

# Transgenic Sunflower (*Helianthus annuus* L.) with Enhanced Resistance to a Fungal Pathogen *Alternaria helianthi*

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## ABSTRACT

Sunflower (*Helianthus annuus* L.) is mainly grown for its oil all over the world. Like any other crop it also suffers major yield losses due to viral, bacterial and fungal diseases. Overexpression of PR proteins leads to increased resistance to pathogens in several crops. The PR-2 protein,  $\beta$ -1,3-glucanase contributes to plant defenses against fungal infection. We report in this paper, overexpression of a tobacco  $\beta$ -1,3-glucanase gene in transgenic sunflower and its resistance towards *Alternaria helianthi*. Molecular analysis by Southern dot blots, PCR analysis confirmed stable integration of the  $\beta$ -1,3-glucanase gene in sunflower transgenics. When these transgenic plants screened for resistance against *A. helianthi*, the transgenics showed not only reduction in the number of spots but also delay in the onset of *Alternaria* blight. The results demonstrate the potentiality of a PR protein from a heterologous source in developing resistant sunflower to *Alternaria* blight.

**Keywords:**  $\beta$ -1,3-glucanase gene, fungal resistance, *in planta* transformation, putative transformants

## INTRODUCTION

Sunflower is a marketable crop product. The crop originated in subtropical and temperate zones, but selective breeding has made sunflower highly adaptable, especially to warm temperate regions. Although the sunflowers themselves are generally not exported, the products derived from them are. Sunflower has oil content of 40-50% (Entire fruit), 50-60% (kernel only) with 90% of oleic, linoleic acid and 20-30% of protein (Lide 1991; Murphy 1994). Sunflower farms play an important role in the world economy and the oncoming oil crisis may determine a further importance of sunflower farms if petroleum product alternatives are more fully researched and required (Ikeda *et al.* 2005). However, its productivity is majorly influenced by biotic stress.

Thus, in breeding programs, increased disease resistance and high oil content have been the main aim for sunflower (Weber *et al.* 2000). Among the economically important diseases, leaf spot incited by *Alternaria helianthi* Tub. Nish. is a major foliar disease causing severe yield losses in the tropics and subtropics. The major limitation for genetic improvement of cultivated sunflower is the lack of acceptable levels of resistance to this disease in the cultivar germplasm. In the plants, the PR proteins are strongly induced when plants respond to wounding or infection by fungal, bacterial, or viral pathogens, and there is compelling evidence that  $\beta$ -1,3-glucanases alone contribute to plant defenses against fungal infection (for review, see Kombrink and Somssich 1997; Leubner-Metzger and Meins 1999; Neuhaus 1999).  $\beta$ -1,3-Glucan, a major component of the cell walls and resting structures of most fungi, is degraded by a PR protein (PR-2 family) (Van Loon, 1999),  $\beta$ -1,3-glucanases by hydrolysis (Pitson *et al.* 1993). Keeping these points in view crops plants have been genetically engineered with a different PR protein gene from a heterologous source in developing disease resistant tobacco (for review see Linthorst 1991), groundnut (Rohini and Rao 2001; Sundaresha *et al.* 2010), mustard (Mondal *et al.* 2007), rice (Ganapathi Sridevi *et al.* 2008), safflower (Manoj Kumar *et*

*al.* 2009b).

