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Using leaf explants for transformation of *Chrysanthemum morifolium* ramat. mediated by *Agrobacterium tumefaciens*

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Abstract

An *Agrobacterium*-mediated transformation for chrysanthemum using leaf explants of two different cultivars was conducted. Leaves were first infected with *Agrobacterium tumefaciens* LBA4404 strain, harbouring a binary vector pBI121 carrying the GUS reporter gene and the *nptII* as the selectable marker gene. 30 seconds for infection of leaves proved to be the optimum duration. Best results were achieved when explants were co-cultivated with *A. tumefaciens* for two days. Shoot induction medium was supplemented with 7 mgl^{-1} kanamycin and 300 mgl^{-1} cefotaxime. Putative transgenic plants were obtained after rooting on 1/2 MS medium containing 7mgl^{-1} kanamycin in the absence of cefotaxime. Transformation efficiency of 'Reagan Elite Salmon' (29.1%) was higher than 'Resomee Splendid' (9.1%). Presence of the *gus* gene was verified with histochemical GUS assay and polymerase chain reaction (PCR) of transformed plants.

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Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.), as a member of the Asteraceae family (Anderson, 2007), is one of the most important ornamentals worldwide. Its cultivation and classical breeding dates back over 2000 years (Teixeira da Silva, 2007). Modern cultivars are hexaploids with a loss or gain of a number of chromosomes which present self-incompatibility (Shinoyama *et al.*, 2012b). Classical breeding methods face with difficulties in many ornamentals –such as chrysanthemums- because of their high ploidy levels, high chromosome numbers and incompatibility (Petty *et al.*, 2003; Müller, 2011). Fortunately, during the last decades genetic engineering has implemented specific alterations of single traits (Lütken *et al.*, 2012).

Susceptibility of chrysanthemums to *Agrobacterium tumefaciens* was first detected in the mid-1970s. Thereafter, transformation mediated by *Agrobacterium* has become the most frequently used method in regard to this plant, globally (Teixeira da Silva *et al.*, 2013) with the ability of creating desirable changes in different cultivars, without altering their important ornamental traits (Sherman *et al.*, 1998; Chung and Park, 2005).

During development of transformation protocols, a selectable marker gene and a reporter gene are inserted in the transformation vector. Afterwards, the reporter gene is substituted with the target gene. Reporter genes are included in vectors to enable easy detection of probable transformants during the development of a transformation protocol (Deo *et al.*, 2010). Among these, the ß-glucuronidase gene (GUS) is the most frequently applied reporter gene in chrysanthemum transformation. Furthermore, the *Cauliflower mosaic virus* promoter (CaMV35S), is the most frequently used promoter (Teixeira da Silva *et al.*, 2013). Leaves are the main explants used for *Agro*-infection and stems rank among the seconds (*Shinoyama et al.*, 2012a). Moreover, kanamycin has been the most important selection agent of transformed tissues and cells in chrysanthemums (Shinoyama *et al.*, 2012b). The timing of *Agrobacterium* infection is important for improvement of the infection efficiency and thereby raising the plant transformation efficiency (Shinoyama *et al.*, 2002).

Complete removal of the bacteria from explants after co-cultivation is necessary; otherwise it may interfere with the future growth and organogenesis of the explants. Overgrowth of bacteria results in death of the explants. Antibiotics are used for elimination of bacteria. The selected antibiotic should efficiently remove the bacteria while does not prevent the growth and organogenesis of the explants (Swarnapiria, 2009).

Various protocols have developed in regard to optimizing Chrysanthemum transformation using GUS reporter gene (Van Wordragen *et al.*, 1991; Trigiano and May, 1992; Annadana *et al.*, 2002; Kudo *et al.*, 2002; Aida *et al.*, 2005; Teixeira da Silva, 2005; Sun *et al.*, 2009; Song *et al.*, 2012); however, successful transformation of this ornamental species depends on the cultivar (Boase *et al.*, 1998; Teixeira da Silva *et al.*, 2014). Therefore, attempts were made to optimize the transformation protocol for two chrysanthemum cultivars using leaf explants in order to pave the way for further genetic manipulations of this valuable ornamental.

