Transgenic in Pulse Crops: Present Scenario and Future Strategies

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Abstract

Transgenic techniques have made it possible to transfer any desirable gene from any organism to a plant species. Use of molecular biology techniques in combination with novel gene transfer technology has broadened the gene pool and crossed sexual crossability boundaries. Efforts are underway to produce transgenics having resistance genes to a variety of insect-pests and diseases using *Agrobacterium*-mediated gene transfer or by direct DNA transfer methods. Transgenics possessing resistance to insect-pests have been demonstrated in plants expressing insecticidal genes such as δ endotoxins from *Bacillus thuringiensis* (Bt), protease inhibitors, enzymes, secondary plant metabolites and plant lectins. Bt genes have been deployed in several crops on global basis while other alternative genes have received considerably less priority. While cereals, vegetables and commercial crops have received greater importance in developing transgenics, pulse crops like chickpea, pigeonpea, lentil, pea, cowpea, *etc.*, have received considerably less attention. Keeping in view the regional importance of these crops, there is urgent need for development and deployment of transgenics for management of *Helicoverpa armigera*. In this review current status of transgenics in pulses, particularly dealing with biotic stresses, has been discussed.

1. Introduction

The major pulse crops under cultivation and consumption include chickpea, pigeonpea, pea, lentil, mungbean, urdbean, common bean and cowpea. Their yield levels are not very encouraging and often limited by a number of biotic and abiotic stresses. The present production of pulse crops is 13-14 million tons. The present Indian population of about 1015 million is expected to rise to 1350 million by 2020. Keeping in view the dietary requirement of proteins, a minimum of 27 million tons of pulses is required by 2020. This can only be achieved by expanding area under pulses, raising yield genetically and minimizing the losses caused by biotic and abiotic stresses. Pulse crops are infested by many diseases and insect-pests (Table 1). Conventional methods of pulse improvement have paid little dividends in developing insect-pest resistant varieties basically because of unavailability of suitable donor parents and efficient screening techniques. Besides, chemical method of insect-pest control is also polluting environment and disturbing ecological balance. Hence, the development of

Table 1: Major biotic stresses identified in pulse crops

Crops	Diseases	Insect-pests
Chickpea	*Fusarium* wilt, *Ascochyta* blight	Pod borer, bruchids
Lentil	Rust, *Fusarium* wilt	--------
Pea	Powdery mildew, rust	Pod borer
Pigeonpea	*Fusarium* wilt, *Phytophthora* blight	Pod borer, pod fly
Mungbean	Mungbean yellow mosaic virus, *Cercospora* leaf spot	Storage pests
Urdbean	Mungbean yellow mosaic virus, *Cercospora* leaf spot	Storage pests

genetically resistant/tolerant varieties through biotechnological means is the only viable, economic and eco-friendly approach.

2. Biotechnological Approaches

Rapid advances in recombinant- DNA technology and plant transformation methods have allowed introduction of any foreign gene from any source into almost any plant species. It is now possible to genetically manipulate the plant species as per requirement. Agricultural biotechnology has greater potential to improve crop productivity, to decrease dependency on harmful chemicals such as pesticides, antibiotics and fertilizers and also complements the traditional breeding approaches. To break undesirable genetic linkages or to assemble desirable genes in specific population through the traditional breeding is a laborious and time-consuming process. In addition, the chances of success are also quite unpredictable. With these conventional methods, the available gene pool is further restricted by the sexual incompatibility of many interspecific and intergeneric crosses (Nisbet and Web, 1990). The new transgenic technology has substantially broadened the gene pool, and has allowed the transfer of genes governing well-defined traits. The transgenic development requires availability of routine regeneration and transformation system, suitable gene constructs, recovery and multiplication of transgenic plants, molecular and genetic characterization of transgenic plants for stable and efficient gene expression, and evaluation of transgenic plants with respect to specific stress under the controlled environmental conditions.

