

Effect of Urea-Type Cytokinins on the Adventitious Shoots Regeneration from Cotyledonary Node Explant in the Common Ice Plant, *Mesembryanthemum crystallinum*

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Abstract : *Mesembryanthemum crystallinum* (common ice plant) was used as a model plant to study the regulatory properties of Crassulacean acid metabolism (CAM) and tolerance to abiotic stresses. Although transformation is a useful genetic approach, it has not been established in this species due to recalcitrancy for regeneration. To establish an efficient procedure for regeneration of *M. crystallinum*, we examined the effects of urea-type cytokinins, thidiazuron (TDZ) and forchlorofenuron (CPPU) on the adventitious shoot induction. Adventitious shoots were generated only from explants obtained from the cotyledonary node, not from explants obtained from the cotyledon, hypocotyl and roots. Urea-type cytokinins, TDZ and CPPU were more effective for the induction and the morphogenesis of adventitious shoots than adenine-type cytokinin, 6-benzyladenopurine (BA). We have found that the 2.5 mg L⁻¹ TDZ induced the largest number of multiple shoots and the highest frequency of adventitious shoot induction from single explant. In addition, fewer hyperhydric shoots were produced on the medium containing TDZ than in that containing BA and CPPU in the presence of 1.0 mg L⁻¹ NAA. The regenerated shoots rooted on the MS medium within one month, and the rooting was promoted by replacing the agar medium with vermiculite or Florialite. The fertile plant with normal morphological properties was harvested for four months after sowing. Using the improved regeneration procedure with TDZ, we successfully introduced a kanamycin-resistant gene (*nptII-HPH*) into the cotyledonary node mediated by *Agrobacterium tumefaciens*. These results indicated that this regeneration procedure using cotyledonary node explants and TDZ could be useful for the genetic engineering of *M. crystallinum*.

Key words : Cotyledonary node, Hyperhydricity, *Mesembryanthemum crystallinum* L., Organogenesis, Thidiazuron.

Mesembryanthemum crystallinum L. (the common ice plant, Aizoaceae, Caryophyllales), a facultative halophyte, is widely used as a model plant to study the response to environmental stresses under aspects of genetics, physiology and biochemistry (Adams et al., 1998; Bohnert and Cushman, 2000). This species arose initially from the discovery of its ability to shift from C₃ photosynthesis to Crassulacean acid metabolism (CAM) when exposed to salinity, drought, and temperature extremes (Bohnert and Cushman, 2000). Many studies on CAM and mechanism of stress tolerance including signaling events (Taybi and Cushman, 1999; Taybi and Cushman, 2002), circadian regulation of CAM (Taybi et al., 2000; Dodd et al., 2003; Boxall et al., 2005), and scavenging system of reactive oxygen species (Ślesak et al., 2002) have been investigated in this species. More recently more than 15,000 expressed sequence tags (ESTs) have been sequenced and large-scale cDNA microarray analysis of the expression profiles of genes

that respond to salinity stress is currently in progress using the EST clones (Agarie et al. unpublished). However, despite the importance of the model stress plant, the procedure for production of transgenic ice plant, which is essential for functional genomics, has not been established.

Early attempts to transform the ice plant such as transformation of roots with *Agrobacterium rhizogenes* (Andolfatto et al., 1994) and transformation of calli with *Agrobacterium tumefaciens* (Ishimaru, 1999) were not successful. One of the main reasons is that the ice plant is recalcitrant for *in vitro* regeneration.

The efficiency of regeneration ice plant shoots using these procedures was poor (Meiners et al., 1991; Abou-Mandour, 1992; Wang and Lüttge, 1994; Cushman et al., 2000). A regeneration protocol for establishment with hypocotyl-derived calli was recently reported (Cushman et al., 2000). However, the regeneration system via callus induction has proven to be a multi-

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Abbreviations: BA, 6-benzylaminopurine; CAM, crassulacean acid metabolism; CPPU, *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (Forchlorofenuron); IAA, indole-3-acetic acid; KN, kinetin; MS, murashige and skoog media; NAA, α -naphthalen acetic acid; PGRs, plant growth regulators; TDZ, *N*-phenyl-*N'*-(1, 2, 3-thidiazol-5-yl) urea (Thidiazuron); ZT, zeatin; 2,4-D, 2,4-dichlorophenoxyacetic acid.

step procedure, which requires a long time interval for the development of whole plants and there is a risk of somaclonal variation when the procedure involves a callus phase (Larkin and Scowcroft, 1981). Thus the procedure via a direct organogenetic pathway, which may possess less somaclonal variation, is more appropriate for regeneration of *Agrobacterium*-mediated transformation in the ice plant.

Direct organogenesis from different types of explants including the hypocotyl, cotyledon and cotyledonary node has been described in the ice plant (Meiners et al., 1991). In the study, the effects of type and combination of plant growth regulators on the direct organogenesis have also been investigated (Meiners et al., 1991), but their frequency of the regeneration of adventitious shoot was low ranging from 0.5% to 41%.

