

A Simple and Efficient Method for Somatic Embryogenesis and Plant Regeneration from Leaves of Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura]

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Abstract

A simple and efficient method for somatic embryogenesis of chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] was established. The best result of somatic embryogenesis was obtained with MS medium containing 2.0 mg l⁻¹ 2,4-dichlorophenoxyacetic acid and 1.0 mg l⁻¹ kinetin. Embryos with roots 1 to 2 mm long gave the highest frequency for conversion of embryos to plantlets. Root meristems appeared to be formed in somatic embryos by 20 days after culture initiation, while the shoot apices formed after replanting in phytohormone-free medium, resulting in a bipolar structure. The embryo-specific ECP63 gene was expressed in the somatic embryos, as in zygotic embryos, confirming that the plant regeneration occurred via embryogenesis. The growth habits and leaf and flower morphology of the regenerated plantlets were normal, but the day of flowering differed from that of cutting controls. The present somatic embryogenesis method was applicable to 8 out of 13 cultivars studied.

Key words: chrysanthemum, leaf segment, phytohormone, somatic embryo.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; ECP63, embryogenic cell protein 63; FAA, formalin acetic acid; GA₃, gibberellic acid A3; 2iP, 2-iso pentenyl adenine; Kin, kinetin; SEM, scanning electron microscope.

Introduction

Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] was introduced to Japan from China during the Nara Era (A.D. 710–794), and now it is one of the most important ornamental plants. Seedlings for commercial production are obtained by vegetative propagation of stem cuttings. *In vitro* propagation via adventitious shoot formation from various tissues and organs is also possible (Hill, 1968; Iizuka *et al.*, 1973; Earle and Langhans, 1974; Khalid *et al.*, 1989). Tissue culture methods provide means to eliminate various pathogens (Ahmed *et al.*, 1987; Horst, 1990) and to induce and

select desirable mutations (Broertjes *et al.*, 1976; Preil *et al.*, 1983; De Jong and Custers, 1986; Dalsou and Short, 1987; Huitema *et al.*, 1987).

Steward *et al.* (1958) and Reinert (1958) first recognized somatic embryogenesis in plants. They reported that somatic cells of carrot cultured *in vitro* formed somatic embryos. Somatic embryogenesis has since been extensively investigated as a model system for understanding the mechanism of zygotic embryogenesis, because the morphological changes in somatic embryos are similar to those in zygotic embryos. Somatic embryogenesis has been reported in several plant species, including chrysanthemum (Earle and Langhans, 1974; May and Trigiano, 1991; Pavingerova *et al.*, 1994), coffee (Nakamura *et al.*, 1994), potato (De Garcia and Martinez, 1995), melon (Tabei *et al.*, 1991), carnation (Frey *et al.*, 1992), alfalfa (Xu and Bewley, 1992), cucumber (Lou and Kako, 1995), and sunflower (Krasnyanski and Menczel, 1993). Somatic embryogenesis is an *in vitro* process for plant regeneration that is potentially a very efficient system for the clonal mass propagation of plants (Sharp *et al.*, 1982; Lutz *et al.*,

1984; Ammirato and Styer, 1985). In addition, somatic embryos are generally thought to arise from a single cell (Nomura and Komamine, 1985), so this technique should be useful for the dissolution of chimeras in mutation breeding and gene recombination breeding.

Somatic embryogenesis is induced by transferring somatic tissue cultured on a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) to 2,4-D-free medium. 2,4-D is a strong herbicide that is thought to be a stress agent in producing somatic embryogenesis (Kato and Takeuchi, 1966; Nomura and Komamine, 1985; Zimmerman, 1993). Alternatively, somatic embryos of carrot can also be induced by treating apical shoot tips with osmotic stress such as high concentrations of sucrose (Kamada *et al.*, 1989, 1993), NaCl (Kiyosue *et al.*, 1989) and heavy metal ions (Kiyosue *et al.*, 1990), or heat stress (Kamada *et al.*, 1994) in phytohormone-free medium. By these methods, somatic embryos are formed directly on the surface of plant segments, without visible callus formation, after the segments have been subcultured in phytohormone-free medium without these chemicals or at high temperature. Nomura and Komamine (1985) and Zimmerman (1993) suggested that 2,4-D would trigger the induction of somatic embryogenesis, which is not formed by the other chemicals or high temperature, by forming some suitable substance. This substance is believed to induce many physiological changes in segments before and after treatments that are not directly involved in somatic embryogenesis.