Various methods are routinely used for introducing genes into a wide range of crop plants (Potrykus,1991; Dale *et al.*,1993). Early attempts were made towards the development of *Agrobacterium*-based vector for the introduction of exogenous DNA into the host genome. *Agrobacterium* contains a plasmid, called the Ti-plasmid, part of which gets integrated into a plant cell's DNA upon infection. This bacterium, which is capable of inserting new DNA into the host cell, is a natural genetic engineer.

Biotechnologists alter the Ti-plasmid to contain the desired gene which they wish to incorporate into the host plant cell. When this bacterium infects the plant cell, the new gene is incorporated into the genome of the recipient cell. Thus, altered plant cells are grown into a complete plant. In this way, crops can be developed that are naturally resistant to insect-pests.

Among various biotic and abiotic stresses, gram pod borer (*Helicoverpa armigera* Hubner) is the most important. Helicoverpa affects wide variety of crops. It is very widely distributed across the country. Extensive damage by Helicoverpa has been reported in cotton, pigeonpea, chickpea, sunflower, and vegetables. In tropics, total crop losses due to Helicoverpa may exceed US $ 2 billion annually and the cost of insecticides used to control *H. armigera* is estimated to be over US $500 million per year (Sharma, 2001).

There are many insecticidal proteins and molecules, which can be safely expressed in transgenic plants for insect control. Some examples are protease inhibitors, lectins, α-amylase inhibitors, enzymes such as chitinase and peroxidase and δ-endotoxins of *Bacillus thuringiensis* (*Bt*). Some efforts have been made to develop transgenic legume plants by inserting a gene from *Bacillus thuringiensis* (*Bt*). The gene produces a protein (δ-endotoxin) that is lethal to many lepidopteran insects. Since the specific binding sites and appropriate pH are not found in any other insects or animals midgut, the protein is harmless to them. The use of Bt is environment friendly and avoids the use of chemical pesticides. Similarly, non-Bt insecticidal proteins are also being used to develop transgenic legume plants.

3. Present Status

Development of transgenic technology has shown promises in mitigating many biotic stresses. Many transgenic varieties have already been developed. The major target insect-pests, which require priority in pulses, are listed in Table 1. Various genes are now being deployed in legume crops. The development of transgenic and expression of insecticidal resistance genes in crop plants has emerged as one of the potential methods to control insect pest. Genes from *Bacillus thuringiensis, Bacillus sphaericus,* protease inhibitors, and plant lectines have been used alone or in combination with other conventional host plant resistance to develop crop specific cultivars to provide a high level of resistance against a range of insect-pests (Hilder and Boulter, 1999; Kumar, 2003). Insect resistant Bt-transgenic crops were first grown commercially in 1996 (Krattiger, 1997). Since then the area under Bt–crops has increased steadily. In 1999, an estimated 26% of corn and 32% of cotton (Carpenter and Gianessi, 2000) grown in USA contained insecticidal protein derived from Bt. While such transgenic crops have considerable advantages both for environment and for biological safety,

transgenic plants to be successful in IPM, they have to substitute completely or partially for the use of insecticides in crop production.

4. Genes with Insecticidal Activities

4.1 *Bt* Insecticidal Proteins

Bt has been the most commonly used gene for developing insect resistant varieties. These crystal proteins are highly toxic to specific insects, mites, nematodes, flatworms or protozoans (Fietelson *et al.*, 1992). Bt has a wide insecticidal spectrum ranging from *Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga* and extending up to nematodes, mites and protozoa (Kumar *et al.*,1996; Schuler *et al.*,1998). Bt produces two different types of insecticidal activities which are agronomically important, the most widely known one is being called δ-endotoxin or insecticidal crystal protein (ICP).

The ICP usually exists as protoxin of high molecular weight (135-138 kDa). Upon ingestion by insect larvae these are proteolytically processed into small molecular weight toxin in highly alkaline midgut of the larvae.The toxin molecules bind to specific receptor present in the membranes of midgut epithelium and cause pore formation. This causes disruption of the electrical, K+ and pH gradients across the membrane leading to the death of larvae (Kumar *et al.*, 1996). The presence of specific receptors in the insect classes is what determines the lack of activity of Bt δ–endotoxins towards mammals and other organisms including beneficial insects. Genetic engineering has facilitated stable expression of Bt genes in crop plants with considerable success (Schuler *et al.*, 1998).