Materials and methods

Plant materials

Stem segments with 2-3 nodes were collected from donor plants of two chrysanthemum cultivars, 'Resomee Splendid' and 'Reagan Elite Salmon', which were grown in a greenhouse. After 30 minutes rinsing under tap water in the laboratory, these were surface sterilized with 70% ethanol for 30 seconds, followed by immersion in 2.5% sodium hypochlorite solution with Tween 20 for 10 minutes. Finally, three rinses in sterile distilled water were done and nodal segments were cut from 0.5 cm above and below the axillary buds. These explants were then cultured in Murashige and Skoog (MS) (1962) medium supplemented with 0.25 mgl^{-1} benzylaminopurine (BAP) with the aim of

proliferating *in vitro* stock plants. Cultures were kept at 25±1°C under 27 µmol m^{-2} s^{-1} illumination with a 16/8 light/dark photoperiod. After 6-7 weeks of culture, leaves from the *in vitro* developed shoots were cut into about 5 mm^2 and used as explants for the transformation experiments.

Agrobacterium strain

Transformations were done with the *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121 obtained from National Institute of Genetic Engineering and Biotechnology (Iran). This construct contained GUS reporter gene under control of CaMV35S promoter and neomycin phosphotransferase (*nptII*) selectable marker gene for kanamycin resistance (Fig. 1).

Determining kanamycin concentration in culture media

Before starting transformation experiment, optimum concentration of kanamycin to be used as the selectable agent in the medium was determined. For this purpose, leaves and petioles were cultured on an optimized medium achieved from a preliminary experiment consisted of MS medium fortified with 1.5 mgl^{-1} BAP and 1 mgl^{-1} NAA. This medium was also supplemented with 5, 7, 10, 30, 60 or 90 mgl^{-1} kanamycin.

Agrobacterium-mediated transformation

Leaf explants were initially submerged in an *Agrobacterium* suspension with OD_{600}=0.6 for 5 or 1 minutes, 30, 15 or 10 seconds. Afterwards, excess liquid was thoroughly removed from explants by sterile filter papers and co-cultured for two or three days in dark on media containing 1.5 mgl^{-1} BAP, 1 mgl^{-1} NAA and 7 mgl^{-1} kanamycin. Thereafter, explants were transferred to shoot induction medium (SIM) which was the same as co-cultivation medium, with the addition of 300 mgl^{-1} cefotaxime. Subcultures were done every two weeks. After seven weeks, regenerated shoots were transferred to 1/2 MS free of hormones and cefotaxime as root induction medium (RIM) which still contained kanamycin. 30 gl^{-1} sucrose and 6.5 gl^{-1} agar were added to all the media.

Polymerase Chain Reaction (PCR) analysis

Plant genomic DNA was extracted from leaves of *in vitro* rooted plants by using a modified Cetyltrimethyl ammonium bromide (CTAB) method (Porebski *et al.*, 1997; Saghai-Maroof *et al.*, 1984) and PCR was conducted with the following pairs of primers (5'→ 3'):

Fw	GUS+2	GGTGGTCAGTCCCTTATGTTAGG
Rv	GUS-4	CCGGCATAGTTAAAGAAATCATG
Fw	35S	GCTCCTACAAATGCCATCA
Rv	GUS-4	CCGGCATAGTTAAAGAAATCATG

PCR analysis was carried out using 50 ng of genomic DNA per 25 µl reaction volume. The PCR amplification cycles using GUS gene primers was performed by one cycle of initial denaturation at 95°C (4 min) followed by 30 cycles at 94°C (1 min), annealing at 58°C (1 min), extension at 72°C (1 min) and ultimately one cycle at 72°C (5 min) for extension. The same steps holds true while using the other pair of primers except for the annealing temperature which was 60°C. PCR products were run on 1% (w/v) agarose gel. Finally, we transferred gels into the Gel Doc to visualize bands under UV light.

