Transgenic Plant Journal ©2008 Global Science Books

Transgenic Peanut (*Arachis hypogaea* L.) Plants Expressing *cryIEC* and Rice Chitinase cDNA (*Chi 11*) Exhibit Resistance against Insect Pest *Spodoptera litura* and Fungal Pathogen *Phaeoisariopsis personata*

Madappattuparambil Ravindran Beena[1] • Rakesh Tuli[2] • Aparna Dutta Gupta[3] • Pulugurtha Bharadwaja Kirti[1*]

[1] Department of Plant Sciences, University of Hyderabad, Hyderabad-500046, India
[2] National Botanical Research Institute, Lucknow-226001, India
[3] Department of Animal Sciences, University of Hyderabad, Hyderabad-500046, India

Corresponding author: * **pbksl@uohyd.ernet.in**

ABSTRACT

Spodoptera litura has been identified as a common insect pest of peanut, causing severe yield losses. A synthetic *cry1EC* gene had been specially designed and proven effective against the larvae of this polyphagous Lepidopteran insect. We have successfully introduced the synthetic *cry1EC* gene alone and along with a rice chitinase cDNA, *Chi11* into JL-24, a popular variety of peanut in India. The transgenic plants were raised from cotyledonary node explants through *Agrobacterium tumefaciens*-mediated transformation. The synergistic effect of both genes was studied in enhancing mortality of the target insect. The presence and expression of the synthetic *cry* gene and selectable marker (*npt*II) gene in regenerated plants were confirmed by PCR, Southern and Western blot analyses. Inheritance of the transgene was confirmed in the T_1 and T_2 generations. Insect bioassays on the transgenic plants carrying both genes showed enhanced resistance against the target insect larvae compared to plants harboring the *cry* gene alone. A fungal bioassay on plants carrying both genes showed varied resistance against the fungal pathogen *Phaeoisariopsis personata*, which causes late leaf spot disease, in addition to resistance against the insect pest *Spodoptera*. This is the first report on peanut engineered with both *cry* gene and chitinase against herbivores and leaf spot disease.

Keywords: 35S promoter, *Agrobacterium tumefaciens*, gene pyramiding, insect herbivore, leaf spot disease

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important cash/oil crop of the world, which suffers severely from biotic stresses. Peanut, an important cash crop, is an annual legume. Its seeds are a rich source of edible oil (43-55%) and protein (25-28%). About two thirds of world production is crushed for oil and the remaining one third is consumed as food. Its cake is used as feed or for making other food products and haulms provide quality fodder. It is presently cultivated in 108 countries of the world. Asia with 63.4% area produces 71.7% of world groundnut production followed by Africa with 31.3% area and 18.6% production, and North-Central America with 3.7% area and 7.5% production (www.icrisat.org/Text/coolstuff/crops/gcrops4.html).

Spodoptera litura, which is common in warm and humid climates, is one of the notorious insects that attack young leaves and flowers of peanut plants, before fruiting, thereby affecting the productivity severely. Various chemical and cultural practices have not improved the situation. Moreover, existing pest management practices escalate the input costs. Integrated pest management (IPM) is the most reliable and cost-effective strategy that has been proposed to make pest management an environmental friendly practice. Host plant resistance is one of the important tactics in IPM. Though wild germplasm with insect and disease tolerance were identified in the genus *Arachis* (Holbrook and Stalker 2003), its use in peanut improvement has so far not become possible because of problems in gene introgression. The number of transformation protocols available for various plants have prompted biotechnologists to attempt complementing conventional breeding practices in developing insect (Singsit *et al.* 1997) and disease resistant (Rohini and Sankara Rao 2001; Chenault *et al.* 2005) peanut plants.

The first report in peanut expressing a *Cry* gene [*cry1A(c)*] is that of Singsit *et al.* (1997), who had used particle bombardment for gene transfer, where a range of resistance responses against lesser cornstalk borer was observed. Singh *et al.* (2004) developed a hybrid δ-endotoxin *cry1EC* encoding a chimeric protein that is more toxic against *S. litura* than the parental toxins, Cry1Ca and Cry1Ea. They demonstrated that complete protection against the target insect pest was obtained when this gene was deployed in cotton and tobacco using *Agrobacterium tumefaciens*-mediated gene transfer system using the strain LBA4404. Surekha *et al.* (2005) also observed similar results when *cry1EC* was introduced into pigeon pea plants with significant reduction in damage due to *Spodoptera* using *A. tumefaciens* and the binary vector used by Singh *et al.* (2004) as ascertained by Singsit *et al.* (1997). Recently, Tiwari *et al.* (2008) reported the efficiency of *cry1EC* against *S. litura* in transgenic peanut plants using *A. tumefaciens*-based vectors.

Chitinase expression in insect gut occurs normally during moulting and the presence of chitinase at an inappropriate time could be detrimental for the insects. When fed on chitinase-expressing tobacco transgenic plants coated with sub-lethal concentrations of a *Bacillus thuringiensis* toxin, the plants exhibited enhanced resistance against the larvae, which were significantly stunted relative to larvae fed on toxin treated non-transgenic controls (Ding *et al.* 1998). This suggests that if a chitinase gene is simultaneously expressed along with a *Cry* gene in transgenic plants,

Received: 4 May, 2008. Accepted: 1 July, 2008.

Original Research Paper

enhanced resistance against the target insect could be achieved. Downing *et al.* (2000) achieved increased toxicity against the stem borer, *Eldana saccharina* of sugarcane by transforming it with *cry1Ac7* and a chitinase gene (*ChiA*). Such gene stacking also would delay the development of resistance by insects (Zhao *et al.* 2003).

Chitin is an important component of the cell wall of fungal species. Hydrolytic enzymes like chitinase and glucanase that degrade the fungal cell wall components pose as attractive candidates for developing disease resistant crop plants (Eapen 2003). Rohini and Sankara Rao (2001) introduced a tobacco chitinase gene in peanut and reported these plants to exhibit resistance against the late leaf spot-causing pathogen. Chenault *et al.* (2005) developed transgenic peanut plants using two anti-fungal genes (a rice chitinase and/or an alfalfa glucanase). Other transgenic crop plants carrying a chitinase gene against fungal pathogens reported were Broglie *et al.* (bean endochitinase, 1989) and Kellman *et al.* (a class II chitinase, 1996) in tobacco, Lin *et al.* (rice chitinase, 1995) in rice, Yamamoto *et al.* (rice chitinase, 2000) in grape vine, Krishnaveni *et al.* (rice chitinase, 2001) in sorghum, and Kumar *et al.* (rice chitinase, 2004) in pigeon pea.

Efforts were made in our studies to develop transgenic peanut plants expressing *cry1EC* and its synergistic effect on the larvae of *Spodotera litura* when used along with a rice chitinase cDNA (*Chi11*). Also, we aimed to study whether rice chitinase expressed in plants is capable of conferring resistance against the fungus, *P. personata*, which causes leaf spot disease. These results are the focus of this communication.