With the development of molecular methods for transfer of specific genes and their expression in the new host species since 1983, there is a major interest in developing transgenic plants. Transgenic technology that involves insertion of foreign DNA sequence has tremendous potential for improvement of legume plants. Both *Agrobacterium* mediated and direct gene transfer methods have been used (Table 2). All the major pulse crops can be infected by wild type *Agrobacterium* and produce tumors. So far, most of the transformation in pulse crops are limited to the transfer of marker genes, except in soybean and forage legumes. In most cases the reports do not indicate regeneration of transgenic plants and/or inheritance of the transferred gene(s). Efforts are on to develop transgenic plants resistant to pod borer in chickpea and pigeonpea using Bt crystal protein gene. Early results obtained at various centres in the country are very encouraging. In chickpea few reports are available on genetic transformation. Transformed callus was obtained using wild strains of *Agrobacterium* at Indian Agricultural Research Institute, New Delhi. Besides, transformed chickpea

Table 2: Genetic transformation of pulse crops

Crop	Type of vector	References
Vigna spp	*Agrobacterium tumefaciens*	Muthukumar *et al.* (1996)
Pigeonpea	*Agrobacterium tumefaciens*	Lawrence and Koundal (2001)
Chickpea	*Agrobacterium tumefaciens*	Srinivasan (1991), Kar *et al.* (1996), Krishnamurthy *et al.*(2000),
Chickpea	Gene gun	Kar *et al.* (1997)

plants possessing Cry1Ac construct known for resistance to *Helicoverpa armigera* was also reported from the same centre. However, inheritance of the transgene was not demonstrated in these studies. There is one recent report on chickpea transformation from National Chemical Laboratory (NCL), Pune. This demonstrates transmission of a recombinant gene to the progeny for the first time. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad has already made substantial progress towards development of *Bt* transgenic chickpea and pigeonpea. Besides, GB Pant University of Agriculture and Technology, Pantnagar and National Botanical Research Institute (NBRI), Lucknow are also conducting research work in the same direction. At Indian Institute of Pulses Research (IIPR), Kanpur, transformed callus and plantlets possessing *npt*II gene have been obtained through *Agrobacterium* mediated transformation. In the case of pigeonpea, transformed callus and plantlets possessing foreign genes have been reported from various institutes such as IARI, IIPR, NBRI, NCL and Bose Institute.

Grain legumes are one of the least amenable groups to transformation amongst dicotyledonous crops, although they are usually susceptible to *Agrobacterium* infection. Important parameters for successful transformation of grain legumes include characteristics of the *Agrobacterium* strain used for inoculation of target plant tissues, the vectors, which the bacterial strain carries, the co-cultivation period and a selection system. Although most efforts have centered upon the use of *Agrobacterium* for introducing genes into grain legumes, there are also reports of the use of biolistics. Shoot apical meristems of mature seeds or whole embryos have been used extensively as target tissues for direct gene transfer by particle bombardment in *Glycine max, Phaseolus vulgaris* and with limited success in *Vigna* species. In the majority of the cases, explants from near the shoot apex or the apex itself have been the targets. Apical meristems permit rapid multiple shoot production with minimum tissue culture compared with other types of tissues. More importantly, the genotype has less influence on plant regeneration. The transformation frequency in case of biolistics is usually low compared to *Agrobacterium*-mediated gene transfer, however, it has been reported

that particle bombardment may be the preferred option for gene introduction into large-seeded grain legumes, circumventing the host specificity of many grain legumes to infection by *Agrobacterium*.

