African Journal of Biotechnology Vol. 11(37), pp. 9141-9148, 8 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.267
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Use of petal explants for successful transformation of *Dendranthema × grandiflorum* Kitamura 'Orlando' mediated by *Agrobacterium tumefaciens*

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Accepted 5 March, 2012

An *Agrobacterium*-mediated genetic transformation system for chrysanthemum 'Orlando' using petal explants was developed. After decontamination, petals were divided into two parts; terminal (position 1) and middle (position 2) parts, and were pre-cultured for two days on Murashige and Skoog (MS) medium containing 1.0 mg·L^{-1} indole acetic acid (IAA), 1.0 mg·L^{-1} 6-benzylaminopurine (BA), and 0.1 mg·L^{-1} kinetin (SIM, shoot induction medium). Then, the explants were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA-4404 harboring a binary vector pCAMBIA 2301 carrying the reporter gene β-glucuronidase (GUS), and the marker gene neomycin phosphotransferase (NPT II). The highest frequency of transgenic shoot induction was obtained when the explants were co-cultivated with *A. tumefaciens* for two days. The formation of transgenic shoots varied with the position of explant. The highest regeneration frequency (13.3) was obtained when position 2 explants were cultured on the shoot induction medium supplemented with 7.5 mg·L^{-1} kanamycin and 250 mg·L^{-1} cefotaxime. Putative transgenic plants were obtained after rooting on the MS medium supplemented with 20 mg·L^{-1} kanamycin and 250 mg·L^{-1} cefotaxime. Histochemical GUS assay and polymerase chain reaction analysis of transgenic plants confirmed the presence of the GUS gene. Thus, successful transformation of chrysanthemum 'Orlando' petal explants mediated by *A. tumefaciens* was confirmed.

Key words: Chrysanthemum, co-cultivation, GUS, kanamycin, NPTII, petal explants.

INTRODUCTION

The genus Chrysanthemum (Asteraceae) consists of around 30 species of perennial flowering plants, native to Asia and Northeastern Europe. It is one of the most popular cut flowers and potted plant crops with economic importance in the floriculture industry. This situation has promoted studies on the development of an efficient transformation protocol in chrysanthemum. To introduce biotechnological methods in chrysanthemum breeding, the establishment of *in vitro* plant regeneration protocol is a prerequisite. For this reason, studies on regeneration of chrysanthemum have been promoted and achieved in a number of explant sources including stems (node and internode), axillary buds, leaves, shoot tips or apical meristems, protoplasts, roots, pedicels, and flowers (Barthomeuf et al., 1996; Boase et al., 1998; Lindsay and Ledger, 1993; Oka et al., 1996, 1999; Park et al., 2005; Renou et al., 1993; Rout et al., 1997; Sauvadet et al., 1990; Song et al., 2011; Tian et al., 1993; Zheng et al., 2001; Zito and Tio, 1990).During the last decade, transgenic chrysanthemums have been developed by an *Agrobacterium*-mediated transformation technique (Aida

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Abbreviations: IAA, Indole acetic acid; **BA,** 1.0 mg·L^{-1} 6-benzylaminopurine; **SIM,** shoot induction medium; **GUS,** gene β-glucuronidase; **RIM,** root induction medium; **NPT II**, neomycin phosphotransferase.

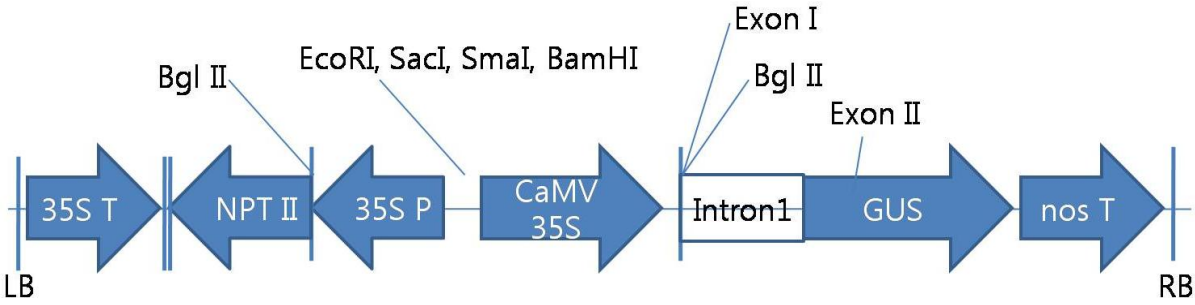

Figure. 1. A linear map of pCAMBIA 2301 with GUS gene used for plant transformation. RB, Right border; T*35S*, 3′ signal of CaMV 35S; *NPTII*, neomycin resistance gene; P*35S*, CaMV 35S promoter; GUS, GUS marker gene; Nos T, Nos terminator; LB, left border.

