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Agrobacterium-mediated Genetic Transformation of Rice Chitinase (*chi*II) for Fungus Resistance in Chrysanthemum cv. 'Snow Ball'

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ABSTRACT

Plant regeneration and genetic transformation techniques have been described in internode tissue of chrysanthemum (*Dendranthema grandiflora* Tzelev) cv. 'Snow Ball'. Callus was developed on Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA) and 1 mg L^{-1} α-naphthalene acetic acid (NAA). Highest shoot regeneration from callus was obtained with 0.5 mg L^{-1} BA, 0.25 mg L^{-1} NAA and 1 mg L^{-1} gibberellic acid (GA_3). *Agrobacterium*-mediated genetic transformation was achieved using internode explants and rice chitinase gene (*chi*II). Highest callus induction was achieved on selective medium containing 10 mg L^{-1} hygromycin (Hyg) and 300 mg L^{-1} cefotaxime (Cef) after 48-hr pre-conditioning following 96-hr co-cultivation. Highest number of shoots per callus was observed when MS medium was supplemented with 0.5 mg L^{-1} BA, 0.5 mg L^{-1} NAA, 1 mg L^{-1} GA_3, 10 mg L^{-1} Hyg and 300 mg L^{-1} Cef. Shoots were elongated and multiplied on MS medium containing 0.5 mg L^{-1} BA, 0.5 mg L^{-1} indole-3-acetic acid and 1 mg L^{-1} GA_3. Rooting was accomplished on half-strength MS medium supplemented with 0.2 mg L^{-1} indole-3-butyric acid, 0.2% activated charcoal and 5 mg L^{-1} Hyg. The putative shoots were hardened with 82% survival in a glasshouse. The transformed plants were analysed for the presence and integration of *chi*II gene by PCR and southern blot analysis.

Keywords: *Agrobacterium tumefaciens*, callus, *Dendranthema grandiflora*, internode, *in vitro*, regeneration
Abbreviations: BA, 6-benzyladenine; **CIM**, callus induction medium; **Cef**, cefotaxime; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Hyg**, hygromycin; **NAA**, α-naphthalene acetic acid; **SRM**, shoot regeneration medium; **TDZ**, thidiazuron (*N*-phenyl-*N*'-1,2,3-thidiazol-5-ylurea)

INTRODUCTION

Chrysanthemum is one of the most important ornamental plants in the world. It is native of China and reached America, India and other countries in 19th century (Arora 2005). Chrysanthemum is the second largest cut flower after rose among the ornamental plants in the global market (Kumar *et al*. 2006). 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. On the other hand, major destructive fungi are developing resistance to most classes of fungicides and environmental pollution caused by these chemicals is a serious problem (Plimmer 1984; Mohan Babu *et al*. 2003). In view of the environmental hazards, modern biotechnological tools could be of much significance to alleviate the negative effect of such chemicals. Being an important commercial crop, application of tissue culture and plant genetic engineering in chrysanthemum cultivars is of special value to obtain improved or desired traits like disease and insect resistance.

Although desirable traits have been introduced by classical breeding, there have been some limitations to this technique due to narrow gene pool. Thus genetic transformation has the potential to hasten the production of new genotypes and broaden the available gene pool (Ainsley *et al*. 2001). Genetic transformation provides an alternative means for elucidating gene function and for making targeted single trait improvement in clonally propagated crops. Genetic transformation of chrysanthemum with various marker, reporter and desirable genes has already been reported (Fukai *et al*. 1995; Teixeira da Silva 2003a, 2003b, 2004a, 2004b; Aida *et al*. 2004; Aswath *et al*. 2004; Narumi *et al*. 2005; Chung and Park 2005; Teixeira da Silva 2005, 2006; Chandler and Tanaka 2007; Choi *et al*. 2008; Soh *et al*. 2009; Wang *et al*. 2009).

