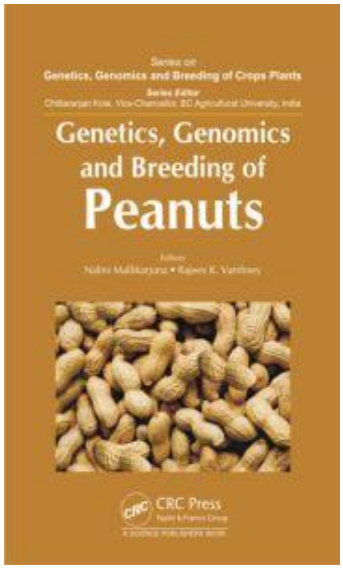

Chapter 9: Transgenic Interventions in Peanut Crop Improvement: Progress and Prospects

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Transgenic Interventions in Peanut Crop Improvement: Progress and Prospects

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Abstract

Legumes rank third in world crop production in which the major constraint to crop productivity is attributed to biotic and abiotic stress. Peanut, also known as groundnut (*Arachis hypogaea* L.) is a

major oilseed crop in the world, both for oil and as a protein source. Host plant resistance provides the most effective and economic option to manage stress tolerance in peanut which is also time consuming involving expensive agronomic practices. However, for many biotic and abiotic stresses, effective resistance gene(s) in cultivated peanut have not been identified. Success in breeding for better adapted varieties to biotic/abiotic stresses depend upon the combined efforts of various research domains like plant and cell physiology, molecular biology, genetics and breeding. Moreover, availability of known genotypes with natural resistance to stresses is a prerequisite for the successful breeding program. With a few exceptions, crop improvement in peanut programs through conventional breeding has received little progress.

Over the years, biotechnology has emerged as a promising tool to overcome both biotic and abiotic stresses in plants. Biotechnology applications include potential approaches, especially where the existing germplasm lacks the required traits for conventional breeding and provide promising ways to increase peanut productivity, either through improved seed quality or stress resistance. However, the progress has been very limited in legumes till date since these approaches require the identification of genes that control important agronomical traits, the understanding of gene regulation and metabolic pathways, along with methods of delivering genes or small RNAs into peanut plants. A new tool of engineering of multiple genes or regulatory machinery involving transcription factors has emerged for controlling the expression of different stress-responsive genes instead of inserting single genes for a single trait. Hence, researchers have focused their research on peanut functional genomics and biotechnology, and have achieved great strides during the recent decades. In this chapter, we discuss the recent progress and the current status of transgenic technology in peanut which offers the best option in host plant resistance breeding to combat various economically important biotic/abiotic stresses and its use in the crop improvement for stress tolerance.

Keywords: Transgenic breeding, Genetic transformation, Regeneration**,** Somatic embryogenesis, Genetically modified plants, Overexpression, Gene silencing, RNAi technology

9.1 Introduction

Legumes, rich sources of proteins and minerals, are referred to as "poor man's meat" in certain cultures. In order of importance, peanut, cowpea and beans represent about 80% of the production and cultivated area of food legumes, which are essential staples in the diets of millions. Peanuts share approximately 10% among production of 286.7 million metric tons of the world total of oilseeds behind soybeans (53%), rapeseed (15 %) and cotton seeds (12 %).

Peanut production process from planting to harvest is affected by different types of biotic and abiotic stresses that cause annual yield losses of over US$ 3.2 billion (Dwivedi et al. 2003). Since the mid-1970s, edible peanuts have increased in both domestic consumption and export trade in India. In contrast, production in Africa has declined by 17% over the last two decades. Acreage, production and productivity of peanut in India has shown large amount of fluctuations since 1993-94 to 2006-07. The productivity of peanut in India suffers mainly since 80% of the crop is grown under rainfed conditions by resource-poor farmers (Kaushik 1993). Lack of irrigation facilities to protect the crop from soil-moisture deficit during breaks in rainfall in the monsoon season affects germination. Rainfall pattern during the presowing months and availability of substitute high-value oilseed crops like soybean and sunflower with short durations requiring less water had a significant negative impact on acreage allocation decisions of the farmers (Patil et al. 2009). Resource-poor farmers who obtain low yields of 500-800 $kg.ha^{-1}$ due to various biotic and abiotic constraints grow about 93.8% of the world's production of peanut. Moreover, a big gap exists between the realized yield and potential yield of peanut at both subsistence and commercial systems of production in Asia and Africa.

The decrease in peanut productivity is mainly affected by various biotic, abiotic and economic factors. The economic status of the small and marginal farmers restricts them to use poor quality local seed in addition to minimum or no fertilizer applied during cultivation, which is essential as peanut is mostly grown in marginal and poor soils of low fertility. Use of complex fertilizers may also add to deficiencies of nutrients such as calcium and sulfur affecting the yields.

The major abiotic factors affecting peanut production include drought, high temperature, low soil fertility, low soil pH and iron chlorosis. Among the biotic factors, fungal diseases, virus diseases, bacterial wilt disease, aflatoxin contamination, nematodes, foliar insect pests, and soil insect pests, pod borer (*Helicoverpa* spp.) play a significant role in yield reduction (Sharma and Oritz 2000; Dwivedi et al. 2003). The plant disease management technologies are greatly influenced by environmental pollution, deleterious effects of chemicals on nontarget organisms, resurgence of pesticide resistance among pathogens and outbreak of secondary pathogens. Hence, there is an urgent call for increased crop production to cater to the needs of the increasing population. In order to reconcile with the demands of intensive agriculture with maintenance of the ecosystem, pest control strategies employed in the future must be environmentally compatible and selective to target pests.

9.2 Rationale for Transgenic Peanut Breeding

From USDA estimates (FAS 2000), peanuts ranked third in production among oilseeds and 90% of world peanut production was accounted by developing countries (ERS 2001) with 2.5% increase annually. Though the world harvested area of peanut has changed very little since 1970s with an annual growth of only 0.1% (between 1972-1990) and 1.2% (between 1991-2000), the production has increased from 0.8 metric tons (during 1972) to 1.37 metric tons (during 2000) i.e., 1.9% increase per year (Revoredo and Fletcher 2002). It is generally accepted that the average yield of peanut is below its presumed potential, and efforts to improve the productivity of this crop by conventional breeding means have not been very effective. The major reason behind this is the lack of sufficient and satisfactory levels of genetic variability within the germplasm of

cultivated peanut. Many wild annual *Arachis* species, which possess a wealth of agronomically desirable genes, are sexually incompatible with the cultivated varieties. Several advanced research institutes or groups are working with ICRISAT and other partners to apply modern biotechnology to the problems of peanut improvement in developing countries. Biotechnology tools such as marker-assisted breeding, tissue culture, *in vitro* mutagenesis, embryo rescue and genetic transformation have contributed to solve or reduce some of these constraints. Major yield increases could be achieved by development and use of cultivars addressing abiotic and biotic stresses. Comprehensive reviews on the history of molecular marker development in peanut were provided by Stalker and Mozingo (2001) and Dwivedi et al. (2003). However, only limited success has been achieved so far. The emergence of "omics" technologies and the establishment of model legume plants such as *Medicago truncatula, Glycine max* and *Lotus japonicus* (Cannon et al. 2009) are promising strategies for understanding the molecular genetic basis of stress resistance, which is an important bottleneck for molecular breeding. Understanding the mechanisms that regulate the expression of stress-related genes is a fundamental issue in plant biology and will be necessary for the genetic improvement of legumes (Bertioli et al. 2011).

Transgenic research has opened exciting opportunities in plant protection which result in prolonged benefit in sustainable agriculture with a high degree of safety which is also an important part of second green revolution. The techniques of genetic modification will allow breeders to access new gene pools, particularly those of wild *Arachis* species, bringing valuable traits into the modern cultivated peanut that cannot be addressed by conventional means. Development of transgenic peanut therefore has a good potential for its improvement. Advances in biotechnology have provided alternative pest control strategies that are based on natural biological processes. Tissue culture and genetic engineering have proven as important powerful tools in biotechnology that have been extensively used, either by taking advantage of naturally occurring defense mechanisms, which confer disease resistance of avoidance or by modifying plant genome to develop pest resistance.

