort has been devoted to the transfer of such genes from wild *Arachis* species into the cultivated gene pool (Stalker and Moss 1987). A major challenge in such research programs is the identification of recombinant genotypes that contain the introgressed chromosomal segment in a desirable, adapted genetic background. Traditionally, plant breeders and cytogeneticists have selected such desirable recombinants on the basis of plant phenotype. Molecular and biochemical markers are being used to create a linkage map in *Arachis* (P. Lanham, SCRI, UK, personal communication). The establishment of a linkage between an easily scored molecular or biochemical marker and a trait of interest will provide a more direct means of selecting and identifying desirable recombinant genotypes.

Backcross breeding procedures have been used to develop near-isogenic lines (NILs) in a number of crop plants (Young et al. 1988). In this situation a genotype is obtained that carries the target gene in a background that is nearly identical to that of the recurrent genotype. However, small segments of DNA flanking the target gene will persist due to the phenomenon of linkage drag (Zeven et al. 1983). The product of backcrossing will, therefore, be sets of NILs that are similar except for a region near the target gene. In this situation the introgressed segment can be used as a target to determine whether a given RFLP probe or isozyme marker is linked to the target gene. DNA or biochemical markers that are located outside the introgressed segment will exhibit identical electrophoretic profiles between the NILs while markers located within the introgressed segment, i.e., linked to the target gene, will exhibit polymorphism. Using this approach, it is possible to rapidly identify marker genes that are linked to important major genes. Young et al. (1988) used NILs to identify RFLP markers tightly linked to the *Tm-2a* locus conferring resistance to tobacco mosaic virus in tomato. Sarfatti et al. (1989) used a similar approach to locate RFLP markers tightly linked to the *Fusarium oxysporum* resistance gene (I2) in tomato. NIL analysis was also used by McMullen and Louie (1989) to link RFLP markers to a resistance gene for maize dwarf mosaic virus.

The polyploid nature of certain crops allows such organisms to tolerate the loss and gain of chromosomes (aneuploidy). Where they exist, aneuploid chromosomal types have been used extensively in the chromosomal location of genes and in the formulation of linkage maps. These methods are best illustrated in wheat (*Triticum aestivum*), but have also been used in oats (*Avena sativa*), tobacco (*Nicotiana tabacum*), and species of cotton (*Gossypium*) (reviewed by Khush 1973). In these crops cytogenetic techniques have allowed whole chromosomes to be manipulated (Gale et al. 1988) and have allowed the development of single-chromosome recombinant inbred lines (Law 1966). These genetic stocks provide an opportunity to associate quantitatively-determined traits with individual wheat chromosomes (Worland and Law 1986). The availability of RFLP markers in wheat will allow greater precision in intervarietal chromosome manipulations. Techniques for the incorporation of alien chromosome segments into wheat based on homoeologous chromosome pairing are also well advanced. The exploitation of marker-based technology in combination with cytogenetic techniques represents a new resource for use in the analysis of alien gene transfer and wheat chromosome manipulation (Sharp et al. 1989). Although not as advanced as wheat, *Brassica* species are also characterized by the formation of amphidiploids following the hybridization of diploid species. A series of monosomic *B. oleracea* chromosome addition lines have been constructed in the genetic background of *B. campestris* (McGrath and Quiros 1990). Seven of the nine possible *B. oleracea* addition groups were characterized with molecular markers (McGrath et al. 1990). A number of DNA sequences were identified that were duplicated on more than one chromosome, i.e., they physically mapped to different chromosomes. These studies indicate that the duplicated nature of the *B. oleracea* genome may allow interspecies gene transfer to occur. The availability of molecular markers will allow this approach to be exploited more fully in *Brassica* improvement programs.

Beta procumbens chromosome addition lines have been created in a *B. vulgaris* background. Cytogenetic mutants have been generated from the original addition lines in which a *B. procumbens* chromosome fragment possessing a gene for resistance to beet cyst nematode (*Heterodera schachtii*) has been identified (Jung and Wricke 1987). *B. procumbens* species DNA probes have been used to screen for resistance to beet cyst nematode in hybrid progeny (Schmidt et al. 1990). Jung et al. (1990) have used pulse field gel electrophoresis to identify *B. procumbens* chromosome-specific probes. The physical mapping of the nematode resistance gene may, in combination with the cloning of large DNA fragments and transformation, allow the isolation of this gene.

Manipulation of Quantitative Traits

Many of the traits manipulated by plant breeders exhibit continuous variation. Yield, maturity date, and tolerance to abiotic stress are examples of important traits that usually exhibit a quantitative mode of inheritance and are a consequence of the joint action of several genes. Mather and Jinks (1982) have stressed that polygenes or quantitative trait loci (QTL) are inherited in just the same way as the genes of major effect; they segregate, recombine, and exhibit linkage. They show the same range of properties in transmission and action as do the genes of major effect. In other words, the features of metrical traits arise from the action of Mendelian genes. However, we cannot monitor directly by conventional methods individual genes conditioning quantitative traits. A biometrical approach is necessary to partition the total variation into genetic and nongenetic components. This approach has contributed significantly to our understanding of quantitative variation and has provided the genetical framework for many plant breeding strategies (Jinks 1981).

The dissection and manipulation of quantitatively controlled characters are important objectives in both basic and applied genetic research. Stuber (1989) has stated that the plant breeder has limited information on the number of genetic or effective factors involved in the expression of the trait, the chromosomal location of these factors, and the relative size of the contribution of these factors to trait expression.

Biometrical procedures for estimating the number of effective factors controlling a trait have been developed (Mather and Jinks 1982; Powell et al. 1985a; Cockerham 1986; Zeng et al. 1990), but these are of little practical value. The most important challenge is to identify specific regions of the genome that enhance the expression of quantitatively controlled characters. The theoretical basis for interpreting the association of marker loci with QTL has been outlined by Thoday (1961), Mather and Jinks (1982), Gelderman (1975), Tanksley et al. (1982), and Beckman and Soller (1983). Essentially, the theory exploits the fact that the marker locus identifies a chromosomal segment and enables that segment to be monitored in subsequent generations of crossing or selfing. Although the importance of character dissection in plants has been recognized for some time, progress has been hampered by the lack of suitable marker loci. Many of the earlier studies of character dissection in *Drosophila* (Breese and Mather 1957, 1960) and plants (Rasmusson 1933; Everson and Schaller 1955) were based on morphological markers and therefore had limited practical applications in plant and animal breeding. The availability of molecular markers has allowed the theoretical approaches developed by earlier workers to be used effectively to analyze quantitative variation.

Isozymes were the first molecular markers to be used for character dissection in plants. Tanksley et al. (1982) used 12 isozyme loci to locate factors influencing four quantitatively inherited characters in a backcross population derived from *L. esculentum* × *L. pennellii*. Vallejos and Tanksley (1983) reported linkages between segregating enzyme loci and genetic factors for cold tolerance. Isozymes have also been used to map QTL in soybean (Graef et al. 1989) and maize (Stuber et al. 1982, 1987; Edwards et al. 1987). These studies, which have been based on F_2 or backcross generations, demonstrate the potential of molecular markers for studying and manipulating quantitative traits. The majority of these studies have been based on single marker associations. The advent of more complete genetic linkage maps based on RFLPs has allowed intense investigations of quantitative traits. The most detailed genetic linkage maps exist for tomato and maize and it is, therefore, not surprising that the most exhaustive evaluation of RFLPs and QTL have been conducted in these crops. Paterson et al. (1988) investigated a backcross population of a wild tomato species, *Lycopersicon chmielewskii*, to the cultivated tomato. An analysis of fruit weight and the concentrations and pH of soluble solids revealed six QTLs affecting fruit weight, four affecting soluble solids concentration, and five affecting pH. The approach used in this is based on maximum likelihood and exploits the availability of many markers on a chromosome. Quantitative trait loci are more likely to be detected between pairs of flanking markers and Lander and Botstein (1989) have used interval mapping, which assesses the effects of genetic segments rather than effects associated with individual loci. These researchers claim that interval mapping reduces the confounding effects of recombination between marker loci and a QTL, efficiently exploits the information from the RFLP linkage data, and provides greater precision than was previously attainable. A computer program, MAPMAKER-QTL, that implements interval mapping is available (Lander and Botstein 1989).

It is of interest to note that in both tomato and maize, RFLP markers associated with QTL are often chromosomally linked to extreme phenotypic mutants. For example, the three traits analyzed by Paterson et al. (1988) have large effects that map to chromosome 6 and are linked to spinelessness (sp), a

morphological marker that alters plant development. Helentjaris and Shattuck-Eideus (1987) have identified RFLP markers on chromosome 9 of maize that have a significant impact on plant development. These molecular markers are located near the centromere adjacent to a known gibberellic acid biosynthetic dwarf mutant (d_3). Robertson (1989) has postulated that extreme mutant phenotypes that map to specific chromosomal regions may also be expected to be implicated in the expression of quantitatively-controlled characters. Although this hypothesis requires further consideration, it does emphasize the desirability of chromosomally mapping morphological and biochemical mutants in plants.

The majority of the studies designed to analyze QTL in plants have involved a limited number of crosses and have been conducted in a single environment. Tanksley and Hewitt (1988) have stressed the dangers inherent in evaluating the effects of molecular markers in a single cross. Chromosome segments introduced into tomato, from *L. chmielewskii* were identified as having an effect on soluble solids content. The effect of the introduced segment was dependent on genetic background and emphasizes the need to analyze a range of genotypes. Environment and genotype by environment (G×E) interaction may also complicate the identification and interpretation of such experiments. The influence of genetic background (both nuclear and cytoplasmic) and G×E on the role of RFLPs in character dissection warrants further investigation.

Studies on the analysis of genes that condition quantitative traits by the use of molecular markers share a number of common features.

First, in tomato, interspecific crosses were used to maximize the number of polymorphic markers available in the segregating crosses. Although the level of electrophoretic variability for molecular loci in maize is higher than in tomato, the parents were selected to maximize the number of allelic differences in the segregating crosses. Such experiments have been successful in identifying regions of the genome that can account for a substantial portion of the phenotypic variance in a segregating generation. However, this approach may not predict which blocks of genes will be of use in intervarietal transfer in plant breeding.

Second, a requirement for the identification of linkage between a marker and a QTL is linkage disequilibrium. The majority of the studies to date have focused on the use of F_2 or backcross generations to measure the effects of marker loci on QTL. This approach, although valid, assumes that there will be no further breakdown of linkage groups in subsequent selfing and crossing. It is likely that further rounds of gametogenesis will result in the attainment of linkage equilibrium. This indicates that the early segregating generations of a cross may not represent the optimal strategy for the complete evaluation of linkage between a marker locus.

Finally, further complications arising from the use of F_2 and backcross generations include the difficulty of observing accurate estimates of the environmental and genetical components of variance. The presence of dominance can also introduce a bias to the estimate obtained when the unit of replication is based on single plants (Powell et al. 1986). Intergenotypic competition is also an important factor influencing the assessment of quantitative traits (Powell et al. 1985b).

Many of the problems associated with the assessment of segregating populations can be overcome by the use of recombinant inbred lines. Bailey (1971) and Burr et al. (1988) have outlined the advantages of recombinant inbred lines (RIL) in gene mapping. These include the fact that RILs represent a permanent population that can be used indefinitely for mapping. Thus, new loci can be continually added to the linkage map. In some cases, RILs are generated by several rounds of selfing so that homozygosity is approached, e.g., single-seed descent (Brim 1966). Alternatively, double haploids (DH) can be generated from F_1 hybrids. DHs extracted from F_1 hybrids are likely to exhibit a higher linkage disequilibrium relative to other generations due to the reduced opportunities for recombination. Furthermore, the greater additive genetic variation associated with DHs, together with the absence of within-family segregation, indicates that this approach is well suited to relating variation detected at the nucleic acid level to QTL. This approach has been used previously to analyze the effects of morphological (Powell et al. 1985c, 1985d; Powell et al. 1990a), and isozyme loci (Powell et al. 1990b) on quantitative traits in random inbred lines of barley. Powell et al. (1990b) established that over 9% of the genetic variation for single plant yield in DH progenies of a spring barley cross could be accounted for by allelic variation at the α-*Amy-1* locus on chromosome 6H. DHs were also used to demonstrate associations between alleles at the ribosomal, r-DNA (*Nor-H3*) locus on chromosome 5H, and a number of agronomic and quality characters in barley (Powell et al., unpublished data). In particular, allelic variation at the *Nor-H3* locus was responsible for over 28% of the additive genetic variation for milling energy—a measure of grain hardness. This is illustrated graphically in Figure 8 where two distinct frequency distributions are evident. This study indicates that the

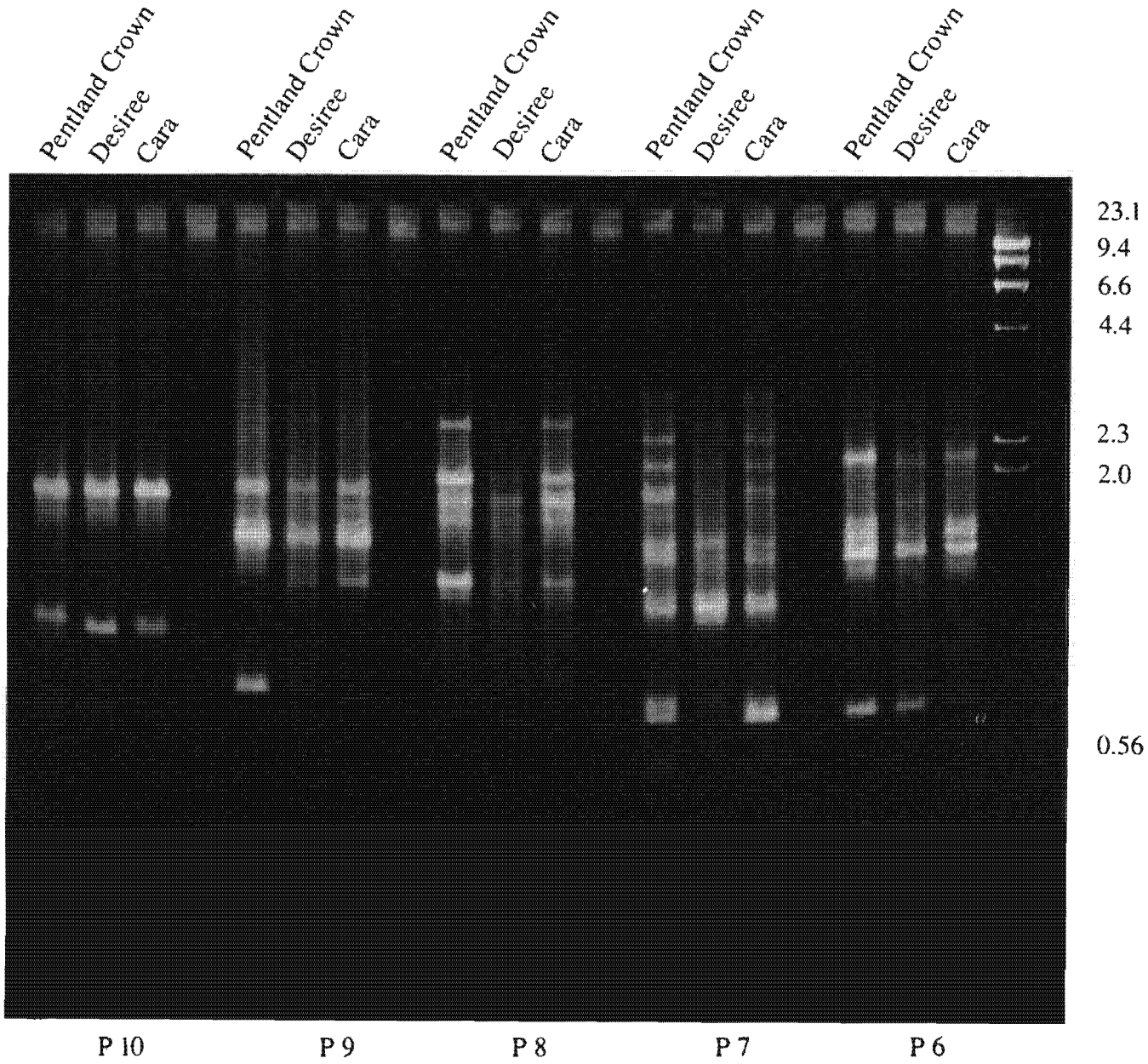

Figure 9. DNA from Pentland Crown, Desiree, and Cara revealing polymorphism when amplified with primers 6 to 10.

Conclusions and Perspectives

The tools of molecular biology now provide the opportunity to develop large numbers of phenotypically neutral genetic markers in any organism from which DNA can be extracted. In plants there have been two dominant features associated with linkage map production. The ability of plants to tolerate and generate genetic diversity together with the ease of interspecific hybridization (Walbot and Cullis 1983) have been vital for the rapid progress achieved in linkage map creation during the last decade. Perhaps significantly, the future challenge for RFLP research lies not in linkage map creation per se but in the exploitation and application of knowledge gained from investigations on genome organization and gene mapping. The control of recombination is a fundamental aspect of the release of genetic variation and is central to the many activities associated with gene marker-based technology. The availability of detailed linkage maps will allow the mechanism(s) responsible for the disruption or preservation of gene complexes to be studied. Questions of importance to plant breeders about recombination are summarized below. Genetic markers will allow many of these issues to be addressed.

- How much recombination occurs in specific chro-

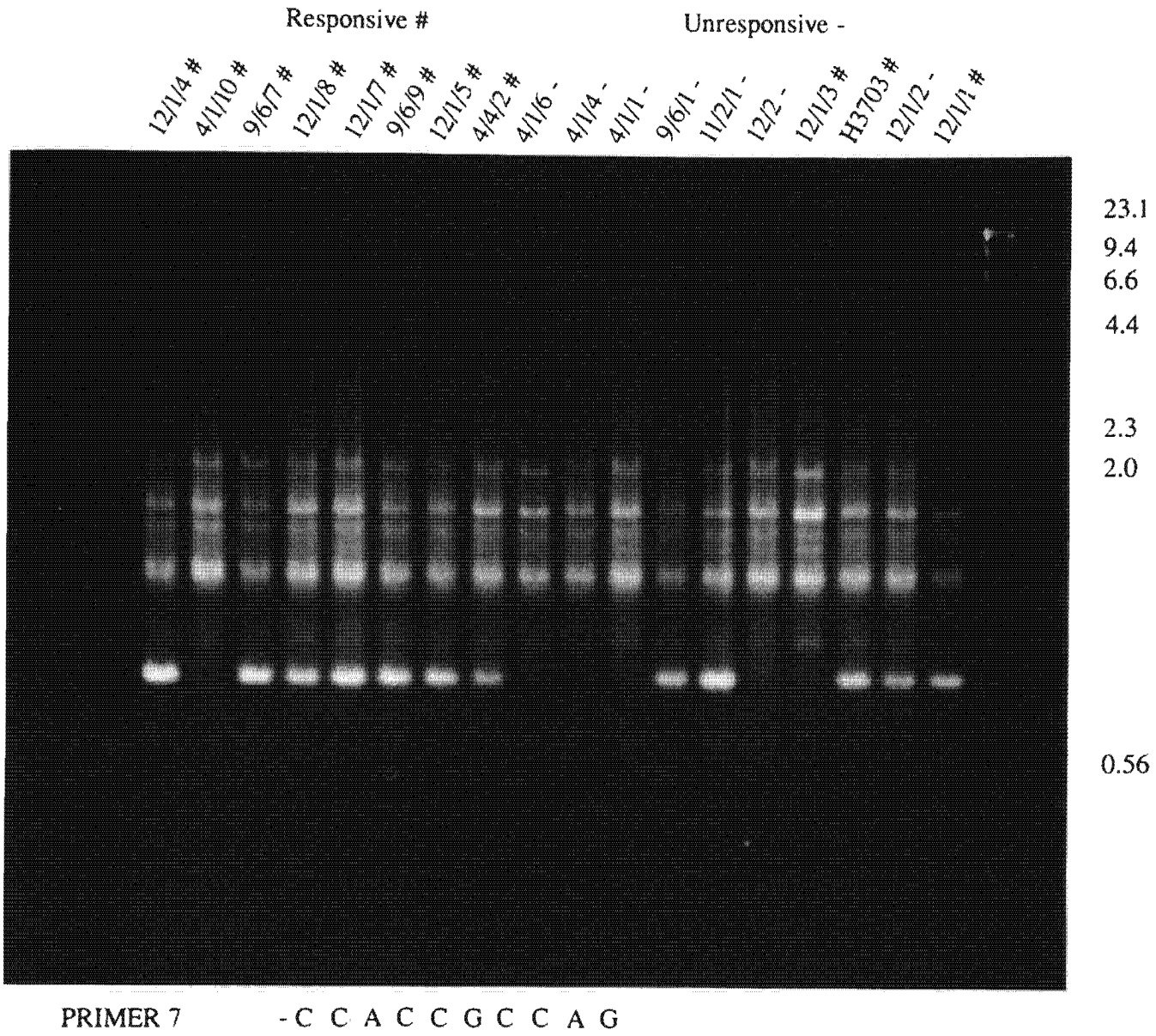

Figure 10. Segregation of a RAPD marker in anther culture derivatives of potato. The 0.8 kb amplified DNA fragment segregates as a null allele.

mosome segments during breeding cycles?

