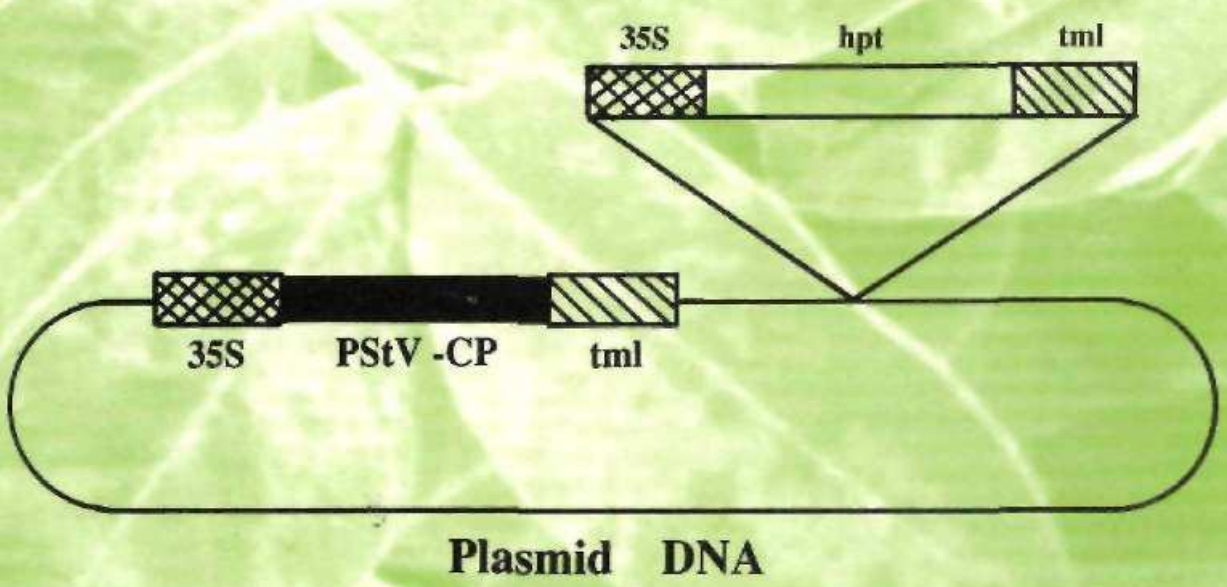
Transformation and Regeneration of Groundnut, and Utilization of Viral Genes to Induce Resistance to Virus Diseases



Plasmid DNA

International Crops Research Institute for the Semi-Arid Tropics

Abstract

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At a meeting organized by ICRISAT in cooperation with the Virology Department, Wageningen Agricultural University, The Netherlands, 14 scientists from six countries met to discuss transformation and regeneration of groundnut and utilization of viral genes to induce resistance to virus diseases. There was a session on transformation and regeneration, in which research on the use of *Agrobacterium tumefaciens,* particle bombardment, and electroporation of protoplasts were presented and discussed. The third session reviewed progress in utilization of viral genes for resistance to groundnut rosette, peanut stripe, peanut clump, and bud necrosis diseases. The final Plenary Session summarized the state of research and indicated where cooperation could be encouraged. A set of recommendations was prepared.

Résumé

Transformation et régénération de l'arachide et l'utilisation de gènes viraux pour induire la résistance aux viroses: compte rendu et recommandations d'une réunion. **Une réunion organisée par l'Institut international de recherche sur les cultures des zones tropicales semi-arides (ICRISAT), conjointement avec le Département de Virologie, Université agricole de Wageningen, Les Pays-Bas, a réuni 14 chercheurs venant de six pays pour faire le point sur la transformation et la régénération de l'arachide et l'utilisation de gènes viraux pour l'induction de la résistance aux maladies virales. La session sur la transformation et la régénération a vu la présentation et la discussion des travaux de recherche sur l'utilisation d'*Agrobacterium tumefaciens*, le bombardement des particules et l'électroporation des protoplastes. La troisième session a examiné les progrès réalisés dans l'utilisation de gènes viraux pour la résistance à la rosette de l'arachide, à la maladie des stries de l'arachide, au rabougrissement de l'arachide et à la maladie de la nécrose des bourgeons de l'arachide. La Session finale plénière a fait le point sur l'état de recherche et a indiqué les domaines où la coopération pourrait être favorisée. Enfin, la réunion a permis l'élaboration de plusieurs recommandations.**

Cover: Plasmid construct containing peanut stripe virus coat protein gene and hygromycin phospho transferase gene superimposed on groundnut leaves with typical blotch symptoms of peanut stripe virus disease.

Transformation and Regeneration of Groundnut, and Utilization of Viral Genes to Induce Resistance to Virus Diseases

Summary and Recommendations of a Meeting,

24-27 Apr 1992.