9.3 Genetic Transformation in Peanut

Successful genetic transformation of plants, including peanut, generally requires a reproducible tissue culture system to regenerate whole fertile plants from single cells (totipotency) as well as a method to deliver the gene(s) of interest to those regenerating cells. Transformation frequencies are directly related to the tissue culture response, and therefore highly regenerative cultures are often transformation competent. The inefficient, inconsistent and genotype dependent published protocols for peanut regeneration have emboldened some researchers in adopting non tissue culture-based approaches, that do not depend on the regeneration of adventitious shoot buds for generating transgenic plants of peanut (Rohini and Rao 2000). *In vitro* regeneration of whole plants of economically important commercial cultivars of peanut from explants such as protoplasts, cell suspension cultures, callus tissue or organized tissue such as embryonic axes, mature and immature embryonic axes (Atreya et al. 1984; Hazra et al. 1989; Brar et al. 1994; Baker et al. 1995), cotyledons (Atreya et al. 1984; Ozias-Akins 1989) and leaves (Baker and Wetzstein 1992; Livingstone and Birch 1995) either by organogenesis or embryogenesis have been reported with different culture media containing different phytohormone combinations (Table 9.1, 9.2).

Regeneration by organogenesis occurs either by direct development of shoots from the surface of cultured explants (Hazra et al. 1989; McKently et al. 1991) or by an intervening callus phase (Bajaj et al. 1981; Bajaj and Gosal 1983, 1988). The reports of organogenesis from de-embryonated cotyledons, immature leaflets, seed explants, epicotyls, hypocotyls and anther-derived callus (Mroginski and Fernandez 1980; Mroginski et al. 1981; Narasimhulu and Reddy

Table 9.1: Responses of various explants and hormones on *in vitro* shoot regeneration in peanut

Explant	Medium	Growth regulators	Morphogenic response	Genotype/ cultivar	Reference
Ovaries	MS	BA (0.5 mg/l) + NAA (2 mg/l)		MK 374, M 13, TMV 2, Robut-33-1	Sastri et al. 1980
Ovules	MS	Kinetin+ GA_3	Shoots and roots		Martin 1970
Immature embryos	-	TDZ (10 mg/l)	-	New Mexico Valencia	Kanyand et al. 1994
	B5	Picloram (0.5- 1 mg/l)	Shoots with roots	Several varieties	Ozias- Akins et al. 1992
Cotyledonary nodes	MS	NAA (1 mg/l) +BA (3 mg/l)	Multiple shoots		Banerjee et al. 1988
De-embryonated cotyledons	MS	Zeatin (4 mg/l) or kinetin (4 mg/l)	Multiple shoots	MK 374, M 13, TMV 2, Robut-33-1	Sastri et al. 1980
	MS	2,4-D (2 mg/l)+ kinetin (2 mg/l)	Multiple shoots	ICG 4367, US 48, TMV 2, TG 19B	Narasimhulu and Reddy 1983
	Moist cotton wool	BA (1 mg/l)	Multiple shoots	TG-17	Bhatia et al. 1985
Mature cotyledons	MS + B5 organics	BA(20μM)+ 2,4-D (10μM)	Multiple shoots	JL-24,J-11, ICGS-11, ICGS-44, Robut 33-1	Sharma and Anjaiah 2000
Embryo axis	MS	None	Shoots regenerated into plantlets		Atreya et al. 1984
Epicotyl	MS	Casein hydrolysate	Multiple shoots, roots		Bajaj 1982
	MS	BA (10 mg/l)+ NAA (1 mg/l)	Organogenesis	New Mexico Valencia	Cheng et al. 1992
	MS	None	9-28% shoots	ICG 4367, US 48, TMV 2, TG 19B	Narasimhulu and Reddy 1983
Mesocotyl	MS	IAA (11μM)+ kinetin(2.3μM)	Shoots with roots		Bajaj 1982

Hypocotyl	MS	IAA (2 mg/l)+ kinetin (2 mg/l)	Shoots	ICG 4367, US 48, TMV 2, TG 19B	Narasimhulu and Reddy 1983
Apical meristem	MS +B5 vitamins	NAA (10μM)+ BA (0.1μM)	Single shoots with many roots		Kartha et al. 1981
		NAA (10μM)+ BA (1μM)	Shoots without any further development		Kartha et al. 1981
Plumule	MS	BA(30μM)+ NAA(5μM)+ brassin (1μM)	Multiple shoots	Okrun	Ponsamuel et al. 1998
Immature leaflets	MS + Gamborg vitamins	NAA (1 mg/l)+ BA (1 mg/l)	50% shoots		Pitman et al. 1983
	MS	NAA (4 mg/l)+ BA (5 mg/l)		JL24	Chengalrayan et al. 1994
	MS	NAA (2 mg/l)+ BA (4 mg/l)	Shoots	NC-7	Utomo et al. 1996
Leaflets	MS	NAA (1 mg/l)+ BA (1 mg/l)	Organogenic callus		Mroginski et al. 1981
		BA (2 mg/l)+ NAA (0.5 mg/l)	Shoot primordia	TMV2	Venkatachalam et al. 1999

MS: Murashige and Skoog (1962)

1983; Pittman et al. 1983; McKently et al. 1990; Willcox et al. 1991; Li et al. 1994) had a very low frequency of transformation. However, not much success with genetic transformation of peanut genotypes was achieved until recently (Sharma and Anjaiah 2000) due to the lack of efficient protocols to obtain whole plants through *in vitro* regeneration of adventitious shoot buds from the transformed tissues. Direct regeneration systems favors easy accessibility for *Agrobacterium*-mediated genetic transformation because of advantages of de novo production of shoot primordia, synchronous with the period of cellular differentiation, rapidity of morphogenesis and lack of requirement for frequent subcultures. Sharma and Anjaiah (2000) obtained success of high-frequency direct shoot regeneration from mature cotyledon explants in various peanut genotypes. Shoot organogenesis and plants were also successfully obtained using immature leaflets (McKently et al. 1991; ICRISAT unpubl. data).

Regeneration via somatic embryogenesis also has been reported (Gill and Saxena 1992; Zhuang et al. 1999; Cucco and Jaume 2000) which has been used in transformation studies in peanut (Ozias-Akins et al. 1992, Sellars et al. 1990, Chengalrayan et al. 1994, 1997). However, conversion of somatic embryos into plants remains inefficient and limits the application of somatic embryogenesis in many systems, including genetic transformation (Wetzstein and Baker 1993).

Developments in genetic transformation for incorporation of novel genes into the peanut gene pool have emboldened researchers with new opportunities for crop improvement in this important legume to pursue the development of transgenic peanut plants resistant to various diseases, insect pests, enhanced nutritional quality and abiotic stresses (Sharma and Anjaiah 2000; Rohini and Rao 2001). Transformation of plants involves the stable introduction of desirable DNA/gene sequences into the nuclear genome of cells, which are capable of giving rise to a whole transformed plant. Transformation and regeneration are interdependent and the totipotency (i.e., single cell capable of giving rise to a whole plant *in vitro*) of the somatic plant

cells via organogenesis or somatic embryogenesis under appropriate hormonal and nutritional conditions (Skoog and Miller 1957) is the essential feature for development of an efficient tissue culture techniques. Totipotent cells give rise to adventitious shoots or somatic embryos, which are both competent and accessible for gene transfer and will give rise directly to nonchimeric transformed plants. Development of an efficient transformation system for the introduction of genes into the crop plants also depends on the various factors such as development of reliable and reproducible tissue culture regeneration systems, selection and preparation of suitable gene constructs and vectors, recovery and multiplication of transgenic plants, molecular and genetic characterization of transgenic plants for stable and efficient gene expression, transfer of genes to elite cultivars by conventional breeding methods if required, evaluation of transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses in the field condition, biosafety assessments including health, food and environmental safety and deployment of genetically modified plants.

