Plant Pathology Journal

ISSN 1812-5387

science alert

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Plant Pathology Journal 12 (1): 1-10, 2013
ISSN 1812-5387 / DOI: 10.3923/ppj.2013.1.10

Agrobacterium Mediated Genetic Transformation of Chrysanthemum (*Dendranthema grandiflora* Tzvelev) with Rice Chitinase Gene for Improved Resistance Against *Septoria obesa*

Shweta Sen, Surinder Kumar, Minerva Ghani and Manisha Thakur
Department of Biotechnology, University of Horticulture and Forestry,
Solan-173230, Himachal Pradesh, India

Abstract: Chrysanthemum is an important commercial cut flower crop. It is severely infected by leaf spot disease caused by *Septoria obesa* resulting in huge yield loss. A simple and efficient protocol for the *Agrobacterium* mediated transformation of chrysanthemum cv., Snow Ball has been developed. The initiation of direct shoots was achieved from four week old internodal segments maintained on MS medium supplemented with 2 mg L^{-1} 6-benzyladenine (BA) and 0.25 mg L^{-1} naphthaleneacetic acid (NAA). To develop transgenic chrysanthemum resistant to fungal diseases, an attempt was made to transfer rice *chitinase* gene in chrysanthemum cv. Snow Ball. The rice *chitinase* gene harboured in the plasmid pCAMBIA-*ubi*-*chi*II (13.8 kb) was delivered via the *Agrobacterium*-mediated method to the internodal segments followed by subsequent development of complete plants on selection medium containing 10 mg L^{-1} hygromycin used as selective agent. Four independent putative transformed plants were recovered with stringent selection pressure. Molecular analysis of these plants confirmed the integration of *chi*II gene in three transformed plants. Transformation frequency of 2.2% was obtained. No plant was classified as resistant, one plant showed slight symptoms whereas two plants exhibited moderate symptoms when the transformants were tested with spore suspension of *Septoria obesa* under glasshouse conditions.

Key words: *Agrobacterium tumefaciens,* chitinase, chrysanthemum, resistance, transformation

INTRODUCTION

Chrysanthemums are very popular cut flowers and ornamental pot plants of high economic value. It is the second largest cut flower after rose among the ornamental plants in the global flower market (Kumar *et al*., 2006). Being an important commercial crop, application of tissue culture and genetic engineering techniques in chrysanthemum cultivars is of special value to obtain improved or desired traits like disease and insect resistance. Chrysanthemum is severely infected by leaf spot disease caused by *Septoria obesa* during warm and humid conditions resulting in 15-20% yield loss. The use of fungicides to control the disease is often ineffective because the pathogen spreads rapidly under favourable conditions. The crop production heavily relies on chemicals for protection which is not viable as these chemicals provide ephemeral benefits often with adverse side effects (Kumar *et al*., 2008). The major destructive fungi, on the other hand, are developing resistance to most classes of fungicides and environmental pollution caused by these chemicals is a serious problem (Moham *et al*., 2003). In view of the environmental hazards, genetic transformation provides an alternative mean for making targeted single trait improvement in clonally propagated crops. Genetic engineering techniques have been used by many workers to improve traits in chrysanthemum (Petty *et al*., 2000; Da Silva, 2004; Shinoyama and Mochizuki, 2006).

In the present study, it was planned to use rice chitinase gene for production of fungal resistant chrysanthemum. Among various plant resistance genes, chitinase genes are among the most promising as this enzyme degrades the substrate chitin found in cell walls of most of the fungi. Hence, genetic engineering of plants with chitinase gene is attractive as control mechanism to fungal pathogens. Several reviews and research articles have also stressed the advantage of using chitinase in plant protection because these enzymes are fungicidal and are part of the plant defense system (Collinge *et al*., 1993).

A rice chitinase gene *chi*II was isolated from a monocotyledon and it has already been used in transformation experiments of dicotyledons successfully

Corresponding Author: Shweta Sen, Department of Biotechnology, University of Horticulture and Forestry, Solan-173230, Himachal Pradesh, India

to enhance resistance (Ganesan *et al*., 2009; He *et al*., 2008). In this study, we introduced rice chitinase gene (*chi*II) in chrysanthemum cv. Snow Ball and evaluated the resistance of transgenic plants to leaf spot disease caused by *Septoria obesa* Syd.

