Plant Biotechnology 25, 77–80 (2008)

Technical Note

Improvement of regeneration and transformation systems for *Cyclamen persicum* using somatic embryo culture

Teruhiko Terakawa*, Tomomichi Yamamura, Toshio Murayama

Central Research Laboratories, Hokko Chemical Industry Co., 2165 Toda, Atsugi, Kanagawa 243-0023, Japan
* E-mail: terakawa-t@hokkochem.co.jp Tel: +81-46-228-5881 Fax: +81-46-228-0164

Received October 10, 2007; accepted November 30, 2007 (Edited by M. Nakano)

Abstract *Cyclamen persicum* is one of the most important pot plants in the world. We developed an effective method for somatic embryo-mediated plant regeneration and genetic transformation using *Agrobacterium*, with somatic embryos as the source of plant material. Numerous somatic embryos were formed from the leaf segments of *Cyclamen* cv. "Fragrance Mini" 16 weeks after initiation on a 2,4-D- and kinetin-containing medium. The maximum regeneration frequency from the somatic embryos was achieved using a regeneration medium containing 0.1 mg l^{-1} benzylaminopurine, 0.01 mg l^{-1} α-naphthaleneacetic acid, and 0.2 mg l^{-1} gibberellin. After co-cultivating the somatic embryos with *Agrobacterium* strain LBA4404 (the plasmid vector pIG121Hm) that harbors genes for hygromycin phosphotransferase, neomycin phosphotransferase, and β-glucuronidase, hygromycin-resistant clones were selected on a medium containing 5 mg l^{-1} hygromycin. Hygromycin-resistant plantlets were regenerated from 14 hygromycin-resistant embryos. Using this transformation system, 74 independent plants were obtained from 1-ml packed cell volume, approximately 2,000 embryos.

Key words: *Cyclamen persicum*, transformation, somatic embryo, regeneration.

The perennial plants in the family *Primulaceae*, genus *Cyclamen*, which naturally originated from the coastal Mediterranean, are one of the most important pot plants in the world. The genus *Cyclamen* includes about 20 species that have different characteristic features. *Cyclamen persicum* Mill, e.g., can display several flower colors, ranging from white through red, pink, and purple; this plant variety is the only economically significant pot plant.

Traditional Cyclamen breeding methods have produced a large number of cultivars with improved floral characteristics and disease tolerance. Further genetic enhancement is conceivable via biotechnological applications such as micropropagation, cell fusion, induction of somaclonal variation, and genetic transformation. However, there are a few reports on the transformation of Cyclamen. Transgenic *C. persicum* have been produced using *Agrobacterium tumefaciens*, and etiolated petioles of aseptic seedlings are used as a target for transformation (Aida et al. 1999; Boase et al. 2002). However, normal (not etiolated) petioles and somatic embryos have not been used as the starting material. There are two essential elements for *Agrobacterium*-mediated transformation: stable introduction into the plant genome and regeneration of transgenic plants. Somatic embryos may be one of the best sources to produce a large number of transgenic Cyclamen plants. Somatic embryogenesis is a very efficient system for clonal mass propagation of plants. Although *C. persicum* somatic embryos have been reported (Kiviharju et al. 1992; Otani and Shimada 1991; Takamura et al. 1995; Takamura and Tanaka 1996; Wicart et al. 1984), the frequency of plant regeneration from somatic embryos is low.

In this study, we developed an efficient method for plant regeneration via somatic embryos in Cyclamen. Moreover, we describe *Agrobacterium*-mediated genetic transformation using somatic embryos as the source of plant material.

A commercial variety of *Cyclamen* cv. "Fragrance Mini" (FM) cultivated in a greenhouse was used as the plant material. FM is recognized as a highly competent variety for somatic embryos (personal data). Leaves were soaked in 70% ethyl alcohol for 1 min, surface sterilized with 1% sodium hypochlorite for 30 min, and then rinsed with sterilized distilled water. The leaves were cut into segments (1 cm×1 cm) and used for somatic embryo formation.

