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BIOTECHNOLOGY : MIRACLE OR MIRAGE TRANSGENIC PLANTS : PROGRESS AND POTENCE

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Contents

Summary

Transgenic plants lodging a foreign gene (transgene), incorporated into their genome have been produced by a variety of in-vitro means viz. indirect method using vector mainly *Agrobacterium* or direct method using either chemical transfer techniques i.e. $CaPO_4$ Co-precipitation, polycation DMSO, DEAE-dextran, polyethylene glycol, or physical transfer techniques i.e. electroporation, electrofection, microinjection, biolistics, laser microbeam, liposome fusion, silicon carbide fibre, sonication, protectifer, macroinjection. The foreign genes transferred confer a specific improvement to the resident genotype eg. resistance to pests, fungi, virus, insecticides, fungicides, weedicides, environmental stresses etc. without altering its genome.

Whereas, mjority of these transgenes have offered innumerable advantages to the humans, they pose some serious ethical, economical, technological and biological risks. In addition to narrowing the biodiversity, the transgenic plant cultivation will lead to gene wipeoff, genetic erosion and to the genotype-vulnerability to abiotic and/or biotic stresses. Thus many novel viral, fungal, insect strains having resistance to transgenic plants may arise. The resistance gene (s) may introgress into the genomes of wild weedy species by chance outcrossing. This will enhance wild weedy plant invasion into cultivated fields and make their elimination arduous. Stability of the transgene expression in some transgenics is low. Therefore, transgenic plants and their tailored-genomes are still on strict trial. Before releasing transgenic plants, thorough evaluation of risk assesments for both the humans, animals, plants as well as the environment are absolutely essential. This enhances the responsibility and accountability of the individuals and institutions releasing the transgenic plants and/or animals. Hence, stringent checks and controls are necessary before transgenics are released for commercial utility.

Keywords : Genetic transformation; Neo-species; Transgene incorporation, amplification and silence.

1. Introduction

Plant breeding started with nomadic agriculture, plant-domestication, cereal-selection, preferential-cultivation and intensive-domestication of plants. Whereas, selection of the best genotypes and their subsequent cultivation led gradually to the plant improvement, the rediscovery of Mendelian laws, the basis of variation and the development of recombination and the utilization of variation for plant improvement, led to the art, science and technique of useful plant-trait improvement the plant breeding. The early plant breeders remained principally interested in improving the genetic potential of crops in order to maximize economic gain per unit of input and land. Later on, the breeders goal was to improve plants to yield-better, to grow faster and to stay disease-, pest-, stress- and drought free or resistant. To achieve one or many of these gains, the breeders used hybridization, introgressions, nuclear and cytoplasmic mutations and substitutions, variation in chromosome numbers and/or form, and their combinations and recombination using breeding techniques like artificial crossings, back-crosses, mutagenesis, in-vitro cultures and cell fusions. Though the success in plant improvement has been phenomenal, no present day crops or other economically useful plants are perfectly and ideally suited to human needs[1]. Therefore, the traditional plant breeding moved to novel breeding, the molecular breeding which uses recombinant DNA technology, gene cloning, genetic transformation, protoplast fusion and in-vitro regeneration. Use of the gene vectors allows molecular breeders to remove peices of DNA from an organism, study its function and insert the gene into the currently grown elite cultivars and thereby genetically rectify the defect as well as improve the genotype. Another major advantage of the molecular breeding is that when a particular gene has been isolated and reconstructed, it can be first tested in model plants and later it can be used in a variety of cultivars of different crops. The molecular breeding which uses the methods and concepts of biotechnology has been able to improve agronomic traits and has produced plants with increased vigour and yield, high degree of tolerance or resistance to pests (insects, nematodes etc) diseases (virus, fungi, bacteria) or climatic stresses (drought, heat, cold, salinity etc.) Such production of genetically manipulated plants using one or more foreign genes the transgenics, is being profusely used and holds promise for the future. The transgenic plants are also used as an analytical tool to explore unique aspects of gene regulation and serve important focus for unifying the basic plant science research in plant breeding, pathology, biochemistry and physiology with molecular biology, as the production of transgenic needs the expertise of all these areas of life sciences. Due to its multiarea based foundation, a large number of transgenics have been produced and many have been released after stringent environmental and field tests. Increased productivity through resistance against pests and disease, *enhanced* efficiencey of photosynthesis and other physiological processes, improved nutritional and other qualities and improved resistance or tolerance to biotic and abiotic factors are

the major advantageous features the transgenics have. Details of these facets in transgenics research comprises the text of this paper. The description given reveals that the transgenics have greatly complemented plant breeding programs to meet the increasing demands of food production needed for the ever growing human population, especially in the developing countries.

2. Herbicide resistant transgenics (Table 1)

Herbicides, widely used in modern agriculture, are chemical compunds that kill or inhibit the growth of plants. But though basically applied to control weeds, they also have deleterious effects on crop plants. Selective and rapid breakdown are not always obtained and left-over herbicides applied to weeds before a crop is planted persist in the soil and decrease crop yield. A promising alternative approach is the development of herbicide tolerant plants for use with broad spectrum or totally non-specific herbicides. Three strategies have been adopted to obtain herbicide resistant plants[2-5], (i) herbicide target modification (ii) target enzyme overproduction (iii) herbicide detoxification.

2.1 Herbicide target modification

Herbicide targets are proteins and the action is two-fold; it either inhibits photosynthesis or amino acid biosynthesis. The most common herbicides used which inhibit photosynthesis are the triazines (atrazine and simazine). These herbicides block electron transport at PII by binding to the Qb protein present in the thylakoid membrane and encoded by the psbA gene of chloroplast (cp) DNA[6]. A single amino acid substitution (serine to glycine) at position 264 in the 32 kDa protein, results in decreased herbicide binding[7]. Triazine-resistant mutants having one altered amino acid have been identified from naturally occuring resistant weed biotypes or microbial species[8]. Manipulation of the resistant chloroplast genome by transfer through sexual hybridization or by developing a chloroplast transformation system are the possible ways to obtain atrazine resistant plants. Beversdorf[9] transferred atrazine resistant chloroplast from *Brassica campestris* to *Brassica napus* by back-crossing the resistant plant to the female parent of *B. napus*. After 8 backcross generations, the nuclear genome is almost isogenic. A faster approach was developed by Cheung *et. al*[10]. In this, tobacco cells were transformed by *Agrobacterium* vector harbouring a psbA gene encoding a triazine insensitive Qb protein, fused to the transit peptide of a nuclear encoded chloroplast protein. Transgenic plants showing increased tolerance to atrazine were produced.