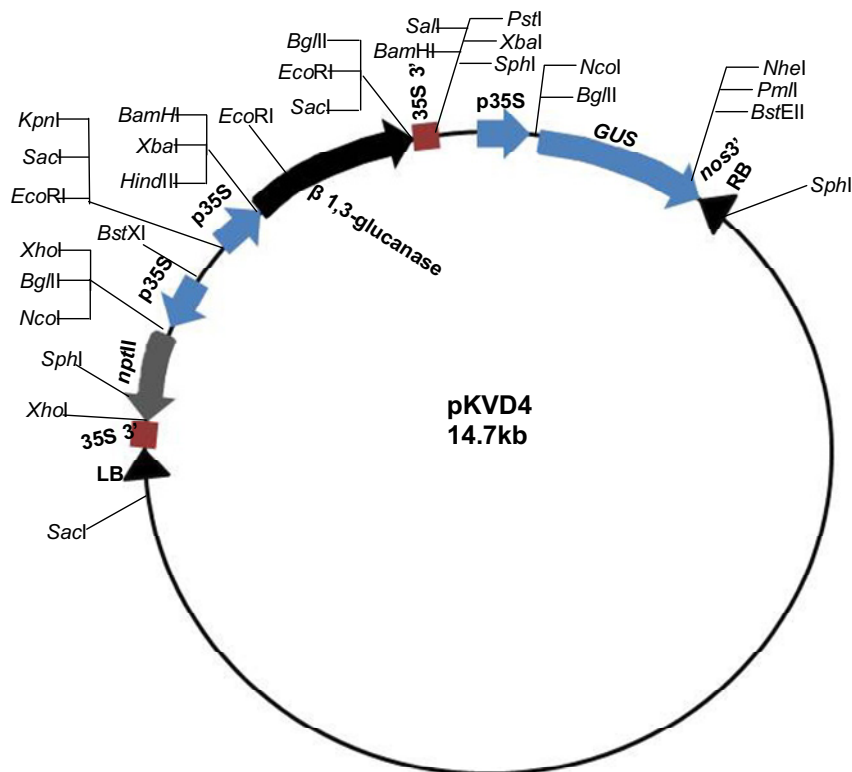
In recent years, biotechnological techniques such as tissue culture and transgenic technologies have been used for improvement of sunflower, but these techniques are mainly limited by the tissue culture response of commercial varieties (Nestares *et al.* 2002). However, reports on sunflower shoot regeneration from hypocotyls, cotyledons, leaves and meristematic tissues of young plantlets are available, but regeneration ability and precocious flowering, vitrification or poor rooting (Sujatha and Prabakaran 2001) pose problems. Further-more, regeneration quality was either low with abnormal morphogenesis, frequency by organogenesis essentially depends on genotype and its interaction with culture conditions (Ozyigit *et al.* 2006). Development of a method to obtain transformants, which is independent of the problems inherent to tissue culture of sunflower, would represent a major accomplishment. One such technique is *in planta* transformation method developed at our laboratory (Sankara Rao and Rohini 1999; Rohini and Sankara Rao 2000a, 2000b, 2001; Keshamma *et al.* 2008; Manoj Kumar *et al.* 2009a, 2009b) was adopted to generate transgenics in sunflower harboring a tobacco PR-2 protein,  $\beta$ -1,3-glucanase.

## MATERIALS AND METHODS

### Plant material and bacterial strains

Genetic transformation studies were carried out using sunflower cv. 'DRSF 110' seeds, obtained from the Directorate of Oil Seeds Research, Hyderabad, India. Mature seeds were soaked overnight in distilled water and were surface sterilized first with 1% Bavastin for 10 min and later with 0.1% HgCl<sub>2</sub> for a few seconds and washed thoroughly with distilled water after treatment with each sterilant. The seeds were later placed for germination in Petri dishes at 28°C. Two-day-old seedlings were taken as explants for *Agrobacterium* infection.

The disarmed *Agrobacterium tumefaciens* strain LBA4404 was obtained from Invitrogen (Carlsbad, CA, USA), harbouring



**Fig. 1** Binary vector pKVD4 harboring *nptII::β-1,3-glucanase::uidA* obtained from Madurai Kamraj University, TN, India was used for sunflower transformation. The β-1,3-glucanase coding region was inserted as a *BglII-BamHI* fragment between the 35S promoter and the 35S polyA terminator in the pKVD4 vector. The *nptII* gene conferring resistance to kanamycin was used as selectable marker and *uidA/gus* as reporter for sunflower transformation. Binary plasmid, pKVD4 was mobilized into *Agrobacterium tumefaciens* (LBA4404) through electroporation and bacterial transformation was confirmed by restriction digestion using appropriate enzymes.

the binary vector, pKVD4 (14.7 kb) obtained from Prof. K. Veluthambi, Madurai Kamraj University, Madurai, Tamil Nadu, India and stored in  $-20^{\circ}\text{C}$ . pKVD4 contains a β-1,3-glucanase gene (2.5 kb) under the control of the CaMV35S promoter and terminator, the β-1,3-glucanase gene was cloned into the *BamHI* and *BglII* sites of the vector pKVD4. The screenable and selectable marker, *uidA*, and the *nptII* gene, were regulated by the CaMV35S promoter and terminator (Fig. 1). Binary plasmid, pKVD4 was mobilized into *A. tumefaciens* (LBA4404) through electroporation (Bio-Rad Laboratories, USA) and bacterial transformation was confirmed by restriction digestion using appropriate enzymes. A single positive colony, LBA4404/pKVD4 was grown in LB medium (pH 7.0) containing  $50\ \mu\text{g mL}^{-1}$  kanamycin. The bacterial culture (3 ml) was later resuspended in 100 ml of Winans' AB medium (pH 5.2) (Winans *et al.* 1988) and grown for 18 h. For *in vitro* gene induction treatments, wounded tobacco leaf extract (2 g in 2 ml sterile water) was added to the *Agrobacterium* suspension in Winans' AB medium, 5 h before infection (Cheng *et al.* 1996).