MATERIALS AND METHODS

Plasmid constructs and *Agrobacterium tumefaciens* strains

A chitinase expression cassette (1.55 kb) with its own polyadenylation signal and CaMV 35S promoter was released from PUC18 by digesting it with *Hin*dIII enzyme and was cloned at the *Hin*dIII site of binary vector pPK202, already harboring the *Cry* (*cry1EC*) and selectable marker, *npt*II (*neomycin phosphotransferase* II), gene to create the double construct (**Fig. 1**). *Agrobacterium tumefaciens* strains LBA4404, and GV2260 were transformed with this confirmed plasmid DNA using the freeze-thaw method (Chen *et al.* 1994) and were used in peanut transformation studies.

Agrobacterium suspension for co-cultivation

Single colonies of individual *Agrobacterium* strains carrying the above mentioned constructs were grown overnight in 25 ml LB medium (Himedia, India) in a rotary shaker at 200 rpm and 28°C. At $OD_{600\ nm}$ = 0.5-0.8, the bacterial suspension was centrifuged at 3000 rpm for 1 min and the pellet was re-suspended in an equal volume of sterile double distilled water (SDDW) and incubated at 4°C for 30 min. This suspension was used for co-cultivation.

Transformation of peanut

Mature seeds of peanut (*Arachis hypogaea* L.) cultivar 'JL-24' were surface sterilized with 0.1% (w/v) $HgCl_2$ for 7 min and rinsed four to five times with SDDW. The seeds were soaked in water for 3-4 h and were incubated for germination on wet blotting paper wicks in culture tubes (250 × 15 mm, Borosil, India). Three to four day old germinating seeds were used for the experiments. The cotyledonary node explants were prepared as explained in Beena *et al.* (2005). The prepared explants were dipped in the bacterial suspension in Petri dishes for 5 min, briefly dried between folds of sterile filter paper to remove excess bacterial suspension and cultured on shoot regeneration medium containing 4.0 mg l^{-1} BAP (6-benzylaminopurine, Sigma-Aldrich, USA) + 0.2 mg l^{-1} NAA (α-naphthalene acetic acid, Sigma-Aldrich) with the cut end inserted in the medium. Co-cultivation was carried out for 72 h after which the explants were transferred to shoot regeneration medium supplemented with 250 mg l^{-1} cefotaxime (Hoechst, India) to inhibit the growth of *Agrobacterium*. After five days of recovery for the explants or when the shoot buds started showing their first signs of emergence, they were sub-cultured onto shoot regeneration medium supplemented with 125 mg l^{-1} kanamycin (Kan, Sigma-Aldrich; selection medium). Two to three subcultures were done on a selection medium with 10-15 days' interval. After 30 to 40 days, the explants were continuously sub-cultured on medium containing lower concentrations of BAP (3.0, 2.0, 1.0, 0.0 mg l^{-1}). All cultures were done in a sterile hood and were incubated in standard culture room conditions.

Rooting

All green and elongated shoots were incubated for rooting on medium supplemented with 0.8 mg l^{-1} NAA. Rooted plantlets were transferred to sterile vermiculite: sand mixture (1:1). After 10 days at culture room conditions, the survived/acclimatized plants were taken to a transgenic glasshouse with natural light at 28 ± 2°C and 80% relative humidity, where they were kept covered with clear poly bags (22.5 X 30 cm) for 7–10 d. The acclimatized plants were then transferred to bigger pots with soil: sand (1:1) mixture.

All transgenic plants harboring the *cry* gene alone will be denoted as *Bt* plants and those harboring both the *cry* gene and chitinase cDNA as *BtChi* plants in this communication.

Molecular characterization of putative transgenic plants

Polymerase Chain Reaction

At 2-3 week old stage the second leaf from the shoot tip of putative T_0, T_1 and T_2 transgenic plants were collected and DNA was isolated using the CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle 1990). For PCR analysis 20 ng of this DNA was used in a cocktail of 200 µM dNTPs, 1.5 mM $MgCl_2$, 2 µM of each *npt*II primer and 0.5 units of Taq DNA polymerase (MBI Fermentas, St. Leon Rot, Germany). The primers used were: Forward: 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and Reverse: 5′-ATC GGG AGC GGC GAT ACC GTA-3′, which amplifies a complete length (700 bp) of the *npt*II gene. The amplification was carried out in a thermal cycler (Eppendorf® Master Cycler) under the following conditions: initial denaturation at 94°C for 4 min, denaturation at 94°C for 1 min, annealing temperature of 59°C for 45 s, extension at 72°C for 1 min for each cycle and a final extension time of 15 min at 72°C. The amplification was conducted for 30 cycles and the products were electrophoresed on 0.8% agarose (molecular biology grade, Sigma-Aldrich) gel stained with ethidium bromide (0.02 mg/80 ml).

Southern blot analysis

In order to analyze the total genomic DNA by Southern blot analysis (Sambrook *et al.* 1989) 10-15 µg of DNA was digested using *Hin*dIII and electrophoresed on a 0.8% agarose gel. The enzyme *Hin*dIII has a single site on the T-DNA between the *npt*II and the *cry1EC* genes (in the double construct, it releases the chitinase cDNA that was cloned at the *Hin*dIII site). The separated fragments were transferred onto a nylon membrane (N+, Amersham Biosciences, UK). The PCR-amplified 700 bp *npt*II fragment and

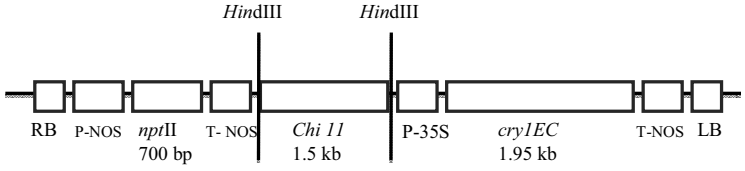

Fig. 1 Partial map of vector pPK202 with chitinase cDNA, *Chi 11* (1.55 kb) cloned at the *Hin*dIII site between *cry1EC* (1.951 kb) and *npt*II (700 bp) genes.

plasmid containing *Chi11* were used to prepare probe using the Hexalabel™ DNA Labeling Kit (MBI Fermentas) using α-^{32}P dATP following the manufacturer's instructions. After hybridization and thorough washes, the nylon membrane was exposed to X-ray film (Amersham Biosciences, UK) depending on the count given by the monitor. After the required exposure time, the X-ray film was developed and observed for the hybridization pattern.

Western blotting

Freshly isolated total protein of the control and transgenic plants using Tris buffer (50 mM Tris-Cl and 1 mM PMSF) was estimated by Bradford's method (1976) and fractionated on an 8.0% SDS polyacrylamide gel (Laemmli 1970). For Western analysis these fractionated proteins were transferred to a PVDF membrane (Amersham Biosciences, UK) or nitrocellulose membrane (Millipore) as per the manufacturers' specifications. The Cry protein was detected using the rabbit anti-Cry1C serum and a goat-anti rabbit IgG (Bangalore GENEI, India) coupled to alkaline phosphatase as secondary antibody. The bands were visualized after staining with BCIP/NBT (Bangalore GENEI, India).

