Journal of Plant Biology, March 2005, 48(1) : 136-141

Development of an *Agrobacterium*-Mediated Transformation System for Regenerating Garland Chrysanthemum (*Chrysanthemum coronarium* L.)

Kwi-Mi Chung and Young-Doo Park*
Graduate School of Biotechnology, KyungHee University, Yongln-Si 447-501, Korea

Although efficient shoot regeneration and selection are essential for genetic transformation mediated by *Agrobacterium*, success has been limited with the garland chrysanthemum (*Chrysanthemum coronarium* L.). In this study, we developed a useful protocol for shoot regeneration with leaf disk explants. The optimal concentrations of NAA and BA were 0.2 mg L^{-1} and 0.5 mg L^{-1}, respectively. To optimize the selection system for regenerating plants from genetically transformed tissues, we tested the effects of four antibiotics (kanamycin, hygromycin, carbenicillin, and cefotaxime). Among them, 5 mg L^{-1} hygromycin proved adequate as a selectable marker, whereas 500 mg L^{-1} carbenicillin was effective in eliminating excessive *Agrobacterium* after co-cultivation. Transgenic plants were obtained by first co-culturing garland chrysanthemum leaf disks with *A. tumefaciens* strain EHA105, which harbors plasmid pRCVII containing the hygromycin resistance (*hpt*) and β-glucuronidase (*GUS*) genes. After the transgenic plants were confirmed via Southern analysis, they were rooted in soil and appeared phenotypically normal. Our report is the first to describe the optimum conditions for producing transgenic plants of this species.

Keywords: Agrobacterium tumefaciens, antibiotics, β-glucuronidase gene, garland chrysanthemum, hygromycin resistance gene

Garland chrysanthemum (*Chrysanthemum coronarium* L., 2n = 18) is one of more than 100 *Chrysanthemum* species in the Compositae family, along with chicory and lettuce. Apart from the valuable ornamental and insecticidal properties it shares with other *Chrysanthemum* species, it is also commonly cultivated as an Asian vegetable known as 'ssukkat' (Korea), 'shungikee' (Japan) or 'kor tongho' (China) (Fukai et al., 1995a, b). Moreover, it is considered a healthful food because its indented leaves contain not only fresh aroma, but also higher contents of vitamins, flavonoid, and quercetin than other crops. Efficient genetic transformation is necessary when introducing foreign genes into plants. Although desirable traits can be acquired through classical breeding, that technique is limited by a narrow gene pool. In addition, breeding a useful variety by conventional methods is time-consuming and expensive, and can be hindered by incompatibility and barriers for wide crosses. Moreover, in the case of floral crops, characteristics such as uniform growth and synchronous flowering are polygenically inherited. This polygenic sexual crossing may also alter the delicate balance of factors determining plant growth and development (Mol et al., 1989).

Agrobacterium-mediated transformation is an ideal technique for producing plants that are valuable for agronomic and scientific purposes, because it is very efficient in yielding desirable changes in cultivars without disturbing their important ornamental traits (Saito et al., 1992; John et al., 1998). However, to successfully introduce useful and interesting foreign genes into plant genomes via *Agrobacterium*, a reproducible plant regeneration system is required.

Because reports on the tissue-culturing of garland chrysanthemum have been quite scarce, one of our research objectives was to develop a simple, reliable, and efficient genetic engineering system for regenerating plants from leaf explants and improving the existing cultivars. *Agrobacterium*-mediated genetic transformation requires, selectable marker genes that confer antibiotic resistance. In the presence of such a selective agent, only genetically transformed cells then divide and regenerate. Therefore, another objective was to determine the effectiveness of antibiotics, commonly used in *Agrobacterium* transformation. Finally, we investigated the possibility of producing transgenic shoots by optimizing the composition of our introduction media.