Virology Department, Wageningen Agricultural University, The Netherlands

Edited by

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International Crops Research Institute for the Semi-Arid Tropics
Patancheru, Andhra Pradesh 502 324, India

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Overseas Aid for Biotechnology in Agriculture

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Biotechnology could have a tremendous impact on the agricultural productivity of the developing world, and this makes it an important issue in North/South relations.

There has been much discussion in the literature on what is considered to be the undue concentration of biotechnology research in the developed world, and on unfair terms of trade for the Third World in relation to biotechnology. It is clear that most biotechnology research for agriculture is in the developed world, and is aimed at assisting developed country agriculture. It is argued that Third World genetic resources are exploited free of charge by the developed world and genes from them are patented. There are fears of the substitution of Third World agricultural commodities by biotechnology products. Although some of these arguments that have been put forward to support the Third World viewpoint appear to be overstated, they are certainly not groundless, and there is a need to change the direction of research towards developing-country problems. Overseas aid can be used to help redress this imbalance, and aid agencies must work with both the private and public sectors to do this. A cost-effective approach is to use overseas aid to exploit patented genes in the private sector for developing countries.

Projects in biotechnology offer solutions in the reasonably short term in relation to the time scale of conventional plant breeding. This is because genes, not available in the crops' germplasm, can be transferred into already-adapted and popular cultivars, to impart resistance against an important pest or disease, and can be targeted to solve well-defined and important agricultural problems.

There is a great need for information exchange to prevent duplication of research, and the establishment of more networks for the biotechnology of developing-country crops is desirable.

The acceptance of biotechnology as a major research area for overseas development will inevitably mean a change in priorities for overseas aid that are based on production values and poverty considerations.

Biotechnology projects can be considered as offering excellent value for money and therefore deserving a high priority for overseas aid funding. They may prove to be more cost effective than conventional breeding projects on tropical crops. When the costs of a biotechnology project and a conventional breeding program based in a developing country are compared, then the biotechnology project will emerge as an attractive option.

Transformation and Regeneration

Chairperson: J.P. Moss

Regeneration and Transformation of Embryogenic Groundnut Cultures

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Embryogenic cultures of groundnut can be induced from immature embryos (cotyledons and embryo axes) on media containing an auxin-type growth regulator. We routinely use the growth regulator, picloram, at a concentration of 0.5-3 mg L^{-1} in Murashige and Skoog's medium. Somatic embryos and embryogenic "callus" can be observed 3 weeks after culture initiation (Ozias-Akins 1989). Embryogenic callus can be maintained on the same medium for at least 2 years without losing the capacity for plant regeneration. Embryogenic callus consists of clusters of somatic embryos that are asynchronous in their development. Callus masses double to triple over one 4-week subculture period.

Regenerable cultures that can be aseptically maintained provide a continuous supply of tissues for transformation experiments and offer a considerable advantage over individual shoot meristems in terms of numbers of treatable units and selection strategies. Disadvantages are genotypic differences in in vitro response (Ozias-Akins et al. 1992a) and the amount of time required for plant regeneration from embryogenic cultures (4-5 months). Transient expression monitored by GUS gene expression 24 h postbombardment is high in differentiating somatic embryos and embryogenic calluses. For stable transformation experiments, we are bombarding embryogenic calluses with a plasmid construct containing the hygromycin phosphotransferase *(hpt)* gene driven by the CaMV 35S promoter. Bombarded tissues are either placed under hygromycin selection on picloram-containing medium or transferred to nonselective regeneration medium. Hygromycin at 10 mg L^{-1} is sufficient to suppress the growth of nontransformed embryogenic callus. Kanamycin must be used at much higher levels (400-1000 mg L^{-1}) and thus may not be suitable as an efficient selectable marker. Hygromycin-resistant, embryogenic lines have been selected that show PCR amplification of the chimeric *hpt* gene using one primer specific to the promoter region and one specific to the *hpt* coding region. Integration of the foreign DNA remains to be confirmed by Southern analysis.

Our approach to regeneration from somatic embryos of groundnut has evolved from maturation and conversion (Ozias-Akins et al. 1992b) to a combination of embryogenesis

and organogenesis. Somatic embryos and embryogenic calluses are transferred directly from maintenance medium (in dark) to a regeneration medium containing cytokinins (in light) with subsequent transfers onto media containing cytokinins plus gibberellin. One typical regeneration experiment resulted in 913 shoots from 160 callus pieces (50 mm^2) for an average of 5.7 plants per callus. Regeneration of putative transformants is in progress.

Ozias-Akins, P. 1989. Plant regeneration from immature embryos of peanut. Plant Cell Reports 8(4):217-218.

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Ozias-Akins, P., Singsit, C., and Branch, W.D. 1992b. Interspecific hybrid inviabtlity in crosses of *Arachis hypogaea* x *A. stenosperma* can be overcome by in vitro embryo maturation or somatic embryogenesis. Journal of Plant Physiology 140(2):207-212.