### Transformation and recovery of transformants

An *in planta* transformation protocol (Rohini and Sankara Rao 1999; Keshamma *et al.* 2008; Manoj Kumar *et al.* 2009a, 2009b) was followed to obtain sunflower transformants. Briefly, the seedlings with just emerging plumule were infected by 5-6 pricks at the apical meristem with a sterile needle and subsequently immersed in the *Agrobacterium* culture for 60 min. Following infection, the seedlings were transferred to autoclaved Soilrite<sup>®</sup> moistened with water (25: 40, w/v) for germination under aseptic conditions in a growth room maintained with 95% RH. Four seedlings per jar were placed in wide mouth capped glass jars of 300 ml capacity. After 6-7 days, the seedlings were transferred to Pro-trays<sup>®</sup> (60 × 15 cm) containing Soilrite<sup>®</sup> and were allowed to grow under growth room conditions for a week. The growth chamber was maintained at  $28 \pm 1^{\circ}\text{C}$  under a 14-h photoperiod with fluorescent light (FL40S.W, Mistubishi, Tokyo) of  $35\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  intensity. Week-old seedlings were later transplanted to 45 × 30 cm diameter earthen pots containing autoclaved red loamy soil (volume

~12 kg) and a dose of 120 N: 80 P: 50 K (Nagarjuna Fertilizers Pvt. Ltd., Hyderabad, India) was applied to the soil. These  $T_0$  plants were shifted to the greenhouse with an optimum temperature of  $28 \pm 1^{\circ}\text{C}$  and 85% RH.

### Expression of β-glucuronidase

Phenotypic GUS expression was determined by staining shoot apices of putative  $T_0$  seedlings (putative transformants). The excised shoot apices were surface sterilized with 0.1%  $\text{HgCl}_2$  for a few seconds and washed thoroughly thrice with distilled water just before they were taken for the assay. The method of Jefferson (1987) was used to assess histochemical *uidA* gene expression in the tissues of putative transformants, using 5 days post co-cultivated tissues that were incubated overnight at  $37^{\circ}\text{C}$  in a solution containing 0.1 M phosphate buffer, pH 7.0, 2 mM, β-glucuronidase (X-Gluc) (5-bromo-4-chloro-3-indole-β-D-glucuronide; Sigma-Aldrich, St. Louis, USA), 5 mM each of potassium ferricyanide and ferrocyanide and 0.1% Triton X-100. The tissues were later soaked with 75% ethanol to clear chlorophyll.

The mature  $T_0$  generation seeds were collected, dried and stored till use. The  $T_1$  generation plants were obtained by germinating  $T_0$  seeds. The molecular and bio-efficacy analysis was carried out in  $T_1$  plants.  $T_1$  sunflower plants were grown in a greenhouse following a recommended package of instructions ([http://www.ikisan.com/links/ap\\_cultivation](http://www.ikisan.com/links/ap_cultivation)) and the plants were labeled with aluminium tags.

### Southern dot blot analysis of putative transgenic plants in $T_1$ generation

Total genomic DNA was isolated from young leaves of putative  $T_1$  transformants and untransformed (wild type) plants using the CTAB method (Dellaporta *et al.* 1983).

For Dot blot analysis, 5 μg genomic DNA was blotted onto nylon membrane (Sambrook *et al.* 1989) and hybridized with the PCR product of *nptII* gene. In order to analyze the total genomic DNA for transgene integration, 15 μg of total genomic DNA was

digested overnight with the appropriate restriction enzymes, *Xba*I and *Nhe*I. The digested DNA samples were electrophoresed on a 0.8% agarose gel. The separated fragments along with the uncut DNA were transferred onto a positively charged nylon membrane (Pall Pharmed Filtration Pvt. Ltd., Mumbai, India). The membrane was hybridized with  $\alpha$ -<sup>32</sup>P-radioactive probe which was made from purified *35S-UidA* PCR product labeled by random prime labeling (Fermentas Inc., USA). Hybridization was carried out at 65°C in Church buffer (Church and Gilbert, 1984) for 18 h. Membranes were washed for 30 min each in 2x SSC, 0.1 % SDS; 0.1x SSC, 0.1% SDS at 65°C (Sambrook *et al.* 1989). Membrane was wrapped, placed overnight on FUJI Image Plate (IP) and IP was read using phosphor imager (FUJI FILM FLA-5100, Fuji Photo Film Co. Ltd., Tokyo, Japan).

