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Embryogenic Cell Culture, Protoplast Regeneration, Cryopreservation, Biolistic Gene Transfer and Plant Regeneration in Japanese Cedar (*Cryptomeria japonica* D. Don)

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Abstract

Somatic embryogenesis in *Cryptomeria japonica* was initiated at a relatively high frequency from immature seeds collected from the end of June to mid-July. Induction of embryogenic cultures was possible on media with or without plant growth regulators, and the initiation frequency varied from 5 to 16%. Embryogenic cell lines have been maintained and proliferated for more than 2 years in solid and liquid media. For long-term storage embryogenic cells were cryopreserved using a simple freezing method. Cotyledonary embryos were obtained mostly on maturation media containing abscisic acid (ABA) and polyethylene glycol (PEG) as osmotic agent, however, the plant conversion rate was still low. Plants regenerated from somatic embryos continued growing in a greenhouse. Furthermore, a procedure for the individual culture of protoplasts isolates from embryonal masses, and an approach for microprojectile bombardment-mediated transformation using pIPT and pMAT vectors was also described.

1. Introduction

Japanese cedar (*Cryptomeria japonica* D. Don), commonly called sugi, is the most important forest tree in Japan. It is the only species in the genus and its natural distribution lies between Aomori Prefecture (40° 42' N) and Yaku Island, Kagoshima Prefecture (30° 15' N) (Fukuhara and Kikuchi, 1981). Artificial planting of this species began more than 500 years ago and today more than 10 million hectares have been cultivated (Ohba, 1993). As a result of wide cultivation, pollen allergies have become a serious social problem in Japan. In recent years, plant genetic engineering for transgenic sugi with allergen-free pollen has been investigated as a solution to the sugi pollinosis problem. A stable and efficient plant regeneration system is important for genetic engineering. Somatic embryogenesis is the most attractive plant regeneration system for microprojectile bombardment-mediated transformation. Since somatic embryogenesis and plantlet regeneration of gymnosperm woody species was first reported in Norway spruce (*Picea abies* L. Karst.) (Hakman *et al.*, 1985; Chalupa, 1985; Hakman and von Arnold, 1985), studies in many other conifers have been reported (Tautorus *et al.*, 1991; Attree and Fowke, 1993; Gupta and Grob, 1995). However, information on somatic embryogenesis in sugi is still scarce and restricted to the production of early somatic embryos without plant regeneration (Ogita *et al.*, 1999).

In recent years, advances in conifer tree transformation by microprojectile bombardment techniques have been reported for *Picea glauca* (Ellis *et al.*, 1993; Bommineni *et al.*, 1993), *P. mariana* (Charest *et al.*, 1996), *P. abies* (Walter *et al.*, 1999), *Larix laricina* (Klimaszewska *et al.*, 1997), *Cupressus sempervirens* (Lambardi *et al.*, 1998) and *Pinus radiata* (Walter *et al.*, 1994; Walter *et al.*, 1998). However, information concerning to transformation of Japanese conifer trees has been limited to the recent report of Mohri *et al.* (2000).

In this report we describe plant regeneration through somatic embryogenesis from immature seeds and a biolistic approach for transformation of *C. japonica* using the pIPT5, pIPT10, and MAT21 plasmid vectors.

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2. Materials and methods

2.1 Somatic embryogenesis

2.1.1 Plant material

Several plant materials were tested in preliminary experiments for the initiation of embryogenic cultures (**Table 1**). Shoots, female flower buds, and male flowers were collected from late February to early March in 1997. For the experiment with frozen storage shoots, branches were collected and stored at -5 °C and -15 °C for 1, 3, and 6 months. Immature seeds were collected in the middle of June. Mature seeds collected in October 1995 were stored at -5 °C.

Shoot and female flower bud explants were disinfected first by a 2 min immersion in 70% ethanol followed by 10 min immersion in 0.1% (w/v) mercuric chloride solution and then rinsed seven times with sterile distilled water. Male flowers were disinfected in 70% ethanol for 3 min, dried on sterile filter paper inside the laminar flow cabinet, and then dissected to remove the pollen explants. Immature seeds were disinfected with 1% (w/v available chlorine) sodium hypochlorite solution for 15 min and then rinsed five times with sterile distilled water. Stored mature seeds disinfected with 70% ethanol for 5 min and subsequently in a 0.3% (w/v) mercuric chloride solution for 15 min were rinsed seven times and kept in sterile distilled water at 4 °C for 48 h before the dissection.