- Can large segments of the chromosome remain intact over several breeding cycles?
- Does the amount of recombination between specific loci vary with different breeding strategies?
- Is the amount of recombination in a chromosome segment affected by selection for specific genes on the segment?
- Do current breeding methods efficiently exploit the potential variation from crosses?

Significant progress in the introgression of exotic germplasm into well-adapted cultivars is also anticipated. The use of PCR technology to rapidly amplify genomic DNA fragments will also play a significant role. However, it is unlikely that this technology will completely replace conventional RFLPs but RAPDs and isozymes should be regarded as complementary to RFLPs. Studies with a range of crops have already demonstrated that a significant portion of the additive genetic variation for quantitative traits can be determined by relatively small regions of the genome. Recombinant inbred lines, particularly DH, will play an increasingly important role in linkage map creation and in the manipulation of quantitative traits in plants.

Further advances in gene mapping will demand an integrative, multidisciplinary approach in which the skills of the plant breeder to identify appropriate well-characterized germplasm is of paramount importance. Both national scientists and the staff of the international research centers have a vital role to play in the

identification of objectives and in the exploitation of appropriate technology to address important scientific objectives.

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DNA Markers and Marker-mediated Applications in Plant Breeding, with Particular Reference to Pearl Millet Breeding

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Abstract

The paper discusses the various markers available for the plant breeder to use, their characteristics, and their advantages and disadvantages. Besides covering the types of markers presently being employed in breeding experiments, it also covers the newer DNA-based and antibody detection technologies which promise to provide more comprehensive genetic maps that will allow extended applications of markers.

The following biochemical and molecular marker systems are discussed: protein markers, isozymes and storage proteins; monoclonal antibodies (McAbs); restriction fragment length polymorphism (RFLPs); specific polymorphic locus amplification test (SPLATs); and randomly amplified polymorphic DNA (RAPDs).

The use of markers in plant breeding is discussed extensively, with emphasis on the applications of tagging major genes in specific breeding methods, including recessive genes during backcross and pedigree breeding; dominant and multiple genes in backcross breeding, and selection for the recurrent parent genotype in backcross breeding.

Backcrossing in pearl millet to introgress two characters, downy mildew resistance, probably dominant, and photoperiod insensitivity, which is recessive, is used as an example.

The application of markers to tag genes for quantitative characters and indirect selection using quantitative trait loci (QTLs) can be done with high efficiency.

Evaluating parental inbred lines for a large number of markers allows the prediction of heterozygosity in the F_1 and the prediction of hybrid yield. Similarly, combining ability can be predicted.

Introduction

The use of genetic markers and genetic maps in breeding, much discussed since DNA markers have become available, is by no means new. The possibility of using linked markers—or 'gene tags' in todays' jargon—to apply indirect selection for agronomic traits has long been known. Varietal distinction and definition, or fingerprinting, has always relied on heritable, often trivial, traits. Those who were not wedded to the concept of 'polygenes', which effectively precluded the genetic characterization of complex quantitativly expressed traits (QTLs), were well aware of the possibility of identifying and mapping the factors controlling characters as intransigent as yield itself (Thoday 1961). The problem was, of course, that there were never enough markers available to the geneticist/breeder to develop these concepts into practical tools. To make matters worse many of the few markers that we did have were unacceptable for the development of generally applicable marker-aid techniques in commercial plant breeding programs.

Among the characteristics of an 'ideal' marker locus are, that it has a phenotype which is irrelevant to the gross agronomic phenotype, and that it does not affect, directly or pleiotropically, the traits for which the breeder is selecting. This immediately rules out

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most morphological characters, such as inflorescence type or plant habit, almost all disease resistance genes, and most of the many recessive mutants that, until recently, formed the backbone of the genetic maps in the few plant species in which they existed. A further characteristic of ideal markers is that they should be expressed codominantly, i.e., the heterozygote should be distinguishable from either homozygote. The emergence of protein markers, particularly those provided by isozyme analysis that emerged in the 1960s, seemed to meet both of the above criteria. Indeed, biochemical markers have been particularly useful both in breeding practice (Ainsworth and Gale 1987) and the further development of marker-aided technology (Stuber et al. 1987). However, even this panacea was eventually found wanting. There turned out to be a very finite number of systems, i.e., classes of protein, which could be analyzed in any single laboratory, and the degree of polymorphism was often not adequate for use in a breeding program using relatively closely related genotypes as parents. Moreover, isozyme expression was often found to be dependent on the developmental stage of the tissue from which they were extracted, which led to ambiguity in interpretation of the gels, and, since each enzyme requires a different staining system and often its own electrophoretic conditions, considerable skill, a large range of chemicals, and a battery of electrophoretic equipment is needed to effectively use a number of different isozyme marker systems.

Use of variation in the DNA itself, initially as restriction fragment length polymorphism (RFLP) first in animal systems (Botstein et al. 1980) and throughout the 1980s in an increasing number of crop plants, overcame many of the problems that have limited the use of biochemical markers. The number of loci available is effectively infinite; for example, in the pearl millet genome any length of DNA can be cloned from its 2 billion base pairs and be used as an RFLP probe. The degree of polymorphism realized is greater than for any other type of marker because differences as small as only a single base-pair change can be observed. There are no developmental effects, provided methylation-sensitive restriction sites are avoided, so the DNA from, say, the tip of a grain, will give the same result as DNA from the tip of a root. Finally a major advantage is that only a few methodologies are needed to visualize variation in this DNA at any locus, i.e., once a few protocols are mastered a single laboratory can expect to be able to use all DNA markers for which the relevant information is publicly available.

The same criteria apply to conventional RFLP analysis or the use of polymerase chain reaction (PCR) mediated applications such as the random amplified polymorphic DNA (RAPD) system (Williams et al. 1990).

DNA markers, of course, do have defects for general use in breeding programs. They are expensive to use, relatively time consuming to assay, and require a substantial and relatively sophisticated laboratory set up. Each of these difficulties are, and will continue to be, alleviated with the development of new methodologies such as automated DNA extraction and analysis and the development of visualization systems that do not rely on radiochemicals.

The Overseas Development Administration (ODA), UK, have initiated a project to construct a DNA marker and isozyme linkage map in pearl millet (*Pennisetum glaucum* L.Br.) with the purpose of identifying probes that will be of direct help in pearl millet breeding programs. The project involves collaborative research between ICRISAT, the Cambridge Laboratory (CL), Institute of Plant Science Research, Norwich, and the Centre for Arid Zone Studies (CAZS), University of Wales, Bangor. The initial project targets will be markers for downy mildew resistance and a photoperiod-insensitivity gene as well as constructing a high-density linkage map. The DNA and isozyme work is being done in the CL, the field work at ICRISAT, and the screening for downy mildew resistance at ICRISAT and CAZS.

In this review we list the types of markers presently being employed in breeding experiments, and the newer DNA-based and antibody detection technologies that promise to provide the more comprehensive genetic maps which will allow extended applications of markers. The methods themselves are covered in detail elsewhere in this publication (Powell 1992) so here we restrict ourselves to a brief list of advantages and disadvantages of the various species of marker systems. The methods are summarized in Figure 1.

Genetic Markers

Below we list only biochemical and molecular marker systems. These are summarized in Figure 1 with an indication of the time required for the various systems, which also differ in the level of technical skills and resources needed. Although the use of most conventional morphological markers is not usually practicable in breeding programs, those that are available should not be ignored. Some, for example antho-

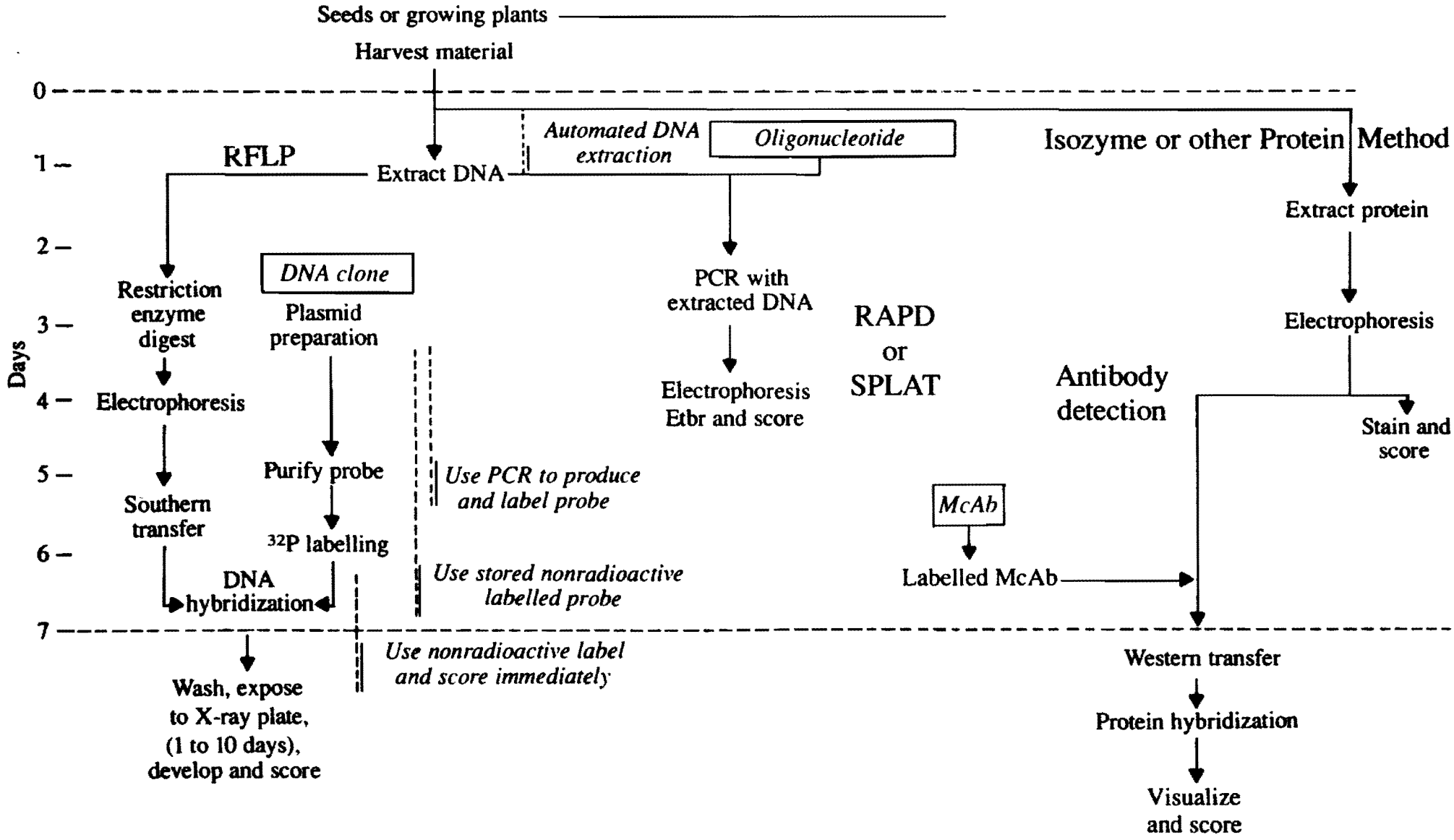

Figure 1. Methods of detection of biochemical and molecular markers and an indication of the time required for their use. Some needed technical enhancements are shown in italics (⋮ = existing time, | = reduced time).

cyanin pigmentation of coleoptiles or ligules, are trivial in terms of the breeders' ideotype but very conveniently and economically assessed. Another class of markers which fall into this category are disease resistance alleles effective only against races that are not, or no longer, relevant in the region served by the program. These genes are often easily scored by breeders with conventional backup from their pathology laboratories.

Protein Markers, Isozymes, and Storage Proteins

Pro: Simple extraction and staining; several protocols often available; rapid and economical for mass screening; very useful for wide-cross applications; variation can be used in physiological experiments.

Con: Number of marker loci still limiting; protocols system specific; considerable experience of individual systems required for analysis and interpretation; interpretation confounded by developmental effects; polymorphism levels often limiting in varietal comparisons.

Monoclonal Antibodies (McAbs)

McAbs to random plant proteins, of which electrophoretic variants can be identified on Western blots without any further knowledge of the actual protein under investigation, offer potential alternatives to the use of random DNA clones for RFLP analysis or random oligonucleotides for RAPD analysis.

Pro: Potential number of systems large; polymorphism from protein properties in addition to electrophoretic differences; a single detection system for all markers; tests can be adapted to easily transported 'kit' formulation.

Con: Antibody production requires experiments with animals; system relatively untested with random libraries; relative levels of polymorphism unknown.

Restriction Fragment Length Polymorphism (RFLPs)

Pro: Numbers effectively infinite; single protocol for all markers; polymorphism levels can be enhanced by use of wide range of restriction enzyme digests; use of known-function clones can relate variation to function; transferable between species.

Con: Relatively slow, particularly DNA extraction and Southern transfer; detection system dependent on genome size; polymorphism still limiting in some species; still requires use of radioactivity although nonradioactive methods now being developed for universal use.

Specific Polymorphic Locus Amplification Test (SPLATs)

SPLATs are essentially second generation RFLPs. Once an important diagnostic RFLP locus has been identified, the polymorphic fragment can be cloned and end-sequenced. Then appropriate oligonucleotide primers may be raised that will amplify only the specific fragment in a PCR test.

Pro: Relatively rapidly screened; detection of null alleles without the need for electrophoresis; transportable 'kit' development possible; very small amounts of genomic DNA required.

Con: Formulation and production of primers expensive; requires DNA extraction.

Randomly Amplified Polymorphic DNA (RAPDs)

Pro: Relatively rapid; primers available cheaply; polymorphism easily detected; many primers can be screened on a single PCR run; very small amounts of DNA required.

Con: Mainly dominant markers and thus less efficient for many application; primers may detect different loci in different crosses; experimental conditions highly critical, to some extent PCR-machine dependent.

Use of Markers in Plant Breeding

The uses of RFLPs and isozyme markers in plant breeding are numerous and the amount of information required to use them varies considerably (Table 1). For most fingerprinting applications no information is required on the linkage groups of the markers, or their linkage relationships with genes controlling traits. In contrast, efficient tagging of quantitative traits requires a high density RFLP linkage map, and the positioning of quantitative trait loci (QTL) on the map. Between these extremes intermediate amounts

Table 1. Some applications of markers in plant breeding and requirements for linkage data.

Application	Need			Trait loci linkage to:	
	None	Helpful	Essential	Marker	Map
Fingerprinting					
Organization of germplasm	X	X			
Identifying parental lines/cultivars	X				
Analysis of pedigrees	X	X			
Testing hybrid seed purity	X				
Testing inbred line/cultivar purity	X	X			
Testing similarity of isogenic lines	X	X			
Testing similarity in mutation breeding	X	X			
Quantification of Heterozygosity					
Tests of breeding systems	X				
Prediction of F_1 hybrid yield	X	X			
Cytogenetic Applications					
Aneuploid analysis			X[1]		
Tagging alien chromosome segments	X				
Intergenomic and specific relationships				X	
Engineering chromosome segments			X		
Tagging Major Genes					
For single major genes	X[2]	X[3]	X[2]	X[3]	
For multiple genes (pyramiding)	X[2]	X[2]	X[3]	X[2]	X[3]
Backcross Breeding					
Recurrent parent genotype	X	X	X[4]		
Quantitative Inheritance					
Tagging QTLs	X	X			

1. Much cytology can be avoided, and only linkage groups need be known.
2. Applies to single marker tags.
3. Applies to flanking marker tags.
4. If selection targeted against donor parent genes around donor gene, when several (or more) flanking markers are necessary.

of information are required, but many applications for which linkage information is unessential can benefit from it. For example, in pedigree analysis, linkage information can be used to determine how differences detected between cultivars are distributed on the chromosomes, and in heterozygosity studies linkage information can localize heterozygosity in the genome.

Fingerprinting

It is clear that RFLP markers are a powerful tool to discriminate between inbred lines, cultivars, and landraces, with many potential uses to the plant breeder. Two examples, which will be studied in the project, are given where fingerprinting alone will provide useful information.

In pearl millet, a highly cross-pollinated crop, it is difficult to determine whether mutation breeding on inbred lines has been successful because of mutation or because of selection for existing variation. Useful genetic variation could be due to mutation or outcrossing of the line in a previous generation. If the inbred line is largely homozygous, and the product of mutation breeding is almost identical to the original line then we can assume that the new product is the result of mutation and not outcrossing.

A similar purpose is checking on the similarity of isogenic lines, particularly if the approach to homozygosity can be verified over generations.

Genotypic Selection (Indirect Selection)

In a conventional breeding program selection is made on the phenotypes of individual plants, or families of plants. In genotypic or indirect selection markers are used to tag the desired genes. Markers are selected that are closely linked to the genes that control the desired character. The degree of response in indirect selection is a function of the effects of the genes and the tightness with which they are linked to the markers.

Gene Tagging of Major Genes

There are general advantages in being able to tag major genes. These include the ability to select for a trait without the need for an environment that allows expression of the trait, juvenile testing for markers at the early stages of plant growth before many traits are expressed, the ability to simultaneously select for several genes that control the same character (gene pyramiding), and the ability to detect recessive genes in heterozygotes.

Environmental stability. Plant breeders can select for a trait, once linkage between RFLPs and the trait of interest is known, without regard to the environment. This has great advantages in pearl millet where off-season nurseries are used, or when the specific environments which allow traits to be expressed are expensive to provide, such as in smut susceptibility in pearl millet, or when there is unreliable expression of the character. A good example is the use of the linked isozyme marker, endopeptidase-1 (*Ep-D1*), to detect the eyespot (*Pseudocercosporella herpitricoides*) resistance gene, *Pch1*, which was transferred to wheat from *Aegilops ventricosa*. The precision of selection using *Ep-D1* as a marker is excellent. Where there was disagreement between the classification of resistance using disease screening or the isozyme, retesting of the genotypes revealed that the isozyme classification was invariably correct (Summers et al. 1988).

A breeder can also select for resistance to races or diseases that are not available at the site of testing. It is hoped that eventually resistance to African pathotypes of *Sclerospora graminicola* can be selected in India by using molecular markers.

Juvenile testing. Juvenile testing for markers associated with mature phenotypes has the greatest advantage in pearl millet in testing seedlings for genes that restore or maintain cytoplasmic-genetic male sterility. In topcross pollinator breeding, selection for fertility restoration is required, but the ability of a genotype to restore or maintain cannot be determined conventionally until the subsequent generation when the fertility of testcross progeny is established.

The example of eyespot resistance in wheat also demonstrates the use of juvenile testing. This disease is conventionally assessed in 10-week-old plants after seedling inoculation (Hollins et al. 1985). *Ep-D1* can be assessed in young leaves for the allele corresponding with resistance and those plants not homozygous for *Ep-D1* discarded. *Ep-D1* is particularly efficient as a marker because in F_5 and F_6 progenies from resistant × susceptible crosses no recombination was observed between *Pch-1* and *Ep-D1b* (Summers et al. 1988; Worland et al. 1988).

Gene pyramiding. Indirect selection is particularly valuable in selecting plants with two or more independent genes which give a similar phenotype, a process known as gene pyramiding. For example, it might be advantageous to include more than one resistance gene in a cultivar in the expectation that resistance will be more durable. However, simultaneous selection for more than one gene is difficult since the phenotype is often the same however many resistance genes are present. In conventional breeding, the inbred progeny produced by selfing from an individual plant must be tested in order to be able to determine the number of resistance genes each plant has. The resources required to do this would normally be prohibitive. However, once linkage of the individual disease resistance genes to RFLP markers is established, individual plants can be scored for the presence of one, two, three, or more resistance genes. No progeny testing is required, and multiple genes for more than one disease can be simultaneously screened in a single plant. The technique of gene pyramiding can be applied in any method of plant breeding, e.g., backcross or pedigree breeding.