Recently, some studies showed that synthetic phenylurea derivatives such as *N*-phenyl-*N'*-(1, 2, 3-thidiazol-5-yl) urea (TDZ) and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU) have higher cytokinin activity to induce adventitious shoots than adenine derivatives such as kinetin and 6-benzylaminopurine (BA) (Mok et al., 1987; Huetteman and Preece, 1993; Kaneda et al., 1997). It has been shown that TDZ provided an efficient stimulus for the induction of *in vitro* shoot regeneration in several plant species (Liu et al., 1994; Murthy et al., 1998; Uchida et al., 2003; Ipekci and Gozukirmizi, 2004; Gu and Zhang, 2005), and CPPU was effective for the induction of embryonic callus in grape (*Vitis labruscana*) (Nakajima et al., 2000) and *Citrus* (Fiore et al., 2002), somatic embryogenesis in peanut seedlings (Murthy and Saxena, 1994) and golden pothos [*Epipremnum aureum* (Linden & Andre) Bunt.] (Zhang et al., 2005) and the shoot formation in raspberry (*Rubus idaeus* subsp. *vulgatus* Arrhen.) (Millan-Mendoza and Graham, 1999) and lavender (*Lavandula vera* DC) (Tsuro et al., 1999).

The objectives of the present research were: (1) to examine the efficiency of explant type for regeneration, (2) to compare morphogenetic response of the ice plant to adenine with that to and urea-type cytokinin and (3) to introduce a foreign gene using the regeneration methods and *Agrobacterium tumefaciens*.

Material and methods

1. Plant materials

Mesembryanthemum crystallinum L. seeds were obtained from the wild-type ice plant that had been grown in a greenhouse. The seeds were sterilized in 2.0 % sodium hypochlorite solution for 6 min. After sterilization, seeds were put onto germination medium (pH 5.7) containing 4.6 g L⁻¹ MS salts (Murashige and Skoog, 1962), 1 x B-5 vitamins (Gamborg et al., 1968), 3.0% sucrose and 0.8% agar. Seedlings were grown in a growth chamber at 25°C with 16h/8h (light/dark) photoperiod under cool-white fluorescent light (70–80 μmol m⁻² s⁻¹).

2. Plant regeneration procedure

Cotyledonary node explants were dissected under stereomicroscope aseptically from 5 to 7 days old *in vitro* seedlings. Similarly, cotyledon, hypocotyl and root explants were cut into small pieces (2–3 mm). The excised explants were placed on solid medium containing MS basal medium supplemented with 3.0% sucrose and 0.8% agar and various concentrations of plant growth regulators. The medium for the culture of hypocotyl contained 0.2 or 2.0 mg L⁻¹ 6-benzylaminopurine (BA); 0.5, 5.0 or 10 mg L⁻¹ kinetin (KT); 0.5, 1.0 or 5.0 mg L⁻¹ zeatin (ZT); 0.005, 0.5, 1.0, 2.0 or 4.0 mg L⁻¹ forchlorofenuron (CPPU) or 1.0, 2.5 or 5.0 mg L⁻¹ thidiazuron (TDZ). The medium for the cotyledonary node culture contained with α -naphthalene acetic acid (NAA) (0, 0.1 or 1.0 mg L⁻¹) plus BA (0, 0.5, 1.0, 2.5, or 5.0 mg L⁻¹), CPPU (0, 0.5, 1.0, 2.5, or 5.0 mg L⁻¹) or TDZ (0, 0.5, 1.0, 2.5 or 5.0 mg L⁻¹). Explants were subcultured on fresh MS medium containing 0.5 mg L⁻¹ BA every 10–14 days in 15 × 100 mm plastic disposal Petri dishes sealed with surgical tapes. The Scheffe's test with Stat View v.5 (SAS Institute Inc.) was used for data analysis.

Elongated shoot were excised from explants and cultured on rooting medium consisted of MS medium without plant growth regulators (PGRs). Rooted plants were transferred to plant box (60 mm × 60 mm × 100 mm) filled with vermiculite or Florialite (a mixture of vermiculite and cellulose fibre; Nissinbo Industries, Inc.) containing the solution of MS salts. Plants were then transferred to plastic boxes (200 mm × 350 mm × 100 mm) covered with plastic cling film under the same conditions as described above. The regenerated plantlets were acclimatized by transferring to the decreased humidity condition in the green house for a week.