Insect bioassay

Detached leaf feeding tests were done on control and transgenic plants for testing their resistance against the insect using the 1st (1-2 day(s) old) and 2nd (4-5 days old) instar larvae of the pest *Spodoptera litura*. Mature leaves collected from 4-6 weeks' old plants were washed thoroughly using DDW, blot dried, and were placed on wet filter paper in sterile Petri dishes. Five larvae were released into each Petri dish, sealed and observations were made every 12/24 h regarding mortality, mobility, growth/weight of the larvae and the area of leaf damaged. The set up was kept under observation for a maximum of five days. For each transgenic plant three replicates were conducted and the experiment was repeated twice for the positively transformed plants.

Fungal assay

Spores of *Phaeoisariopsis personata* were collected from infected peanut plants using a spore collector and were used immediately or stored at –20°C until use. The spores were suspended in 1.0 ml of SDDW. One drop of this was taken for spore count using a haemocytometer. After the spore count, the concentration of the suspension was adjusted by dilution so that the final spore count was 25,000 spores/ml. To this suspension Tween 20 was added to make a final concentration of 0.01% (v/v). Leaves from the transgenic and control non-transgenic peanut plants were collected and washed thoroughly in DDW. They were blot dried and placed on wet filter paper in Petri dishes. The leaves were painted, approximately 250 µl on both sides of one leaflet (peanut leaf has four leaflets) with the spore suspension using a soft brush. The set up was incubated in the culture room conditions. The filter paper was wet occasionally to maintain humidity. The plates were maintained until the control non-transgenic leaves were covered with infection i.e., approximately 20 days. As for the insect assay, for each transgenic plant three replica plates were maintained and the experiment was repeated twice.

PCR, Southern blot analyses, insect and fungal bioassays were performed up to the T_2 stage and Western blot analyses up to the the T_1 stage. The pictures provided are of experiments conducted during any one of these stages.

Statistical analysis

The experimental design was completely random. One-way analysis of variance was calculated using online ANOVA program (http://www.physics.csbsju.edu/cgi-bin/stats/anova). F- (**Table 2**) and *t*-tests were applied manually as explained in Khan and Khanum (1994) for the bioassays.

RESULTS

The protocol for transformation and elongation of transformed shoot buds standardized for peanut was repeatable. On 4.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA, the cotyledonary node explants produced an average of 24.2 shoot buds within 20-30 days time (**Table 1**). ANOVA was performed on the observations made on different groups of experiments using different combinations of plant growth regulators. The analyses showed that the difference among the samples of the same group was significant (F=2.691) but was not significant among the groups. When the best combination of growth regulators was selected for the transformation, other parameters were taken into consideration other than the number of shoots regenerated from a single explant. Though on medium with only BAP the number of shoots developed was similar to that developed on medium containing a combination of NAA and BAP the latter combination medium was preferred as clumping of shoots were not observed. Moreover, the shoots developed on combination medium were easy to elongate and root. The shoots developed on medium containing BAP alone were sturdy and did not elongate faster compared to the shoots developed on medium containing BAP and NAA. On a medium containing 2,4-D, callus developed along with the shoot buds which made rooting and further acclimatization difficult. These results on transformation are similar to our earlier results observed on regeneration (Beena *et al* 2005).

Under a selection pressure of 125 mg l^{-1} Kan, the control experiment and the untransformed shoot buds from the *Agrobacterium*-treated explants showed similar responses. They either bleached, mainly near the base of the stem and petiole region or turned brown and died. To confirm that there were no escapes, the shoot buds were sub-cultured 3-4 times onto same medium containing 125 mg l^{-1} Kan at 10

Table 1 Effect of different concentrations of BAP in combination with different concentrations of NAA and 2,4-D on peanut cotyledonary node explants after 60 days of transformation.

Sample №	Plant growth regulators (mg l^{-1})			Average number of shoot buds per explant ± SD	% explants responded
	BAP	NAA	2,4-D		
1	3.0			17.8 ± 0.66	63
2	4.0			25.6 ± 2.16	76
3	5.0			17.2 ± 2.19	58
4	3.0	0.1		12.4 ± 0.66	50
5	4.0	0.1		7.7 ± 2.10	25
6	5.0	0.1		10.2 ± 4.04	65
7	3.0	0.2		6.1 ± 0.94	25
8	4.0	0.2		24.2 ± 2.52	69
9	5.0	0.2		12.3 ± 1.41	50
10	3.0		0.5	6.1 ± 1.22	67
11	4.0		0.5	3.8 ± 1.09	80
12	5.0		1.0	15.8 ± 1.77	60
13	3.0		1.5	10.8 ± 1.06	50
14	4.0		1.5	2.2 ± 0.98	20
15	5.0		1.5	5.0 ± 1.67	40

Fig. 2 Flowering in *Bt* (**A**) and *BtChi* (**B**) transgenic plants.

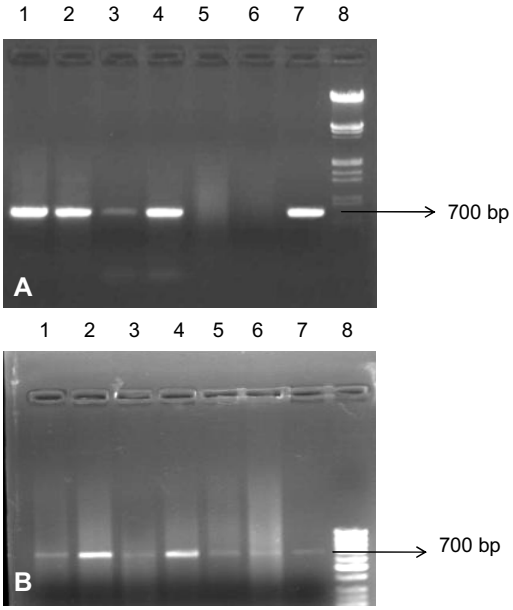

Fig. 3 PCR analyses done on *Bt* (top gel) and *BtChi* (lower gel) plants. Gel shows a 700 bp-amplified fragment of the *npt*II gene from the genomic DNA of transgenic peanut plants. (**A**) Lanes 1-5: *BtChi* transgenic plants; Lane 6: negative control (DNA from non-transgenic control plant; Lane 7: positive control; Lane 8: Molecular weight marker. (**B**) Lanes 1-6: *Bt* transgenic plants; Lane 7: positive control (plasmid containing the gene); Lane 8: Molecular weight marker.