In pearl millet, it is possible that there are several genes controlling resistance to different pathoypes of the downy mildew pathogen. Some inbred lines display stable across-location resistance in both India and Africa, whereas other lines although resistant in some locations, are moderately or very susceptible in

others. If inbred lines that have stable across-location resistance are used as resistance donors then the genes for resistance can only be transferred if screening is done in most or all generations across a wide range of environments. Such a program would be expensive and time consuming. However, using well-controlled and well-replicated experiments and a range of inoculum sources it may be possible to find two or more resistance genes that are linked to RFLPs. It should then be possible to use the gene pyramiding technique for downy mildew resistance breeding in pearl millet.

Applications of Tagging Major Genes in Specific Breeding Methods

Recessive Genes and Backcross Breeding

Markers can be used to improve the efficiency of selection for a recessive gene during a backcross breeding program. Backcross breeding often involves alternating generations of backcrossing, selfing and progeny testing. The progeny testing is done to determine which of them carry the desired recessive gene. A recessive allele could be indirectly selected, however, by selecting for a linked marker or flanking markers so that progeny testing is rendered unnecessary. Resources are saved as plants can be eliminated from the breeding program after nondestructive seedling testing.

The use of an RFLP marker requires a very small number of plants. If as few as four plants are sampled for the RFLP marker there is a 94% chance of finding a plant that carries it. If the first four plants that are sampled do not have the marker, then further plants can be sampled. Essentially, in 94% of the generations it will only be necessary to sample four plants. If flanking markers are not available, and if the linkage between the recessive gene and a single RFLP marker is not that tight, then if two or three independent pedigrees in the backcross program are followed in this way it will be almost certain that one of them carries the gene.

Recessive Genes and Pedigree Breeding

The advantages of gene tagging in pedigree breeding is less than in backcross breeding, because generation advance by selfing increases homozygosity and hence the proportion of individuals which express recessive alleles increases in each generation. However, the general advantages of juvenile testing, environmental stability, and gene pyramiding remain.

Dominant and Multiple Genes in Backcross Breeding

Indirect selection for dominant genes will be profitable in a backcross program when the general advantages of gene tagging apply. Moreover, the benefits of marker-based selection increase when more than one character is simultaneously backcrossed into the recurrent parent. With conventional breeding it is usually necessary to transfer characters individually by backcrossing. These products of the individual backcross breeding programs are then intercrossed to combine the new traits. By using markers, more than one gene can be incorporated simultaneously while still screening a low number of plants. The cost per donor gene of screening for RFLPs decreases when more than one gene is transferred, as the same DNA sample can be used to test for many RFLPs on a single plant basis.

Dominant and Multiple Genes in Pedigree Breeding

The advantages of tagging for dominant genes and multiple traits are less in pedigree breeding as compared to backcross breeding. Selection for multiple traits is easier in pedigree breeding because multi-environment progeny testing can easily be incorporated into the breeding program without increasing the number of generations required, and the complication of recovering the recurrent parent genotype is removed. However, the normal advantages of gene tagging remain.

Selection for the Recurrent Parent Genotype in Backcross Breeding

If a number of RFLPs between the donor and recurrent parent are determined that are not linked to the donor trait, then genotypic and phenotypic selection can be used for the recurrent parent genotype. Progeny are selected that carry the target allele, and very strong selection pressure for markers closest to the recurrent parent can then be applied without risk of losing the donor gene.

In backcross breeding the recurrent parent genotype is frequently not recovered for genes near to the donor gene. When a high-density RFLP map is available, the recurrent parent genotype can be selected near the gene of interest. Neinhuis et al. (1987) used RFLPs in this way to select in tomato for donor genes for insect resistance, and to more rapidly recover the recurrent parent genotype.

Backcrossing in Pearl Millet

Two characters in pearl millet are commonly handled by introgression in backcross breeding programs. One of these characters, downy mildew resistance, is probably dominant, and the other, photoperiod insensitivity, recessive. In both cases indirect selection using RFLPs would be beneficial.

In pearl millet, backcross breeding for downy mildew resistance is required particularly to rescue male-sterile lines that become susceptible to downy mildew. Good male-sterile lines are difficult to breed and once a successful line is available it is economically worthwhile to extend its life using backcross breeding. Attempts to do this so far have not been successful, as the resistance gene has invariably been lost due to the selection of disease-free plants that did not carry the resistance gene, but had merely escaped the disease.

Resistance to downy mildew is believed to be controlled by dominant genes, although reliable data concerning the genetic control of this disease is very limited. Downy mildew resistance is difficult to measure conventionally. The disease has to be scored as a percentage of infected plants and not as severity of symptoms, the susceptibility of plants varies with age and the source of inoculum that is used, and environmental factors greatly affect the disease incidence. Consequently, a large number of replications are required for accurate measurements and only progenies, not individual plants, can be reliably assessed with these conventional methods. Individual plants cannot be used as they will have scores of either 0% or 100%, whereas most genotypes have intermediate values. The selection of RFLP markers that are tightly linked to the gene or genes that control downy mildew resistance avoid these difficulties, and will greatly help to improve selection efficiency. First the linkage between resistance genes and RFLP markers will have to be determined in extremely well-replicated and well-controlled experiments. Once linked RFLP markers are determined indirect selection for them will be more effective than selecting for the disease.

The e_1 gene is recessive and imparts earliness and photoperiod insensitivity. It can be used to improve the synchrony of hybrid parents by introducing the gene into the later parent by backcross breeding. As long as the earlier parent does not have the e_1 gene the phenotype of the hybrid will be unchanged. A marker for the recessive gene will speed up the process of conventional backcrossing. The gene can be detected without selfing and screening the progeny under extended-daylength environments between each generation of backcrossing. The environmental stability of the marker will also avoid the complications created by the reduced expression of the e_1 gene in some genetic backgrounds.

It would also be helpful to have RFLP markers for the recessive d_2 gene, and for genes for fertility restoration and sterility maintenance.

Of course, in both the case of downy mildew resistance and the e_1 gene there will be considerable benefits in tagging these genes in pedigree breeding, or in population improvement.

Application of Tagging Genes for Quantitative Characters

Indirect Selection Using Quantitative Trait Loci (QTLs)

Most traits that the plant breeder selects for are quantitatively inherited. Analysis of biochemical and DNA markers in crosses between parents that differ for a quantitative trait can be used to find RFLPs linked to the genes that control the quantitative traits (quantitative trait loci or QTLs). Individual marker alleles can then be assigned breeding values according to the realized effect of the QTL to which they are linked. The realized QTL effect is a function as to how large an effect the QTL has, and how tightly it is linked to the marker or flanking markers. Selection can then be exerted for a number of markers simultaneously which will have the effect of selecting for QTLs with a positive effect on the quantitative trait (Paterson et al. 1988). This can be done with high efficiency because of the ability to score for several RFLP markers simultaneously in a single plant in a manner which is free from environmental influence or gene interactions.

Stuber and Edwards (1986) reported a marker-based selection procedure in maize. They used 15 isozyme marker loci which had been assigned breeding values, and this indirect genotypic selection was

as effective for improving the trait under selection (grain yield) as phenotypic selection. If a larger number of markers could be used then genotypic selection would be more effective (but probably not more cost effective) than phenotypic selection.

However, of the potential uses of RFLP markers for indirect selection, its use for quantitative traits that do not have a high heritability is likely to be the most problematic. For certain traits such as yield, which have many components, the important QTLs and thus the RFLPs linked to them will differ in different crosses, as the genetic architecture of each cross will differ. For example, QTLs could account for a large amount of the variation for yield because of differences between the parents for highly heritable traits, such as time to flower or disease resistance, which are highly correlated to yield. Unless other genotypes of related pedigree have already been analyzed, this means that genotypic selection can only be used after a complex and resource-consuming analytical procedure to determine linkage of markers and QTLs. Also, during the selection for quantitative traits relatively rare transgressive segregants are selected for. Consequently large numbers of plants need to be sampled, and the cost of this may be too high when compared to conventional phenotypic selection. However, the number of plants can be reduced by selecting for genotypes that carry all of the desired alleles, even if they are in the heterozygous condition. The required transgressive segregants can then be selected for in the subsequent generation or generations. Indeed, because of the ability to identify different genotypes that may be phenotypically identical, markers can be used effectively in smaller populations than conventionally required. Finally, in crops such as pearl millet where the environment is less controlled than in the case of maize grown under high inputs, and where differences between environment are very large, less of the variation may be accounted for in QTLs, and the QTLs controlling yield limiting components are likely to differ greatly with environment.

Prediction of Hybrid Yield

Heterozygosity. Evaluating parental inbred lines for a large number of markers allows the prediction of heterozygosity in F_1 hybrids of the parents. Since heterozygosity is related to yield it should be possible to predict those crosses with the highest heterozygosity and hence yield. Frei et al. (1986) made hybrids from inbreds for which RFLP analysis had been done. The results analyzed by Walton and Helentjaris (1987) showed that those hybrids that gave a low dissimilarity (<50%) for the 34 RFLP loci considered, could safely be discarded as low yielding. It would still be necessary to yield test within the high dissimilarity group because the extent of dissimilarity above 50% was not well correlated to yield.

Combining ability. Another use of the linkage between RFLPs and QTLs is in the prediction of combining ability. Since RFLPs can be identified that are positively correlated to important traits, inbred lines can be selected with RFLPs that should give high general combining ability. Walton and Helentjaris (1987) evaluated 39 inbreds of maize for 42 RFLP loci. A preliminary analysis revealed that 13 RFLP loci had significant effects on grain yield at harvest. Using these 13 loci to predict grain moisture of hybrids at harvest was successful. Of course, after selection of parental lines with high general combining ability combinations can be chosen that are complementary (i.e., maximise heterozygosity in the F_1). However, problems associated with this type of analysis remain; in that the RFLPs identified may differ with the set of crosses analyzed, and may differ with the target environment.

Introgression

In several crops chromosome segments carrying beneficial genes from wild species are introgressed into the cultivated species. The chromosome segments carry genes (either qualitative or quantitative in nature) that are desirable, such as disease resistance genes in the case of wheat, and genes controlling soluble solids in tomato. RFLP markers can very easily be used to trace the presence or absence of the chromosome segments in plants, as there is more polymorphism between than within species for most markers.

For the method to work universally in improving the character the genes in the alien chromosome segments should have the same effect in different genetic backgrounds in the cultivated species, i.e., they have the same effect in different crosses. Tanksley and Hewitt (1988) found that one chromosomal segment (Aco-2) from *Lycopersicon chmielewskii* interacted significantly with the genetic background of *L. esculentum* in which it was placed. In some cases it significantly increased solids, whereas in others its effect was neutral or caused a decrease in solids.

In wheat, a translocation between the 1BL and 1RS chromosomes has a desirable effect on yield and yield

stability (Rajaram et al. 1983). There are now many markers (Javornik et al. 1991), including disease resistance genes, cytological differences, and biochemical and DNA markers which can be used to detect and select for the translocation.

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RFLP Analysis for QTL Identification and Chromosomal Localization: Homozygous and Heterozygous Populations

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Abstract

The recognition of chromosomal segments carrying useful genes by means of molecular genetic markers—isozymes, Restricted Fragment Length Polymorphisms (RFLPs)—may offer the possibility of speeding up selection programs and of reducing field work. On theoretical grounds the main advantages of this approach are: the high prediction power with regard to the genetic composition of a segregating population obtained from characterized parents, selection applied at a very early plant stage, and reduction of the effects of environmental variations. The present paper considers the practical potentiality of these methodologies in connection with plant breeding programs, taking into account the type of the characters studied and the genetic structure of the population in maize.

The efficiency of RFLP analysis based on recombinant inbreds is discussed on the basis of the results obtained from the study of pollen competitive ability (PCA) and cellular membrane thermostability (CMS) in maize. PCA is a character affecting the mating system, the genetic analysis of which requires complex progeny testing. For this character RFLP analysis detected at least six quantitative trait loci (QTLs) localized on different chromosomal segments. About 70% of the genetic variability can be monitored by controlling the segregation of six RFLP loci. Similar results were obtained for CMS, a physiological index widely used to evaluate thermostability in plants. The analysis of the matrices of correlation between RFLP loci—either within or between chromosomes—could be used to detect false chromosomal assignments.

A highly heterozygous population, simulating early generations in selection programs, was also studied to evaluate the efficiency of the RFLP analysis. The results obtained for different traits show that the amount of the genetic variability that can be monitored by RFLP loci is greatly affected by the type of gene action controlling differences between families.

Introduction

The use of RFLP markers to detect chromosome segments carrying quantitative trait loci (QTLs) is one of the most promising technologies for plant breeding (Paterson et al. 1988; Helentjaris and Burr 1989). Efficiency depends mainly on the availability of densely saturated genetic maps and on a high level of polymorphism in populations to be used as basic material for selection. On theoretical grounds, the chromosome localization of QTLs and the availability of close-linked RFLP markers permit good power of prediction of the genetic composition of a segregating population, application of selection at a very early plant stage, and detection of fragments of DNA for gene isolation and molecular characterization (Ganal et al. 1989).

In selection programs concerned with single-gene traits, only a close-linked marker is required for efficient use of this method. Where quantitative traits are concerned, efficiency depends to a large extent on the type of gene action controlling the variability and on the estimation of the genotypic values of the segregating individuals. Consequently, for plant breeding purposes such as assisted selection, RFLP analysis must be carried out using the most suitable material and experimental design.

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Replicated Progeny

Many applications of RFLP analysis for QTL mapping have been based on single plants of segregating populations. On the other hand, high efficiency of genotypic value estimation is obtained by using replicated progeny of family structured populations, such as recombinant inbreds (RIs) and F_3. In particular, use of RIs provides a number of advantages (Burr et al. 1988). The RIs constitute a permanent population that can be used indefinitely for mapping both new molecular markers and QTLs; both interloci and interallelic interactions are reduced so that linkage analysis is simplified; and the expanding map resulting from the several rounds of recombination events allows detection of recombination between close-linked markers. On the other hand, RIs are not readily available for most of the populations to be used by the breeder; owing to the high level of homozygosity, they do not yield information about gene action contributing to specific combining ability; and, in allogamous species, the information obtained may be biased by inbreeding effects.

In our laboratory, RI-based QTL mapping has been used to study a number of traits in maize. The results obtained can be used to discuss the potential of the procedure. For this purpose, data obtained from the analysis of pollen competitive ability (PCA), measured as pollen tube growth rate (PTGR) in vivo, and cellular membrane stability (CMS) will be considered.

PTGR is a major component of pollen fitness affecting the genetic structure of both sporophytic and gametophytic populations of many Angiosperm species (Ottaviano and Mulcahy 1989). In maize, the variability of the character can be explained as the expression of at least two categories of genes: genes specific for pollen development, germination, and tube elongation and genes controlling metabolic processes common to pollen and sporophyte. For the first category, nine different genes (Ga: Gametophytic Factor) have been described in maize. Tube growth rate of Ga-carrying pollen is faster than that of Ga-carrying pollen: in mixed pollination, Ga pollen, in competition with pollen carrying the ga allele, fertilizes close to 100% of the ovules. All alleles detected for these loci show major phenotypic effects and chromosome mapping requires complex progeny tests. For the second category of genes, results obtained by means of biochemical and molecular analysis (isozyme, messenger RNA (mRNA)) of gene expression indicate that a very large number of genes may be involved (see Ottaviano and Mulcahy 1989, for a review).

RFLP analysis of PTGR was based on a set of 44 RIs obtained by selfing the segregating progeny of the F_1 obtained by crossing two inbred lines (T232×CM37). These RI lines have been characterized with regard to 200 RFLP loci, giving the possibility of monitoring most of the plant genome (Burr et al. 1988). The PTGR of each line is measured by means of a mixed pollination technique: pollen of given lines is mixed in equal proportion with the pollen of a standard line carrying a genetic marker for colored aleurone and used to pollinate an unrelated hybrid plant. Since in maize the style length varies according to the position of the flowers on the ear—increasing from the top to the base—relative PTGR is expressed by the regression of the proportion of uncolored kernels on ear segments (Ottaviano et al. 1988).

The results obtained show that the variability of the character has a typical quantitative pattern, that differences between inbreds cannot be accounted for by segregation of Ga alleles having major effects, and that the heritability of the character is very high ($h^2 = 0.77$). The detection of the chromosome segments carrying loci affecting this variability was carried out by means of regression analysis. The PTGR is taken as the dependent variable and RFLP loci as regressor. For each locus, value 1 and 2 was attributed to the CM37 allele. For heterozygous combinations, the value was 1.5. The value of the coefficient of regression measures the effect of the allele substitution and the sign indicates the distribution of the alleles in the two parental lines: + indicates that the allele that increases the character is from CM37. R^2 values estimate the amount of variation between inbred means explained by the variation of that specific locus.

The analysis detected 29 significant values ($P < 0.05$) that cluster in six chromosomal regions (Table 1). However, this information does not allow a straightforward unambiguous estimate of the number of QTLs, or a precise mapping, because in each cluster, one or more QTLs may be located. Moreover, when the RI set is not very large, random drift may result in apparent localization of the same QTL in two or more unlinked sites. Most of this indeterminacy can be removed on the basis of the analysis of the matrix of correlation between RFLP loci, either within or between chromosomes, and of the sign of the effects on each cluster. On the basis of this analysis about 60% of the genetic variability is shown to be the result of the segregation of at least six QTLs.

Table 1. RFLP loci showing significant effects on pollen tube growth rate (PTGR).

Chromosome	No. of RFLP loci	R^2	b
3	2	0.136	–1.45
5	5	0.179	–2.30
6	1	0.188	2.31
7	6	0.240	2.46
8	6	0.145	2.04
9	9	0.256	–2.57

R^2 and b values given are those of the locus showing the highest correlation.

Only one of them (chromosome 6) cannot be assigned unambiguously; the molecular marker is highly correlated to that in cluster 4.

A very important aspect of these results is shown when they are compared with the chromosomal localization of Ga factors obtained by classical mapping procedures based on alleles producing major effects; three of these are found in the chromosomal region detected by RFLP analysis. As these alleles do not segregate in the RI set, it means that in a maize population the ga loci are present as isoalleles having small phenotypic effects not detectable by means of standard genetic analysis.

Cellular membrane stability (CMS) of RIs was evaluated as the increase of electrical conductance produced by the electrolyte diffused in deionized water by the injured tissue (Sullivan 1971; Ottaviano et al., 1991). Heat treatment (47°C for two hours) was applied to leaf disks incubated in a thermostatically controlled bath. Although the molecular basis of the within-species variability is not known, structural analysis of the membranes suggests that differential thermostability can be produced by differences of saturation of membrane lipids, membrane protein and protein-lipid ratios, protein-lipid configuration, substances (proteins, phenols, growth regulators) possessing a natural affinity to membranes, and enzymatic fatty acid desaturation (McDaniel 1982).

Analysis of differences between RIs by standard statistical procedures indicates that the variability of the character is largely genetically controlled. The pattern of the variability is that of the typical quantitative traits, indicating a large number of segregating genetic factors. The difference between the two parental lines is 4.6 standard deviations. The estimate of minimum number of genes, according to Taylor (1976), is two.

Results obtained by RFLP analysis are summarized in Table 2 and Figure 1. It shows that the variability of CMS is attributable to a minimum QTL number not less than six, unambiguously distinguished. The inspection of the matrices of correlation between chromosomes indicates that in this specific study no false assignment of QTL was made.

For the predictive purposes of selection work, a model including six RFLP loci highly associated with the character would account for 39% of the variability between mean values of RIs (Table 2). As RFLP variability is not affected by environmental factors, and assuming that these factors act at random on the quantitative traits (the experimental design legitimates this assumption), the R^2 value obtained by fitting this model is an estimate of the proportion of the genetic variance between RI means explained by the variability of RFLP loci included in the model. Taking into account the proportion of genetic variance between RI family means $h^2_B = 0.73$, a selection based on the six RFLP markers included in the model would monitor 0.39/0.73 or .53% of the genetic variability of the character. The remaining portion is likely to be due to QTLs of minor effect, the contribution of which is not statistically detected.