3. *Agrobacterium* and plasmid

Agrobacterium tumefaciens strain EHA 101 harboring a binary vector pBI was used. The binary vector contained genes for a neomycin phosphotransferase (*nptII*), an enhanced green fluorescent protein (*EGFP*) and a hygromycin phosphotransferase gene (*HPH*) in the T-DNA region. *nptII*, *EGFP* or *HPH* were driven by NOS, CaMV35S or CaMV35S promoter, respectively and all of them were terminated by NOS terminator. *Agrobacterium* was grown in YEP (10 g L⁻¹ Bacto peptone, 10 g L⁻¹ Bacto yeast extract, 5.0 g L⁻¹ NaCl, 15 g L⁻¹ agar; pH 5.2) with 50 mg L⁻¹ kanamycin and 50 mg L⁻¹ hygromycin. Then *Agrobacterium* cells were collected by centrifugation for 5 min at 20°C at 12,000 g and then resuspended in 30 mL of MS liquid medium containing 1.0 mg L⁻¹ TDZ, 0.25 mg L⁻¹ NAA, and 10 mg L⁻¹ acetosyringone (OD₆₀₀ = 0.2, pH 5.2) for transformation as described below.

Table 1. Morphogenetic responses of hypocotyl culture of *M. crystallinum* on MS medium supplemented with different concentrations of cytokinins.

Cytokinin type	Concentration (mg L ⁻¹)	Number of explants	Number of shoots	Callus response of the explants ^a	Callus color ^b
BA	0.2	50	0	++	W, Y, or R
	2	50	0	++	W, Y, or R
KT	0.5	50	0	++	W or Y
	5	50	0	++	W or Y
	10	50	0	++	W or Y
ZT	0.5	50	0	++	W or Y
	1	50	0	++	W or Y
	5	50	0	++	W or Y
CPPU	0	200	0	++++	W or SG
	0.005	200	1	++++	W or SG
	0.5	200	0	++++	W or SG
	1	200	1	++++	W or SG
	2	200	2	++++	W or SG
	4	200	0	++++	W or SG
TDZ	0	50	0	+++	W, SG, or G
	1	50	0	+++	W, SG, or G
	2.5	50	0	+++	W, SG, or G
	5	50	0	+++	W, SG, or G

^a Observations after 20 days in culture: ++, callus on the edges; +++, developed callus; +++++, well developed callus.

^b Callus color: G, green; R, red; SG, slightly green; Y, yellow; W, white.

4. Transformation of the ice plant

The cotyledonary node explants were sonicated for 2 sec in 1.5 mL tube in an ultrasonic bath and then incubated with the *Agrobacterium* cells in MS liquid medium prepared as described above for 5 min at 25°C. The cotyledonary node explants had been precultured on MS medium containing 1.0 mg L⁻¹ TDZ, 0.25 mg L⁻¹ NAA, 30 g L⁻¹ sucrose and 8.0 g L⁻¹ agar and incubated at 25°C under a 16-h photoperiod at 70–80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided from cool white fluorescent lamps for 5 days. They were then transferred to the co-culture medium containing 10 mg L⁻¹ acetosyringone and 8.0 g L⁻¹ agar for 5 days in the dark at 25°C. To remove *Agrobacterium*, we transferred the infected cotyledonary node explants to MS medium containing with 1.0 mg L⁻¹ TDZ, 0.25 mg L⁻¹ NAA, 100 mg L⁻¹ calbenycilin, 300 mg L⁻¹ vancomycin and 8.0 g L⁻¹ agar, and cultured for 3 days at 25°C under a 16 h photoperiod at 70–80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided from cool white fluorescent lamps.

5. Selection and regeneration of transformed plants

After co-culturing with the *Agrobacterium* cells

harboring pBI, the cotyledonary node explants were transferred to shoot induction medium containing 1.0 mg L⁻¹ TDZ, 0.25 mg L⁻¹ NAA, 500 mg L⁻¹ carbenicillin, 300 mg L⁻¹ vancomycin, 20 mg L⁻¹ hygromycin and 8.0 g L⁻¹ agar, and the cultured for 4 week at 25°C under a 16 h photoperiod at 70–80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided from cool white fluorescent lamps. Thereafter, they were incubated on shoot induction medium containing 1.0 or 2.5 mg L⁻¹ TDZ plus 0.1, 0.25 or 0.5 mg L⁻¹ NAA and 8.0 g L⁻¹ agar for 3–4 week.

6. DNA extraction and polymerase chain reaction analyses

Genomic DNA was extracted from fresh leaf tissue of putative transformants (T₀) according to the cetyltrimethylammoniumbromide (CTAB) method (Murray and Thompson, 1980), and the presence of the *nptII-HPH* gene was confirmed using PCR. The PCR analysis was carried out in 1 x PCR buffer, 0.2 mM dNTP (Takara ExTaqTM), 0.05 U mL⁻¹ Taq polymerase (Takara ExTaqTM), DNA (4.5 ng) and oligonucleotid primers to a final concentration of 0.5 μM . To amplify the *nptII-HPH* gene, (834 bp), we used the following

Table 2. Effect of BA on morphogenetic response of various explants in *M. crystallinum*^a.

Organ type	Explants forming shoots (%) ^b	Number of buds developing into shoots per explants ^b	Callus response of the explants ^c
Cotyledon	0	0	++
Cotyledonary node	59.7 ± 6.97	2.03 ± 0.21	-
Hypocotyl	0	0	++
Root	0	0	++

^a Culture medium = MS + BA (2.5 mg L⁻¹). Data are means of five replications with 10 explants per petri dish after 2 weeks of culture.