*Corresponding author; fax +82-31-202-8395
e-mail ydpark@khu.ac.kr

MATERIALS AND METHODS

Plant Materials

Seeds of garland chrysanthemum (Heung-nong, Korea) were sterilized in 70% ethanol for 1 min, then, with vigorous shaking, for 15 min in 30% commercial Clorox (1.6% hypochlorite) plus 0.1% Tween-20. After a thorough rinsing in sterile distilled water, they were placed on germination media [Murashige and Skoog (MS) basal medium, 3% sucrose, and 8 g L^{-1} agar, (pH 5.8)] in mayonnaise bottles (about 20 seeds per bottle). The *in vitro* seedlings were incubated in a climate chamber at 25°C under a 16-h photoperiod. Each month, plants with expanded leaves and roots were sub-cultured to fresh media.

Effect of NAA and BA Concentrations on Shoot Induction from Leaf Disk Explants

We used 25-mm^2 leaf disks of garland chrysanthemum to examine the effect of various α-naphthaleneacetic acid (NAA) and benzyladenine (BA) concentrations on shoot induction. Plant regeneration tests were conducted in 90-mm petri dishes containing 25 ml MS medium that was solidified with 0.8% agar and supplemented with different levels of NAA (0.0, 0.1, 0.2, 0.5, or 1.0 mg L^{-1}) and BA (0.0, 0.5, 1.0, or 1.5 mg L^{-1}). These growth regulators were filter-sterilized and added to the media after autoclaving. After the explants were positioned (seven per plate), the petri dishes were sealed with Para film and incubated as described above. Their newly developed adventitious bud primordia and shoots were counted after 30 d.

Effect of Antibiotics on Shoot Induction from Leaf Disk Explants

After we had determined the best combination of growth regulators for shoot regeneration, that optimized medium was used to examine the effect of antibiotics on shoot regeneration. For each experiment, the treatments included kanamycin B at 0, 10, 20, 30, 40, 50, 100, 200, or 300 mg L^{-1}; hygromycin at 0, 1, 3, 5, 7, 10, 20, or 30 mg L^{-1}; carbenicillin at 0, 500, or 1000 mg L^{-1}; and cefotaxime at 0, 200, or 400 mg L^{-1}. All antibiotics were filter-sterilized and added to the cooled media after autoclaving. Petri dishes containing the leaf explants (seven per plate) were then exposed to a 16-h photoperiod as described above. All experiments were repeated three times. Data were also recorded after 30 d for the number of shoots exceeding 5 mm in length. Values were expressed as the means of triplicate experiments, and error bars on the graphs represented standard deviations.

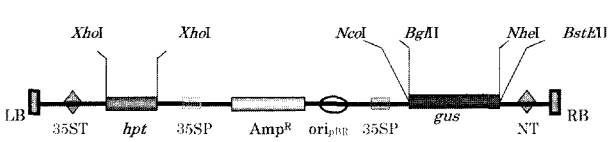

Figure 1. Plasmid map of binary vector pRCV2 used in genetic transformation study. LB, left border; RB, right border; 35ST, 3'signal of CaMV 35S; *hpt*, hygromycin resistance gene; 35SP, CaMV 35S promoter; Amp^R, ampicillin-resistance gene active in *E. coli*; Ori_{pBR}, origin of replication of ColE1; NT, 3' signal of nopaline synthase.

Agrobacterium-Mediated Genetic Transformation of Garland Chrysanthemum

To confirm that transgenic shoots could be induced on media containing determined concentrations of hormones and antibiotics, leaf disks of garland chrysanthemum were genetically transformed with *A. tumefaciens* carrying binary vector pRCVII (Fig. 1) containing the *hpt* (hygromycin resistance gene) and *gus* (β-glucuronidase gene) (Kim, 2002). This *Agrobacterium* was grown on a solid YEP medium with kanamycin (50 mg L^{-1}) at 28°C for 48 h prior to selection. One colony was then grown in 5 ml of a YEP liquid medium, containing 50 mg L^{-1} kanamycin on a shaker at 28°C for 48 h. Afterward, 1 ml of the bacterial culture was transferred to 50 ml of YEP containing 50 mg L^{-1} kanamycin and was cultured for 24 h (A_{600} = 0.8) before use. This culture was centrifuged at 5000 rpm for 15 min at 4°C and the pellet was then suspended in 50 mL of new YEP. After the suspended cells were re-centrifuged and the supernatant was discarded, the pellet was re-suspended in 30 mL YEP for explant infection.

