International Journal of Biotechnology and Biomedical Sciences
p-ISSN 2454-4582, e-ISSN 2454-7808, Volume 3, Issue 1; January-June, 2017 pp. 18-22

http://www.krishisanskriti.org

Genetic Transformation in Chrysanthemum cv. 'Snow Ball' for Fungus Resistance

Shweta Sen*[1] and Surinder Kumar[2]

[1,2]Department of Biotechnology, University of Horticulture and Forestry, Solan-173 230 (H.P.), India
E-mail: [1]drshwetathalwal@gmail.com, [2]skhf@rediffmail.com

Abstract—*We report here the genetic transformation studies in leaf explant of chrysanthemum (Dendranthema grandiflora Tzelev) cv. 'Snow Ball'). Callus was developed on Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} kinetin and 10 mg L^{-1} α-naphthalene acetic acid (NAA). Highest shoot regeneration from callus was obtained with 0.5 mg L^{-1} BA, 0.1 mg L^{-1} IAA and 1 mg L^{-1} gibberellic acid (GA_3). Agrobacterium-mediated genetic transformation was achieved using leaf explants and rice chitinase gene (chiII). 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} IAA, 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, 0.1 mg L^{-1} indole-3-acetic acid and 1 mg L^{-1} GA_3. Rooting was accomplished on half-strength MS medium supplemented with 0.25 mg L^{-1} indole-3-butyric acid, 0.2% activated charcoal and 5 mg L^{-1} Hyg. The putative shoots were hardened with 60% survival in a glasshouse. The transformed plants were analysed for the presence and integration of chiII gene by PCR and southern blot analysis.*

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, thidiauron*

1. INTRODUCTION

Chrysanthemum is one of the most important ornamental plants in the world.Chrysanthemum is the second largest cut flower after rose among the ornamental plants in the global market [4]. 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 [5]. 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 [12,6]. 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 [1]. Genetic transformation provides an alternative means for elucidating gene function and for making targeted single trait improvement in clonally propagated crops [15].

Chitinases are low molecular weight pathogenesis related proteins which are extracellular, acid soluble and protease resistance [8]. In some cases where this mechanism is too week to protect the plant, engineering constitutive expression of the defence protein can boost tolerance to fungal pathogens [2,17]. Transgenic cucumber plants with *Chi*II gene showed enhanced resistant against gray mold (*Botrytis cinerea*) [14]. On the other hand, there are some reports showing that transgenic plants expressing chitinase do not have resistance to some fungal diseases [9,10]. 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* leaf explant, is described.

2. MATERIALS AND METHODS

2.1 Explant source

Leaf explant (0.5 cm^2) 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*.

2.2 Culture medium and culture conditions

To achieve callus induction, leaf explants were cultured in 100-ml flasks (Borosil, Bombai, India) on MS medium [7] supplemented with vitamins, 30 g L^{-1} (w/v) sucrose, 8 g L^{-1} (w/v) Difco bacto agar (LobaChemie, Mumbai, India) and 1.0 mg L^{-1} kinetin and 10 mg L^{-1} NAA. This medium was named as callus induction medium. 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, 0.1 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., Bombai) to get complete plantlets. The regenerated plants were acclimatized as described earlier [5].

2.3 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 [16] containing 50 mg L^{-1} kanamycin sulphate (Kan) and 50 mg L^{-1} streptomycin (both from Himedia, Mumbai, India).

2.4 Genetic transformation

The leaf explant (0.5 cm^2 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. Pre-conditioned explants were dipped into bacterial suspension (bacterial pellet dissolved in 10 ml of MS liquid medium) 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} IAA, 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.

2.5 Statistical analysis

Each treatment consisted of at least 20 explants and each experiment was repeated thrice. Data recorded for different parameters were subjected to completely randomized design [3]. Statistical analysis based on mean values per treatment was made using ANOVA.

2.6 Polymerase chain reaction

Genomic DNA was isolated from the leaves of Hyg-resistant plants following the method of [11]. 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. 94°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.

2.7 Southern hybridization

Southern blot analysis was done according to Sambrook *et al*. (1989)[13]. 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).

3. RESULTS AND DISCUSSION

After one week of inoculation, the callus was initiated on the cut ends of leaf 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, 0.1 mg L^{-1} IAA 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 0.25 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 0.1 mg L^{-1} IAA was more effective in inducing shoots than any other combination (**Table 1**). BA was more effective than TDZ in inducing shoots from callus.

Leaf segments were pre-cultured on CIM for 48 h, then co-cultuvated with *A. tumefaciens* for 96 h and then transferred to fresh selective CIM containing antibiotics. Callus formed after one week at the cut edges of the leaf explant and also at the wound site; where the tissue was damaged during inoculation (**Fig. 2A&B**). 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. 2C&D**). Nine independent Hyg-resistant lines were selected in the presence of hygromycin; these were elongated and multiplied. The hygromycin-resistant shoots were transferred onto rooting medium for rooting (**Fig. 2E**) and acclimatized. The transformed plants were carefully nurtured in the glasshouse .