Heterozygous Populations

The basic material to be used for selection, in the majority of plant breeding programs, is composed of highly heterozygous populations. The objectives of the work are the selection of genotypes in which most of the positive alleles are fixed and, for hybrid varieties, the production of parental lines maximizing combining ability.

Table 2. RFLP loci showing significant effects on cellular membrane stability (CMS).

Chromosome	No. of RFLP loci	R^2	b
1	1	0.099	–0.096
2	4	0.157	0.126
4	1	0.099	–0.087
8	1	0.229	0.136
9	4	0.170	–0.133
10	1	0.142	–0.118

R^2 and b values given are those of the locus showing the highest correlation.

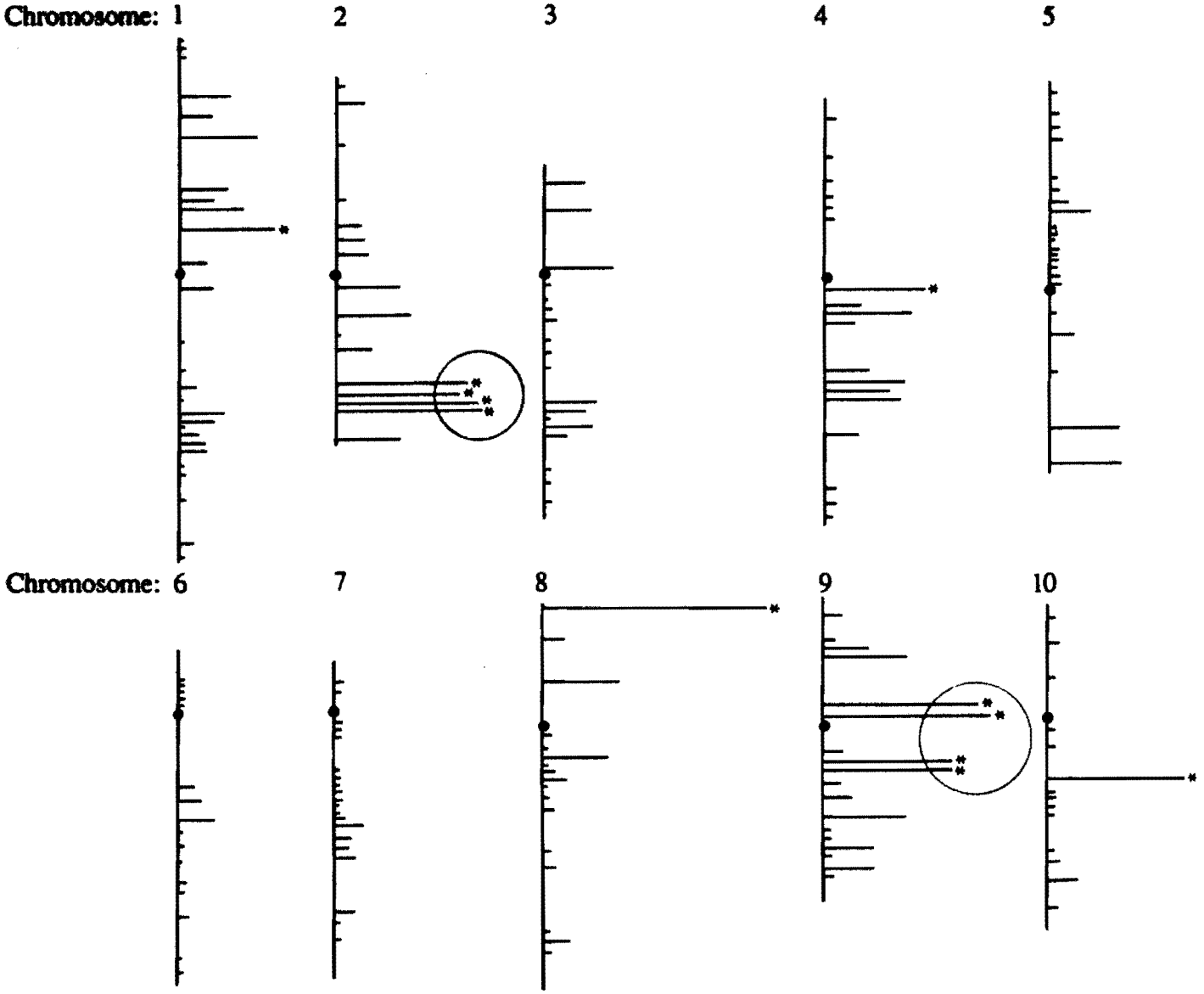

Figure 1. RFLP analysis for temperature injury in recombinant inbreds from T32xCM37. Bars indicate degree of correlation (R^2) between RFLP loci and CMS (cellular membrane stability). * indicates significant values ($P<0.05$). Clusters of significant values (circled) indicate the presence of a single QTL (quantitative trait locus).

The possibility of increasing the efficiency of selection for both these aspects, by means of genetic molecular markers, was studied in a single-cross hybrid set produced by intercrossing the RIs used for the experiments reported in the previous section. The design was a partial balanced diallel cross between 44 inbreds yielding a genetically balanced set of 74 hybrid combinations. The field layout was a complete block design with three replications. Measurements were made for a number of quantitative traits. The results of this study will be published elsewhere: here only data concerning mean kernel weight (KW) are reported. Standard combining ability analysis indicates that the character shows a large amount of genetic variability and that this is due to both general and specific combining ability (GCA and SCA) effects.

RFLP analysis was carried out on kernel mean values according to the procedure used for RIs; this is the equivalent of considering the additive model for genetic effects. Results obtained show that 20 different chromosomal regions carrying loci contribute to single cross hybrid differences and that the effects of the QTL in these regions account for 90% of the genetic variability between hybrid means. However, for plant breeding purposes, it is important to dis-

criminate between additive (fixable) and nonadditive (not fixable) genetic effects and to take into account the fact that the latter are lost during inbreeding. In fact, for many crop plants, selection is applied to produce inbred genotypes to be used either as variety (only in autogamous species) or as parental lines for hybrid production (in both autogamous and allogamous species). Moreover, the analysis of differences between genotypes (between hybrids in this case) does not yield information useful to assist selection for combining ability.

The information useful in these cases can be obtained by partitioning the genotypic values into general and specific combining effects (GCA and SCA), so as to obtain two different vectors of data, one for each type of genetic effect. Each vector constitutes the dependent variable to be regressed on the vector of RFLP loci values. For example, the analysis applied to SCA effects detects eight chromosomal regions carrying genes for combining ability (Table 3). Moreover, the analysis distinguishes the alleles in each region as having positive or negative effects.

Table 3. RFLP loci showing significant effects on specific combining ability for mean kernel weight (KW).

Chromosome	No. of RFLP loci	R^2	b
2	2	0.062	–0.714
3	2	0.104	0.956
4	2	0.071	–0.571
5	6	0.100	–0.832
6	1	0.081	0.749
7	4	0.122	–0.839
9	1	0.074	–0.698
10	1	0.098	–0.796

R^2 and b values given are those of the locus showing the highest correlation.

Conclusions

The results discussed in this paper support the view that RFLP markers represent a powerful method for increasing the efficiency of selection for quantitative traits. However, the application of the method requires a suitable experimental design defined on the basis of the objectives of the breeding program. Since field testing of the selected progeny is needed to select traits not monitored by molecular markers and for the general agronomic evaluation, the type of families to be considered in defining the experimental design, and, consequently, the model for RFLP analysis, should be determined on the basis of the criteria used for classical breeding methods.

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Organization and Expression of Plastid and Mitochondrial Genomes

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Abstract

The chloroplast genome consists of circular DNA molecules, which are 120 to 190 kbp long depending on the species considered and which usually comprise a region present twice but in opposite orientation (inverted repeat), a large single-copy region, and a small single-copy region. The complete nucleotide sequences of the chloroplast DNA of Marchantia *(liverwort), tobacco, and rice have been determined.*

The chloroplast genome of higher plants codes for 4 ribosomal RNAs, 30 different transfer RNAs (which allow translation of the 61 sense codons), and over 60 identified or putative proteins, but there are also over 20 unidentified or open reading frames (ORFs). Six tRNA genes and 10 protein genes contain intervening sequences (introns).

Transcription in chloroplasts involves promoters that are located 10 and 35 bp upstream of the transcription start and which show very high sequence similarity with prokaryotic promoters. Some transcripts are monocistronic, and some are polycistronic. The mechanisms of translation in chloroplasts have many common features with those operating in prokaryotes.

The size of the plant mitochondrial genome varies from about 200 to over 2000 kbp depending on the species considered. The presence of repeated sequences allows recombination events to occur, generating subgenomic molecules. DNA of nuclear and chloroplastic origin can be found inserted in plant mitochondrial DNA and in some cases can be expressed in the mitochondria.

Identified plant mitochondrial genes code for 3 ribosomal RNAs, about 20 transfer RNAs (12 mitochondrial tRNAs are encoded by the nuclear DNA and must be imported), and about 15 proteins (subunits of the complexes involved in the respiratory chain and ribosomal proteins). Some of these protein genes contain introns.

Plant mitochondrial messenger RNAs undergo editing, a post-transcriptional modification of several cytidines into uridines causing a change in the amino acid encoded, which results in the conservation of protein sequences.

Introduction

All eukaryotic cells contain mitochondria where oxidative phosphorylation allows the conversion into adenosine triphosphate (ATP) of the energy liberated by the transfer of electrons along the respiratory chain. In addition, photosynthetic organisms contain another type of organelles, chloroplasts, where light energy can be converted into ATP. In fact, plants contain several kinds of plastids (chloroplasts, chromoplasts, amyloplasts, etc.), but the organization and expression of the plastid genome has been so far mainly studied in the case of chloroplasts, so this chapter will focus attention essentially on the plastid genome in green leaves.

Both chloroplasts and mitochondria contain their own DNA—which is different from nuclear DNA—and their own systems for the replication of DNA, for the transcription of DNA into RNA, and for the translation of messenger RNAs into proteins (for reviews

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see Bohnert et al. 1982; Crouse et al. 1984; Gray et al. 1984; Dyer 1985; Tzagoloff and Myers 1986; Steinmetz and Weil 1987; Weil 1987).

The existence of genetic information in the chloroplasts was postulated about 80 years ago on the basis of observation made on non-Mendelian mutants (Baur 1909), but the presence of chloroplast DNA was demonstrated for the first time less than 30 years ago (Ris and Plaut 1962). In fact, most of the proteins found in chloroplasts or mitochondria are encoded by nuclear genes, synthesized in the cytoplasm as precursors, and imported into the organelle. These precursors are somewhat larger than the corresponding mature polypeptides, because they have at their N-terminus an extra peptide sequence (up to a few dozen amino acids) that allows the import of the polypeptide into the organelle and is cleaved to yield the mature polypeptide (Chua and Schmidt 1978; Smith and Ellis 1979; Tzagoloff and Myers 1986; Schatz 1987). This mature polypeptide of nucleo-cytoplasmic origin is often a subunit of an oligomeric enzyme or complex comprising a number of polypeptide chains, some of nucleo-cytoplasmic origin, and some of organellar origin. This is the reason organelle biogenesis requires a concerted expression of nuclear and organellar genes whose products must assemble to form functional enzymes, complexes, or particles in the organelle.

A minority of organellar proteins are therefore encoded either by the mitochondrial (mt) or the chloroplast (cp) genome. Some of these organellar genes have been identified and sequenced, as we shall see when reviewing what is known about the organization of the organellar genomes.

Organization of the Chloroplast (cp) Genome

The cp genome consists of circular DNA molecules that all have the same length in a given plant species (this is not the case for plant mitochondrial (mt) DNA) and that usually have a size ranging from 120 to 190 kilobase pairs (kbp), depending on the species considered. For instance, the cp genome of *Marchantia polymorpha* is about 120 kbp (Ohyama et al. 1986), while that of tobacco is about 155 kbp (Shinozaki et al. 1986b).

In most species studied so far (and this includes a unicellular green algae such as *Chlamydomonas reinhardtii*, as well as higher plants), cp DNA comprises (see Fig. 1) a region present in two copies, in opposite orientation (inverted repeat), carrying an operon of the ribosomal RNA (rRNA) genes. This repeated region has a size which differs depending on the species considered, for instance 10 kbp in *Marchantia polymorpha*, but 25 kbp in tobacco. The two copies of the inverted repeat are separated by:

- A large single-copy region, which is about 80 kbp long in *Marchantia polymorpha* and tobacco.
- A small single-copy region, which is about 20 kbp long in *Marchantia polymorpha* and tobacco.

There are some exceptions to this general organization:

- In several legumes, e.g., pea and broad bean, and in pine, there is only one rRNA operon. Comparisons between the organization of the genes in these legumes, and in the legumes having inverted repeats, suggest that one repeat has been lost during the gene rearrangements that have occurred during evolution.
- In *Euglena* there are three and a half rRNA operons in the same (direct) orientation. Strains have been described which have 1 or 5 rRNA operons.

Identification of Chloroplast Genes

To characterize genes present in chloroplast DNA, several approaches have been used, after digestion of the DNA with restriction endonucleases (which cut the DNA at specific sites) and determination of the position of the radioactively labeled restriction fragments on the chloroplast genome.

The position of the gene coding for rRNAs and transfer RNAs (tRNAs) can easily be determined by hybridization of the radioactively labeled RNAs to the various restriction fragments of chloroplast DNA.

The localization of the gene(s) coding for a protein is somewhat more difficult to determine. When the protein is purified, antibodies can be raised and then used to identify this protein among the polypeptides synthesized in vitro upon translation of chloroplast mRNAs. Hybrid-arrested translation and hybrid-released translation will allow identification of the DNA fragment(s) containing the gene(s) for this protein.

When the partial sequence of the protein is known (the sequence of a dozen amino acids may be sufficient), or when the sequence of the protein has been determined in another species (protein and DNA sequences are now obtainable from databases), an oligodeoxynucleotide coding for this sequence can be synthesized and used as a probe to locate the corresponding gene on the various restriction fragments of chloroplast DNA.

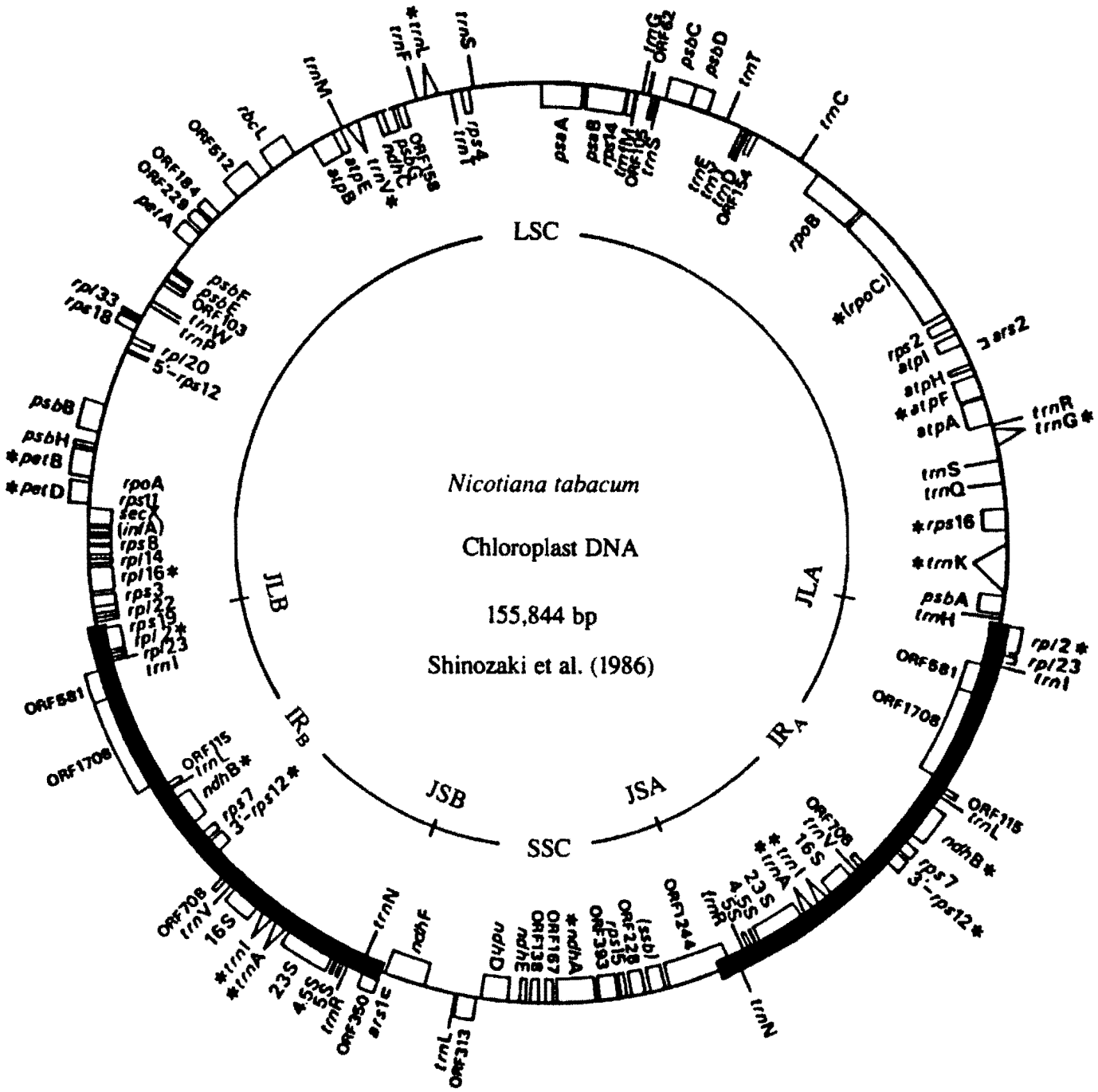


Figure 1. The tobacco chloroplast genome (Source: Shinozaki et al. 1986). LSC = large single copy region; SSC = small single copy region; IR_A and IR_B = inverted repeat regions.

When a gene has been localized in the chloroplast DNA of one species, this gene can be used—after cloning and amplification—as a heterologous probe to localize the corresponding gene on the chloroplast genome of other plant species, because of the high degree of sequence conservation of chloroplast genes.

Finally, progress in the methods for cloning and sequencing DNA has culminated in the determination of the complete nucleotide sequence of chloroplast DNA from *Marchantia polymorpha* (Ohyama et al. 1986), tobacco (Shinozaki et al. 1986b), and rice (Hiratsuka et al. 1989). Because of the high degree of homology between chloroplast and prokaryotic genes, computer comparison of chloroplast DNA and *Escherichia coli* DNA sequences has allowed the localization of some chloroplast genes on the basis of sequence homology.

For instance, in tobacco, identified and localized chloroplast genes code for 4 rRNAs: 23S, 16S, 5S, and 4.5S RNAs; 30 different tRNAs, and about 50

proteins. In addition, a dozen putative genes have been located which might code for proteins, as well as approximately 20 Open Reading Frames (ORFs) or Unidentified Reading Frames (URFs) comprising over 70 codons each.

The genes coding for the four rRNAs are clustered in the inverted repeat region and are thus present twice in the tobacco chloroplast genome. The genes for the 23S, 16S, and 5S rRNAs are very similar to those in *E. coli*; for instance there is about 75% homology for the 16S RNA (Schwarz and Kössel 1980) and 70% for the 23S RNA (Edwards and Kössel 1981; Shinozaki et al. 1986b), between the maize chloroplast and *E. coli*. The 4.5S RNA is a typical chloroplast RNA species, but it corresponds to the 5' end of the 23S RNA in *E. coli*.

Thirty different tRNA genes have been localized, and since 7 of them are located in the repeated region, there are 37 tRNA genes. In theory, a minimum of 32 tRNAs are necessary to translate the 61 sense codons of the 'universal' genetic code, but if one considers the possibility of a U-N wobble and/or a 'two out of three' mechanism for codon-anticodon base pairing, protein synthesis can take place in the chloroplast without import of tRNAs (Pfitzinger et al. 1990). The tRNA genes are scattered over the chloroplast genome in higher plants (Weil et al. 1982), but in *Euglena* most of them are clustered (Hallick et al. 1984).