Another group of herbicides - sulphonylureas and imidazolinones block and inhibit amino acid biosynthesis. The target enzyme is acetolactate (ALS)[11-13]. Mutant forms of ALS resistant to sulphonylureas and/or imidazolinones having one altered amino acid from the wild type sensitive ALS have been identified and isolated. Transgenic tobacco plants resistant to sulphonylurea through expression of a mutant ALS gene from *Arabidopsis*

Table 1 Herbicide resistant transgenic plants.

	Species modified	Transgene source	Transgene product	References
1.	*Target modification:*			
	Beta vulgaris	*Arabidopsis thaliana*	Acetolactate synthase	106
	Brassica napus.	*A. thaliana*	(as above)	11, 12
	Festuca arundinaceae.	*E. coli*	Hygromycin phosphotransferase.	108
	Linum usitatissimum.	*A. thaliana*	Acetolactate synthase	109
	Nicotiana tabaccum	*A. thaliana*	(as above)	14, 15, 16.
2.	*Enzyme Overproduction :*			
	Glycine max.	Plant and microbial genes	Analogue of EPSP synthase	110, 111
	Linum usitatissimum.	(as above)	(as above)	112
3.	*Enzyme Detoxification :*			
	Agrostis palustris	*Streptomyces hygroscopicus*	Bialaphos	113
	Beta vulgaris	(as above)	Phosphinothricin acetyltransferase	106
	Brassica napus.	(as above)	(as above)	27
	B. oleracea.	(as above)	(as above)	27
	Festuca arundinaceae	(as above)	(as abovce)	108
	Gossypium hirsutum	*Alcaligenes eutrophus*	2, 4-D monooxygenase	114
	Hordeum vulgare	(as above)	(as above)	20
	Lycopersicon esculentum	*Streptomyces hygroscopicus*	Phosphinothricin acetyl transferase	27
	Medicago sativa	(as above)	(as above)	115
	Nicotiana tabaccum	*Alcaligenes eutrophus.*	2, 4-D monooxy genase	32, 33
	Solanum tuberosum	*Streptomyces hygroscopicus*	Phosphinothricin acetyl transferase	27
	Triticum aestivum	(as above)	(as above)	116

were obtained which could tolerate four times herbicide concentrations[3,14,15]. Maize plants tolerant to imidazolines correlated with the presence of an altered ALS enzyme have also been produced[13].

2.2 Target enzyme overproduction

i) Glyphosate : During the last decade, rapid progress has been made in developing herbicides which degrade rapidly and are non-toxic to animals. One of the most potent broad spectrum herbicide is glyphosate (N-phosphonomethyl glycine), marketed under the trade name of Roundup. It interferes amino acid biosynthesis by inhibiting the enzyme 5-enolpyruvylshikimate-3-phophate synthase (EPSP). High level herbicide tolerance has been obtained in plants by overproduction of EPSP in the chloroplast[16-17]. An EPSP synthase cDNA isolated from a glyphosate tolerant *Petunia hybrida* cell line and joined to a CaMV 35S promoter and Ti nos (nopaline synthase) 3' polyadenylation signal was used for *Agrobacterium* mediated transformation of *Petunia* cells. Overproduction of EPSP synthase resulted in transgenics which could tolerate high doses enough to kill wild type *Petunia* plants[18]. In-vitro construction and transfer of a chimeric gene with plant EPSP synthase transit peptide joined to a bacterial EPSP coding region led to transgenic tobacco[19-20] and tomato due to the accumulation of stable glyphosate-resistant enzyme in the chloroplasts. The combination of the chloroplast transit peptide sequence of petunia cDNA clone and *E. coli* mutant resistant enzyme gave rise to fully resistant plants in tomato, potato, tobacco, soybean, brassica and sugarbeet[22-23].

ii) *Phosphinothricine (PPT) :* Phosphinothricine is a non-selective herbicide and inhibits glutamine synthase (GS)[24]. Inactivation of GS leads to accumulation of ammonia which is toxic to the cells. An alfalfa cell line resistant to PPT due to amplification of GS gene was reported[25] suggesting that an overexpression of the enzyme overcomes the toxic effect of the inhibitor. Insertion of the overexpressing GS gene from alfalfa lead to transgenic tobacco plants[26-27].

2.3 Detoxifying enzymes :

Herbicide detoxifying enzymes counteract the affect of several herbicides by inactivating them before they are able to inhibit the target enzyme. The most successful example is the detoxification of phosphinothricine phosphate. Murakami *et al*[28], isolated the *bar* gene from *Streptomyces hygroscopius* confering resistance to PPT by encoding an enzyme phosphinothricine acetyl transferase (PAT). This enzyme is able to inactivate PPT by acetylation of the free NH_2 group[29]. The *bar* gene was inserted into an *Agrobacterium* vector and used for transforming several plants. Transgenic plants showing increased tolerance were obtained[30].

Other enzymes are glutathione-5 transferase (GST) which modifies triazineherbicides[31] and 24-D dic hlorophenoxyacetate monooxigenase involved in 2,4 D degradative pathway. Transgenic tobacco plants were

produced through genetic engineering of *tfd* gene of soil bacterium *Alcaligens eutrophus* which encodes the first 2,4-D dichlorophenoxyacetate monooxigenase[32,33]. Table 1 summarises the list of herbicidal resistant transgenic plants developed after using the foreign gene sources.

3. Insect resistant transgenics (Table 2)

Control of insect pests has been an integral part of the development of agricultural practices as crop damage caused by insects is a major economic factor in agriculture in tropic and temperate regions of the world. Modern high intensity agriculture which has been responsible for the tremendous increase in food production, is dependent on the use of chemical pesticides. But the drawbacks of this strategy are that pesticides are often highly toxic to non-target organisms, strong selection pressure on insect populations imposed by insecticides causes rapid acquirement of resistance to such compounds and overuse of pesticides decrease the vigour of the crop and makes it more susceptible to an insect attack. Hence, attention was focused on improving the inherant resistance of the crop plant to insect attack which resulted in the development of transgenic plants able to protect themselves against insects by expressing insecticidal proteins or a protinease inhibitor gene. These plants offered advantages :

(i) absence of non-proteinaceous residues in soil or ground water.

(ii) high specificity with respect to the target organism

(iii) protection of plant parts such as roots which are difficult to reach by conventional methods

Two methods of control have been developed in transgenic plants.

3.1 Use of bacterial toxin gene

Gram positive bacterial *Bacillus thuringiensis* contain peptide toxins as crystals in their spores which when ingested by insects are cleaved by the proteases in the intestines resulting in the conversion of the protoxin into the active toxin. This toxin impairs digestion and midgut paralysis as a result of which the insect stops feeding and ultimately dies[34]. Successful transformations with *B thuringiensis* have been reported[35-37].