2.1.2 Initiation of embryogenic cultures

Immature, open-pollinated cones were collected in 1997, 1998, and 1999 from seed orchards at the Forestry and Forest Products Research Institute, Tsukuba, Japan. Experiments to induce embryogenic cultures were carried out using mostly megagametophytes containing zygotic embryos.

The excised explants were cultured in two media: SM1 (Standard Embryonic Tissue Capture medium) (Smith, 1996), and LPm (modified from Quorin and Lepoivre's medium) (Aitken-Christie and Thorpe, 1984) containing basal salts reduced to half concentration from the standard, 10 g l^{-1} sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 3 μM 6-benzylaminopurine (BAP), 500 mg l^{-1} L-glutamine (filter sterilized), and solidified with 3 g l^{-1} gelrite. The pH of the media was adjusted to 5.8 prior to autoclaving for 15 min at 121 °C. The cultures were kept in darkness at 25 ± 1 °C. The presence or absence of embryonal masses from the explants was observed under the stereomicroscope fortnightly for up to 3 months, and the induction of embryogenic cells was recorded if distinct early stages of embryos characterized by an embryonal head (dense cells) with suspensor system (elongated cells) proliferated after the first subculture.

2.1.3 Maintenance and proliferation of embryogenic cultures

For maintenance and proliferation of embryogenic cultures, the following media were tested: (1) MSs (standard Murashige and Skoog's medium) (Murashige and Skoog, 1962), (2) MSm (modified Murashige and Skoog's medium; basal salts were reduced at half concentration from the standard, and replacing NH_4NO_3 with 1,000 mg l^{-1} L-glutamine), (3) LPs (standard Quorin and Lepoivre's medium) (Aitken-Christie and Thorpe, 1984), (4) LPm (modified Quorin and Lepoivre's medium; basal salts were reduced at half concentration from the standard and supplemented with 500 mg l^{-1} L-glutamine), (5) MSG3 (MSG3 medium) (Becwar *et al.*, 1990), (6) SM1 (Standard Embryonic Tissue Capture medium) (Smith, 1996; without activated charcoal), (7) SM2 (Standard Embryogenesis medium) (Smith, 1996), (8) EM (Embryo Maturation medium) (Maruyama, unpublished; components in mg l^{-1}: KNO_3 1,000, $NaNO_3$ 60, KH_2PO_4 70, $MgSO_4.7H_2O$ 500, $CaCl_2.2H_2O$ 75, $Ca(NO_3)_2.4H_2O$ 60, $NaH_2PO_4.2H_2O$ 160, KCl 750, $MnSO_4.4H_2O$ 20, H_3BO_3 40, $ZnSO_4.7H_2O$ 25, KI 1, $CuSO_4.5H_2O$ 2.4, $Na_2MoO_4.2H_2O$ 0.2, $CoCl_2.6H_2O$ 0.2, $FeSO_4.7H_2O$ 30, Na_2EDTA 40, myo-inositol 1,000, thiamine 5, pyridoxine 0.5, nicotinic acid 5, glycine 5, sucrose 30,000, casein hydrolysate (Sigma C-9386) 500, L-glutamine 1,000, asparagine 500, arginine 250, citrulline 40, ornithine 40, lysine 30, alanine 20, proline 20), and (9) 1/2 EM (half-strength EM medium; basal salts and vitamins were reduced to half concentration from the standard and the concentration of KCl was reduced to 75 mg l^{-1}, casein hydrolysate and all aminoacids were replaced with 1,500 mg l^{-1} L-glutamine).

Media were supplemented with 0-10 μM 2,4-D plus 0-5 μM BAP (**Table 3**). Liquid and solid media (3 g l^{-1} gelrite) were tested. Amino acid stock solutions were filter sterilized and added to the medium after autoclaving.

Embryogenic tissues were subcultured at 4 week intervals on 90 x 20 mm petri dishes containing solid media (30-40 m*l* per petri dish), and every 2 weeks in 100 m*l* flasks containing liquid media (30-40 m*l* per flask) on rotary shakers at 100 rpm. All the cultures were maintained in darkness at 25 ± 1 °C.

To determine the effect of liquid culture method on embryogenic cell proliferation, experiments with a rotary shaker, Rollacell and jar fermentor were

carried out. About 0.04 g FW of embryogenic cells were suspended in 100 m*l* flasks containing 40 m*l* of LPm medium supplemented with 10 μM 2,4-D plus 1 μM BAP and cultured in darkness at 25 ± 1 °C on a rotary shaker at 100 rpm (5 flasks were considered as a replication). Proliferation of embryogenic cells in Rollacell (0.25 g suspended in 2 l bottle containing 250 m*l* of medium) and jar fermentor (1 g suspended in 2 l vessel containing 1 l of medium) were tested at 50 rpm. The fresh weight proliferation rate was calculated after 18 days of culturing.