Among the plastid genes coding for proteins, one can distinguish those coding for polypeptides found in the stroma, and those coding for polypeptides of the thylakoids. In the first group, one finds the genes coding for:

- The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This enzyme consists of 8 chains of the large subunit and 8 chains of the small subunit. The latter is coded in the nuclear genome, synthesized in the cytoplasm as a precursor that is imported into the chloroplast and is processed to yield the mature small subunit that associates with the large subunit to form the complete enzyme (Chua and Schmidt 1978; Smith and Ellis 1979).
- Twenty-one of the approximately 60 chloroplast ribosomal proteins, i.e., 12 r-proteins of the small particle (30S) and 9 r-proteins of the large particle (50S). The 40 or so other r-proteins are therefore imported from the cytoplasm. There is a rather high homology between these r-proteins and their counterparts in *E. coli*. The nucleotide sequences of the genes (and the structures of the proteins, as revealed by cross-immunological reactions) can reach homologies as high as 75%. The clustering of some genes is also homologous; for example proteins L23, L2, S19, L22, S3, L16, L14, and S8 have their genes clustered in this order in both the chloroplast genome and *E. coli* genome (Ohyama et al. 1986; Shinozaki et al. 1986b).
- Translation initiation factor IF-1 (Ohyama et al. 1986) and elongation factor EF-Tu, at least in *Euglena* (Montandon and Stutz 1983), are encoded by chloroplast DNA.
- The determination of the complete chloroplast genome sequence has revealed, in *Marchantia* and in tobacco (Ohyama et al. 1986; Shinozaki et al. 1986b), the presence of genes homologous to those in *E. coli* which code for the α, β, and β' subunits of RNA polymerase.

The genes coding for ribosomal proteins—polypeptides of the RNA polymerase and translation factors—are sometimes considered together with those coding for rRNAs and tRNAs as forming a group of genes coding for products involved in the expression of the chloroplast genome.

The second group of plastid genes comprises those coding for thylakoid proteins that participate in photosynthesis (Ohyama et al. 1986; Shinozaki et al. 1986b):

- Proteins of photosystem I (PS I).
- The apoproteins A_1 and A_2 of P 700 (the reaction center of PS I).
- Proteins of photosystem II (PS II)—at least 11 proteins, among them:
- - the 32 kDa quinone-binding protein, responsible for the sensitivity to herbicides such as atrazine (in resistant mutants, sequencing of this gene has shown that only one nucleotide is different).
  - the apoprotein of P 680
  - the 44 kDa protein
  - the D_2 protein
  - cytochrome b_{559}
- Proteins of the cytochrome b_6/f complex:
  - cytochrome f
  - cytochrome b_6
  - subunit IV
- Proteins of the chloroplast ATPase complex:
  - 6 of the 9 subunits of this complex, namely α, β, ε, I, III, and IV.

On the other hand, the proteins of the 5th thylakoid complex (the light-harvesting protein-chlorophyll complex) appear to be coded in the nuclear genome.

In addition, a dozen putative genes and about 20 ORFs have been located in tobacco, which could also code for polypeptides functioning in the chloroplast, thus bringing the total to over 120 sequences (24 of

them are located in the repeat region and are therefore present twice in the cp genome).

Among the ORFs there are 6 sequences homologous to those coding in the mitochondria for the subunits of NADH dehydrogenase (Ohyama et al. 1986; Shinozaki et al. 1986b), so that one wonders whether a respiratory chain, including this NADH dehydrogenase, exists and functions in the chloroplasts.

In tobacco (Shinozaki et al. 1986a and 1986b), a small number of chloroplast genes (6 tRNA genes and 10 protein genes) contain introns, while in *Marchantia* these numbers are 6 tRNA and 12 protein genes (Ohyama et al. 1986). Some of these are very long (several hundreds of base-pairs), even in the case of genes coding for tRNAs (although tRNAs have only 70-80 nucleotides), as illustrated by the split $tRNAs^{Lys}$ gene which has a 2526 bp intron. In *Euglena* chloroplasts, on the contrary, tRNA genes have no introns, whereas multiple introns are found in some protein genes which have no intron in higher plants, such as the gene coding for the large subunit of RubisCO which has 9 introns, or the gene of the 32 kDa protein of PS II which has 4 introns (for a compilation, see Crouse et al. 1985).

Expression of Chloroplast Genes

Chloroplast gene expression requires four steps: transcription, post-transcriptional modifications, translation, and post-translational modifications.

Transcription

The transcription of chloroplast genes can be studied by the analysis of the transcripts obtained either in vivo or in vitro (for reviews, see Crouse et al. 1984; Briat et al. 1986). To transcribe exogenous DNA (usually cloned fragments of chloroplast DNA), cell-free systems have been prepared from chloroplasts, but heterologous systems can also be used (*E. coli* RNA polymerase has often been used). The sites of transcription, initiation, and termination can be localized using S1 nuclease, which hydrolyzes unpaired DNA sequences and therefore reveals the limits of DNA sequences engaged in a DNA-RNA hybrid (formed between the coding DNA strand and the transcript).

In prokaryotes, the RNA polymerase recognizes, on the DNA, promoters comprising two regions that have very conserved sequences (consensus sequences); these are located 10 and 35 bp upstream of the transcription start and are separated by an optimal distance of 17 ± 1 bp.

Studies performed on sequences found upstream of about 60 chloroplast genes have shown a very high homology between *E. coli* and chloroplast consensus sequences (Briat et al. 1986).

	–35	–10
E. coli consensus sequence:	TTGaca	TAtaaT
Chloroplast consensus sequence:	TTGaNT	TAtaaT

Mutagenesis experiments in vitro (base substitutions, deletions, insertions) performed on the consensus sequences of a number of chloroplast genes have shown that these –10 and –35 sequences are required for an accurate transcription in vitro (Link 1984; Gruissem and Zurawski 1985), thus confirming the promoter activity of these sequences which are very similar to prokaryotic promoters.

It is interesting to note that in higher plant chloroplasts the same promoter sequences are found upstream of the three classes of genes (genes coding for rRNAs, tRNAs, and mRNAs), which suggests that these three types of RNAs are transcribed by the same RNA polymerase (as in *E. coli*), whereas in eukaryotes the promoters recognized by RNA polymerases, I that transcribes RNAs, II that transcribes mRNAs, and III that transcribes tRNAs, are different.

Chloroplast RNA polymerase has been difficult to purify, but recent results suggest that it resembles the *E. coli* enzyme. This has already been suggested by computer-assisted comparisons that have revealed chloroplast DNA sequences showing similarity with those coding for *E. coli* RNA polymerase subunits α, β, and β'.

Some chloroplast mRNAs are polycistronic (as in prokaryotes), but there are also mono-cistronic mRNAs. Cotranscription occurs in the case of rRNAs (Strittmatter and Kössel 1984) that are transcribed together with two tRNAs (a $tRNA^{Ala}$ and a $tRNA^{Ile}$), whose genes are located in the spacer between the genes of the 23S rRNA and 16S rRNA, and in the case of tRNAs, mainly in *Euglena* where clustering of tRNA genes is a general feature (Hallick et al. 1984). This also occurs in higher plants where three chloroplast tRNA genes have been shown to be clustered and cotranscribed (Ohme et al. 1985). As far as protein genes are concerned, cotranscription apparently occurs in the case of clustered genes coding for polypeptides participating in the same complex, as for

instance in the case of ATPase subunits α and I, of ATPase subunits β and ε (whose genes even overlap), and of some ribosomal proteins (Shinozaki et al. 1986a and 1986b).

As far as termination of transcription is concerned, just upstream of the termination site (identified using S1 nuclease) of a number of plastid genes, short inverted repeat sequences have been observed which would allow the formation of hairpin structures and resemble termination signals in prokaryotes (Zurawski et al. 1981).

A control of the expression of chloroplast genes in various cell types can occur at the level of transcription, as shown in the case of the gene coding for the large subunit of RubisCO, which is transcribed in the bundle-sheath cell chloroplasts of maize, but not in mesophyll cell chloroplasts (Link et al. 1978).

But there are also mechanisms that operate post-transcriptionally, by modulating the stability of individual cp mRNAs during plant development, and it seems that inverted repeats present in the 3' non-translated regions of cp genes could play a role in mRNA stability (Gruissem et al. 1988).

Post-transcriptional Modifications (Maturation of RNAs)

Primary transcripts in prokaryotes as well as in eukaryotes are usually longer than the final products (rRNAs, tRNAs, mRNAs) and must therefore be shortened, which is achieved by the action of specific nucleases. Chloroplast mRNAs have neither a cap (methylated guanylic nucleotide) at their 5' end, nor a long poly A tail at their 3' end; this is in contrast to eukaryotic mRNAs.

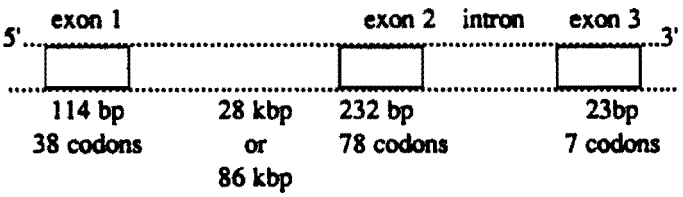
Maturation implies specific processes in the case of transcripts corresponding to genes interrupted by introns. In tobacco chloroplasts, 16 identified genes (6 tRNAs genes and 10 protein genes) have introns (Shinozaki et al. 1986a and 1986b). Some of them are several hundred bp long and the intron present in the gene coding for $tRNA^{Lys}$ has over 2500 bp. For some of them, primary transcripts and final transcription products have been characterized. The mechanisms responsible for the splicing of introns in chloroplasts have not yet been elucidated, but it has been proposed that chloroplast introns be classified into three groups (Shinozaki et al. 1986b).

- The group I introns, such as that found in $tRNA^{Leu}$ (UAA) (Bonnard et al. 1984), have a secondary structure similar to that of the self-splicable intron of the *Tetrahymena* rRNA precursor. These introns could be spliced either by an autocatalytic process, or by an enzymatic process involving, for instance, a maturase as in mitochondria.
- The group II introns can be folded so as to have a secondary structure similar to that of the introns present in the genes coding for yeast or maize mitochondrial cytochrome oxidase subunit II and for yeast cytochrome b. In some of these introns, ORFs have been found, which can code for maturases involved—as the name suggests—in the maturation of pre-mRNA into mRNAs.
- The introns found in 12 out of the 16 tobacco chloroplast split genes belong to group III; they are present in genes coding for tRNAs or proteins and have conserved sequences at their 5' and 3' boundaries that resemble the conserved sequences found in introns present in nuclear genes:

Chloroplast introns (group III)	...5'GTGCGNY...ATCNRYYNYYAY3'
Nuclear introns	.....GTGRAGT.....YYYYYYNCAG...

The gene coding for tobacco chloroplast ribosomal protein S12 is a special case: it consists of three exons, but the first one (the 5' exon) is located very far from the other two (the 3' exons). These two 3' exons are present in the repeat region, so that the 5' exon is in fact located:

- 28 kbp downstream of the 3' part of the gene present in one of the repeat regions (on the same strand),
- 86 kbp downstream of the 3' part of the gene present in the other repeat region (on the opposite strand).



This gene has been called a 'divided gene' and its maturation apparently implies a special mechanism of 'trans-splicing' (Zaita et al. 1987).

The chloroplast genes of *Euglena* often differ from the corresponding genes in higher plant chloroplasts; for instance, there is no intron in the gene coding for ribosomal protein S12 or in the tRNA genes in *Euglena* chloroplasts. However, other genes which have no intron in higher plant chloroplasts, such as the genes coding for the large subunit of RubisCO or for the 32 kDa protein of PS II, have introns in *Euglena* chloroplasts. Introns seem to represent as much as 20% of the chloroplast genome in *Euglena* (Koller and Delius 1984).

Translation

The first step of protein biosynthesis in chloroplasts, i.e., the aminoacylation of tRNAs, involves chloroplast-specific tRNAs coded in the chloroplast genome, which are different from the cytoplasmic tRNAs and resemble prokaryotic tRNAs. The sequence homology is often about 70%, the modified nucleotides are often identical, and generally chloroplast tRNAs can be aminoacylated in vitro using chloroplast or prokaryotic enzymes; whereas, they are not aminoacylated by the enzymes from the cytoplasm of the same cell (Weil and Parthier 1982).

Chloroplasts contain 30 tRNAs that are sufficient to translate the 61 sense codons of the universal genetic code (in contrast to mammalian or fungal mitochondria, chloroplasts use the universal genetic code), if one considers the possibility of a U-N wobble and/or of codon-anticodon pairing involving in some cases only two nucleotides out of three (Pfitzinger et al. 1990).

In the chloroplast the enzyme that catalyses the attachment of an amino acid to the corresponding tRNA—called aminoacyl-tRNA synthetase—differs from its cytoplasmic counterpart in its catalytic, structural, and immunological properties (Colas et al. 1982; Dietrich et al. 1983). However, it seems that the chloroplast aminoacyl-tRNA synthetases are coded by the nuclear genome, as are the cytoplasmic aminoacyl-tRNA synthetases. The initiation of protein biosynthesis in chloroplasts involves, as in *E. coli*, a formyl-methionyl-tRNA (Burkard et al. 1969), whereas in eukaryotic cytoplasm the initiator methionyl-tRNA is not formylated.

As in prokaryotes, the attachment of the 30S ribosomal particle on the mRNA involves the pairing of the 3' end of the 16S RNA (of the 30S particle) to a complementary sequence (called the Shine-Dalgarno sequence) found upstream of the initiation codon (AUG) on the mRNA.

Chloroplast ribosomes strongly resemble prokaryotic ribosomes. They are of the 70S type (whereas the ribosomes of eukaryotic cytoplasm are of the 80S type), prokaryotic ribosomal RNAs (70-75%), and ribosomal proteins. In addition, homology has also been observed between chloroplast and prokaryotic initiation factors (IF), and also between the elongation factors (EF).

Generally speaking, one can say that translation in chloroplasts very much resembles translation in prokaryotes. Furthermore, it is sensitive to the same antibiotics (chloramphenicol or streptomycin, for instance), whereas it is resistant to antibiotics (cycloheximide, for instance) that inhibit protein synthesis in eukaryotic cytoplasm.

Codon usage in chloroplasts shows a clear preference for U and A in the 3rd position (wobble position) within a family of synonymous codons (Steinmetz and Weil 1987). A correlation has been observed between the concentrations of the various chloroplast isoaccepting tRNAs and the frequency of usage of the corresponding codons (Pfitzinger et al. 1987).

Post-translation Modifications (Maturation of Proteins)

Little is known at present of the post-translational modifications of the proteins synthesized in the chloroplasts. However it has been shown that some proteins lose a few amino acids, either at their N terminus, for instance, in the case of the large subunit of RubisCO (Amiri et al. 1984) and of cytochrome *f* (Alt and Hermann 1984) or at their C terminus, for instance, in the case of the 32 kDa protein of PS II (Marder et al. 1984), during the maturation processes, prior to their assembly to form active enzymes or complexes. On the other hand, some chloroplast proteins are phosphorylated (for a review, see Steinmetz and Weil 1987).

Conclusions Concerning the Chloroplast Genome

The chloroplast genome shows many similarities to the prokaryotic genomes.

As far as the structure and the organization of the genes are concerned, there are important homologies not only in the sequences of the genes (coding for rRNAs, tRNAs, and proteins) but also in the organization of certain genes that are found in the same cluster; sometimes genes even appear in the same order in the chloroplast genome, and in the *E. coli* genome (for instance, in the case of several ribosomal protein genes).

As far as gene expression is concerned, important similarities have also been observed between chloroplasts and prokaryotes. The sequences involved in the initiation (promoters) and termination (terminators) of transcription in chloroplasts are very similar to those identified in prokaryotes. The mechanisms of translation also have many common features: homology of tRNA sequences, cross-reactions possible between chloroplast and prokaryotic tRNAs and amino-

acyl-tRNA synthetases, similar size of the ribosomes, homology of rRNA and r-protein sequences, use of a formylated methionyl-tRNA in the initiation process, presence on the mRNAs upstream of the initiation codon of a sequence complementary to that of the 3' end of 16S mRNA allowing the attachment of the 30S particle to the mRNA, and sensitivity to the same antibiotics.

These similarities are in favor of the theory of an endosymbiotic origin of the chloroplasts, according to which these organelles have evolved from prokaryotic ancestors (*Cyanobacteria* and *Prochloron*, for instance). But the presence of introns in some chloroplast genes suggests that, if this theory is correct, the prokaryotic ancestors might have had introns, at least some of which have been maintained in the chloroplast genomes, whereas they have disappeared from the eubacterial genomes. Conversely, introns may have appeared after the endosymbiotic event.

The chloroplast genome contains the information necessary for the synthesis of chloroplast rRNAs and tRNAs and for the synthesis of a relatively limited number of chloroplast proteins (about 50 have been identified, and this number could go up to 80 if the ORFs are coding for proteins). Most chloroplast proteins are, therefore, coded in the nuclear genome, synthesized in the cytoplasm, and imported into the chloroplasts. When one examines the chloroplast proteins coded in the chloroplast genome, one sees that in most cases they must become associated with polypeptides imported from the cytoplasm in order to form functional enzymes or complexes (RubisCO, ribosomes, photosystems I and II, cytochrome b_6/f, ATPase). Chloroplast biogenesis and function therefore require a control of gene expression in the chloroplast and in the nucleocytoplasmic compartments, in order to ensure a concerted synthesis of polypeptides that must assemble in the chloroplast to form functional oligomeric complexes. The understanding of these regulation mechanisms is presently one of the major goals of plant molecular biology.

Chloroplast Transformation

For the past years, many efforts have been made to use the techniques of genetic engineering in order to obtain transgenic plants having improved properties, such as resistance to herbicides or to various pathogens. Very promising results have been obtained by introducing new genes into the nuclear genome of plants, but transformation of chloroplasts has only become possible recently, through the use of the particle gun. We shall therefore have to wait to see whether crop improvement can be achieved by introducing DNA into chloroplasts.

Organization of the Plant Mitochondrial Genome

The mitochondrial genome is about 16 kb in mammals and about 78 kb in *Saccharomyces cerevisiae*. In plants, it is much larger and can vary from about 200 kb in *Brassica* (Palmer and Shields 1984) to over 2400 kb in the watermelon (Ward et al. 1981).

When DNA is extracted from the mitochondria of a given plant species, a heterogeneous population of DNA molecules (having different sizes) can be observed. This is due to the presence in plant mt DNA of repeated sequences allowing recombination events that generate subgenomic circles (Palmer and Schields 1984; Lonsdale et al. 1984; Quetier et al. 1985) that can derive from one large circular molecule called the master-circle or master-chromosome. So far only one plant has been described, *Brassica hirta*, whose mitochondria contain only one type of circular DNA molecule of 208 kb (Palmer and Herbon 1987).

In addition to very high molecular weight DNA, plant mitochondria can also contain shorter DNA molecules, either circular or linear, and double-stranded RNAs (Lonsdale 1989).

Plant mitochondrial genomes contain chloroplast or nuclear DNA insertions (Stern and Lonsdale 1982; Schuster and Brennicke 1988; Breiman and Galun 1990). Some of the sequences can be expressed in mitochondria (Marechal et al. 1987; Leon et al. 1989; Sangare et al. 1990).

Identification of Mitochondrial Genes

As in the case of chloroplasts, most mitochondrial proteins are encoded in the nucleus, synthesized as precursors in the cytoplasm and imported into the mitochondria. However, plant mitochondrial DNA contains genes coding for three ribosomal RNAs, 26S, 18S, and 5S, a number of transfer RNAs, and several proteins. While the two large rRNAs are also encoded by mt DNA in mammalian and fungal mitochondria, 5S RNA is only encoded by mt DNA in plants.

Plant mt DNA, in contrast to mammalian and yeast mt DNA and also in contrast to cp DNA, does not code for a complete set of transfer RNAs. In po-

tato for instance, 20 tRNA genes have been identified in the mt genome, while 11 mt tRNAs are encoded by the nuclear genome and must be imported into the mitochondria (Marechal-Drouard et al. 1990). Among the 20 tRNAs coded for by mt DNA, 5 are chloroplast-like and are probably transcribed from promiscuous cp DNA sequences inserted in the mt genome. It should be stressed that while some mt tRNAs (such as $tRNA^{Gly}$ or $tRNA^{Val}$) are encoded for by mt DNA in potato, this is not the case in wheat or maize (Joyce et al. 1988; Sangare et al. 1990). An unusual situation exists in the case of an isoleucine-specific mt tRNA, encoded by an mt tRNA gene possessing a CAT (methionine) anticodon, which is post-transcriptionally modified into a LAU (isoleucine) anticodon (Weber et al. 1990).