MATERIALS AND METHODS

Explant source and culture conditions: Internodal segments (0.8-1.0 cm) from aseptic cultures (4 weeks old) of *Dendranthema grandiflora* Tzelev cv., Snow Ball maintained in the Department of Biotechnology, University of Horticulture and Forestry, Solan, India were used as explants. For Direct Shoot Regeneration (DSR) the explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA) (Table 1), 30 g L^{-1} sucrose and solidified with 8 g L^{-1} agar (Difco bacto, LobaChemie, Mumbai, India). The pH of the medium was adjusted to 5.8 with 1 N HCl and/or 1 N NaOH before autoclaving at 1.1 lbs $inch^{-2}$ for 15 min. The shoots were transferred after 2 weeks to Shoot Elongation Medium (SEM) comprising of 0.5 mg L^{-1} BA, 0.1 mg L^{-1} Indole-3- acetic acid (IAA) and 1 mg L^{-1} gibberellic acid (GA_3). Micro-shoots (2.5-3.0 cm long) developed after 4 weeks from the subcultured explants, were kept for rooting in culture tubes (25×150 mm) containing Root Induction Medium (RIM) comprising of half strength MS salts, 0.2 mg L^{-1} Indole-3-butyric Acid (IBA), 2 g L^{-1} activated charcoal, 30 g L^{-1} sucrose and solidified with 8 g L^{-1} agar. All the cultures were maintained at 25±2°C under 16 h photoperiod (irradiance of 50 μmol m^{-2} sec^{-1}) provided by white, cool fluorescent tubes (Philips India, Mumbai, India). After 4 weeks well developed root system developed. For acclimatization, the roots were washed thoroughly and transferred to potting mixture containing sterile sand, soil and Farm yard Manure FYM (1:1:1, w/w). The plants were acclimatized by gradually exposing to the natural environment prior to transfer to the glasshouse.

***Agrobacterium* strain and plasmid:** Disarmed *Agrobacterium tumefaciens* strain LBA 4404 (provided by Dr. S Muthukrishnan, Kansas State University, USA) harbouring plasmid pCAMBIA *bar-ubi-chi*II (13.8 kb) (Fig. 1) with two selectable markers, phosphinothricin

Table 1: Effect of BA and NAA on explants producing shoots and number of shoots per explant (%)

Treatment (mg L^{-1})			
BA	NAA	Explant producing shoots[1]	No. of shoots per explant[2]
0.0	0.00	0 (1.00)	(1.00)
1.0	0.50	3.33 (1.77)	0.33 (1.13)
1.0	0.75	0 (1.00)	0 (1.00)
1.0	1.00	1.67 (1.48)	0.25 (1.10)
1.5	0.25	0 (1.00)	0 (1.00)
1.5	0.50	0 (1.00)	0 (1.00)
1.5	0.75	9.33 (2.77)	0.33 (1.13)
1.5	1.00	6.00 (2.45)	0.33 (1.13)
2.0	0.25	52.67 (5.25)	2.66 (1.27)
2.0	0.50	48.60 (6.90)	0.66 (1.52)
2.0	0.75	39.38 (4.75)	1.33 (1.41)
2.0	1.00	6.67 (2.71)	0.33 (1.13)
$LSD_{0.05}$		(0.15)	(0.41)

[1]Values within parentheses are arc sine transformed values, [2]Values within parentheses are square root transformed values, BA: Benzyladenine, NAA: Naphthalene acetic acid

