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Resistance to *Alternaria* Leaf Spot Disease in Transgenic Safflower (*Carthamus tictorius* L.) Harboring a Rice Chitinase Gene

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ABSTRACT

Safflower (*Carthamus tinctorius* L.) is an important oilseed crop of semi-arid regions. Yield losses due to fungal diseases are enormous in the cultivation of this crop. Overexpression of pathogenesis-related (PR) proteins leads to increased resistance to pathogenic fungi in several crops. The PR protein chitinase hydrolyses a major cell wall component, chitin of pathogenic fungi and acts as a plant defense barrier. We report in this paper, overexpression of a rice chitinase in transgenic safflower cv. 'A-1' and its resistance towards *Alternaria carthami*. PCR was used to confirm stable integration of the *chitinase* gene in transgenic safflower plants. When screened for resistance against *A. carthami*, these plants showed not only a reduction in the number of spots but also a delay in the onset of disease. Overall the method resulted in a transformation efficiency of 7.72% on analysis of T_1 plants. The results demonstrate the potential of a PR protein from a heterologous source in developing fungal disease-resistant safflower.

Keywords: *Alternaria carthami*, fungal resistance, *in planta* transformation

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a valuable oilseed crop cultivated in India, Mexico Argentina, Australia, Canada, China, Spain, Italy, Turkey, Iraq, Iran, Egypt, and Ethiopia and is an alternative oil crop for the dry lands of these countries. India occupies premier position in safflower in the world as it was cultivated over an area of 364,000 ha (50% of world area) and had a production of 229,000 tons (27% of world production) during 2005-06 (Anonymous 2007). Even the peak average seed yields of 660 Kg/ha in India were attained in 2007. The crop has 35% oil content with a high amount of linoleic acid, which is of a very high therapeutic value (Nikam and Shitole 1999). Its flowers are used for colouring, flavouring foods and making dyes exclusively as a source of red dye carthamin extracted from its florets (Li and Mündel 1996).

Successful utilization of plant biotechnology for plant improvement requires the development of an efficient shoot regeneration system. Plant regeneration systems were optimized using hypocotyls, immature embryos, seedling leaves, roots (Nikam and Shitole 1999), cotyledonary explants (Tejovathi and Das 1997; Nikam and Shitole 1999; Mandal and Gupta 2001; Neetika *et al*. 2005) and direct somatic embryogenesis (Mandal *et al*. 1995, 2001, 2003). However, multiplication frequency and rooting of safflower were low (Nikam and Shitole 1999). Although several reports of *in vitro* regeneration of safflower have been published (Mandal *et al*. 1995; Baker and Dyer 1996), an efficient plant regeneration system applicable to a wide group of genotypes/cultivars is still lacking. Development of a method to obtain transformants, which is independent of the problems inherent to tissue culture of safflower, would represent a major accomplishment. One such technique is *in planta* transformation methods that target the *Agrobacterium* to the apical meristem or the meristems of axillary buds. So far this protocol has been successfully standardized for maize (Chumakov *et al*. 2006), wheat (Putu Supartana *et al*. 2006), rice (Supartana *et al*. 2005), buckwheat (Kojima *et al*. 2000), kenaf (Kojima *et al*. 2004), soybean (Chee *et al*. 1989) and mulberry (Ping *et al*. 2003). This technique is advantageous because it does not involve regeneration procedures and therefore the tissue culture-induced somaclonal variations are avoided. The present study has been undertaken to develop a stable genetic transformation system for introducing transgene(s) into safflower using the *in planta* method of *Agrobacterium*-mediated transformation, developed in our laboratory (Sankara Rao and Rohini 1999; Rohini and Sankara Rao 2000a, 2000b, 2001; Keshamma *et al*. 2008; Manoj Kumar *et al*. 2009) and successful production of transgenic safflower with a fungal resistant transgene affording enhanced resistance against *Alternaria* leaf spot, one of the import fungal diseases caused by *Alternaria carthami* Chowdhury. It would be useful to develop transgenics in safflower harboring PR proteins. In this paper we present the development and analysis of safflower transformants harboring the *chitinase* gene.