Although many insecticidal genes have been transferred to different crop species, the most satisfactory system in terms of field resistance is the one based on Bt. Bt toxins have been expressed in at least 30 different plant species (Schuler *et al.*, 1998). However, the level of resistance they confer depends on whether native (wild type) or truncated, modified genes have been used (Kumar *et al.*, 1996). The prokaryotic codon used in Bt genes needs to be modified towards that of higher plant genes. In addition, features that can destabilize the transcripts in higher plant cells need to be removed. As of today, three Bt–transgenic crops are under commercial cultivation (De Maggad *et al.*, 1999). Bt insecticidal proteins have been expressed in soybean, alfalfa and peanut for resistance to their respective pests. A native *Cry1Ac* gene has been expressed in chickpea to confer protection against *Helicoverpa armigera* (Kar *et al.*, 1997). Development of pod borer larvae was affected when fed on transgenic tissues. A synthetic gene encoding *Cry1Ac* toxin was introduced in soybean by particle bombardment and the transgenic plants were observed to be resistant to corn earworm (*Helicoverpa zea*), soybean looper (*Psuedoplusia includens*) and velvetbean caterpillar (*Anticarsia gemmatalis*) (Stewart *et al.*, 2001). Similarly, a synthetic *Cry1Ac* gene was transferred to alfalfa for resistance to *Spodoptera littoralis* (Strizhov *et al.*, 1996). The transgenic plants produced Bt-IC to the extent of 0.01-0.2% of total soluble protein and were resistant to cotton leaf worm and beet army worm. Transformation of peanut by a synthetic *Cry1Ac* gene resulted in various levels of resistance to the lesser corn stalk borer, from complete larval mortality to a 66% reduction in larval weight (Singist *et al.*, 1997).

4.2 Protease Inhibitors

Currently, there are two major groups of plant-derived genes used to confer insect resistance on crops: inhibitor of insect digestive enzymes (proteinase and α-amylase inhibitors) and lectins. Plant protease / proteinase inhibitors are polypeptides or proteins that occur naturally in a wide range of plants and are a part of the plants natural defence system against herbivores. However, these proteins are expressed at very low levels in their native state. Hyper-expression of proteinase inhibitors in transgenic plants would lead to significant levels of insect protection. Fourteen different plant proteinase-inhibitor genes have been introduced into crop plants. Table 3 exhibits the list of legume crop species transformed with different proteinase inhibitor genes.The most active inhibitor identified to date is the cowpea trypsin inhibitor (CpTi), which has been transferred to ten different crop species. Experiments with transgenic plants

Table 3: Insect resistant legume crops

Crop	Target pest	References
Bt insecticidal protein *Cry1Ac*		
Soybean	*Helicoverpa zea*	Stewart *et al.* (2001)
Peanut	Lesser corn stalk borer	Singist *et al.* (1997)
Chickpea	*Helicoverpa armigera*	Kar *et al.* (1996)
Alfalfa	*Spodoptera littoralis*	Strizhov *et al.* (1996)
Proteinase inhibitors		
Pea	*Helicoverpa armigera*	Charity *et al.* (1999)
Alfalfa	*Spodoptera litoralis*	Narviez-Vasquez *et al.* (1992)
Alfalfa	*Frankliniella spp.*	Thomas *et al.* (1994)
Bean amylase inhibitor		
Pea	*Callosobruchus maculatus*	
Pea	*Bruchus pisorum*	Shroeder *et al.* (1995)
Adzuki bean	*Callosobruchus maculatus*	Ishimoto *et al.* (1996)

and artificial diets have sown that CpTi affects a wide range of Lepidopteran and Coleopteran species (Gatehouse and Hilder, 1994). The serine-proteinase inhibitors (from soybean) when expressed in transgenic tobacco and potato resulted in considerable larval mortality of *Spodoptera littoralis*. In addition to serine-proteinase inhibitors, one cysteine-proteinase inhibitor from rice has been introduced into several other crops. Recently, a gene encoding multi-domain proteinase inhibitor precursor was expressed in transgenic pea under the control of Rubisco small subunit promoter (Charity *et al.*,1999). Insect feeding trials have shown that the mortality of *Helicoverpa armigera* larvae was high as compared to controls. Protease inhibitor from insects have also been expressed in plants. In *Manduca sexta* (Tobacco hornworm), several protease inhibitors are present in hemolymph. One of these proteins inhibits the activity of the enzyme elastase. Expression of this inhibitor in transgenic alfalfa has resulted in reduced thrips *(Frankliniella spp.)* infestation.