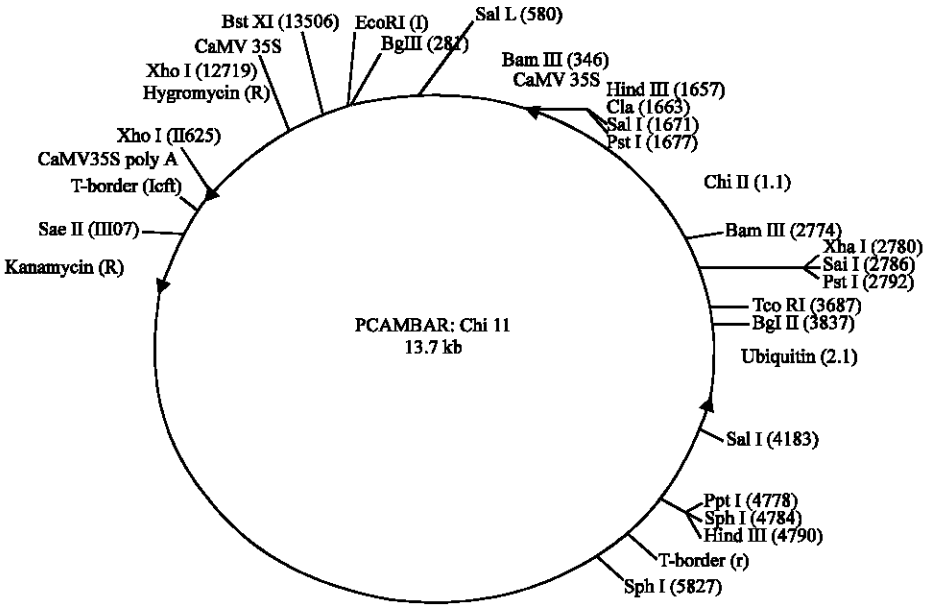

Fig. 1: Map of pCAMBIA *bar-ubi-chi*II transformimg vector

acetyltransferase (*bar*) and the hygromycin phosphotransferase (*hpt*), was used for genetic transformation. Both selectable marker genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter. The chitinase gene-*chi*II (1.1 kb) was controlled by the maize ubiquitin I-Ubi I (2 kb). Bacteria were maintained on Yeast Mannitol Broth YMB medium (Vincent, 1970) containing 50 mg L^{-1} kanamycin sulphate and 50 mg L^{-1} streptomycin.

Sensitivity of hygromycin: Tolerance limits (sensitivity) of the internode segments for hygromycin (Hyg) were determined by culturing these on direct shoot regeneration medium supplemented with different concentrations of Hyg (0-10 mg L^{-1}). Hyg was filter sterilized and added to the medium after autoclaving followed by cooling at 40°C.

Co-cultivation and selection of transformants: Fresh internode segments were inoculated on DSR medium and pre-conditioned for 48 h. A single bacterial colony was inoculated into 10 mL of YMB broth fortified with filter sterilized kanamycin sulphate (50 mg L^{-1}) and streptomycin (50 mg L^{-1}). Bacterial culture was incubated at 28°C on an orbital shaker at 120 rpm for 24 h and used for co-cultivation in the late log phase (A_{540} = 0.520). The bacterial culture was centrifuged at 5000 xg for 10 min. The pellet so obtained was dissolved in 10 mL of liquid MS basal medium. Pre-conditioned explants were dipped into this suspension for 20 min with pricking and incubated on DSR medium for co-cultivation at 25±2°C under dark conditions for 96 h. After co-cultivation, explants were washed three times with sterile distilled water. The explants were blotted dry using sterile filter paper and cultured on selection medium [DSR+300 mg L^{-1} cefotaxime (Cef) containing Hyg (10 mg L^{-1})]. Explants not inoculated with *Agrobacterium* were cultured on selection medium as negative control. The cultures were incubated at temperature of 25±2°C under 16 h photoperiod and sub-cultured every week. Twenty co-cultivated explants per Petri plate were cultured with three replicates and the experiment was repeated three times (total number of explants = 180). Escapes were removed by sub-culturing green shoots on selective DSR medium at an interval of 4 weeks (two cycles). The shoots (2.5-3 cm long) thus obtained were transferred for rooting to RIM supplemented with 5 mg L^{-1} Hyg to maintain selection pressure. The rooted shoots were subsequently transferred to pots and moved to a glasshouse after acclimatization (as described earlier).