^b Means number of shoots ± SE.

^c Callus response: ++, callus on the edges; -, no callus.

primer pair: forward primer 5'-GTG GAG AGG CTA TTC GGC TAT GAC TGG GCA-3' and reverse primer 5'-TCA TAG AAG GCG GCG GTG GAA TCG AAA TCT-3'. Reaction started with denaturing at 94°C for 30 sec, 63.7°C for 30 min, 72°C for 1 min. The program was terminated by an extension at 72°C for 7 min. The amplification products were analyzed by electrophoresis on 1.2% agarose-ethidium bromide gels (0.07% ethidium bromide) with KODAK 1D image analysis software.

Results

1. Shoot induction from hypocotyl explant culture

Hypocotyl explants have previously been used to induce shoots via somatic embryogenesis of ice plant (Wang and Lüttge, 1994; Cushman et al., 2000), and we first examined the effects of urea-type cytokinins on somatic embryogenesis in hypocotyl explants (Table 1). Callus was produced in the medium, but embryos were not. Calli showed different color depending on the type of cytokinin. For example, green callus and slightly green callus were obtained only in the medium with TDZ and CPPU, respectively. Red callus was obtained only in the medium with BA. Callus formed more easily in the medium with the urea-type cytokinins, TDZ and CPPU than with BA, ZT and KN. However, calli did not produce somatic embryos or organs even after culture for 6 months (Table 1). Only in the medium with CPPU (0.005, 1.0 and 2.0 mg L⁻¹), small shoots formed directly at the cut ends of the hypocotyl explant after 1 week of the culture. However, the regeneration frequency was very low.

2. Influence of explant type on morphogenetic responses

To explore the possibility of generating large numbers of shoots in more defined manner, we inoculated different types of explants derived from the seedlings, which had been grown *in vitro* for 7 days, on the media supplemented with 2.5 mg L⁻¹ BA (Table 2). The frequency of shoot formation was clearly influenced by the type of the explant tissue. After 1 week of culture, all explants derived from hypocotyl,

cotyledon and root formed friable calli. These calli did not induce adventitious shoots. However, in the cotyledonary node explants, the frequency of direct shoot organogenesis was high (59.7%), indicating that the cotyledonary node should be the optimal explant for regeneration of the ice plant.

3. Influence of the type of cytokinins on direct shoot organogenesis from cotyledonary node explants

The effect of TDZ and CPPU on the regeneration frequency of the explants derived from cotyledonary nodes, the effect of the combination of plant growth regulators (NAA plus TDZ, CPPU or BA) on the regeneration frequency was investigated (Fig.1). In the absence of plant growth regulators in the medium, a single shoot (definite bud) was formed from cotyledonary node explants. In the present study, explants, which originated more than two shoots were counted as the regenerated plant. The rate of adventitious shoot formation was more than 60% on the media containing BA except the medium containing 5.0 mg L⁻¹ BA and 1.0 mg L⁻¹ NAA. On the medium containing 1.0, 2.5 or 5.0 mg L⁻¹ CPPU without NAA, adventitious shoot formation rate was similar to that on the medium containing BA but addition of NAA decreased the rate except the medium containing 2.5 mg L⁻¹ CPPU plus 0.1 mg L⁻¹ NAA. The highest rate of adventitious shoot formation was 85.7% on the medium containing 2.5 mg L⁻¹ TDZ, but the rate was decreased by the addition of 1.0 mg L⁻¹ NAA though not as great as in the case of medium containing CPPU (Fig. 1).

Fig. 2 shows that average number of regenerated shoots from the cotyledonary node cultured on MS medium supplemented with BA, CPPU or TDZ together with or without NAA. All cotyledonary node explants produced more than two adventitious shoots. Especially, on the medium containing 0.5 mg L⁻¹ TDZ, 2.5 mg L⁻¹ TDZ or 5.0 mg L⁻¹ TDZ plus 0.1 mg L⁻¹ NAA, all cotyledonary node explants produced more than four adventitious shoots. NAA in combination with CPPU or TDZ tended to reduce shoot regeneration from the cotyledonary node.