MATERIALS AND METHODS

Plant material and bacterial strains

Genetic transformation studies were carried out using safflower cv. 'A-1' 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% $HgCl_2$ 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 37°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 the binary vector, pKG2 (13.4 kb) obtained from Prof. K. Velu-

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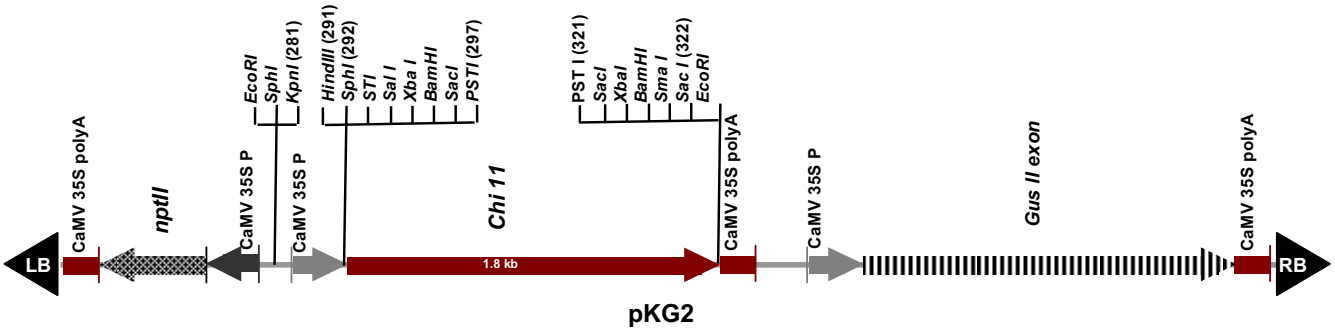

Fig. 1 pKG2 derivative Ti-plasmid construct *chi11*::*npt*II::*uidA* used for safflower transformation. The *chi11* coding region was inserted as an *Eco*RI-*Sph*I fragment between the 35S promoter and the 35S polyA terminator in the pKG2 vector. The *npt*II gene conferring resistance to kanamycin was used as selectable marker and *uid*A/*gus* as reporter for safflower transformation.

thambi, Madurai Kamraj Univeristy, Madurai, Tamil Nadu, India. pKG2 contains a chitinase11 gene (1.8 kb) under the control of the CaMV35S promoter and 35S polyA terminator, the *chi11* gene was cloned into the *Eco*RV and *Sma*I sites of the vector pKG2. The screenable and selectable marker, *uid*A, and the *npt*II gene, were regulated by the CaMV35S promoter and terminator (**Fig. 1**). LBA4404/pKG2 was grown in LB medium (pH 7.0) containing 50 μgml^{-1} kanamycin. The bacterial culture (3 ml) was later re-suspended in 100 ml of Winans' AB medium (pH 5.2) (Winans *et al*. 1988) and grown for 18 h. For *vir* 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 2001; Keshamma *et al*. 2008; Manoj Kumar *et al*. 2009) was followed to obtain safflower 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® 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 Protrays® (60 × 15 cm) containing Soilrite® and were allowed to grow under growth room conditions for at least 7 days. The growth chamber was maintained at 28 ± 1°C under a 14-h photoperiod with fluorescent light (FL40S.W, Mistubishi, Tokyo) of 35 µmol m^{-2} s^{-1} intensity. Seven-day 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 on optimum temperature of 28 ± 1°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% $HgCl_2$ 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 *uid*A gene expression in the tissues of putative transformants, using 7 days post co-cultivation tissues that were incubated overnight at 37°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 T_0 plants in the greenhouse grew normally, flowered and set seed. The T_1 plants were analysed for the transformants.

DNA extraction and PCR analysis

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

The presence of the *npt*II gene in the putative transgenic safflower plants was detected by PCR using two *npt*II primers: forward (5′-GGGCAGGCCAGCGTATCGTG-3′) and reverse (5′-TCCCGCTAGTGCCT TGTCCAGTT-3′) specific to *npt*II gene (Topfer *et al.* 1989). PCR was performed to amplify a 750 bp *npt*II gene fragment in the putative transformants. In order to amplify the *npt*II gene fragment, PCR was initiated by a hot start at 94°C for 7 min followed by 30 cycles of 94°C min^{-1}, 58°C1.5 min^{-1} and 72°C min^{-1} with a 72°C10 min^{-1} final extension. Similarly, to reconfirm the integration of the *gus* transgene, a 35S CaMV promoter-specific (Bevan 1984) forward primer (5′-TGTAGAAA CCCCAACCCGTGAAAT-3′) and *uid* A reverse (5′-TCCCGCTA GTGCCTTGTCCAGTT-3′) primer (*35S-uid*A) 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_1 and T_2 transformants. The conditions for *35S-uid*A 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 $MgCl_2$, 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 pKG2 vector as a positive control while the reaction mix without DNA as water blank. The products were run on 1% agarose ethidium bromide gels.