Strategies for the Microprojectile Bombardment of Groundnut

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In order to design an efficient, reliable protocol for the transformation of groundnut via microprojectile bombardment, it is essential to maximize the targets of tissue that will produce fertile plants. Two approaches are currently being undertaken to increase the likelihood of success. The first approach involves bombarding a tissue that is capable of regeneration, and inducing plant formation under selection. One suitable explant type that we employ is immature leaflets, which readily undergo organogenesis to form fertile plants. The planar geometry of the leaflets make them well suited to bombardment and selection. We have recovered stably transformed callus lines from the leaflets at a rate of 0.024-0.25 callus lines per leaflet, depending on the genotype. The regeneration of stably transformed plants from this material has yet to be achieved. We are also bombarding embryogenic callus material, that can readily be induced to proliferate and regenerate, and is amenable to selection by hygromycin. Bombarded embryos can transiently express B-glucuronidase (GUS) at a very high level. Bombarded embryogenic material is currently proliferating in the presence of hygromycin. Any surviving material will be regenerated and tested for stable integration of the introduced gene.

In our second approach, germ line tissue is targeted to produce chimeric plants yielding stably transformed offspring. Since the goal is to produce chimeric plants, GUS histological staining, rather than a selective agent, is used to identify plants that may yield stably transformed progeny. This method is therefore labor intensive, but it provides little chance for the somaclonal variation and loss of ability to regenerate often encountered with long-term tissue culture. We are targeting the apical domes of both somatic and zygotic embryos. Zygotic embryos, which require the shortest culturing period, are excised from dry seeds, and the apical domes housing the germ lines are easily exposed for bombardment. Fertile plants are rapidly produced from the embryos on hormone-free medium. Somatic embryos can transiently express GUS at a high rate, while GUS expression by zygotic embryos is moderate. Regeneration of bombarded embryos is presently underway.

It is hoped that by employing a variety of approaches, the stable transformation of groundnut will soon be achieved.

Evaluation of Plant Regeneration and Genetic Transformation Systems in Groundnut

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Genetic transformation technologies to introduce foreign genes into cells are essential both for basic studies of gene function, and for the production of agronomically valuable transgenic plants. At the Scottish Crop Research Institute (SCRI) we are developing an efficient and reliable genetic transformation method for groundnut using *Agrobacterium*-mediated transformation. Initially, we used two virulent strains of *Agrobacterium tumefaciens* - C 58 and A 586, to assess their natural ability to infect wounded tissues of groundnut plants, and subsequently to initiate tumours. It was observed that tumours can be readily produced at the point of infection and tumour lines, which are free from *Agrobacteria* , can be grown on a hormone-free medium for several months, confirming their true tumorigenic nature. However, attempts to regenerate transgenic plants from these tumorigenic calluses were not successful. The two most important prerequisites in developing *Agrobacterium-mediated* transformation are (1) the ability to regenerate plants from such explant tissues as cotyledon or leaf, and (2) the availability of a suitable selection system to obtain transgenic tissues which can be produced by transgenic plants. With a view to obtaining transgenic plants, we therefore used disarmed forms of *Agrobacterium* strains C 58 and A 856 possessing a binary vector, KIWI 105. This binary vector contains two marker genes, GUS and either NPT II or HPT under the control of the 35S promoter and NOS terminator.

Plant regeneration from two different explant tissues, mature cotyledons, and young leaflets of several Indian and African cultivars and breeding lines, has been established. However, the precise response of explants of these cultivars and lines is genotype-dependent. To develop an efficient antibiotic-based selection system to produce transgenic tissues and plants, both kanamycin and hygromycin were tested, and hygromycin was found more effective. In the transformation studies, both cotyledon and leaf tissues were inoculated with overnight-grown *Agrobacterium* in the presence of acetosyringone (100 µM). Assays for GUS activity showed that the gene was expressed in the cotyledonary and leaf tissues 48 h after inoculation and after 2 weeks of culture. Recently, we performed the GUS fluorogenic assay on transgenic calluses, further confirming their transgenic nature. However, our attempts to obtain transgenic plants from these transgenic tissues have not been successful to date.

Our ultimate aim is to produce transgenic groundnut plants with viral resistance properties against groundnut rosette and Indian peanut clump viruses induced by the coat protein- or satellite-mediated resistance mechanisms.

Acknowledgements

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Current Research on Regeneration and Transformation of Groundnut at ICRISAT

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Research on regeneration of *Arachis* started at ICRISAT as a part of a wide hybridization program in which many hybrid plants were regenerated by organogenesis from calluses derived from a single hybrid embryo (ICRISAT 1987). Initial research indicated that Murashige and Skoog's (MS) basal medium was satisfactory, and shoots could be regenerated from calluses derived from hybrid embryos using naphthalene acetic acid (NAA) and benzylaminopurine (BAP). Embryos were derived from wide crosses using hormone application. Heart-shaped embryos gave rise to plants, but the numbers of plants recovered

from globular or torpedo embryos were small. Shoots were then rooted on filter paper bridges over liquid medium (Moss et al. 1988).