et al., 2004, Chung and Parlc, 2005; Narumi et al., 2005; Soh et al., 2009). Although there are now transgenic chrysanthemums that confer β-glucuronidase (GUS) and kanamycin resistance and some other genes because of the generally low transformation efficiencies, further development of transformation studies is still required. In most studies, transgenic shoots have been achieved using leaf, pedicel, and stem explants (Teixeira da Silva, 2003). One report describes genetic transformation of chrysanthemum using 'petal explant'-derived callus (Mino et al., 2007). In this paper, the effects of kanamycin concentration, co-cultivation periods, and petal explant positions on shoot/root formation and transformation were determined to establish a transformation system for chrysanthemum 'Orlando' using petal explants through direct shoot formation.

MATERIALS AND METHODS

Plant materials, culture media and experimental conditions

Flowering was induced by a short day treatment in a greenhouse. After floral initiation, flowers were isolated, were thoroughly washed in running tap water, and subsequently were surface-sterilized with a 70% (v/v) ethanol solution for 30 s and 1.5% (v/v) sodium hypochlorite solution for 10 min, followed by three rinses with sterilized water. After sterilization, single petals were removed from the flower buds and were divided into two parts of 6 mm in length; a terminal part (position 1) and middle part (position 2). The abaxial sides of explants were placed down to contact the regeneration medium. For shoot regeneration, petals were cultured in a Petri dish containing a 25 ml shoot induction medium (SIM). The SIM composed of MS medium supplemented with 1.0 $mg \cdot L^{-1}$ indole acetic acid (IAA), 1.0 $mg \cdot L^{-1}$ 6-benzylaminopurine (BA), 0.1 $mg \cdot L^{-1}$ kinetin, 3% (w/v) sucrose, and 0.8% (w/v) agar (Park et al., 2005). Unless specified, all cultures were maintained for 14 days at 25±1°C in darkness and then exposed to light of 45 µmol m^{-2} s^{-1} with a light/dark cycle of 16/8 h.

Agrobacterium strain and culture for transformation

Transformations were done with the disarmed *A. tumefaciens* strain LBA4404 with the binary vector pCAMBIA 2301 (Figure 1) obtained from Dr. Kim (Gyeongsang National University, Korea). The construct (p35SGUS intron) contains a NOS-driven neomycin phosphotransferase (NPT II) selection gene and a 35S-driven GUS marker gene. To prevent expression of the marker gene in *A. tumefaciens,* the GUS gene was interrupted by a plant intron that can be removed by plants and not by the bacterium, thus restricting GUS activity to transformed plant cells only. Pre-cultured *Agrobacterium* cells were transferred in to a YEP liquid medium (10 $g \cdot L^{-1}$ bacto peptone, 10 $g \cdot L^{-1}$ yeast extract, and 5 $g \cdot L^{-1}$ NaCl) supplemented with 20 $mg \cdot L^{-1}$ kanamycin and 50 $mg \cdot L^{-1}$ rifampicin, and then incubated at 28±2°C in a rotary shaker (180 rpm) for 1one to two days.

Effect of antibiotics on shoot regeneration and rooting

In order to examine the effect of concentration of kanamycin, two experiments were conducted. In experiment 1, petal explants were cultured on a SIM supplemented with 0.0, 2.5, 5.0, 7.0 or 10.0 $mg \cdot L^{-1}$ kanamycin for shoot induction. Explants that were able to induce callus and healthy shoot buds were considered kanamycin resistant, while explants that failed to induce callus and had shoots with mottled or bleached leaves were considered to be kanamycin sensitive. In experiment 2, elongated shoots were cultured on a MS medium supplemented with 0.0, 5.0, 10.0, 15.0 or 20.0 $mg \cdot L^{-1}$ kanamycin in combination with 250 $mg \cdot L^{-1}$ cefotaxime for rooting under a 16 h photoperiod. Ten replicates (dishes) were used for each experiment and data were recorded after four weeks for the number of shoots per explant and roots per shoot.