Somatic embryogenesis in chrysanthemum has been reported (Earle and Langhans, 1974; May and Trigiano, 1991; Pavingerova *et al.*, 1994). Earle and Langhans (1974) noted that 'embryoids' were formed in callus cultures of *Chrysanthemum morifolium* Ramat. (*Dendranthema × grandiflorum*), but did not present substantial evidence. The other two papers noted that a 'somatic embryo' was formed directly from leaf segments on MS basal medium containing 2,4-D and 6-benzylaminopurine (BAP), or 2,4-D, BAP, 2-iso pentenyl adenine (2iP), and gibberellic acid A3 (GA₃). Unfortunately, the frequency of plant regeneration from somatic embryos using those protocols is low and highly dependent on the genotype. Furthermore, the procedures are complicated and time-consuming, as they require several regular changes of the medium and in the culture conditions. In addition, detailed data warranting that plants regenerate from the somatic embryos are lacking.

This study developed a method for plant regeneration via somatic embryogenesis in chrysan-

themum that is simple, efficient and independent of genotype. We succeeded in establishing the method in the cv. Shuho-no-chikara and other major Japanese cultivars. We also confirmed that plants were regenerated from somatic embryos, based on histological observations and by the expression of an embryo-specific gene.

Materials and Methods

Plant materials

The chrysanthemum [*Dendranthema × grandiflorum* (Ramat.) Kitamura] cv. 'Shuho-no-chikara' and 12 other cultivars were used. Plants growing in a greenhouse were surface-sterilized in 70% ethanol, and then in 1% sodium hypochlorite solution for 15 min, before being rinsed three times with sterile distilled water. *In vitro* cultures were established from meristem cultures on Murashige and Skoog (1962) basal medium (MS medium) containing 3% sucrose and 0.3% Gellan Gum (Pure Chemical, Inc. Kyoto). The medium was adjusted to pH 5.8 before autoclaving at 121°C for 15 min. The *in vitro* culture was maintained at 25°C under a 16-h photoperiod using cool-white fluorescent lamps and 25°C darkness for 8 h. The lamps provided a photosynthetic photon flux [PPF (400–700 nm)] of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Optimizing the phytohormones for somatic embryogenesis

We used three phytohormones: 2,4-D, kinetin (Kin), and BAP. To optimize the conditions for somatic embryogenesis from leaves, combinations of these phytohormones were examined. The MS medium was supplemented with 1.0, 2.0, or 4.0 mg l⁻¹ 2,4-D in factorial combination with 0, 0.1, 0.5, 1.0, or 2.0 mg l⁻¹ Kin or BAP, and used to test for the formation of somatic embryo. One- to 1.2-cm long young leaves were excised from plants and cut into 3x3-mm segments, excluding the distal and basal portions of the lamina. Nine segments were placed with the abaxial surface in contact with the above mentioned medium, and the segments were then incubated under a 16-h photoperiod with cool-white fluorescent lamps that provided a photosynthetic photon flux [PPF (400–700 nm)] of 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$. We counted the number of somatic embryos in each segment. This procedure was repeated three times.

Plantlet regeneration from somatic embryos

Shoots at several stages were used; globular stage (root length 0 mm), oval stage (1 to 2 mm), early elongated root stage (3 to 6 mm), and late elongated

root stages (7 to 10, 11 to 15, 16 to 20, and over 20 mm). They were transferred to phytohormone-free MS medium, and the number of shoots that regenerated plantlets was counted.