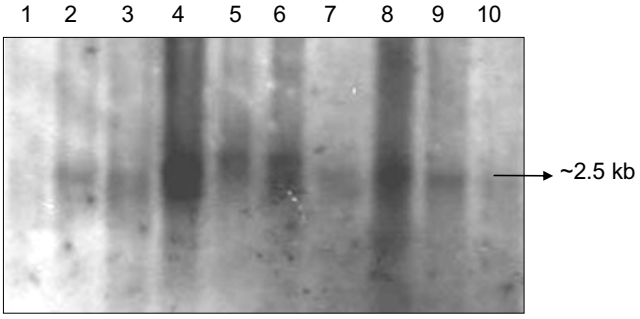

Fig. 4 Southern analysis performed on T_0 *Bt* and *BtChi* transgenic plants. Genomic DNA digested with *Hin*dIII enzyme and hybridized with radioactive probe prepared with 700 bp *npt*II fragment. Lane 1: Control non transgenic plant; Lanes 2-6: Transgenic *Bt* plants (B1, B2, B3, B4, B5); Lanes 7-10: Transgenic *BtChi* plants (BC2, BC3, BC5, BC4).

days interval. Incubation of cultures on high concentration of Kan for 40 days (at 125 mg l^{-1} with subcultures every 10 days) eliminated most untransformed shoot buds. This was evident from PCR and Southern blot analyses. More than 90% of the plantlets showed the corresponding bands for the presence of the gene. Kan was not included in the medium for further shoot elongation and rooting. When a medium devoid of Kan was provided, the shoots showed a distinct increase in growth and development. Two to three shoots elongated and grew healthily for every sub-culture done. Repeated sub-cultures onto lower (3.0, 2.0, 1.0, 0.0 mg l^{-1}) BAP-containing medium helped in elongating most of the shoot buds in a month.

From different experiment and transformation events 29 *Bt* and 36 *BtChi* plants were recovered. Flowering commenced after 20 to 30 days of transfer and establishment of the plants (**Fig. 2**). In 90 to 100 days time the pods became mature and were harvested.

Though most of the plants produced as little as 1-3 pods, a maximum of 18 and 13 pods were produced by individual T_0 *BtChi* and *Bt* plants, respectively. A few of them were shriveled but all the seeds were fertile.

PCR and Southern analyses

PCR analysis showed that 93.1% of *Bt* and 91.66% of *BtChi* plants, respectively that survived in the glasshouse were positive for the *npt*II gene. The oligonucleotide primers specifically designed for amplifying the *npt*II gene amplified an expected fragment size (700 bp) from the genomic DNA of the T_0 and T_2 transgenic plants (**Fig. 3**).

Southern hybridization was performed on T_0 and putative transgenic plants to confirm the integration and integrity of the results obtained in the PCR analysis. Digestion with *Hin*dIII and probing with *npt*II should yield a fragment greater than 1.3 kb in size for confirming integration. All the plants showed the presence of *npt*II in their genomes. Two plants showed different integration patterns while others showed a similar integration pattern irrespective of having the *cry* gene alone or together with Chitinase cDNA. Two fragments, 2.5 and 2.8 kb in size, hybridized with the probe indicating different integration of the transgene (**Fig. 4**). The hybridization pattern in T_2 plants also was similar to T_0 plants showing fragments of approximately 2.6 kb.

Southern hybridization was carried out on T_1 and T_2 *BtChi* plants with radiolabeled plasmid containing the rice chitinase expression cassette. *Hin*dIII digestion of the genomic DNA released the cassette from the T-DNA. An expected fragment size of 1.5 kb was observed in all the positively transformed *BtChi* plants along with two copies of small sized native chitinase gene, which were also present in the control, non-transgenic sample.

Western analysis

Western analysis confirmed that all the plants that were positive for the presence of the *npt*II gene in Southern analysis also expressed the *Cry* gene to produce the Cry protein. All T_0 and T_1 plants that showed the presence of the gene in Southern analysis also showed an approximately 70 kDa protein on immunoblotting and color detection against a negative control (**Fig. 5**).

Insect bioassay

Feeding tests were done on the mature leaves of the young T_0 and T_1 plants using the 1st instar larvae of the target insect, *S. litura*. Both *Bt* and *BtChi* plants responded in a similar fashion in the beginning, but larval mortality was faster in the *BtChi* plants. Within 72 h 100% mortality was observed in all the plants harboring the *cry* gene as well as chitinase cDNA. For plants harboring the *cry* gene alone, it took one or two days more to achieve the same level of mortality. The leaf area damaged was significantly less in

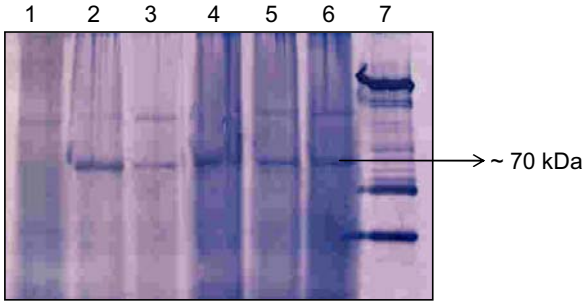

Fig. 5 Western analysis done on freshly extracted proteins from the transgenic peanut plants showing a ~70 kDa band. Lane1: Control, non-transgenic plant; Lanes 2-4: transgenic *Bt* plants (B1, B2, B3); Lanes 5 and 6: transgenic *BtChi* plants (BC5, BC4); Lane 7: Total protein extracted from *E. coli* expressing Cry1C. Anti Cry1C serum was used as primary antibody and Goat anti-rabbit IgG ALP conjugate was used as secondary antibody and the bands were visualized after staining with BCIP/NBT, which is an ALP conjugate substrate.

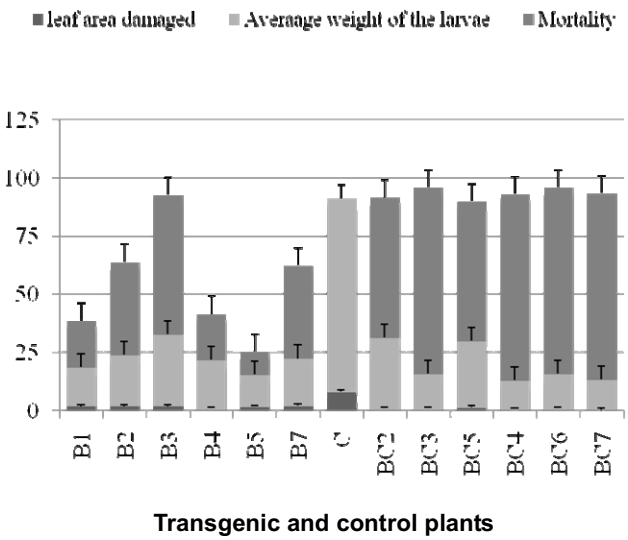

Fig. 6 *Bt* vs. *BtChi* plants. Chart showing a comparison between *Bt* (1-6: B1, B2, B3, B4, B5 and B7) and *BtChi* (7-12: BC2, BC3, BC5, BC4, BC6 and BC7) plants with regard to the leaf area damaged, average weight of the larvae, and percent mortality in 72 h. C: control non-transgenic plants.