A chimeric gene with the structure CaMV35S promoter/*B. thuringiensis* toxin coding sequence/ Ti nos 3' termination sequence was constructed. This gene was placed in a Ti vector and tomato leaf disc cells were transformed by co-cultivation with *A. tumefaciens*[36]. The transgenic tomato plants were protected against feeding damage by larvae of the lepidopterans specifically *Manduca sexta*. Also, significant control of tomato fruitworm *(Heliothis zea)* and the tomato pinworm *(Keiferia lycopersicela)* have been obtained[38,39]. Many other transgenic plants have also been produced (Table 2) which express the bacterial toxin in their vegetative and floral organs and are thereby effectively protected against attack by some insects. *Bt* toxin genes have been isolated from a number of different bacterial strains, but genetic

engineering has been carried out only on strains active against lepidoteran pests. But as some insect strains show resistance to one type of endotoxin and are sensitive to another eg. *Plodia interpuctella*[40], it is plausible to construct transgenic plants with two types of protein against the same insect. A further improvement in insect control by transgenic plants is increased endotoxin protein production which not only kills susceptible larva but also reduces the fertility of the mature insects thus reducing their progeny.

3.2 Use of plant proteinase inhibitor gene

Proteinase inhibitors conferring endogenous resistance to insect attack are widespread among higher plants. In addition, they have anti-metabolic activity in a wide range of insects which provides an attractive strategy to make plants resistant to herbivorous insects by introducing genes for certain protease inhibitors.

Expresssion of a plant derived proteinase inhibitor-cowpea trypsin was reported from transgenic tobacco. The cowpea trypsin inhibitor (C_pTI) is an insecticidal component preventing development of the larvae of field and

Table 2. Insect resistant transgenic plants.

Plant species	Insect pest	Gene source	Transgene product	Reference
1. Resistance through bacterial toxin (Bt) gene :				
Lycopersicon esculentum.	*Manduca sexta, Keiteria lycopersicella*	*Bacillus thuriengi-ensis. (as above)*	Bt-insecti cidal protein	35,36,38, 39 117,118
Gossypium hirsutum	*Petinophora gossypiella*	*(as above)*	Bt-insecti cidal protein ein	38
Zea mays	*Heliothis zea*	*(as above)*	Bt-insecti cidal protein.	119
2. Resistance through proteinase inhibitor gene :				
Nicotiana tabaccum	*Manduca sexta.*	*Vigna ung-uiculata*	Trypsin Inhibitor protein.	41, 42

Table 3. Virus resistant transgenic plants.

Host Plant	Coat protein source	Protection against	References
1. VIRAL COAT PROTEIN MEDIATED RESTISTANCE :			
Lycopersicon esculentum	TMV, TMVI	TMV, TMVI	43, 121, 122
	A1MV	A1MV	123
Medicago sativa	*A1MV*	*A1MV*	*124*
Nicotiana tabaccum	*TMV*	*TMV*	*125*
	A1MV	A1MV	123, 126, 127
	CMV	CMV	52, 128
	TSV	TSV	127, 129
	TRV	TRV	129, 130
	PVX	PVX	131,
	SMV	TEV, PVY	46,134
Solanum tuberosum	*PVX or PVY*	*PVX* or PVY	133, 139
	PLRV	PLRV	137, 138, 140
Carica papaya	PRV	PRV	135, 136
Cucumis sativus	*CMV*	CMN	141, 142
2. NON-STRUCTURAL VIRAL GENE MEDIATED RESTSTANCE :			
Nicotiana tabaccum	TMV-V1	TMV	47
3. SATELLITE RNA GENE MEDIATED RESTISTANCE :			
Nicotiana tabaccum	TRV, CMV	TRV, CMV	48, 49
4. ANTISENSE RNA MEDIATED RESTSTANCE :			
Nicotiana tabaccum	CMV, PVX, TMV	CMV, PVX, TMV	52, 53, 125, 131

Abbreviations used : ALMV = Alfalafa Mosaic virus, CMV = Cucumber mosaic virus, PVX = Potato virus X, PVY = Potato virus Y, PRV = Papaya ringspot virus, PLRV = Potato leaf roll virus, PMV = Pepper mottle virus, SMV = soybean mosaic virus, TMV = Tobacco mosaic virus, TRV = Tobacco ringspot virus, TSV = Tobacco streak virus, TEV = Tobacco etch virus.

storage pests. It has the advantage of insignificant toxicity as it is present in the cowpea seeds itself which is consumable. A library of cDNA clones was produced from mRNA isolated from developing cowpea seeds. A cauliflower mosaic virus (CaMV) promoter and a nopaline synthase gene from *Agrobacterium tumefaciens* providing a polyadenylation signal sequence and a transcription terminator was added to the cDNA to be transcribed and translated in a transgenic plant. The synthetic gene was incorporated into an *Agrobacterium* binary vector and was used to transform tobacco plants. The transgenic CpTI expressing plant

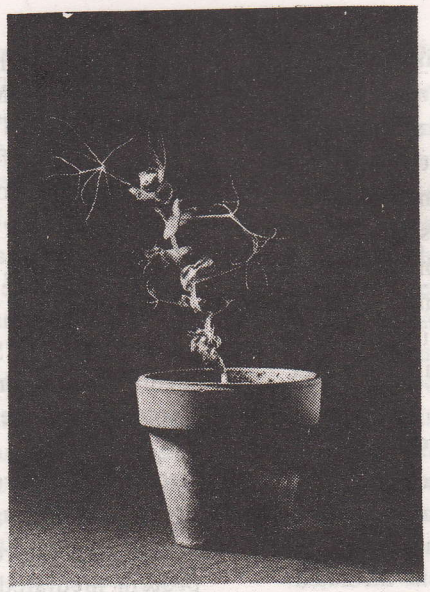

Fig. 1. Coat protein mediated virus resistant transgenic pea.