Grid PCR analyses of putative transgenic plants in T_1 generation

Seeds from each individual plant were maintained as separate lines. T_1 safflower plants were grown in a greenhouse following a recommended package of instructions (Anonymous 2000) and the plants were labeled with aluminium tags. They were divided into different grids containing 100 plants each so that there were 10 plants each along the rows or columns. Samples from 10 plants either along a row or column formed a composite sample. As a result, from each grid of 100 plants numbered from 1 to 100, 20 composite samples originated (Keshamma *et al*. 2008).

The above grid PCR analyses was used for preliminary screening of putative transgenic plants in T_1 generation whereas T_2 generation plants were analyzed using individual PCR to confirm transgene integration.

Alternaria resistance screening

Isolation and purification of *Alternaria carthami* cultures was done from fresh infected parts of safflower collected from control plots of the University of Agricultural Sciences, GKVK, Bangalore, India. *A. carthami* 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^{-1} was autoclaved at 1 atm for 20 min) in 9 cm Petri dishes for fur-

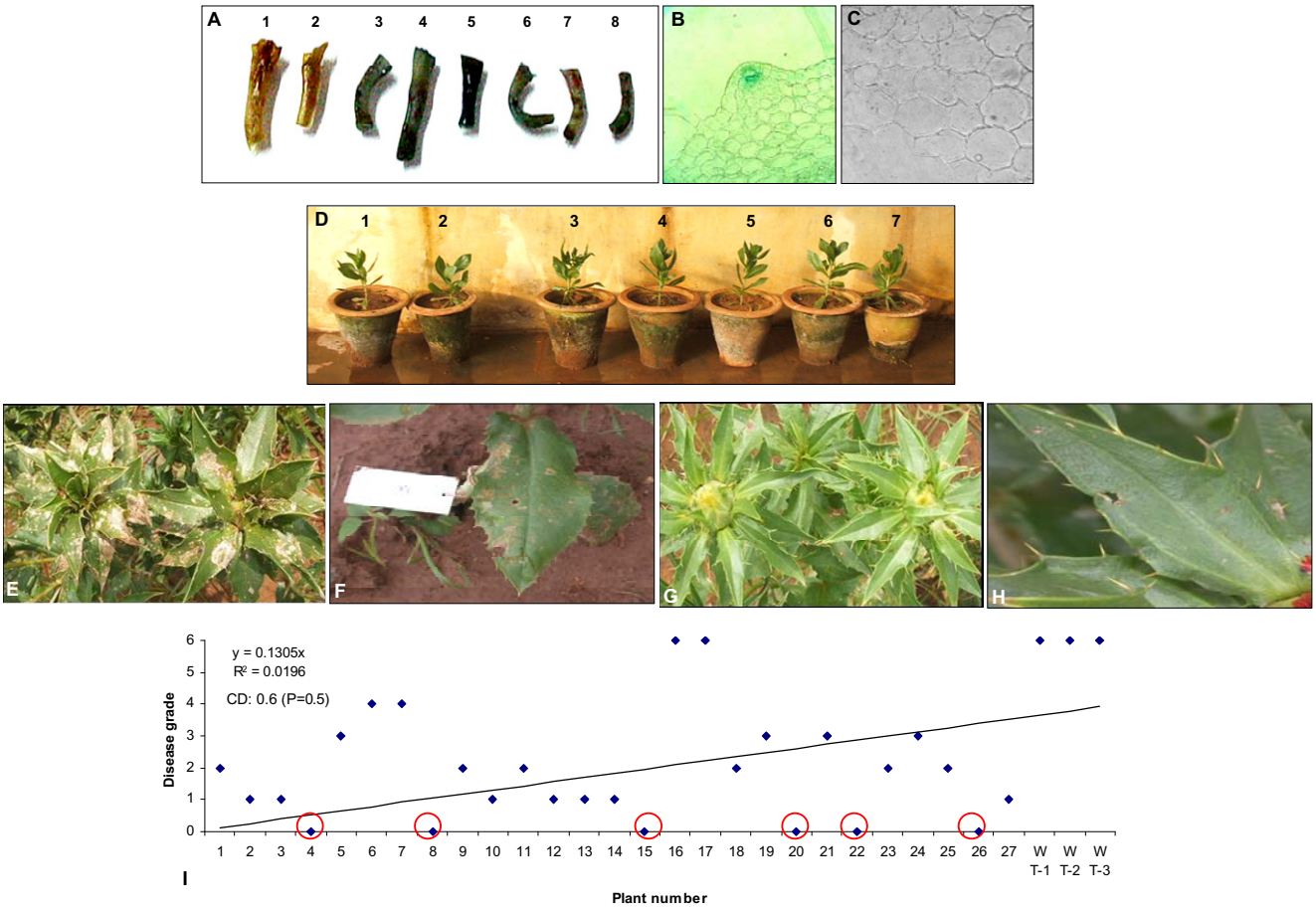

Fig. 2 (**A**) GUS histochemical analysis of the primary transformants. 1) Wild type 2-8) T_0/primary transformants. (**B**) Expression of GUS in the transgenic seedlings of safflower and localization in the cells. (**C**) Wild type-untransformed cells. (**D**) T1 generation plants of safflower. 1-2) Untransformed plants. 3-7) Putative transformants. (**E to H**) Bioassay of the safflower plants against *Alternaria carthami.* (**H, I**): untransformed plant. (**J, K**): Resistant transformed plant. (**I**) Disease grading 0-6 scale (0 = no symptoms resistant type; 6 = 91-100% spots and susceptible type); Circled ones are resistant type.

ther study. A nine-day-old *A. carthami* 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 5×10^5 spores ml^{-1}. 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. carthami*, the PCR-positive T_1 generation plants were challenged with *A. carthami* in a complete randomized design (CRD). A suspension of the spores (10^6 conidial spores per ml^{-1} sterile water) was prepared from sporulating mycelium of *A. carthami* maintained on PDA plates for nine days. The abaxial surface of the leaves of 25-day old T_1 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 rated up to 30 days after inoculation. 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.

Statistical procedures

The experiment was conducted in a CRD. The resultant data was subjected to analysis of variance (ANOVA) followed by mean separation by the Student Newman-Keul's test (p=0.05). All analyses were performed using the SAS (Version 6.0) (1996) package.

RESULTS

