International Journal of

Plant Breeding and Genetics

ISSN 1819-3595

Academic
Journals Inc.

www.academicjournals.com

International Journal of Plant Breeding and Genetics 6 (4): 168-181, 2012
ISSN 1819-3595 / DOI: 10.3923/ijpbg.2012.168.181
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Agrobacterium-mediated Genetic Transformation of Chrysanthemum (*Chrysanthemum morifolium* Ramat.) with an Aphidicidal Gene, *gcs* (Gamma-cadinene Synthase)

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ABSTRACT
Florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) belongs to the Asteraceae family and represents the second most important floricultural crop in the world. Unfortunately most genotypes are sensitive to aphids. Internode explants of 1581 and 4043 genotypes were incubated with *A. tumefaciens* strain AGL-0 containing pBIN plasmid with the *npt* gene as a selectable marker for kanamycin resistance and *gcs* gene as an aphicidal gene with rbcS promoter. Kanamycin resistant shoots were induced from internodes after 3 weeks. Finally, the shoots were rooted on MS medium containing 30 mg L^{-1} kanamycin. Incorporation and expression of the transgenes were confirmed by PCR and RT-PCR analysis. Genotype 4043 has been transformed in this study for the first time. Transformation frequency for GCS was 6.25 and 5% for genotypes 1581 and 4043, respectively.

Key words: Florists' chrysanthemum (*Chrysanthemum morifolium*), genetic transformation, aphid resistance, rbcS promoter, internode explant, gamma-cadinene synthase

INTRODUCTION
Cultivated chrysanthemum (*Chrysanthemum morifolium* Ramat), also classified as *Dendranthema* x *grandiflora* (Anderson, 1987) belongs to the *Asteraceae* family (Salinger, 1991). It is also known as florist's chrysanthemum or autumn queen and predominantly sold as a cut flower in many countries of the world (Erler and Siegmund, 1986). After rose it is globally the second economically most important floricultural crop and they are appreciated for their long vase life. The wide spectrum of colours and shapes and their ability to produce desired grades and types at anytime during the year adds to their economic importance.

Control of aphids (*Myzus persicae* (Sulzer) and spider mites (*Tetranychus urticae* Koch) on mature chrysanthemums is a critical problem. Flowers are easily damaged by chemical sprays, coverage is often inadequate due to dense foliage and spray residues are undesirable. Since pests quickly develop resistance to commonly used chemicals, there is a need for continual screening of new materials for phytotoxicity to flowers (Marouski, 1971) and for pest control. Safe control measures are not always available and flowers with aphids and mites are frequently marketed. So introduction of genes inducing pest resistance to this plant could be a promising solution. Genetic transformation of dicotyledonous plants is still most efficiently achieved by using the natural gene

transfer system of *Agrobacterium*. The susceptibility of chrysanthemum to *Agrobacterium* has already been demonstrated (Miller, 1975; De Cleene and De Ley, 1976). A successful regeneration protocol in chrysanthemum is a prerequisite for the recovery of morphologically and developmentally normal control and transgenic plants. The ability to regenerate whole plants from adventitious shoots without an intermittent callus phase has been previously achieved in *Dendranthema* from various explant sources: Leaves, stems, shoot tips, flower parts or pedicels and protoplasts (Rout and Das, 1997). Tissue culture studies on chrysanthemum were first initiated by Morel and Martin (1952). They used meristem tip culture to obtain virus free plants. Ben-Jaacov and Langhans (1972) obtained rapid multiplication of chrysanthemum plants *in vitro* by proliferation of callus from shoot tips in liquid medium followed by shoot differentiation and elongation. Single or multiple shoots and leafy basal callus were observed in shoot tip culture by Earle and Langhans (1974). Bush *et al.* (1976) obtained plant regeneration from petal-derived callus. Some attempts were made by Grewal and Sharma (1978) and Wambugu and Rangan (1981) and Levy (1981). Slusarkiewicz-Jarzina *et al.* (1982) have reported plant regeneration from leaf-derived callus. Plant regeneration from tissue cultures of various parts of *C. cinerariaefolium* has been described by Zeig *et al.* (1983). Urban *et al.* (1994) reported regeneration of shoots from the leaf segments of Iridon and Helka cultivars of *Chrysanthemum*. Mityushkina *et al.* (1995) regenerated adventitious shoots from leaf explants of 32 of 40 *Chrysanthemum morifolium* cultivars tested, while Kim and Kim (1998) regenerated shoots directly from internodes and leaf segments of *D. indicum* and *D. zawadskii* on basal media supplemented with 3.0 mg L^{-1} BAP and 0.2 mg L^{-1} IAA. Oka *et al.* (1999) reported that adventitious buds were mainly formed at the cut ends of primary leaves of garland chrysanthemum after 6-9 days culture on MS medium supplemented with 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA. Datta *et al.* (2002) stated that leaf explants of chrysanthemum differentiated shoot buds in the presence of BA and IAA. Da Silva and Fukai (2003) used a single auxin or cytokinin, or numerous permutations of each, in conjunction with Thin Cell Layer (TCLs) explants of chrysanthemum to manipulate the callogenic, caulogenic, rhizogenic and somatic embryogenic pathways/programs *in vitro*. A high level of transgenic shoots and transformation efficiency was achieved due to the small size of TCLs, despite a lower shoot regeneration capacity, when *Agrobacterium* (ideal vector) infection of TCLs was followed by a high (30 mg L^{-1}) selection (kanamycin) pressure.