Histochemical Gus assay

GUS expression was assayed on intact leaves of kanamycin resistant rooted plants which were also PCR positive. Staining was performed according to Mousavi *et al.*, (2009) using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate (Jefferson, 1987), with some modifications. Samples were inoculated at 37°C for 48 h after staining. Then explants were bleached in 70% ethanol by several washes to remove chlorophyll and facilitate blue color detection.

Transformation efficiency calculation

Transformation efficiency was calculated according to Teixeira da Silva (2007) as [(Y/Z) ×100] % where Y= Number of rooted *in vitro* explants on medium containing 7 mgl^{-1} kanamycin and Z= Total number of initial explants.

Results and discussion

Optimal concentration of kanamycin

It was revealed that shoot induction from some explants occurred only in medium supplemented with 5 mgl^{-1} kanamycin while in other concentrations was failed. Hence, the first higher concentration -7 mgl^{-1} kanamycin- was determined as the optimal concentration for selection of transformed shoots in both chrysanthemum cultivars. Regeneration was associated with yellowing and complete removal of chlorophyll content of leaf tissues (Fig 2.), though similar reports was not found in this case.

Table 1. Transformation efficiency of two chrysanthemum cultivars after transformation of leaf explants with LBA4404 harboring pBI121 plasmid.

cultivar	total no. explants	leaf no. explants	regenerated no. rooted plants	*in vitro* no. positive plants	PCR no. positive plants	GUS transformation efficiency (%)
'Resomee Splendid'	120	49	11	3	0	9.1
'Reagan Elite Salmon'	120	59	35	15	3	29.1

High sensitivity of chrysanthemums to kanamycin has been reported in some investigations with lethal concentrations of 10 to 50 mgl^{-1} dependant on the cultivar (Renou *et al.*, 1993; de Jong *et al.*, 1994; Urban *et al.*, 1994). Song *et al.* (2012) used petal explants for transformation of chrysanthemum and reported 7.5 mgl^{-1} of kanamycin as the best concentration for selection of the transformed shoots.

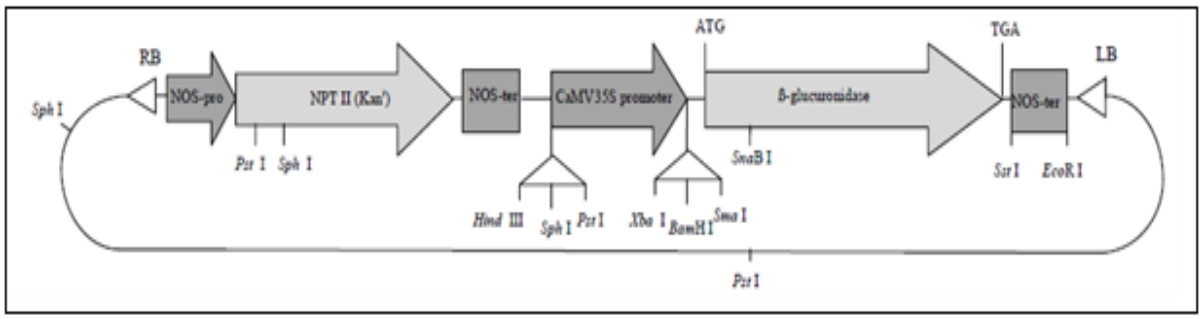

Fig. 1. A schematic map of pBI121 with GUS marker gene. RB, Right Border; NoS-pro, NoS promoter; NPT II, Neomycin Phosphotransferase marker gene; Nos-ter, NoS terminator; LB, Left Border.

Regeneration of transgenic shoots

Based on the results, after three days of co-cultivation, high percentage of leaf explants showed contamination that could not be prevented even with cefotaxime wash. Therefore, 2-day co-cultivation proved to be better which is commonly used in transformation experiments of other plant species (Horsh *et al.*, 1985; Sangwan *et al.*, 1992; Villemont *et al.*, 1997). Song *et al.* (2012) also confirmed 2-day co-cultivation as the best period for transformation o chrysanthemum using petal explants.