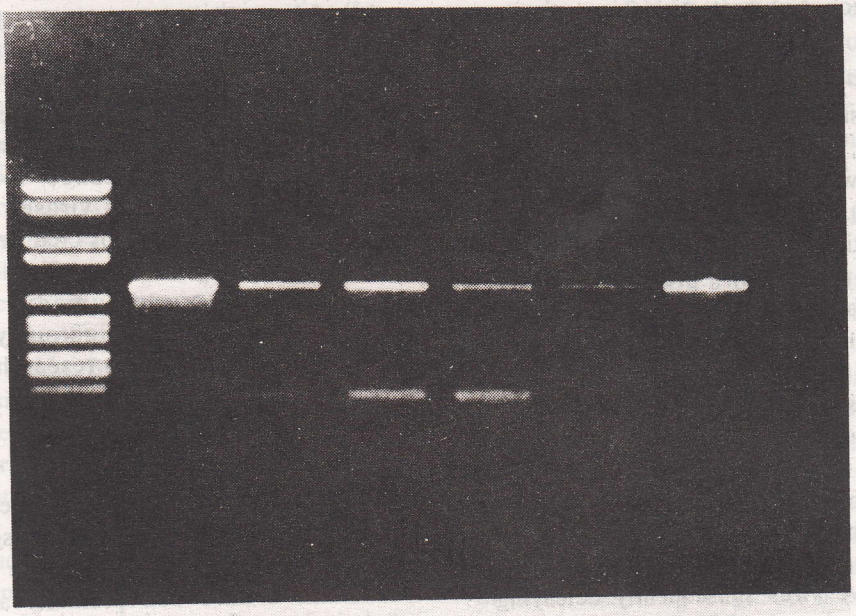

Fig. 2. Agarose gel electrophoresis of amplified DNA in transgenic pea.

showed a significant resistance to a wide spectrum of insects[40,41], indicating the potential of such genes. The advantage of using these genes lies in their broad spectrum of activity in many different insects and their nontoxicity as such inhibitors are found in the food of humans and animals. The major disadvantage is the high level of protein needed to kill the insect larvae.

4. Virus resistant transgenics (Table 3)

Significant progress has been made in protecting crops against a number of viral diseases through genetic engineering using the following (i) coat protein mediated protection (ii) protection by non-structural viral gene (iii) protection via satellite RNA expression (iv) protection via antisense RNA

4.1 Coat protein mediated protection

The most promising and successful way to produce virus resistant plants is to insert and allow expression of a viral coat protein gene (s). The principle is based on the observation that infection of a plant with one viral strain protects against superinfection by another related strain. This phenomena is also referred to as cross-protection. Although the molecular mechanism involved are not as yet clear, it is assumed that the mild virus which infected the cell first, produces excessive amount of protein which remains in a free state. The unbound protein inhibit the uncoating of the RNA of the second aggressive virus subsequently deleaying or preventing the RNA expression and replication[42]. Powel-Abel *et al*[43] were the first to describe the expression of tobacco mosaic virus (TMV) coat protein when infected with TMV in transgenic tobacco. A cDNA coding for the TMV coat protein under the control of a CaMV 35S-promoter was transferred to tobacco. The progeny of the engineered plants expressing the viral coat protein gene either showed delayed disease symptoms or failed to develop symptoms at all. Since then, coat protein mediated virus protection has also been introduced in other plants (Table 3, Fig 1-2). According to Beachy *et al*[44, 45], two steps are operative one at early infection and the other during the spread of infection.

An interesting feature of coat protein mediated protection is that in some cases the plant is not only protected against an infection of the virus from which the coat protein was received but also against other seriol ogicaly non-related viruses. Stark and Beachy[46] demonstrated that expression of coat protein of SMV in transgenic tobacco led to resistance to two seriologically unrelated viruses PVY and TEV. The strategy of coat protein mediated virus protection offers great potentials to control many viral diseases and is being vigorously and successfully pursued by researchers.

4.2 Non-structural viral gene protection

Using a non-structural gene from TMV strain U1, Golemboski *et al*[47] were able to produce highly virus resistant transgenic tobacco plants. the chimeric gene used contained a CaMV35S promoter and a coding region of an open reading rame which encodes a 54 kD protein. Transgenic

plants containing the chimeric 54kDa ORF showed complete resistance even when inoculated with high concentrations of the virus. Analysis of the chimeric gene expression in these plants showed the presence of mRNA but not the 54 kDa protein which makes it unclear whether the resistance is due to the protein or its mRNA[48]. This question can be answered by in-vitro mutagenesis experiments.

4.3 Satellite RNA modification

Satellite RNAs are small RNA molecules encapsulated by plant viruses and packaged together with a viral genome. Certain plant RNA viruses harbour these molecules. Such molecules are unable to replicate independently but require assistance from the virus. The ability of certain satellite RNA to modify and ameliorate plant viral disease symptoms aroused attention for using it as a means for controlling viral disease This led Harrison *et al*[48] and Gerlach *et al*[49] to incorporate genomes of satellite RNA CMV and TRV under the control of constitutive promoters into tobacco plants. The transgenic plants which expressed the satellite RNA when infected with the corresponding virus exhibit significant delay in symptom development compared to the plants devoid of the gene coding RNAs. But there are potential risks for using satellite RNA for genetic engineering as (i) satellite RNA that are ameliorative in one species may be lethal to another[50], (ii) satellite RNA mutate rapidly and a single nucleotide change introduced in in-vitro mutagenesis can make an ameliorative satellite necrogenic[51]. Hence deeper understanding of their molecular biology is needed for their safe use in genetic engineering experiments.

4.4 Antisense RNA resistance

An antisense RNA is an RNA complementary to the mRNA strand and contains base sequences complementary to the target (sense) RNA transcripts. When present together, they anneal to form duplex RNA molecules thereby blocking translation. Attempts have been made to produce virus resistant plants by using antisense RNA against viral coat protein gene[52-54]. Plants showing some resistance to low inoculum have been produced for CMV, PVX and TMV. Experiments with CMV were not very optimistic as out of 12 lines, only one transgenic line showed some resistance to CMV while the others were as susceptible as control plants. A higher rate of resistance can be expected if the antisense RNA's expressed are complementary to key regions involved in the regulation of replication of gene expression. Use of antisense RNA molecules need more understanding, both of RNA metabolism and viral life cycles.

5. Fungal resistant transgenics (Table 4)

Fungal diseases are a major problem in agriculture causing enormous world-wide economic losses. Though a variety of plants have a natural mechanism to resist attack of pathogenic fungi either through preformed barriers, such as cell walls and cuticle or through the production of defense enzymes, the pathogens have somehow also developed ways to evade these defense mechanisms[55-57]. In order to combat the diseases, control by fungicides is essential but sometimes problematic. Therefore new approaches of introducing resistance genes

to plants have been taken up. But in comparision to virus and insect resistance, methods of obtaining resistance against fungi and bacteria are less developed. The methods were developed taking into account, the natural defense related genes present in plants which is triggered by pathogen attack, environmental stress and by biotic and abiotic elicitors. Bowles[55] grouped the defense genes into three classes, (i) genes synthesizing compounds involved in cell wall modification, (ii) genes encoding defense related proteins exhibiting antimicrobial activities or catalyse the synthesis of products that retard microbial activity and (iii) genes encoding the pathogen related (PR) proteins whose appearance is correlated with defense responses.