In the last studies, transgenic plants of chrysanthemum have been produced by using an *Agrobacterium*-mediated transformation technique (Ledger *et al.*, 1991; Wordragen *et al.*, 1991; Renou *et al.*, 1993; Urban *et al.*, 1994; De Jong *et al.*, 1994; Fukai *et al.*, 1995; Da Silva, 2005; Sherman *et al.*, 1998a) and transgenic chrysanthemums with practical characteristics have also been produced (Sherman *et al.*, 1998b; Takatsu *et al.*, 1999).

In several cases, the diverse phytochemicals responsible for insect resistance are produced by glandular trichomes. Methyl ketones from *Solanum habrochaites* f. sp. *glabratum*, sesquiterpene carboxylic acids from *S. habrochaites* and acyl-glucose esters from *Solanum pennellii* are examples of such compounds that possess toxic properties against Lepidoptera or aphids (Williams *et al.*, 1980; Goffreda *et al.*, 1990; Juvik *et al.*, 1994; Frelichowski and Juvik, 2001). Some plant lectins or agglutinins are toxic to sap-sucking insects (Rahbe and Febvay, 1993). Transgenic tobacco-expressing *Galanthus nivalis* agglutinin (GNA) can significantly inhibit the population development of peach-potato aphids (Hilder *et al.*, 1995). Additionally, transgenic potato (Gatehouse *et al.*, 1996), rice (Rao *et al.*, 1998; Nagadhara *et al.*, 2003) and wheat (Stoger *et al.*, 1999) expressing GNA have been produced and also exhibit significant resistance to Homopteran

pests-like aphids and planthoppers. Another plant agglutinin gene pta, which is from *Pinellia ternate*, has also been introduced into tobacco and its expression in transgenic tobacco plants confers enhanced resistance to peach-potato aphids (Yao *et al.*, 2003). In addition to GNA and concanavalin A (ConA; Gatehouse *et al.*, 1999), a lectin from the seeds of *Amaranthus caudatus*, named *Amaranthus caudatus* agglutinin (ACA), reportedly has the ability to decrease the survival rate and inhibit the development of the aphids tested at a concentration of 68 μg mL^{-1}, the median lethal concentration (LC_{50}), indicating that this lectin could be a potential aphid-resistant protein (Rahbe *et al.*, 1995).