Optimum infection duration was 30 sec because of the severe contamination of explants at higher concentrations. Reported infection periods using LBA4404 strain of *Agrobacterium* are disparate among investigators. Boase *et al.* (1998) soaked leaf explants in *Agrobacterium* suspension for 30-60 sec; while Tsuro *et al.* (2005) selected 10 min infection time at OD_{600}=0.1 for the same explant.

Regeneration from leaf explants was drastically decreased after transformation in both cultivars (Data not shown). Hypermethylation is a defense reaction shown by animals or plants in response to pathogen attack (Schmitt *et al.*, 1997). Addition of antibiotics to the medium may mimic a pathogen attack and result in induction of the mentioned reaction with

subsequent reduction of regeneration capacity (Teixeira da Silva and Fukai, 2001). However, susceptibility to antibiotics is cultivar dependent (Chung and Park, 2005).

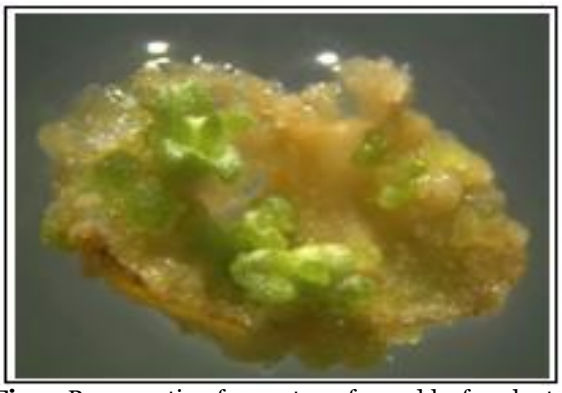

Fig.2. Regeneration from a transformed leaf explants in medium supplemented with 5 mgl^{-1} kanamycin along with chlorophyll removal of the initial explants.

Another surprising observation after transformation was hyperhydricity of some regenerants especially in 'Reagan Elite Salmon' (Fig. 3), which could not further develop roots. Based on some reports, one disadvantage of cefotaxime is that it can enhance hyperhydicity and necrosis of shoots, thereby reducing their regeneration potential (Schroder *et al.*, 1991; Chung and Park, 2005).

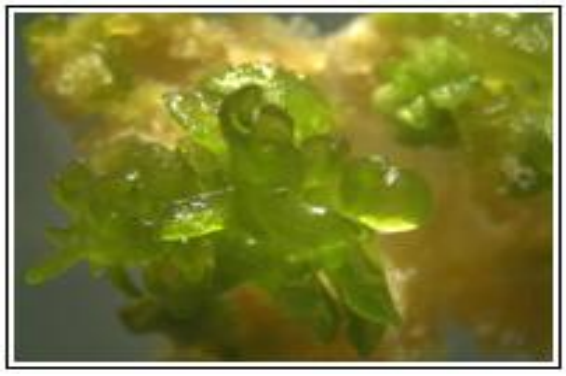

Fig. 3. Hyperhydricity of regenerated shoots following transformation of leaf explants with LBA4404 harboring pBI121 in 'Reagan Elite Salmon' in SIM supplementd with 7 mgl^{-1} kanamycin and 300 mgl^{-1} cefotaxime.

In order to investigate probable negative effect of cefotaxime in current study, its concentration was halved in further transformations. Using this approagh, number of abnormal regenerants was reduced by approximately up to zero.

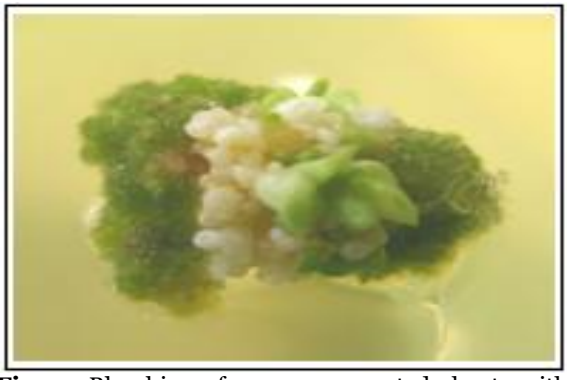

Fig. 4. Bleaching of some regenerated shoots with LBA4404 harboring pBI121 after transformation in the medium supplemented with 7 mgl^{-1} kanamycin.