Plant transformation and recovery of transformants

About 50 seedlings were subjected to *in planta* transformation using LBA4404/pKG2 of which 15 seedling were subjected to GUS histochemical assay. The remaining 35 T_0 seedlings were transferred into pots and shifted to a greenhouse. These plants nevertheless showed healthy vegetative growth (**Fig. 2D**), flowered 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_0 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 safflower to *in planta* transformation strategy as an indication of transformation. **Fig. 2A** shows GUS expression in the shoot apex of the primary transformants 7 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 *uid*A gene within the cells and not in the apoplastic region (**Fig. 2B**). The section of the wild-type tissue did not show any staining/blue colour (**Fig. 2C**). This indicates the integration and expression of the transgene.

PCR analysis

Thirty five primary transformants harboring the chitinase gene could be established in the greenhouse. The group of 350 T_1 plants was divided into 70 composite grid samples. PCR analysis resulted in a possibility of 56 plants being putative transgenics (**Fig. 2E**). Repeated PCR analysis resulted in 27 plants showing consistent PCR amplification with *npt*II primers giving a 750 bp amplification product of *npt*II, which was similar to that of the positive control pKG2 vector (**Fig. 2F**). No such bands were observed in the untransformed control and water blanks under similar conditions. This observation clearly indicates the presence of the transgene in the genomes of T_1 transformants. The grid PCR positives were taken for further characterization by individual PCR analysis using 35S-*uidA* primers to amplify a 687 bp product (**Fig. 2G**).

Disease screening of transgenic plants

For future cultivation of promising transgenic safflower plants, we evaluated resistance of the transgenic lines to *A. carthami*, an important tropical disease. The disease selected for this evaluation was leaf-localized caused by the fungal pathogen. All 27 PCR-positive T_1 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 A-1 plants.

T_1 transgenic lines exhibited various levels of enhanced resistance to *A. carthami*. Particularly, significant *chi*-induced enhancement of resistance to *A. carthami* was observed when compared to untransformed controls. The six high *chi*-expressers (lines 3, 8, 14, 17, 20 and 27) showed no symptoms compared to the susceptible untransformed control plants which however, showed higher disease severity (**Fig. 2L**). Transgenic lines with medium or low levels of transgene expression, showed slightly higher severity than wild-type plants whereas two lines i.e. 9 and 21 showed disease symptoms as good as untransformed controls at the end of 30 days after inoculation (DAI).