About a dozen protein genes have been identified in plant mt DNA. Most of them have also been found in the mt genomes of other organisms, e.g., mammals and fungi, such as the genes coding for subunit I, II, and III of cytochrome oxidase, for cytochrome b, for subunits 6 and 9 of ATPase, or for subunits 1, 2, 3, 4, and 5 NADH dehydrogenase. However, in plants, several mt genes have been identified that code for proteins that are nuclear-encoded in other organisms. This is the case, for instance, for the gene coding for subunit α of ATPase (Hack and Leaver 1983; Braun and Levings 1985) and for the genes coding for ribosomal proteins S12, S13, and S14 (Bland et al. 1986; Gualberto et al. 1988; Wahleithner and Wolstenholme 1988).

Genes coding for a maturase or reverse transcriptase have been identified in *Oenothera* (Schuster and Brennicke 1987) and in broad bean (Wahleithner et al. 1990). This could play a role in the splicing of class II introns present in several mt genes or in the integration of chloroplast and nuclear sequences into the mt genomes. In addition, in a variety of plant mt genomes, a number of ORFs have been observed and some of them have been shown to be expressed, but since they do not show any homology with known genes listed in the available data banks, they have not been identified yet. We shall discuss later the mt genes that are characteristic of cytoplasmic male-sterile plants.

The total number of functional genes present in plant mitochondria can be deduced either from the two-dimensional electrophoretic analysis of labeled proteins synthesized in organello that yield between 30 and 50 spots (Hack and Leaver 1983)—but some of them could be resulting from post-translational modifications or degradation—or from the analysis of a transcription map. The latter approach used in the study of one of the simplest plant mt genomes—that of *Brassica campestris* which has only about 200 kb (Makaroff and Palmer 1987)—revealed 24 abundant transcripts, 9 of which correspond to already known genes. While it seems that plant mt genomes code for more proteins than mammalian or yeast mt genomes, it is too early to have an exact figure and it should be kept in mind that some proteins (such as the ribosomal proteins) may be encoded by the mt genome in some plants but not in other plants.

While the structure of most plant mt genes is generally simple, consisting of only one uninterrupted reading frame, some genes contain introns (such as *cox*II, *nad*1, *nad*4, *nad*5). In wheat mitochondria the *nad*4 gene contains three introns, one of which is more than 3 kb long.

Expression of Plant Mitochondrial Genes

Transcription

The transcription patterns of plant mt genes are often quite complex, probably because of the presence of multiple transcription initiation sites or of processing intermediates. While most transcripts correspond to one gene, some genes are cotranscribed and yield multiple transcripts (Wissinger et al. 1988; Gaulberto et al. 1988).

So far little is known about the signals controlling transcription (and maturation of transcripts) in plant mitochondria, although it appears that the expression of plant mt genes can be regulated during plant development (Young and Hanson 1987). Although repeated sequences have been localized close to the 5' end of several mt transcripts (Schuster and Brennicke 1989; Gualberto et al. 1988, 1990), their role as promoters of transcription has not been demonstrated. Hairpin structures have been found at the 3' end of several mt transcripts, but here also there is no experimental evidence that they function as terminators of transcription.

Editing

Comparisons between the sequences of several plant mt genes and of the corresponding cDNA sequences (resulting from reverse transcription of the corresponding mt mRNAs, using specific oligodeoxyribonucleotides as primers) has revealed that a

number of cytosine residues present in the coding region of the gene are changed to uracil in the mRNA (Gualberto et al. 1989; Covello and Gray 1989; Lamattina et al. 1989). Such a change, affecting a cytosine at the first or at the second position (or both) of a codon, results in a change of the amino acid specified by this codon, so that the plant mt protein shows more similarity to the corresponding mt protein in other organisms, e.g., mammals and fungi. In other words, these modifications enhance the conservation of mt protein sequences. Depending on the gene considered, between 4 and 20 cytosine residues are changed to uracil (in the genes so far studied), but it should be stressed that the editing sites vary when different plants are considered (in some plants the consensus amino acid is specified by the sequence of the gene, so that no editing is necessary).

Editing appears to be a post-transcriptional phenomenon, as unedited or partially edited transcripts can be observed, as shown in the case of wheat *nad*3 and *rps* 12 genes. The mechanisms of editing have not yet been elucidated and it is not known if they involve deamination or base substitution, or cleavage of the sugar-phosphate backbone, replacement of a cytidylic nucleotide by a uridylic nucleotide, and religation.

It is not yet known either how the editing sites are recognized, in other words how the editing machinery distinguishes the cytosine residues that have to be modified into uracil from those cytosines which should remain unchanged. In trypanosome mitochondria (where editing consists of the addition and deletion of uracil residues), a model has been proposed (Blum et al. 1990), based on the complementarity between the mRNA to be edited and an antisense RNA called guide RNA (gRNA). Although studies of the sequences surrounding editing sites in wheat *cox*III transcripts have shown the existence of families of consensus sequences that could be involved in RNA-RNA interactions assuming G-U base pairing (Gualberto et al. 1990), there is so far no report demonstrating the presence of antisense RNA in plant mitochondria.

Cytoplasmic Male Sterility

In a cytoplasmic male-sterile plant, no fertile pollen is being shed and this is a useful trait for the production of hybrids, since it prevents self-pollination that otherwise has to be avoided by manual emasculation of male flowers (a time-consuming procedure if it has to be used in a maize field, for instance). In a number of plant species, cytoplasmic male sterility (cms) has been shown to result from modifications in the mitochondrial genome, but the fact that nuclear restorer genes can restore fertility suggests that cms is in fact due to a nuclear mitochondrial incompatibility. There are several cms lines of maize, but one of them, cms-T, has been particularly well studied, including at the molecular level. This cms line is sensitive to the toxin of *Helminthosporium maydis*, and this caused a major catastrophe in the USA several years ago, because the cms-T line had been generally adopted in hybrid production, and there were severe losses due to an infection.

In the mitochondrial genome of the cms-T line of maize, a chimeric gene called *urf-13* was found that is not present in the normal line (see Fig. 2). This genome results from the fusion of parts of the genes coding for the 26S rRNA subunit 6 of ATPase (Dewey et al. 1986); this chimeric gene is expressed into a 13 kDa protein, responsible for the cms phenotype (Forde et al. 1978; Dewey et al. 1987). A clear correlation was established between the cms phenotype, the presence of this chimeric gene and of its product (the 13 kDa protein) in maize mitochondria, and the sensitivity to the *Helminthosporium* toxin and to a chemical called methomyl. Furthermore, when this chimeric gene was introduced into either *E. coli* or yeast (using the technology of genetic engineering), some of the symptoms observed in the mitochondria of cms-T maize exposed to the toxin or to methomyl (inhibition of respiration, swelling, leakage of ions) were well observed in the transformed bacterial or yeast cells exposed to methomyl (Lewing and Dewey 1988; Glab et al. 1990). In maize plants containing nuclear restorer genes, the expression of the chimeric mitochondrial gene is inhibited and the production of viable pollen is restored.

Similarly, cms in petunia is also related to the presence and expression of a chimeric mt gene called *pcf*, resulting from the fusion of parts of the genes coding for cytochrome oxidase subunit II and for ATPase subunit 9 (Fig. 1; Young and Hanson 1987).

Conclusions Concerning the Transformation of Plant Mitochondria

Considering the importance of mitochondria in plant metabolism and the role of the mt genome in an agronomically-important trait (cms), it is not surprising that attempts have been made to transform plant mitochondria. As in the case of chloroplasts, the par-

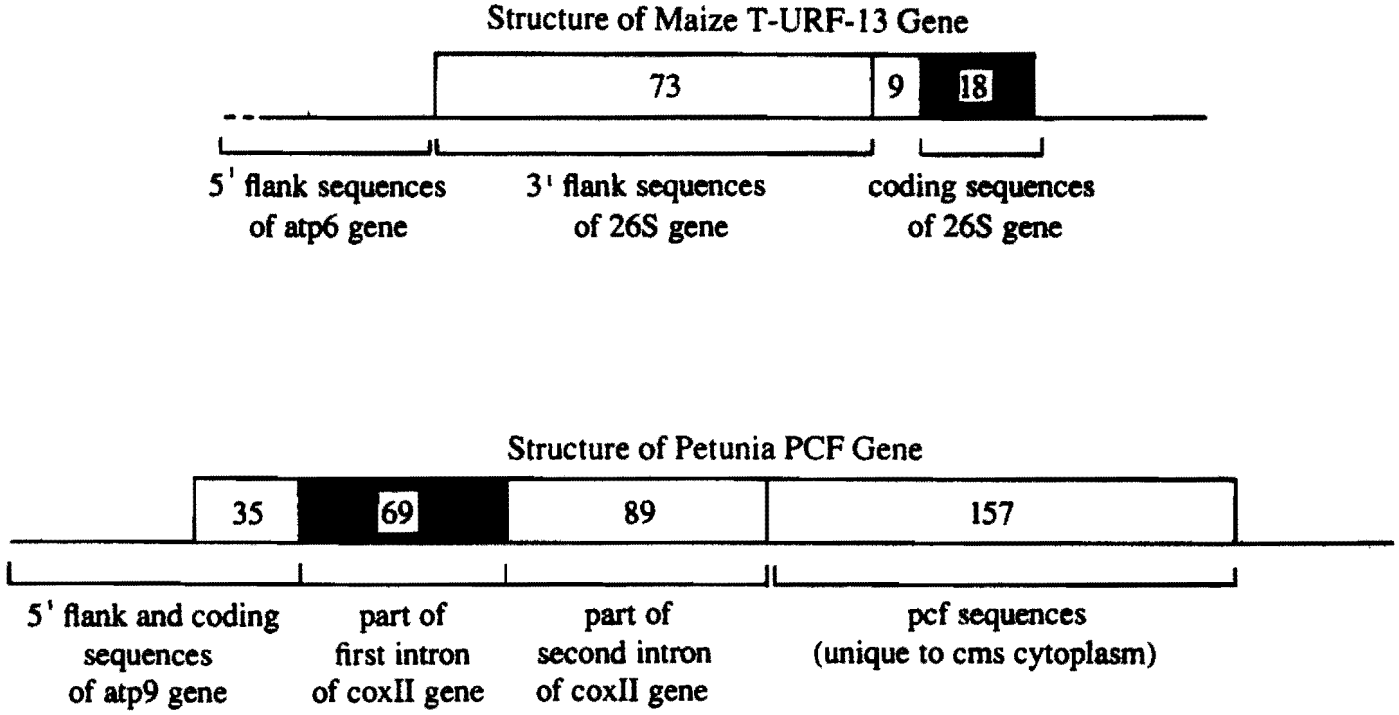


Figure 2. Chimeric genes found in the mitochondrial genome of cms lines of maize and petunia.

ticle gun has enabled delivery of exogenous DNA into *Chlamydomonas mitochondria*, a promising result that opens new possibilities of crop improvement through transformation of plant mitochondria.

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Screening of Male-sterile and Maintainer Cytoplasms of Pearl Millet [*Pennisetum glaucum* (L.) R.Br.] by Restriction Fragment Length Polymorphism in Mitochondrial DNA

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Cytoplasmic male sterility (CMS) is used extensively in hybrid seed production and the conventional method of classifying male-sterile cytoplasms is slow and laborious. There is a need to incorporate diverse cytoplasmic backgrounds in hybrid seed production to avoid disease epidemics. This requires the development of rapid and reliable methods of detecting new CMS sources. Mitochondrial DNA (mtDNA) from several CMS (SA) and maintainer (B) lines were purified and digested with several restriction endonucleases. Southern blot hybridizations with pearl millet mtDNA probes containing 18S-5S rRNA and Cox I genes revealed fragment length variations among cytoplasms. The 10.9 kb and 13.6 kb clones hybridized to 6.0 kb Bam HI and 7.5 kb Xho I fragments in CMS-A lines 67, PMC 23, DSA 105, DSA 118, DSA 134, DSA 144-1, and all maintainer lines, while CMS A-lines of PMC 30, 5141, 81, and 843 (SA-1) were characterized by the absence of these fragments. SA-1 lines had a distinct 7.5 kb Sma I fragment, hybridizing to 13.6 kb and 9.7 kb clones whereas the other cytoplasms showed a 6.0 kb fragment instead. Hybridization of the 9.7 kb clone with Hind III digests of mtDNA from PMC 30A, DSA 118A, and DSA 105A lines differed from those of B lines. Autoradiograms of Hind III digests of 5141A and B mtDNAs hybridized with the same probe produced a pattern distinct from other lines. A unique 4.3 kb fragment was present in the cytoplasms of 67A, PMC 23A, and DSA 144-1A when Pst I digests of mtDNA were probed with maize Atp 6 clone. MtDNAs of 81A4 and 88001 showed patterns distinct from each other as well as from all other lines. Hybridization of Pst I generated mtDNA fragments with labeled Atp 6 clone differentiated the range of cytoplasms used in the present study.

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Role of Mitochondria in Cytoplasmic Male Sterility in Sorghum

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It has now been established that organelle genomes show a certain degree of autonomy and participate partially in controlling cellular processes sometimes leading to a phenotypic trait. One such trait is cytoplasmic male sterility (CMS) which is maternally inherited and has been used in the production of F_1 hybrid seeds in certain crop plants. Recent studies on CMS suggest that genetic determinants that control this phenotypic expression—at least in maize, sorghum, and pearl millet and possibly in other plants—are mostly located on the mitochondrial genome (Dixon and Leaver 1982; Pring et al. 1982; Ricard et al. 1986; Erickson et al. 1986). Various subunits of *Cox I, Cox II, ATPase*, and *nad3* genes encoded by mitochondria have been shown to undergo changes in their structures and/or copy number in male-sterile lines of different plant lines. However, no substantial correlation could be established so far between these observed changes in the gene structure and functional aspect of mitochondria. In order to understand mitochondrial genome organization and its correlation to mitochondrial function in CMS we have initiated studies to compare the isolated mitochondria of the male sterile, 2219A, and its restorer fertile line, 2219B, of sorghum with respect to biochemistry, genome organization, transcriptional, and translational products. Results of mitochondrial electron transport, cytochrome stoichiometry, ATP export from mitochondria, and endonuclease restriction pattern are reported here, which present part of our study in relation to CMS.

The *Sorghum bicolor* male-sterile line, 2219A, and its restorer line, 2219B, were obtained from the All-India Sorghum Improvement Project, Hyderabad, and multiplied at our institute. All the experiments were performed on 5-6 day old etiolated seedlings from both lines and the results were compared. For the purpose of mitochondrial electron transport, the mitochondrial membranes were prepared following the method of Arora and Sane (1989). The electron transport of whole chain (NADH → oxygen) and partial reactions (NADH → cyt.C, NADH → ferricyanide, and ascorbate → oxygen) were carried out at various temperatures (15-40°C). The reactions were initiated by adding respective substrates in the presence of suitable inhibitors for partial reactions. The amount of oxygen consumed in the case of the whole chain and the ascorbate → oxygen reaction was monitored in the Gilson Oxygraph. Using a SLM Aminco spectrophotometer at 340 nm and 550 nm, the amount of NADH oxidized or the cyt.C reduced was monitored.

The amount of various cytochromes was determined by difference spectroscopy as described by Peterson et al. (1977) and Rickwood et al. (1987). The reduced minus oxidized spectra was recorded with a baseline set between 500-650 nm. The amount and the stoichiometry of cytochromes was calculated using a series of formulae.

For the purpose of ATP translocation studies across the mitochondrial membrane, intact mitochondria were prepared utilizing a sucrose density gradient. The export of ATP from the mitochondria through ADP-ATP translocator was determined following the method of Liu et al. (1988) where the amount of NADPH formed as a result of coupled reactions of glucose phosphate dehydrogenase and hexokinase was directly correlated to the ATP exported outside mitochondria. The 'Km' of ADP for ADP-ATP translocator was also determined in the same way using varying concentrations of ADP in the reaction mixture.

The mitochondrial DNA was isolated following the basic procedure of Chase and Pring (1985). The isolated DNA was subjected to various purification processes that included CsCl density gradient, high salt-urea treatment, and sephacryl S-1000 column chromatography. The purified DNA was subjected to

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various endonuclease digestions as per manufacturer's protocol and the restricted fragments were resolved electrophoretically on 0.8% agarose gel.

The mitochondrial electron transport in both lines showed a temperature optima generally at 25°C for both the whole chain and the partial reactions. The uncoupled rates of electron transport in case of the NADH → oxygen was found to be lower by 40-50% in cases of sterile lines when compared to that of the fertile lines. Similarly, lower rates were also observed to an extent of 40-50% in cases of partial reactions, i.e., NADH → ferricyanide, NADH → cyt.C, and ascorbate → oxygen. The rates of electron transport measured in all the cases were expressed in terms of cyt.aa3. These results suggested that fertile-line mitochondria were capable of respiring more efficiently than sterile-line nitrochondua. However, respiratory control ratio (RCR) and P/O ratio did not show variations in two lines indicating that phosphorylation efficiencies in both lines are not changed.

Difference spectroscopy revealed that stoichiometry of cytochromes was not the same in both lines. The male-sterile line possessed almost 50% of the cyt.Cl when compared to the fertile line and that changed the stoichiometry from aa3:b:cl:c as 1:2:2:1 in the fertile line to 1:2:1:1 in the sterile line. This change in stoichiometry was further supported by other biochemical experiments.

Using 80-85% intact mitochondria, it was observed that the export of ATP was more efficient, by at least double in fertile-line mitochondria as compared to the sterile-line. Possibly the whole mitochondria was inhibited and sluggish in the sterile line in order to produce comparable amounts of ATP to be exported at rates similar to those observed in the fertile line. However, the 'Km' for ADP that was much lower in the fertile line (almost 50% of the sterile line), did also indicate that the problem may lie in the efficiency of the translocator.

The restriction digest of mitochondrial DNA from both the lines showed diversities in their total numbers of fragments as well as location. The EcoRI restriction gave at least three additional bands of approximately 2.3kbp, 6.4kbp, and 9.5kbp in 2219B. However, digestion with HindIII showed the presence of a 5kbp band in 2219A not detected in 2219B; alternatively, two bands of about 2.3kbp and 20kbp were absent in 2219A. The AvaI restriction produced a unique band of 20kbp in 2219A, however, KpnI restriction gave a unique band of 9kbp in 2219B.

These results suggest that a high degree of polymorphism and/or deletions might be taking place while achieving cytoplasmic male sterility in sorghum, which need extensive investigations in the light of mitochondrial functions. Existence of a newly generated chimeric gene, *TURF13*, in the cytoplasm of CMS-T maize (Dewey 1987) and role of its 13kd polypeptide in inhibiting ATP synthesis presents an excellent example of this kind.

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Part III

A Glossary of Selected Biotechnology Terms

Appendix I: List of Participants

Appendix II: Workshop Recommendations

Index

A Glossary of Selected Biotechnology Terms

A.K. Weissinger[1] and J.P. Moss[2]

This glossary is intended to help those who are not familiar with all branches of biotechnology to get the most out of this publication. It is based on a glossary prepared by A.K. Weissinger, and expanded by J.P. Moss, who thanks A.K. Weissinger for offering his glossary, and staff of the Cellular and Molecular Genetics and Virology Groups at the Scottish Crops Research Institute for help, and authors whose clear definition of terms in their papers made compiling a glossary an easier task.

Agrobacterium - A genus (group) of disease-causing soil bacteria some of which have been exploited for the genetic engineering of plants. *Agrobacterium tumefaciens* has the ability to transfer some of its DNA to its plant host as a part of the process by which it produces crown gall disease. It can be modified so that it no longer causes disease, but still transfers its DNA to plants. Although other methods for plant transformation have been developed, *Agrobacterium* infection and DNA transfer is still the most commonly used procedure for transformation of crop plants. *Agrobacterium* typically does not infect grasses efficiently, and therefore is not well suited for transformation of cereals such as corn and wheat. (See also T-DNA, Ti plasmid.)

Androgenesis - The process which gives rise to progeny which have only paternal chromosomes. It can occur in vivo when the female gamete nucleus does not fuse with the male gamete, and takes no further part in development, or disintegrates after fertilization, and in vitro when pollen is cultured, and gives rise to haploids.

Aneuploid - An individual having more or fewer chromosomes than the normal, euploid, complement, which is an exact multiple of the haploid chromosome number.