5.1 Defense related proteins

An attack by a pathogenic fungi triggers a number of active responses in plants. One of the most important response is the synthesis of defense related proteins which include amylase proteinase inhibitors, thiomins, hydrolytic emzymes such as B-1, 3 glucanase, chitinase and enzymes involved in the synthesis of phytoalexins[56-57].

(i) Phytoalexins : Phytoalexins, a secondary metabolism product, are low molecular weight antimicrobial compounds which act as broad spectrum antibiotics and play an important role in arresting the growth of fungal pathogens[58-59]. Expression of phytoalexin genes may be due to elicitors produced by host and fungal cell wall breakdown or by abiotic agents such as mechanical injury, ultraviolet irradiation and heavy metals. The first phytoalexin to be purified and identified was pisatin from pea[60]. Others include medicarpin, maakiain, phaseolin, phaseollidin, kievitone from legumes and lubinin and rishitin from potato. Indirect proof for the role of phytoalexins in disease resistance has been supplied by genetic

Table 4. Fungal disease resistant transgenic plants.

Plant species transformed	Transgene source	Transgene product	Resistance against	Reference
Brassica napus	*Phaseolus vulgaris*	Bean endo chitinase	*Rhizoctonia solani*	64
Nicotiana tabaccum	(as above)	(as above)	(as above)	64
N. tabaccum	*Serratia marcescens*	Chitinase	*Alternaria longipipes*	143
N. tabaccum	*Hordeum vulgare*	Ribosome inhibiting or inactivating protein	*Rhizoctonia solani*	72

experiments utilizing pathogen strains that vary in their virulence and ability to degrade a particular plant phytoalexin[61-62]. Hain[63] demon-strated that introduction of the key biosynthetic gene for a peanut stilbene phytoalexin into tobacco plants enables them to produce the peanut phytoalexin - resveratol. It is predicted that plants able to make large amounts of foreign phytoalexins would be resistant to pathogens that could detoxify the new chemical structures.

(ii) Chitinase : A common natural mechanism of plants to resist fungal attack is secretion of the enzyme chitinase which attacks the cell wall of the fungus. This enzyme chitinase is very stable, resistant to heat and inhibits fungal growth in-vitro. Strains of *Serratia marcescens* are effective in the biocontrol of a number of pathogenic fungi eg. *Scerotium rolfsii* due to the secretion of chitinase. Introduction of a microbial chitinase gene into tobacco plants[64,65], has shown promise for the control of certain fungal pathogens. There are additional novel avenues suggested to develop disease resistant plants such as to introduce gene(s) that detoxify pathogen toxins, inhibit essential pathogen enzymes and encode antimicrobial peptides. Such genes have been described from plants[66-71]. Though considerable information is known about the mechanisms determining plant disease resistance, greater understanding will be acheived when disease resistance genes are finally cloned and characterized in higher plants. This will dramatically improve disease control in the field through their systematic transformation into various crop plants.

(iii) Ribosome inhibiting proteins : Seeds of various cereals contain proteins that are toxic to some pathogen. For example, barley contains a ribosome inhibiting protein (RIP) which is a glycosylase that inhibits ribosome function by cleaving a glycosyl form the 60S subunit of ribosomes thus preventing peptide elongation. This protein while not toxic to plants, inhibits the growth of a number of pathogenic fungi. Using a chimeric gene capable of expressing barley RIP in the stem and roots of tobacco, Logemann *et al*[72] developed transgenic plants capable of resisting attack by *Rhizotonia solani.*

6. Stress tolerant transgenics (Table 5)

Biological stress refers to any change in environmental conditions that might reduce or adversly change a plant's growth or development. Crop plants are subjected to a variety of environmental extremes such as drought and temperature stresses and breeders have long faced problems selecting for stability of performance over a range of environments, using extensive testing and an intricate biometrical approach. Environmental stress alters gene expression[73] and permits isolation of stress related genes. Improving resistance to environmental stress thus requires a combination of breeding, physiological

and biotechnological approaches to understand (i) the structure and enzymatic functions of stress proteins (ii) mechanisms regulating the stress genes and (iii) identification, isolation and transfer of these genes by various transformation schemes. Although precise molecular basis of stress phenomena is poorly understood, monitoring of level of plant tolerance to cold, heat, drought and salts has been possible to some extent. Three most common types of stresses the plant face are (i) water streass (ii) temperature stress and (iii) salt stress.

6.1 Water stress : Water stress is mainly considered in terms of drought stress. Survival of the plant depends upon its ability to function under water scarcity. This can be circumvented by drought-avoidance requiring a short growing season or dehydration-avoidance where the plant maintains sufficient tissue hydration for metabolic functioning. Abscisic acid (ABA) concentration effects yield under water stress conditions[73,74]. Thus selection for plants having high ABA accumlation should form an important selection criterion for drought resistance.

6.2 Temperature stress : Temperature stress refers to any temperature outside the optimum for growth and development. It depends on growth stage, the most severe perturbation occuring at germination and fruit formation. Three different types of temperature stress are heat-stress, chilling-stress and freezing-stress.

(i) Heat tolerance : Thermal tolerance is viewed in terms of stress degree exposure duration and developmental stage. Of all biological processes, the reproductive-stage is most sensitive to heat leading to floral abscission, pollen sterility and poor fruit-set. All these lead to yield reductions. Heat shock response is characterized by (i) decrease in protein

Table 5. Stress tolerant transgenic plants.

Species	Genetic modification	Transgene Source	Reference Product	
Lycopersicon esculentum	Frost protection	Fish, *Pseudo pleuronectes americana*	*Antifreeze* protein	144
Nicotiana tabaccum	Cadmium tolerance	Mouse	Metalloth ionein binding protein	145
N. tabaccum	Cold tolerance	*Arabidopsis thaliana*	Glyderol - 3 phosphate acyl transferase	146

synthesis, (ii) production and accumulation of large amount of heat shock protein (iii) gradual decline in heat shock protein synthesis and return to normal synthesis. Though plant breeders have been able to manipulate thermal tolerance as a heritable agronomic trait, the relationship between thermal tolerance in-vivo is ambiguous. As genetic resources for heat tolerance exist in rice, potatoes, soybean and tomato, for other crops, these need to be explored.

(ii) Chilling stres : It is the most severe environmental stress that reduces germination and growth rate, vegetative and reproductive growth and leads to deformed fruit formation and/or failure of fruit and/or seed set. Chilling telerance becomes operational below O^0C. Genetic resources for chilling tolerance have been found in maize[75]. Such genes have been introduced in tomato from exotic germplasm[76] and transgenic tomato resistant to chilling stress have been developed.