Polymerase chain reaction: Putative transformants and one control (non-transformed) were used for PCR-Southern Blot analysis. Genomic DNA was extracted from the leaf tissue using Offringa and Lee (1995) protocol. The integrated rice chitinase gene was detected by PCR using two oligonucleotide forward and reverse primers 5'-GGACGCAGTCTCCTTCAAGA-3' and 5'-ATGTCGCAGT AGCGCTTGTA-3', respectively. PCR reaction mixture consisted of 10.2 μL milli Q water, 0.4 μL *Taq* DNA polymerase, 2 μL 10 X buffer, 1.8 μL of 25 mM $MgCl_2$, 1.6 μL dNTPs, 1 μL of each primer and 2 μL of genomic DNA. The amplification conditions for the *chi*II fragment were: 94°C for 4 min as pre-heating, 32 cycles at 94°C for 60 sec as denaturation, annealing at 55°C for 90 sec and extension at 72°C for 120 sec and final extension at 72°C for 5 min. The amplified products were separated on a 1.2% agarose gel and photographed using gel-documentation system (Alpha imager). DNA isolated from the plasmid served as positive control.

Southern blot analysis: The genomic DNA was isolated from the leaves of four PCR-positive hardened plants and one control plant. 15 μg of genomic DNA was digested with *Hind*III (MBI, Fermentas Life Sciences, USA) and subjected to electrophoresis on 0.8% agarose gel under submerged conditions. The DNA was then transferred to nylon membrane (Bright Star Plus, Ambion Ltd, USA) by capillary blotting. The membrane was cross-linked in UV cross linker (Spectrolinker XL-1000 UV Crosslinker Spectronic Corporation) and probed with 3.1 kb chitinase-ubi fragment (obtained by *Hind*III restriction of plasmid DNA harboured in *Agrobacterium* strain LBA-4404). Hybridisation was carried out at 55°C. All other procedures were carried out according to the manufacturer's instructions.

RT-PCR analysis: Reverse transcription followed by the polymerase chain reaction (RT-PCR) for the amplification of specific RNA sequence in cDNA form was carried out on the transgenics for the expression of *chi*II gene. Total RNA from the transgenes was isolated using PLANT Total RNA Kit (GeneAid) and subsequent cDNA synthesis was carried out using cDNA synthesis Kit (Life Technologies) according to the manufacturer's protocol. Direct PCR amplification of RNA was carried out to rule the possibility of having amplified contaminant DNA in the samples. One such sample served as negative control and plasmid DNA from pCAMBIA *bar-ubi-chi* II (13.8 kb) was used as the positive control. The amplified fragments (using the same components, primers and conditions for *chi*II gene described earlier for PCR) were separated on 1.2% agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

Evaluation of resistance to leaf spot disease in transgenic chrysanthemum: *Septoria obesa* (local isolate) procured from the Department of Mycology and Plant Pathology,

University of Horticulture and Forestry, Solan, India was used as pathogen. Spore suspension was obtained from the 15 days old cultures of *Septoria obesa* on PDA medium by scrapping the conidia into sterile distilled water. The spore concentration was adjusted at 1×10^4 spore mL^{-1} and Tween-20 (0.1%) was used as the sticking agent. Plants (2 months old) expressing *chi*II gene and non-transformant (control) were sprayed with spore suspension under glasshouse conditions at 25±2°C and 80% RH in three independent trials. They were observed daily for one month following inoculation and the disease symptoms were recorded based on the reaction of individual regenerates to the *S. obesa.* Resistance was categorized into four classes (based on visual observations) with (1) indicating no symptoms, (2) indicating very slight symptoms, (3) indicating moderate symptoms and (4) indicating severe symptoms after 15 days of inoculation.

Statistical analysis: Each experiment consisted of 20 explants and each experiment was replicated thrice. Data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez, 1984). The statistical analysis based on mean value per treatment was made using ANOVA.

RESULTS AND DISCUSSION

Fungal, bacterial and viral diseases are the main limiting factor in large scale cultivation of crops (Nadarajah *et al*., 2009; Farrag and Abo-Elyousr, 2011; Sharma *et al*., 2012). Fungal diseases are the most destructive world-wide and are responsible for causing huge loss by reducing the yield and quality of flowers (Hammond *et al*., 2006). During the last few years efforts have been made on the transgenic expression of plant fungal chitinases in crop plants and significant improvements in the resistance to fungal diseases have been made (Rajasekaran *et al*., 2005). Several reports have also stressed the advantage of using chitinases for plant protection because these fungicidal enzymes are not harmful to the plants as the substrate chitin is not found in the plants (Lorito *et al*., 1998). The enhanced resistance against fungal pathogens was obtained when the plants were engineered with chitinase genes (Tabei *et al*., 1998; Takatsu *et al*., 1999). Mostly chitinases cause hyphal tips to lyse *in vitro*. Some chitinases have isozymal activities and can hydrolyze the peptides in the bacterial cell wall, whereas others have exohydrolytic activity (Shinshi *et al*., 1990). In our study a protocol for adventitious shoot regeneration and genetic transformation was developed for introducing rice chitinase (*chi*II) gene for developing transgenic plants resistant to leaf spot pathogen.