A suitable system for selection of transgenic tissues and plants is one of the most important aspects of any transformation system. The utility of any particular gene construct as a transformation marker varies depending on the plant species and explant involved. Promoters are essential to control expression of the gene and also provide valuable insights about the overexpression or silencing of any gene in response to external stimuli. The most commonly developed transgenic plants use either the constitutive promoters like 35S of the Cauliflower Mosaic Virus (CaMV) or the maize ubiquitin or potato ubiquitin (Yang et. 2003; Joshi et al. 2005) to drive expression of the gene of interest in their gene constructs. These promoters being constitutive in nature sometimes results in expression of the downstream transgenes in all organs and at all the developmental stages, which can be metabolically expensive leading to undesirable pleiotropic effects (Bhatnagar-Mathur et al. 2008). Hence, use of inducible or tissue-specific promoters is increasing in recent years for enhancing targeted gene expression, which also

safeguards against biosafety and regulatory concerns to a certain extent. Use of these tissue-specific constructs is also important in RNAi technology to augment gene silencing strategies (Bhatnagar-Mathur et al. 2008).

The transformation and regeneration protocols for peanut are now well-established. Transformation techniques and plant regeneration from *in vitro* cultured tissues have been described for many species (Lindsey and Jones 1989; Dale et al. 1993; Birch 1997). There are numerous reports of tissue culture and transformation of peanut from various explants (Kartha et al. 1981; Sastri and Moss 1982; Kanyand et al. 1994). Regeneration via somatic embryogenesis has also been reported as one of the promising methods for transformation studies in peanut (Ozias-Akins et al. 1993, Sellars et al. 1990, Baker and Wetzstein 1995, Chengalrayan et al. 1994, 1997).

9.4 Transfer of Genetic Material

Different methods of DNA transfer have been developed for the production of transgenic peanut over the last few years. The most commonly used means of DNA delivery or transferring novel genes into either organogenic or embryogenic cultures of plant cells/ peanut are either biologically by *Agrobacterium tumefaciens* or by direct gene transfer using microprojectile/ particle bombardment or by electroporation (Table 9.2). Research is being carried out globally with single or multiple gene introductions to produce disease resistant, pest-resistant, healthier and high-quality peanuts. Peanut tissues are susceptible to infection by wild-type strains of *A. tumefaciens* (Lacorte et al. 1991). The choice between using microprojectile bombardment or *Agrobacterium* as the means by which to deliver DNA is determined by several factors including the laboratory facilities and technical skills available, the species and/or cultivar to be transformed (many monocots are still recalcitrant to transformation with *Agrobacterium*, although this is improving all the time), and the regeneration system.

Table 9.2: Update on Genetic transformation in peanut

Explant	Gene delivery system	Gene introduced	Transformation frequency/status	Strain/Plasmid	Reference
Cotyledon	*Agrobacterium*		3.30%		Rohini and Rao 2000
	Agrobacterium	*uidA, nptII*	47%	LBA4404, pBI121	Venkatachalam et al. 2000
	Biolistic	*uidA, hph*	1.6%	pCAMBIA-1301	Yang et al. 2001
	Biolistic	*uidA,hph*	168 hygomycin resistant lines	pMOG617/ pxVGH	Wang et al. 1998
	Agrobacterium	*gus, nptII*	T2 generation viable seeds		ICRISAT 1994
Leaf	*Agrobacterium*	*Gus,nptII*	0.2-0.3%	pBI121	Cheng et al. 1997
Embryonic axis	*Agrobacterium*	*uidA,nptII*	9%	EHA101/ pMON9793	McKently et al. 1995
	Agrobacterium	*Bar* and *PSTV genome*	Putative transformants		Cassidy and Ponsamuel 1996
	Biolistic / Particle bombardment	*MerApe9, hph/MerApe9* ,mercuric ion reductase		pAC2MR/ pACH2MR	Yang et al. 2003
	Biolistic		0.9-1%		Brar et al. 1994
Leaf, epicotyl	*Agrobacterium*	*uidA*	12-36% (leaves), 15-42% (epicotyls)	EHA 101	Egnin et al. 1998
Leaf Discs	*Agrobacterium*	*uidA, nptII*	6.7% putative shoots;	pBI121	Eapen and George 1994
Embryonic axis,	*Agrobacterium*	*uidA, nptII*		pTiBo542/ pTiT37	Lacorte et al. 1991
cotyledon, leaf, petiole explants,	*Agrobacterium*	*IPCV (coat protein)*	55%	pBI121/pROKII: IPCVcp	Sharma and Anjaiah 2000
Mature cotyledons	*Agrobacterium*	*H protein gene*			Khandelwal et al. 2003

Seedling explants	*Agrobacterium*	*Gus, nptII*	Second generation callus colonies		Li et al. 1996
Protoplast	Electroporation	*PstV coat protein*	Protoplast derived callus colonies		Li et al. 1996
	Electroporation	*Gus, nptII*			Li et al. 1996
Embryonic leaflets	Electroporation				Padua et al. 2000
Epicotyl	Biolistic	*uidA, hph*		pKYLX80-N11 pTRA140	Magbanua et al. 2000
Embryonic callus	Biolistic	*Luc, hph*	54 independent transgenic lines	pDO432/pHygr/ pGIN	Livingstone and Birch 1995
	Biolistic	*hph*	1%		Ozias- Akins et al. 1993
Shoot meristem of embryonic axis	ACCELL (biolistic)	*Gus, bar, TSWV nucleocapsid protein*	Transgenic plants up to R2 generation		Brar et al. 1994
Somatic embryos	Biolistic	*hph gene, nucleocapsid protein gene of TSWV*	52 hygromycin resistant cell line	pCB13-N+ pCB13-N++	Yang et al. 1998
Immature cotyledons	Biolistic	*cry1AC*			Singsit et al. 1997
Mature Zygotic embryos	Biolistic	*GFP*		p524EGFP.1	Joshi et al. 2005

Gus/uidA: gene encoding glucoronidase activity*; hph*: gene conferring resistance to hygromycin*; nptII*: gene conferring resistance to neomycin and kanamycin*; TSWV*: tomato spotted wilt virus*; PStV*: peanut stripe virus*; PCV*: peanut clump virus*; bar*: gene conferring resistance to herbicide resistance

9.4.1 Direct Gene Transfer

Direct DNA transfer methods can circumvent the genotype dependence of *Agrobacterium* infection. Direct gene transfer has been accomplished by several methods such as microprojectile bombardment, electroporation of protoplasts and intact tissues, microinjection of protoplasts or meristems and polyethylene glycol-mediated transformation of protoplasts. Among these, microprojectile bombardment is the most commonly used method for genotype-independent genetic transformation (Sharma et al. 2005).

Particle bombardment was developed by Sanford and coworkers (Sanford et al. 1987; Klein et al. 1988; Sanford 1990) and has been the most commonly used method for direct introduction of genes into a number of plant species including peanut. Transient expression (Li et al. 1995) was reported from cultures developed through bombardment of callus lines from immature peanut leaflet tissue (Clemente et al. 1992) and leaflets (Schnall and Weissinger 1995). However, bombardment of 1-2-year-old embryogenic callus derived from immature embryos followed by stepwise selection for resistance to hygromycin in semi-solid and liquid media produced transgenic shoots at a frequency of 1% (Ozias-Akins et al. 1993), while the shoot meristems of mature embryonic axis produced transgenic plants at a relatively low transformation frequency of 0.9-1.0% (Brar et al. 1994). Transgenic peanut plants using the somatic embryos were developed from immature cotyledons by transforming the *cry1Ac* gene for resistance to the cornstalk borer (*Elasmopalpus lignosellus*) (Singsit et al. 1997). Similarly, Livingstone and Birch (1995) obtained efficiently transformed Spanish and Virginia types of peanut by particle bombardment into embryogenic callus derived from mature seeds. More recently, cobombardment of embryogenic callus derived from mature seeds was used to develop peanut lines exhibiting high levels of resistance to Peanut Stripe Virus (PStV) (Higgins et al. 2004). Similarly, using particle bombardment transient expression of GUS and 2S albumin gene from Brazil nut was observed in peanut (Lacorte et al. 1997).