(iii) Freezing stress : In crop plants, considerable genetic variability occurs in freezing tolerance below O^0C. Considered to be genetically conditioned, the inheritance pattern of freezing stress tolerance is scantily known in wheat and rice. Frost hardiness is considered a quantatively inherited trait controlled by a complex interaction of several genes[77].Plasma membrane is regarded as the target site for freezing because disruption of cellular membranes is the first symptom of freeze injury in plants. Steponkus *et al*[78] opine increased tolerance as related to change in plasma lipid composition. Series of biochemical alterations follow freezing viz. increase in proline and organic acids, sugar and soluble proteins. The expression of cold regulated genes parallels freezing tolerance in plants[77]. But knowledge about molecular genetics of cold acclimatization and freezing tolerance is needed before biotechnological manipulations are possible to develop freezing tolerant plants.

6.3 Salt stress

Efforts to develop salt resistant varieties have been unsuccessful due to multipartite nature of stress that makes difficult to predict the extent of stress in a saline environment as salinity causes (i) water stress from the osmotic effects (ii) mineral toxicity of the salt and (iii) interruptions of the mineral nutrition of the plant. Also saline fields are inherently variable in their salt distribution and thus require an appropriate strategy to breed for high yields.

In-vitro selection and cell lines with enhanced salt resistance have been isolated[79], but this is rarely associated with resistance at the whole plant level[80,81].

7. Protein modifications in transgenics (Table 6)

Seeds of higher plants contain large quantities of storage proteins which upon germination are hydrolyzed providing nitrogen for growth. But such proteins are deficient in amino acids that are essential for human and livestock nutrition[82]. Cereals are most limited in tryptophan, threonine and lysine and legumes in the sulphur containing amino acids methionine and

Table 6. Transgenic plants with improved quality.

Plant species	Genetic modification	Transgene source	Transgene product	Reference
A *Food processing quality :*				
Brassica napus	Increased stearic acid	*Brassica rapa*	Antisense stearoyl ACP desaturase	147
	Increased methionine	*Bertholletia excelsa.*	Seed storage protein	86, 148
	Increased lysine	*Cornybacterium dap, E. coli*	Aspartokinase, dibydrodipico linic acid synthetase	173
Glycine max	Increased methionine	*Bertholletia excelsa*	Seed storage protein	149
	Increased lysine	*Cornybacterium* dap, *E. coli*	Aspartokinase, dihydrodipico-linic acid synthetase	173
Lycopersicon esculentum	Improved storage	*Lycopersicon esculentum*	Antisense poly-galacturonase	150
	Flavour enhanced	Artificially synthesised	Synthesised monellin	152
Medicago sativa	Improved protein quality	Chicken	Chicken ovalbumin	153
Solanum tuberosum	Increased starch content	*E. coli*	ADP-glucose pyrophospho-rylase	154
Nicotiana tabaccum	Increased manitol	*E. coli*	Mannitol dehydrogenase	155, 156
B *Speciality chemicals :*				
Arabidopsis thaliana	Biodegradable thermoplastic	*Alcaligenes eutrophus*	Polyhydroxy butyrate (PHB)	157
Brassica napus	Increased lauric acid	*Umbellularia californica*	Lauroyl-ACP thioesterase	158
	Enkephalins	Chimeric gene part from *Homo sapiens* & *Arabid-opsis thaliana*	Leu-enkephalin	137
Petunia hybrida	Flower colour	*Zea mays* *Gerbera* spp	Dihydraflavonol 4-reductase (DFR)	159, 160
Solanum tuberosum	Serum albumin	*Homo sapiens*	Human serum albumin	161
	Cyclodestrins	*Klebsiella pneumoniae*	Cyclodextrin glycosyltra-nsferase	162

cysteine[83]. Conventional plant breeding techniques to increase amino acids in crops have met with low success[84]. Storage proteins are the products of multigene families comprising about 10 or more members, tightly linked at a given locus[85]. Plant genetic engineering aims at modifying seed storage proteins to improve nutritional properties of seeds. Two approaches followed in altering seed amino acid composition by molecular means are :

(i) to find a naturally occuring seed storage protein with high levels of the desired amino acid, clone the corresponding gene and allow its expression.

(ii) to modify seed storage protein genes by recombinant DNA or in-vitro mutagenesis so that they encode proteins that are similar to wild-type proteins, but contain higher levels of essential amino acids.

Tissue specific, developmentally regulated expression of both dicot and monocot seed storage protein genes have been demonstrated in dicot transgenic plants. For instance in *Brassica napus*, both the chimeric and modified storage protein transgenes have expressed fully[86-88] Likewise, a chimeric phaseolin transgene is expressed in tobacco[89]. Modified storage protein genes exhibiting differential accumulation of four phaseolin glycoforms successfully expressed in transenic tobacco[90]. and influencive role of the polypeptide glycan in post-translational processing and transport of barley lectin to vacuoles in transgenic tobacco[91] clearly indicate the decisive role and demonstrable expression of storage protein genes in transgenic plants.

Despite the modifications in seed storage protein genes in transgenic plants, the manipulation of seed quality through genetic engineering faces several obstacles. The addition of one gene to plant genomes may not be very effective in improving the plant phenotype due to the strong expression of the rest of the multigene family. Despite this, the multiple codons for lysine and tryptophan in a 19 kD zein cDNA by site directed mutagenesis have been successfully incorporated[92]. Although the engineered zein gene was corrected in its deficiency in essential amino acids, the protein was not stably incorporated into its normal cell compartment in the endosperm of maize. Another major experimental constraint in modification of seed storage proteins is the time required to regenerate and obtain seeds from the tranformed plants. In contrast to bacterial or cell culture systems in which modified proteins can be tested in a matter of days or weeks, in plants, it may approach a year or sometimes even more. Infact, expression of modified storage proteins in transgenic plants is still in its infancy and requires development of reliable systems for quick testing of modified storage proteins[94-96]. No data are available about what effect overexpressing proteins rich in a particular amino acid will have on amino acid pools or other physiological factors Lumen[93] suggested that protein quality is not the only parameter of importance and the interaction of changes in protein quality and quantity with changes in oil or starch has to be closely monitored. All should be balanced in such a way as to provide a useful product accepted by the market.

7.1 Production of high value protein

Pharmaceutically useful high value peptide-leuenkephalin was produced by inserting its gene sequence

into a seed of *Arabidopsis thalians*[87]. The seeds of this plant had the polypeptide in abundance. In fact ten to several hundred grams/ha of this polypeptide was obtained in the seeds produced by the transgenic rape seeds having the polypeptide transgene[94]. The synthesis of human albumin by the tubers of transgenic potato and the production of immunoglobins in transgenic tobacco plant represents two more major examples of production of high molecular weight useful proteins by transgenic plants.