2.1.4 Maturation of somatic embryos

Proliferated embryogenic cells were cultured in liquid SM3 medium (Embryo Development medium) (Smith, 1996) for two weeks before plating on maturation media. Cultures were maintained in darkness at 25 ± 1 °C on a rotary shaker at 100 rpm. Maturation media containing basal salts, vitamins and the amino acids of the SM3 medium, and supplemented with different concentrations of sugar and polyethylene glycol 4,000 (Wako Pure Chem. Ind., Ltd.) (PEG) were tested (**Table 4**). Amino acid stock solutions and racemic abscisic acid (ABA)(Wako Pure Chem. Ind., Ltd.) were filter sterilized and added to the medium after autoclaving.

About 500 mg FW of embryonal masses suspended in 2-3 m*l* were plated on filter paper disks (Advantec No.2, 70 mm in diameter) over 90 x 20 mm petri dishes containing maturation media (30-40 m*l* per petri dish), sealed with Novix-II film (Iwaki Glass Co., Ltd.) and cultured in darkness at 25 ± 1 °C for 8 to 12 weeks. Three petri dishes for each treatment were tested.

2.1.5 Germination and plant conversion

Somatic embryos were collected from maturation media, and transferred to LPmAC medium (modified LP medium containing basal salts reduced at half concentration from the standard, 20 g l^{-1} sucrose, 5 g l^{-1} activated charcoal, and solidified with 12.5 g l^{-1} Wako agar). The cultures were kept at 25 ± 1 °C under photon flux density of about 65 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (100 V, 40 W; Toshiba Co.) for 16 h daily. The number of somatic embryos germinated (root emergence) and converted to plantlets (emergence of both root and epicotyl) was recorded after 8 weeks. The germinants were transferred into 300 m*l* Erlenmeyer flasks containing 100 m*l* of fresh medium and kept under conditions described above. Emblings that showed root and epicotyl growth were transferred into flasks containing vermiculite fertilized with 0.1% (v/v) Hyponex plant food solution (The Hyponex Co., Inc.) containing (w/v): 5.0% N. 4.36% P, and 4.15% K, and cultured for about 4-8 weeks before *ex vitro* acclimatization. Developed emblings were transplanted into pots filled with vermiculite and acclimatized in a growth cabinet. During the first 2 weeks emblings were kept under high relative humidity in plastic boxes with transparent covers inside the growth cabinet and irrigated with tap water. After that, the cover was opened gradually and pots were fertilized with a nutrient solution modified from Nagao (1983) containing in mg l^{-1}: NH_4NO_3 143, $NaH_2PO_4.2H_2O$ 55.1, KCl 47.1, $CaCl_2.2H_2O$ 52.5, $MgSO_4.7H_2O$ 61, Fe-III EDTA 25, Cu EDTA 0.1, Mn EDTA 0.1, Zinc EDTA 0.1, H_3BO_3 1.5, KI 0.01, $CoCl_2.6H_2O$ 0.005, and MoO_3 0.005. Covers were removed completely about 6 weeks after transplanting.

2.1.6 Statistical analysis

Analyses of variances were performed to test the effect of liquid culture method on embryogenic cell proliferation and the effect of media on somatic embryo maturation. Statistically significant mean differences were determined by Waller-Duncan's multiple range test.

2.1.7 Cryopreservation of embryogenic cultures

For the cryopreservation of embryogenic cultures two protocols as described elsewhere: (1) Hargreaves and Smith (1992), and (2) Sakai *et al.* (1991), were tested. In protocol (1), embryogenic cells were removed from the proliferation medium and cultured in liquid media containing 0.4 M sorbitol for 24 h. About 150 mg FW of pretreated cells suspended in 0.5 m*l* were transferred to 1.5 m*l* cryovials (held on ice) containing 0.5 m*l* of 20% dimethylsulphoxide (DMSO) solution, and then kept at -30 °C for 2 h prior to immersion in liquid nitrogen (LN). To reinstate growth, vials were removed from LN and immersed in a water bath at 40 °C for about 2 min. Vial contents were poured onto a nylon screen and the contents of each vial was rinsed with about 100 m*l* of proliferation medium. In protocol (2), about 150 mg FW of embryogenic cells removed from proliferation medium were transferred to cryovial and treated with a cryoprotectant mix solution (20% glycerol and 15% sucrose) for 10 min at 25 °C and then cooled in a freezer at -30 °C for 30 min prior to immersion in LN. After thawing, the cryoprotectant mix solution was drained from the vials and replaced with medium containing 40% (w/v) sucrose and kept for 20-30 min.