A range of explants from cultivated groundnut and from wild *Arachis* spp were used to regenerate plants. Young leaflet segments cultured on MS + 1 mg L^{-1} NAA or MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP gave rise to calluses and shoots. Wild species responded well. *Arachis pusilla* leaflet segments and root discs produced shoots on MS media supplemented with NAA + BAP, NAA + kinetin (Kn), or Kn + gibberellic acid (ICRISAT 1982).

Cotyledons from mature seeds were readily available and responsive explants. When cultured on MS + BAP at 25.0 mg L^{-1} + NAA 2.0 mg L^{-1} (Nalini Mallikarjuna et al. 1992), cotyledons regularly formed multiple shoots, though the frequency of response and number of shoots formed depended on the genotype.

This regeneration technique was used in experiments on transformation of groundnut using *Agrobacterium tumefaciens* with kanamycin resistance, and we were able to regenerate shoots that survived on a medium containing kanamycin.

One of ICRISAT's aims is to produce plants resistant to important constraints limiting groundnut yields in the semi-arid tropics (SAT). When conventional solutions are not available, where they are inappropriate, or where a more stable, durable or environmentally friendly solution is required, we will use wide hybridization or transformation to access appropriate genes.

ICRISAT has well-developed Breeding, Virology, Entomology, Pathology, and Physiology Units, and the Cell Biology Unit aims to incorporate transformation into the crop improvement program, taking advantage of ICRISAT's multidisciplinary team's knowledge of constraints in the SAT, and its ability to screen for resistance to those constraints either at ICRISAT Center, or through its networks and international trials.

At present, genes of major interest are coat protein genes for groundnut rosette virus (GRV) and peanut clump virus (PCV), and we have collaborative research projects with the Scottish Crop Research Institute (SCRI) for GRV, and the Centre de cooperation internationale en recherche agronomique pour le developpement (CIRAD) for PCV. We also have a collaborative project with SCRI, linked to the GRV project, to develop techniques for transformation.

We hope to develop additional links with other institutions, and to develop the capability at ICRISAT to characterize and clone genes of interest.

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Genes with Potential for Utilization in Transformation

Chairperson: M.A. Mayo

Genes with Potential for Utilization in Transformation: An Overview

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Transformation of plants with genes derived from virus genomes has been shown to confer to transgenic plants, agronomically useful levels of resistance to virus infection for a number of virus-host combinations. This overview outlines the range of possibilities, some not attempted as yet, to exploit our increasingly detailed knowledge of plant virus genomes in applying this nonconventional approach to obtain virus-resistant plants.

The source of the gene in transformation can be a plant genome, a virus genome, or a molecule synthesized in vitro. Where a plant gene is known to confer virus resistance, it is possible that this could be transferred to a susceptible host plant by transformation. Equally, where a gene confers susceptibility, it would be a target for inactivation by transformation with interacting sequences. However, neither approach has yet been successful. Synthetic genes are obtained from nucleotide sequences synthesized to be complementary to the target gene, either as antisense or as ribozyme RNA. The great majority of transgenes used to date have originated from the virus genome.

Four types of genes have been used, and in an oversimplified model they can be thought of as affecting different stages in the life cycle of a virus. The commonest gene, which was thought to be targeted at early stages of virus multiplication, is the coat protein gene of the target virus. Transformation with the coat protein gene has introduced resistance against at least 15 viruses in 11 virus groups. Many workers are attempting this method with a wide range of viruses and crops. The effects of transformation with coat protein genes can be summarized as a delay and attenuation of virus-induced symptoms, and decreases in the effective infectivity of an inoculum and in the amount of virus synthesized in those plants which are infected. The effect has been shown to be dependent on the virus and host being used, on the construction of the vector used for transformation, and on the environmental conditions of the experiment. The effect obtained is sometimes, but not always, correlated with the amount of coat protein made in the transgenic tissue. Some coat protein genes are effective even when no protein is made,

Antisense RNA, either alone or in conjunction with a catalytic ribozyme domain, has been found to confer resistance when expressed from a transgene. It has proved possible to

clone sequences encoding the complementarity-determining regions of mouse monoclonal antibodies, and to transform plants with the sequences such that plant cells make antibody-like molecules. This approach is potentially capable of disrupting virus infection by interfering with virus gene products.

Satellite and defective interfering (DI) RNA compete with genome RNA for viral replicase and thereby inhibit multiplication. Both effects have been shown to work in plants expressing the satellite or DI RNA from a transgene. This effect is self-amplifying and its efficacy does not depend on high levels of expression.

A large majority of attempts to induce virus resistance by transformation have been with coat protein genes. This approach remains a first choice, but other approaches have been shown to work and provide either an ancillary method to reinforce the coat protein gene effect, or an alternative method to overcome particular problems such as target virus sequence variability.