T_2 generation

Sixty seeds from six PCR-positive plants with no disease i.e. resistant type T_1 generation plants were selected and germinated in the greenhouse. Genomic DNA was extracted from one-month-old T_2 plants along with non-transgenic plants and PCR analysis was performed as mentioned above using promoter and gene specific *35S-uid*A primers. All the T_2 generation plants yielded a 687-bp amplification product of *35S-uid*A, which was analogous to that of the positive control. No amplification was seen in non-transgenics and water blank (**Fig. 3A, 3B**).

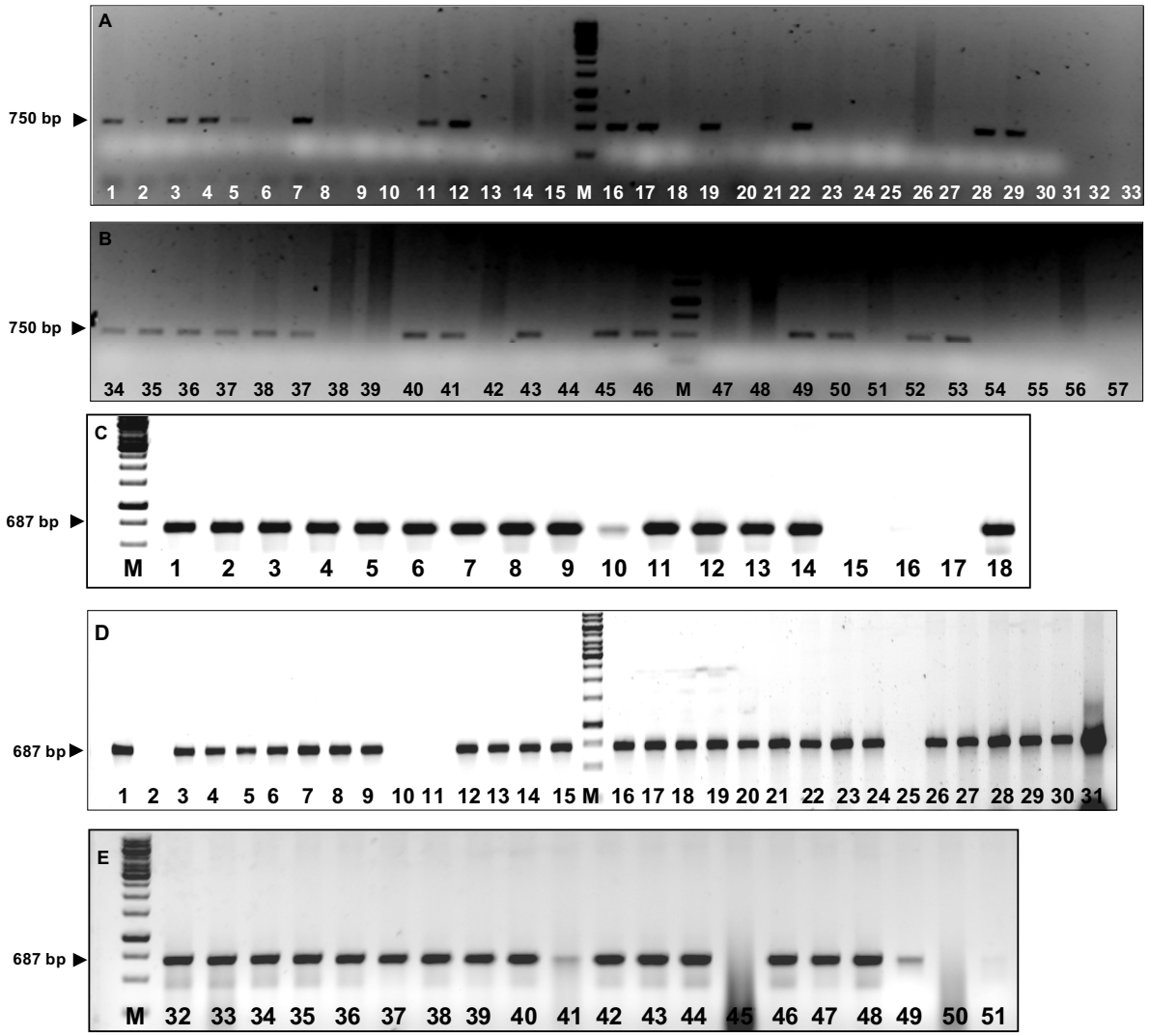

Fig. 3 PCR analysis of T_1 generation plants. (**A, B**) Grid PCR (a representative gel) analysis: PCR was performed to amplify the 750 bp *npt*II gene fragment. Lane 1: binary vector (plasmid DNA); Lanes 2-55: putative transformants; Lane 56: untransformed control plant (negative control); Lane 57: water blank. (**C**) Individual PCR analysis for the confirmation of *gus* gene integration using *35S-uid*A primers in the selected grid PCR-positive plants. Lanes 1-15: selected T_1 transformants; Lane 16: untransformed control; Lane 17: water blank; Lane 18: binary vector; PCR analysis of T_2 generation plants. (**D, E**): PCR was performed as described in the text using *35S-uid*A primers. Lanes 1-30 and 32-49: T_2 transformants; Lane 31: binary vector; Lane 50: untransformed control; Lane 51: water blank Lane M: 1 kb marker (Bangalore Genei Pvt Ltd., Bangalore, India).

DISCUSSION

In recent years, much attention has been focused on understanding the complex defence mechanisms of plants in response to pathogenic infection. The rapid accumulation of host-coded 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 chitinases and B-1,3 glucanases 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). Introduction of these 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). Chitinase was detected in up to five leaves above the infected leaf of cucumber plants (Matraux *et al*. 1988), and higher chitinase activity and earlier accumulation in resistant cultivars than susceptible one (Rasmussen *et al*. 1992; Ignatius *et al*. 1994; Neha *et al*. 1994). However, with the rapid advancement in genetic transformation resistance to pathogen infection can be improved by regulation of pathogen resistant genes such as chitinase 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 one such genetic transformation protocol i.e., an *in planta* method that was adopted based on our method standardized earlier for sunflower (Sankara Rao and Rohini 1999), safflower (Rohini and Sankara Rao 2000b), groundnut (Rohini and Sankara Rao 2000a), cotton (Keshamma *et al*. 2008) and bell pepper (Manoj Kumar *et al*. 