Hydrolytic enzymes are known to play a significant role in the antifungal activity of the plant defence system (Tabaizadeh *et al*. 1999; Yamamoto *et al*. 2000). Chitinases are low molecular weight pathogenesis related proteins which are extracellular, acid soluble and protease resistant (Neuhaus 1999). In some cases where this mechanism is too weak to protect the plant, engineering constitutive expression of the defence protein can boost tolerance to fungal pathogens (Garison *et al*. 1996; Warkentin *et al*. 1998). Transgenic cucumber plants with *Chi*II gene showed enhanced resistant against gray mold (*Botrytis cinerea*) (Tabei *et al*. 1997). Similarly, 13-43% reduction in the development of black spots in transgenic rose plant was observed with chitinase gene (Marchant *et al*. 1998). On the other hand, there are some reports showing that transgenic plants expressing chitinase do not have resistance to some fungal diseases (Neuhaus *et al*. 1991; Nielson *et al*. 1993). With a long term plan to develop transgenic plants of chrysanthemum with resistance to fungal pathogen, *Agrobacterium*-mediated genetic transformation of chitinase gene using *in vitro* internode explant, is described.

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MATERIALS AND METHODS

Explant source

Internodes (0.8-1 cm) from aseptic cultures (six-weeks old) of *Dendrenthema grandiflora* Tzelev cv. 'Snow Ball' maintained on MS (Murashige and Skoog 1962) medium supplemented with 1 mg L^{-1} 6-benzyladenine (BA) and 0.2 mg L^{-1} α-naphthalene acetic acid (NAA) in the Department of Biotechnology, University of Horticulture and Forestry, Solan, India were used as explants. In the present study, the cultivar 'Snow Ball' was selected as it is highly susceptible to leaf spot disease caused by *Septoria obesa*.

Culture medium and culture conditions

To achieve callus induction, internode explants were cultured in 100-ml flasks (Borosil, Mumbai, India) on MS medium supplemented with vitamins, 30 g L^{-1} (w/v) sucrose, 8 g L^{-1} (w/v) Difco bacto agar (LobaChemie, Mumbai, India) and 0.5 mg L^{-1} BA and 1 mg L^{-1} NAA. This medium was named as callus induction medium (CIM). The pH of the medium was adjusted with 1 N HCl and/or 1 N NaOH to 5.8. Medium was autoclaved at 1.1 kg cm^{-2} for 15 min at 121°C. The cultures were maintained at 24 ± 2°C with a 16-h photoperiod (50-60 µmol m^{-2} s^{-1}) provided by white, cool fluorescent lamps (40 W each, Philips, India). After four weeks on CIM, the calli were subcultured on the same medium for proliferation. The callus pieces (0.8-1 cm^2) were transferred to the shoot regeneration medium (SRM) consisting of MS medium supplemented with various concentrations of plant growth regulators (PGRs) alone or in combination such as BA, thidiazuron (TDZ), NAA, indole-3-acetic acid (IAA) and gibberellic acid (GA_3). A total of 20 concentrations and combinations of PGRs were used, although only the treatments that produced shoots are presented in **Table 1**. The shoots elongated and were multiplied on MS medium supplemented with 0.5 mg L^{-1} BA, 0.5 mg L^{-1} IAA and 1 mg L^{-1} GA_3. This medium was named as shoot elongation medium (SEM). The regenerated shoots (2.5-3 cm long) were separated and individual shoots were transferred to root induction medium (RIM) consisting of MS medium (1/2 strength) containing 0.2 mg L^{-1} indole-3-butyric acid (IBA) and 0.2% activated charcoal (E. Merck (India) Ltd., Mumbai) to get complete plantlets. The regenerated plants were acclimatized as described earlier (Kumar *et al*. 2008).

Agrobacterium strain and plasmid

Disarmed *Agrobacterium tumefaciens* strain LBA 4404 containing binary vector pCAMBIA *bar-ubi-chi*II (13.8 kb; **Fig. 1**) with chitinase gene (Dr. Muthukrishanan, Kansas State University, USA) was used for genetic transformation. Selectable markers were the phosphinothricin acetyltransferase gene (*bar*) and the hygromycin phosphotransferase (*hpt*). 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) promoter. Bacteria were maintained on YMB medium (Vincent 1970) containing 50 mg L^{-1} kanamycin sulphate (Kan) and 50 mg L^{-1} streptomycin (both from HiMedia, Mumbai, India).