Another notable observation after transformation was bleaching of some regenerated shoots after few days in the medium containing kanamycin which apparently indicates that those shoots were not transformed (Fig. 4.).

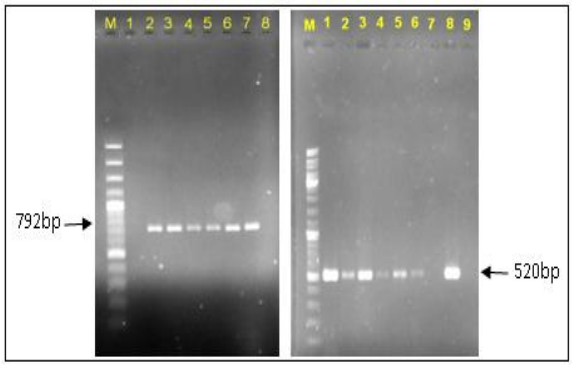

Fig. 5. PCR analysis of putative transgenic lines of chrysanthemum with (right): GUS gene primers; M, DNA ladder; 1, pBI121plasmid as positive control; 2-6 and 8, putative transgenic lines; 7, non-transformed plant; 9, wild-type plant as negative control; (left): Fw 35S and Rv gus primers; M, 100-bp plus DNA ladder; 1, wild-type as negative control; 2, pBI121 plasmid as positive control; 3-7, putative transgenic lines; 8, non-transformed plant.

PCR analysis results

PCR analysis of kanamycin resistant rooted plants showed that all the plants which confirmed presence of the reporter gene after electrophoresis with specific primers of the *gus* gene; revealed the same results while using the Fw 35S and Rv gus primers. The expected size of PCR product on the gel with specific primers of the *gus* gene was 520 bp and with the

other pair of primers was 792 bp (Fig. 5.) which were confirmed after gel electrophoresis of putative plants. As tabulated in Table 1, transformation efficiency in leaf explants of 'Reagan Elite Salmon' (29.1%) was extremely higher than 'Resomee Splendid' (9.1%). It has been reported that various cultivars show different reactions to genetic manipulation, hence; an optimized protocol is not necessarily appropriate for a broad range of cultivars (Teixeira da Silva and Fukai, 2001). In almost 50 reports of *Agrobacterium*-mediated transformation of chrysanthemum, leaf was the most frequent initial explants used (Teixeira da Silva, 2007). Transformation efficiency of chrysanthemum leaf explants using LBA4404 varied in different studies and it was reported 0.8% (Ledger *et al.*, 1991), 0-23.9% (Shinoyama *et al.*, 2002) or 3.3% (Tsuro *et al.*, 2005) based on the cultivar.

Fig. 6. GUS expression on a broad part of a transformed leaf tissue.

Histochemical GUS assay

A number of leaves from the plants with positive results in PCR analysis showed blue color (Table 1) which indicated success in GUS gene expression. As illustrated in Fig. 6, blue color was seen in a broad part of the leaf blade. Existence of blue color areas suggests high gene transfer efficiency (Song *et al.*, 2012). Some plants with positive results in PCR analysis did not show blue color after staining, which might be due to gene silencing (Shinoyama *et al.*, 2012).

In current study we tried to optimize *Agrobacterium*-mediated transformation of two chrysanthemum cultivars using LBA4404 harboring pBI121 plasmid. Due to high sensitivity of chrysanthemums to kanamycin it is proposed to investigate effects of other selectable agents in future. Furthermore, it seems that despite the vast use of cefotaxime in different crops such as chrysanthemums, its application results in reduced regeneration and also some other problems which were mentioned in the text. Therefore, it is suggested to make a comparison among various antibiotics to detect their influences on this plant species.

References

Aida R, Nagaya S, Yoshida K, Kishimoto S, Shibata M, Ohmiya A. 2005. Efficient transgene expression in chrysanthemum, *Chrysanthemum morifolium* Ramat., with the promoter of a gene for tobacco elongation factor 1α protein. Japan Agricultural Research Quarterly **39(4),** 269-274.