Agrobacterium-mediated transformation

For *Agrobacterium* inoculation of petal explants, petal explants were pre-cultured on the SIM. After two days, explants were submerged in 25 ml *Agrobacterium* suspension (OD 0.8) for 30 min, transferred onto sterile filter paper to remove the excess liquid and co-cultured for one, two or three days on SIM at 25°C in the dark. After co-cultivation, the explants were rinsed three times with MS liquid medium containing 500 $mg \cdot L^{-1}$ cefotaxime, blot-dried and transferred onto SIM with 7.5 $mg \cdot L^{-1}$ kanamycin and 250 $mg \cdot L^{-1}$ cefotaxime for the first selection of the transformants. After four weeks, regenerated shoots were transferred onto root induction medium (RIM) containing 20 $mg \cdot L^{-1}$ kanamycin and 250 $mg \cdot L^{-1}$ cefotaxime for the second time selections. The RIM composed of MS basal nutrients and vitamins supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. All antibiotics were filter-sterilized and added to the cooled media after autoclaving.

GUS assay

Expression of GUS gene was assayed on leaves of kanamycin

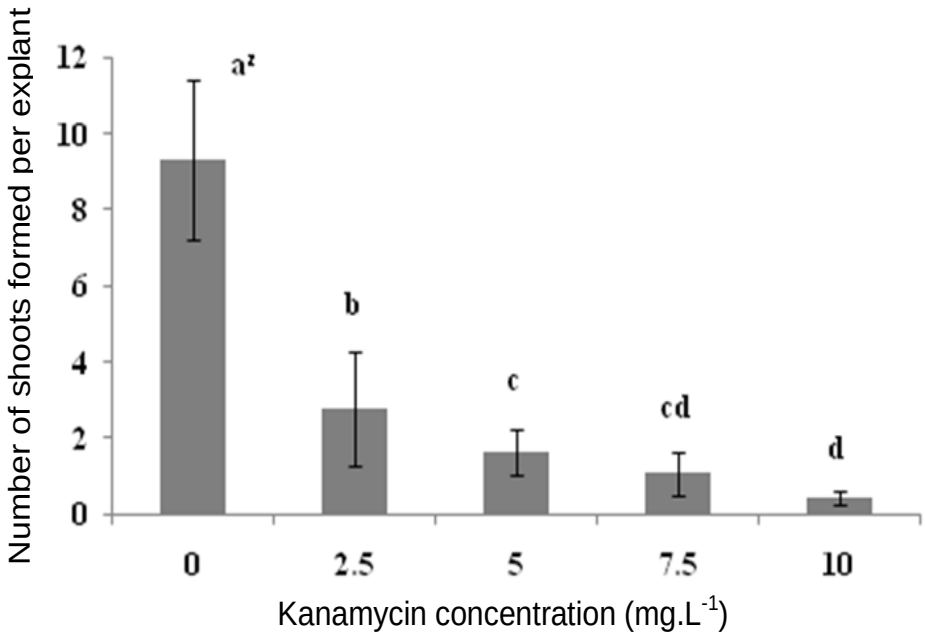

Figure 2. Effects of kanamycin concentration on shoot induction from petal explants of chrysanthemum 'Orlando'. [z]Mean ± SE followed by same letter are not significantly different ($p<0.05$).

resistant rooted plants as described by Rueb and Hensgens (1989) with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate (Jefferson, 1987). The leaves were infiltrated with the buffer consisting of 0.5 mg·L^{-1} X-Gluc (5-bromo-4-chloro-3-indolylbeta d-glucuronic acid), 2.0 mM potassium ferricyanide and potassium ferrocyanide, 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) and 50 mM sodium phosphate buffer at pH 7.0. The reaction mixture was incubated overnight at 37°C and cleared of chlorophyll by four washes with 70% (v/v) ethanol. GUS expression was examined under a stereomicroscope.