Genetic transformation

The internode segments (0.8-1 cm in size) were inoculated on CIM and pre-conditioned for 48 h where the explants undergo a physiological and developmental shift to enter for morphogenetic competency. A single bacterial colony was inoculated into 10 ml of liquid YMB medium containing 50 mg L^{-1} Kan and 50 mg L^{-1} streptomycin (filter sterilized) and incubated at 28°C on a shaker at 120 rpm for 24-h and used in the late log phase A_{540} at 0.520. The bacterial culture was centrifuged at 5000 × *g* for 10 min and MS liquid medium was added to the bacterial pellet to make up a volume of 10 ml. Pre-conditioned explants were dipped into this suspension for 20 min, blotted on sterile filter paper and transferred to CIM medium for co-cultivation. After co-cultivation for 96 h, explants were subsequently transferred to fresh CIM-Cef medium comprising of CIM supplemented with 300 mg L^{-1} cefotaxime (Cef: Ranbaxy, India) to inhibit bacteria. To determine the toxic levels of concentration of hygromycin (Hyg: HiMedia, Mumbai, India) for effective selection of putatively transgenic plants, control explants were cultured on CIM medium with different concentrations of Hyg (1-10 mg L^{-1}). At 10 mg L^{-1} and above, the explants did not grow further and turned brown. Therefore, 10 mg L^{-1} was used as selection pressure for the culture of co-cultivated explants. After one week plants cultured on CIM-Cef medium were transferred to the selection medium, comprising of CIM supplemented with 10 mg L^{-1} Hyg and 300 mg L^{-1} Cef. The cultures were maintained under a 16-h photoperiod and subjected to stringent selection on the selection medium for 4 weeks, after which the callus pieces (0.8-1 cm^2) were subcultured on selective SRM containing MS salts supplemented with 0.5 mg L^{-1} BA, 0.5 mg L^{-1} NAA, 1 mg L^{-1} GA_3, 10 mg L^{-1} Hyg and 300 mg L^{-1} Cef. The regenerated shoots were rooted on RIM containing 5 mg L^{-1} Hyg and acclimatized as described earlier.

Table 1 Shoot regeneration frequency of chrysanthemum from internode explants.

Treatment (mg L^{-1})					Shoot regeneration (%)	Average number of shoots
BA	TDZ	NAA	IAA	GA_3		
0	0	0	0	0	0 (1.0)	0 (1.0)
1	0	0	0	0	1.25 (6.41)	0.3 (1.13)
0.5	0	0.25	0	1	82.08 (64.96)	4.7 (2.37)
0.5	0	0.50	0	1	72.90 (58.91)	4.3 (2.30)
1	0	0.25	0	1	54.16 (47.39)	3.3 (2.07)
1	0	0.50	0	1	57.71 (30.55)	3.7 (2.15)
0	0.5	0	0.1	1	25.41 (28.09)	2.2 (1.82)
0	1	0	0.1	1	17.90 (26.28)	1.7 (1.63)
0.5	0	0	0.1	1	73.30 (58.24)	4.9 (1.73)
1	0	0	0.1	1	67.08 (54.99)	2.0 (2.15)
0	0.5	0.25	0	1	13.30 (21.41)	0.7 (1.27)
0	0.5	0.50	0	1	11.23 (19.60)	0.4 (1.13)
0	1	0.25	0	1	8.33 (16.77)	0.7 (1.13)
0	1	0.50	0	1	5.41 (13.44)	0.2 (1.17)
$CD_{0.05}$					(0.19)	(0.06)

Figures within parentheses are arc sine and square root transformed values

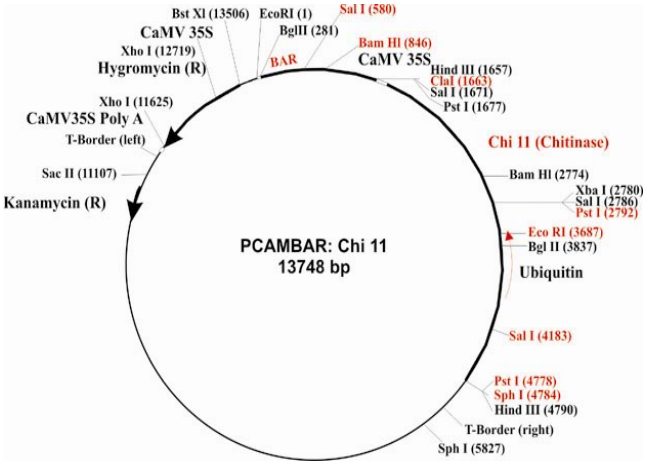

Fig. 1 Map of pCAMBIA *bar-ubi-chi*II transforming vector used for genetic transformation.