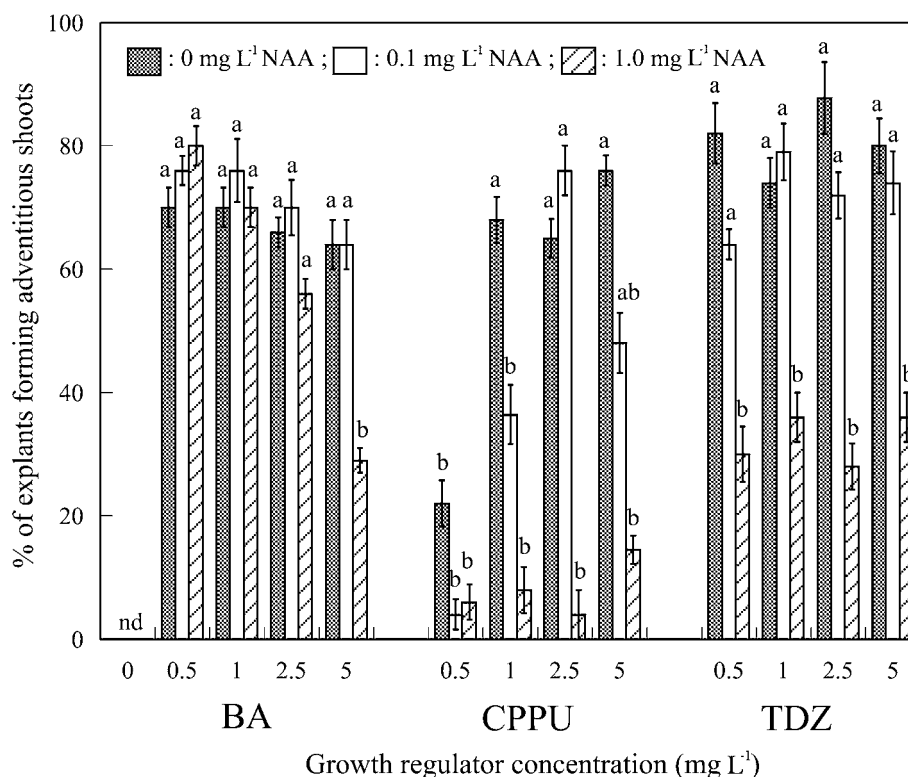


Fig. 1. Effect of the combinations of NAA and BA, CPPU or TDZ on frequencies of adventitious shoot initiation on cotyledonary node explants from *M. crystallinum*. Results were scored after 10 days of culture. nd: not detected. Values are means of five replications with 10 explants per petri dish \pm standard error. Bars with different letters are significantly different at the 5 % level by Scheffe's test.

4. Effects of auxin and cytokinin on hyperhydric shoot from cotyledonary node explants

Fig. 3 shows the effects of the combination of auxin (NAA) and cytokinins (BA, CPPU, or TDZ) on the formation of hyperhydric adventitious shoots from cotyledonary node explants. On the medium containing BA together with 0.1 mg L⁻¹ NAA, the frequency of hyperhydric shoots was relatively low ranging from 3 to 35%, but it was significantly increased by the addition of 1.0 mg L⁻¹ NAA. The effect of explants with CPPU treatment showed similar response with those of BA. On the medium with CPPU alone the frequency was lower than that on the medium containing BA plus 0.1 mg L⁻¹ NAA, but on the medium including 1.0 mg L⁻¹ NAA plus 1.0 or 5.0 mg L⁻¹ CPPU, the number of hyperhydric shoot was higher than that in the media containing BA and 1.0 mg L⁻¹ NAA. On the other hand, on the medium containing TDZ, the highest frequency of hyperhydric shoots was shown in the presence of 0 or 0.1 mg L⁻¹ NAA and the lowest frequency in the presence of 1.0 mg L⁻¹ NAA. Overall, the frequency of hyperhydricity on the medium containing TDZ was lower than that in the medium with the other cytokinins. The growth evaluated by fresh weight of multiple shoots was also higher on the medium with the TDZ than that on

the medium with BA and CPPU, and the shape of shoots was healthy on the medium with TDZ (data not shown).

Regenerated shoots were separated and subcultured on MS medium without plant growth regulators to induce roots. Roots were formed at the base of excised shoots in 2 weeks (Fig. 4). Fertile plants were obtained four months after sowing.

5. Transformation of ice plant

The 6-day-old cotyledonary node explants were co-cultured with an *Agrobacterium* culture for 3 days, and the explant was transferred onto the medium for removal of *Agrobacterium* and antibiotic selection. At 4 weeks after the selection, the introduced *nptII-HPH* gene was confirmed by PCR (Fig. 5). Genomic DNA was isolated from young shoots of regenerants grown on the selection medium containing kanamycin and hygromycin for 4 weeks. Amplified DNA band for *nptII-HPH* of expected length (834 bp) have been detected only in the genomic DNA isolated from the transformed plant (Fig. 5). The ratio of survived plants cultured on the medium with antibiotic was 8.4% (25 per 299 plants) and 6.4% (19 per 299 plants) at the 1 and 4 weeks after the selection, respectively. Among the selection medium containing 1.0 or 2.5 mg L⁻¹