After 4 weeks, the shoot buds started originating directly from the internode explant cultured on Direct Shoot Regeneration (DSR) medium. Percent explants forming shoots with average number of shoots per explant were recorded with the highest response on MS medium supplemented with 2 mg L^{-1} BA and 0.25 mg L^{-1} NAA. About 52.67% internode explants regenerated shoots on DSR medium, showing conformity with the results obtained earlier (Park *et al*., 2005; Hua and Hua, 2007). Shoot elongation and multiplication were achieved on MS medium supplemented with 0.5 mg L^{-1} BA, 0.1 mg L^{-1} IAA and 1 mg L^{-1} GA_3. Shoots (2.5-3 cm long) were excised and cultured on ½ strength MS medium containing 0.2 mg L^{-1} IBA and 2 mg L^{-1} activated charcoal for root induction. The rooted plantlets were transferred to pots containing a mixture of sand:soil:FYM (1:1:1) and acclimatized.

Among the various concentrations and combinations of BA and NAA tested for adventitious shoot regeneration, 2 mg L^{-1} BA in combination with 0.25 mg L^{-1} NAA was found to be most effective in inducing shoots than any other combinations (Table 1). Sun *et al*. (2009) reported best shoot regeneration with 0.2 mg L^{-1} NAA and 1 mg L^{-1} BA on MS medium in ground cover chrysanthemum. Mishra *et al*. (2004) obtained 4.43 shoots per shoot tip explant with 0.2 mg L^{-1} NAA and 2 mg L^{-1} BA in chrysanthemum. A number of workers have reported direct adventitious shoot regeneration from different explants in chrysanthemum (Bo *et al*., 2005; Hua and Hua, 2007; Datta *et al*., 2001; Waseem *et al*., 2009).

The use of proper type and concentration of selection agent in the selection medium is essential to enhance transformation frequency, in which the selection agent allows only transformed cells or plants to survive. In the present study Hyg was used as selection agent as phosphinothricin (PPT) selection may potentially lead to higher frequency of spontaneous PPT resistant mutants which is caused by single gene mutation. Thus, the effective transformation frequency using PPT selection could be less than observed in comparison to hygromycin selection (Leung *et al*., 1990).

Hygromycin has been extensively used as a selective antibiotic in transformation experiments mainly because hygromycin resistant gene is present as a selectable marker in several plant transformation vectors. Initially, the explants were placed on direct shoot regeneration medium which contained different concentrations of hygromycin (1-10 mg L^{-1}). The cultures not supplemented with hygromycin were considered as controls. Our results showed that increased concentration of hygromycin led to a gradual decline in growth of the explants (Fig. 2). The minimum growth of the explant was observed on

10 mg L^{-1} hygromycin. Therefore, this concentration was used in selection medium for regeneration of transformants. Similar results were reported by Shirasawa *et al.* (2000). Hygromycin B is an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes (Bakkar, 1992). The *hpt* gene codes for a kinase (hygromycin phosphotransferase, HPT) that inactivates hygromycin B through phosphorylation.

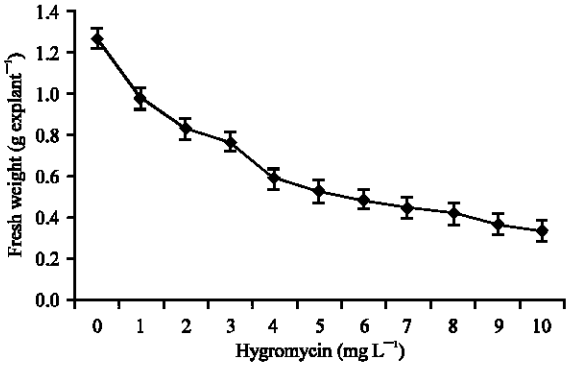

Fig. 2: Effect of hygromycin on the survival of internode explants. Horizontal bars represent standard error