Anderson NO. 2007. Flower breeding and genetics: Issues, challenges and opportunities for the 21st century. Springer, 822 p.

Annadana S, Beekwilder MJ, Kuipers G, Visser PB, Outchkourov N, Pereira A, Udayakumar M, De Jong J, Jongsma MA. 2002. Cloning of the chrysanthemum *UEP1* promoter and comparative expression in florets and leaves of *Dendranthema grandiflora*. Transgenic Research **11,** 437-445.
http://dx.doi.org/10.1023/A:1016313924844

Boase MR, Bradley JM, Borst NK. 1998. Genetic transformation mediated by *Agrobacterium tumefaciens* of florists' chrysanthemum (*Dendranthema* × *grandiflorum*) cultivar 'Peach Margaret'. In Vitro Cellular and Developmental Biology- Plant **34,** 46-51.

Chung KM, Park YD. 2005. Development of an *Agrobacterium*-mediated transformation system for regenerating Garland Chrysanthemum (*Chrysanthemum coronarium* L.). Journal of Plant Biology **48(1),** 136-141.
http://dx.doi.org/10.1007/BF03030573

De Jong J, Mertens MMJ, Rademaker W. 1994. Stable expression of the GUS reporter gene in Chrysanthemum depends on binary plasmid T-DNA. Plant Cell Reports **14,** 59-64. http://dx.doi.org/10.1007/BF00233300

Deo PC, Tyagi AP, Taylor M, Harding R, Becker D. 2010. Factors affecting somatic embryogenesis and transformation in modern plant breeding. The South Pacific Journal of Natural and Applied Sciences **28,** 27-40. http://dx.doi.org/10.1071/SP10002

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT. 1985. A simple and general method for transferring genes into plants. Science **221,** 1229-1231. http://dx.doi.org/10.1126/science.227.4691.12.29

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal **6,** 3901-3907.

Kudo S, Shibata N, Kanno Y, Suzuki M. 2002. Transformation of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) via *Agrobacterium tumefaciens*. Acta Horticulturae **572,** 139-147.

Ledger SE, Deroles SC, Given NK. 1991. Regeneration and *Agrobacterium*-mediated transformation of chrysanthemum. Plant Cell Reports **10,** 195-199. http://dx.doi.org/10.1007/BF00234294

Lütken H, Clarke JL, Müller R. 2012. Genetic engineering and sustainable production of ornamentals: current status and future directions. Plant Cell Reports **31,** 1141-1157. http://dx.doi.org/10.1007/s00299-012-1265-5

Mousavi M, Mousavi A, Habashi AA, Arzani K. 2009. Optimization of physical and biological parameters for transient expression of *uidA* gene in embryogenic callus of date palm (*Phoenix dactylifera* L.) via particle bombardment. African Journal of Biotechnology **8(16),** 3721-3730.

Müller R. 2011. Physiology and genetics of plant quality improvement. Doctoral Dissertation, University of Copenhagen.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum **15,** 473-497.

Petty L, Harberd N, carré I, Thomas B, Jackson S. 2003. Expression of the Arabidopsis *gai* gene under its promoter causes a reduction in plant height in Chrysanthemum by attenuation of the gibberellins response. Plant Science **164,** 175-182. http://dx.doi.org/10.1016/S0168-9452(02)00380-1

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

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel BS. 1992. Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in Arabidopsis thaliana. Plants **188,** 439-456. http://dx.doi.org/10.1007/BF00192812

Schmitt F, Oakeley EJ, Jost JP. 1997. Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tobacum* plants. Journal of Biological Chemistry **272,** 1534-1540. http://dx.doi.org/10.1074/jbc.272.3.1534

Schroder M, Dixelius C, Rahlen L, Glimelius K. 1991. Transformation of *Brassica napus* by using the *aadA* gene as selectable marker genes. Physiologia Plantarum **92,** 37-46. http://dx.doi.org/10.1111/j.13993054.1994.tb06652.x

Sherman JM, Moyer JW, Daub ME. 1998. A regeneration and *Agrobacterium*-mediated

transformation system for genetically diverse chrysanthemum cultivars. Journal of American Society for Horticultural Science **123,** 189-194.