The leaf segments were placed onto a somatic embryo formation (SEF) medium, consisting of the MS medium (Murashige and Skoog 1962) supplemented with 4 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 0.1 mg l^{-1} kinetin, 3% (w/v) sucrose (pH 5.8), and 0.2% Gelrite, and were cultured in the dark at 25°C. After 3 months, somatic embryos that formed directly on the surface of the segments were transferred into test tubes containing a hormone-free MS liquid medium

supplemented with 3% (w/v) sucrose. One milliliter of the liquid MS medium containing somatic embryos was transferred onto a plant regeneration (PR) medium consisting of the MS medium supplemented with 3% (w/v) sucrose, 0.5% Gelrite, and different concentration combinations of benzylaminopurine (BA), α-naphthaleneacetic acid (NAA), and gibberellin (GA_3). *A. tumefaciens* strain LBA4404 harbors the binary vector plasmid pIG121Hm, which contains selectable marker genes for hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (*npt* II). β-glucuronidase gene (GUS), which is controlled by the 35S promoter of the cauliflower mosaic virus, was utilized as the reporter gene. *Agrobacterium* strains were grown overnight at 28°C on a Luria broth (LB) liquid medium containing 50 mg l^{-1} kanamycin and 30 mg l^{-1} hygromycin (Hyg). The cells were collected by centrifugation and resuspended in a hormone-free one-third strength MS liquid medium. Somatic embryos were inoculated with an *Agrobacterium* suspension for 5 min prior to transfer to a solid MS medium containing 0.3% Gelrite and 4 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 3% (w/v) sucrose, 50 mg l^{-1} rifampicin, and 10 mg l^{-1} acetosyringone at 28°C for 7 days. After co-cultivation, the somatic embryos were transferred to a selection medium consisting of the MS medium supplemented with 4 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 50 mg l^{-1} rifampicin, 30 mg l^{-1} Hyg , 3% (w/v) sucrose (pH 5.8), and 0.2% Gelrite, and were cultured in the dark at 25°C. One month after selection, the developing secondary somatic embryos were transferred onto a new selection medium for 4 weeks for further selection of the transformed plants. The resulting developing secondary somatic embryos were transferred to a regeneration medium containing 5 mg l^{-1} Hyg. Following shoot and root induction on this medium, plants were transferred to a hormone-free one-third strength MS medium containing 0.3% Gelrite. The plantlets were transplanted into pots containing growing soil with vermiculite and perlite, and maintained under high humidity. Transgenic plants were grown in a growth chamber at 20°C with 16 h of light per day.

Total DNA was extracted from the leaf tissue of putative shoots using cetyltrimethylammonium bromide (CTAB) (Murray and Thompson 1980). Approximately 10 mg of leaf tissues were homogenized with 200 μl of CTAB buffer. Integration of *hpt* was confirmed by polymerase chain reaction (PCR) with the *hpt*-specific primers: 5′-ATGAAAAAGCCTGAACTCACCGCGA-3′ and 5′-TCCATCACAGTTTGCCAGTGATACA-3′. The amplification reaction was conducted by preheating at 94°C for 2 min: 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s.

After 4 weeks of culture, the leaf segments darkened in color and compact embryogenic calli appeared along the cut edges of the leaf segments on the SEF medium. White somatic embryos were observed on the embryogenic calli after 10–12 weeks of culture. Furthermore, numerous somatic embryos formed 16