In the isoprenoid biosynthesis pathway, Farnesyl Diphosphate Synthase (FDS) catalyzes two consecutive condensations of isopentenyl diphosphate with dimethylallyl diphosphate and the resultant geranyl diphosphate (Cane, 1990). The ultimate product of these two reactions, farnesyl diphosphate (FDP), is utilized in the biosynthesis of sterols, dolichols, mitochondrial electron transfer chain components, prenylated proteins and a wide range of secondary sesquiterpenoids (Chappell, 1995). Recently cDNAs encoding FDS have been isolated from a number of plant species, including *Arabidopsis thaliana* (Delourme *et al.*, 1994), *Lupinus albus* (Atkinson *et al.*, 1995), *A. annua* (Matsushita *et al.*, 1996) and *Gossypium arboreum* (Liu *et al.*, 1998). Studies with specifically labeled mevalonic acid (MVA) or acetate demonstrated the folding pattern of farnesyl diphosphate (FDP) required for gossypol formation (Masciadri *et al.*, 1985; Stipanovic *et al.*, 1986). Subsequently, the enzymatic product of the cyclization of E,E-FDP in cotton extracts was identified as (+)-δ-cadinene (Benedict *et al.*, 1995; Davis and Essenberg, 1995; Chen *et al.*, 1995). Four cDNAs of CDN synthase (CDN1-C1, CDN1-C14, CDN1-A and CDN1-C2) have been isolated from *Gossypium arboreum* (Chen *et al.*, 1995, 1996). There are several cadinane sesquiterpenoids and heliocides (sesterterpenoids) deposited in pigment glands in cotton plants that function in pathogen and insect resistance (Stipanovic *et al.*, 1999).

Townsend *et al.* (2005) addressed the enzyme (+)-δ-cadinene synthase (CDNS), as the first step in the biosynthesis of sesquiterpenes, such a gossypol, that provide constitutive and inducible protection against pests and diseases. RNAi silencing of this gene led to a drastic reduction of gossypol levels in a seed-specific manner, without reducing this compound and other related terpenoids in somatic tissues (Sunilkumar *et al.*, 2006). Cotton plants accumulate gossypol and related toxic sesquiterpene aldehydes to protect themselves against pathogens and insect herbivores. Gossypol is synthesized via the sesquiterpene biosynthesis pathway. Enzymes that catalyze three consecutive steps in gossypol synthesis were identified, including farnesyl diphosphate synthase (FDS), (+)-delta-cadinene synthase (CAD) and (+)-delta-cadinene-8-hydroxylase (CYP706B1).

In this study we transformed genotypes 1581 and 4043 (for the first time) of chrysanthemum using *Agrobacterium tumefaciens* strain AGL-0 containing the binary vector pBIN carrying *npt* and *gfp* (selectable markers) or *gcs* (aphidicidal gene) overexpression constructs with rbcS promoter. For *gcs* the plasmid contained two genes including *fds* that produces the substrate (FDP) and *gcs* that produces gamma-cadinene. Transformed plants were tested by PCR and qRT-PCR.

MATERIALS AND METHODS

Plant materials and culture conditions: Sterile florist's chrysanthemum cultivars 1581 and 4043 were provided by Plant Research International (PRI) of Wageningen University (Netherlands) and multiplicated on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar (w/v). The pH value of the medium was adjusted to 5.8 before autoclaving

at 121°C for 15 min. Internode explants with length of 3-5 mm were used for transformation. All cultures were maintained in a tissue culture room under a photoperiod regime of 14 h light (3000 lux) and 10 h darkness at a constant temperature of 25°C. Transgenic plants were grown in the greenhouse with supplementary high-pressure sodium light under 16/8 h light/dark rhythm and temperature regime of 21/18°C and used for molecular analysis.

Sensitivity test of explants to kanamycin: In order to check the sensitivity of different chrysanthemum genotypes to kanamycin, internode explants of chrysanthemum were put on regeneration medium containing different concentration of kanamycin (0, 10, 20, 30 and 40 mg L^{-1}). Regeneration frequencies were compared after 20 days of culture.