8. Tasty and long lived fruits and vegetables in transgenics

More than half of the fresh fruits and vegetables produced annually are lost due to spoilage caused mainly by ethylene formation that triggers fruit ripening. To delay the ripening, sequestrants of ethylene are used or fruits are harvested long before they are ripe. Whereas sequestering ethylene involve the use of chemicals and consequently results in price rise, harvesting ripened fruit produces unpleasant taste on use. One of the best examples of producing tasty and long lived transgenic fruits is tomato.

Tomatoes become mushy due to the production of a softening enzyme called polygalaturonase (PG) which causes pectin breakdown in the cell pulling apart the cells. All other changes associated with tomato ripening such as flavour and colour development, are not affected by this enzyme. The Calgene scientists cloned complementary DNA to tomato PG and inserted into tomato, DNA in the antisense orientation. These transgenic tomatoes exhibit decreased PG level upto 99% increasing thereby the shelf life of the tomato. Most interesting and improtant is that this transgenic tomato does not have a foreign gene but its own genome in reverse form The transgenic tomato is more resistant to mechanical stress associated with handling, packaging and transport without losing compressibility. This tomato is the first geneticlly engineered food crop released in the US market under the name "Flavr Savor"

9. Engineering male sterility in transgenics

Male sterility obviates artificial and chemical emasculation, enhances possibility of outcrossing and ensures hybrid production[97].

Commercial outcrossing of the usable male sterility is limited by its prevalent high instability, delicately balanced genetic requirements and profound environmental sensitivity. Therefore, creation of usable stable male sterility by using gene technology is an asset for commercial hybrid production. This was achieved by Mariani *et al*[98].

A strategy to engineer male sterility in tobacco and oil rape seed was devised[98]. This strategy takes advantage of the tapetal specific transcriptional activity of the tobacco TA 29 gene and an RNase/RNase inhibitor defense system utilized by bacteria *Bacillus amyloliquifaciens* The chimeric RNase gene T1 and barnase gene containing the tobacco TA 29 gene promoter induced male sterility in both these plants. This TA29 RNase gene selectively destroys the tapetal cell layer, prevents pollen formation and results in male sterility. The barstar is produced intracellularly and protects the bacteria from the lethal effects of barnase by forming a stable complex with barnase in the cytoplasm. Mariani *et al*[99] restored male fertility in the genetically engineered male sterile oilseeed rape plants by introducing the barstar gene in the male sterile plants.

This introduction was done by conventional crossing using male steriles as females and those having barstar gene as males. Thus, both the creation of male sterility and restoration of male fertility in male steriles, when fertility restorer genes are not available or traceable, has been done by genetic engineering; the fertility restoration being done by a proteinaceous inhibitor of the RNase under the control of the same tapetum specific promoter and introduced in plants[100]. This inhibitor suppresses the tapetum destroying RNase activity fully and pollen fertility gets restored.

10. Transgenic plants as bioreactors

Development of transgenic plants with altered low molecular weight (e. lipids, sugars, secondary methabolited) and high molecular weight compounds (proteins, carbohydrates, polymers, fibres) is in rapid progress[95]. Nearly 30% of the amino acids of this seed protein are sulpur amino acids. The Brazil nut seed protein contributed upto 8% of the total seed protein in the transgenic tobacco plants resulting in a significant increase in the methionine content. If this gene is transferred to legumes, a major improvement in seed protein quality will be achieved and the biological value, efficiency ratio and digestibility of the legume proteins will enhance dramatically.

11. Conclusions

Transgenics, the neospecies, are the organisms harbouring new genes within their genome. Included in these are also the organisms in which the resident genes have either been silenced or replaced by refined foreign genes. This foreign gene incorporation is done by direct or indirect methods. The vector mediated indirect method utilizes *Agrobacterium* whereas direct method uses many chemicals or physical techniques for the gene transfer[101]. Of the various techniques utilized, the *Agrobacterium* mediated gene transfer has been most widely used (Table 10). The genes commonly introduced are those inducing resistance against herbicides, insecticides, viruses, fungi and environmental stress. In addition, this in-vitro breeding technology has produced transgenic plants with aminogram in cereals and legumes and plants producing economically useful proteins and biopharmaceuticals[102]. Moreover, both male sterility induction and male fertility restoration has also been engineered by the transgene technology. This paves an easy, reliable and useful way for commercial hybrid production and the consequent increased productivity. Currently, transgenic plants range from forest and fibre plants to cereals, fruits, ornamentals and vegetables.

Despite numerous brilliant successes and breakthroughs in transgenic development technology, several obstacles exist in the transgene cloning, transfer, expression and stability. Moreover, many transgenes stay silent either immediately after the transfer or get silenced after some generations of expression[103]. In addition, the transgenics pose grave ethical, economical, ecological and technological risks. Transgenes, especially those conferring resistance to pests, diseases, herbicides and stress may get transferred by cross pollination to sexually compatible wild weedy species offering them a selective advantage over the cultivated ones. Moreover, repeated transformations of a genome, pyramiding of several transgenes following multiple rounds of transformations and elimination of ancillary sequences are the major issues facing global transgene marketing strategy. May be transgenics lead to proliferation of new viral, fungal and insect strains that gain resistance to transgen[illegible] resistant plants. This can have serious impacts on humans, birds,

Table 7. Transgenic plants used for research investigations.

Plant species	Genetic modification	Transgene source	Transgene product	Reference
Beta vulgaris	Plant persistence	*Streptomyces hygroscopicus.* & *E. coli.*	Phosphinothricin acetyltransferase & Neomycin phosphotransferase	163
Brassica napus.	Male sterility	*Bacillus amyloliquefaciens*	Ribonuclease & Ribonuclease inhibitor	98, 99
	Gene expression	*E. coli*	Chloramphenicol acetyl transferase	164
	(as above)	(as above)	Neomycin phosphotransferase	165
	Pollen dispersal	*Streptomyces hygroscopicus*	Phosphinothricin acetyl transferase	166
	Plant persistence	(as above)	(as above) & Neomycin phosphotransferase	163
Gossypium hirsutum	Pollen dispersal	*E. coli*	(as above)	167
Lycopersicon esculentum.	Gene regulation	(as above)	ADP glucose pyrophonsphorylase	168
		Zea mays	Sucrose phosphate synthase	169
Nicotiana tabaccum	Gene regulation	*E. coli*	Chloamphenicol acetyl transferase	170
Solanum tuberosum	Gene expression	(as above)	Neomycin phosphotransferase	171
	Gene regulation	(as above)	ADP glucose pyrophosphorylase	168
	Pollen dispersal	*Arabidopsis thaliana*	Acetolactate synthase	172
	Plant persistence	*Streptomyces hygroscopicus,*	Phosphinothricin acetyltransferase & Neomycin phosphotransferase.	163
		E. coli		

Table 8. Field released transgenic plants.