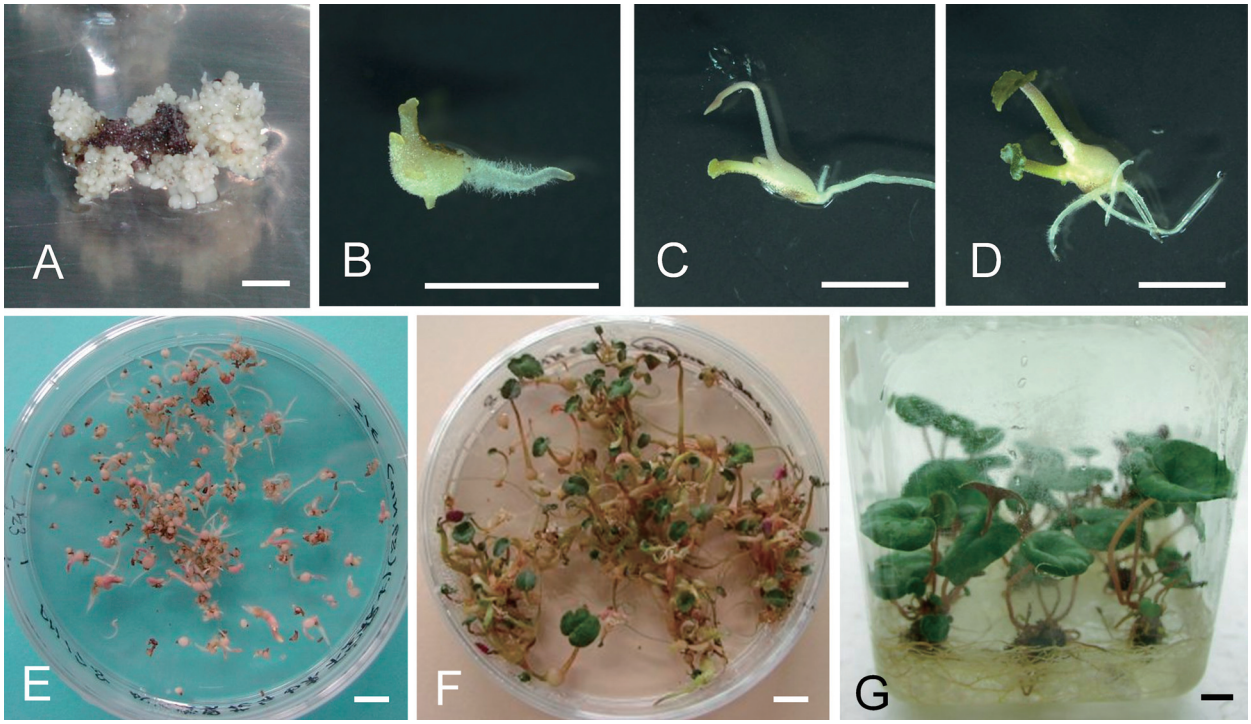

Figure 1. Induction and regeneration of somatic embryos. (A) Somatic embryos induced from leaf segment. Appearance of regenerated plants after (B) 14 days, (C) 30 days, and (D) 50 days. (E) Appearance of somatic embryos spread on the regeneration medium. (F) Typical appearance of regeneration from somatic embryos. (G) Appearance of plantlets in the culture box. Bar=(A–D), 5 mm; (E–G), 1 cm.

weeks after initiation (Figure 1A). In preliminary experiments, the highest frequency of embryogenesis was apparent on the SEF medium containing 4 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin (data not shown). A PR medium with and without GA_3 was used to establish a plantlet regeneration system. Table 1 shows the effects of BA and NAA in combination with GA_3 on regeneration from somatic embryos after 7 weeks of culture. The maximum regeneration frequency (86%) was achieved when cultured on a PR medium containing 0.01 mg l^{-1} NAA, 0.1 mg l^{-1} BA, and 0.2 mg l^{-1} GA_3. In the absence of GA_3, the frequency of plant regeneration was <10%. There was, however, no significant difference in the regeneration frequency due to the concentrations of the plant growth hormones BA and NAA. In this case, regeneration was not apparent on the hormone-free PR medium. Figure 1B, C, and D shows the various stages during the process of germination of the somatic embryos. After 2–3 weeks, somatic embryos formed a tuber-like tissue (Figure 1E). After 4–5 weeks of growth under fluorescent lighting (approximately 3,000 lux), the regenerated plantlets had normal growth of the shoots and normal formation of tubers (Figure 1F). After transfer to the hormone-free medium, plantlets displayed normal growth in the growth chamber (Figure 1G).

Table 1. Effect of plant growth regulators on plant regeneration of somatic embryos

Plant growth regulator (mg l^{-1})			No. of plants per 0.05ml PCV of somatic embryos*1
BA	NAA	GA_3	
0	0	0	0
0.1	0	0	5
0.1	0.01	0	1
0.1	0.1	0	4
0.1	0	0.2	39
0.1	0.01	0.2	82
0.1	0.1	0.2	58
1	0	0	0
1	0.01	0	0
1	0.1	0	3
1	0	0.2	38
1	0.01	0.2	40
1	0.1	0.2	42
0	0	0.2	38
0	0.01	0.2	31
1	0	0.2	38

*1 0.05 ml PCV contained about 100 somatic embryos were used for each medium.