Molecular analysis of the putative transformants was carried out by PCR and Southern blot hybridization. Out of a total nine transformants, four 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 other five hygromycin resistant 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 2.22% (**Table 2**). 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 four transformants that were positive for PCR. The hybridization signals for the *chi* II gene was detected in three plants as distinct single copy integration (**Fig. 4**). However, the confirmed transgenic plant after multiplication were subjected to fungal disease assay with *Septoria obesa* to test the effectiveness of the rice chitinase gene against the leaf spot pathogen and transgenic plants were not found to be completely resistant to the pathogen infection. In the present study, transgenics of chrysanthemum with rice chitinase (*chi*II) gene were developed with a long-term plan to develop resistance to fungal pathogen.

4. ACKNOWLEDGEMENTS

Authors are grateful to Dr. Muthukrishanan, Kansas State University, USA for providing the pCAMBAR-chi II-ubi construct.

REFERENCES

[1]Ainsley PJ, Collins GG, Sedgley M (2001) Factors affecting *Agrobacterium* mediated gene transfer and the selection of transgenic calli in paper shell almond (*Prunus edulis* Mill). *Journal of Horticultural Science and Biotechnology* 76, 522-528

[2]Garison R, Grezes-Besset BB, Schneider M (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric gene. *Nature Biotechnology* 14, 643-646

[3]Gomez KA, Gomez AA (1984) *Statistical Procedures for Agricultural Research*, John Wiley and Sons, New York, pp 328-332

[4]Kumar S, Prasad KV, Choudhary ML (2006) Detection of genetic variability among chrysanthemum radiomutants using RAPD markers. *Current Science* 90, 1108-1113

[5]Kumar S, Negi SP, Kanwar JK (2008) *In vitro* selection and regeneration of chrysanthemum (*Dendranthema grandiflorum* Tzelev) plants resistant to culture filtrate of *Septoria obesa* Syd. *In Vitro Cellular and Developmental Biology – Plants* 44, 474-479

[6]Mohan Babu R, Sajeena A, Seetharaman K, Reddy MS (2003) Advances in genetically engineered (transgenic) plants in pest management – an overview. *Crop Protection* 22, 1071-1086

[7]Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* 15, 473-479

[8]Neuhaus JM (1999) Plant chitinase (PR-3, PR-4, PR-8, PR-11). In: Datta SK, Muthukrishnan S (Eds) *Pathogenesis Related Proteins in Plants*, CRC Press, Bola Raton, pp 77-105

[9]Neuhaus JM, Ahl-Goy P, Hinz U, Flores S, Meins Jr F (1991) High level expression of a tobacco chitinase gene in *Nicotiana sylvestris* susceptibility of transgenic plants to *Cercospora nicotianae* infection. *Plant Molecular Biology* 16, 141-151

[10]Nielsen KK, Mikkelsen JD, Kragh KM, Bojsen K (1993) An acidic class I chitinase in sugar beet: Induction by *Cercospora beticola*, charaterization and expression in transgenic tobacco plants. *Molecular Plant-Microbe Interactions* 6, 495-506

[11]Offringa R, Lee F (1995) Isolation and characterization of plant genomic DNA sequences via (inverse) PCR amplification. In: Jones H (Ed) *Methods in Molecular Biology, Plant Gene Transfer and Expression Protocols*, Humana Press Inc, NJ, pp 181-195

[12]Plimmer JR (1984) Chemical for agriculture. *IAEA Bulletin* 26, 13-16

[13]Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edn), Cold Spring Harbor Laboratory, New York

[14]Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1997) Cucumber plants harbouring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). *Plant Cell Reports* 17, 159-164

[15]Teixeira da Silva JA (2002) Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflora* (Ramat) Kitamura) using stem segments. *Breeding Science* 45, 179-184

[16]Vincent JM (1970) *A Manual for Practical Study of Root Nodule Bacteria*, IBP Handbook no. 15, Blackwell Scientific, Oxford, pp 164

[17]Warkentin D, Chai B, Liu AC, Hajela RK, Zhong H, Sticklen MB (1989) Development of transgenic creeping bentgrass (*Agrostis palustris* Huds) for fungal disease resistance. In: Sticklen MB, Kenna MP (Eds) *Turfgrass Biotechnology, Cell and Molecular Genetics Approaches to Turfgrass Improvement*, Ann Arbor Press, MI, pp 153-161