Internode segments were pre-cultured on DSR medium for 48 h, then co-cultivated with *A. tumefaciens* for 96 h, followed by culturing on fresh selection medium containing antibiotics (MS+2 mg L^{-1} BA+0.25 mg L^{-1} NAA+10 mg L^{-1} Hyg+300 mg L^{-1} Cef). Direct shoots formed after 4 weeks at the cut edges of the internode explant and also at the wound site, where the tissue was damaged during inoculation (Fig. 3a). These shoots were sub-cultures for two more cycles (4 weeks $cycle^{-1}$) on the same medium to remove escapes. A high selection pressure was maintained in first two cycles, as a low selection pressure might allow the induction of escape shoots. Some authors suggested that the origin of escape shoots may be from the transiently transgenic cells (Ledger *et al.*, 1991; Fukai *et al.*, 1995) or due to

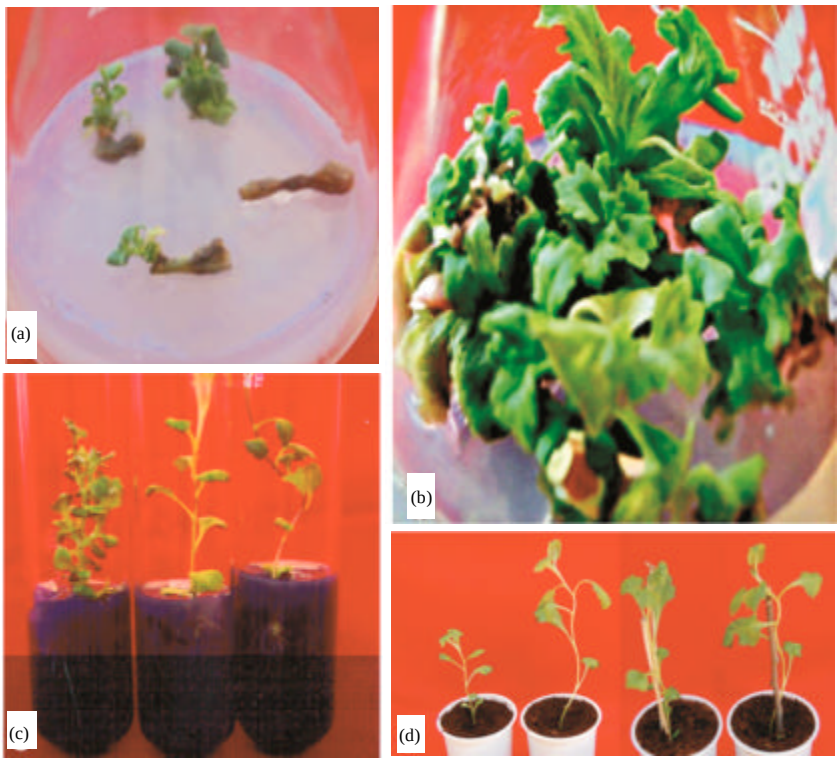

Fig. 3(a-d): Plant regeneration and genetic transformation studies in chrysanthemum cv. Snow Ball from internode tissue, (a) Adventitious shoot regeneration from internode segments on selective shoot regeneration medium after 4 weeks, (b) Multiplication and elongation of shoots on shoot elongation medium, (c) Rooting of regenerated shoots on root induction medium after 4 weeks of inoculation and (d) Hardened putative transformed plants

lack of stable T-DNA transfer (Lowe *et al*., 1993). The non-transformed (control) tissue did not survive on the selective medium. Four independent hygromycin resistant shoots were obtained on the DSR medium containing 10 mg L^{-1} Hyg. The Hyg resistant shoots were elongated on SEM (Fig. 3b) and subsequently transferred to rooting medium for rooting (Fig. 3c) and acclimatized. The transformed shoots were carefully nurtured in the glasshouse (Fig. 3d).