Transformation protocol: Internodes of *Chrysanthemum* cultivars 1581 and 4043 were pre-cultured on regeneration medium (MS supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA) for 2 days. The highly virulent *A. tumefaciens* strain AGL-0 (Hood *et al.*, 1986) with a binary vector pBIN was used in this experiment. The effectiveness of this strain and plasmid in transient gene expression in chrysanthemum tissue has been confirmed (De Jong *et al.*, 1994). Single colony of bacteria was obtained on LB medium containing 50 mg L^{-1} Kanamycin and Rifampicin after overnight culture under 28°C. One single colony was cultured in 5 mL liquid LB containing 50 mg L^{-1} Kanamycin and Rifampicin and grew at 28°C on shaker overnight. The culture diluted 1/100 in fresh LB medium with the same antibiotics and was used for transformation after overnight culture under 28°C. Pre-cultured internodes were kept in 29 mL liquid MS to prevent being dry. When all the internodes have been collected 0.6 mL *Agrobacterium* culture (OD_{600} = 0.8-1) and 30 μL acetosyringone (0.1 M) added and incubated for 30 min. All the explants were put on sterile filter paper for a few minutes to remove excessive bacteria and transferred to regeneration medium with 100 μM acetosyringone. The cultures incubated in 25°C and dark for 2 days. After co-culture the explants were transferred to selection medium (regeneration medium+400 mg L^{-1} vancomycin+250 mg L^{-1} cefotaxime+30 mg L^{-1} kanamycin) and incubated under light for selection. All explants were transferred to fresh selection medium every 21 days afterwards and maintained for 65 days after inoculation. Green regenerated shoots were transferred to rooting medium (½ MS+200 mg L^{-1} Van.+125 mg L^{-1} Cef). Rooted plants were transferred to the greenhouse after being hardened and *Agrobacterium* free tested.

DNA and RNA analysis: Genomic DNA was isolated from young leaves as described by Pereira and Aarts (1998) and was used primarily for PCR screening. Primers with product size of 100 bp were designed according to the sequence in the constructs using website http://www.genscript.com for PCR and qRT-PCR. The plasmid contained two genes: *fds* that produces the substrate (farnesyl diphosphate) for GCS and *gcs* that produces gamma-cadinene. Primers were designed for both genes to check their expression level by qRT-PCR that were as follows: FDS-Forward: TCACCACCTTTGATGGAGAA; FDS-Reverse: CGCAAGCAACAGGAAGATAA; GCS-Forward: TGAAAGAGTTTGCCACAGATG; GCS- Reverse: TTGTGTTTGATCGAGGCATT. A volume of 4 μL DNA was used for PCR, adding 0.5 μL superTaq polymerase and 2.5 μL 10×buffer, 1 μL 10 mM specific forward and reverse primers, 0.25 μL 10 mM dNTP and water to a final volume of 25 μL. Amplification was performed in the GeneAmp PCR system at the following conditions: 94°C, 5 min, 35 cycles of 94°C, 30 sec and 55°C, 30 sec; 72°C, 20 sec; then finally 72°C, 7 min with a drop to 4°C). Positive lines were analysed by qRT-PCR to show the level of gene expression. Total RNA

was extracted by the TriPure™ small sample method. cDNA synthesis was done using the TaqMan™ Reverse Transcription Reagents. Reverse transcription was performed in the GeneAmp PCR system at the following conditions: 25°C, 10 min; 48°C, 30 min; 95°C, 5 min. A volume of 1 μL of cDNA (2 μg) was used for qPCR, adding 10 μL BIO-RAD iQ™ SYBR® Green Supermix, 2 μL 3 μM specific forward and reverse primers and a volume of 20 μL water. Primers designed for housekeeping gene actin as the reference gene (Actin-Forward: CCTCTTAATCCTAAGGCTAATCAG; Actin-Reverse: CCAGGAATCCAGCACAATACC). Amplification and real-time measurement were performed in the iCycler iQ5 (Bio-Rad, USA) (95°C, 3 min, 40 cycles of 95°C ,10 sec and 60°C, 30 sec; 95°C, 1 min; 60°C, 1 min). The results were analyzed using the IQ5 Optical System Software and $2^{-äCt}$ CT Method (Livak and Schmittgen, 2001).

RESULTS

Sensitivity test of explants to kanamycin: In order to check the effect of kanamycin on regeneration of different chrysanthemum genotypes, internode explants were cultured on regeneration medium containing different concentrations of kanamycin. In concentration of 10 mg L^{-1} regeneration only occurred for genotype 1581. So it seems 20 mg L^{-1} would be suitable for selection of this genotype and 10 mg L^{-1} would be enough for selection of other genotypes. All genotypes were regenerated on regeneration medium without kanamycin (Fig. 1).