Crop species	Genetic modification achieved
Beta vulgaris (Sugar beet)	Sulfonylurea (HR), Glufosinate (HR)
Brassica napus (Rape seed)	Bt protein (IR), Glufosinate (HR), Glyphosate (HR), Seed storage protein, Oil composition, Male sterility Bar marker gene, nptII marker gene
Brassica oleracea (Cauliflower)	Male sterility
Carica papaya (Papaya)	Papaya ring spot virus (VR)
Chrysanthemum	Flower colour
Cichorium intybus (Chicory)	Male sterility
Cucumis melo (Cantalope, melon)	Cucumber mosaic virus (VR)
Cucurbita pepo (Squash)	Cucumber mosaic virus (VR)
Glycine max (Soybean)	Glufosinate (HR), Glyphosate (HR), Soybean mosaic virus (VR), Seed storage protein
Gossypium hirsutum (Cotton)	Bt protein (IR), Bromoxynil (HR), Glyphosate (HR), Sulfonylurea (HR), npt II marker gene
Helianthus annuus (Sunflower)	Seed storage protein, male sterility
Juglans regia (Walnut)	Bt protein (IR)
Linum usitatiss-immum (Flax)	Glyphosate (HR), sulfonylurea (HR)
Lycopersicon esculentum. (Tomato)	Tobacco mosaic virus (VR), Tomato mosaic virus (VR), Bt protein (IR), Glyphosate (HR), Sulfonylurea (HR), Bromoxynil (HR), Glufosinate (HR), Fruit ripening Maize transposon AC/DS
Medicago sativa (Alfaalfa)	Alfalfa mosaic virus (VR), Glufosinate (HR), Lectin protein (IR)
Nicotiana tabaccum (Tobacco	Tobacco mosaic virus (VR), Bt protein (IR), Tobacco etch virus (VR), Sulfonylurea (HR), Glufosinate (HR) Glyphosate (HR), Bromoxynil (HR), Heavey metal tolerance, CAT marker gene.
Oryza sativa (Rice)	Marker genes, Bt protein (IR), Seed protein storage genes, male sterility
Petunia hybrida (Petunia)	Flower colour pattern genes, male sterility
Populus (popular)	CAT marker gene
Prunus domestica. (Prune-plum)	Plum pox virus (VR)
Solanum tuberosum (Potato)	X and Y viruses (VR), Potato leafroll virus (VR) Bt protein (IR), Bromoxynil (HR), Glufosinate (HR), Increased starch content gene, Sulfonylurea (HR), npt II marker gene
Zea mays (Maize)	Bt protein (IR), European cornborer (IR), Glufosinate (HR), Bromoxynil (HR), sulfonylurea (HR), Glyphosate (HR), Modified protein gene, Male sterility.

HR = Herbicide Resistance, IR = Insect Resistance,
VR = Virus Resistance.

Table 9. Total number of fieldtested transgenics in different countries upto 1995.

Argentina 08 Australia 13 Belgium 52 Canada 63 Chile 08 *China 07 Costa Rica 05 Denmark 09 Finland 17 France 104 *Germany 19 Israel 14 Italy 09 *Japan 13 Mexico 09 New Zealand 17 Spain 16 Sweden 12 Switzerland 19 The Netherlands 28 United Kingdom 37 United States 193 Total 672

*Complete information not available due to government or people resistance, for the development or release of transgenics.

Table 10. Major successful transformation methods for obtaining transgenic plants.

Transformation Method.	Species transformed*
Agrobacterium mediated gene transfer	*Actinidia deliciosa, Alocasarina verticillata, Apium graveolens, Arabidopsis thaliana, Arachis hypogea, Armoracia rusticana, Asparagus officinalis, Beta vulgaris, Brassica carinata, B. juncea, B. napus, B. oleracea, B. rapa, Carica papaya, Citrullus lanatus, Cucumis melo, C. sativus, Daucus carota, Dendrathema indicum, Dianthus caryophyllus, Frageria vesca, Gossypium hirsutum Glycine max Helianthus annuus, Ipomoea purpurea, Juglans regia Kalachoe lacinata, Lactuca sativa, Linum usitatissimum Lotus corniculatus, Lycopersicon esculentum, Medicago sativa, M. varia,Musa acuminata, Nicotiana tabaccum, Passiflora edulis,* Phaseolus vulgaris
	Pisum sativum, Petunia hybrida, Poncirus trifoliata Populus nigra Prunus armeniaca, P. domestica, Pyrus malus, Solanum melongena, S. muricatum, S. tuberosum, Stylosanthes humilis, Synapsis alba, Vicia narbonensis, Vigna aconitifolia, Vitis rupestris, V. vinifera.
Direct DNA transfer to protoplast	*Agrostis alba, Brassica oleracea B. napus, Dactylis glomerata, Festuca arundinacea, Glycine max Lactuca sativa, Oryza sativa, Triticum aestivum, Zea mays.*
Biolistics	*Agrostis palustris, Avena sativa, Carica papaya, Glycine max Gossypium hirsutum, Hordeum vulgare, Musa sapientum Nicotiana tabaccum, Oryza sativa Phaseolus vulgaris Picea glauca Populus nigra, Saccharum officinarum Secale cereale, Sorghum bicolor, Triticum aestivum, Zea mays*
Electroporation	*Asparagus officinalis, Oryza sativa, Phaseolus vulgaris Zea mays.*

insects and other animals that feed over these plants. Overgrowth of transgenic plants in habitats where ind genous relatives of these plants ordinarily grow will diminish indigenous species, genetic richness and consequently reduce biodiversity. This paves way for the species extinction from this globe. Thus transgenics still stand on trial, time and tedious test. A vindication of plant transgenics and the support they need from industry and government are emphasised by Dixon[104] and Hoyle[105] as the transgenic plants have not only been utilized in multifacted investigations (Table 7), but are released out of necessity because of their immense utility (Table 8). In the developed world (Table 9), the major transformant being the vector *Agrobacterium* (Table 10).

With 30 million Department of Biotechnology, Government of India's budget, the demand-driven research in biotechnology encompasses the development and release of transgenic organisms in India. This will be a step towards the global competitiveness and innovation. Of course, this research demands rigorous peer review with emphasis on scientific exellence and thorough field and stability testing of transgenics.

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