Histological observation of somatic embryos

The segments with somatic embryos were fixed in formalin acetic acid (FAA), dehydrated in a graduated ethanol concentrations of 50, 70, 85, 95, and 100% by volume, and then transferred to n-butyl alcohol. The time of immersion in each solution was 1 h. After this process, they were embedded in paraffin (Merck Co. Germany). Serial of 10- μ m sections obtained with a JUNG RM2045 (Leica, Germany) were double-stained with Delafield's hematoxylin solution and eosin-acetic acid solution.

Scanning electron microscopy

Somatic embryos at different stages were fixed in FAA. Samples were dehydrated in graduated ethanol concentrations of 50, 60, 70, 80, 90, 99.5, and 100% by volume. The time of immersion in each ethanol solution was 1 h. Ethanol was then replaced by 3-methylbutyl acetate for 15 min for dehydration. This procedure was repeated twice. The solvent exchanged with liquid CO₂, and the samples were critical-point dried. Sections were coated with gold-palladium on a Quick Cool Coater SC-701MCY (Sanyu-Denshi, Japan) and examined using a JSM-5410LV scanning electron microscope (JOEL, Japan) (Xu and Bewley, 1992).

Northern blot analysis of ECP63 expression

In order to investigate whether the regeneration observed here was embryogenesis, a cDNA clone containing the full length of ECP63 gene isolated by Zhu *et al.* (1996) was used. This gene encodes embryogenic cell protein 63 (ECP63) of carrot (*Daucus carota* L.), which specifically exists in embryogenic cells and somatic embryos, and responds to ABA only in embryonic organs. Total RNA was extracted from 100 mg of fresh somatic embryos, leaves, petals, and zygotic embryos of cvs. Shuho-no-chikara and Yamate-shiro. The materials were homogenized in liquid nitrogen using a ceramic mortar and pestle, and extracted using a Qiagen RNeasy Plant Total RNA Kit (Qiagen, USA). Twenty micrograms of RNA were fractionated by formaldehyde gel electrophoresis through 0.8% agarose and ethidium bromide in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Equal loading was verified by the ethidium bromide-staining. The gel was then blotted onto a positively-charged nylon

membrane (Roche & Boehringer Mannheim, Germany). The pre-hybridization, hybridization, and detection conditions were made as described by Southern (1975). Total RNA was isolated from each shoot using the guanidinium thiocyanate protocol described previously (Newman *et al.*, 1993) using a 520-bp DIG-labeled cDNA (313 to 833 bp) that corresponds to ECP63 gene (Zhu *et al.*, 1996) provided by Prof. H. Kamada, University of Tsukuba.

Morphological observation of regenerated plants in a greenhouse

The regenerated plantlets were planted into a cell tray with vermiculite and covered with a plastic sheet for acclimation in the greenhouse at 25°C. After 2 weeks, the plantlets were repotted in soil and grown in the greenhouse. On flowering, about six months after acclimation, we evaluated stem length, number of leaves, diameter of flower and days to flowering.

Results

Optimizing the phytohormone combinations for somatic embryogenesis

The color of leaf segments turned yellow 2 to 3 days after culture initiation. A white to pale yellow primary callus was formed along the cut edges of the segments and the subepidermal parts by 5 to 7 days of the culture. Somatic embryogenesis was observed on the medium containing a combination of 2,4-D and BAP or Kin, but not on the medium with 2,4-D alone (**Table 1**). Kin appeared to be more effective than BAP. The number of somatic embryos increased linearly with the Kin concentration from 0.1 to 1.0 mg l⁻¹. Embryogenesis was inhibited at 2.0 mg l⁻¹ Kin. The highest yield of somatic embryos was obtained on the medium containing 2.0 mg l⁻¹ 2,4-D and 1.0 mg l⁻¹ Kin. This medium is designated hereafter D2K1 medium (see **Table 1**).