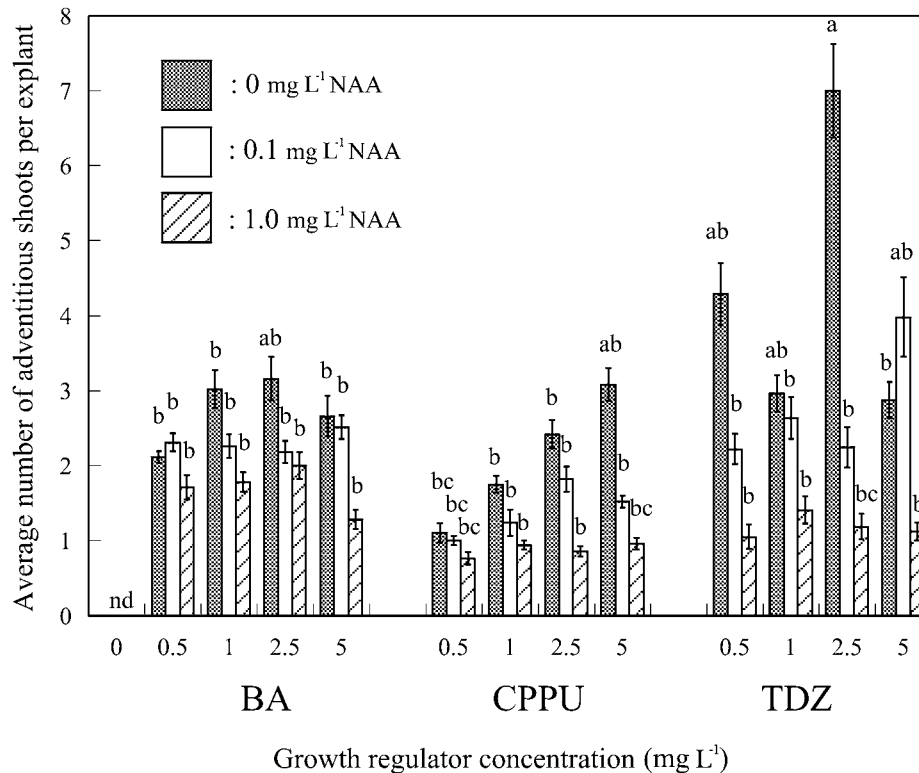


Fig. 2. Effect of the combinations of NAA and BA, CPPU or TDZ on adventitious shoot formation in *M. crystallinum*. nd: not detected. Results were scored after 10 days of culture. Values are means of five replications with 10 explants per petri dish \pm standard error. Bars with different letters are significantly different at the 5% level by Scheffe's test.

TDZ plus 0.1, 0.25 or 0.5 mg L⁻¹ NAA, the transgenic shoots were obtained only in the medium including 1.0 mg L⁻¹ TDZ and 0.25 mg L⁻¹ NAA. The transformation efficiency of ice plant from cotyledonary node explants was 0.3% (1 per 299 plants).

Discussion

The frequency of shoot induction in the present study was greater than in other studies, in which the shoots were developed through somatic embryogenesis (Abou-Mandour, 1992; Wang and Lüttge, 1994; Cushman et al., 2000) and organogenesis (Meiners et al., 1991) in the ice plant. Cushman et al. (2000) showed an improved procedure for the embryogenesis of the ice plant, but only 0.5-1.0% of the explants produced embryogenic calli. Meiners et al. (1991) have initially used hypocotyls as an explant for organogenesis in the ice plant, and obtained a relatively high efficiency of regeneration (23-34%). In our study, however, the frequency of regeneration in the organogenesis from hypocotyl explant was very low (Table 1 and 2). Although the hypocotyl has been used as an explant to induce callus in many herbaceous and woody species, it showed additional callus formation and subsequent shoot regeneration seldom occurred.

The cotyledonary node was the most appropriate

explant for regeneration in the ice plant, and a maximum regeneration frequency was 85.7% (Fig. 1). Meiners et al. (1991) also demonstrated multiple shoot formation on cotyledonary nodes, but the frequency of the regenerated plants (21-41%) was similar to that on hypocotyls (23-34%). The high regeneration frequency in the present study was probably due to the use of the cotyledonary node, which had been excised precisely under a stereomicroscope. The axillary meristems at the junction of the cotyledon and the embryo axes contain many active cells that are competent for developing new organs. The cotyledonary node should be a useful target for gene delivery in the ice plant as in many recalcitrant regeneration species such as legumes (Chandra and Pental, 2003).

We could obtain adventitious shoots in a short period. In our procedure, regenerated plants were obtained within four months, which was more rapid than embryogenesis via a callus system described in elsewhere (Wang and Lüttge, 1994; Cushman et al., 2000). The first adventitious shoot formation from cotyledonary node explants was observed within 3-5 days and another shoots began to form quickly.