Leaf disks were cut into 25 mm^2 segments, wounded with a sterile razor blade, and infected by immersing them in the bacterial inoculum for 5 min. Afterward, they were placed on sterile filter paper to remove any excess bacterial culture, then embedded in a co-cultivation medium (pH 5.8) comprising MS salts, 0.2 mg L^{-1} NAA, 0.5 mg L^{-1} BA, 3% sucrose, and 0.8% agar.

After 3-d of co-cultivation in the dark at 25°C, the inoculated leaf segments were washed with an MS liquid medium containing 500 mg L^{-1} carbenicillin to eliminate the bacteria. The explants were then transferred to a selection medium (pH 5.8) containing MS

salts, 0.2 mg L^{-1} NAA, 0.5 mg L^{-1} BA, 3% sucrose, 500 mg L^{-1} carbenicillin, 5 mg L^{-1} hygromycin, and 0.8% agar. Shoots were transferred to fresh selection media 4 weeks after co-cultivation, then transferred to a rooting medium (pH 5.8) with 1/2 MS salts, 3% sucrose, 500 mg L^{-1} carbenicillin, and 0.8% agar.

Plant Genomic DNA Isolation

Plant genomic DNA was isolated according to the method described by Macouch et al. (1988). Leaf tissue (0.5 g) was ground with liquid nitrogen in a well-chilled mortar and pestle. This fine powder was then mixed with extraction buffer [0.5 M NaCl, 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), and 1.25% SDS] and incubated at 65°C for 20 min. The mixture was extracted with phenol and chloroform, and precipitated in isopropanol. Precipitated DNA was picked out and washed in 70% EtOH; then, dried and dissolved in TE buffer (1M Tris-Cl, 100 mM EDTA, pH 8.0).

Southern Hybridization Analysis

Ten μg of genomic DNA was digested with *Xho*I or *Bam*HI, separated by electrophoresis on a 0.9% agarose gel, and transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech, USA). The separated plant DNA was probed with a ^{32}P-labeled 0.7-kb *Xho*I fragment of the *hpt* gene from pRCVII plasmid DNA to confirm integration of pRCVII into the plant genome. Random primer-labeling of the probe was conducted per manufacture's instructions for the Ladderman TM Labeling Kit (Takara, Japan).

RESULTS AND DISCUSSION

Effect of NAA and BA Concentrations on Shoot Induction from Leaf Disk Explants of Garland Chrysanthemum

To successfully introduce useful and interesting foreign genes into plant genomes via *Agrobacterium*, an efficient regeneration system is required. In doing so, it is important to optimize the composition for media used in tissue culture because transformation depends on the combined effect of endogenous hormones, gene expression, and responsiveness to external stimuli (Annadana et al., 2000).

In this study, we tested the effect of plant growth regulators on shoot regeneration from leaf explants, using 20 combinations of NAA and BA (Fig. 2). In the absence of BA from the medium, roots and calli were induced, instead of shoots. In contrast, when NAA was lacking, calli were induced, but then turned black. Sharma et al. (1990) reported that BA had an important role in shoot differentiation from *Brassica* cotyledons. However, NAA also was needed to regenerate shoots from *B. campestris* cotyledons (Hachey et al., 1991). Therefore, it is the optimal combination of both auxin and cytokinins that influences shoot induction from explants. Here, we determined that the ideal concentrations were 0.2 mg L^{-1} NAA plus 0.5 mg L^{-1} BA.

Effect of Antibiotics on Shoot Induction from Leaf Disk Explants of Garland Chrysanthemum.