Pre-conditioning and co-cultivation duration had a marked effect on the production of hygromycin resistant explants. The 48 h pre-conditioning followed by co-cultivation for 96 h duration, resulted in 17.5% of hygromycin resistant explants with 4-5 average number of shoots. Aswath *et al*. (2004) suggested a pre-conditioning time of 2 day followed by 3 day co-cultivation for obtaining a maximum putative transformation rate in *Dendranthema grandiflorum*. Co-cultivation period of more than three days has been successfully used in chrysanthemum cultivars while transferring various marker and/or desirable genes (Kudo *et al*., 2002; Mitiouchkina *et al*., 2006).

The plant regeneration system from internode explants used presently was reliable and reproducible as direct shoot regeneration does not involve a long dedifferentiation phase and thus the problem of chimeral shoots due to somaclonal variations is reduced. Direct regeneration also required less time to produce the whole plant from the explant. Survival rate of the *in vitro* regenerated plantlets was over 85% in control experiments and a total of four independent, healthy putatively transgenic plants were produced through *Agrobacterium* mediated rice chitinase gene transfer.

Molecular analysis of the putative transformants was carried out by PCR and Southern blot hybridization. All four hygromycin resistant plants were found to be positive for the amplification of the 237 bp fragment of the *chi*II gene by PCR (Fig. 4a). No amplification was

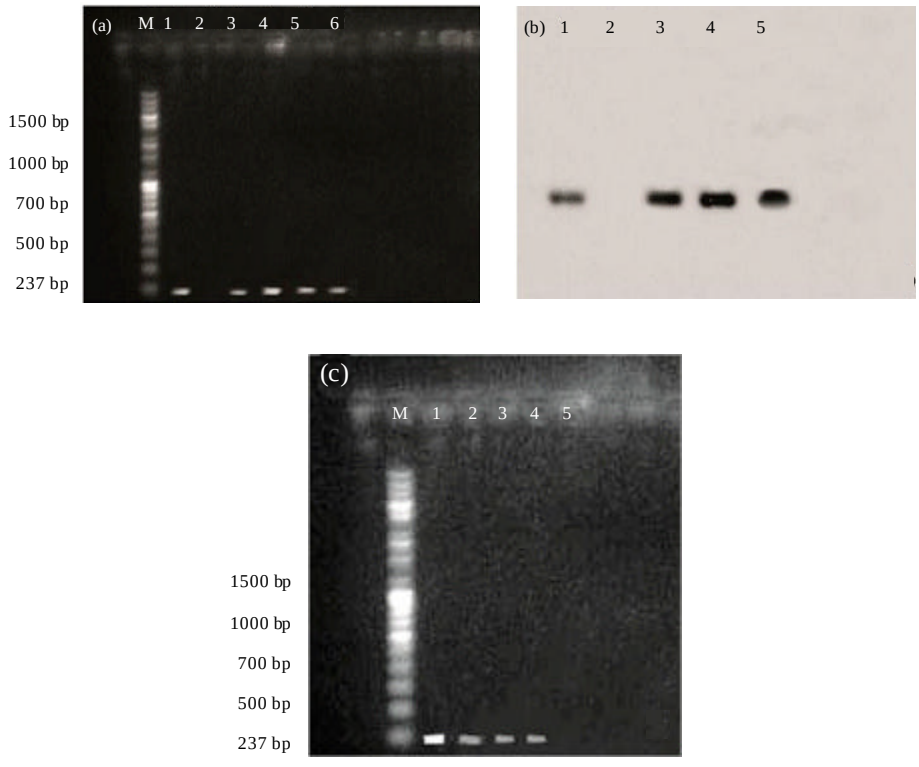

Fig. 4(a-c): Molecular characterization of transgenic chrysanthemum plants (a) PCR analysis with *chi*II gene specific primers; M DNA size marker (100 base pair ladder); Lane 1: Positive control (plasmid pCAMBIA *bar-ubi-chi*II; 13.8 kb), Lane 2: Negative control (untransformed plants), Lane 3-6: Transgenic chrysanthemum plants, (b) Southern blot hybridization Lane 1: Positive control (plasmid pCAMBIA *bar-ubi-chi*II; 13.8 kb), Lane 2: Negative control, Lanes 3-5: Transgenic chrysanthemum plants and (c) RT- PCR analysis of the transgenic lines, M DNA size marker (100 base pair ladder), Lane 1: Positive control, Lane 2-4: Transgenics showing expression of *chi*II gene, Lane 5: Negative control (untransformed tissue)