Statistical analysis

Each treatment consisted of 20 explants and each experiment was repeated thrice. Data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez 1984). Statistical analysis based on mean values per treatment was made using ANOVA. The LSD multiple range test ($P \leq 0.05$) was used to determine differences between treatments.

Polymerase chain reaction

Genomic DNA was isolated from the leaves of Hyg-resistant plants following the method of Offringa and Lee (1995). PCR

analysis was carried out to detect the presence of *chi*II gene using forward and reverse primers 5′-GGACGCAGTCTCCTTCAAGA-3′ and 5′-ATGTCGCAGTAGCGCTTGTA-3′, respectively. The primers were designed by Bangalore Genei, Bangalore, India. Each PCR reaction mixture (20 µl) consisted of 10.2 µl Milli Q water, 0.4 µl of 2 U *Taq* polymerase, 2 µl of 10X *Taq* DNA polymerase buffer, 1.8 µl of 25 mM $MgCl_2$, 1.6 µl of dNTPs, 1 µl of 5 pM each primer and 2 µl of 50 ng genomic DNA. The PCR was carried out for 32 cycles using a thermal cycler (GeneAmp PCR System 8600, USA) under the following conditions. 84°C denaturing for 1 min, 55°C annealing for 1 min, extension at 72°C for 2 min and another 5 min at 72°C for final extension. The amplified products were separated on a 1.2% agarose gel and stained with ethidium bromide to visualise under UV light.

Southern hybridization

Southern blot analysis was done according to Sambrook *et al*. (1989). The method involved digestion of 40 µg of genomic DNA with *Hind*III (MBI, Fermentas Life Sciences, USA), electrophoresing the digestive product on a 0.8% agarose gel and then blotting DNA fragments on to a nylon membrane (Hybond N+, Amersham). This was followed by hybridization with probe DNA. The probe was prepared by restricting plasmid DNA with *Hind*III and resulting 3.1 kb chitinase-ubi fragment was eluted from gel using Qiagen gel extraction kit (Qiagen Inc., USA) and used as a probe after radiolabelling (Decalabel^{TM} DNA labeling kit, Fermentas Life Sciences, USA).

RESULTS AND DISCUSSION

After one week of inoculation, the callus was initiated on the cut ends of internode explants and the entire surface of the explant was covered with callus after four weeks of inoculation. Shoots started originating from calli subcultured on SRM after four weeks. Per cent calli forming shoots and number of shoots per callus were recorded, with the highest response on MS medium supplemented with 0.5 mg L^{-1} BA, 0.25 mg L^{-1} NAA and 1 mg L^{-1} GA_3. Shoot elongation and multiplication were achieved on MS medium supplemented with 0.5 mg L^{-1} BA, 0.5 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 supplemented with 0.2 mg L^{-1} IBA and 0.2% activated charcoal. The rooted plantlets were transferred to pots containing a mixture of sand: soil: FYM (1: 1: 1) and acclimatized.

Among the various PGRs tested on MS medium for shoot regeneration, BA and TDZ in combination with NAA or IAA were able to form shoots from callus. 0.5 mg L^{-1} BA in combination with 0.25 mg L^{-1} NAA was more effective in inducing shoots than any other combination (**Table 1**). BA was more effective than TDZ in inducing shoots from callus. Mitiouchkina *et al*. (2000) found MS medium supplemented with 1 mg L^{-1} BA, 0.1 mg L^{-1} IAA and 1 mg L^{-1} GA_3 as the best medium for *in vitro* micropropagation of chrysanthemum cv. ‘White Snowdon’. Maximum frequency of shoots from leaf and stem segment derived callus was achieved with 0.2 mg L^{-1} IAA and 0.2 mg L^{-1} BA in *Chrysanthemum morifolium* (Bhattacharya *et al*. 1990). Fukai *et al*. (1995) reported 0.1 mg L^{-1} IAA and 1 mg L^{-1} BA as the best medium for shoot multiplication of *Dendranthema grandiflorum*. Aswath *et al*. (2004) achieved 4-7 shoots per callus mass with 0.25 mg L^{-1} NAA and 2.5 mg L^{-1} BA. Sarkar and Shaheen (2001) reported best response for callus induction and regeneration of shoots with 5 mg L^{-1} BA and 0.5 mg L^{-1} kinetin in cv. ‘Snow Ball’. Jaramillo *et al*. (2008) reported shoot regeneration in three varieties of chrysanthemum ‘White Albatross’, ‘Yellow Albatross’ and ‘Escapade’ on MS medium supplemented with 4.83 µM NAA and 4.44 or 13.32 µM BA. Sun *et al*. (2007) reported best shoot regeneration on MS medium supplemented with 0.2 mg L^{-1} NAA and 1 mg L^{-1} BA in ground cover chrysanthemum ‘Cheng Yun’.