Suspensions of 7 to 10-day-old cultured embryo-

genic cells of two cell lines were used. For subsequent growth recovery after storage in LN, embryogenic cells were cultured in liquid proliferation medium (1/2 EM medium supplemented with 10 μM 2,4-D and 3 μM BAP) in darkness at 25 ± 1 °C on a rotary shaker at 100 rpm.

Growth recovery and morphological characteristics of cryopreserved embryogenic cells were observed under the inverted microscope weekly for up to 6-8 weeks and using an epifluorescence microscope (Nikon Optihot, Tokyo, Japan) under U-excitation fluorescence light after staining with 4', 6-diamidino-2-phenylindole (DAPI). Staining with DAPI was performed as described elsewhere (Kinoshita *et al.*, 1991). Cells were stained for 1 h with DAPI solution containing 0.5 μg ml^{-1} DAPI, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 100 mM NaCl, and 10 mM 2-mercaptoethylamine hydrochloride, in a moist chamber at 25 °C in the dark.

2.1.8 Isolation and culture of protoplasts from embyogenic cultures

Protoplasts were isolated from suspension cultures maintained for 1-2 years. Cells from 10 to 15-day-old embryogenic suspension cultures were collected on 100 μm nylon Falcon cell strainer (Becton Dickinson & Co.) and incubated in a enzyme solution containing 1% (w/v) Cellulase Onozuka RS (Yakult Honsha Co., Ltd.), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd), and 0.6 M mannitol for 5 h at 25 °C. The digest was filtered through 40 μm cell strainer and then centrifuged at 100 x g for 3 min. The supernatant was removed and the protoplasts were resuspended in 0.6 M mannitol solution and centrifuged once more. The yield of protoplasts was counted with haemacytometer and their viability was checked by fluorescein diacetate (FDA). Protoplasts were cultured at a density of 1x 10^2 ml^{-1} to 5 x 10^4 ml^{-1} in 96 well tissue culture plates containing 0.6 M mannitol proliferation media supplemented with various combinations of 2,4-D (0.1, 0.3, 1, 3, 10 μM) and BAP (0, 0.1, 0.3, 1 μM). Plates containing protoplast cultures in wells and sterile distilled water in outspaces between wells (to maintain relative humidity) were sealed with Novix-II film and kept at 28 ± 1 °C in darkness. The plating efficiency percentages (number of colonies / initial plating density x 100) were determined after 6 weeks of culturing.

2.1.9 Proliferation of embryonal masses from individually cultured protoplasts

Isolated protoplasts were picked up using a micromanipulator system (NT-88NE Narishige Co., Ltd.). Micropipettes (Capillary tubes MC-06 Nihon Rikagaku Kikai Co., Ltd., 70 μm in diameter at the tip) were sterilized by dry-heating in an electric oven at 160 °C for 2 h before use. Picked up protoplasts were placed one by one in 96 well tissue culture plates containing 50 μl of liquid medium per well. Protoplasts (1 per well) were cultured individually in media and conditions described above.

2.2 Biolistic approach for plant transformation

2.2.1 Plant material

Open-pollinated cones were collected in 1998 from July 28 to September 31. Seeds removed from the cones were soaked overnight in distilled water before two step disinfection. Seeds were disinfected by agitating them in 5 % (w/v available chlorine) sodium hypochlorite solution for 15 min and then rinsed five times with sterile distilled water. Zygotic embryos removed from seeds under a stereomicroscope inside the laminar flow cabinet were then immersed in 5% (v/v) hydrogen peroxide for 5 min and rinsed three times with sterile distilled water, surface dried on sterile filter paper and transferred to petri dishes containing woody plant medium (WPM) (Lloyd and McCown, 1980) with no exogenous plant growth regulators and solidified with 3 g l^{-1} gelrite. Embryos were then cultured for 5 days before the bombardment experiments.