2009). In our method, *Agrobacterium* is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, *Agrobacterium tumefaciens* transfers 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_0) being chimeric in nature. Hence, analysis of the transgenic plants should be carried out in the T_1 generation. As a preliminary analysis that is high throughput selection by grid PCR was followed to screen 350 T_1 generation plants using *npt*II primers. Further, the selected 27 grid PCR positives were individually analyzed for transgene integration by promoter- and reporter-specific primers (*35S-uid*A). Similar screening was adopted in transgenic groundnut to select true transformants using marker (*npt*II)-specific primers in the T_1 generation (Keshamma *et al*. 2008).

Nevertheless, analysis of the T_0 generation plants was carried out with an objective to know whether chimeras were produced. The *uid*A gene used in the study, which expresses only upon transfer to a plant system, facilitated the identification of the chimeras. The first indication of the transformability and chimeric nature in T_0 plants was obtained by GUS histochemical analysis of the shoot region as seen in **Fig. 2A**. Manoj Kumar *et al*. (2009) also reported a similar kind of observation in the T_0 generation of bell pepper transformants. Based on this initial affirmation, T_1 transformants were subjected to grid PCR as described above.

The selected plants based on molecular analysis were subjected to a fungal bioassay against *A. carthami* to select good performing plants. It was observed that the susceptible plants developed symptoms on the 6th 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, 6 plants were identified as resistant (**Fig. 2H-K**).

Our results indicated the viability and reproducibility of the *in planta* transformation protocol of our earlier work (Rohini and Sankara Rao 2000b) in safflower genetic engineering. The stable integration and inheritance of the transgenes until the T_2 generation was also 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_0 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_1 generation that are stable transformants can vary between the chimeric T_0 plants as it depends on how many of the transformed cells develop into germ cells. This is evident in the present experiment in which 6 resistant lines were obtained from 350 T_1 plants. The transformation efficiency in the present experiment was 7.72%. However, 6 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_1 transformants. This indicates that the transformation efficiency depends on the number and kind of cells that are transformed and that produce germ cells. A similar transformation efficiency based on PCR analysis was also reported in other crops (Chumakov *et al*. 2006; Putu Supartana *et al*. 2006; Supartana *et al*. 2005; Keshamma *et al*. 2008; Manoj Kumar *et al*. 2009) where *in planta* transformation methodology was used.

The present study demonstrates the efficacy of the chitinase gene against an important safflower fungal pathogen *Alternaria carthami* and therefore possible protection against the fungus.

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