### PCR analysis

The presence of the *nptII* gene in the putative transgenic sunflower plants was detected by PCR using two *nptII* primers: forward (5'-GGG CAGGCCAGCGTATCGTG-3') and reverse (5'-TCCC GC TAGTGCCT TGCCAGTT-3') specific to *nptII* gene (Topfer *et al.* 1989). PCR was performed to amplify a 750 bp *nptII* gene fragment in the putative transformants. In order to amplify the *nptII* gene fragment, PCR was initiated by a hot start at 94°C for 7 min followed by 30 cycles of 94°C min<sup>-1</sup>, 58°C 1.5 min<sup>-1</sup> and 72°C min<sup>-1</sup> with a 72°C 10 min<sup>-1</sup> final extension. Similarly, to reconfirm the integration of the *gus* transgene, a 35S CaMV promoter-specific forward primer (5'-TGTAGAAACCCCAAC CCGTGAAAT-3') and *uid A* reverse (5'-TCCCCTAGTGCC T TGTCCAGTT-3') primer (*35S-uidA*) was used to amplify a 687 bp product. PCR was performed with both sets of primers to check for the co-integration of the transgenes in T<sub>1</sub> and T<sub>2</sub> transformants. The conditions for *35S-uidA* were same as above, except that the annealing temperature was 60°C. The PCR reaction mixture (20 µl) contained 0.3 U *Taq* DNA polymerase, 1X assay buffer (10 mM pH 9.0 TRIS-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin), 150 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 0.25 µM and 100 ng template DNA. The DNA extracted from untransformed plants was used as a negative control, the pKVD4 vector as a positive control. The products were checked on 1% agarose ethidium bromide gels.

The above southern dot blot analyses was used for preliminary screening of putative transgenic plants in T<sub>1</sub> generation whereas T<sub>2</sub> generation plants were analyzed using individual PCR to confirm transgene integration.

### *Alternaria* resistance screening

Isolation and purification of *A. helianthi* cultures was done from fresh infected parts of sunflower collected from control plots of the University of Agricultural Sciences, GKVK, Bangalore, India. *A. helianthi* isolates were purified by the hyphal tip method and were maintained at 15°C on potato dextrose agar (PDA) (Himedia Laboratory Pvt. Ltd., Mumbai, India) (at 40 g L<sup>-1</sup> was autoclaved at 1 atm for 20 min) in 9 cm Petri dishes for further study. A nine-day-old *A. helianthi* culture was ground in 40 ml of sterilized distilled water aseptically. This was aseptically filtered with sterilized muslin cloth and spore concentration was adjusted to 5 × 10<sup>5</sup> spores ml<sup>-1</sup>. Freshly prepared 25 ml of culture suspension was sprayed to the seedlings during 20-25 days after transplantation and it was repeated a second time 15 days after the first spray.

In order to analyze the efficacy of the transformed plants against *A. helianthi*, the Southern dot blot and PCR-positive T<sub>1</sub> generation plants were challenged with *A. helianthi* in a complete randomized design (CRD). A suspension of the spores was prepared from sporulating mycelium of *A. helianthi* maintained on PDA plates for nine days. The abaxial surface of the leaves of 25-day old T<sub>1</sub> generation putative transgenics and untransformed control plants maintained in the greenhouse were sprayed with this suspension. Inoculated plants were kept at 28 ± 1°C in the greenhouse and covered with polythene paper for initial 2 days (to maintain high humidity) and were removed later. The treated plants were rated for the disease symptoms up to 30 days after inoculation (DIA). The spore suspension was administered again, 15 days after the first inoculation and observations were continued

for 2 more weeks. The severity scores ranged from 0 to 6, where 0 = no symptoms and 6 = 91–100% of leaf area affected and/or extensive stem damage or dead plant.

From the resistant T<sub>1</sub> generation plants mature seeds were collected, dried and allowed to grow as T<sub>2</sub> generation plants in greenhouse according to recommended package of instructions. The PCR analysis was carried out in T<sub>2</sub> plants using *35S-UidA* primers.

## RESULTS

### Plant transformation and recovery of transformants

The primary transformants were predominantly developed by following the protocol standardized earlier (Rao and Rohini 1999). About 33 seedlings were subjected to *in planta* transformation using LBA4404/pKVD4 of which 15 seedling were subjected to GUS histochemical assay. The remaining 18 T<sub>0</sub> seedlings were transferred into pots and shifted to a greenhouse. These plants nevertheless showed healthy vegetative growth, flowered (Fig. 2A) and set seed normally.