The advantages of particle bombardment system is that DNA may be transferred directly to cells by the introduction of multiple DNA fragments or multiple plasmids by cobombardment without using specialized or binary vectors, thus eliminating the necessity of constructing a single large plasmid containing multiple transforming sequences. However, the biolistic-based system is labor intensive since it requires bombardment of large number of explants for obtaining few stable transformation events. It may also result in the integration of multiple copies of the transgene, thereby leading to gene silencing which is the major drawback.

9.4.2 Agrobacterium-Mediated Genetic Transformation

The naturally-evolved unique system of *Agrobacterium* transfers the foreign DNA sequences precisely into plant cells using Ti plasmids. *Agrobacterium*-mediated transformation is the preferred method over microprojectile bombardment for gene delivery as it results in higher frequency of stable transformation with single or fewer integrated transgene copies, thus reducing the risk of gene silencing and transgene rearrangements. Moreover, when compared to direct DNA delivery system, *A. tumefaciens* infections are less complex and *Agrobacterium*-mediated transformation is generally precise in transferring and integration into the plant genome as it delivers long stretches of T-DNA between the right and left borders.

Several reports have been published for transforming peanut using *A. tumefaciens* method using hypocotyl explants (Dong et al. 1990; Lacorte et al. 1991; Mansur et al. 1993), leaf explants (Eapen and George 1994), and embryonic axes from mature seeds of peanut (McKently et al. 1995). High transformation frequency was reported by using precultured cotyledons as explants (Venkatachalam et al. 1998, 2000), or leaf segments with 0.3% frequency of fertile transgenic plants (Cheng et al. 1997), whereas stable 3% transformation frequency was reported using a nontissue-culture based *Agrobacterium* transformation involving direct cocultivation of cotyledon attached embryo axis supplemented with wounded tobacco leaf extract (Rohini and Rao 2000). Sharma and Anjaiah (2000) reported an efficient transformation system with >55%

transformation frequency using cotyledon explants. Recently, promoter tagged peanut transgenics using the cotyledonary nodes as explants and a promoter-less fusion gene *nptII:gus* were produced (Anuradha et al. 2006).

9.5 Selection of Transformed Plants

Uptake of DNA transferred by either method only occurs in a minority of cells and selection of those cells is crucial. Most vectors used for the genetic transformation of plants carry marker genes that allow selection and screening of the transformed cells. More than 50 marker genes and molecular techniques were reported to screen for genetic transformation (Liang et al. 2010), which are divided into two categories: a) Selectable markers, and b) Screenable (scorable, reporter, visible) markers. Marker genes are usually co-introduced into a plant genome along with the transgenes in a single plasmid (Curtis et al. 1995), or as separate effector (for genetic transformation) and reporter (for screening) plasmids (Sakuma et al. 2006a). Protocols with selectable markers have yielded 10-fold higher frequency of recovered transgenic events compared to marker-free protocols (Birch 1997; de Vetten et al. 2003; Darbani et al. 2007) and so the use of marker genes is advantageous. Positive selectable marker genes promote the growth of transformed tissue whereas negative selectable marker genes inhibit growth or kill the nontransformed tissue (Liang et al. 2010).

Inclusion of selectable marker genes encoding resistance to an antibiotic such as kanamycin or hygromycin or to a herbicide such as phosphinothricin, glyphosate, bialaphos and several other chemicals (Wilmink and Dons 1993) in addition to the gene(s) of interest, allows the selection of such cells, by addition of the compound to the nutrient medium. Cells that express the resistance gene can proliferate while the untransformed cells die. Judicious choice of antibiotic and concentration levels may be an important criterion for the recovery of transformed cells, because too high a level would be deleterious even to the transformed cells at initial stages of screening. For peanut, hygromycin B is the most appropriate compound for the selection of

transformed cells whereas kanamycin was also reported to be an effective selection agent to select stably transformed callus tissue obtained from immature leaflets of peanut (Clemente et al. 1992). The herbicide Basta® (active ingredient phosphinothricin) has also been used to select transgenic peanut tissue (Brar et al. 1994).

Screenable (reporter) genes have also been developed from bacterial genes, which encode proteins that are used for easy detection in a sensitive, specific, quantitative, reproducible and rapid manner, to measure transcriptional activity and are used to investigate promoters and enhancers of gene expression and their interactions. Some of the reporter genes reported include chloramphenicol acetyltransferase (CAT; Herrera-Estrella et al. 1983), a bacterial enzyme that transfers radioactive acetyl groups to chloramphenicol; Luciferase (LUC/ LUX; Olsson et al. 1988), a firefly enzyme that oxidizes luciferin and emits photons; Green fluorescent protein (GFP; Reichel et al. 1996), an autofluorescent jellyfish protein; β-galactosidase (GAL), a bacterial enzyme that hydrolyzes colorless galactosides to yield colored products; β-glucuronidase (GUS; Beason 2003) (an enzyme that hydrolyzes colorless glucuronides to yield insoluble colored products) and nopaline synthase, and octopine synthase (Herrera-Estrella et al., 1988). ß-glucuronidase or GUS (Jefferson 1987) is the most commonly used reporter gene in plant genetic transformation studies including peanut. Assays for screenable markers can be destructive or nondestructive, in terms of the need to sacrifice the test material. GFP in peanut was reported as a nondestructive gene which requires no exogenous substrate to fluoresce by Joshi et al. (2005).

Identifying the small proportion of transformed cells in a large experimental cell population, using only screenable markers is tedious and time consuming. Hence, screenable markers are usually coupled with selectable markers in transformation systems as in almost all commercialized transgenic crops (Liang et al. 2010).

9.6 Future Roadmap for Transgenic Peanut

Genes for transformation can be broadly divided into those that will be used to overcome agronomic limitations (high yield potential, resistance to biotic and abiotic stresses) and ones that could be used to enhance value-added traits (Schnall and Weissinger 1995). Although major emphasis is currently being placed on improving the primary constraints, the manipulation of value-added traits, such as flavor and nutrition will be of much concern for peanut improvement using transgenic technology. Transgenic technology could conceivably be used in peanut for the introduction of disease and pest resistance as well as value-added traits such as improved vitamin, protein and oil quality, enhancing the crop product value, quality and safety. The genus *Arachis*, which itself is a repository for most of the valuable pest and disease resistance genes, could be used to transform cultivated peanut varieties (Bhatnagar-Mathur et al. 2008). Current efforts include incorporating immunity or very high resistance to several viral and fungal diseases through transformation of peanut cultivars that have very high demand for which no adapted resistant peanut genotypes are available. Improved crop protection through the transfer and expression of disease resistance genes will decrease or eliminate the usage of pesticides, which are costly to the grower and may be harmful to the environment.

9.6.1 Abiotic Stress Tolerance

Drought is the major cause for low and erratic pod yield in peanut that contributes to over 6.7 million t loss in annual world peanut production (Subbarao et al. 1995), resulting in estimated monetary losses of over US$ 520 million annually (Sharma and Lavanya 2002). Yield losses in peanut due to water deficits vary depending on timing, intensity and duration of the deficit, coupled with other location-specific environmental stress factors such as high irradiance and temperature (Nigam et al. 2001). Due to the scarcity of available water in semi-arid tropics regions, drought management strategies, whether agronomic or genetic, therefore need to focus

on maximizing extraction of available soil moisture and the efficiency of its use in crop establishment, growth, biomass and grain yield (Serraj et al. 2005).