Observing somatic embryo and plantlet development

Globular somatic embryos were observed 14 to 21 days after initiation of the culture on D2K1 medium (**Fig. 1A-C**). At this stage, the root meristem and vascular bundles had already formed. When these somatic embryos were transferred to phytohormone-free MS medium (**Fig. 1D**), they formed shoot and root meristems, obtained morphological bipolarity after 10 days (**Fig. 1E**), and regenerated into plantlets after 20 days (**Fig. 1F**). Twenty-eight days after the initiation of culture on D2K1 medium, the roots of somatic embryos continued to grow (**Fig.**

Table 1 Effects phytohormone combinations on induction of somatic embryos in chrysanthemum cv. Shuho-no-chikara.

Medium code	Additional substances			Callus formation	No. of somatic embryo per leaf segment
	2,4-D (mg l ⁻¹)	BAP (mg l ⁻¹)	Kin (mg l ⁻¹)		
Control	0.0	0.0	0.0	-	-
D1	1.0	0.0	-	±	0.0 ± 0.0
D1B01	1.0	0.1	-	+	0.8 ± 1.2
D1B05	1.0	0.5	-	+	1.2 ± 2.1
D1B1	1.0	1.0	-	++	2.0 ± 1.6
D1B2	1.0	2.0	-	+	2.3 ± 1.3
D1K01	1.0	-	0.1	+	5.2 ± 0.2
D1K05	1.0	-	0.5	+	4.4 ± 0.4
D1K1	1.0	-	1.0	++	6.0 ± 0.6
D1K2	1.0	-	2.0	++	3.7 ± 0.4
D2	2.0	0.0	-	±	0.0 ± 0.0
D2B01	2.0	0.1	-	+	0.5 ± 1.2
D2B05	2.0	0.5	-	+	3.2 ± 2.2
D2B1	2.0	1.0	-	+	4.8 ± 1.6
D2B2	2.0	2.0	-	++	3.1 ± 1.7
D2K01	2.0	-	0.1	+	4.8 ± 0.5
D2K05	2.0	-	0.5	+	10.0 ± 1.2
D2K1	2.0	-	1.0	+	21.3 ± 1.2
D2K2	2.0	-	2.0	++	6.0 ± 1.8
D4	4.0	0.0	-	±	0.0 ± 0.0
D4B01	4.0	0.1	-	+	0.0 ± 0.0
D4B05	4.0	0.5	-	+	0.0 ± 0.0
D4B1	4.0	1.0	-	+	0.0 ± 0.0
D4B2	4.0	2.0	-	+	0.1 ± 0.6
D4K01	4.0	-	0.1	+	2.2 ± 0.5
D4K05	4.0	-	0.5	+	7.6 ± 0.6
D4K1	4.0	-	1.0	+	4.2 ± 0.9
D4K2	4.0	-	2.0	+	2.9 ± 1.8

Nine leaf segments were put onto each Petri dish.

Data represent mean ± SE of 27 samples.

1G). These shoots were easily detached from the segment with forceps, whereas adventitious shoots and roots had to be cut off with a scalpel. This difference was a good way to distinguish somatic embryos from adventitious shoots and roots. After being subcultured in phytohormone-free MS basal medium, shoots were formed on the apex of the somatic embryos and the roots were allowed to continue to grow (**Fig. 1H-L**). The somatic embryos developed into plantlets on D2K1 medium 40 to 50 days after culture initiation (**Fig. 1M, N**), but the frequency of plant regeneration was very low. Somatic embryos with roots of different lengths were transferred to phytohormone-free medium and the number of somatic embryos that developed into plantlets was counted (**Table 2**). Somatic embryos

at the oval stage (1 to 2 mm root length) had the highest frequency of plant regeneration. Conversely, the rate was very low for somatic embryos at the later root elongation stages (up to 7 mm) or those that were not transferred to phytohormone-free MS medium.