Nonconventional Approaches to Develop Resistance to Tomato Spotted Wilt Virus

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In the past few years, numerous outbreaks of tospoviruses have been reported in many crops and ornamental plants. In most cases, the outbreaks are caused by isolates of tomato spotted wilt virus (TSWV), the type species in the newly created genus Tospovirus within the arthropod-borne Bunyaviridae. Several other distinct and possible virus species within this genus have been reported recently, e.g., impatiens necrotic spot virus (INSV, exclusively in ornamentals), and there are indications that groundnut can host at least three different tospovirus species, i.e., groundnut bud necrosis virus (GBNV) and groundnut yellow spot virus (GYSV) (both reported from India) in addition to TSWV.

We determined complete sequences of all three genomic RNA segments of TSWV. The larger (L) RNA is 8897 nucleotides long and entirely of negative polarity, encoding the 331.5 kD viral polymerase ("transcriptase") in the viral complementary (vc) strand. The two other RNA segments are ambisense, each containing a gene in the viral (v) strand and another in the vc strand. The medium (M) RNA comprises 4821 nucleotides and encodes a common 127.4 kD precursor to both envelope glycoproteins Gl and G2 and a nonstructural protein, NSm, of 33.6 kD. The S RNA (2916 nucleotides) specifies the nucleocapsid (N)

protein of 28.8 kD and a second nonstructural (NSs) protein of 52.4 kD. Detailed molecular knowledge on the TSWV genome, and the cloning of all viral genes, have facilitated investigation as to whether these genes could be used to create engineered resistance to TSWV. Transformation of tobacco with the gene encoding the viral N protein indeed leads to high levels of resistance. Strikingly, prior introduction of a frame-shift mutation, and removal of the ATG translational start codon of this virus gene did not significantly influence the resistance levels obtained in transgenic plants, indicating that the resistance observed is primarily RNA-mediated. Further, transgenic tobacco plants are also resistant to inoculation using viruliferous thrips, *Frankliniella occidentalis,* one of the most important natural vector species. This implies that the engineered resistance is also operational under natural inoculation conditions. The transgenic plants are only protected to isolates and strains of TSWV and not to other tospoviruses, although sharing considerable (= 80%) nucleotide sequence homology in their N genes to TSWV. It is concluded therefore that the approach used here for TSWV can be successfully extended to other tospoviruses, but only if homologous N genes are used for transformation of the crop to be protected.

Nonconventional Approaches to Develop Resistance to Groundnut Rosette Disease

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The symptoms of groundnut rosette disease in Africa are caused by a satellite RNA which depends on groundnut rosette virus (GRV) for its multiplication. Both the satellite RNA and GRV depend on a luteovirus, groundnut rosette assistor virus (GRAV), for transmission by *Aphis craccivora.* Moreover, the GRAV-dependent transmission of GRV does not occur in the absence of the satellite RNA. Different forms of rosette disease (e.g., green, chlorotic, etc.) are caused by different variants of the satellite RNA. Some mild variants cause few or no symptoms in groundnut; but protect the plants against the expression of disease symptoms by virulent ones. No natural resistance to GRAV is known in groundnut but natural resistance (not immunity) to GRV, and therefore to the satellite RNA, occurs in some long-duration cultivars and seems to be determined by two recessive genes. This form of resistance is difficult to introduce into short-duration cultivars.

GRV does not seem to produce virus particles, its RNA becomes packaged in the coat protein of GRAV in mixed infections, to produce particles transmissible by *A. craccivora.*

The size of the genomic RNA of GRAV is 6 kb, that of GRV is 4.5 kb, and that of the satellite RNA is 0.9 kb. The nucleotide sequences of several variants of the satellite RNA have been determined; all possess an open reading frame of 372 nucleotides that could encode a protein of 124 amino acids.

Nonconventional approaches to developing groundnut plants resistant to rosette disease could be made by transforming them with regions of nucleotide sequence from any of the three agents involved. The most obvious approach is to use sequences derived from the coat protein gene of GRAV. Another is to use sequences derived from mild forms of GRV satellite RNA: plants transformed with these sequences might be protected against infection with, or the expression of disease symptoms by virulent forms of the satellite. Other approaches would involve the replicase genes of GRV or GRAV, or any other genes that may yet be discovered by sequencing these molecules.