We have found that the urea-type cytokinin such as TDZ and CPPU were more effective than adenine

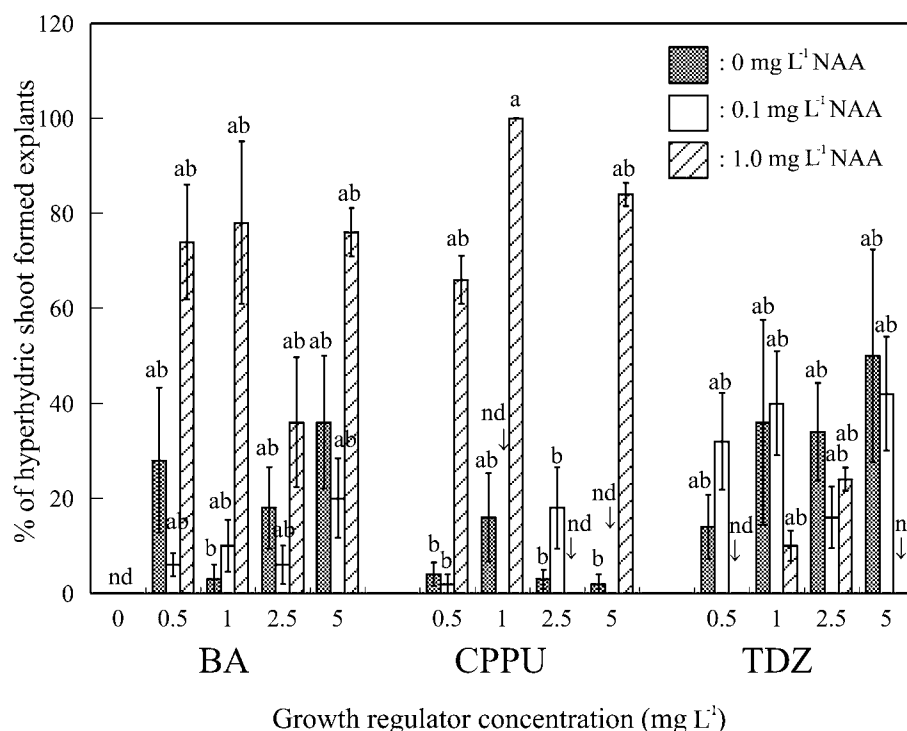


Fig. 3. Effect of the combination of NAA and BA, CPPU or TDZ on hyperhydric shoots derived from cotyledonary node explants of *M. crystallinum*. Results were scored after 10 days of culture. Values are means of five replications with 10 explants per petri dish \pm standard error. Bars with letters are significantly at the 5% level by Scheffe's test. nd: not detected.

type cytokinin (BA) for the induction of adventitious shoots on the cotyledonary node in the ice plant (Fig. 1). Especially, TDZ was more effective than CPPU for shoot formation and development of healthy adventitious shoots. TDZ induced more adventitious shoots than BA, especially at the concentration of 2.5 mg L⁻¹ in the medium. The urea-type cytokinin has higher biological activity than adenine-type cytokinins (BA and KN) in many plant species (Mok et al., 1987; Huetteman and Preece, 1993; Kaneda et al., 1997), and enables micropropagation of the most recalcitrant species (Huetteman and Preece, 1993). The high stability of TDZ due to its resistance to cytokinin oxidase might be a reason for its efficacy (Mok et al., 1987). Another possible reason for the higher activity of TDZ could be substitutive activity for both auxin and cytokinin required for shoot formation (Visser et al., 1992). Murthy et al. (1995) and Hutchinson et al. (1996) observed that endogenous auxin and cytokinins accumulated in tissues treated with TDZ. Indeed, CPPU has been shown to possess a stronger cytokinin-like activity than BA in some species (Millian-Mendoza and Graham, 1999). However, our results indicated that TDZ induced significantly more shoots than CPPU in the ice plant. Similarly Guo et al. (2005) showed that TDZ was more efficient than either BA, KT or CPPU for induction of shoots from cotyledon and leaf

segments in stem mustard (*Brassica juncea* var. *tsatsai*). We conclude that the use of cotyledonary node and TDZ alone was the most suitable combination for inducing adventitious shoots of the ice plant.

Hyperhydricity tended to be lower in the plants treated with TDZ treatment. The medium supplemented with 1.0 mg L⁻¹ NAA plus BA or CPPU had a higher percentage (70–100%) of hyperhydric shoots than TDZ alone or TDZ plus NAA. On the medium containing TDZ without NAA, the frequency of hyperhydricity was 50%, but on the medium containing 1.0 or 2.5 mg L⁻¹ TDZ, addition of 1.0 mg L⁻¹ NAA decreased the frequency of hyperhydricity. Furthermore, there was no hyperhydricity symptom on the medium containing 1.0 mg L⁻¹ NAA plus 0.5 or 5.0 mg L⁻¹ TDZ. On some media, for example, containing TDZ with or without 0.1 mg L⁻¹ NAA, the ratio of hyperhydricity was slightly higher than on the medium containing CPPU. However, the shoot regenerated from explants, which had been cultured on the media supplemented with TDZ was healthy, e.g., the shoot shape, length, thickness, color and number of shoots were superior to those on the medium with CPPU (Sunagawa et al., 2006). As mentioned above, the number of adventitious shoots were reduced by the addition of 1.0 mg L⁻¹ NAA. Therefore, the appropriate NAA concentration was suggested to be around 0.1