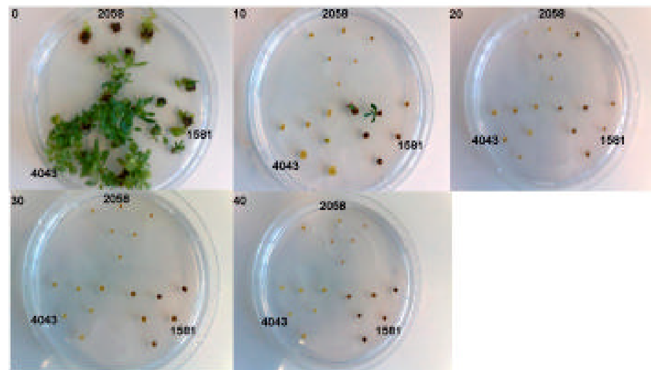

Fig. 1: The effect of kanamycin concentrations (0, 10, 20, 30 and 40 mg L^{-1}) on regeneration of different chrysanthemum genotypes

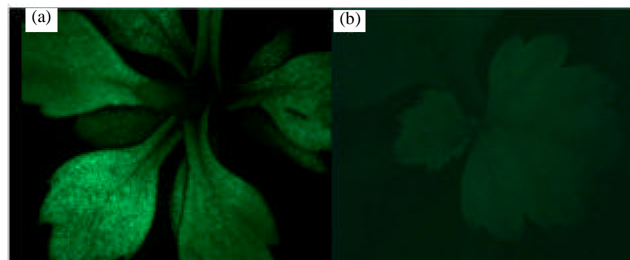

Fig. 2: Regenerated shoots with and without *gfp* expression photographed under a fluorescent microscope, (a) *gfp* transgenic plant, (b) wild type or non-transgenic plant

Regeneration and transformation: For regeneration of *Chrysanthemum* MS medium containing 0.1 mg L^{-1} IAA+ 1 mg L^{-1} BAP was used. Internode explants were transformed using *Agrobacterium tumefaciens* strain AGL-0 containing the binary vector pBIN carrying *npt* and *gfp* or *gcs* overexpression constructs with rbcS promoter. Regeneration in inoculated explants by *Agrobacterium* occurred on selection medium 3 weeks after incubation. Regeneration and transformation frequency in genotype 1581 was higher than 4043. Transformation frequency was 6.25 and 5% for GCS in genotypes 1581 and 4043, respectively. In transgenic plants some abnormalities were observed including dwarfing and thin leaves that we could easily identify transgenic plant from non-transgenic.

GFP gene expression analysis: *In vitro* shoots regenerated and kept green on the selective medium were analyzed by UV microscope for fluorescence screening. The expression of *gfp* in the leaves of regenerated shoots could be observed by eye due to the fact that the protein expressed so high and resulted in light green leaves. We used ImpactVector1.4-GFP that targets the GFP expression into plastids of leaf tissue. So under the fluorescence microscope leaves of transgenic plant looked green instead of red (for wild type) using a filter allowing all wavelengths above 420 nm. Using specific GFP filter with no normal light positive plants could be seen strongly shiny green while negative plants were dark (Fig. 2).

Molecular analysis of transgenic plants: DNA was extracted from leaves of transformed plants and PCR performed using designed primers for *gcs* with product size of 100 bp. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The result of PCR for some transgenic lines has been shown in Fig. 3. Total RNA of positive plants confirmed by PCR was extracted and cDNA was produced. For checking the expression level of *gcs* and *fds* in different transgenic lines qRT-PCR was performed using the same primers as used in PCR. The expression level of gamma-cadinene was much higher in genotype 1581 but the expression level of farnesyl diphosphate was lower than genotype 4043 (Fig. 4).

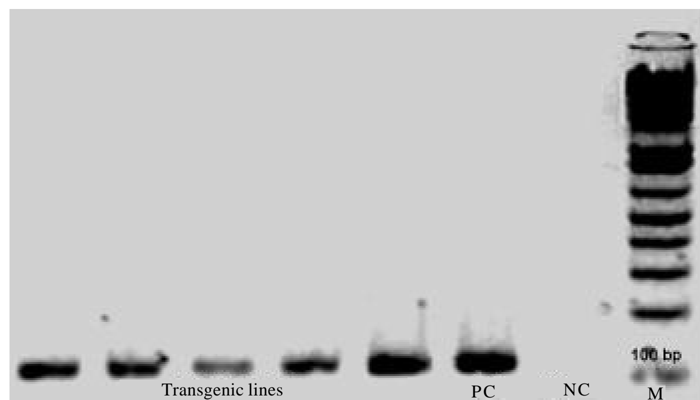

Fig. 3: PCR analysis for the presence of the *gcs* gene in transgenic chrysanthemum plants. M: Size marker, PC: Positive control, NC: Negative control