Northern blot analysis of ECP63

Total RNA was extracted from somatic embryos of cvs. 'Shuho-no-chikara', 'Monroe', 'Symbol', 'Kin-fusya', and 'Hiroshima-beni', and from other tissues, including leaves and petals of 'Shuho-no-chikara' and zygotic embryos of 'Shuho-no-chikara' x 'Yamate-shiro', and subjected to Northern blot analysis. With the specific 520-bp probe encoding ECP63 gene (**Fig. 2**), the somatic and zygotic

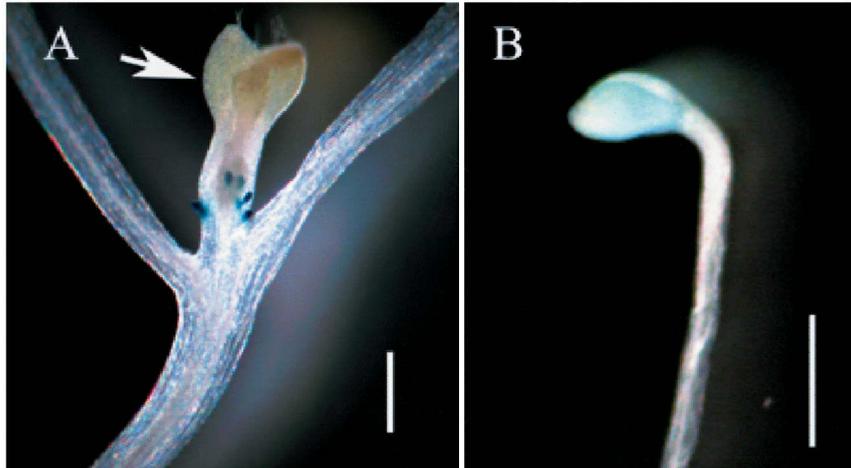


Fig. 4 Expression pattern of *PABI3-GUS* gene in *Arabidopsis* plant grown with sugar abundance (A) or depletion (B) condition.

Seedlings grown on vertical (Condition 1; sugar abundance) or oblique (Condition 2; sugar depletion) dishes containing 2% sucrose under dark condition for 14 days were examined. Arrow in panel (A), indicates a pair of true leaves, indicating “leaf development”. Appearance of the first pair was scored as positive leaf development. Stained spots in (A) are stipule primordia; compare GUS staining in the shoot apex and cotyledons in (B). Bar = 1 mm.

Table 2 Effects of developmental stages on the frequency of somatic embryos conversion to plant of chrysanthemum cv. Shuho-no-chikara.

Root length ¹⁾ (mm)	No. of somatic embryos	No. of regenerated plants	Frequency of conversion (%)
0 (globular)	50	18	36.0
1-2 (oval)	50	48	96.0
3-6	50	27	54.0
7-10	50	3	6.0
11-15	50	4	8.0
16-20	50	2	4.0
>20	50	1	2.0

¹⁾ Root length of somatic embryos at the time when transferred to phytohormone free MS medium.

When the somatic embryos were kept on the D2K1 medium without being transferred to phytohormone-free MS medium, the number of somatic embryos, that of regenerated plants and the frequency of conversion was 50, 1 and 2.0 (%), respectively.

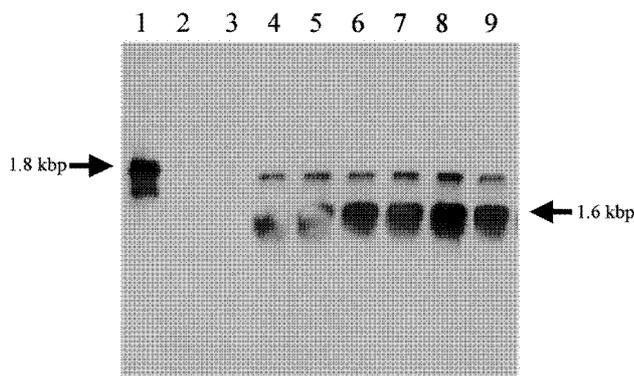


Fig. 2 Comparison of ECP63 genes expression in somatic embryos of chrysanthemum by Northern blot analysis. Total RNA was isolated from somatic embryos and other tissues, and approximately 20 μ g total RNA was applied in each lane. The probe was the 520 bp specific fragment of carrot embryogenic cell protein 63 (ECP63) gene.

Lane 1: pGEM-t easy-Carrot ECP63.

Lane 2: Leaves of 'Shuho-no-chikara'.

Lane 3: Petals of 'Shuho-no-chikara'.