2.2.2 Vector constructs

The pIPT5, pIPT10, and pMAT21 vector constructs used in this study, offered by Central Research Laboratory of Nippon Paper Industries Co., Ltd., were shown in **Fig. 6**. The pMAT21 vector for generating marker-free transgenic plants (MFTPs) included a special sequence in which removal of the *ipt* gene is efficiently mediated by the site-specific recombination system *R*/RS from *Zygosaccharomyces rouxii*, in place of the maize transportable element *Ac* (Sugita *et al.*, 1999). The GUS reporter gene and the NPT II gene are controlled by the CaMV35S promoter. The presence of β-glucuronidase (GUS) introduced was histochemically verified with a GUS assay.

2.2.3 Biolistic transformation parameters

The biolistic particle delivery device (NK System PIGG-1, Japan)(**Fig. 9A**) was used in transformation experiments. DNA for bombardment was isolated using the Plasmid Midi Kit 100 (QIAGEN Co. Ltd.). Gold particles (1 μm, Tokuriki Honten Co., Ltd.) were coated with plasmid DNA solution as described elsewhere (Christou, 1992). The bombardment parameters (NK System PIGG-1 manual)

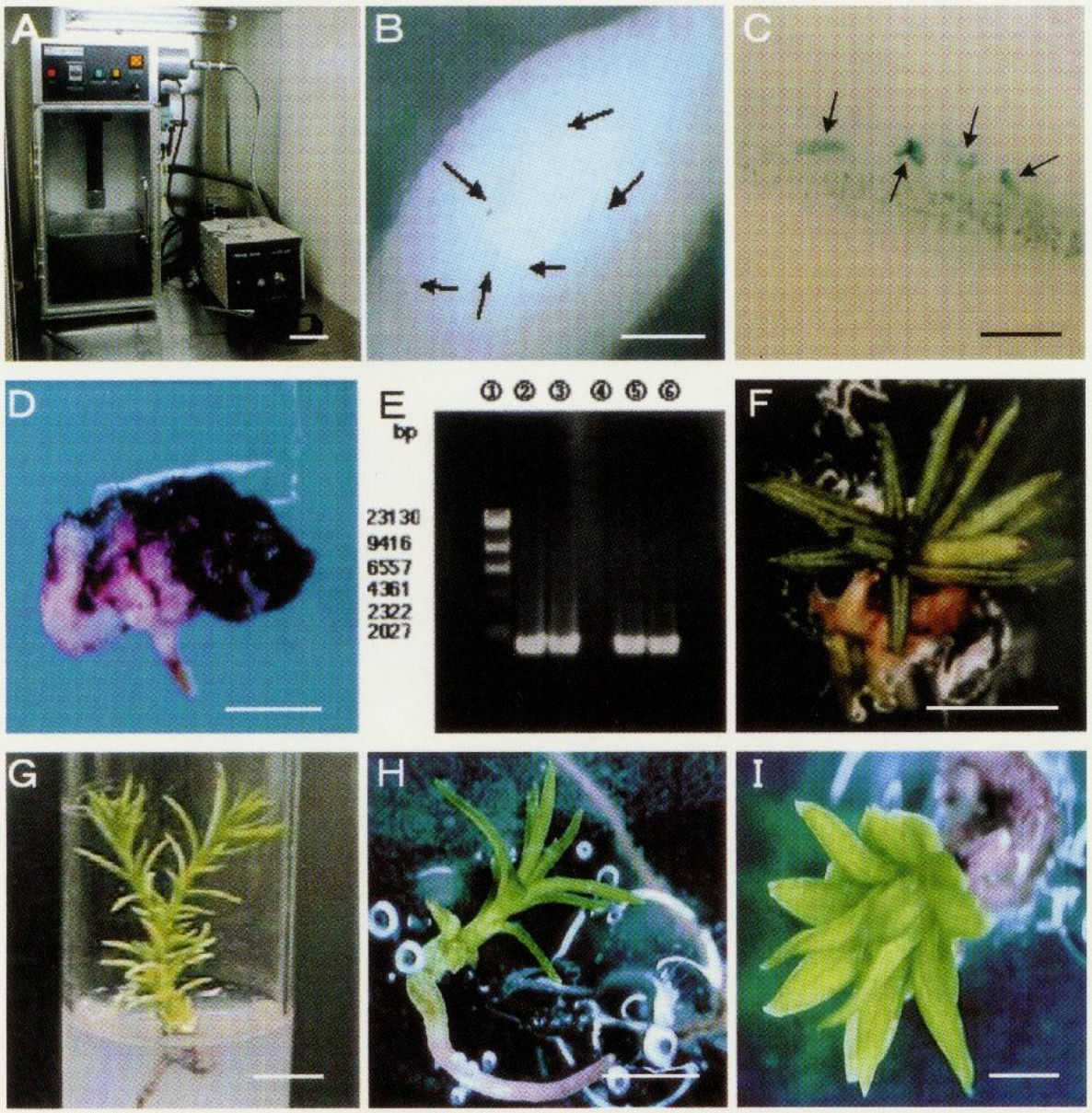

Fig. 9 A biolistic approach for transformation of *Cryptomeria japonica*. **A** : The biolistic particle delivery device used (bar = 10 cm). **B** and **C** : Blue spots observed by staining with x-Gluc on microprojectile bombarded embryos (bar = 1 mm). **D** : Deformity in embryo after microprojectile bombardment using pIPT 5 vector (bar = 5 mm). **E** : PCR analysis; 1 = DNA size marker (λ-Hind III), 2 and 3 = pIPT10 transformants, 4 = nontransformants, 5 and 6 = pMAT21 transformants. **F** : Mutiple bud formation from microprojectile bombarded embryo using pIPT10 vector after culturing on plant growth regulator-free medium (bar = 1 cm). **G** : Rooted shoot (bar = 1 cm). **H** : A nonbombarded embryo (control) germinating and growing on medium without plant growth regulators (bar = 1 cm). **I** : A nonbombarded embryo (control) showing multiple bud formation from its develoled apical region after culturing on medium supplemented with BAP (bar = 1 cm).

were: acceleration pressure 4-8 kg cm^{-2}, target distance 30 mm, vacuum in chamber 675 mm Hg.

2.2.4 Histochemical analysis of GUS expression

Histochemical GUS assays were performed according to Walter *et al.* (1994). Bombarded tissues were flooded with a staining solution containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Wako Pure Industries Co., Ltd.) and incubated at 37 °C for 3 days. The presence of blue spots was observed under a stereomicroscope (Mz8; Leica, Germany).

2.2.5 PCR analysis