Somatic embryos are not limited to the leaf segments and can be induced from the flower scaposes. Furthermore, it is possible to culture from aseptic scaposes of explants in the culture box. In this study, the rate of regeneration markedly improved using plant growth hormones, especially GA_3, for regeneration from the somatic embryos. Somatic embryos formed in leaf calli can be regenerated on a medium containing 1 mg l^{-1} NAA and 1 mg l^{-1} kinetin to develop tubers (Otani and Shimada 1991). Meanwhile, our result may reflect the stabilization of germination due to dormancy release in the somatic embryos.

The plasmid vector pIG121Hm, which harbors *hpt* and *npt*II as selective markers and *GUS* as a reporter gene, was introduced into the somatic embryos. Approximately 2,000 embryos were co-cultured with *Agrobacterium* strain LBA4404 utilizing a selection medium containing 5 mg l^{-1} Hyg and 300 mg l^{-1} carbenicillin, which was optimal for the selection of transformation. For the selection of transformants, 10 mg l^{-1} Hyg was very strong (data not shown). In two separate experiments, Hyg-resistant embryogenic clones that represented

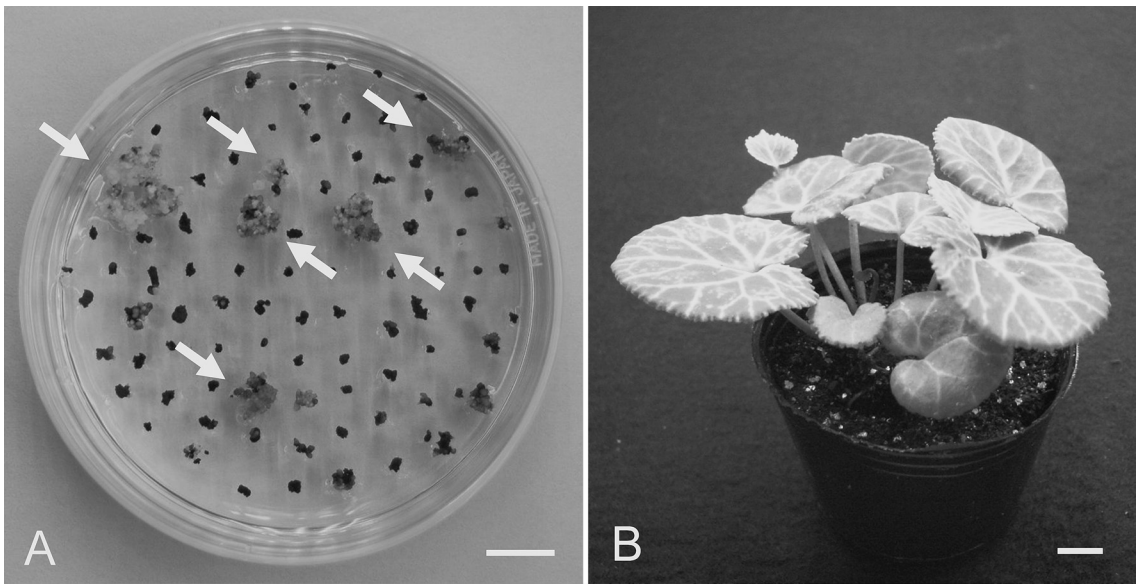

Figure 2. *Agrobacterium*-mediated transformation and regeneration of transgenic plants. (A) Hyg-resistant clones. (B) Transgenic plants. Arrows indicate Hyg-resistant clones. Bar=1 cm.

secondary embryos appeared on the selection medium after 10 weeks of selection (Figure 2A). Fourteen Hyg-resistant clones were selected (Table 2). All were regenerated by transfer to the regeneration medium containing BA, NAA, and GA_3. A total of 74 independent plants that were regenerated from 14 Hyg-resistant clones were examined using PCR. Thirty-seven plants were PCR-positive. The efficiency of transformation based on the number of transgenic hygromycin-resistant calli in 1-ml packed cell volume was about 0.7% when *A. tumefaciens* LBA4404 was used. All the regenerated plants grew normally and developed into intact plants (Figure 2B).