Genomic DNA isolation and polymerase chain reaction (PCR) analyses

To detect the presence of the GUS gene in the putatively transgenic plants, plant genomic DNA was extracted from 0.4 g (fresh weight) of plant tissue using the GeneAll DNA extraction kit (ExprepTM Plasmid SV; GeneAll Biotechnology, Seoul, Korea). The isolated genomic DNA was used as a template for a GUS-specific polymerase chain reaction (PCR). The following GUS gene primers 5′-ACCTGCGTCAATGTAATGTTCTGC-3′ and 5′-TCACCG-AAGTTCATGCCAGTCCAG-3′ were used to detect the presence of GUS gene (anticipated amplicon size, 461 bp). PCR analysis of genomic DNA was carried out using 200 ng of genomic DNA employing reagents from Elpisbio Biotechnology (Taejeon, Korea) in a 25 µL reaction volume. The PCR amplification was performed by initial denaturation at 95°C (5 min) followed by 30 cycles at 94°C (30 s), annealing at 60°C (30 s), extension at 72°C (40 s) and finally holding at 72°C (7 min) for extension employing a Gene Amp PCR system 9700. The PCR products were run on 1% (w/v) agarose gel in 1x TBE (Tris base, boric acid, 0.5 mM EDTA), then stained with ethidium bromide (0.1 mg/ml). The gels were visualized under ultraviolet light.

Statistical analysis

A completely randomized design with three replications was used. The experimental results were subjected to an analysis of variance (ANOVA) by using the SAS program (Statistical Analysis System, V. 9.1, Cary, NC, USA).

RESULTS AND DISCUSSION

Effect of antibiotics on shoot regeneration and rooting

In preliminary experiments, petals of chrysanthemum 'Orlando' were cultured on a SIM with 0.0, 10.0, 15.0, 20.0, 25.0, 50.0 or 100.0 mg·L^{-1} kanamycin to determine the optimal concentration for the selection of transformed shoots. The explants did not induce shoots and turned brown when the medium was supplemented with kanamycin. Thus, we tested lower concentrations of kanamycin (0.0, 2.5, 5.0, 7.5 or 10.0 mg·L^{-1}). Of the different kanamycin concentrations tested, the mean number of shoots per explant decreased with increasing kanamycin concentration (Figure 2).

At 7.5 mg·L^{-1} kanamycin, the average number of shoots decreased drastically and exhibited leaf chlorosis within 4 weeks of culture. Explants that did not develop shoots became brownish and died when the medium was supplemented with a high concentration of kanamycin (10.0 mg·L^{-1}). Takatsu et al. (1998) also reported that adventitious shoot regeneration from stem segments of

Table 1. Rooting response of shoots of chrysanthemum 'Orlando' cultured on MS medium supplemented with kanamycin.

Kanamycin concentration (mg·L^{-1})	Number of roots formed per explant	Root induction (%)
0.0	3.8±1.9^{az}	100.0±0.0^{a}
5.0	1.0±0.3^{b}	57.7±0.5^{b}
10.0	0.1±0.0^{c}	6.0±0.2^{c}
15.0	0.1±0.0^{c}	4.8±0.2^{c}
20.0	0.0±0.0^{c}	0.0±0.0^{c}

^{z}Mean separation within columns by 5% Duncan's multiple range test (DMRT).

Table 2. Effect of co-cultivation time on shoot induction from petal explants of chrysanthemum 'Orlando'.

Co-culture period (A) (day)	Position (B)	Total number of explant	Total number of regenerated explants	Regeneration frequency (%)
1	1	125	6.0	4.8b^{z}
	2	241	10.0	4.1b
2	1	113	12.0	10.6a
	2	255	30.0	13.3a
3	1	115	4.0	3.5c
	2	283	6.0	2.1c
(A)				***^{y}
(B)				NS
(A)*(B)				*

^{z}Mean separation within columns by 5% *Duncan's multiple range test* (DMRT); ^{y}NS, *, *** indicate non-significant or significant at P = 0.05, 0.01 or 0.001, respectively.

cv. `Yamabiko' was suppressed by kanamycin at 10.0 mg·L^{-1}. In this study, 7.5 mg·L^{-1} kanamycin was chosen for the first selection of putative transformants, although shoot formation diminished completely at 10.0 mg·L^{-1} kanamycin. A number of studies have reported that chrysanthemum is very susceptible to kanamycin (lethal concentrations of 10 to 50 mg·L^{-1}, depending on the cultivar), and many escape shoots were obtained in transformation experiments (De Jong et al., 1994; Renou et al., 1993; Urban et al., 1994). Therefore, in order to reduce escape shoots, we investigated the effect of various concentrations of kanamycin on root formation. When the rooting medium was supplemented with 5.0 to 15.0 mg·L^{-1} kanamycin, the frequency of root induction and mean number of roots per shoot were reduced drastically (Table 1). However, at 20.0 mg·L^{-1}, kanamycin completely inhibited rooting and browning or death of shoots after four weeks of culture; thus, 20.0 mg·L^{-1} was used for the second selection of putative transformants.