Genomic DNA was isolated from transformed tissue using modified CTAB method according to Walter *et al.* (1998). PCR (Polymerase Chain Reactions) were performed in a TaKaRa PCR Thermal Cycler SP using a PCR profiles consisting of 30 cycles of 1 min denaturation at 94 °C, a further 30 cycles of 1 min annealing at 60 °C, and 30 cycles of 2 min extension at 72 °C. A forward primer (5'-GCACCGAAGTTCATGCCAGT-3') and another reverse one (5'-GTGGAATTGATCAGCGTTGG-3') were synthesized on the basis of the sequence of GUS gene. After amplification, PCR products were separated on 0.6% agarose gel by electrophoresis (100 V for 35 min), stained with ethidium bromide solution (0.5 μg m^{-1}) and then photographed on a UV-transilluminator.

3. Results

3.1 Initiation of somatic embryogenesis

As shown in **Table 1**, in preliminary experiments, the embryogenic cell induction was achieved only on megagametophyte explants from immature seeds. In some megagametophyte isolates from stored mature seeds, the extrusion of embryogenic-like tissue from the micropylar end was observed on media containing plant growth regulators but without further proliferation of embryogenic masses. The response of cultured female flower bud, pollen, and shoot explants was mostly nonembryogenic callus formation, and elongation in shoot explants on media without plant growth regulators. Formation of embryo-like structures on frozen storage shoot explants, as described for *Larix* (Bonga, 1996), was not observed in sugi shoots.

For further experiments, only explants from immature seeds were used (**Fig. 1A**). Medium was not a critical factor for embryogenic cell induction. The initiation of embryonal masses was observed after 2-4 weeks of culture on both media with (LPm) or without exogenous plant growth regulators (SM1)(**Fig. 1B**). Initiation frequencies varied according to the collection dates and media from 1.3% to 14.7% from megagametophyte explants collected between June 13 and July 16. The highest response was achieved with explants collected on July 16 and cultured on LPm medium (14.7% for megagametophytes and 16.0% for zygotic embryos).

Highest response on MS1 medium was also achieved with seeds collected on July 16 (12.0% for both megagametophytes and zygotic embryos) (**Table 2**). At this time, the beginning of germination was observed in some explants simultaneously with embryonal mass and/or embryo-like structure formation. Since the physiological maturation of a seed is determined by its ability to germinate, this result indicates that the zygotic embryos from immature seeds collected on July 16 became mature and suggests that the seed collection in mid-July was the critical limit for embryogenic cell induction on medium with no plant growth regulators. Although most of the somatic embryogenesis studies in conifer trees have reported the use of plant growth regulators for embryogenic cell induction, for sugi the presence or absence of exogenous plant growth regulators into media was not a critical factor for embryogenic cell induction.