### GUS expression

Infection of the already differentiated embryonic tissue with *Agrobacterium* may result in random gene integration and hence the T<sub>0</sub> plants will be chimeric. However, some of the tissues developed from transformed cells should show gene integration. The extent of transformation was ascertained based on GUS histochemical assay. For this, the tissues that were tested free of residual *Agrobacterium* were used. GUS histochemical analysis of the primary transformants was therefore used as the first proof for the amenability of sunflower to *in planta* transformation strategy as an indication of transformation. Fig. 2B shows GUS expression in the shoot apex of the primary transformants 5 days after infection whereas endogenous GUS-like activity was not seen in the untransformed controls. Sections of the GUS-stained tissues revealed the expression of the *uidA* gene within the cells and not in the apoplastic region (Fig. 2C). The section of the wild-type tissue did not show any staining/blue colour (Fig. 2D). This indicates the integration and expression of the transgene.

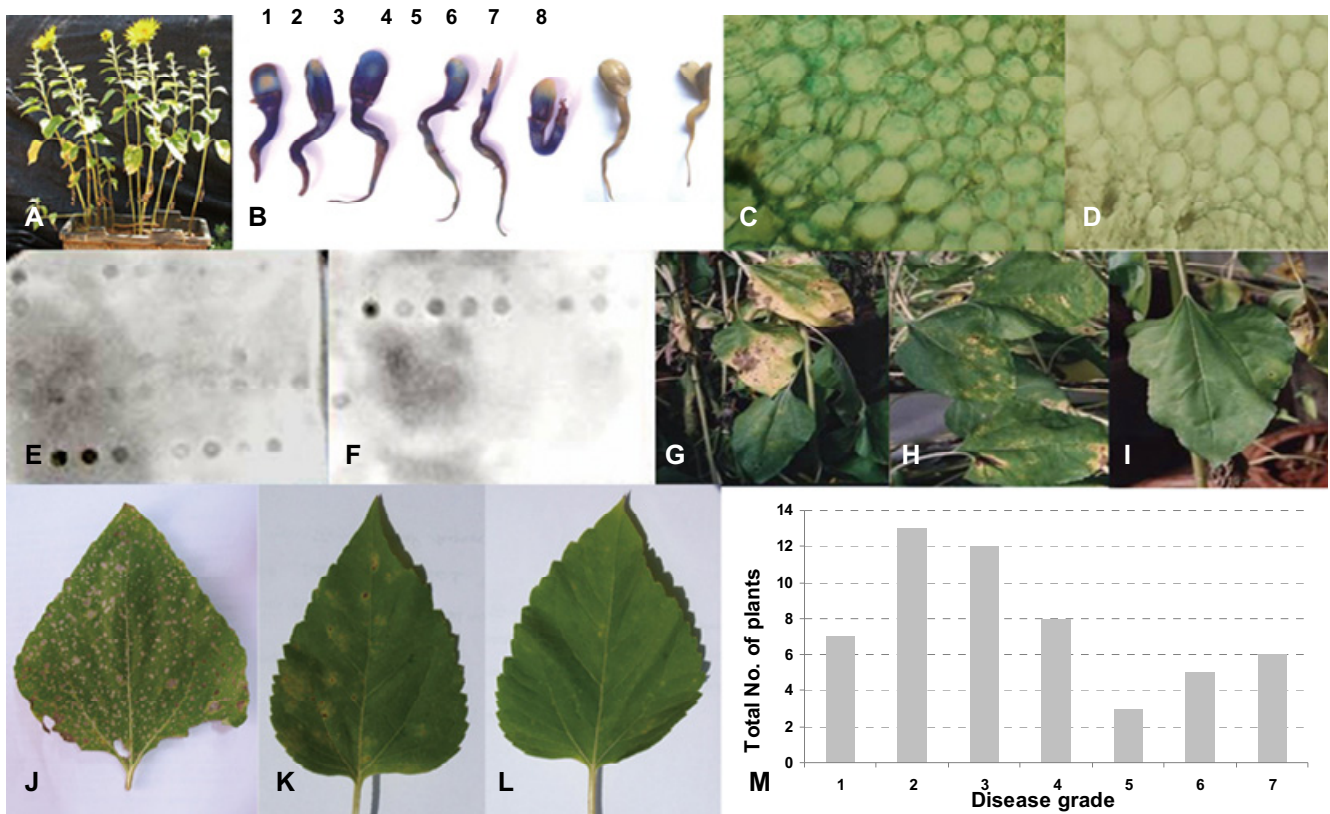
### Molecular characterization of T<sub>1</sub> transformants

Eighteen primary transformants (T<sub>0</sub> generation) harboring the glucanase gene could be established in the greenhouse. A total of 360 T<sub>1</sub> generation plants were raised from the 18 primary transformants in the greenhouse and preliminary analysis was carried out by dot blot Southern analysis. Genomic DNA was extracted from the leaf samples of all the 360 plants plus untransformed control and 5 µg of DNA was used for the dot blot Southern analysis. The DNA when hybridized with *nptII* probe indicated putative transgenic nature in 51 plants (Fig. 2E-F). No such signal was observed in the untransformed control DNA.