Regeneration of transgenic shoots

The effect of the co-cultivation period on induction of transgenic shoots was investigated. As shown in Table 2, differences among the co-cultivation periods were detected. When the petal explants were transferred onto selective shoot regeneration medium after a day co-cultivation period, the frequency of shoot induction was 4.8. The highest frequency of shoot formation was obtained with a 2-day co-cultivation period, which is commonly used for transformation of various plants (Horsch et al., 1985; Sangwan et al., 1992; Villemont et al., 1997). However, a 3-day co-cultivation period decreased the frequency of shoot formation. Additionally, most of the developed shoots did not induce roots and died after they were transferred onto the rooting medium with 20.0 mg·L^{-1} kanamycin.

Longer co-cultivation period resulted in high levels of transient expression (Ok et al., 2007). In contrast, longer co-cultivation period did not improve the transformation frequency. Thus, a 2 day co-cultivation period was selected for transformation of chrysanthemum using petal explants. A co-culture period of 2-day has also been found to be optimum in other plant species such as *Antirrhinum majus* (Holford et al., 1992), *Cajanus cajan* (Mohan and Krishnamurthy, 2003), *Glycine max* (Li et al.,

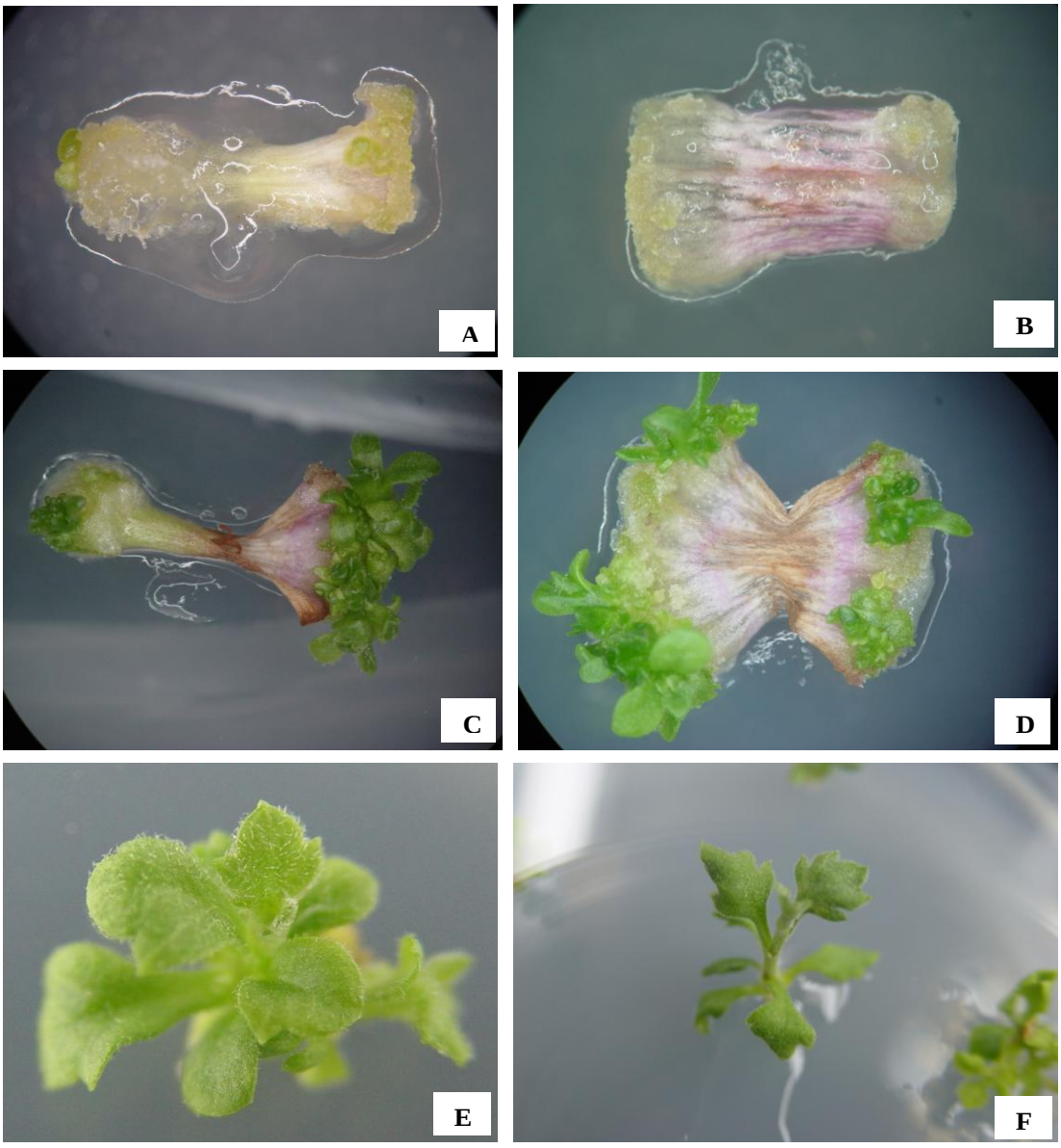

Figure 3. Regeneration of putative transgenic plantlets from petal explants. A, callus induction in position 1; B, callus induction in position 2; C, induced shoot in position 1; D, induced shoot in position 2; E, elongated shoot; F, plantlet having well-developed roots and shoots.