Many genes that display altered expression patterns in response to environmental stresses have been identified over the last 10 years (Bray 2004; Shinozaki and Yamaguchi-Shinozaki 2007) and the functions of some of these genes have been studied in detail (Vinocur and Altman 2005; Lemaux 2008, 2009; Mittler and Blumwald 2010). Several genes that confer drought tolerance have been tested in the field for many years (Yang et al. 2010) among which a few are waiting for the approval of commercial release at US federal regulatory agencies (Castiglioni et al. 2008; Yang et al. 2010).

Transgenic research using transcription factors has been the most widely used technology in developing drought-tolerant varieties (Dubouzet et al. 2003; Pellegrineschi et al. 2004; Oh et al. 2005; Behnam et al. 2006; Xiao et al. 2006; Wang et al. 2008; Morran et. 2011). At ICRISAT, efforts for enhancing drought tolerance in peanut through genetic engineering was initiated as early as 2003 through *Agrobacterium*-mediated genetic transformation of drought sensitive cultivar of peanut, JL 24, using the transcription factor *At*DREB1A driven by constitutive CaMV35S promoter as well as a drought-responsive promoter rd29A, which resulted into ~18 35S:DREB1A and 50 rd29A: DREB1A T_0 transformants. Fourteen transgenic events showing high levels of stress tolerance were screened under contained greenhouse (Bhatnagar-Mathur et al. 2004, 2006) and field conditions (Bhatnagar-Mathur et al. 2013). Substantial yield improvement of at least 17% was observed under drought-stress conditions in a field trial across a wide range of vapor pressure deficits, where one of these transgenic events showed 40% higher transpiration efficiency than the control plants under water-limiting conditions (Bhatnagar-Mathur et al. 2007, 2009, 2013).

Another study revealed that transgenic plants having *AtNHX1* gene are more resistant to high concentration of salt and water deprivation than the wild type plants in which salt and

proline level in the leaves of the transgenic plants were also much higher than that of wild type plants (Asif et al. 2011). Similarly, regulated expression of isopentenyl transferase gene (*IPT*) in peanut significantly improved drought tolerance under both laboratory and field conditions (Qin et al. 2011).

9.6.2 Resistance to Biotic Stresses

Diseases attack by different pathogens which include primarily fungi, bacteria, viruses, mycoplasma, nematodes, insect pests and parasitic flowering plants are major constraints to peanut production throughout the world causing majority of economic losses of yield up to 40 to 60%. Although, many diseases infect the crop, only a few cause significant reduction in yields. Comparatively low annual yields have been reported in developing countries (~825 kg/ha) to developed countries (2,650 kg/ha). The major biotic stresses for peanut include the foliar fungal diseases, leaf spot (early and late) and rust. Seed and soil-borne diseases like collar rot, stem rot and dry root rot have also been identified as important. Among viral diseases, bud necrosis (BND), peanut mottle (PMV) and peanut clump (PCV) are important. With regard to insect pests, a wide range of pests like leaf miner, tobacco caterpillar, white grub, jassids, thrips, aphids, red hairy caterpillar and termite are known to cause serious damage to peanut crop (Ghewande et.al. 1987; Basu 1995).

However, crop improvement by conventional breeding lacks to meet the demands of increasing population, especially in seed quality improvement and developing virus and insect-resistant varieties. Therefore, in peanut the Expressed Sequenced Tags (EST) would be a quick and economical approach to identify important peanut genes involved in defense response against fungal infections and also provide data on gene expression and regulation (Houde et al. 2006; Nelson and Shoemaker 2006). Utilizing genomic and proteomic tools, genes and proteins associated with *A. parasiticus* and drought stress were identified (Luo et al. 2005; Guo et al. 2006, 2008). Identified genes could be used for enhanced fungal disease resistance in peanut through marker-assisted selection in breeding or by direct up or down regulation of the target

gene using genetic engineering. Identification of novel promoter and enhancer elements will also be critical to achieving efficacious expression of antifungal/anti-mycotoxin genes. The protocol for genetic modification is now standardized and available for routine applications (Sharma et al. 2000; Bhatnagar-Mathur and Sharma 2006). Hence the major focus lies on developing transgenic peanut varieties for resistance to insect pests/fungal pathogens/important viruses.

9.6.2.1. Fungal Diseases

Poor realization of potential yields has been mainly attributed to diseases in peanut (Ghuge et al. 1981, Chohan 1974). Fungal diseases in peanut are the most significant limiting factor causing more than 50% yield losses throughout the world. Among the foliar fungal diseases Early Leaf Spot (ELS) caused by *Cercospora arachidicola* S. Hori (*Mycosphaerella arachidis* Deighton), Late Leaf Spot (LLS) caused by *Phaeoisariopsis personata* Berk. & M.A. Curtis (*M. berkeleyi*), rust (*Puccinia arachidis*), crown rot (*Aspergillus niger* Teigh.), collar rot caused by *Aspergillus* spp., root rot caused by *Macrophomina phaseolina*, stem rot caused by *Sclerotium rolfsii* and Yellow mold (*Aspergillus flavus* and *A. parasiticus*) causing aflatoxin contamination are the major fungal diseases affecting peanut crop. (Subrahmanyam et al. 1985; McDonald et al. 1985) (Table 9.3). Infection by these fungal pathogens results in severe yield losses and generates poor quality seeds (Pretorius 2005). The use of disease resistant peanut cultivars is the only means of controlling fungal diseases in peanut. Genetic enhancement in peanut through conventional breeding and chemical control has yielded only limited success (Nigam et al. 2012) and the narrow genetic base of the cultivated peanut *Arachis hypogaea* L. hampers the development of improved varieties through conventional breeding leaving with the development of transgenics as the only option.

9.6.2.1.1 Leaf spots: The annual economic losses caused by LLS and rust account for over US$ 599 m and US$ 467 m, respectively (FAO 2004) by causing yield loss of 50-70% (Gibbons 1980; Subrahmanyam et al. 1980a, b, 1984). These diseases damage the plant by reducing the green leaf

Table 9.3: Genetic Transformation of peanut against major fungal diseases/ pathogens.

Disease/pathogen	Gene	Source	Reference
Late leaf spot by *Phaeoisariopsis personatum*	*Chitinase*	Tobacco	Rohini and Rao 2001
	Chitinase	Rice	Chenault et al. 2005
	Glucanase	*Alfa alfa*	
Early Leaf spot by *Cercospora arachidicola*	*Glucanase*	Tobacco	Sundaresha et al. 2010
	Chitinase	Bacteria	Iqbal et al. 2011
	Chitinase	Rice	Iqbal et al. 2012
	Chitinase	Rice	ICRISAT unpublished
A. flavus	*Glucanase*	Tobacco	Sundaresha et al.2010
	mod1,	Maize	Weissinger et al. 2003
	D5C,		Weissinger et al. 1999
	anionic peroxidase		
	synthetic peptide D4E1	Tomato	Ozias-Akins et al. 2000
Cercospora arachidicola Hori. and *Phaeoisariopsis personata*	*SniOLP*	*Solanum nigrum*	Vasavirama and Kirti 2010
	Rs-AFP2	Radish (*Raphanus sativus*)	
	defensin	mustard	Anuradha et al. 2008
Sclerotinia blight	*oxalate oxidase gene*	barley	Livingstone et al. 2005
	Chitinase	Tobacco	Rohini and Rao 2001
	Chitinase	Rice	Chenault et al. 2005
	Glucanase	*Alfa alfa*	
A. flavus and aflatoxin biosynthesis	*Loxl*	Soybean	Ozias-Akins et al. 2000
	Nonheme chloroperoxidase gene(cpo)	*Pseudomonas pyrrocinia*	Niu et al. 2009
	nonheme chloroperoxidase gene	bacteria	Ozias-Akins et al. 2003
	PnLOX3	Peanut	ICRISAT Unpublished

area available for photosynthesis and by stimulating leaflet abscission leading to extensive defoliation (McDonald et al. 1985) which results in lower seed quality, reduced seed size and oil content besides affecting the haulm production and quality.