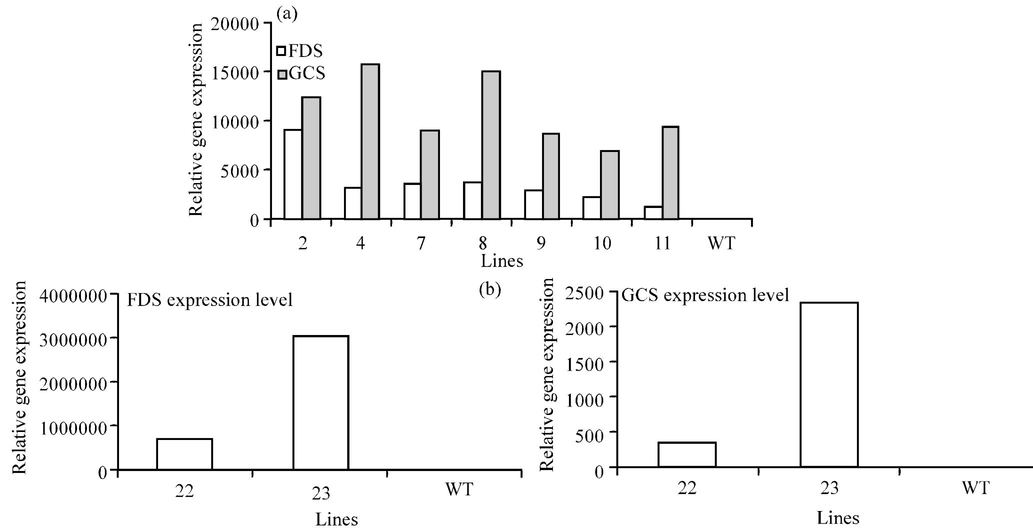

Fig. 4: Relative expression level of *gcs* and FDS in transgenic lines of chrysanthemum, (a) Genotype 1581, (b) Genotype 4043, WT: Wild type or non-transgenic plant

DISCUSSION

In this study we used a direct shoot regeneration system using internode explants. It is believed that adventitious shoot regeneration derived from an initial callus phase may result however in somaclonal variation (Larkin and Scowcroft, 1981) while direct shoot regeneration from leaf or stem explants may eliminate such an undesirable (Kaul *et al.*, 1990). However, a reliable plant regeneration protocol determines the successfulness of any transformation trial. Therefore, plant regeneration conditions have to be optimized for a given plant species/cultivar and type of explant (Uddin *et al.*, 2004). Sreeramanan *et al.* (2006) reported that several days of unselected period prior to selection greatly enhanced transgenic plant recovery. We also applied a 2 days pre-culture period before selection process. The frequency of regeneration in inoculated explants on selection medium was lower than that of non-inoculated explants on regeneration medium without kanamycin. *Agrobacterium* infection is also known to negatively affect shoot regeneration from leaf explants of chrysanthemum (Jong *et al.*, 1993). Regeneration and transformation capacity are inversely related in various *Dendranthema* cultivars (De Neve *et al.*, 1993), while genotype-dependence further hinders the broad application of a single regeneration system to the genetic transformation of chrysanthemum (Da Silva and Fukai, 2002). Also different strains of *Agrobacterium* used in transformation study could influence the regeneration and transformation of plants (Shahriari *et al.*, 2006).

Variation in shoot regeneration potential among genotypes has been reported (Kaul *et al.*, 1990). In the study the frequency of regeneration and transformation was also different for genotypes 4043 and 1581. The frequency of regeneration on selection medium was higher in genotype 1581. One reason could be less sensitivity of this genotype to kanamycin. Also addition of acetosyringone to the bacterial resuspension medium as well as co-cultivation medium can result in significant increase in transformation frequency (Raveendar and Ignacimuthu, 2010; Ming *et al.*, 2007). We also used 100 μM acetosyringone in co-culture medium to increase transformation frequency.