As shown in **Fig. 2** and **3**, no great difference in induction frequencies among mother trees or as function of the seed collection year was observed. Similar maximum induction frequency of around

Table 1. Response of various explants tested to initiate embryogenic cultures in *Cryptomeria japonica*.

Media	Shoot tip	Frozen storage shoot	Shoot primordia	Female flower bud	Pollen	Immature seed	Stored mature seed
LPm-	E, C	C	E, C	C	C	EM, C	G, C
LPm+	C	C	C	C	C	EM, C	C

E, elongation ; C, nonembryogenic callus formation ; EM, embryonal mass formation ; G, germination. Explants were cultured on LPm medium (modified from Quorin and Lepoivre's medium, as described in section 2. 1. 2) containing no plant growth regulators (LPm-) or supplemented with 10 μM 2,4-D plus 3 μM BAP (LPm+).

Table 2. Effect of seed collection date, media, and culture explants on induction of embryogenic cells in *Cryptomeria japonica*.

Seed collection date	Media	Megagametophyte		Zygotic embryo	
		T	EC (%)	T	EC (%)
1997, June 13	SM1	75	1(1.3)	NT	...
	LPm	75	3(4.0)	NT	...
1997, June 24	SM1	60	3(5.0)	NT	...
	LPm	90	7(7.8)	NT	...
1997, July 04	SM1	60	6(10.0)	NT	...
	LPm	90	10(11.1)	NT	...
1997, July 16*	SM1	75	9(12.0)	25	3(12.0)
	LPm	75	11(14.7)	25	4(16.0)

T, number of isolated explants ; EC, number (%) of explants proliferating embryonal masses ; NT, not tested ; ..., no data ; *, the beginning of germination was observed on SM1 medium.

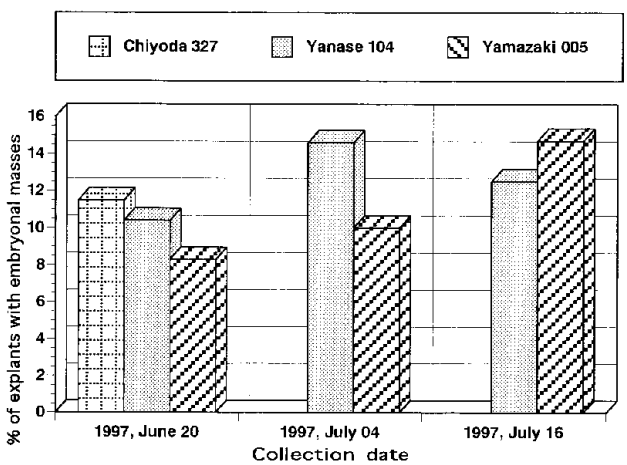

Fig. 2 Induction of embryonal masses from megagametophyte explants of different *Cryptomeria japonica* mother trees.

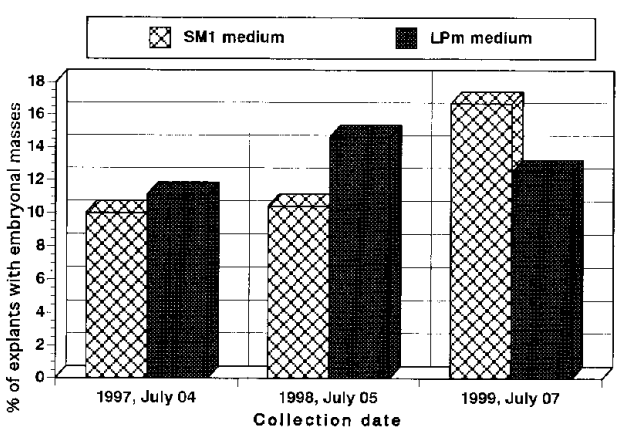

Fig. 3 Induction of embryonal masses from megagametophyte explants of different *Cryptomeria japonica* as function of the seed collection year.

15% was obtained in megagametophytes explants from seeds of both Yanase 104 and Yamazaki 005 mother trees, but with a variation between seed collection dates. That is to say, the initiation frequency in Yanase 104 was maximum on July 4 (14.6%) whereas for Yamazaki 005 was scored 12 days after (14.7% on July 16). This result suggests a putative variation in the developmental stage of embryos at same time between maternal sources.