9.6.2.1.1.1 Early Leaf Spot:

Early Leaf Spot, caused by *Cercospora arachidicola* was first reported from Japan in 1919 (Hemingway 1955). Interestingly, transgenic approaches using bacterial and rice chitinase genes for resistance to early leaf spot in peanut showed fairly good positive correlation between chitinase activity and fungal pathogen resistance (Iqbal et al. 2011, 2012) in which two lines transformed with bacterial chitinase gene showed 56-62% suppression of disease over the nontransgenic controls. Similarly, use of tobacco chitinase gene (Sundaresha et al. 2010) for developing transgenic peanuts against *Cercospora arachidicola* resulted in 16 plants which performed well against infection in the *in vitro* leaf bioassay against *Cercospora*, seven transgenic plants that showed the lowest percent disease index (i.e. 0-25% of leaf area was covered by spots) and delay in the onset of disease were considered to be resistant and were selected for analysis for further generations (Sundaresha et al. 2010).

9.6.2.1.1.2 Late Leaf Spot:

Late Leaf Spot, caused by *Phaeoisariopsis personatum* was first described in the USA in 1885 (Jenkins 1938; Kolte 1985). Transgenic peanuts expressing tobacco chitinase gene (Rohini and Rao 2001), rice chitinase and an alfalfa glucanase gene (Chenault et al. 2005) have been shown to possess enhanced resistance to the late leaf spot. More recently, transgenic peanut plants carrying mustard defensin gene showed variable increased disease resistance to *Cercospora arachidicola* and *Phaeoisariopsis personata* in detached leaf assays and greenhouse evaluations using conidial suspensions (Anuradha et al. 2008). Similarly, over expression of SniOLP (osmotin like protein cloned from *Solanum nigrum*) and Rs-AFP2 (defensin gene from Radish (*Raphanus sativus*)) in a double construct resulted in enhanced resistance against *Cercospora arachidicola* and

Phaeoisariopsis personata in transgenic peanut (Vasavirama and Kirti 2010). At ICRISAT efforts are carried out for developing peanut transgenics using rice chitinase gene which resulted at about >50% decrease in disease incidence (Prasad et al. 2012).

9.6.2.1.2 Rust: Rust, caused by *Puccinia arachidis* is another potential peanut disease of economic importance not only in India but also in Africa, Asia, Oceania and Australia (Hammons 1977, Mayee 1982, 1986, 1987a, 1989, Mayee et al. 1977). At ICRISAT efforts have been made to develop peanut transgenics using rice chitinase gene that resulted in over 50% decrease in disease incidence (Prasad et al. 2012).

9.6.2.1.3 Sclerotinia blight: Blight disease is caused by soil borne fungus *Sclerotinia minor* and *Sclerotinia sclerotiorum*. Transgenic peanut expressing a tobacco chitinase gene (Rohini and Rao 2001), rice chitinase and an alfalfa glucanase gene (Chenault et al. 2005) has been shown to possess enhanced resistance to Sclerotinia blight, respectively. Transgenic events developed using somatic embryos of the Okrun cultivar (Chenault et al. 2002, 2005) were tested over a 3 year period (2000-2002) under field conditions where 14 transgenic lines showed up to 43 to 100% reduction in disease incidence compared to their parent line Okrun showing increased resistance to Sclerotinia blight. Similarly, overexpression of barley oxalate oxidase gene in transgenic peanut developed from embryogenic cultures of Virginia peanut cultivars, showed enhanced resistance to oxalic acid producing fungi, *Sclerotinia minor* (Livingstone et al. 2005). Detached leaflet bioassays carried out under laboratory conditions indicated reduction in the lesion area ranging from 75 to 97% in these transformed plants when compared to their respective nontransformed control cultivars. These transgenic peanut lines identified with partial resistance to *Sclerotinia* blight might be useful in traditional breeding programs for fungal resistance.

9.6.2.1.4 Aflatoxin: Peanuts are susceptible to aflatoxin contaminations which are toxic, carcinogenic substances produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Since conventional breeding methods for controlling aflatoxin are only partially effective, novel

biotechnological methods for enhancing host plant resistance to preharvest *A. flavus* invasion and aflatoxin contamination is considered to be the most cost-effective control measure. Besides, a complete knowledge of the resistance associated proteins/genes and their contribution to host plant resistance (comparative proteomics) is critical to harness their cumulative or complementary benefits in peanut for *A. flavus* infection and aflatoxin contamination.

Peanut produces stilbene phytoalexins in response to fungal infection. Organ-specific expression of multiple copies of a gene for stilbene synthesis (Stilbene synthase) has proven to inhibit fungal growth and spore germination of *Aspergillus* species and aflatoxin contamination. Hydrolytic enzymes such as chitinases and glucanases, which degrade the fungal cell wall, also pose as attractive candidates for development of disease-resistant peanut plants (Eapen 2003). Similarly, glucanase gene from tobacco introduced into peanut (PR protein from heterologous source) showed enhanced disease resistance to *in vitro* seed colonization (IVSC) and no accumulating aflatoxin (detected by HPLC) (Sundaresha et al. 2010). Maize and peanut transgenic expressing synthetic version of maize ribosome inhibiting protein gene, *mod1*, showed enhanced resistance to *A. flavus* and reduced aflatoxin contamination (Weissinger et al. 2003).

The aflatoxin biosynthetic pathway *in vitro* has been shown to be suppressed by enzyme encoded by soybean *loxl* gene that catalyzes the formation of a specific lipoxygenase metabolite of linoleic acid, (13S)-hydroperoxyoctadecadienoic acid ((13S)-HPODE). Transgenic peanut expressing soybean *loxl* gene under the control of carrot embryo specific promoter (DC3) (Ozias-Akins et al. 2000) resulted in reduction in the aflatoxin content. Efforts are being carried out at ICRISAT for generation of peanut transgenics with the rice chitinase gene (Prasad et al. 2012) and peanut lipoxygenase gene (*PnLOX3*). Work is being carried out at ICRISAT in developing construct for use in RNAi approach to suppress 9-hydroperoxide fatty acid producing lipoxygenases since incorporation of plant antisense genes for the 9-hydroperoxide fatty acid producing lipoxygenases also reduces mycotoxin contamination. Other antifungal genes such as

D5C (Weissinger et al. 1999), tomato anionic peroxidase (tap 1), and synthetic peptide D4E (Ozias-Akins et al. 2000) are transformed into peanut and evaluated for antifungal activity against *A. flavus*. However, pure D5C showed strong activity against *A. flavus in vitro*, due to phytotoxicity of D5C, transgenic peanut callus showed poor recovery of plants. Expression of *cry1A(c)* (Ozias-Akins et al. 2002) in transgenic peanut lines could also be an effective means of inhibiting *A. flavus* infection by reducing the damage into peanut pods by lesser cornstalk borer (LCB) *Elasmopalpus lignosellus*, since it has been clearly reported that aflatoxin contamination can increase with insect damage (Lynch and Wilson 1991). Similarly, Ozias-Akins et al. (2003) reported 60-70% reduction in *A. flavus* colony growth in transgenic peanut lines expressing the bacterial chloroperoxidase gene (Rajasekaran et al. 2000). Niu et al. 2009 reported antifungal activity in transgenic peanut by transforming with a non-heme chloroperoxidase gene from *Pseudomonas pyrrocinia.*