2004), *Nicotiana tabacum* (Uranbey et al., 2005), *Vigna radiate* (Jaiwal et al., 2001), and *Vigna unguiculata* (Muthukumar et al., 1996).

In this study, the formation of transgenic shoots varied with the position of explant. Of the different explants positions, position 2 revealed higher shoot formation than position 1. We reasoned that position 2 had a broader cut edge compared with position 1. As we expected, shoots emerged from the cut edges and most of the transient activity was confined to an area close to the wound, thus, extension of cut edges can be affected by the transformation efficiencies. Gamesan and Keerti (2001) reported that the bacterial cell would be in closer contact with the adaxial side of the explants and the cut edge. Perhaps, the physiological state of the abaxial epidermal cells was more favorable for *Agrobacterium* infection, leading to faster T-DNA transfer. Accordingly, the epidermal cells on the adaxial side, as well as the cells in the middle of the two epidermal layers (mesophyll and vascular) showed strong shoot formation activity after two days of culture. In addition, all the potential escape shoots were bleached and discarded with the first selective shoot regeneration medium. However, in the second selective medium, few green shoots did not induce roots and died within four weeks of culture. These escape shoots may be transiently transgenic cells (Fukai et al.,

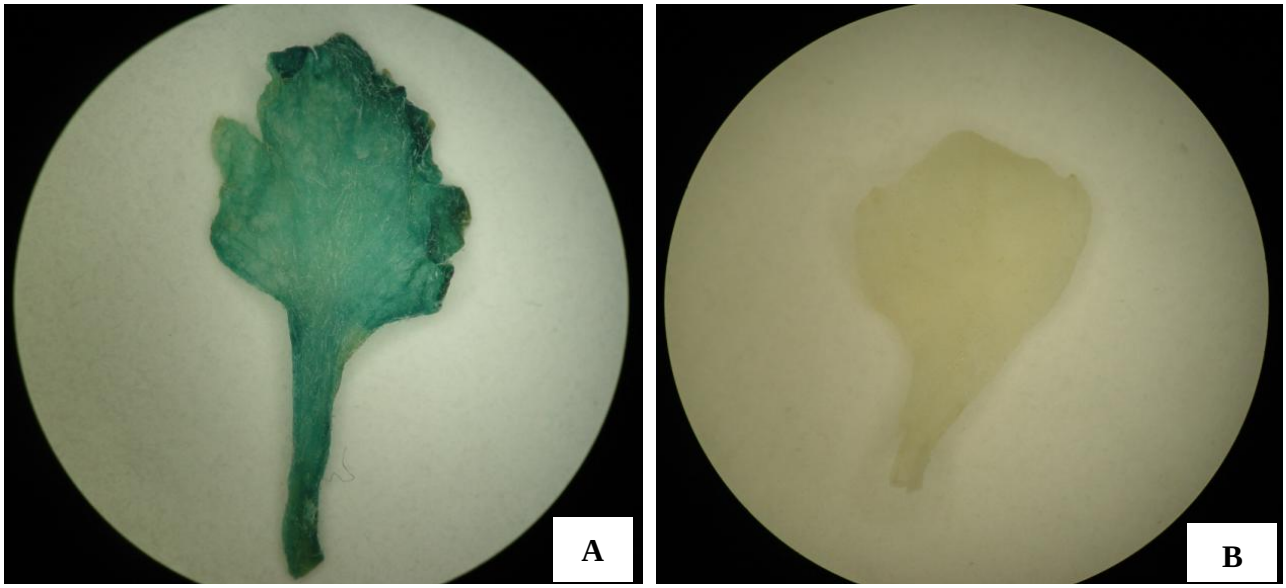

Figure 4. GUS expression on a leaf of a putative transgenic plantlet of Chrysanthemum. (A), GUS staining with leaf of putative transgenic plantlet; (B), non-stained leaf of non-transgenic plantlet.