Whichever method of DNA transfer is implemented,

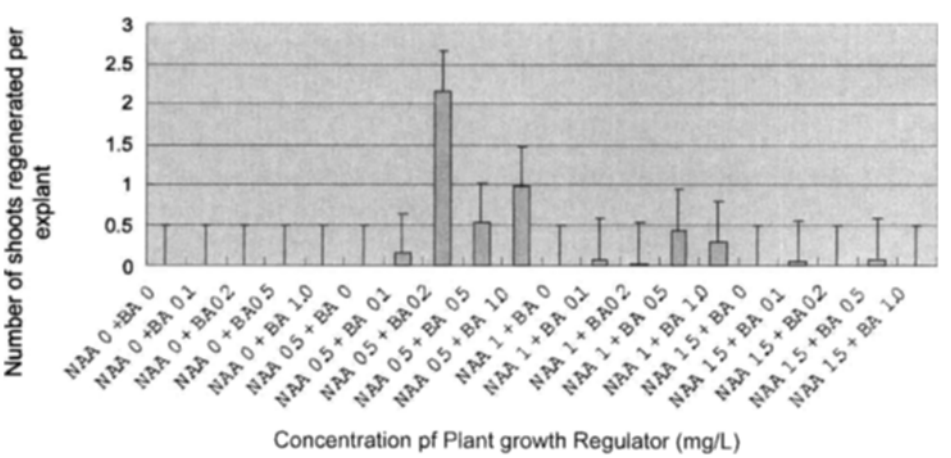

Figure 2. Effects of NAA and BA concentrations on shoot induction from leaf disk explants of garland chrysanthemum. Values are means of triplicate experiments and error bars represent standard deviations.

a strong selection regime combined with high levels of resistance-gene expression is necessary for isolating genetically transformed plants; because, tissues frequently show a high level of natural resistance to those antibiotics commonly used to select transformants (Hauptmann et al., 1988).

Kanamycin had a significant effect on callus formation and shoot regeneration of our garland chrysanthemum. The development of calli was not affected by kanamycin at 40 mg L^{-1}, but was dramatically hindered at 50 mg L^{-1}, and completely inhibited at 100 mg L^{-1} (data not shown). Likewise shoot regeneration was unaffected by kanamycin at 10 mg L^{-1}, but the number of shoots produced was significantly reduced at 20 mg L^{-1}, and no shoots were regenerated from any explants at 40 mg L^{-1} or higher (Fig. 3A). In fact, leaf explants were bleached and turned white when exposed to a toxic concentration of kanamycin. Because callus formation and shoot regeneration were inhibited at 50 mg L^{-1} and 40 mg L^{-1} kanamycin, respectively, garland chrysanthemum can be considered a kanamycin-sensitive species. In our experiments with hygromycin, callus formation was not affected by a level of 1 mg L^{-1}, but was significantly reduced in the presence of 3 mg L^{-1} hygromycin, and completely inhibited at 5 mg L^{-1} or higher (data not shown). Shoot regeneration from leaf explants was also completely prohibited at those higher concentrations (Fig. 3B). Moreover, sensitive tissues blackened and died, in contrast to the kanamycin-treated tissues that turned white. Therefore, this gene-encoding resistance to hygromycin may also be used as a suitable selective marker for garland chrysanthemum transformation.

Sensitivity to antibiotics depends upon genotype. For example, transgenic *Dendranthema indicum* plants were obtained from leaf explants on a selection medium containing 10 mg L^{-1} kanamycin (Ledger et al., 1991). However, Lowe et al. (1993) reported that only a concentration of 15 mg L^{-1} kanamycin was effective, whereas. Renou et al. (1993) obtained only escapes on media containing 25 mg L^{-1} kanamycin