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Protoplast Regeneration and Transformation of Wild *(Arachis paraguariensis)* and Cultivated *(A. hypogaea)* Groundnuts with Peanut Stripe Virus Coat Protein Gene (PStV-CP)

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Efficient plant regeneration from protoplasts of a perennial wild groundnut *(Arachis paraguariensis)* has been achieved using a nurse culture technique. Protoplasts isolated from cell suspensions were immobilized in agarose beads and co-cultured in MS medium with actively growing nurse cells derived from a long-term suspension culture of the same species. Approximately 20% of the protoplasts formed microcalluses within 3 weeks. Protoplast regeneration frequency was affected by the plating density and by the length of nurse culture duration. Optimal plating efficiencies were obtained from protoplast cultures with a plating density of 2×10^4 protoplasts mL^{-1} or from protoplasts co-cultured with

nurse cells for 14 days. Multiple shoots and shoot primordia were produced from protoplast-derived calluses, 40-50 days after transfer to the regeneration medium. Protoplasts isolated from leaf-derived calluses and immature cotyledons of 12 cultivated groundnut *(A. hypogaea)* genotypes were also co-cultured with *A. paraguariensis* nurse cells and produced plating efficiencies ranging from less than 1% to 20%. Protoplast-derived calluses from two cultivars produced shoot primordia and shoots at low frequencies.

Using the established protoplast regeneration system, two plasmids, one containing the hygromycin resistance gene *(hph)* and the B-glucuronidase gene (GUS); the other containing the *hph* gene and the PStV-CP gene, were introduced into protoplasts of A. *paraguariensis* and *A. hypogaea* by treatment with polyethylene glycol (PEG). In both plasmids, the expression of all three genes was under the control of the cauliflower mosaic virus 35S promoter. Hygromycin-resistant colonies were identified by selection on 20 mg L^{-1} hygromycin B for 10 days. An apparent transformation frequency of up to 5% of the protoplast colonies selected was obtained. The expression of the GUS gene, as determined by histochemical staining, indicated the genetic transformation in the majority of hygromycin-resistant colonies. DNA analysis is in progress to further confirm the integration of the transferred genes into the groundnut genome. The successful regeneration of transgenic groundnut plants will allow the evaluation of their resistance to PStV infection after introduction of the viral coat-protein gene.

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Cloning and Expression of the Capsid Protein Gene from a Blotch Isolate of Peanut Stripe Virus[1]

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The 3' terminal 1367 nucleotides (nts) of a blotch isolate of the potyvirus, peanut stripe virus (PStV), were cloned and sequenced as a step toward developing coat protein mediated protection in *Arachis hypogaea* L. This region included the 861 nts (287 amino acids) of the PStV capsid protein (CP) gene. Subclones containing the coding sequences of the CP gene (amino acids 1-287, 17-287, 17-113, and 106-287) were constructed. Potyviral CPs result from proteolytic processing of a larger polyprotein; therefore each PStV CP sub-

1. Paper presented by J.W. Demski, University of Georgia, Griffin, USA. on behalf of the authors.

clone was engineered with an initiator methionine at the amino terminus. Expression of the expected CP polypeptide in *Escherichia coli* for each construct was demonstrated by Western blot analysis using polyclonal and monoclonal antibodies against PStV CP.

Nicotiana tabacum cv Xanthi was transformed using *Agrobacterium tumefaciens* (strain LBA 4404) containing the binary vector pGA 482 into which the PStV CP subclones had been litigated. R_O transformants were tested for marker gene (NPT II) expression by enzyme-linked immunosorbent assay and for the expression of the expected PStV CP polypeptide by Western blot analysis. Selected transgenic lines were selfed and the resulting R_1 generation tested for segregation of the marker gene. Segregating F_2 populations of transgenic tobacco lines expressing portions or all of the PStV CP gene are being challenged with potato virus Y (PVY), tobacco etch virus (TEV), and peanut mottle virus (PMV) to determine the level of heterologous virus resistance of these plants. *Nicotiana benthamiana* has also been transformed with the entire PStV CP gene and will be challenged with PStV to test for resistance.

Nonconventional Approaches to Develop Resistance to Peanut Stripe Virus

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This project arises as a direct result of two other Australian Centre for International Agricultural Research (ACIAR) projects on groundnut improvement in Indonesia. These projects identified the cause of the blotch disease of groundnuts in Indonesia as peanut stripe virus (PStV), quantified its effects, and attempted control by host-plant resistance through conventional means. No sources of resistance to PStV were found when 10 000 accessions from the ICRISAT *Arachis hypogaea* world germplasm collection were screened in Indonesia.

Genetic engineering offers the opportunity to improve existing cultivars by selectively adding specific new traits such as virus resistance. The most successful strategy to date for the control of plant viruses is the expression of viral coat protein genes in transgenic plants. Significant progress has been made in the development of gene transfer systems that allow stable introduction and expression of foreign genes in plants, including legumes, using gene transfer mediated by either *Agrobacterium* or DNA-coated microprojectiles.