both the transgenes compared to the control non-transgenic plants, which were completely consumed by the larvae (**Fig. 6**). When a *t*-test was conducted using data observed on the leaf area damaged by larvae on *Bt* and *BtChi* plants, the calculated *t* value (4.03165) was greater than the tabulated *t* value (2.015) (p = 0.05) and hence the difference between the two types of samples was significant. *BtChi* plants showed less injury than the *Bt* plants (**Fig. 7A, 7B**). The experiment was repeated with 2nd and late 2nd instar larvae to confirm the results. With late 2nd instar larvae the mortality rate was similar to the experiment when 1st instar larvae were used except that in almost all the plants it took 24 to 48 h more for the larvae to die. The average weight of the larva fed on the leaves of control plant was 83 mg. The average weight of the larvae fed on *BtChi* plants were below 20 mg except those fed on two of the transgenic plants. A four times higher growth rate of the larvae fed on control non-transgenic plants than those fed on the transgenic plants and the efficiency of *cry1EC* along with rice chitinase cDNA in controlling *Spodoptera* in peanut. Insect bioassay on T_1 peanut plants were identical to the experiments done on T_0 plants.

Fungal assay

Twenty-five transgenic T_1 peanut plants were analyzed for both fungal as well as insect resistance. Spots started appearing on the leaves within 12 days of inoculation. After 15 days the difference between the transgenic and non-transgenic control plants became obvious (**Fig. 7D-F**). Three transgenic plants (BC4-2, BC4-6, BC2-2) showed no spots even after 20 days of inoculation. These three plants showed 40, 90 and 40% mortality of the larvae, respectively when the bioassay was done using 2nd instar larvae of *S. litura*. Plants (12%) that were susceptible to *P. personata* were also susceptible to *S litura* (**Fig. 8**). However, in BC1-1, although the number of lesions appeared was less than 10 none of the larvae died on insect bioassay. Two plants, BC4-5 and BC4-7, showed equal susceptibility/resistance towards both insect herbivory and fungal pathogen. Three plants (BC4-1, BC4-9, and BC4-20) showed only one lesion after 15 days of inoculation. Transgenic peanut plants viz., BC4-3, BC4-8, BC4-11, BC4-12, and BC4-13 developed only two to four lesions after 20 days of inoculation. Transgenic plant BC4-15 was totally covered by spots and appeared similar to the control non-transgenic leaf. The significance of difference between the samples along with control was checked using F-test. The calculated F-value (3.45) is greater than the tabulated F-value (3.0) and hence the difference between the treatments is statistically significant at 5% (**Table 2**). Out of 25 plants tested for fungal resistance 12% were completely resistant, 80% were partially resistant and 8% were susceptible.

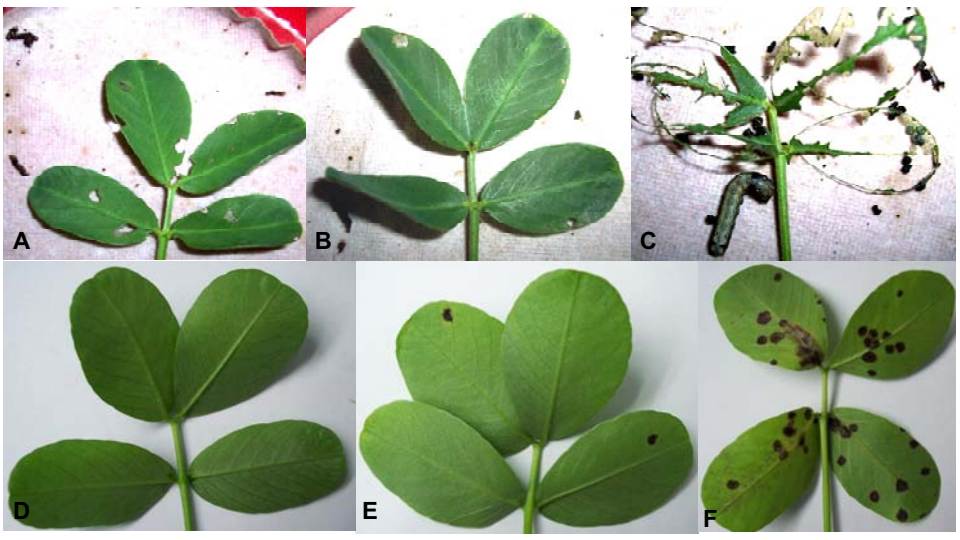

Fig. 7 (**A-C**): Bioassay/detached leaf, feeding test done on transgenic *Bt* and *BtChi* peanut plants. (**A, B**) *Bt* and *BtChi* plants, respectively; (**C**) non-transgenic control plant. (**D-F**): Fungal bioassay conducted on trans-genic peanut plants having rice chitinase cDNA (*chi 11*) as well as *cry 1EC* gene using *P. personata* after 15 days of inoculation. **A** and **B**: trans-genic plants; **C**: non-transgenic control plant.