Table 1 Shoot regeneration frequency of chrysanthemum from leaf 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	2.08 (8.20)	0.6 (1.24)
0.5	0	0.25	0	1	62.50 (52.24)	4.3 (2.30)
0.5	0	0.50	0	1	74.16 (59.45)	3.1 (2.07)
1	0	0.25	0	1	50.40 (45.24)	2.7 (1.91)
1	0	0.50	0	1	54.16 (49.56)	1.8 (1.82)
0	0.5	0	0.1	1	25.80 (30.27)	1.4 (1.62)
0	1	0	0.1	1	22.16 (25.04)	5.7 (1.52)
0.5	0	0	0.1	1	84.16 (76.04)	4.7 (2.37)
1	0	0	0.1	1	82.91 (65.59)	1.0 (2.58)
0	0.5	0.25	0	1	17.08 (24.41)	0.7 (1.41)
0	0.5	0.50	0	1	18.75 (25.66)	0.5 (1.27)
0	1	0.25	0	1	14.16 (22.21)	0.3 (1.27)
0	1	0.50	0	1	8.33 (16.77)	0.3 (1.27)
$CD_{0.05}$					(0.19)	(0.06)

Figures within parentheses are arc sine and square root transformed values

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

Number of explants infected	180
Number of hygromycin resistant shoots	09
Number of PCR-positive shoots	04
Transformation frequency* (%)	2.22

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

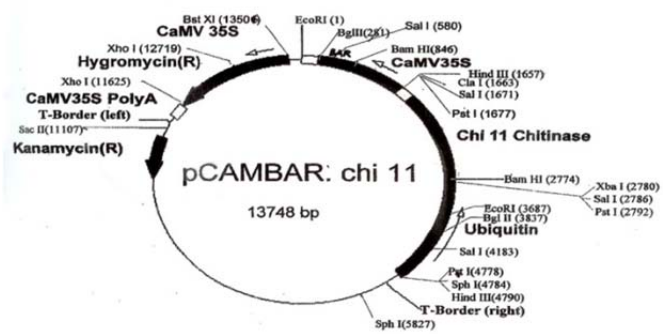

Fig. 1 Map of pCAMBIA *bar-ubi-chi*II transforming vector used for genetic transformation

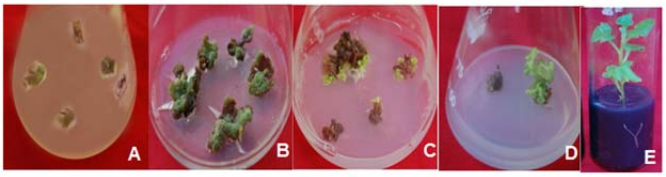

Fig. 2 Plant regeneration and genetic transformation studies in chrysanthemum (*Dendranthema grandiflora* Tzelev Cv. 'Snow Ball') leaf explant. (**A & B**) callus formation on selective callus induction medium in leaf explant; (**C &D**) shoot regeneration on selective shoot regeneration medium after four weeks; (**E**) rooting of regenerated shoots after five weeks of culture..

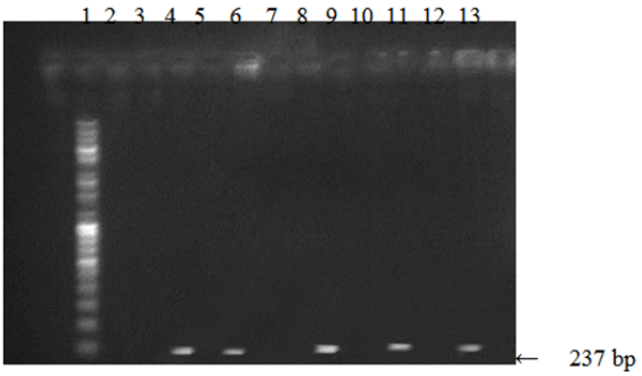

Fig. 3: PCR amplification of *chi*II gene fragment in transformed chrysanthemum plants. Lane 1, a marker (1 kb DNA ladder); Lane 2 &3, DNA sample of non-transformed control plants; Lane 4, plasmid DNA (positive control); Lanes 5-13, DNA samples from transformants (237 bp DNA fragment corresponding to *chi*II gene).

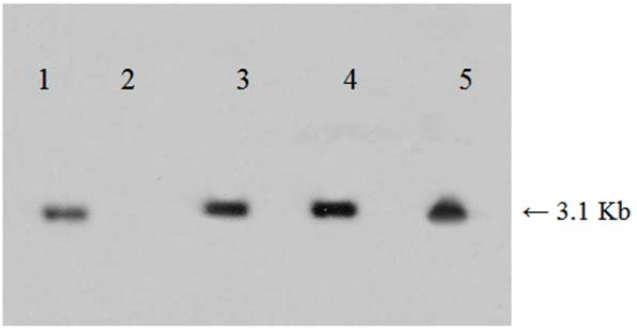

Fig. 4 Southern blot hybridization of PCR positive plants of chrysanthemum. Lane 1, plasmid DNA positive control (plasmid pCAMBIS bar-ubi-chiII (13.8 kb) digested with *Hind*III to release *chi*II (1.1 kb) fragment with *ubi* promoter (2 kb); Lane 2, DNA sample of non-transformed control; Lanes 3, 4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