Lane 4: Zygotic embryos of 'Shuho-no-chikara' x 'Yamate-shiro'.

Lane 5: Somatic embryos of 'Shuho-no-chikara'.

Lane 6: Somatic embryos of Monroe.

Lane 7: Somatic embryos of Symbol.

Lane 8: Somatic embryos of Kin-fusya.

Lane 9: Somatic embryos of Hiroshima-beni.

embryos produced two bands (1.6 and 1.8 kbp), indicating that the organs of interest were somatic embryos. By contrast, ECP63 gene expression was not observed in leaves or petals.

Growth habitat of regenerated plants in a greenhouse

Regenerated plants developed from somatic embryos of chrysanthemum were cultivated in a green-

house at 25°C to flowering (for up to 60 days). Then the stem length, number of leaves, diameter of the flowers and time to flowering were determined. Plants propagated by cutting, as reported elsewhere (May and Trigiano, 1991), were used as the control. The results are summarized in **Table 3**. The stem lengths of lines SE-3, 4, 14, 19, 20, and 24 from somatic embryos were significantly shorter than those of controls. The numbers of leaves and flower diameters of regenerated plants and controls were similar. The time to flowering of regenerated plants differed greatly from that of controls, being shorter than controls in lines SE-2, 6, and 8, and longer in lines SE-4, 14, and 24. The SE-1 and 7 were developed poorly and died before flowering.

Genetic influence on somatic embryogenesis

To verify the applicability of the established protocol (see **Fig. 3**) to other chrysanthemum cultivars, somatic embryogenesis was studied in 12 popular commercial cultivars and was observed in 7 out of the 12 (**Table 4**). ECP63 gene expression in the somatic embryos was tested at the oval stage by Northern blot analysis, and all of them expressed ECP63 gene (**Fig. 2**). From 0.7 to 72.0 somatic embryos were formed on a leaf segment, and two cultivars ('Monroe' and 'Kin-fusya') produced more embryos than 'Shuho-no-chikara' did. The highest frequency of conversion of embryos to plantlets was obtained in 'Kin-fusya'. No somatic embryos were obtained with 'Pinky', 'Bingo', 'Rosanna', 'Swan' and 'Peach'. Reason for this is still nuclear.

Table 3 The growth of regenerated plants via somatic embryogenesis of cv. Shuho-no-chikara in greenhouse

Lines	Stem length (cm)	No. of leaves	Diameter of flower (mm)	Days to flowering (days)
SE-1	-	-	-	-
SE-2	92.5 ± 0.3	42.1 ± 0.2	83.0 ± 0.2	64.0 ± 0.2 *
SE-3	68.0 ± 1.5 *	55.0 ± 0.1	85.0 ± 0.1	66.0 ± 0.1
SE-4	78.4 ± 1.4 *	44.9 ± 0.6	86.0 ± 0.6	88.0 ± 0.6 *
SE-5	82.7 ± 2.2	35.2 ± 0.3	79.0 ± 0.3	66.0 ± 0.3
SE-6	85.2 ± 3.1	36.2 ± 0.3	88.0 ± 0.3	64.0 ± 0.3 *
SE-7	-	-	-	-
SE-8	87.5 ± 0.9	38.5 ± 0.1	86.0 ± 0.1	62.0 ± 0.1 *
SE-9	90.4 ± 0.3	38.5 ± 0.6	87.0 ± 0.6	66.0 ± 0.6
SE-10	87.3 ± 2.2	38.1 ± 1.1	84.0 ± 1.1	69.0 ± 1.1
SE-11	94.0 ± 2.6	38.9 ± 1.3	86.0 ± 1.3	69.0 ± 1.3
SE-12	97.6 ± 1.6	37.3 ± 0.5	85.0 ± 0.5	66.0 ± 0.5
SE-13	86.5 ± 2.5	39.0 ± 0.5	88.0 ± 0.5	72.0 ± 0.5
SE-14	75.5 ± 1.4 *	48.0 ± 0.3	89.0 ± 0.3	85.0 ± 0.3 *
SE-15	80.8 ± 2.2	36.7 ± 0.6	90.0 ± 0.6	73.0 ± 0.6
SE-16	82.7 ± 1.6	36.9 ± 0.9	89.0 ± 0.9	74.0 ± 0.9
SE-17	85.1 ± 1.8	36.4 ± 0.9	86.0 ± 0.9	72.0 ± 0.9
SE-18	82.0 ± 0.6	34.0 ± 1.5	85.0 ± 1.5	68.0 ± 1.5
SE-19	79.5 ± 1.2 *	35.0 ± 1.3	89.0 ± 1.3	73.0 ± 1.3
SE-20	80.0 ± 2.2 *	35.5 ± 0.7	88.0 ± 0.7	80.0 ± 0.7
SE-21	83.2 ± 2.2	36.6 ± 0.4	87.0 ± 0.4	74.0 ± 0.4
SE-22	103.6 ± 0.6	40.3 ± 0.7	88.0 ± 0.7	65.0 ± 0.7
SE-23	81.0 ± 1.6	36.4 ± 0.2	89.0 ± 0.2	77.0 ± 0.2
SE-24	79.3 ± 1.3 *	46.0 ± 0.1	92.0 ± 0.1	95.0 ± 0.1 *
Cutting	99.3 ± 1.6	40.3 ± 0.9	91.0 ± 0.9	73.0 ± 0.9