Production of insect resistant transgenic pea plants was first reported by Puonti-Kaerlas *et al.* (1990) using *Agrobacterium* as a vector. The first *Agrobacterium*-mediated transformation in lentil was reported by Warkentin and McHugan (1992). Transgenic plants have been reported for enhanced resistance against predators by expression of enzyme inhibitors. Bean α-amylase inhibitors derived from *Phaseolus vulgaris* was found to be effective against *Callosobruchus maculatus* in pea. Later

this enzyme inhibitor was also reported effective against *Bruchus pisorum* and *Callosobruchus chinensis* (Shroeder *et al.*, 1995). Similarly, tobacco proteinase inhibitor showed enhanced resistance against *Helicoverpa armigera* in transgenic peas (Charity *et al.*, 1999).

4.3 Amylase Inhibitors

Inhibitors of α-amylases are the second type of enzyme inhibitors used to modify crop plants. α-amylase inhibitor from the common bean (*Phaseolus vulgaris*) forms a complex with and inhibits α-amylases in the midgut of coleopteran and storage pests of the genera *Callosobruchus* and blocks larval development (Ishimoto *et al.*, 1996). Genes for three α-amylase inhibitors have been expressed in pea and Adzuki bean. The gene encoding α-amylase inhibitor under the control of a seed specific promoter in peas showed significant level of insect protection. Similar results were also obtained when Adzuki bean was transformed with α-amylase inhibitor gene and tested for protection against bruchid beetles (Ishimoto *et al.*,1996). It would be very useful if α-amylase inhibitor genes are expressed in pulses like chickpea and pigeonpea which suffer from losses due to variety of storage pests.

4.4 Lectins

Lectins are carbohydrate-binding proteins, some of which are toxic to insects. Various lectins have shown some toxicity against species of the insect order Homoptera, Coleoptera, Lepidoptera and Diptera. The mode of action of lectins against insects remains unclear, but it has been shown that at least some binds to insect midgut epithilium cells. However, some insecticidal lectins also show significant mammalian toxicity, including lectins from *P. vulgaris*, winged bean, soybean and wheat germs. Other lectins, for example those from pea and snowdrop, have demonstrated insecticidal activity and are innocuous to mammals (Gatehouse and Hilder, 1994).

Lectins from snowdrop *(Galanthus nivalis)* have been shown to be very effective against aphids and rice brown planthopper (Powell *et al.*,1995). It has been expressed in nine different crops including potato and tomato. Laboratory tests with engineered potatoes showed that snowdrop lectin did not increase the mortality or development time of potato aphid but considerably reduced fecundity (Down *et al.*,1996). Results of experiments with potato peach aphid were similar, but in addition, the establishment of aphids on transgenic potatoes was reduced (Gatehouse *et al.*,1996). Snowdrop lectin also enhanced the resistance of potato to larvae of tomato moth (*Lacanobia oleracia*). The effect of snowdrop lectin is antifeedant rather than insecticidal (Gatehouse *et al.*, 1997).

4.5 Toxins from Predators

Spiders and scorpions produce powerful neurotoxins that have been expressed in transgenic organisms (Barton and Miller, 1991). Transgenic plants of tobacco have been developed containing an insecticidal spider peptide gene, and some of these plants have shown resistance to *H. armigera* (Jiang *et al.*,1996). The role of neurotoxins from insects and spiders needs to be studied in greater detail before they are deployed in other organisms and plants because of their possible toxicity to mammals.