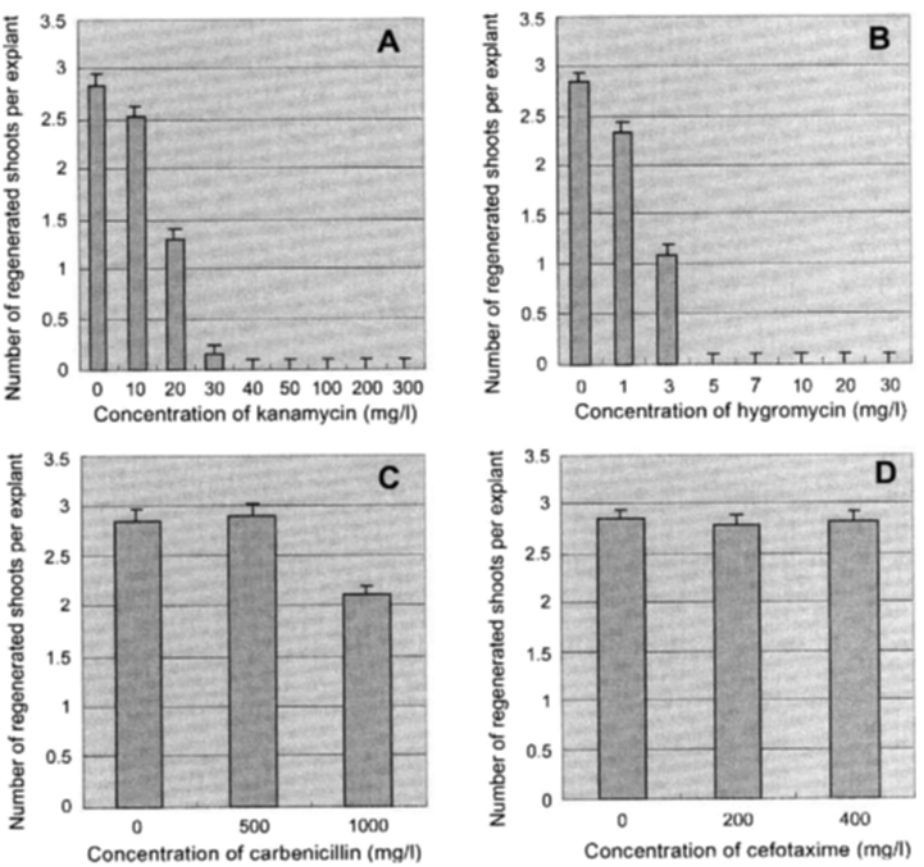

Figure 3. Effects of kanamycin **(A)** , hygromycin **(B)**, carbenicillin **(C)** and cefotaxime **(D)** on shoot induction from leaf disk explants of garland chrysanthemum. Values are means of triplicate experiments and error bars represent standard deviations.

and noted that shoot regeneration was fully depressed at a level of 50 mg L^{-1} kanamycin. In contrast, hygromycin showed inhibitory activity against both prokaryotic and eukaryotic cells by interfering with their protein synthesis (Cabanas et al., 1978; Gonzalez et al., 1978). Moreover, its effectiveness for selection of transgenic plants was optimized at a concentration lower than the one identified in current experiment. For these above reasons, we used the *hpt* gene as a selectable marker for truly transgenic plants.

Carbenicillin and cefotaxime, the cephalosporin type of antibiotics, are generally used to eliminate *Agrobacterium* after co-cultivation. Ideally, they must be lethal to the bacteria while harmless to plant material. Because both have low toxicity to garland chrysanthemum and, especially because the incorporation of 500 mg L^{-1} carbenicillin induced more shoots here, we determined that the latter was suitable for removing *Agrobacterium* during our transformation experiments (Fig. 3C and D). One disadvantage of cefotaxime is that it can enhance the vitrification and necrosis of shoots, thereby decreasing their regeneration potential, whereas carbenicillin can reduce the negative effects of silver nitrate (de Block et al., 1989; Schroder et al., 1991). The latter has also been proven superior to vancomycin, another selective agent, with regard to shoot regeneration (Schroder et al., 1991).

In summary, the ability of our leaf explants to regenerate shoots was significantly influenced by diverse antibiotics. Furthermore, certain selectable markers were detrimental to plant regeneration, and their effective concentrations depended on genotype. Our results demonstrated that an application of 5 mg L^{-1} hygromycin was adequate for selecting genetically transformed tissues and that carbenicillin, at 500 mg L^{-1}, showed low toxicity to garland chrysanthemum plants.