SE; means somatic embryo

Data represent mean ± SE of 20 samples.

* Significant at the 5% level against the cutting of 'Shuho-no-chikara'.

Discussion

We established a method for somatic embryogenesis and plant regeneration from chrysanthemum that is simpler and more efficient than previous methods (May and Trigiano, 1991; Pavingerova *et al.*, 1994), and is somewhat independent of cultivar. As summarized in **Fig. 3**, our protocol requires only two subculturing steps, two kinds of media and a constant light condition for plantlet regeneration via somatic embryogenesis. In contrast, the protocol reported by May and Trigiano (1991) requires complicated procedures, such as changes in the sucrose concentration depending on the cultivar and altering the light/dark conditions for plantlet regeneration. Their protocol was applicable to 5 out of 23 cultivars, whereas our protocol was applicable to 8 out of 13 cultivars. The protocol of Pavingerova *et al.* (1994) requires both semi-solid and liquid media, and only 3 to 5 somatic embryos formed per

explant, whereas with our protocol yielded up to 72 embryos per explant. For the remaining 5 cultivars which did not form somatic embryos by our protocol, combination of phytohormones and/or other substances should be examined in the future.

In general, somatic embryos at the initial stage have a round shape. This is called 'the globular stage' or 'globular embryo' in somatic embryogenesis. The morphological polarity of the embryo is established when the round embryo becomes oval. Cotyledon initiation occurs at one end of the oval somatic embryo at the late globular stage, which is called 'the heart stage' or 'heart-shaped embryo'. At this stage, somatic embryos should have bipolar structures, with both shoot and root meristems. Then, the cotyledon elongates and the radicles are formed, which are called 'the torpedo stage' or 'torpedo-shaped embryo' (Xu and Bewley, 1992). In our case, the somatic embryos had a unique developmental process. The somatic embryos were round-shaped initially, and then

