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–benzyaminopurine; ECP63, embryogenic cell pro‑
tein 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 (Krasynianski 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.,
We produced chimeras using papers formed 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 affected 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-isopentenyl 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 chrysanthemum 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 μmol m⁻² 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 μmol m⁻² 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 regen-
erated plantlets was counted.

**Histological observation of somatic embryos**

The segments with somatic embryos were fixed in
formalin acetic acid (FAA), dehydrated in a gradu-
ated 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-μm
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 etha-
nol 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 dehydra-
tion. This procedure was repeated twice. The sol-
vent 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 mate-
rials 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 frac-
tionated by formaldehyde gel electrophoresis
through 0.8% agarose and ethidium bromide in
MOPS buffer (20 mM MOPS, 5 mM sodium ace-
tate, 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, Ger-
many). 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℃.
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 concen-
tration 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 plant-
lets 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 of phytohormone combinations on induction of somatic embryos in chrysanthemum cv. Shuho-no-chikara.

<table>
<thead>
<tr>
<th>Medium code</th>
<th>Additional substances</th>
<th>Callus formation</th>
<th>No. of somatic embryo per leaf segment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.4-D (mg L⁻¹)</td>
<td>BAP (mg L⁻¹)</td>
<td>Kin (mg L⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>D1</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>D1B01</td>
<td>1.0</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>D1B05</td>
<td>1.0</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
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<td>1.0</td>
<td>1.0</td>
<td>-</td>
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<td>0.1</td>
</tr>
<tr>
<td>D1K05</td>
<td>1.0</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
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<td>-</td>
<td>1.0</td>
</tr>
<tr>
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<td>-</td>
<td>2.0</td>
</tr>
<tr>
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<td>0.0</td>
<td>-</td>
</tr>
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<td>0.1</td>
<td>-</td>
</tr>
<tr>
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<td>2.0</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
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<td>1.0</td>
<td>-</td>
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<td>0.1</td>
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<tr>
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<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>D2K1</td>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
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<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>D4</td>
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<td>0.0</td>
<td>-</td>
</tr>
<tr>
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<td>4.0</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>D4B05</td>
<td>4.0</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>D4B1</td>
<td>4.0</td>
<td>1.0</td>
<td>-</td>
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<td>0.1</td>
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<td>4.0</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
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<td>4.0</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>D4K2</td>
<td>4.0</td>
<td>-</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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 PAB3–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.

<table>
<thead>
<tr>
<th>Root length (mm)</th>
<th>No. of somatic embryos</th>
<th>No. of regenerated plants</th>
<th>Frequency of conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (globular)</td>
<td>50</td>
<td>18</td>
<td>36.0</td>
</tr>
<tr>
<td>1 - 2 (oval)</td>
<td>50</td>
<td>48</td>
<td>96.0</td>
</tr>
<tr>
<td>3 - 6</td>
<td>50</td>
<td>27</td>
<td>54.0</td>
</tr>
<tr>
<td>7 - 10</td>
<td>50</td>
<td>3</td>
<td>6.0</td>
</tr>
<tr>
<td>11 - 15</td>
<td>50</td>
<td>4</td>
<td>8.0</td>
</tr>
<tr>
<td>16 - 20</td>
<td>50</td>
<td>2</td>
<td>4.0</td>
</tr>
<tr>
<td>&gt;20</td>
<td>50</td>
<td>1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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 μg 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 greenhouse 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 else where (May and Trigiano, 1991), were used as the control. The results are summarized in Table 3. The stem lengths of 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 ‘Pink’, ‘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