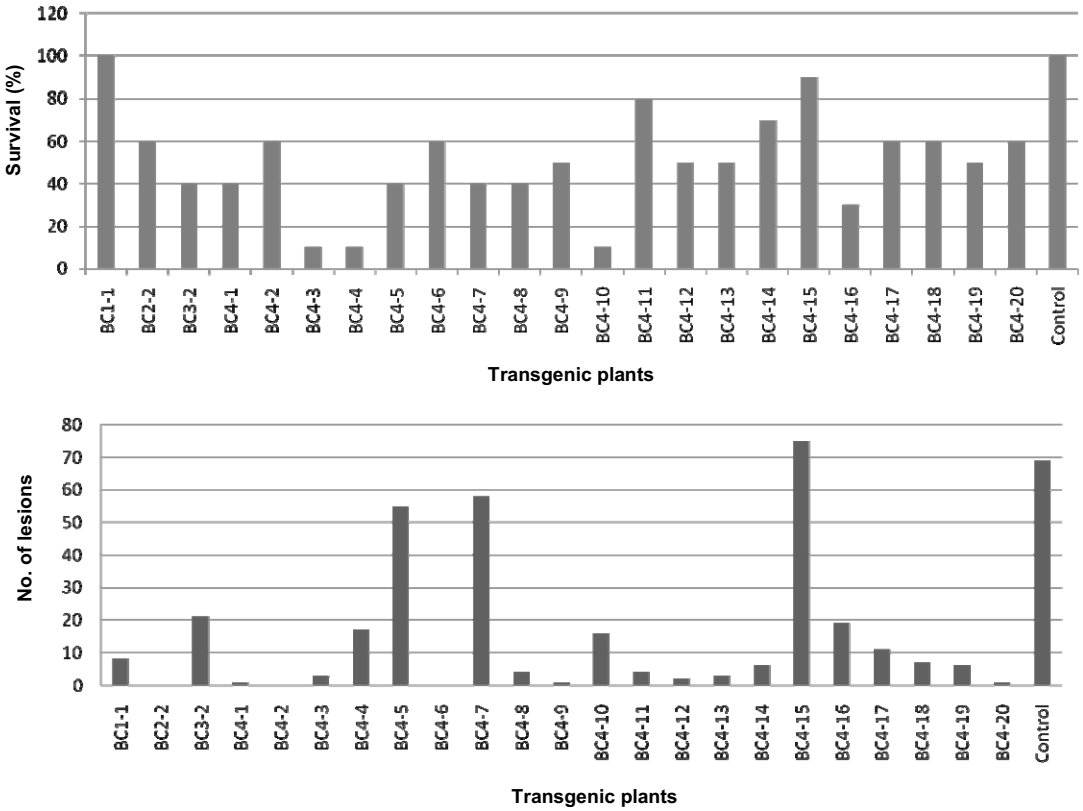

Fig. 8 Data on fungal and insect bioassay done on transgenic peanut plants having both rice chitinase c-DNA (*chi 11*) as well as *cry 1EC* gene; using *P. personata* and *S. litura*. BC2-2, BC4-2, and BC4-6 showed complete resistance against *P. personata*. BC4-3, BC4-4, and BC4-10 showed 90% mortality of the *S. litura* larvae. BC1-1 showed resistance against the fungal pathogen but not against the insect larvae. Top graph: Survival percentage of larvae of *S. litura* in 72 h. Bottom graph: Number of lesions appearing on the transgenic and control plants after 15 days of spore inoculation.

Table 2 F-test performed on the data obtained after fungal assay on the *BtChi* plants using the pathogen *P. personata* that causes leaf spot disease in peanut. Number of lesions observed in three replicates of the transgenic plants and control non-transgenic plants were compared. The calculated F value is 3.45 and the tabulated F value is 3.0 (p = 0.05).

Source of variation	Degrees of Freedom (d.f)	S. S.	M. S.	F
Between samples	c-1 =3-1=2	B-D = 20813.19-18677.55 = 2135.641	(B-D)/c-1 = 2135.641/2 = 1067.8205	(B-D)/c-1 (A-B)/c(r-1) = 1067.82/309.89 = 3.45
Within samples (Residual)	C(r-1) = 3(26-1) = 75	A-B = 44055.00-20813.19= 23241.81	(A-B)/c(r-1) = 23241.81/75 = 309.89	
Total	Cr-1 = 78-1 = 77	A-D = 44055.00-18677.55 = 25377.45		

S.S – Sum of squares, M.S – Mean Square, A – Σx^2, B – $(\Sigma x)^2/nc$, D – Correction Factor = $\Sigma x^2/n$, c – no. of columns, r – no. of rows, d.f- degrees of freedom.

DISCUSSION

The shoot regeneration protocol and subsequent rooting standardized was amenable to *Agrobacterium*-mediated transformation and could produce a minimum of 12-13 plantlets from a single explant. During regeneration experiments on this plant, the number of shoot buds obtained was as high as 104 ± 13.8 within 60 days of culture on medium containing 4.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA (Beena *et al*. 2005). However after *Agrobacterium* treatment and further culture on cefotaxime- and kanamycin-containing medium, the number of shoot buds reduced to 25-26 and among them 12-13 shoot buds elongated, rooted and developed into plantlets. Cotyledonary node explants appeared to be less damaged by the *Agrobacterium* treatment. Once the explants were transferred to medium supplemented with cefotaxime, the cotyledonary node explants regained their regeneration vigor faster than the cotyledon explants. It was necessary to excise or damage the axillary buds properly to get a large number of shoot buds from the explants. The presence of even a single, undamaged bud allowed the explants to develop it into a thick and healthy shoot within a week, suppressing the *de novo* regeneration of shoot buds from adjacent regions. This is similar to apical bud dominance where the development of axillary buds into shoots is suppressed. When the axillary bud was removed, more shoot buds develop from the nodal region, i.e. axillary buds. Hence, explant preparation appears to be crucial to regenerate a maximum number of shoot buds from an explant. BAP and NAA combinations were observed to be better when compared with BAP and 2,4-D and hence all the further cultures after transformation were done on medium containing BAP and NAA. This corroborates the results we obtained during our regeneration experiments with the same plant 3 years earlier (Beena *et al*. 2005)

The high selection pressure given for more than 30 days after co-cultivation eliminated 90% of the development of escapes. This was evident from the PCR amplification (**Fig. 3**) where DNA of the expected 700 bp *npt*II gene was amplified in most of the glasshouse-survived T_0 plants. Also, most of the shoots developed from the shoot bud clumps without selection pressure, sub-cultured after harvesting of well developed shoots, were also found to be transgenic. When Southern analysis was performed on the PCR-positive T_0 plants developed from different transformation events and experiments, almost all showed a similar integration pattern. There is a possibility for the shoots to be regenerated from the remaining cells of shoots already taken for rooting, as the shoot bud clumps were repeatedly sub cultured onto shoot elongation medium after harvesting the elongated shoots. Such repeated shoot production helps in producing more transgenic shoots without the intervention

of callus phase, when the aim is to produce more number of homogeneous transgenic shoots rather than getting more plants from individual transformation events. But this does not explain the similar integration pattern observed in genome of transgenic plants produced from different experiments. Hence, there is a greater possibility that there exist hot spots, also called fragile sites that are target sites for DNA integration, in the host genome and that host plant genome also plays an important part in T-DNA integration as explained by Somers and Makarevitch (2004). According to them the transgene locus structure is more likely to be determined by the plant genome factors than how or in what form the DNA is delivered to the nucleus. This happens when double-strand break repair enzymes are involved in the transgene integration. Gelvin (2000) too indicated that T-DNA integration does not occur where a repair mechanism is absent. In the present case, seven among the T_0 plants tested in Southern analysis showed a similar integration pattern with *Hin*dIII. A similar integration pattern in primary transformants were also observed in *Medicago truncatula* (Trinh *et al.* 1998), sugarcane (Enriquez-Obregon *et al.* 1998) loblolly pine (Tang and Tian 2003), wheat (Patnaik and Khurana 2003), cacao (Maximova *et al.* 2005), and onion (Aswath *et al.* 2006). In T_2 transgenic plants, too the fragment sizes of the *npt*II gene obtained in Southern analyses were similar to those seen in the T_0 plants. This indicates that integration was stable and is inherited in the next generation.

Use of the Kan-resistant gene as the selectable marker was efficient in selecting the transformed shoot buds from the untransformed tissue/shoot buds. All shoot buds that developed on the control explants (not subjected to *Agrobacterium* treatment) either turned brown within 10 days or bleached on medium containing 125 mg l^{-1} kan. These results are in agreement with those of Fontana *et al.* (1993), Kar *et al.* (1997) and Sharma and Anjaiah (2000).