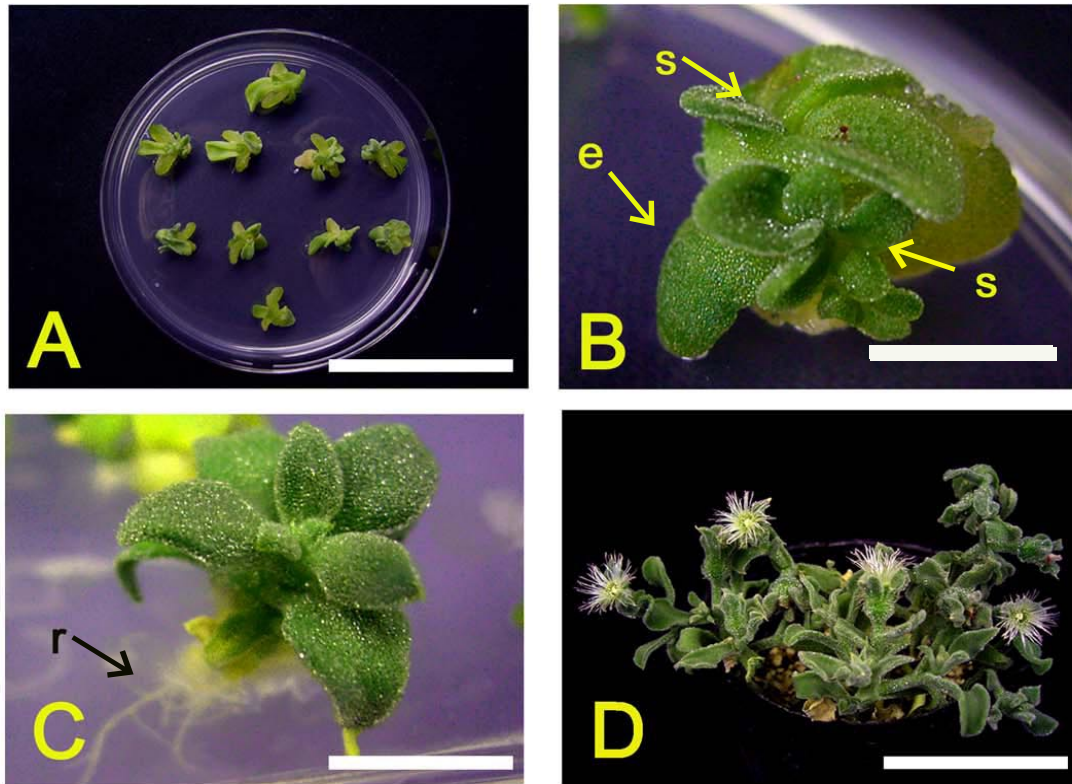


Fig. 4. Plant regeneration *in vitro* from cotyledonary node explant of *M. crystallinum*. A: Shoot formation on MS medium containing 2.5 mg L^{-1} TDZ after 2 weeks of culture (bar = 7.0 cm). B: Close up of explants after 2 weeks of culture (s): adventitious shoot, (e): explant (bar = 5.0 mm). C: Root induction on the shoot 2 weeks after transfer onto hormone-free basal medium, (r): roots (bar = 4.0 cm). D: A regenerated fertile plant in pot established under greenhouse condition (bar = 11.0 cm).

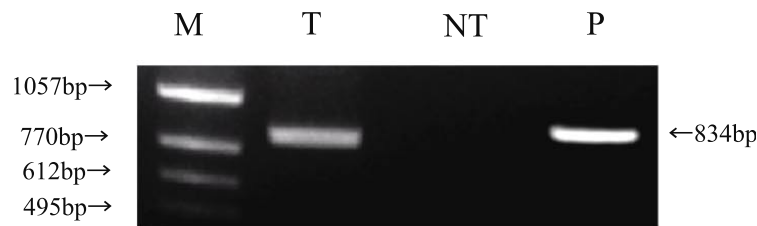


Fig. 5. PCR amplification of *nptII-HPH* gene sequence (834 bp) from independent primary transformants expressing the *nptII-HPH* fusion. M, Maker; T, Transgenic plant; NT, Non-transgenic plant; P, Plasmid.

-1.0 mg L^{-1} . On the medium containing BA, CPPU and TDZ, the mean frequency of hyperhydric shoot was 22.4, 19.2 and 15.9%, respectively, suggesting that exogenous application of TDZ resulted in the minimum frequency of hyperhydricity.

Although the transgenic callus and roots have been produced in the ice plant (Andolfatto et al., 1994; Ishimaru, 1999), cotyledonary nodes have not been used as explants for introducing genes into cells. In this study, we confirmed the introduction of the foreign gene into the cotyledonary node. This regeneration method did not include callus

formation, and it took four months from sowing to transplanting pots via regeneration of adventitious shoots. Somaclonal variation in the regenerants may be reduced by the absence of a callus phase. Thus, we conclude that our regeneration technique can be useful for genetic manipulation of ice plants.

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* In Japanese with English summary.