5. Transgenic Resistance Management

In integrated pest management, host plant resistance is one of the main components. The main purpose of deployment of resistance genes in transgenic plant is to manage the insect pest population and to prevent the development of resistance in insects. The insect pest management strategies are intended to prevent or diminish the selection of rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently at low for insect control. Strategy development generally relies on theoretical assumption and on computer models simulating insect population growth under various conditions. The strategy includes the use of multiple toxins, crop rotations, high or ultra high dosages, and spatial or temporal refugia. The most promising and currently practical strategy is that of using refugia.This strategy reduces the possibility of resistant insects from mating with other resistant insects, thereby preventing the creation of a resistant population. This is achieved by ensuring that there is always plenty of susceptible insects nearby for a few resistant ones to mate with. The basic principle of high dose strategy is to deploy plants with high levels of expression of toxin with the expectation that it would take a long time for insects to overcome the toxins. It assumes that most or all resistance is recessive and that most resistance carriers would be heterozygous. A viable complementary strategy that is best adopted with the above two strategies is the deployment of multiple resistance or pyramiding of resistance genes. This strategy requires more than one resistance gene with different modes of action. It could be achieved either with additional vip protease inhibitor genes or with novel methods of insect resistance, but requires the use of refugia (Gould, 1998). Targeted expression is also complementary to the above described strategies and will become possible in the near future. A toxin gene is expressed only specifically in a certain vulnerable tissue/part of the plant or is expressed both in a certain part of the plant as well as at a particular critical time in the development of the plant. This strategy would allow plenty of susceptible insects to breed normally, thus increasing their predator and parasitic populations, while at the same time it is prevented from causing damage to the critical plant parts or life cycles.

One of the most important tools of resistance management is to apply integrated pest management principles in transgenic crop cultivation. Use of biological control methods (predators, viruses, fungi, *etc.*), botanical pesticides (neem and *Pyrethrum*), crop rotation and sanitation, and traditional methods coupled with minimal application of chemical insecticides will prolong the life of transgenic crops.

6. Future Prospects

Transgenic technology offers opportunity to avoid long gestation period to transfer the desirable genes in a suitable cultivar. Current methodologies are capable only to transfer single or a few genes. The transgenic approach also provides a better solution to the problem of sexual incompatibility of interspecific and intergeneric crosses. For commercial exploitation of this technology it is necessary that it should be economically viable, environmentally safe and easy to use in diverse ecosystems. It should also be harmless to the natural enemies and nontarget organisms. It is also expected that transgenic technology should have lower risk and greater benefits than currently used alternative technologies of insect-pest management.

It seems difficult to incorporate high level of resistance to insect-pests because of the constant development of the immunity in causal organisms. A high level of resistance can be incorporated by transferring the elite genes responsible for coding toxic substances to the insect-pests. *Bt* gene is the best example, which has been found very effective against harmful insect-pests in crops like cotton, tobacco, rice and many vegetable crops. Through traditional breeding it is difficult and time consuming to identify and isolate the desired genes in donors and transfer them to another cultivar by crossing.

The tremendous improvement has taken place in gene transfer techniques and many transgenic plants have been developed in cereals, oilseed crops, vegetables and commercial crops. However, a few legumes like soybean, *Phaseolus*, peanut, and alfalfa could be successfully transformed. Other legumes like pea, lentil, chickpea and pigeonpea still lag behind as far as successful gene transfer is concerned. Apart from the most important category of *Bt* toxin genes, legumes have also been transformed with genes encoding protease inhibitors, α-amylase inhibitor and lectins for insect resistance. However, novel insecticidal proteins and respective genes need to be identified and used in conjugation with Bt to prevent the development of resistant insects. Many grain legumes like pigeonpea and *Vigna* species are yet to be successfully transformed with insecticidal protein genes, though Bt endotoxin genes are available. These legumes are relatively recalcitrant, hence, procedure needs to be developed to improve their regeneration and transformation capacity. There is also urgent need for isolation, characterization and cloning of disease and insect-pest resistance genes from other plants and microbial sources.

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