It is well known fact that the copy number of the gene present influences its expression in transgenic plants. In our studies, all the tested transgenic plants showed single copy integration. This was confirmed by digesting the genomic DNA with rare cutter enzymes like *Apa*I and *Nde*I and performing the Southern analysis, which also showed a single copy of the *npt*II gene (data not shown). This shows the advantage of using *Agrobacterium*-mediated transformation for legume transformation. Moreover all the T_0 plants that survived were fertile and produced seeds. The amenability of using *Agrobacterium* for stable transformations in peanut and thereby producing fertile progenies were also reported by McKently *et al.* (1995), Cheng *et al.* (1996), Venkatachalam *et al.* (2000), Sharma and Anjaiah (2000) and Tiwari *et al.* (2008).

All the positively transformed T_0 plants were subjected to an *in vitro* bioassay. An *in vitro* feeding test using the first, second and late second instar larvae of *Spodoptera litura* showed 100% mortality. But there were differences in the time taken for 100% death, body weight of the larvae and the area of leaf damaged between the *Bt* and *BtChi* plants. *BtChi* plants appeared better with regard to the early killing of the larvae, and the present gene combination would help the plant to recover fast before much damage was done by larvae. Their reduced body weight compared to the larvae fed on *Bt* plants also was significant. A Student's *t*-test was conducted on the observations made using the average weight of the larvae on *Bt* and *BtChi* transgenic plants. The calculated *t*-value is 2.1, which is greater than the tabulated *t*-value (1.476) at P=0.1. This shows that the difference in the weight of larvae fed on these two groups of plants was significant.

When the results were confirmed using immunoblotting, the Cry protein was detected in all the positive plants. The expression of the synthetic *Bt* gene in these plants confirmed that full-length transcripts were generated in these plants ultimately leading to the expression of the gene. The difference in performance of the *Bt* and *BtChi* plants was significant at P=0.05. Similar results were also reported by Wang *et al.* (2005) where insect-resistant *Brassica napus* were produced by transforming it with a chitinase (*Chi*) and scorpion Insect Toxin gene (*Bmk IT*) that were more potent against the target insect. The potential of using other genes together with *Cry* genes in transformation has also been demonstrated by transforming tobacco and potato using Cry1Ac and a proteinase inhibitor against *Heliothis* (Lawrence and Novak 2001), tobacco (Fan *et al.* 1999) and cotton using *Bt* and cowpea trypsin inhibitor against bollworm (Zhang *et al.* 2004) and maize and rice using a fusion protein combining Cry1Ac with the galactose binding domain of the non-toxic ricin B chain in maize against a wide range of insects that are normally not susceptible to *Bt* toxin alone (Mehlo *et al.* 2005).

The fungal and insect bioassay conducted on the transgenic peanut plants carrying rice chitinase c-DNA (*chi 11*) as well as *cry1EC* gene, showed varied response against *P. personata* and *S. litura*. Rohini and Sanakara Rao (2001) also found a varied response (zero to several lesions) by the transgenic peanut plants, transformed using tobacco chitinase gene, against *C. arachidicola*. The intensity of disease symptoms observed on the T_1 plants was variable and can be correlated to chitinase activity as explained by Rohini and Sanakara Rao (2001) and the susceptibility of one of the transgenic plant (BC4-15) could be due to segregation of the gene. Chenault *et al.* (2005) also reported after three years of field study that the transgenic peanut plants carrying anti-fungal genes (a rice chitinase and/or an alfalfa glucanase) depicted partial resistance against sclerotinia blight. Datta *et al.* (2002) reported pyramiding of the insect resistant gene, bacterial blight resistant gene (*xa21*), and a rice chitinase gene in transgenic rice for resistance against *Scirpophaga incertulas* (yellow stem borer), *Xanthomonas oryzae* pv. *oryzae* (bacterial blight) and *Rhizoctonia solani* (sheath blight). Gene pyramiding thus proves to be a more viable strategy for producing transgenic plants that exhibit greater resistance against the insect pests and this would also reduce the chance of development of resistance by the insects against the protein encoded by the introduced transgenes (reviewed by Ye and Smith 2008a, 2008b).

CONCLUDING REMARKS

In our studies, the explant material and shoot elongation strategy used helped us to recover more transformed shoots from the transformation experiments. Though only a limited number of pods were obtained from the primary transgenic plants in the present study, there were neither sterile progenies obtained nor suppression of the gene due to the presence of multi-copy gene. T_1 and T_2 progenies showed the presence of *Bt*, *npt*II and *Chi11* genes demonstrating the inheritance of these genes. Because of the limited number of progeny, the inheritance pattern could not be analyzed. The susceptibility of the cotyledonary node explants for *Agrobacterium*-mediated transformation clearly showed high rates of regeneration after transformation, which is in agreement with the results found by Sharma and Anjaiah (2000). There are no cultivated Indian varieties of peanut which are resistant to leaf spot disease caused by *P. personata* other than one report by Rohini and Shankara Rao (2001) using *in planta* transformation of peanut using a tobacco chitinase gene. The results of this study demonstrate that expression of the rice chitinase c-DNA in peanut genome could control the disease to a varied extent. This study provides a protocol for transformation and recovery of transformed plants within a short span of time (60-70 days for developing T_0 transgenic plants and 10-15 days for establishment in the glasshouse). It also demonstrates the efficacy of gene pyramiding.

ACKNOWLEDGEMENTS

MRB acknowledges the financial support from the Council of Scientific and Industrial Research, Govt. of India in the form of SRF. Financial support in the form of a Research Grant to the lab provi-

ded by DBT, Govt. of India is gratefully acknowledged. Dr. S Muthukrishnan (US) and Mr. B. Madhusudhan, Department of Animal Sciences, University of Hyderabad, is duly acknowledged for providing the rice chitinase cDNA and the anti-rabbit Cry1C serum, respectively.