Agrobacterium-Mediated Genetic Transformation of Garland Chrysanthemum

Transgenic plants were obtained by incubating leaf disks with *A. tumefaciens* strain EHA105 harboring pRCVII, which contains the *hpt* gene as a selectable marker and the reporter gene GUS (Fig. 1). In a separate experiment, leaf disks were immersed in bacterial innocula for 30 sec or, 1, 5, 15, or 30 min. Overall, immersion for 5 min was most effective for inducing transgenic shoots (data not shown).

In our optimized transformation protocol, shoots were produced from leaf disks placed on a selection medium containing 0.2 mg L^{-1} NAA, 0.5 mg L^{-1} BA, 500 mg L^{-1} carbenicillin, and 5 mg L^{-1} hygromycin. After four weeks, they were transferred to fresh media containing 7 mg L^{-1} hygromycin in order to select for escapes (i.e., non-transformants). The grown shoots were then transferred to a rooting medium comprising 1/2 MS salts, 3% sucrose, 500 mg L^{-1} carbenicillin, and 0.8% agar (pH 5.8). Subculturing was done every 4 weeks (Fig. 4).

Southern blot analysis was performed on the total genomic DNA of 10 presumed transformants. It revealed the presence of the expected 700-bp *hpt* gene in our PCR-confirmed transgenic plants, and showed copy numbers of one to three *hpt* genes integrated into the genome for each transgenic line (Fig. 5). In contrast, no hybridization signals were detected in the genomic DNA of the non-transformed controls. This experiment also verified that the concentrations of hormones and hygromycin determined here were ideal for genetic transformation.

Our report is the first to describe the development of an efficient shoot regeneration system for obtaining transgenic garland chrysanthemum plants via *Agrobacterium*-mediated transformation.

ACKNOWLEDGEMENT

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration, Korea.

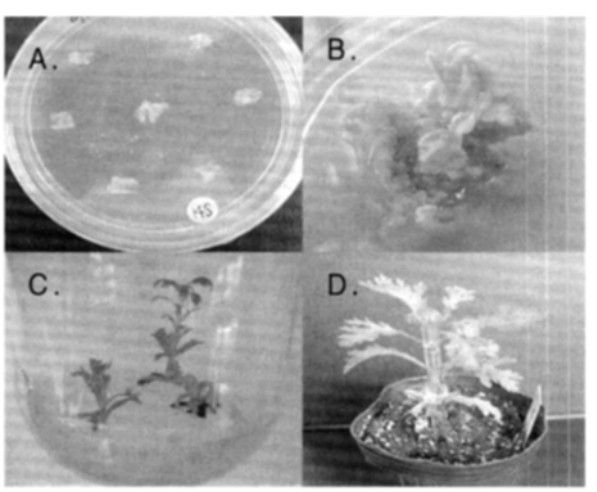

Figure 4. Shoot regeneration of garland chrysanthemum, and the production of transgenic plant. **A**, Leaf disk incubated on selection media. **B**, Transgenic shoot regenerated from leaf disk cultured for 4 weeks. **C**, Rooting of excised shoot. **D**, Acclimation of transgenic plant in pot. Mature plant transferred to soil in greenhouse after 1 week. Phenotypic abnormalities were not detected in regenerated plants.