9.6.2.2 Viral Diseases

Viruses pose a great threat to peanut production throughout the world. Viruses such as the Indian Peanut Clump Virus (IPCV), Peanut Bud Necrosis Virus (PBNV), Groundnut Rosette Assistor Virus (GRAV), Peanut Mottle Virus (PMV), Peanut Stripe Virus (PStV), Tobacco Streak Virus (TSV), and Tomato Spotted Wilt Virus (TSWV) cause considerable damage to the crop. The concept of pathogen-derived resistance (Sanford and Johnston 1985) has stimulated research on obtaining virus resistance through genetic engineering. Since, the insertion of genetic material from the virus had been shown to confer resistance to infection by preventing virus replication and spread in several crop species. Genetic transformation has been used to develop peanut varieties with total resistance and not just tolerance to these viral diseases. The development of new viral control strategies depends on the molecular mechanisms underlying the roles of both dominant and recessive resistance genes (Ritzenthaler 2005). In general, protein-mediated resistance provides moderate protection against a broad range of related viruses while RNA-

mediated resistance has been shown to offer high levels of protection only against closely related strains of a virus (Pang et al. 1993, Lomonossoff 1995, Baulcombe 1996, Dawson 1996). Recent research indicates that pathogen-derived resistance to viruses is mediated, in most cases, by RNA-based Post-Transcriptional Gene Silencing (PTGS) mechanism (Baulcombe 2004) resulting in the degradation of mRNA produced both by the transgene and the virus. RNAi technology (RNA silencing or cosuppression of homologous genes) provides a significant tool for development of virus resistant peanut genotypes (Wang et al. 2000; Colbere-Garapin et al. 2005). The development of genetically transformed peanut cultivars with resistance to viruses and other biotic constraints potentially have tremendous impact on crop productivity, especially in the resource-poor agricultural systems of the semi arid tropics.

9.6.2.2.1 Groundnut rosette disease: Groundnut rosette disease is also one of the major destructive viral disease in sub-Saharan Africa (SSA) resulting in devastating losses to peanut production in Africa. The disease is caused by a complex of three casual agents such as Groundnut Rosette Assistor Virus (GRAV), Groundnut Rosette Virus (GRV) and a satellite RNA (satRNA) and is transmitted by an Aphid, *Aphis craccivora* (Naidu et al. 1998).

At ICRISAT Pathogen-Derived Resistance (PDR) for Groundnut Rosette Disease (GRD) by using GRAV*cp* gene has been exploited to induce host plant resistance to GRD for controlling GRD. Peanut transgenics for resistance to GRAV are being produced in ICRISAT (KK Sharma, unpubl. results) and the molecular characterized transgenic events have been transferred to South Africa for phenotyping under greenhouse conditions. Introduction of GRAV or GRV genomic sequences or genes, or SatRNA–derived sequences that down regulate GRV replication (Taliansky et al. 1996) into suitable peanut cultivars is an ideal RNA-mediated/ gene silencing approach.

9.6.2.2.2 Peanut Stem Necrosis Disease. PSND caused by Tobacco Streak Virus (TSV) was reported in India in 2000 (Reddy et al. 2002). TSV was reported as a frequent occurrence on

peanuts in Brazil (Costa and Carvalho 1961), but it was first noticed on peanut in 1999 in South Africa (Cook et al. 1999).

At ICRISAT, work is being carried out on engineering TSV resistance through *A. tumefaciens*-mediated transformation of popular peanut variety JL 24 (Spanish type) with TSV coat protein gene (*TSV cp* gene), and recovery of transgenic plants that block systemic movement of TSV spread. The resistant transgenic events identified under greenhouse conditions will be evaluated under restricted field conditions in the TSV hot-spots in the near future. Similarly, transgenic peanut lines containing sense and antisense coat protein gene of TSV transformed through *Agrobacterium*-mediated transformation of de-embryonated cotyledons of cultivar JL 24 are under evaluation for their reaction to TSV (Bag et al. 2007).

9.6.2.2.3 Peanut Bud Necrosis: Peanut Bud Necrosis Disease (PBND) is caused by PBNV - transmitted by *Thrips palmi*. Strategies to combat peanut bud necrosis disease (PBND) include development of transgenic peanut plants expressing PBNV nucleocapsid gene at ICRISAT, which showed a modest tolerance to PBND (Chander Rao et al. 2006). Three selected transgenic peanut events of T_1 and T_2 generation showed a 40 to 67% decrease in disease incidence under greenhouse virus challenging experiments. However, under field conditions in a contained on-station trial only one event showed less than 25% disease incidence. The expression of symptoms in some plants was delayed by 40-60 days and 14-21 days under greenhouse conditions and contained on-station trial respectively as compared to the control plants. Because of the unexpected lower frequency of virus resistant events throughout the challenging experiments, an alternate strategy based on RNA interference (antisense and hairpin-RNA) mediated gene silencing is being used as a potential tool to address a complex constraint like PBNV. Currently,, RNAi-mediated resistance approach to counter the effect of NSs gene in the PBNV genome is being pursued.

9.6.2.2.4 Tomato spotted wilt virus: Tomato spotted wilt virus (TSWV), first reported in Brazil (Costa 1941) is transmitted by thrips *Scirthothrips dorsalis* Hood (Mali and Patil 1979) and *Frankliniella schultzei* (Trybom) (Ghanekar et al. 1979).

Due to lack of availability of considerable levels of resistance in germplasm, development of transgenic plants through genetic engineering is the only effective approach for protection against TSWV which is carried over by both RNA and protein-mediated control (Pang et al. 1993). These approaches include using both sense and antisense TSWV nucleocapsid protein gene (*N* gene) expression. Nucleocapsid protein gene (*N* gene) was introduced into a runner and a Valencia type variety (Brar et al. 1994; Chenault and Payton 2003) whereas the *N* gene, was inserted into New Mexico Valencia A peanut, by Li et al. (1997). The field ratings from the study of Yang et al. (1998) indicated that there was a potential to combine nucleoprotein-mediated resistance in transgenic peanut with host-plant resistance that already had been identified in the peanut germplasm. Variety AT 120 transgenics with antisense nucleocapsid gene (Magbanua et al. 2000) and Marc 1 transgenics transformed with coat protein gene of TSWV (Ozias-Akins et al. 2002) showed lower disease incidence than respective nontransformed cultivar or than in moderately resistant cultivar Georgia Green. Transgenic progeny of Marc 1 peanut cultivar also showed lower incidence of spotted wilt in comparison to the nontransgenic controls in field evaluations and under controlled environmental conditions in the USA over years and locations (Yang et al. 2004), indicating its potential use in conventional breeding programs. Use of stable pathogen-derived resistance based on homology dependent RNA silencing for durable TSWV resistance was suggested by Bucher et al. (2003).

9.6.2.2.5 Peanut stripe virus (PStV): PStV is transmitted by seed and also by aphids (*Aphis craccivora, A. gossypii* and *Myzus persicae*). Transgenic plants of peanut varieties with high levels of RNA-mediated resistance to peanut stripe potyvirus (PStV) were obtained following cobombardment of embryogenic callus derived from mature seeds of the commercial cultivars,

Gajah and NC 7, which were transformed with one of the two forms of PStV coat protein (*cp*) gene (an untranslatable, full-length sequence (*cp* 2) or a translatable gene encoding a *cp* with an N-terminal truncation (*cp* 4)) (Higgins et al. 2004). Resistance to PStV was stably inherited over at least five generations in these transgenic plants of Gajah variety (Dietzgen et al. 2004). From the study of Hapsoro et al. 2005, 2007, three different kinds of response to PStV infection were identified-resistant, recovery and susceptible, the transgenic peanut lines cv. Gajah proved stable up to seven generations of selfing and some pure lines were identified. Franklin et al. (1993) reported transformed callus expressing the PStV coat protein gene through *Agrobacterium*-mediated genetic transformation.