REFERENCES

Aswath CR, Mo SY, Kim DH, Park SW (2006) *Agrobacterium* and biolistics transformation of onion using non-antibiotic selection marker phosphomannose isomerase. *Plant Cell Reports* **25**, 92-99

Beena MR, Jami SK, Srinivasan T, Swathi Anuradha T, Padmaja G, Kirti PB (2005) Efficient regeneration from cotyledonary node explants of peanut (*Arachis hypogaea* L. cv. JL-24). *Indian Journal of Plant Physiology* **15**, 131-134

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye interaction. *Annals of Biochemistry* **72**, 248-254

Broglie KE, Biddle P, Cressman R, Broglie R (1989) Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. *Plant Cell* **1**, 599-607

Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. *Biotechniques* **16**, 664-668

Chenault KD, Melouk HA, Payton ME (2005) Field reaction to sclerotinia blight among transgenic peanut lines containing antifungal genes. *Crop Science* **45**, 511-515

Cheng M, Jarret RL, Li Z, Xing A, Demski JW (1996) Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens*. *Plant Cell Reports* **15**, 653-657

Datta K, Baisakh N, Maung Thet K, Tu J, Datta S (2002) Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight. *Theoretical and Applied Genetics* **106**, 1-8

Ding X, Gopalakrishnan B, Johnson LB, White FF, Wang X, Morgan TD, Kramer KJ, Muthukrishnan S (1998) Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Research* **7**, 77-84

Downing KJ, Leslie G, Thomson JA (2000) Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes in sugarcane-associated bacteria. *Applied Environmental Microbiology* **66**, 2804-2810

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**, 12-14

Eapen S (2003) Regeneration and genetic transformation in peanut: current status and future prospects. In: Jaiswal PK, Singh RP (Eds) *Applied Genetics of Leguminosae Biotechnology*, Dordrecht: Kluwer Academic Publishers, pp 165-186

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, de la Riva GA, Housein GS (1998) Herbicide resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. *Planta* **206**, 20-27

Fan X, Shi X, Zhao J, Zhao R, Fan Y (1999) Insecticidal activity of transgenic tobacco plants expressing both *Bt* and *CpTI* genes on cotton bollworm (*Helicoverpa armigera*). *Chinese Journal of Biotechnology* **15**, 1-5

Fontana GS, Santini LS, Caretto S, Frugis G, Mariotti D (1993) Genetic transformation in the grain legume *Cicer arietinum L*. (chickpea). *Plant Cell Reports* **12**, 194-198

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 223-256

Holbrook CC, Stalker HT (2003) Peanut breeding and genetic resources. *Plant Breeding Reviews* **22**, 297-356

Kar S, Basu D, Das S, Ramkrishnan NA, Mukherjee P, Nayak P, Sen SK (1997) Expression of *cry1A(c)* gene of *Bacillus thuringiensis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. *Transgenic Research* **6**, 177-185

Kellman JW, Kleinow T, Engelhardt K, Philip C, Wegener D, Schell J, Schreier PH (1996) Characterization of two class II chitinase genes from peanut and expression studies in transgenic tobacco plants. *Plant Molecular Biology* **30**, 351-358

Khan IA, Khanum A (1994) *Fundamentals of Biostatistics*, Ukaaz Publications, Hyderabad, India pp 408-415

Krishnaveni S, Jeoung JM, Muthukrishnan S, Liang GH (2001) Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. *Journal of General Breeding* **55**, 151-158

Kumar SM, Kumar BK, Sharma KK, Devi P (2004) Genetic transformation of pigeon pea with rice chitinase gene. *Plant Breeding* **123**, 485-489

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685

Lawrence SD, Novak NG (2001) A rapid method for the production and characterization of recombinant insecticidal proteins in plants. *Molecular Breeding* **8**, 139-146

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. *Biotechnology* **13**, 686-691

Maximova SN, Marelli J-P, Yang A, Pishak S, Verica JA, Guiltinan MJ (2005) Over expression of a cacao class chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen. *Planta* **224**, 740-749

McKently AH, Moore GA, Doostdar H, Niedz RP (1995) *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. *Plant Cell Reports* **14**, 699-703

Mehlo L, Gahakwa D, Trung Nghia P, Loc NT, Capell T, Gatehouse JA, Gatehouse AMR, Christou P (2005) An alternative strategy for sustainable pest resistance in genetically enhanced crops. *Proceedings of the National Academy of Sciences USA* **102**, 7812-7816

Patnaik D, Khurana P (2003) Genetic transformation of Indian bread wheat (*Triticum aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo derived calli. *BMC Plant Biology* **3**, 5-16

Rohini VK, Sanakara Rao K (2001) Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. *Plant Science* **160**, 889-898

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edn), Cold Spring Harbour Laboratory Press, New York.

Sharma KK, Anjaiah V (2000) An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens*-mediated genetic transformation. *Plant Science* **159**, 7-19

Singh PK, Kumar M, Chaturvedi CP, Yadav D, Tuli R (2004) Development of a hybrid δ-endotoxin and its expression in tobacco and cotton for control of a polyphagous pest *Spodoptera litura*. *Transgenic Research* **13**, 397-410

Singsit C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P (1997) Expression of a *Bacillus thuringiensis cry1A*(c) gene in transgenic plants and its efficacy against lesser cornstalk borer. *Transgenic Research* **6**, 169-176

Somers DA, Makarevitch I (2004) Transgene integration in plants: poking or patching holes in promiscuous genomes? *Current Opinion in Biotechnology* **15**, 126-131

Surekha Ch, Beena M R, Arundhati A, Singh PK, Tuli R, Dutta Gupta A, Kirti PB (2005) *Agrobacterium* mediated genetic transformation of pigeon pea (*Cajanus cajan* (L.) Millsp.) using embryonal segments and development of transgenic plants for resistance against *Spodoptera*. *Plant Science* **169**, 1074-1080

Tang W, Tian Y (2003) Transgenic loblolly pine (*Pinus taeda* L.) plants expressing a modified δ-endotoxin gene of *Bacillus thuringiensis* with enhanced resistance to *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud. *Journal of Experimental Botany* **54**, 835-844

Tiwari S, Mishra DK, Singh A, Singh PK, Tuli R (2008) Expression of a synthetic *cry1EC* gene for resistance against *Spodoptera litura* in transgenic peanut (*Arachis hypogaea* L.). *Plant Cell Reports* **27**, 1017-1025

Trinh TH, Ratet P, Kondorosi E, Durand P, Kamate K, Bauer P, Kondorosi A (1998) Rapid and efficient transformation of diploid *Medicago truncatula* and *Medicago sativa* ssp. *Falcate* lines improved in somatic embryogenesis. *Plant Cell Reports* **17**, 345-355

Venkatachalam P, Geetha N, Khandelwal A, Shaila MS, Lakshmi Sita G (2000) *Agrobacterium*-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. *Current Science* **78**, 1130-1136

Wang J, Chen Z, Du J, Sun Y, Liang A (2005) Novel resistance in *Brassica napus* developed by transformation of chitinase and scorpion toxin genes. *Plant Cell Reports* **24**, 549-555

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000) Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Reports* **19**, 639-646

Ye G-Y, Smith KF (2008a) Marker-assisted gene pyramiding for inbred line development: basic principles and practical guidelines. *International Journal of Plant Breeding* **2**, 1-10

Ye G-Y, Smith KF (2008b) Marker-assisted gene pyramiding for inbred line development: practical applications. *International Journal of Plant Breeding* **2**, 11-22

Zhang Y-J, Zhao H-Y, Wu K-M, Zhao K-J, Peng Y-F, Guo Y-Y (2004) Expression of Cry1Ac protein in *cry1Ac*/*CpTI* transgenic rice and its resistance in different developmental stages to *Chilo suppressalis*. *Chinese Journal of Agricultural Biotechnology* **1 (3)**, 149-153

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nature Biotechnology* **21**, 1493-1497