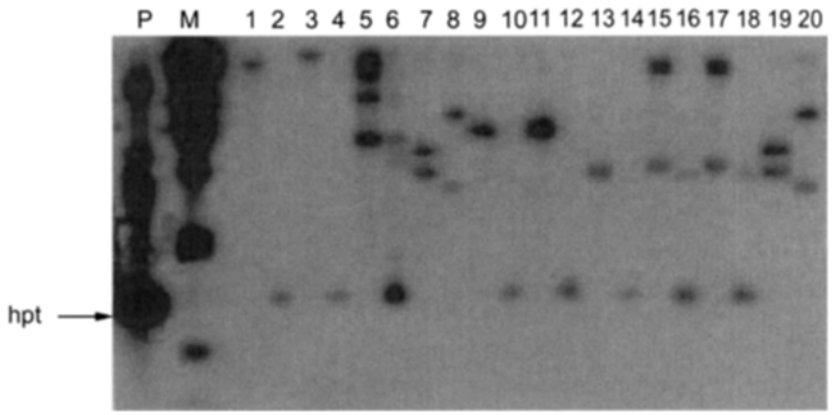

Figure 5. Southern hybridization of DNA from garland chrysanthemum, using pRCVII. Genomic DNA of each transgenic line was digested with *Bam*HI (odd number) and *Xho*I (even number), respectively, then probed with ^{32}P-labeled 700-bp *Xho*I fragment from pRCVII plasmid DNA. M, size marker (lamda DNA digested with *Hin*dIII); P, positive control (RCVII plasmid); lanes 1-20, transgenic plants.

Received September 6, 2004; accepted January 19, 2005.

LITERATURE CITED

Annadana S, Rademaker W, Ramanna M, Udayakumar M, de Jong J (2000) Response of stem explants to screening and explant source as a basis for methodical advancing of regeneration protocols for chrysanthemum. Plant Cell Tiss Org Cult 62: 47-55

Cabanas MJ, Vazquez D, Modolell J (1978) Dual interference of hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. Eur J Biochem 87: 21-27

de Block M, Brouwer D, Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and *neo* genes in the transgenic plants. Plant Physiol 91: 694-701

Fukai S, de Jong J, Rademaker W (1995a) *Agrobacterium*-mediated genetic transformation of chrysanthemum. Acta Horticulturae 392: 147-152

Fukai S, de Jong J, Rademaker W (1995b) Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflorum* Kitamura) using stem segments. Breeding Sci 45: 179-184

Gonzalez A, Jimenez A, Vazquez D, Davis J, Schindler D (1978) Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. Biochim Biophys Acta 521: 459-469

Hachey JE, Sharama KK, Moloney MM (1991) Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured *in vitro*. Plant Cell Rep 9: 549-554

Hauptmann RM, Vasil V, Ozias-Atkins PM, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB, Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol 86: 602-606

John MS, Moyer WJ, Margaret ED (1998) A regeneration and *Agrobacterium*-mediated transformation system for genetically diverse chrysanthemum cultivars. J Amer Soc Hort Sci 123: 189-194

Kim HS (2002) Development and application of plant transformation vector for rescue cloning. M.S. thesis, Kyung Hee University, YongIn

Ledger SE, Deroles SC, Given NK (1991) Regeneration and *Agrobacterium*-mediated transformation of chrysanthemum. Plant Cell Rep 10: 195-199

Lowe JM, Davey MR, Power JB, Blundy KS (1993) A study of some factors affecting *Agrobacterium tumefaciens* and plant regeneration of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat). Plant Cell Tiss Org Cult 33: 171-180

Macouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet **76**: 815-829

Mol JNM, Stuitje AR, Gerats A, van der Krol A, Jorgensen R (1989) Saying it with genes: Molecular flower breeding. Trends Biotech 7: 148-153

Renou JP, Brochard P, Jalouzot R (1993) Recovery of transgenic chrysanthemum (*Dendranthema grandiflora* Tzvelev) after hygromycin resistance selection. Plant Sci 89: 185-197

Saito K, Yamazaki M, Murakoshi I (1992) Transgenic medical plants: *Agrobacterium*-mediated foreign gene transfer and production of secondary metabolites. J Natural Products 55: 149-162

Schroder M, Dixelius C, Rahlen L, Glimelius K (1991) Transformation of *Brassica napus* by using the *aadA* gene as selectable marker and inheritance studies of the marker genes. Physiol Plant 92: 37-46

Sharma KK, Bhowani SS, Thorpe TA (1990) Factors affect high frequency differentiation of shoot and roots from cotyledon explants of *Brassica juncea* (L.) C. Plant Sci 66: 247-253