Successful genetic transformation has been reported through *Agrobacterium tumefaciens* in many plant species, such as, aphid resistance transgenic tobacco expressing pta gene (Yao *et al.*, 2003); freezing resistance lettuce (Pileggi *et al.*, 2001); brown planthopper and green leafhopper resistance indica rice (Nagadhara *et al.*, 2003) and aphid resistance transgenic cotton with *aca* gene (Wu *et al.*, 2006). Transgenic plants expressing *Bacillus thuringiensis* (Bt) crystal protein genes have been generated and showed strong resistance to a number of important insect pests that feed by chewing such as rice stem borers (Cheng *et al.*, 1998, 2007; Tu *et al.*, 2000), tobacco budworms (Barton *et al.*, 1987) and cotton bollworms (Kota *et al.*, 1999; Tabashnik *et al.*, 2002). However, a very important group of insects that suck the phloem of plants including aphids and planthoppers has been proven difficult to control by conventional plant breeding and by Bt technology, a matter made worse by their importance as vectors of plant viruses (Mochida *et al.*, 1979). So transformation of chrysanthemum using *Agrobacterium* seems to be a suitable method for pest resistance induction.

Progress has been made in optimizing the rate of transcription and translation in plant cells (Koziel *et al.*, 1996; Dai *et al.*, 2000; Outchkourov *et al.*, 2003a). However, proteolytic degradation of heterologously expressed proteins is still a limiting factor in the accumulation of many foreign proteins in plants (Dolja *et al.*, 1998; Stevens *et al.*, 2000; Sharp and Doran, 2001a, b). A generally adopted approach to increase heterologous protein accumulation levels in plants is to change the compartmentalization of the expressed proteins by targeting to and retention in the endoplasmic reticulum (Wandelt *et al.*, 1992; Schouten *et al.*, 1996) or chloroplasts (Wong *et al.*, 1992). Three different promoters (CaMV35S, Lhca.3.St1 and rbcS1) have been analyzed for their ability to drive maximal expression of equistatin. The rbcS1 promoter from chrysanthemum (Outchkourov *et al.*, 2003b) yielded the highest average expression level (0.36%). In our study we also used this promoter to produce higher levels of product. For transformation of chrysanthemum first we applied 20 mg L^{-1} kanamycin in selecton medium based on the result of kanamycin sensitivity test but all regenerated shoots could not produce root in rooting medium containing the same concentration of kanamycin and there were many escapes as checked by PCR. So we increased the level of used kanamycin to 30 mg L^{-1} in the next experiments. Other workers (Horsch *et al.*, 1985; Deroles, 1988; Ulian *et al.*, 1988) have also reported that high numbers of shoots are regenerated under kanamycin selection but fail to root in the presence of kanamycin. Two hypotheses have been put forward to account for the high number of escapes. Cells with transient expression of the NPTII gene provide temporary protection from kanamycin allowing non transformed shoots to regenerate. Alternatively, escapes may have an integrated copy of the T-DNA which is initially expressed but is subsequently shut down (Horsch *et al.*, 1985; Deroles, 1988; Velcheva *et al.*, 2005). On the other hand antibiotic used for selection may be partially or completely phosphorylated by cells expressing *npt* gene (Bashir *et al.*, 2004). In transgenic plants some abnormalities observed *in vitro* including dwarfing and thin leaves but when the plant were transferred to the soil and greenhouse the plants grew and turned to normal shape after some days. In the present study, we generated transgenic chrysanthemum plants containing and expressing *gfp* (selectable marker) or *gcs* (aphidicidal gene) genes. Genotype 4043 has been transformed for the first time. PCR and qRT-PCR analysis confirmed their transgenic status. Most of chrysanthemum genotypes are sensitive to insects including aphids and spider mites. So transformation of chrysanthemum using *Agrobacterium* and pyramiding strategies seems to be a suitable method for pest resistance induction.

ABBREVIATIONS

BAP:	6-Benzylaminopurine
IAA:	Indole acetic acid
MS:	Murashige and Skoog's medium
GFP:	Green Fluorescent Protein
rbcS:	Ribulose-1,5-bisphosphate carboxylase
NPTII:	Neomycin phosphotransferase
GCS:	Gamma-cadinene synthase
FDS:	Farnesyl diphosphate synthase
PCR:	Polymerase chain reaction
qRT-PCR:	Quantitative real time polymerase chain reaction
LB:	Luria-Bertani

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