<table>
<thead>
<tr>
<th>Lines</th>
<th>Stem length (cm)</th>
<th>No. of leaves</th>
<th>Diameter of flower (mm)</th>
<th>Days to flowering (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SE-2</td>
<td>92.5 ± 0.3</td>
<td>42.1 ± 0.2</td>
<td>83.0 ± 0.2</td>
<td>64.0 ± 0.2 *</td>
</tr>
<tr>
<td>SE-3</td>
<td>68.0 ± 1.5 *</td>
<td>55.0 ± 0.1</td>
<td>85.0 ± 0.1</td>
<td>66.0 ± 0.1</td>
</tr>
<tr>
<td>SE-4</td>
<td>78.4 ± 1.4 *</td>
<td>44.9 ± 0.6</td>
<td>86.0 ± 0.6</td>
<td>88.0 ± 0.6 *</td>
</tr>
<tr>
<td>SE-5</td>
<td>82.7 ± 2.2</td>
<td>35.2 ± 0.3</td>
<td>79.0 ± 0.3</td>
<td>66.0 ± 0.3</td>
</tr>
<tr>
<td>SE-6</td>
<td>85.2 ± 3.1</td>
<td>36.2 ± 0.3</td>
<td>88.0 ± 0.3</td>
<td>64.0 ± 0.3 *</td>
</tr>
<tr>
<td>SE-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SE-8</td>
<td>87.5 ± 0.9</td>
<td>38.5 ± 0.1</td>
<td>86.0 ± 0.1</td>
<td>62.0 ± 0.1 *</td>
</tr>
<tr>
<td>SE-9</td>
<td>90.4 ± 0.3</td>
<td>38.5 ± 0.6</td>
<td>87.0 ± 0.6</td>
<td>66.0 ± 06</td>
</tr>
<tr>
<td>SE-10</td>
<td>87.3 ± 2.2</td>
<td>38.1 ± 1.1</td>
<td>84.0 ± 1.1</td>
<td>69.0 ± 1.1</td>
</tr>
<tr>
<td>SE-11</td>
<td>94.0 ± 2.6</td>
<td>38.9 ± 1.3</td>
<td>86.0 ± 1.3</td>
<td>69.0 ± 1.3</td>
</tr>
<tr>
<td>SE-12</td>
<td>97.6 ± 1.6</td>
<td>37.3 ± 0.5</td>
<td>85.0 ± 0.5</td>
<td>66.0 ± 0.5</td>
</tr>
<tr>
<td>SE-13</td>
<td>86.5 ± 2.5</td>
<td>39.0 ± 0.5</td>
<td>88.0 ± 0.5</td>
<td>72.0 ± 0.5</td>
</tr>
<tr>
<td>SE-14</td>
<td>75.5 ± 1.4 *</td>
<td>48.0 ± 0.3</td>
<td>89.0 ± 0.3</td>
<td>85.0 ± 0.3 *</td>
</tr>
<tr>
<td>SE-15</td>
<td>80.8 ± 2.2</td>
<td>36.7 ± 0.6</td>
<td>90.0 ± 0.6</td>
<td>73.0 ± 0.6</td>
</tr>
<tr>
<td>SE-16</td>
<td>82.7 ± 1.6</td>
<td>36.9 ± 0.9</td>
<td>89.0 ± 0.9</td>
<td>74.0 ± 0.9</td>
</tr>
<tr>
<td>SE-17</td>
<td>85.1 ± 1.8</td>
<td>36.4 ± 0.9</td>
<td>86.0 ± 0.9</td>
<td>72.0 ± 0.9</td>
</tr>
<tr>
<td>SE-18</td>
<td>82.0 ± 0.6</td>
<td>34.0 ± 1.5</td>
<td>85.0 ± 1.5</td>
<td>68.0 ± 1.5</td>
</tr>
<tr>
<td>SE-19</td>
<td>79.5 ± 1.2 *</td>
<td>35.0 ± 1.3</td>
<td>89.0 ± 1.3</td>
<td>73.0 ± 1.3</td>
</tr>
<tr>
<td>SE-20</td>
<td>80.0 ± 2.2 *</td>
<td>35.5 ± 0.7</td>
<td>88.0 ± 0.7</td>
<td>80.0 ± 0.7</td>
</tr>
<tr>
<td>SE-21</td>
<td>83.2 ± 2.2</td>
<td>36.6 ± 0.4</td>
<td>87.0 ± 0.4</td>
<td>74.0 ± 0.4</td>
</tr>
<tr>
<td>SE-22</td>
<td>103.6 ± 0.6</td>
<td>40.3 ± 0.7</td>
<td>88.0 ± 0.7</td>
<td>65.0 ± 0.7</td>
</tr>
<tr>
<td>SE-23</td>
<td>81.0 ± 1.6</td>
<td>36.4 ± 0.2</td>
<td>89.0 ± 0.2</td>
<td>77.0 ± 0.2</td>
</tr>
<tr>
<td>SE-24</td>
<td>79.3 ± 1.3 *</td>
<td>46.0 ± 0.1</td>
<td>92.0 ± 0.1</td>
<td>95.0 ± 0.1 *</td>
</tr>
<tr>
<td>Cutting</td>
<td>99.3 ± 1.6</td>
<td>40.3 ± 0.9</td>
<td>91.0 ± 0.9</td>
<td>73.0 ± 0.9</td>
</tr>
</tbody>
</table>

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
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 regeneration observed in our study was embryogenesis.

A low frequency of plant regeneration is the most serious problem in somatic embryogenesis (Hann 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

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

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

1) Nine leaf segments were put onto each Petri dish.
embryo before subculturing influences the subsequent frequency of plant regeneration. Oval somatic embryos with 1–2 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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