Internode segments were pre-cultured on CIM for 48 h, then co-cultuvated with *A. tumefaciens* for 96 h and then

Fig. 2 Plant regeneration and genetic transformation studies in chrysanthemum (*Dendranthema grandiflora* Tzelev Cv. ‘Snow Ball’) internode tissue. (**A**) callus formation on selective callus induction medium in internode explant; (**B**) shoot regeneration on selective shoot regeneration medium after four weeks; (**C**) rooting of regenerated shoots after five weeks of culture; (**D**) hardened transformed and control plants of chrysanthemum.

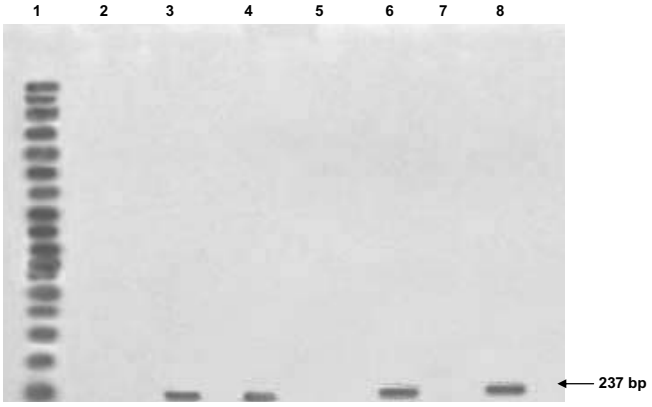

Fig. 3 PCR amplification of *chi*II gene fragment in transformed chrysanthemum plants. Lane 1, a marker (1 kb DNA ladder); Lane 2, DNA sample of non-transformed control plants; Lane 3, plasmid DNA (positive control); Lanes 4-8, DNA samples from transformants T_1, T_2, T_3, T_4 and T_5 (237 bp DNA fragment corresponding to *chi*II gene).

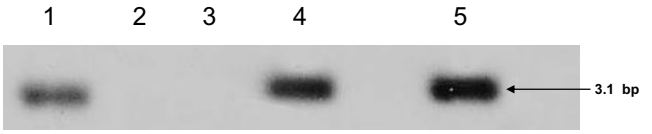

Fig. 4 Southern blot hybridization of PCR positive plants of chrysanthemum. Lane 1, plasmid DNA positive control (plasmid pCAMBIA *barubi-chi*II (13.8 kb) digested with *Hind*III to release *chi*II (1.1 kb) fragment with *ubi* promoter (2 kb); Lane 2, DNA sample of non-transformed control; Lanes 3, 4, and 5, DNA samples from PCR positive plants T_1, T_3 and T_5.

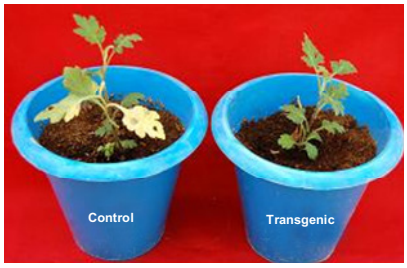

Fig. 5 Bioassay of control and transformed plants of chrysanthemum with spore suspension of *Septoria obesa*.

transferred to fresh selective CIM containing antibiotics. Callus formed after one week at the cut edges of the internode explant and also at the wound site; where the tissue was damaged during inoculation (**Fig. 2A**). The non-transformed (control) tissue did not survive on the selective medium containing 10 mg L^{-1} Hyg and 300 mg L^{-1} Cef. The developing transformed calli were transferred to selective SRM. The explants gave rise to green callus from which shoots developed (**Fig. 2B**). Five independent Hyg-resistant lines (T_1, T_2, T_3, T_4 and T_5) were selected in the presence of

Table 2 Transformation efficiency of *in vitro*-derived internode explants.