observed in case of untransformed plant DNA. The transformation efficiency out of 180 explants used with respect to the amplification of the expected size of the gene fragment was about 2.2% (Table 2). Takatsu (2002) developed an improved protocol for *Agrobacterium*-mediated genetic transformation of chrysanthemum by using stem segments of 23 cultivars. The presence of *npt*II gene in the regenerated chrysanthemum was confirmed by PCR-Southern analysis and transformation efficiency of 2.46% was observed by Doglova *et al.* (1997). Tsuro *et al.* (2005) used *Agrobacterium rhizogenes* for efficient transformation of chrysanthemum and reported that with the *gus* introduction, a significantly higher transformation rate (6%) was achieved. Yu *et al.* (2009) developed a stable and efficient transformation system with high transformation frequency for small flowered pot chrysanthemum. Transformation frequencies of 11.21 and 38.4% have also been reported in chrysanthemum (Wang *et al.*, 2004; Sun *et al.*, 2009). The difference in results may be due to the type of explant, type of gene introduction method, *Agrobacterium* strain and its identification (Teixeira da Silva and Fukai, 2001). Although, the transformation frequency is low, the protocol developed can be used to mobilize genes of agronomic importance in chrysanthemum. The transgene integration pattern in the nuclear genome of the putative transformed plants was confirmed through Southern hybridization analysis of the genomic DNA. The Southern hybridization was carried out in four transformants that were positive for PCR. The hybridization signals for the *chi*II gene were detected in three plants as distinct single copy integration (Fig. 4b). Kudo *et al.* (2002) and Seo *et al.* (2003) developed two confirmed transformants resistant to kanamycin in chrysanthemum. The expression of the *chi*II gene in transgenes was analyzed by reverse transcriptase (RT- PCR). Of the four PCR positive putative transgenics, only three plants tested positive (Fig. 4c). No amplified DNA fragments were detected in RNA samples subjected to direct PCR amplification. Three plants of transgenic chrysanthemum expressing *chi*II gene grown in the glasshouse were used to evaluate susceptibility to leaf spot disease. No plant was classified as highly resistant; one plant showed larger but slight symptoms on only lower 3-4 leaves that did not spread further, even if the incubation period was extended by 15 days (Fig. 5). Transgenic plants were shorter in height as compared to control. Similar results are reported by other workers (Jong-Seong *et al.*, 2000; Chen *et al.*, 2011). Other two plants, showed moderate resistance with the rate of spread slower than the control. The control plants showed severe symptoms. The expression of the *chi*II gene confers improved tolerance to leaf spot disease, although the enhanced resistance is partial and quantitative. Similar results were reported by other

Table 2: Transformation efficiency on *in vitro* derived internode explants

Parameter	Value
No. of explants infected	180.00
No. of hygromycin resistant plants	4.00
No. of PCR positive plants	4.00
Transformation efficiency*	2.2%

*Transformation efficiency is percentage of total number of PCR positive shoots to total number of explants infected

Fig. 5: Chrysanthemum plants, a: Transgenic, b: Control, Slight symptoms shown by the transgenic plant as compared to control plant, arrows indicate leaf spots caused by *Septoria obesa*

workers (He *et al*., 2008; Nirala *et al*., 2010). It has been reported that the plants overexpressing chitinase do not have enhanced resistance to fungal diseases (Neuhaus *et al*., 1991; Nielsen *et al*., 1993).

CONCLUSIONS

We demonstrated the successful regeneration of adventitious shoots from internode segments of chrysanthemum, followed by successful integration of the transferred *chi*II gene into chrysanthemum genome and expression of transferred gene showed an enhanced tolerance to leaf spot disease. These findings suggest that the rice chitinase (*chi*II) gene could be utilized as a genetic source of disease resistance for breeding and improving chrysanthemum. Work is in progress to check the stability of the trait using other molecular and/or proteomic analysis to achieve a clear indications of stable induction of tolerance to *Septoria obesa*.

ACKNOWLEDGMENT

Authors are grateful to Dr. Muthukrishnan, Kansas State University, USA for providing construct (pCAMBAR-chiII-ubi) for carrying out genetic transformation.

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