PCR analysis was carried out for the selected 51 plants and was showing consistent PCR amplification with *nptII* primers giving a 750 bp amplification product of *nptII*, which was similar to that of the positive control pKVD4 vector (Fig. 3B). No such bands were observed in the untransformed control under similar conditions. This observation clearly indicates the presence of the transgene in the genomes of T<sub>1</sub> transformants. The transgenic nature of T<sub>1</sub> generation plants was re-confirmed by PCR for *uidA* gene were taken for further characterization and reconfirmation of transgene by using *35S-uidA* primers to amplify a 687-bp product (Fig. 3A).

### *Alternaria* screening of transgenic plants

For future cultivation of promising transgenic sunflower plants, we evaluated resistance of the transgenic lines to *A.*



**Fig. 2** (A) T<sub>1</sub> generation plants of sunflower. (B) GUS histochemical analysis of the primary transformants. 1-6) T<sub>0</sub>/primary transformants; 7-8) Untransformed (wild type) sunflower. (C) GUS expression in the transverse section of transgenic seedlings of sunflower and its localization in the cells. (D) transverse section of wild type-untransformed seedlings of sunflower. (E-F) Dot blot Southern analysis of putative transgenic (a representative blot): 5 μg of genomic DNA was blotted on to a positively charged nylon membrane and probed with labeled 750-bp *nptII* gene fragment. Strong and weak signal was observed in 51 putative transgenics. (G-I) Bioassay of the T<sub>1</sub> generation sunflower plants against *Alternaria helianthi* at 30 DAI. G: Wild type; (H) Moderately resistant transformed plant; (I) Resistant plant; (J-L) Third nodal leaf (from top) of sunflower plants inoculated with *Alternaria helianthi* at 30 DAI (J) Wild type; (K) Moderately resistant transformed plant; (L) Resistant plant (M) Disease grading 0-6 scale (0 = no symptoms resistant type; 6 = 91-100% spots and susceptible type) on x-axis; Total No. of plants in each disease grade category on y-axis.

*helianthi*, an important tropical disease. All 51 dot blot and PCR-positive T<sub>1</sub> generation transgenic lines plus untransformed control plants were subjected to disease screens to determine whether the transgenic lines displayed increased levels of resistance to the chosen disease compared to wild-type DRSF 110 plants.

T<sub>1</sub> transgenic lines exhibited various levels of enhanced resistance to *A. helianthi* (Fig. 2M). Particularly, significant glucanase-induced enhancement of resistance to *A. helianthi* was observed when compared to untransformed controls. The seven high β-1,3-glucanase-expressers (lines 11, 19, 24, 31, 54, 61 and 66) showed no symptoms compared to the susceptible untransformed control plants which however, showed higher disease severity (Fig. 2G-L). Transgenic lines with medium or low levels of transgene expression, showed slightly higher severity than wild-type plants whereas lines 2, 7, 41-48 and 70 showed disease symptoms as good as untransformed controls at the end of 30 DAI.

### Southern analysis

The seven plants which performed well and showed no reaction in fungal bioassay were checked for integration of the transgene by genomic southern analysis. Genomic DNA of seven high performing plants, wild type plant and vector pKVD4 digested with *Xba*I and *Nhe*I was probed with radiolabelled 687-bp *35S-uidA* sequence. A clear hybridization signal was seen in all the seven transformants at ~3.0 kb region corresponding to the pKVD4 vector insert release of *uidA*, confirming the stable integration of transgene into the genomes of T<sub>1</sub> generation sunflower plants. However, no such hybridization signal was observed in the non trans-