9.6.2.2.6 Peanut Clump Virus (PCV): The disease is soil borne and is caused by peanut clump virus (PCV) that is transmitted by a fungus, *Polymyxa* sp. living in the soil. ICRISAT has developed the first-ever transgenic peanut, resistant to the dreaded Indian Peanut Clump Virus (IPCV) by the introduction of *coat protein (cp)* gene and *replicase (rep)* genes of the target virus IPCV by using *Agrobacterium*-mediated transformation (Sharma and Anjaiah 2000). Field evaluations were carried out twice against IPCV under controlled conditions during the rainy season of 2002-2004 in an on-station sick plot at ICRISAT, Patancheru, India with 10 transgenic lines carrying single gene inserts (five each with *IPCVcp* and *IPCVrep* genes) of which four transgenic events (three with *IPCVcp* and one with *IPCVrep*) showed complete resistance to IPCV.

9.6.2.2.7 Bacterial wilt: It is a soil-borne disease caused by *Ralstonia solanacearum.* A novel approach of introducing microbial toxins (phytotoxins) such as tabtotoxin acetyl transferase and glucose oxidase into the plant has emerged as an efficient way to develop resistance in a wide range of host species (Eapen 2003). This approach can be conveniently used to impart resistance against bacterial wilt of peanut caused by *Burkholderia solanacearum*, formerly known as *Pseudomonas solanacearum.*

9.6.2.3 Insect resistance

Among the insect pests *Spodoptera litura*, *Aproaerema modicella*, *Amsacta* spp., *Heliothis* spp., aphids, jassids, thrips and termites cause major yield losses. Though, a moderate level of resistance against specific pests was observed in wild relatives of peanut cultivars (Stalker and Moss 1987), but is often accompanied by undesirable agronomic features (low shelling and undesirable pod and kernel traits), interspecific reproduction barriers and linkage drag which impedes development of resistant cultivars using traditional breeding approaches. Hence the development of transgenic peanut for resistance to insects is gaining importance. The first transgenic peanut expressing *cry1EC* gene resistance to *S. litura* using de-embryonated cotyledon explants were developed by Tiwari et al. (2008). Leaf feeding bioassay was carried out twice under laboratory conditions on highly expressing transgenic lines, which showed 100% death of larvae at the 2nd instar stage of *S. litura*. Since, besides spodoptera, *Helicoverpa armigera* (Hubner) occasionally occurs on the peanut crop causing defoliation to a limited extent resulting in major crop loss, development of the peanut transgenics cv. TMV-2 expressing a chimeric *Bt* gene, *cry1X*, was reported (Entoori et al. 2008). *In vitro* detached leaf bioassays under laboratory conditions led to more than 50% mortality in 27 transgenic plants, showing not more than 10% damage against *H. armigera* and *S. litura*. Among the insect-pests, Lesser Cornstalk Borer (LCB), *Elasmopalpus lignosellus* (Zeller), is another major pest of peanut in the southern United States causing severe reduction in crop quality. Peanut transgenics against LCB using *cry1Ac* gene (Singsit et al. 1997) showed complete larval mortality to a 66% reduction in larval weight in insect feeding bioassay of transformed plants indicating various levels of resistance.

9.6.3 Biofortification and enhancing quality traits

Besides lysine, threonine and isoleucine, peanut is deficient in the essential amino acid methionine. The dietary and nutritional value of peanut can be improved by either raising the

level of sulfur-containing amino acids of storage proteins or by changing the proportion of methionine-rich proteins already present in the peanut seed. Genetic transformation is an effective and an alternative approach for developing methionine-rich peanuts.

Efforts have been made to identify genes that play an important role in controlling the crucial and important regulatory biochemical steps whose constituents play a major role in determining the quality of peanuts. Attempts have been made to produce transgenic peanut plants with improved protein quality by transferring genes like the Brazil nut 2S albumin gene (Lacorte et al. 1997). Malnutrition due to vitamin A, zinc (Zn) and iron (Fe) deficiencies is a significant public health issue in most of the developing and undeveloped world involving one-third of the world's population (~1.02 billion people) (FAO 2009). Hence providing biofortified staple food with essential amino acids, vitamins and trace elements without imposing any additional cost to the consumer is an alternative and best solution to overcome the problem of vitamin and trace element deficiency for the poor in the population. The success in peanut transformation technology enabled researchers to address more complex and important aspects of biofortification in peanut for enhanced levels of beta-carotene (provitamin A). Work has been initiated at ICRISAT to develop genetically engineered groundnut having enhanced levels of ß-carotene (pro-vitamin A) to combat vitamin A deficiency. Owing to the high oil content >50% in peanut, targeting β-carotene to the oil bodies for enhanced bioavailability was thought to be critical. This has been achieved by using oleosin promoters for driving the carotenoid biosynthetic genes for targeting these to the oil bodies (Bhatnagar et al. 2010, Bhatnagar-Panwar et al. 2013), as has been previously reported in *Arabidopsis* and *Brassica napus* (Siloto et al. 2006; Hu et al. 2009). Over 200 primary transgenic events of groundnut have been developed by introducing the phytoene synthase gene (*psy1*) from maize that resulted in increased ß-carotone levels, in seed oil bodies to an extent of 20-25-folds when compared to the untransformed controls.

9.6.4 Improvement in Quality of Oil

For peanut, oil content, oil quality and storage protein composition are major issues for quality improvement, and genes controlling these important agronomic traits have been the focus of peanut gene cloning. Currently efforts are carried over to increase stability and quality of peanut oil by hydrogenation to reduce the level of polyunsaturated fatty acids, which also has undesirable health and food quality consequences. Peanut's oils contain high levels of monounsaturated fatty acids that are prone to oxidation as compared to other oils with high levels of polyunsaturated fatty acids. Different genes for improving quality of oil have been proposed (Wang et al. 2011) that can be used for developing transgenic peanuts. For enhancing the shelf-life of peanut products, a higher oleic/linoleic (O/L) ratio is considered desirable. The introduction of the double bonds in the plant fatty acids occurs by the action of enzyme delta-12 fatty acid desaturase. Engineering a gene encoding for delta-12 fatty acid desaturase in peanut by antisense or RNAi strategies may help to reduce activity of this enzyme and hence produce oil with higher O/L ratio. Expression of additional copies of the gene for this enzyme may enhance the content of oleic acid and hence the O/L ratio. Several other reported genes which can be used for developing peanut transgenics for improving nutritional quality are listed in Table 9.4.

Table 9.4: Genes proposed for genetic transformation of peanut for nutritional enhancement

Reason for modifications	Gene/ activity engineered	Modifications required	Success status of transgenic research	Reference
Reduction in the risk for artherosclerosis	Antisense of stearoyl-CoA- β-ketoeicosanoyl CoA syhthetase	Reduction in long chain saturated fatty acids	Transgenic *Brassica* by antisense expression of stearoyl-ACP-desaturase gene	Knutzon et al. 1992
Reduction in aflatoxin load	Stilbene synthase	Increase in stilbenes	Transgenic tobacco	Hain et al. 1990
Improvement in nutritive value of protein	Gene encoding Brazil nut methionine-rich protein	Increase in polypeptides rich in S-containing amino acids	Transgenic tobacco	Altenbach et al. 1989
Reduction in flatus properties	Galactinol:sucrose-6-galactosyl transferase	Reduction in raffinose and stachyose	Not yet attempted	-
Prolongation of shelf-life	Stearoyl desaturase	Increase in oleic acid	Transgenic tobacco with yeast and rat genes	Polashock 1992, Garyburn 1992
Improve protein quality	Brazil nut 2S albumin gene	-	Transgenic peanut	Lacorte et al. 1997
Enhancement in carotenoid content	Maize *psy* gene, maize *lycopene cyclase* gene, bacterial *crtB*	Increase in β-carotene content	Transgenic peanut	Sharma K.K. Unpublished

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