Shinoyama H, Kazumi T, Komano M, Nomura Y, Tsuchiya T. 2002. An efficient transformation system in chrysanthemum [*Dendranthema x grandiflorum* (Ramat.) Kitamura] for stable and non-chimeric expression of foreign genes. Plant Biotechnology **19,** 335-343. http://dx.doi.org/10.5511/plantbiotechnology.19335

Shinoyama H, Aida R, Ichikawa H, Mochizuki A, Nomura Y. 2012a. The establishment of a gene transformation system in chrysanthemum (*Chrysanthemum morifolium* Ramat.) and its recent progress. Plant Biotechnology **29,** 1-15.

Shinoyama H, Aida R, Ichikawa H, Nomura Y, Mochizuki A. 2012b. Genetic engineering of chrysanthemum (*Chrysanthemum morifolium*): Current progress and perspectives. Plant Biotechnology **29,** 323-337. http://dx.doi.org/10.5511/plantbiotechnology.12.0521a

Song JY, Sivanesan I, Jeong BR. 2012. Use of petal explants for successful transformation of *Dendranthema × grandiflorum* Kitamura 'Orlando' mediated by *Agrobacterium tumefaciens*. African Journal of Biotechnology **11(37),** 9141-9148. http://dx.doi.org/10.5897/AJB 11.267

Sun L, Zhou L, Lu M, Cai M, Jiang XW, Zhang QX. 2009. Marker-free transgenic chrysanthemum obtained by *Agrobacterium*-mediated transformation with twin T-DNA binary vectors. Plant Molecular Biology Reporter **27,** 102-108. http://dx.doi.org/10.1007/s11105-008-0062-3

Swarnapiria R. 2009. Genetic transformation in ornamentals- A review. Agricultural Reviews **30(2),** 120-131.

Teixeira da Silva JA. 2005. Effective and comprehensive Chrysanthemum (*Dendranthema X grandiflora*) regeneration and transformation protocols. Biotechnology **4(2),** 97-104.

Teixeira da Silva JA. 2007. Chrysanthemum (*Dendranthema × grandiflora*) In: Methods in molecular biology, vol. 344. *Agrobacterium* protocols, (2nd ed.), vol 2, edited by: Wang K, Humana Press Inc., Totowa, NJ, 321-329.

Teixeira da Silva JA, Fukai S. 2001. The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. Journal of Applied Horticulture **3(1),** 3-12.

Teixeira da Silva JA, Shinoyama H, Aida R, Matsushita Y, Raj SK, Chen F. 2013. Chrysanthemum biotechnology: Quo vadis?. Critical Reviews in Plant Sciences **32(1),** 21-52. http://dx.doi.org/10.1080/07352689.2012.696461.

Trigiano RN, May RA. 1992. Laboratory exercises illustrating organogenesis and transformation using chrysanthemum cultivars. HortTechnology **4,** 325-327.

Tsuro M, Kubo T, Shizukawa Y, Takemoto T, Inaba K. 2005. *Agrobacterium rhizogenes* is a useful transporter for introducing T-DNA of the binary plasmid into the chrysanthemum, *Dendranthema grandiflorum* Kitamura, genome. Plant Cell, Tissue and Organ Culture **81,** 175-181. http://dx.doi.org/10.1007/s11240-004-4783-1

Urban LA, Sherman JM, Moyer JW, Daub ME. 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Science **98,** 69-79.

Van Wordragen MF, De Jong J, Huitema HBM, Dons HJM. 1991. Genetic transformation of chrysanthemum using wild type *Agrobacterium* strains, strain and cultivar specificity. Plant Cell

Reports **9,** 505-508.
http://dx.doi.org/10.1007/BF00232106

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Norreel BS. 1997. Role of the host cell in the *Agrobacterium*-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta **201,** 106-172.
http://dx.doi.org/10.1007/BF01007700