Antibiotic Resistance Marker - A gene, usually bacterial in origin, which encodes an enzyme which breaks down, modifies, or otherwise neutralizes an antibiotic. An antibiotic resistance marker typically confers antibiotic resistance to bacterial cells and can, in some cases, provide antibiotic resistance to plant cells into which it has been transformed. (See also Cotransformation, Marker, Plasmid, Reporter Gene, Transient Expression.)

Antisense - An antisense RNA molecule is one which is able to hybridize with its sense counterpart and form a duplex which is unstable in vivo, and is used to degrade mRNA. In plants transformed to produce antisense RNA, the target gene is expressed at low level or not at all.

Assay - A chemical procedure for identifying and/or quantifying a chemical, e.g. a gene product encoded by a transforming gene and expressed in transformed cells. (See also Immunoassay, Reporter Gene.)

Autoradiogram/Autoradiograph - An image produced on x-ray film by direct exposure to radioactive materials. Autoradiography is often used to visually identify cloned genes and gene products. (See also Colony Filter Hybridization, DNA Sequencing, Hybridization, Immunoassay, Probe.)

Bt Toxin - A crystalline protein made naturally by some strains of the bacterium *Bacillus thurengiensis*. Bt Toxin is a potent insecticide which has specific activity against certain orders of insects. This specificity for insect order varies between different strains of the bacterium. A common variety of the toxin is highly active against moth larvae such as the tobacco horn worm. The gene which encodes this bacterial product has been cloned and introduced into tobacco by transformation. Transformed tobacco plants which then produce this protein show high levels of resis-

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tance to the horn worm. The toxin is not know to have any activity in humans, and therefore appears to be a relatively safe alternative to some other forms of insect control. (See also Insecticidal Crystal Protein.)

Binary Vector - A vector containing two plasmids, where one plasmid contains the virulence gene (responsible for transfer of the T-DNA), and another plasmid contains the T-DNA borders, the selectable marker and the DNA to be transferred. (See also Constructs, c-DNA, Ligation, Plasmid, Vector.)

Biolistics - The use of accelerated particles to introduce DNA into cells. (See also Microprojectile Bombardment, Particle Gun.)

Biotechnology - A general term applied to any of a broad range of disciplines by which living systems are modified or manipulated in order to produce novel products, enhance plant or animal productivity, mediate disease resistance, etc.

Blotting - The transfer of DNA or RNA or protein from a gel to a membrane (e.g. nylon or nitrocellulose), to which it binds and is stabilized for further study. (See also Dot Blot, Northern Blot, Southern Blot, Western Blot.)

Callus - Plant cells which proliferate (grow and divide) on a defined medium and lack morphological differentiation. Callus may originate from various differentiated plant structures (e.g., immature embryos) and may possess differing degrees of cellular organization. Embryogenic callus has the capacity to regenerate complete plants, usually via somatic embryogenesis. (See also Embryogenic Callus, In Vitro Selection, Organogenesis, Regeneration, Suspension Culture.)

Capsid Protein - One of the most fascinating and successful uses of transformation in plants has been the introduction into plants of genetic sequences which encode the outer coat or covering protein ("capsid") of certain pathogenic viruses such as Tobacco Mosaic Virus. Plants which express high levels of this alien protein have been shown to resist infection by pathogenic viruses. Cross-protection against several related viruses has also been demonstrated. Since capsid protein from naturally occurring viruses is present in the environment, the production of this novel protein by tobacco probably does not represent a significant environmental risk. It holds great promise for producing crop cultivars with broad resistance to pathogenic viruses. (See also Coat Protein, Containment, Cross Protection.)

Carrier DNA - DNA of undefined sequence content which is added to the transforming (plasmid) DNA used in physical DNA-transfer procedures. This additional DNA has been shown to increase the efficiency of transformation in electroporation and chemically mediated DNA-delivery systems. The mechanism responsible for this effect is not known. (See also Chimeric Gene.)

Chimera - An organism or tissue which has an heterogeneous genetic composition. Some methods for gene transfer can produce transgenic tissues or plants which consist of a mixture of transformed and non-transformed cell lineages. Such tissues or plants are said to be chimeric.

Chimeric Gene - A semisynthetic gene, consisting of the coding sequence from one organism, fused to promoter and other sequences derived from a different source. Most genes used in transformation are chimeric. (See also Binary Vector, Plasmid, Transformation, Vector.)

Chloroplast - A plastid or organelle in plant cells which traps light energy and carries out photosynthesis.

Chromosome - A structure composed of double-stranded DNA and associated proteins which carries the bulk of genetic information in higher plants and animals. Typically, the genetic complement of a plant or animal is carried by many chromosomes (tobacco has 48) which are located within the nucleus of every cell in the organism. As cells divide during growth of the organism, the chromosomes are replicated, a complete set going to each new daughter cell. During transformation, the newly introduced gene is inserted into the chromosomal DNA and becomes an integral part of it (it is said to "integrate").

Cloning - The isolation of DNA sequences (genes), and the incorporation of these sequences into plasmids or other molecules for replication (increase) in bacteria or phage.

Coat Protein - The protein which surrounds the nucleic acid component of virus particles. Also the molecular species of protein(s) that comprise the protective protein layer of viruses. (See also Capsid protein, Cross Protection.)

Colony Filter Hybridization - A technique for the identification of bacterial colonies containing cloned DNA sequences (genes). The bacterial colonies from a petriplate are replicated on the surface of a nitrocellulose membrane and are then ruptured by a combination of enzyme and detergent treatments. DNA from these bacterial colonies is then denatured (made single stranded) and is probed with a radioactive, single-stranded probe and colonies with which the probe hybridizes are identified. Bacterial colonies on the original petriplate which have been identified as carrying the sequence of interest are isolated from them.

Complementary DNA- see c-DNA

Constitutive - Applied to a gene, or its enzyme product, to indicate that it normally functions in a cell, and does not require inducing.

Constructs (Constructions) - DNA molecules, for example, in plasmids, which have been "engineered" for a specific purpose. This term is often applied to DNA molecules used in transformation. (See also Transformation.)

Containment - Procedures, practices, and facilities which prevent the escape to the environment of organisms which carry recombinant DNA or are otherwise potentially hazardous. There has been considerable discussion regarding the safety and desirability of releasing recombinant plants to the environment. "Release" could include growing organisms under normal field conditions where they are free to outcross with other plants, disperse their seeds, or otherwise permit the movement of the introduced gene to other plants, or to allow the possibility that the transformed plant could become established in the environment outside human control. Field trials of transformed plants have been carried out successfully in a large (and growing) number of locations in the U.S., but in most instances all plant residue, including fruits, has been collected and either retained or destroyed in a way which renders it biologically inactive. Release of genetically engineered organisms to the environment is virtually inevitable, but procedures currently in plan should provide adequate protection against release of organisms which present a significant threat to humans or the environment. Risk assessment is largely based on the danger inherent to the introduced gene, and the probability that the transformed plant will transfer the introduced gene to a weedy species or become a weed itself. For example, a gene which encodes a toxic product active in humans would be high risk, while a gene encoding a virus capsid protein — known to be harmless to both man and animals — would be low risk. Similarly, release of transgenic Sorghum in an area where there are weedy sorghum plants is of higher risk than the release of transgenic tobacco which is not known to outcross to other native plants and is not itself prone to becoming a weed. (See also Transformation.)

Conversion - The development of a somatic embryo into a plant. (See also Regeneration, Micropropagation, Organogenesis.)

Cotransformation - The simultaneous transformation of an organism with two or more genes, one or more of which is silent (nonselectable) while at least one gene confers antibiotic resistance or another identifiable or selectable trait. Cotransformation is potentially useful in transformation of plants and animals because one gene allows identification of transformation at the cell level, while the other gene may only be expressed at maturity. In that case, the marker (cotransformed) gene would allow selection of transformed cells or callus prior to regeneration of whole plants. (See also Reporter Gene.)

Cross-protection - The mechanism whereby inoculation with one pathogen confers resistance to another pathogen. It has been shown that infection with one strain of a virus protects against infection or the expression of symptoms when inoculated with a second strain of the same virus. A plant transformed with the coat protein gene of one virus may be resistant to infection with the same or a closely related virus, a phenomenon that mimics cross-protection. (See also Capsid Protein, Coat Protein.)

Defective Interfering Particles - Virus particles which lack part of the nucleic acid component present in standard virus particles. This smaller nucleic acid depends on the genome of the standard virus for its replication and characteristically this replication interferes with and diminishes the extent of the multiplication of the standard virus genome.

Denaturation - A process whereby double-stranded DNA is made single stranded by the disruption of hydrogen bonds which normally join opposing strands of nucleotides. This is accomplished by treatment of DNA at elevated temperature and pH.

Direct DNA Uptake (Direct Gene Transfer) - A transformation procedure in which naked DNA is ad-

ded to a suspension of protoplasts. The protoplasts are then treated chemically (e.g., with polyethylene glycol) or electrically (in the case of electroporation) to allow DNA to pass through the cell membrane, and thus into the cell. (See also Biolistics, Electroporation, Particle Gun, Transformation.)

DNA - Deoxyribonucleic acid. DNA is typically a long, thread-like molecule, composed of two strands which lie side by side and are chemically linked to one another along their length. Each strand is composed of a long series of subunit molecules called "nucleotides" or "bases". There are four bases, Adenine, Guanine, Cytosine, and Thymine. The arrangement of these bases along the DNA strand composes a code which can be "read" by the cell. The information it contains allows cells (and the organisms they make up) to produce all of the products they need to function. (See also Gene, RNA.)

c-DNA - A DNA molecule synthesized by reverse transcription of a mature messenger RNA. Preparation of c-DNAs is often the first step in cloning DNA sequences of interest from developed plant tissues. While c-DNAs usually do not include regulatory or other controlling sequences, they can be used to identify (probe) and isolate genes and their associated sequences from genomic DNA.

DNA Constructs (Constructions) - see Constructs.

DNA "Fingerprinting" - When DNA is cut by a restriction enzyme, an array of fragments of different sizes is produced. Since the places where the DNA is cut by the enzyme are dependent upon the sequence of its bases, and different DNA molecules have different sequences, the fragment array produced when a DNA molecule is cut with a specific enzyme is usually unique to that molecule. This principle is applied to compare DNA from two or more sources (plants, animals, bacteria, etc.) If the DNA molecules of these organisms are the same or similar, this indicates that the two organisms share a significant amount of genetic information, and are probably related. DNA fingerprinting is now being applied to a very large number of biological problems, ranging from gene isolation (for use in genetic engineering) to paternity cases. It has also been used to demonstrate that two named cultivars of a crop were actually identical, and therefore should not have been sold as two distinctly different cultivars. DNA fingerprinting is often referred to by its more technical name, "Restriction Fragment Length Polymorphism (RFLP) Analysis". (See also RFLP.)

DNA Sequencing - The determination of the nucleotide base sequence of a DNA molecule by the base-specific sequential degradation of the DNA, or base-specific termination of DNA synthesis, followed by the separation of the fragments by high-resolution electrophoresis and autoradiography.

Dot Blot (Slot Blot) - A DNA hybridization procedure which is similar to the Southern Transfer process, except that DNA is bound directly to a support membrane without first being subjected to electrophoretic separation. This procedure is used primarily to test for the presence of a particular DNA sequence, or to measure the quantity of a known sequence within a mixture of DNA. (See also Southern Blot.)

Electrophoresis - Separation of particles (usually DNA, RNA, or proteins) in a gel in an electric field.

Electroporation - The introduction of transforming DNA or RNA into protoplasts or other cells by the momentary disruption of the cell membrane through exposure to an intense electric field. Although the precise mechanism of electroporation is poorly understood, it is thought that pores are formed by the local polarization of the cell membrane when it is exposed to a high electric potential. These openings persist for a variable amount of time, depending upon the temperature at which the cell is treated. Macromolecules, such as DNA or RNA, enter through these openings either through diffusion or through electrophoretic movement. The membrane openings then reseal, capturing introduced DNA and preventing escape of the cell contents.

Embryogenesis - The formation of an embryo. Embryo formation can result from the sexual union of gametes (zygotic embryogenesis), or can arise asexually from somatic tissues, usually in culture (somatic embryogenesis). (See also Organogenesis.)

Embryogenic Callus - An undifferentiated cell mass which produces somatic embryos. Embryos derived in this fashion are often a means of recovering plants from cultured tissues, and are widely used as a route to the recovery of transgenic plants.

Endonuclease - An enzyme which catalyses the cleavage of DNA at specific sites within the molecule. (See also Restriction Enzyme, Exonuclease.)

Epitope - The functional region of an antibody with which the antigen combines.

Exonuclease - An enzyme which catalyses the breakage of phosphodiester bonds between nucleotides, operating inward from the termini of a DNA molecule. Some exonucleases degrade only single-stranded DNAs and are thus useful for the removal of protruding single-stranded DNA produced by restriction endonuclease digestion.

Fiber-mediated DNA Transfer - A process whereby DNA is transferred into cells by vortexing a suspension of plant cells with silicon carbide fibers which have been coated with DNA. Subsequently the gene was expressed in the cells, indicating that DNA has been transferred.

Frameshift - The transfer of a ribosome during the translation of one open reading frame to a different reading frame such that the resulting translation product contains sequences encoded by each of the different reading frames joined at the point of the frameshift.

Gametoclonal Variation - Variation observed in individuals which have been produced by tissue culture of gametes. (See also Somaclonal Variation.)

Gene - A series of bases in a DNA molecule which encodes a specific protein product, also usually spoken of as including regulatory sequences which control its expression in the cell. Synthetic genes can be made by connecting coding sequences from one organism to regulatory sequences of another. These synthetic genes, once introduced into the target plant or animal, function as normal part of the recipient organism. (See also Chromosome, Chimeric Gene, Constitutive, DNA, Gene Expression, Genomic Library.)

Gene Expression - When a gene is "decoded" by the cell, and a new protein is produced, the gene is said to be "expressed" by the plant. Some genes are expressed in cells everywhere in the plant and at all developmental stages. Such genes are said to exhibit "constitutive" expression. Other genes exhibit "regulated" expression. Sequences which direct time- or tissue-specific expression have been isolated. When these sequences are associated with other genes and introduced into plants, the transgenic plants typically show time- and tissue-dependent patterns of expression. (See also Tissue-specific Expression.)

Gene Tagging - The detection of a gene by the identification of a closely linked marker. (See also DNA Fingerprinting, Marker, Molecular Marker.)

Genetic Engineering - Like "Biotechnology", this is a somewhat vague and overused term. It is often used to refer to "transformation", as in "...the plant was genetically engineered to produce higher alkaloid levels." Genetic engineering is also used generically to refer to all of the techniques which are used to manipulate DNA, including gene isolation and cloning, plasmid construction, etc.

Genomic Library - A collection of recombinant DNA molecules (plasmid or phage) which together carry sequences representative of an organism's genome. These molecules are propagated in bacteria or phage. The library is an important tool used in the process of isolating genes. Useful genes can then be transferred to other organisms by transformation (g.v.).

Hanging Drop Culture - Culture of cells in a drop of medium suspended on the underside of a glass carrier. Advantages are that only small volumes of media are needed, high cell densities can be achieved, and ease of observation.

Hybridization (of Nucleic Acid) - The re-annealing (joining) of homologous single-stranded regions indicates complementarity of sequence. Hybridization is often used with the Southern transfer technique to identify cloned sequences by hybridizing them to a radioactive probe. (See also Southern Blot.)

Hybridoma - A hybrid cell line produced by the fusion of a lymphocyte with a myeloma cell. Hybridomas are potentially capable of synthesizing monoclonal antibodies.

Immunoassay - A method of determining the type and amount of a protein that exploits the reaction between the protein and antibodies to it. There are many types e.g. radio immunoassay, enzyme-linked immunosorbent assay, immunosorbent electron microscopy, etc.

Inclusion Protein - Protein encoded by a virus genome that forms a microscopically discernable structure (the inclusion body) in the infected cells. Inclusion proteins are almost always not coat proteins. Their morphology is often characteristic for particular groups of viruses.

Insecticidal Crystal Protein (ICP) - A protein that is

toxic to insects. (See also Bt Toxin.)

Insertion Mutagenesis - The disruption of gene function by the insertion of a mobile genetic element into the gene. This process can occur spontaneously through the movements of resident transposable elements within the genome, or can be induced by the introduction of such elements during sexual crosses or via transformation.

In Vitro Selection - Selection for phenotypes (traits) expressed at the cellular or callus level which usually possess genetic changes that control the trait. Trait expression at the cellular level may or may not result in the same expression at the plant level. Traits such as herbicide resistance, salt tolerance, or disease resistance, or grain quality can be selected. Mutants usually occur at low frequency. (See also Somatic Cell Selection.)

Iso-electric Focussing (IEF) - An electrophoretic technique whereby proteins are separated in a pH gradient and are focussed at their isoelectric point. (See also Electrophoresis.)

Isozymes - Different forms of the same enzyme that occur within and between individuals. Isozymes are commonly used as markers. (See also RFLP, Molecular Marker.)

Ligation - The annealing of the termini of two double-stranded DNA molecules to one another, followed by the formation of phosphodiester bonds between terminal nucleotides in each strand of the DNA in the presence of DNA ligase. Ligation is used to join DNA molecules together, such as in the insertion of a gene into a plasmid to construct a transformation vector.

Liposome - A fatty droplet in a cell. Artificial liposomes containing DNA can be produced and fused with protoplasts as a means of DNA transfer.

Macroinjection - Injection of DNA into plants, usually into or near the flower, using a standard hypodermic syringe and needle. DNA is injected into leaf sheaths or flower buds, and not into individual cells. (See also Microinjection.)

Marker - A character which is linked to a gene, but is not the product of that gene. An ideal marker is easily screened, not affected by environment, expressed in hemizygous or heterozygous state, and facilitates the detection of genes whose products or expression cannot be detected. (See also Antibiotic Resistance Marker, Gene Tagging, Isozymes, Random Amplified Polymorphic DNA, Molecular Marker, Reporter Gene, RFLP.)

Marker-based Selection - The use of markers to select individuals with the desired gene, e.g., use of a marker to select resistant plants without the need to expose them to the pathogen.

Microinjection - A DNA delivery technology in which DNA is injected into cells or protoplasts with a microscopic needle drawn from a fine glass tube. While DNA delivery efficiency is very high in animal cells, this procedure has proven to be very troublesome in most plant systems. Efficient delivery is dependent upon introducing the injection needle into the nucleus of the cell to be treated. This is very difficult in plant cells. Also, the procedure can be very time consuming, requiring as much as several minutes to introduce DNA into a single cell.

Microprojectile Bombardment - DNA delivery process in which transforming DNA is associated with microscopic metal particles (tungsten or gold, chosen for their high density) which are then accelerated to high velocity in a "gun". The particles are capable of penetrating the walls of plant cells, and can deliver DNA into the cells without complex treatments such as those required for electroporation. This procedure combines efficient DNA transfer with the capacity to treat very large cell numbers in reasonably short time. (See also Biolistics, Particle Gun.)

Micropropagation - The production of many individuals from a meristem. (See also Regeneration, Somatic Embryogenesis, Conversion.)

Molecular Marker - A marker which is detected at the molecular level, either as a direct gene product (e.g. a protein), or as a product of the detection of specific nucleotide sequences (e.g. RFLP, PCR).

Monoclonal Antibody - An antibody preparation which contains only one molecular species of antibody. They are synthesized by hybridoma cells.

Mycoplasma-like Organism (MLO) - An organism which is intermediate between bacteria and viruses. Synonymous with Spiroplasma.

Near-isogenic Line (NIL) - A genotype, usually de-

rived by backcrossing, which differs from another in only a few genes. NILs are valuable in mapping, as the gene of interest may be flanked by genes which can be used as markers. (See also Recombinant Inbred Line.)

Northern Blot - A procedure for the identification of RNA sequences in which RNA is transferred to a support membrane following electrophoresis. The transferred RNA is hybridized to single-stranded DNA probes. This technique is often used to measure expression (transcription) of a gene for which a specific c-DNA is available for use as a probe. This is useful to determine, e.g., expression of a gene in a particular tissue. (See also Southern Blot, Western Blot.)

Organogenesis - The formation of organs (e.g., leaves). Often used to describe the formation of organs from callus tissues. Organogenesis is a common route for the regeneration of plants from cultured tissues. (See also Embryogenesis, Somatic Embryogenesis.)

Parthenogenesis - Development of an individual from the egg cell without fertilization by the male gamete.