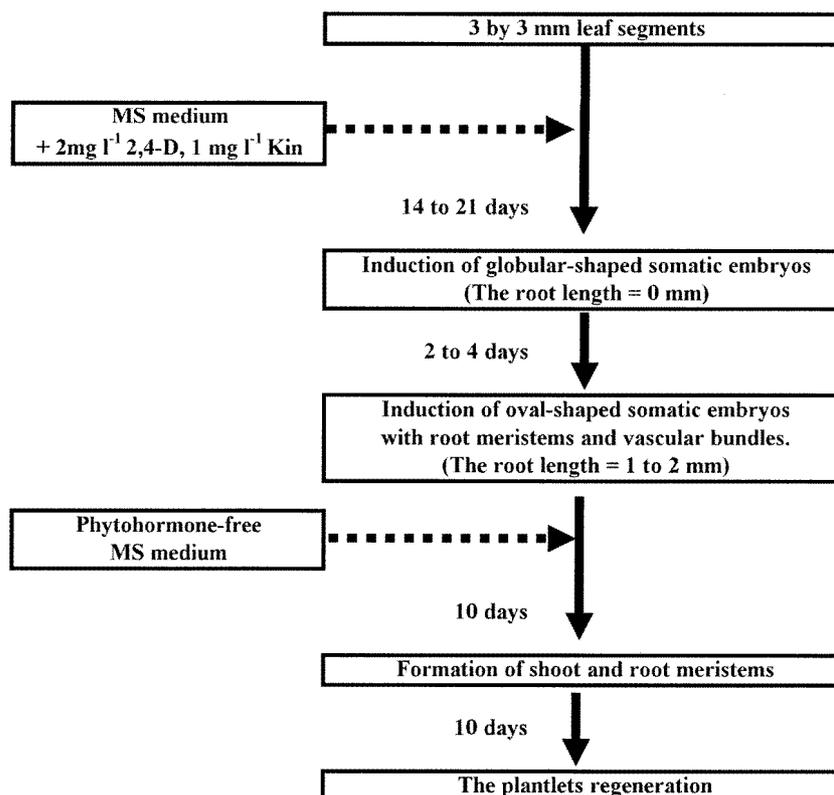


Fig. 3 Schematic illustration of the protocol for somatic embryogenesis of chrysanthemum from leaf segments.

Table 4 Effect of genotypes on somatic embryogenesis and plant regeneration in chrysanthemum.

Cultivars	No. of leaf segments ¹⁾	No. of somatic embryos	Frequency of somatic embryos per segment.	No of regenerated plants	Frequency of conversion (%)
Miss Betty	18	23	1.3	22	95.7
Monroe	18	649	36.1	640	98.6
Symbol	18	20	1.1	19	95.0
Yamate-shiro	18	51	2.8	48	94.1
Hiroshima-beni	18	63	3.5	57	90.5
Pinky	18	0	0.0	-	-
Bingo	18	0	0.0	-	-
Kin-fusya	18	1296	72.0	1280	98.8
Rocky	18	13	0.7	10	76.9
Rosanna	18	0	0.0	-	-
Swan	18	0	0.0	-	-
Peach	18	0	0.0	-	-
Shuho-no-chikara	18	381	21.2	371	97.4

1) Nine leaf segments were put onto each Petri dish.

roots formed from the apex of the globular somatic embryo. Shoot meristems formed on the somatic embryos after being transferred onto phytohormone-free MS medium. At this stage, our somatic embryos had bipolar structures. Northern blot analysis confirmed the expression of the embryo-specific protein (ECP63) gene in our somatic embryos, as in zygotic embryos, suggesting that the plantlet regen-

eration observed in our study was embryogenesis.

A low frequency of plant regeneration is the most serious problem in somatic embryogenesis (Hanning and Conger, 1986; Parrott *et al.*, 1989; Trigiano *et al.*, 1989; May and Trigiano, 1991; Pavingerova *et al.*, 1994). We examined the plant regeneration of somatic embryos at several developmental stages and discovered that the root length of the somatic

embryo before subculturing influences the subsequent frequency of plant regeneration. Oval somatic embryos with 1- to 2-mm roots regenerated significantly more frequent than those at other stages, and more frequently than previously reported values (May and Trigiano, 1991; Pavingerova *et al.*, 1994).

Somatic embryogenesis is highly dependent on genotype, as shown in other species (Hanning and Conger, 1986; Parrott *et al.*, 1989; Trigiano *et al.*, 1989). In previous studies on chrysanthemum (May and Trigiano, 1991), the ability to form somatic embryos from leaf segments was limited to about one-half of the cultivars evaluated, indicating genotype dependency. By contrast, our method was applicable to 8 out of 13 chrysanthemum cultivars, indicating lower genotype dependency. This method should be useful for recovering unique genotypes generated by mutation or genetic engineering techniques in chrysanthemum.

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