We found that somatic embryos succeeded in producing many transgenic Cyclamen plants using *A. tumefaciens* LBA4404 and a hygromycin-resistant selective marker gene. Previous studies utilized etiolated petioles of seedling as explants for transformation (Aida et al. 1999, Boase et al. 2002). Although etiolated petioles are available for producing transgenic Cyclamen, these seedlings were obtained following germination from seeds, which resulted in a heterogeneous population of seedlings. The character of resulting transformants may vary. To eliminate this heterogeneity, we decided to use somatic embryos introduced from a parent plant. Furthermore, somatic embryos allow treatment of large numbers of transformants per experiment. Using our method, 20 transformants can be obtained at the same time, because one leaf segment forms about 1,000 somatic embryos. However, some parent plants are unable to induce a somatic embryo. In such a case, it is suggested that the aseptic adventitious buds induced from an exclusive parent plant can be used. In another experiment not described presently, we successfully tried to generate Cyclamen transformants using aseptic adventitious buds (data not shown).

Table 2. Efficiency of transformation in Cyclamen

Exp.	No. of co-cultivated somatic embryos*1 (ml PCV)	No. of Hyg-resistant clones	No. of regeneration plant	No. of PCR (+) plant
1	0.5	3	27	11
2	0.5	11	47	26

*1 0.5 ml PCV contained about 1,000 somatic embryos.

In conclusion, we successfully established an effective method for *Agrobacterium*-mediated transformation of Cyclamen using somatic embryos (Figure 3). This method, which involves five steps, takes 25–27 weeks. Using this protocol, transformants are produced within 14–15 weeks after co-cultivation. This method may be useful as a genetic engineering technique for introducing a specific gene into an exclusive genotype and for obtaining many transformants at the same time. Finally, the procedure may greatly aid in efforts to improve phenotypic characteristics including flower color, morphology, and disease tolerance.

Acknowledgments

We thank Dr. Kenzo Nakamura, Nagoya University, for providing the plasmid pIG121Hm.

References

Aida R, Hirose Y, Kishimoto S, Shibata M (1999) *Agrobacterium tumefaciens*-mediated transformation of *Cyclamen persicum* Mill. *Plant Sci* 148: 1–7

Boase MR, Marshall GB, Peter TA, Bendall MJ (2002) Long-term expression of the *gus*A reporter gene in transgenic Cyclamen produced from etiolated hypocotyls explants. *Plant Cell Tiss Org Cult* 70: 27–39

Kiviharju E, Tuominen U, Tormala T (1992) The effect of explant material on somatic embryogenesis of *Cyclamen persicum* Mill. *Plant Cell Tiss Org Cult* 28: 605–612

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nuc Acids Res* 8: 4321–4325

Murashige T, Skoog E (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473–497

Otani M, Shimada T (1991) Somatic embryogenesis and plant regeneration from *Cyclamen persicum* Mill leaf cultures. *Plant Tissue Cult Lett* 8: 121–123

Takamura T, Miyajima I, Matsuo E (1995) Somatic embryogenesis of *Cyclamen persicum* Mill. 'Anneke' from aseptic seedlings. *Plant Cell Rep* 15: 22–25

Takamura T, Tanaka M (1996) Somatic embryogenesis from the etiolated petiole of Cyclamen (*Cyclamen persicum* Mill.). *Plant Tissue Cult Lett* 13: 43–48

Wicart GA, Mouras A, Lutz A (1984) Histological study of organogenesis and embryogenesis in *Cyclamen persicum* Mill. Tissue cultures: Evidence for a single organogenetic pattern. *Protoplasma* 119: 159–167

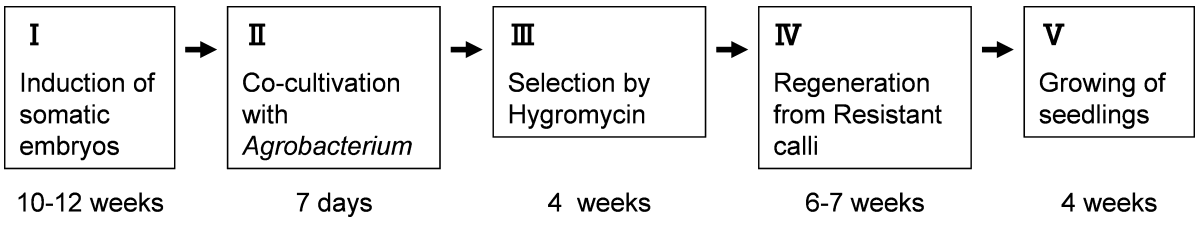

Figure 3. Protocol for *Agrobacterium*-mediated transformation of *Cyclamen persicum*.