1995; Ledger et al., 1991) or lack stable T-DNA transfer (Han et al., 2007; Lowe et al., 1993).

Gus assay

Histochemical analysis of leaves obtained from putative transgenic plants showed a characteristic deep blue color throughout the tissue indicating constitutive GUS gene expression (Figure 4A), but not in non-transformed plants (Figure 4B). In earlier reports, GUS expression was mainly found in the veins and mid-rib. The existence of blue-staining areas suggests high gene transfer efficiency and not a cell-to-cell transport of transcription/translation products of the GUS gene because single blue-stained cells also existed (van Wordragen et al., 1992). Generally, plants that showed GUS activity were considered as transgenic plants because the vector contained GUS intron gene in the T-DNA region, which ensures its expression in plant cells, but not by *A. tumefaciens*. Additionally, the intensity of staining was higher, possibly due to the presence of intron connected to the GUS gene, which was consistent with other reports (Mohanty et al., 1999; Rashid et al., 1996). The intron containing plasmid, the GUS intron, has been reported to increase the level of GUS activity by 80 to 90-fold compared with the non-intron plasmid (Tanaka et al., 1990).

PCR analysis

PCR analysis was conducted using leaves from the 68 surviving kanamycin-resistant shoot lines and the non-transformed control. The expected 461 bp PCR product specific for the GUS gene fragment was observed in the total DNA extracted from 41 independent GUS-positive shoot lines and in the plasmid pGI2301/GUS intron positive control, indicating that the GUS gene had been successfully transformed into these 41 lines. An example is shown in Figure 5.

Transformation with *A. tumefaciens* using petal explants was successful in the establishment of an effective transformation system for chrysanthemum ‘Orlando’ (Figure 3). After sterilization, petals that divided into two parts were pre-cultured for two days on SIM. The explants were then submerged in a 25-ml *A. tumefaciens* suspension (O.D. 0.8), and were shaken for 30 min. Inoculated petal explants were transferred on a SIM and were co-cultivated for two days in the dark, and then were transferred on the first selection medium (SIM supplemented with 7.5 $mg \cdot L^{-1}$ kanamycin and 250 $mg \cdot L^{-1}$ cefotaxime) for shooting (Figure 3C and D), because of the sensitivity to kanamycin. Elongated shoots (Figure 3E) were transferred on the second selection medium (SIM supplemented with 20.0 $mg \cdot L^{-1}$ kanamycin and 250 $mg \cdot L^{-1}$ cefotaxime) for rooting (Figure 3F), because non-transgenic shoots did not induce roots and died. Histochemical GUS assay and polymerase chain reaction analysis of transgenic plants confirmed the presence of the GUS gene. Thus, successful transformation of chrysanthemum 'Orlando' petal explants mediated by *A. tumefaciens* was confirmed.

ACKNOWLEDGEMENT

Ju Yeon Song was supported by a scholarship from the BK21 Program, the Ministry of Education, Science and Technology, Korea.

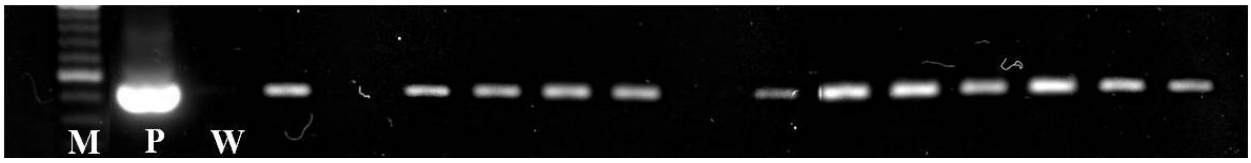

Figure 5. PCR analysis of putative transgenic lines with GUS gene primers. M, 1 kb marker; P, plasmid vector pCAMBIA 2301; W, wild-type plant.

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