The principal aim of this project is to generate genetically engineered transgenic groundnut plants resistant to peanut stripe virus. To achieve this, we intend to propagate

and purify a single local-lesion isolate of PStV from Indonesia and clone its genomic RNA. Complementary DNA clones harboring the viral coat protein gene will be identified and the nucleotide sequence determined. A translational start codon and regulatory sequences for gene expression in the plant host will be introduced into the coat protein gene construct In parallel, a gene transfer and plant regeneration system for the commercial Indonesian groundnut cultivar 'Gajah' will be developed using marker genes. Firstly, embryo culture and the parameters for microprojectile bombardment will be optimized, to give stable transfer into groundnut cells suitable for regeneration. Secondly, a sensitive, nontoxic assay using luciferase reporter genes will be developed to facilitate regeneration of uniformly transformed groundnut plants from single transformed cells.

Once developed, the techniques will be used to transfer the gene controlling synthesis of PStV coat protein into groundnut cells, and to regenerate transformed plants. Transformed plants and their progeny will be assayed for the expression of the viral coat protein gene and evaluated for PStV resistance. Scientists from Indonesia will be trained in molecular biological techniques to enable them to undertake evaluation of the F_2 progeny of virus-resistant lines that will be evaluated under field conditions in Indonesia. They will challenge the plants with natural and/or artificial epidemics of the virus, and replication of the virus will be analyzed.

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Nonconventional Approaches to Develop Resistance to Peanut Clump Virus: West African Isolate

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Regular surveys carried out in Senegal, Burkina Faso, and Mali from 1986 to 1991 enabled us to demonstrate that peanut clump virus (PCV):

- had a distribution in Senegal from the River Senegal to the Gambian border,
- was particularly common on agricultural research stations, and occurred frequently in breeders' trials,
- was found to naturally infect other cultivated plants such as sugarcane *(Saccharum officinarum),* sorghum *(Sorghum bicolor),* and maize *(Zea mays),* and
- caused a wide range of symptoms in groundnut from the classical stunted plant with dark green leaves (the "super clump") on one hand, to normal-sized plants with a different kind of line pattern on the other.

The widespread distribution of the disease, the high levels of seed transmission of the virus (up to 30% in some assays), and the fact that the disease is soilborne, indicate the importance of this virus in Africa.

In view of these new data, and following an external review of the Institute in 1990, it was decided for the next 5 years of research to provide l'Institut des recherches pour les huiles et oleagineaux (IRHO) with the required resources for future groundnut breeding. Some new approaches need to be developed to provide future tools for breeders, such as in vitro culture techniques to overcome problems in gene transfer. This should assist cell biologists, virologists, and pathologists to undertake research aimed at incorporating resistances to fungal, viral, and nematode diseases into agronomically acceptable groundnut cultivars.

It was decided to study PCV variability at the genome level as the first step in a program of groundnut transformation to induce resistance to PCV. This is being done in collaboration with l'Institut de biologie moleculaire des plantes (IBMP) - Centre national de la recherche scientifique (CNRS) laboratory in Strasbourg. Nucleic acids were purified and characterized. Of three isolates studied, one showed deletion in RNA 2 (1.1×10^6 instead of 1.2×10^6 daltons) and the second isolate was cloned. cDNA and riboprobes were obtained and 90% of the RNA 2 was sequenced. Only the 5' end has not yet been sequenced. cDNA probes are now being made to study variability among PCV isolates.

Nonconventional Approaches to Develop Resistance to Indian Peanut Clump Virus

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Peanut clump disease in India is caused by the Indian form of peanut clump furovirus (IPCV). The disease can cause significant crop losses. Distinct isolates of the virus which are only weakly related serologically and by nucleotide sequence homology occur in different parts of India. These isolates of IPCV are only very distantly related to the West African form of peanut clump virus. No genotype of groundnut resistant to IPCV infection was found among the several thousands tested.

The genome of IPCV comprises a 6 kb RNA 1 and a 3.5-4 kb RNA 2 which encodes the coat protein. Complementary DNA copies of IPCV RNA have been cloned in the plasmid pUC19, and from a library of 500 clones, about one-third were specific for RNA 2. The largest of these were arranged by restriction endonuclease mapping to form a contiguous sequence of about 2.5 kb. Further cloning is in progress to extend this sequence to cover the entire RNA molecule. The cDNA map was orientated with respect to RNA 2 by determining which of the two oppositely orientated M 13 ssDNA subclones of part of the cDNA hybridized with IPCV RNA in Northern blots. Sequencing of about 80% of the mapped cDNA has identified parts of two open reading frames and further sequencing is in progress to complete these sequences.

Cloned cDNA representing about 70% of the RNA 1 molecule has also been obtained. The coat protein gene from RNA 2 has obvious potential as a source of material to transform groundnut tissue and to induce resistance to IPCV. The other gene or genes in RNA 2, and sequences from RNA 1, are also potential sources of resistance genes for future work for the diagnosis of IPCV isolates.

Plenary Session

Chairperson: R. Goldbach
Co-chairperson: J.R. Witcombe

Summary: Potentials and Prospects for the Transformation and Regeneration of Groundnut

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Progress in three major techniques to transform plants - *Agrobacterium,* particle bombardment, and protoplast transformation - has been presented at this meeting. Expression of genes in the treated tissues has been reported, but there is no evidence yet that they have been incorporated, nor have transgenic plants been produced from the treated tissues.