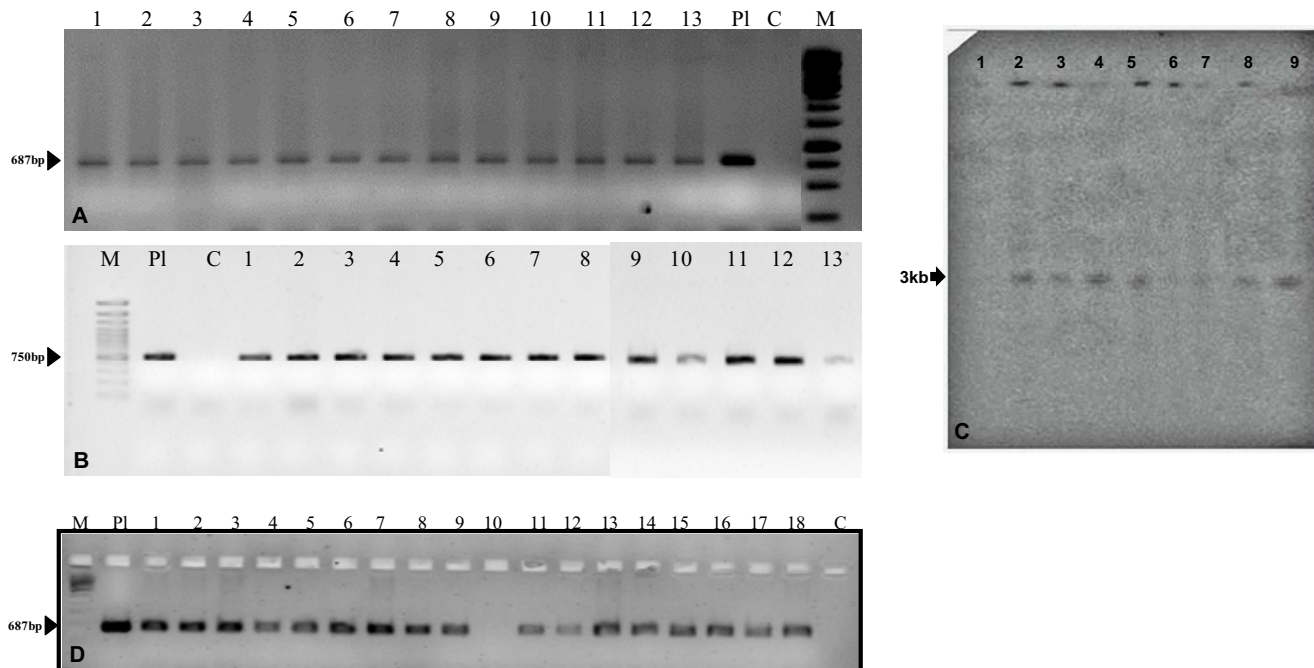
genic control (Fig. 3C). These plants were therefore progressed into the next generation.

### T<sub>2</sub> generation

The stability of the transgenes in the T<sub>2</sub> generation was ascertained by PCR analysis. Ten seeds from seven Southern positive plants with no disease i.e. resistant type T<sub>1</sub> generation plants were selected and germinated in the greenhouse. Genomic DNA was extracted from one-month-old T<sub>2</sub> plants along with non-transgenic plants and PCR analysis was performed as mentioned above using promoter and gene specific *35S-uidA* primers. All the T<sub>2</sub> generation plants yielded a 687-bp amplification product of *35S-uidA*, which was analogous to that of the positive control. No amplification was seen in untransformed sunflower (Fig. 3D). The amplification of the right amplicon confirmed that the transgenes were inherited into the subsequent generation.

### DISCUSSION

In recent years, much attention has been focused on understanding the complex defense mechanisms of plants in response to pathogenic infection. The rapid accumulation of host-encoded proteins, commonly known as pathogenesis-related (PR) proteins, with antifungal activity has been demonstrated in various host-pathogen interactions (Van Loon 1997; Van Loon *et al.* 1998, 2006). Among these proteins are hydrolytic enzymes such as β-1,3 glucanases and chitinases which have been purified and characterized from several plants (Legrand *et al.* 1987; Rasmussen *et al.* 1992; Buchter *et al.* 1997; Yeboah *et al.* 1998). Induction of these



**Fig. 3 Molecular analysis of T<sub>1</sub> generation plants.** (A) PCR analysis to amplify 687-bp *35S-uidA* gene fragment. Lanes 1-13: Dot blot Southern positive transgenics. (B) Amplified product of 750-bp *nptII* gene fragment. Lanes 1-13: PCR positive (*35S-uidA*) transgenics. PI: Binary vector; C: Untransformed; Lane M: Marker. (C) Genomic Southern analysis: The Southern analysis was carried out for 7 lines that showed '0' disease grade in fungal bioassay. 15 µg of genomic DNA digested with *XbaI* and *NheI* was probed with 687-bp *35S-uidA* gene fragment. Lane 1: untransformed control; 2: Plant no. 11; 3: 19; 4: 24; 5: 31; 6: 54; 7: 61; 8: 66; 9: pKVD4 vector as positive control. (D) PCR analysis (a representative gel) of Southern positive T<sub>2</sub> generation plants.

enzymes occurs in different plant species in response to fungal infection (Metraux and Boller 1986; Cachinero *et al.* 1996), wounding (Ignatius *et al.* 1994), and treatment with ethylene or elicitors (Roby *et al.* 1988; Mauch *et al.* 1992; Wubben *et al.* 1996). β-1,3 glucanases enhanced the resistance against *Rhizoctonia solani* in transgenic tobacco (Jach *et al.* 1995) and rice against *Magnaporthe grisea* (Nishizawa *et al.* 2003). However, with the rapid advancement in genetic transformation resistance to pathogen infection can be improved by regulation of pathogen resistant genes such as β-1,3 glucanases and other genes controlling host resistance (Broglie *et al.* 1991; Vierheilig *et al.* 1993; Tabai *et al.* 1998; Datta *et al.* 1999, 2000, 2001; Kumar *et al.* 2003).