Partial Digest - The incomplete digestion of DNA with a restriction enzyme so that some restriction sites remain undigested. Since the cleavage at restriction sites occurs randomly, the effect of partial digestion is to produce DNA fragments of various lengths containing overlapping sequences. By partial digestion, a series of random fragments of a genome can be produced for incorporation into a plasmid or phage to make a complete genomic library containing overlapping sequences which together incorporate a large portion of the entire genome. (See also Genomic Library.)

Particle Gun - The apparatus to accelerate DNA coated particles in order to introduce DNA into cells. (See also Biolistics, Microprojectile, Transformation.)

PEG (Polyethylene Glycol) - A chemical which promotes the formation of cell fusions by partial solubilization of protoplast membranes. Transformation of plant protoplasts has been accomplished by treatment of protoplasts with PEG in the presence of concentrated transforming DNA. (See also Protoplasts, Transformation.)

Plaque - A viral "colony", appearing as a cleared region in a bacterial lawn which has been lysed by the progeny of a single bacteriophage (virus). When genomic or c-DNA libraries are prepared in phage particles, each plaque represents a single cloned sequence. By hybridization of plaques through a procedure similar to colony filter hybridization, plaques (phage) incorporating a desired sequence can be identified.

Plasmid - Nonchromosomal self-replicating (circular) DNA. This term usually refers to self-replicating, nonchromosomal bacterial DNAs, some of which confer antibiotic resistance on their host cells. Plasmids are used in gene cloning and as the basic structures from which transformation vectors are built. (See also Binary Vector, Cloning, Constructs, Ti Plasmid, Vector.)

Plastid - An organelle in the cytoplasm of the cell, e.g. chloroplast, mitochondrium.

Polymerase - An enzyme which causes two molecules to join together, and thus leads to the formation of large molecules. Polymerases are involved in the assembly of nucleotides into DNA molecules.

Polymerase Chain Reaction (PCR) - A procedure for the amplification (numerical increase) of a particular DNA sequence by the repeated synthesis of new nucleotide strands on a template consisting of the desired sequence to which a sequence-specific primer has been attached prior to the beginning of the reaction. The polymerization of the new strand is catalyzed by a thermostable polymerase. This allows the temperature of the reaction mixture to be raised between subsequent polymerization rounds to a temperature at which denaturation of the newly formed double-stranded DNA occurs. This produces two new single-stranded templates upon which two new strands can be synthesized. By repeatedly raising and lowering the temperature of the mixture, a very large amplification of the sequence of interest can be achieved. In this way, detectable and useful amounts of DNA can be obtained from tissues in which the sequence is represented at very low levels. The technique has a multitude of applications ranging from testing transgenic cells for the presence of an introduced sequence, to the isolation of DNA sequences from very small amounts of tissue for forensic analysis. (See also Random Amplified Polymorphic DNA.)

Polymorphism - Having more than one form, usually

referring to variation between individuals in morphology, or in DNA as analyzed by RFLP or PCR, or in isozymes.

Probe - A defined sequence of DNA or RNA, usually radiolabelled, which is used to identify a homologous sequence of interest by hybridization. (See also Northern Blot, Southern Blot, Western Blot.)

Promoters - A region on DNA at which RNA polymerase binds and initiates transcription.

Protoplast - A plant or bacterial cell from which the cell wall has been removed, usually by an enzymatic process. Some plant species can be regenerated from protoplasts. (See also Direct DNA Uptake, Electroporation, Liposome, Microinjection, PEG.)

Pulsed Field Gel Electrophoresis - An electrophoretic technique used to separate large DNA molecules, by the application of pulsed, alternating orthogonal electric fields to a gel. (See also Electrophoresis, IEF.)

Quantitative Trait Locus (QTL) - A locus which affects the expression of a character that is polygenically or quantitatively inherited. QTLs are difficult to detect by conventional genetics, but some QTLs can be detected by marker technology.

Random Amplified Polymorphic DNA (RAPD, Pronounced 'Rapid') - A technique using single, short (usually 10-mer) synthetic oligonucleotide primers for PCR. The primer initiates replication at specific complementary sites on the DNA, producing fragments up to about 2 kb long, which can be separated by electrophoresis, and stained with ethidium bromide. A primer can exhibit polymorphism between individuals, and polymorphic fragments can be used as markers.

Readthrough - The passage of a ribosome during the translation of an open reading frame past the termination codon that normally causes the ribosome to stop. The resulting translation product is the standard protein encoded by the open reading frame with additional sequences at its C-terminal end. Readthrough is usually in the same reading frame but can also be caused by frameshift.

Recombinant DNA - The hybrid DNA formed by combining pieces of DNA from different organisms, e.g., a bacterial plasmid containing a plant DNA sequence is a recombinant-DNA molecule.

Recombinant Inbred Line (RIL) Also Recombinant Inbred (RI) - One of a number of lines derived from the same cross by repeated selfing until each line is near homozygous. RILs which differ with respect to genes of interest can be used to associate genes with markers (gene tagging). (See also Near Isogenic Line (NIL).)

Recombination - The joining of two DNA molecules, often said of the integration of a transforming DNA into the chromosomal DNA of the host organism.

Regeneration - The regrowth of whole plants from cultured cells or callus. This usually occurs via one of two pathways: organogenesis and embryogenesis. In somatic embryogenesis, plant regeneration occurs from callus or suspension cultures by a process similar to embryo formation in vivo. Anatomically, root and shoot axes develop simultaneously. In organogenesis, shoot and/or roots (or other organs) develop independently. Although embryogenesis is the preferred pathway, either method may result in efficient plant recovery. (See also Callus, Embryogenesis, Organogenesis, Suspension Culture.)

Repetitive DNA - DNA which consists of repeated nucleotide sequences.

Replicase - The enzyme, or enzymes, responsible for the synthesis of virus-sense nucleic acid molecules by using as a template the same virus-sense nucleic acid (i.e. replication).

Reporter Gene/Marker Gene - A reporter or "marker" gene is a gene, often derived from bacteria, which does not in itself provide a useful change in transgenic plants, but serves to identify and/or recover transformed plants from among a large sample which may or may not have been transformed. They are also very useful in studies of gene expression, since their presence or absence, and the quantity of the gene's product can be precisely measured. Common marker genes currently in wide use include beta-glucuronidase and neomycin phosphotransferase. Beta glucuronidase (GUS) is an enzyme which can be detected by two sensitive procedures, one of which allows direct observation of transformed cells or tissues. Neomycin phosphotransferase (NPT) can be monitored by quantitative assay, but most importantly it provides protection to transgenic cells and plants

(seedlings) against the antibiotic kanamycin. This allows the direct selection in culture of cells or tissues which are transformed with the gene by simply growing the material on medium containing kanamycin. Progeny of these transformed plants can also be monitored by germinating seeds on medium with kanamycin, where those which express NPT remain green, while those without it turn white and eventually die. Other genes which are not so easily monitored are often introduced along with these markers. By selecting plants which express the marker gene, there is a high probability that these plants will also carry the real gene of interest. (See also Antibiotic Resistance Marker, Binary Vector.)

Restriction Enzymes - Enzymes of bacterial origin which cleave DNA at highly specific locations determined by the random occurrence of specific short nucleotide sequences (usually four or six base pairs). Many restriction enzymes cleave double-stranded DNA in such a way that each resulting molecule end has a protruding, single-strand sequence commonly referred to as a "sticky end". Two molecules with homologous sticky ends can hybridize readily to form recombinant molecules. (See also Restriction Fragment, RFLP.)

Restriction Fragment - A short DNA molecule resulting from the partial or complete cleavage of a larger molecule with a restriction enzyme. The distribution of restriction enzyme cleavage sites in a particular DNA molecule is determined by their sequence of nucleotides. The fragments which result from restriction enzyme digestion have various lengths which reflect the structure of the parent molecule.

RFLP (Restriction Fragment Length Polymorphism) - Although the sequence of DNA from two genotypes of the same species may be very similar and thus produce very similar restriction fingerprints, some polymorphism (variation) in the distribution of restriction sites for a particular restriction enzyme occurs between genotypes. The particular array of restriction fragments produced for each genotype is thus diagnostic of that genotype. Specific fragments may be linked to unidentified but important genes and can thus serve as genetic markers for those traits.

Restriction (Site) Map - A diagram showing the locations of restriction sites relative to one another and to other features of a DNA molecule. It is common practice to characterize the structure of cloned DNA molecules by restriction mapping, a process in which the organization of sites is deduced from the sizes of fragments produced when the molecule is cleaved by single enzymes or combinations of enzymes.

Reverse Genetics - The study of gene action or the phenotype produced by mutating a gene or synthesizing a nucleotide sequence in vitro, and introducing it into a plant.

Ribozyme - A small RNA molecule which will hybridize to a target RNA and thereby cause the excision of the target RNA between two particular nucleotides. The cleavage is independent of protein and is the same mechanism as that whereby some satellite RNA molecule oligomers self cleave during replication.

RNA - Ribonucleic acid. RNA, like DNA, is a long thread-like molecule composed of four different subunit "bases". Like DNA, it incorporates Adenine, Guanine and Cytosine, but Thymine is replaced with another base, Uracil. Different types of RNA have different functions in the cell, but they are primarily involved in "reading" the information encoded by DNA, and the production of proteins. (See also Transcription, Translation.)

Satellite - (1) The region of the chromosome distal to the Nucleolar Organizer, which often appears detached from the chromosome in metaphase preparations.

(2) A virus or nucleic acid that is unable to multiply in cells without the assistance of a specific 'helper' virus, has no appreciable sequence homology with the helper virus, and is not necessary for the multiplication of the helper virus.

Selectable Marker - See Antibiotic Resistance Marker.

Somaclonal Variation - Variation induced by tissue culture in organisms regenerated from somatic tissues. Some types of somaclonal variation are inherited by the progeny.

Somatic Cell Selection - Selection of cells in culture, usually in suspension cultures, by applying a selection pressure that will kill or reduce the viability of susceptible cells. It has been used to produce resistant plants by exposing cultures to filtrates of pathogenic bacteria. (See also In Vitro Selection.)

Southern Blot/Southern Transfer - A technique developed by E.M. Southern for the transfer of (denatured) DNA from an electrophoretic gel to a nitrocellulose membrane, on which the DNA can be bound. The transferred DNA can then be hybridized to single-stranded DNA or RNA probes. An essential technique for the identification of cloned DNA sequences.

Spiroplasma - Synonymous with Mycoplasma Like Organism (MLO)

Stable Transformation - Gene transfer which leads to the integration of introduced sequences into the chromosomes of the recipient plant. Stably transformed cells or organisms pass the newly integrated gene(s) to their progeny.

Suppressor tRNA - A class of tRNA that recognizes termination codons, and causes an amino acid to be incorporated into a growing peptide chain, rather than the termination of the chain.

Suspension Culture - A culture in which cells, clusters of cells and/or small callus pieces are suspended in liquid nutrient media. Plant suspension cultures usually must be aerated by agitation. Suspension cultures are useful as a source of protoplasts for transformation and also for in vitro selection employing herbicides or toxins. Suspension cultures are useful for selection because of the large surface area of cells which are exposed to the medium and the possibility of extremely uniform exposures. Embryogenic suspension cultures are capable of regenerating complete plants via somatic embryogenesis.

T-DNA - Transfer DNA. The DNA which is transferred to the host plant by *Agrobacterium*. It is flanked by two 25bp border regions which are recognized by the virulence gene of *Agrobacterium*.

Tagging - See Gene Tagging

Ti Plasmid - (Tumor inducing plasmid). A plasmid that occurs naturally in *Agrobacterium tumefaciens* and causes tumors in host plants by inserting DNA into the host genome.

Tissue Culture - The growth of plant or animal cells, tissues and organs outside of the organism, usually in glass or plastic containers ("in vitro"). There the "explant" tissue or cells are supplied with the nutrients and other substances required for continued growth. Although it is integral to other kinds of work, such as genetic engineering, tissue culture is now generally considered to be a discipline in its own right.

Tissue-specific Expression (of a Gene or Gene Product) - The production of a gene product in a specific tissue at a specific developmental stage or time. Tissue-specific expression is often desirable when a gene product is only needed at a certain stage of growth or in certain tissues.

Transcription - The production of messenger RNA (mRNA) in the nucleus, using DNA as a template so that the sequence of nucleotides is maintained and can be used in translation (q.v.).

Transformation - The genetic modification induced by the incorporation into a cell of "foreign" DNA purified from other cells or viruses. (See also *Agrobacterium*, Direct DNA Uptake, Genomic Library, PEG, Plasmid, Vector.)

Transient Expression - Expression of a transforming gene by recipient cells over a relatively brief time span. Transient expression, e.g., of antibiotic resistance markers in cells may be due to rapid cell death or the loss of transforming DNA over cell generations in the absence of DNA replication.

Translation - The production of a protein in a ribosome using the sequence of bases of the mRNA to determine the sequence of amino acids in the protein.

Transport Protein - A protein (other than coat proteins) encoded by the virus genome which is essential to the movement of virus infection from one cell to another.

Transposition - The movement of a genetic element from one location within the genome to another.

Transposon (Transposable Element) - A mobile genetic element which can excise from one location in the genome and reinsert at another location. Some transposons, e.g., Ac, are capable of catalyzing their own transposition, including excision and reinsertion. Other elements, such as Ds, require the presence of another element (Ac) in order to accomplish transposition and are incompetent for transposition in the absence of such an element.

Transposon Tagging - The "labelling" of a gene with a transposon through the process of insertion mutagenesis with the transposon.

Vector - A specialized plasmid or other DNA molecule which has been"engineered" to contain marker or other genes for use in transformation. Vectors typically contain sequences which allow their replication and selection in bacteria for amplification of the DNA used in transforming other organisms.

Virion - Synonymous with 'Virus particle'.

Western Blot - A technique in which protein is transferred to a support membrane following electrophoresis. A particular protein molecule can then be identified by probing the blot with a radiolabelled antibody which binds only to a specific protein to which the antibody was prepared. Useful for measurement e.g. of levels of production of a specific protein in a particular tissue or at a particular developmental stage.

Appendix I

International Workshop on Biotechnology and Crop Improvement in Asia

3-7 December 1990
ICRISAT

International Crops Research Institute for the Semi-Arid Tropics
Patancheru, Andhra Pradesh 502 324, India

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Appendix II

Recommendations

The focus on biotechnology's potential contributions to sustainable agricultural production in the region derives from a projected need for dramatic increases in crop productivity in the face of increasing population and natural resource demands. Conventional technologies alone may be inadequate to meet present and future challenges.

Biotechnologies must be applied to achieve specific, well-defined goals, based on the problems which limit production of food crops. It is important to focus first on production of the crop, and let the needs of the crop drive the choice of appropriate technology.

The workshop emphasized the need for the development of multigene, long-lasting, robust strategies for specific problems.

Because these technologies are resource intensive, it is essential to eliminate unnecessary duplication wherever possible.

Potential applications of biotechnology include: (1) the use of tissue culture for clonal propagation, wide crosses, and conservation of elite and disease-free materials; (2) the use of biochemical and molecular techniques for diagnostics and genome characterization; and (3) the use of cellular and molecular strategies in conventional and nonconventional breeding programs.

Networking, Training, and Technology Transfer

The workshop recommends:

- The establishment of a regional plant biotechnology network for all food crops. All interested scientists, institutes, and individuals active in the region should be encouraged to participate in the network. The network members would include scientists from any countries who could contribute to the application of biotechnology to crop improvement in Asia.
- That efforts should be made to coordinate and, where possible, integrate the network activities with those of other networks and relevant organizations. It should have sustained host institution and donor support, with periodic review to evaluate its contributions to regional progress in biotechnology.
- That the network could be based at ICRISAT Center, which has appropriate scientific, information and communications resources for this purpose. The establishment of this network would require a minimal foundation level of funding to support a network coordinator, recurrent administrative expenses and information dissemination costs.
- The formation of a steering committee comprised of national representatives and technical specialists. An informal, regional newsletter could serve as a primary and reliable means of information exchange with individual members.
- That the network strengthens existing links, and develops new links, with other laboratories in order to sustain existing expertise, to acquire newly developed methods, and to promote the transfer of technology to the researchers of Asian countries wherever necessary.
- That the activities of the network include the maintenance of a directory of members and resource people, the gathering and dissemination of information on critical issues in plant biotechnology, the identification of specific training and technology transfer needs and fund raising to support designated activities. Emphasis should be given to the development of bilateral relationships, which build on initial contacts and provide continuing support and exchange of ideas between scientists. The network should involve Universities and other groups, especially those involved in fundamental research that may benefit Asian countries.
- That the network does not stock any organisms, but should maintain a database of such stocks, and include one or more centers which stock strains of *Agrobacterium* and/or plasmids.
- That proper training is vital. There are two main elements, (1) the organization of training courses and (2) exchange of staff with relevant laboratories in other countries. Students from Asian countries could be trained at institutes within Asia, at ICRI-

SAT, or in advanced laboratories in developed countries.
- That training should include participation with industry, and in addition to training in biotechniques, should include aspects of biosafety, the testing of derived lines and release of genetically engineered organisms. The network should disseminate information on biosafety so scientists are aware of the risks involved.

The participants suggested that ICRISAT Center would be a suitable base for the network, as it has appropriate scientific, information, and communications resources for this purpose. The establishment of this network would require a minimal foundation level of funding to support a network coordinator, recurrent administrative expenses and information dissemination costs.

Biosafety

The workshop highlighted the need for country-specific development in regulatory infrastructure to complement and insure the safe, responsible, and timely application of biotechnological advancements in crop improvement.

The workshop recommends
- That the network establishes an ongoing source of information on country activities in this area, related international scientific and legislative developments, and identifies resource people in biosafety.
- That the network commission brief reports on the status of biosafety, for which it should seek outside help where necessary, and provide a link with expertise, and with resource persons.

Intellectual Property Rights (IPR)

The workshop has recognized the involvement of this issue in technology acquisition.

The workshop recommends
- That the network promotes exchange of information, and provides authoritative contacts.
- That the network operates within IPR legislation of partner countries

Private and Public Sector Involvement

The workshop recommends
- That efforts be made to foster contacts with members of both public and private sector scientific communities to identify technical opportunities for interaction and to develop equitable collaborative agreements. The network could provide the necessary case studies and contacts, including links with legal resources.

Genome Characterization and Diagnostics

Genome Analysis

The workshop stressed that RFLP technology is a powerful tool of great potential in plant breeding, to improve the speed and precision of conventional breeding, specially for traits that are difficult to manipulate using conventional approaches. The technology also has proven potential to characterize germplasm.

The workshop noted the existence of a Rockefeller Initiative to develop an RFLP network to support breeding of sorghum and pearl millet in developing countries. The workshop recommends the expansion of collaboration with other Institutes in Asian countries, as well as the initiation of new programs where necessary.

The workshop recommends
- That breeders are involved in the conception and execution of RFLP programs.
- That existing links are reinforced and new links, with laboratories with relevant skills are developed.
- That probes are freely available; database organization may be necessary for this, and a database indicating the nature and source of clones would facilitate their timely and efficient use.

The workshop identified potential for collaboration in the analyses of mitochondrial genomes in pearl millet male-sterile lines.

Diagnostics

The workshop recognized the potential of biotechnology in developing diagnostic procedures for the identification of pathogens, genes, and other biological macromolecules.

The workshop recommends:
- That methods used should include (1) nonradioactive probes, (2) DNA amplification (PCR), (3) synthesis of antibodies in non-mammalian cell

systems, (4) cloning of antigens, and (5) immunocytochemistry with gold-labeled probes.

In Vitro Culture and Transformation

The workshop recommends:

- That in vitro culture and micropropagation procedures are appropriate means of maintaining high-value genetic stocks, as an aid to experimentation and of maintaining gene pools, particularly in vegetatively propagated species. Technologies which maintain the genetic stability of the stored materials must be developed and applied.
- That all efforts be planned and expedited in full coordination with breeding programs that can use the resulting materials effectively. Advanced breeding lines should be used as source material in programs, to develop adapted material of value to the concerned breeders.
- That methods of generating random genetic variation via in vitro culture should be approached with caution unless such systems can be coupled with efficient in vitro selection procedures.
- That although anther culture and parthenogenesis are currently the most commonly used means of producing haploids, but alternative routes should also be considered.
- That there is currently no universally applicable strategy for transformation of all crop species. Practical application requires the development of cost-effective genotype - independent transformation systems, ideally with utility in more than one crop.
- That transformation work would be greatly facilitated by the development and maintenance of a data base that would serve as a guide to availability of expertise and materials, such as *Agrobacterium* strains and plasmids.

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