Number of explants infected	180
Number of hygromycin-resistant shoots	5
Number of PCR-positive shoots	3
Transformation frequency* (%)	1.67

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

hygromycin; these were elongated and multiplied. The hygromycin-resistant shoots were transferred onto rooting medium for rooting (**Fig. 2C**) and acclimatized. The transformed plants were carefully nurtured in the glasshouse (**Fig. 2D**).

Molecular analysis of the putative transformants was carried out by PCR and Southern blot hybridization. Out of a total five transformants, three (T_1, T_3 and T_5) were found to be positive for the amplification of the 237 bp fragment of the *chi*II gene by PCR (**Fig. 3**). There was no amplification observed in case of the T_2, T_4 and untransformed plant DNA. The transformation efficiency (percentage of total number of PCR-positive shoots to total number of infected explants) out of 180 explants used with respect to the amplification of the expected size of the gene fragment was about 1.67% (**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 plants was confirmed by PCR-Southern analysis and transformation efficiency of 2.46% was observed in cvs. 'Shuhou-no-Chikara', 'New Summer Yellow' and 'Yamabiko'. Transgenic plants of five varieties of chrysanthemum with *bt toxin*, *rolc*, *chs* and AFP genes were produced and transformation frequency of 1-4% was recorded (Doglov *et al*. 1997). Teixeira da Silva and Fukai (2002) reported nature and frequency of stable transgene expression in standard cv. 'Lineker' and spray type cv. 'Shuhou-no-Chikara' of chrysanthemum with four different gene introduction methods. Transformation frequency of 5% was observed with Agro-infection in cv. 'Lineker'. Genetic transformation of Korean chrysanthemum has been successfully achieved by co-cultivation with *A. tumefaciens*. Selection with 100 mg L^{-1} Kan led to a transformation frequency of 7.8% with respect to co-cultivated explants (Tosca *et al*. 2000). Yu *et al*. (2009) developed a stable and efficient transformation system with high transformation frequency for small flowered pot chrysanthemum. Tsuro *et al*. (2005) used *Agrbacterium rhizogenes* for efficient transformation of chrysanthemum and reported that with the *gus* introduction, a significantly higher transformation rate (6%) was achieved. Transformation frequency of 11.21 and 38.4% has been reported in chrysanthemum by Wang *et al*. (2003) and Sun *et al*. (2009), respectively. 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 in the present study, 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 three transformants (T_1, T_3 and T_5) that were positive for PCR. The hybridization signals for the *chi*II gene was detected only in two plants (T_3 and T_5) as distinct single copy integration (**Fig. 4**). Kudo *et al.* (2002) and Seo *et al.* (2003) developed two confirmed transformants resistant to Kan. However, the confirmed transgenic plant after multiplication is now being subjected to further genetic analysis and fungal disease assay with *Septoria obesa* to test the effectiveness of the rice chitinase gene against the leaf spot pathogen (**Fig. 5**).

Fungal diseases are the most troublesome worldwide and are responsible for reducing yield and quality of flowers (Hammond *et al*. 2006). The techniques of genetic transformations of plants offer a new perspective to introduce new fungus resistance genes into a crop's gene pool. Mostly, chitinases cause hyphal tips lyse *in vitro*. Some chitinases having isozymal activity and can hydrolyze peptides in bacterial cell wall (Shinshi 1990). Transgenic cultivars expressing chitinase gene have been shown to defend fungal attack in a wide variety of crops (Punja and Raharjo 1996; Bolar *et al*. 2000; Shi *et al*. 2000; Oldach *et al*. 2001; Ganesan *et al*. 2009). In the present study, transgenics of chrysanthemum with rice chitinase (*chi*II) gene were isolated with a long-term plan to develop resistance to fungal pathogen.

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