The present paper describes a genetic transformation protocol i.e., *in planta* method was adopted based on our method standardized earlier for sunflower (Sankara Rao and Rohini 1999), safflower (Rohini and Sankara Rao 2000b; Manoj Kumar *et al.* 2009b), groundnut (Rohini and Sankara Rao 2000a), cotton (Keshamma *et al.* 2008) and bell pepper (Manoj Kumar *et al.* 2009a). In our method, *Agrobacterium* is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, *Agrobacterium tumefaciens* transfer the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary transformants (T<sub>0</sub>) being chimeric in nature. Hence, analysis of the transgenic plants should be carried out in the T<sub>1</sub> generation. As a preliminary analysis, dot blot Southern analysis was followed to screen 360 T<sub>1</sub> generation plants using *nptII* radiolabeled probe. Further, the selected 51 Southern positives were individually analyzed for the transgene integration using two sets of primers i.e. *nptII* gene specific and *35S*-promoter forward-*uidA* reverse primers (*35S-uidA*). A similar kind of screening was adopted in transgenic groundnut to select the true transformants using marker (*nptII*) specific primers in T<sub>1</sub> generation (Keshamma *et al.* 2008).

Nevertheless, analysis of the T<sub>0</sub> generation plants was carried out with an objective to know whether chimeras were produced. *Uid A* gene used in the study that expresses only upon transfer to plant system facilitated the identifica-

tion of the chimeras. The first indication of the transformability and chimeric nature in T<sub>0</sub> plants was obtained by the GUS histochemical analysis of the shoot region as seen in Fig. 2B. In our earlier studies on bell pepper (Manoj Kumar *et al.* 2009a) and safflower (Manoj Kumar *et al.* 2009b), we reported similar kind of observation in the T<sub>0</sub> transformants. Based on this initial affirmation, T<sub>1</sub> transformants were subjected to grid PCR as described above.

The selected plants based on molecular analysis were subjected to fungal bioassay against *A. helianthi* to select high performing plants. It was observed that the susceptible plants developed symptoms on the 6<sup>th</sup> day after inoculation, on par with the non-transformed controls. In plants that showed moderate resistance, symptoms appeared around 9-10 days after inoculation and barely spread. In the resistant plants, the leaves remained healthy even after 15 days after inoculation. Based on the analysis, 7 plants were identified as resistant (Fig. 2I, 2L).

Our results indicated the viability and reproducibility of *in planta* transformation protocol of our earlier work (Sankara Rao and Rohini 1999) in sunflower genetic engineering. The stable integration and inheritance of the transgenes till the T<sub>2</sub> generation has also been verified. The method therefore is advantageous because it not only avoids the need for *in vitro* propagation but also its associated somaclonal variations. The efficiency of transformation in any crop using *in planta* transformation depends on a number of factors and standard percentage efficiency cannot be set for any crop or experiment. Firstly, it depends on the number of chimeras arising from the total number of T<sub>0</sub> plants. The number of chimeras depends on the number and type of cells that integrate the transgene. Secondly, the number of plants in the T<sub>1</sub> generation that are stable transformants can vary between the chimeric T<sub>0</sub> plants as it depends on how many of the transformed cells develop into germ cells. This is evident in present experiment that 7 resistant lines obtained from 51 PCR positive T<sub>1</sub> plants. The transformation efficiency in the present experiment was 14%. However, 7 best lines were selected based on bioefficacy for advancement into the next generation. The percentage efficiency of transformation in this study was calculated based on the

number of PCR positives obtained from T<sub>1</sub> transformants. This indicates that the transformation efficiency depends on the number and kind of cells that are transformed and that produce the germ cells. Similar transformation efficiency based on PCR analysis has also been reported in other crops (Supartana *et al.* 2005; Chumakov *et al.* 2006; Putu Supartana *et al.* 2006; Keshamma *et al.* 2008; Manoj Kumar *et al.* 2009a, 2009b) where *in planta* transformation methodology has been used.

The present study demonstrates the efficacy of the  $\beta$ -1,3-glucanase gene against an important sunflower fungal pathogen *A. helianthi* and therefore the possible protection against the fungus.

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