Agrobacterium-mediated transformation has resulted in GUS activity in callus 2 weeks after inoculation, but plants have not been produced. As yet particle bombardment of immature leaflets has only given transient expression, but there is hope that bombarded apical domes will produce stably transformed plants. Immature cotyledons, a good source of somatic embryos, have been bombarded and GUS activity observed.

The new initiative on protoplasts has produced plants from protoplasts, and has also shown GUS activity in protoplasts.

For all these systems there is a need to verify incorporation of foreign genes, and to improve transformation protocols to give maximum levels of incorporation and expression.

There is also a need to increase efficiency of regeneration. A wide range of explants have been tried, but there are genotypic differences in response. The development of the technique of bombardment of apical domes may overcome this difficulty, but if transformation is to become a routine part of crop improvement programs, the technique must be applicable to all genotypes, repeatable, and cost effective.

This meeting has emphasized resistance to viruses, but there are other constraints to crop production and quality that could be approached in a similar way. These include *Aspergillus flavus* and aflatoxin contamination, insect pests, and abiotic stresses. The nutritional and keeping qualities of the seed could also be addressed. New varieties produced using these techniques would be of value in developing countries, and breeders must be involved in the production and evaluation of transgenic plants. All research must be carried out under suitable containment facilities, with due regard to the regulations of the country in which it is conducted.

Summary: Genes with Potential for Utilization in Transformation

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Some themes emerged during presentations on the progress towards using virus genes for the transformation of groundnut to induce resistance to virus infection.

The viruses being considered as targets for transgenic resistance are diverse. They include; peanut stripe (PStV) and peanut mottle (PMV) potyviruses, West African (WAPCV) and Indian peanut clump (IPCV) furoviruses, tomato spotted wilt tospovirus (TSWV), peanut stunt cucumovirus (PSV), groundnut rosette virus (GRV) (proposed umbravirus group), and groundnut rosette assistor luteovirus (GRAV). These are from six genera in five or more of the proposed or accepted families of viruses and thus represent a wide diversity in mode of genome expression.

Several strategies are being considered to obtain virus-resistant plants. Transformation is planned with coat protein genes (PStV, PMV, WAPCV, IPCV, GRAV), satellite-encoding sequences (GRV), a nucleocapsid gene (TSWV), and a sequence encoding a defective interfering RNA (TSWV). The coat protein gene of PStV has been assembled in a transformation vector, other constructions are yet to be made.

Variation among isolates of PStV, WAPCV, and IPCV suggests the danger of developing a form of resistance which would not be effective against all strains of a virus. The use of two genes against a single virus, possibly operating in different ways, should be considered for such viruses.

The question of the validity of experimentation with *Nicotiana* and other readily transformed model systems to test virus genes for efficacy was contested. It is necessary for experimenters to be especially persuasive with donors if they wish to obtain support for such experiments.

Most work has been, or is, directed toward making transformation vector constructions using the 35S promoter from cauliflower mosaic virus. Other promoters may be important in obtaining expression only in certain tissues, such as roots. Also, when transformation involves inserting two genes at the same time, the use of different promoters may be useful to inhibit the loss of one of the transgenes by homologous recombination. Promoters analogous to the 35S promoter from caulimoviruses that infect legumes, or groundnut itself, are potential candidates.

Attention should be paid to biosafety aspects of the particular gene and vector construction used for a transformation. The possibility of mutation of a satellite sequence to yield a molecule with virulent rather than ameliorative effects, and the possibility of using transgenically expressed coat protein encapsidating and thus enabling transmission of pathogens such as GRV should be considered. Minor modifications of the vector constructions might be enough to overcome any such effects.

Recommendations

The participants decided to establish a Working Group on transformation and regeneration of groundnut and utilization of viral genes to induce resistance to virus diseases. The Working Group agreed to:

- invite participation by other concerned scientists as appropriate,
- request ICRISAT's Legumes Program to coordinate the activity of this group,
- interact through informal exchange of information and material and by means of reports to the International *Arachis* Newsletter and Peanut Research,
- provide information on ongoing projects to members and to potential donors,
- assist members and donors in identification, formulation, and conduct of projects,
- encourage full cooperation and communication with organizations and governments involved in testing transgenic plants,
- encourage continuation and further collaboration between members,
- further the use of biotechnology to produce groundnuts resistant to viruses,
- seek funding to support its activities, and
- meet at least once in 2 years.

Participants at the meeting (left to right): A. Kumar, R. Goldbach, D.V.R. Reddy, J.R. Witcombe, R.G. Dietzgen, D. McDonald, J.M. Dollet, A.F. Murant, J.P. Moss, M.A. Mayo, J.A. Schnall, J.W. Demski, and P. Ozias-Akins.

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