The optimal developmental stage of zygotic embryos as a critical factor in somatic embyogenesis initiation for several conifer species has been reported in terms of seed collection date or time after fertilization (Becwar *et al.*, 1990; Tautorus *et al.*, 1991; Lelu *et al.*, 1994; Jain *et al.*, 1995; Klimaszewska *et al.*, 1997; Lelu *et al.*, 1999; Kim *et al.*, 1999). However, due to the difficulty of determining the precise time of fertilization in open-pollinated cones and that variation in zygotic embryo development depends on weather and location, the criteria of explant collection for embryogenic cell induction cannot be easily generalized. In addition, variation in developmental stage of embryos may be observed among trees and within the same tree and even individual cones. The observation of developmental stage of individual embryos is the most appropriate method to determine the optimal time for embryo selection.

Embryogenic cell induction as a function of the seed collection year was determined over 3 years with seeds collected at the beginning of July. The best initiation frequency (16.7%) was achieved in 1999 on SM1 medium (**Fig. 3**). This result indicates that the use of plant growth regulators is not essential for embryogenic cell induction when explants are cultured at the appropriate development stage. Somatic embryogenesis initiation on medium without plant growth regulators have been reported also for *Pinus radiata* (Smith, 1996), *P. sylvestris*, and *P. pinaster* (Lelu *et al.*, 1999).

Table 3. Effect of media on embryogenic cell proliferation in *Cryptomeria japonica*.

2,4-D+BAP	MSs		MSm		LPs		LPm		MSG3		SM1		SM2		EM		1/2EM	
(μM)	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
0+0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1	2*	2*	NT	NT	NT	NT
1+0	NT	NT	2	2	NT	NT	2	2	NT	NT	NT	NT	2	2	NT	NT	2	2
1+0.5	1	1	3	3	1	2	3	3	NT	NT	2	1	3	3	2	2	3	3
2+1	NT	NT	3	3	NT	NT	3	3	NT	NT	NT	NT	NT	NT	NT	NT	3	3
3+0	NT	NT	2	2	NT	NT	2	2	NT	NT	NT	NT	2	2	NT	NT	2	2
3+1	1	2	3	3	1	2	3	3	3	3	2	1	3	3	2	2	3	3
5+0	NT	NT	3	2	NT	NT	3	2	NT	NT	NT	2	NT	NT	NT	NT	3	2
5+1	1	2	3	3	1	2	3	3	3	3	2	2	3	3	2	3	3	3
10+0	NT	NT	3	2	NT	NT	3	2	NT	NT	NT	1	NT	NT	NT	NT	3	2
10+1	NT	NT	3	3	NT	NT	3	3	NT	NT	2	2	3	3	NT	NT	3	3
10+3	1	2	3	3	1	2	3	3	3	3	2	1	3	3	2	3	3	3
10+5	1	1	2	3	1	2	2	3	NT	NT	1	1	2	3	2	2	2	3
0+1	NT	NT	NT	NT	NT	NT	1	1	NT	NT	NT	1	NT	NT	NT	NT	NT	NT
0+5	NT	NT	NT	NT	NT	NT	1	1	NT	NT	NT	1	NT	NT	NT	NT	NT	NT
1+5	NT	NT	NT	NT	NT	NT	2	2	NT	NT	NT	1	NT	NT	NT	NT	NT	NT

L, liquid media ; S, solid media ; 1, not good growth ; 2, fair growth ; 3, good growth ; (*), tendency to development ; NT, not tested.

3.2 Maintenance and proliferation of embryogenic cultures

Table 3 shows the results of maintenance and proliferation of embryogenic cultures for several media and combinations of plant growth regulators. The maintenance and proliferation of sugi embryogenic cells was possible in 1/2 EM, LPm, MSm, SM2, and MSG3 medium containing a combination of 2,4-D (1-10 μM) plus BAP (0-5 μM). The principal characteristic of these media is the reduction in concentration of inorganic components from the standard and the addition of L-glutamine as organic nitrogen source. On the other hand, the growth of cells when culturing in MSs, LPs, EM, and SM1 medium (medium with high concentration of inorganic components and/or without addition of L-glutamine) was not optimal. These media supported growth only for short culturing and then cell condition deteriorated over time. Organic nitrogen sources have improved the proliferation of embryogenic cells as compared to inorganic nitrogen sources (Boulay *et al.*, 1988; Finer *et al.*, 1989; Tremblay and Tremblay, 1991; Jain *et al.*, 1995). The use of L-glutamine as a substitute for the inorganic nitrogen source in the medium have been reported for somatic embryogenesis in many conifer trees (Becwar *et al.*, 1990; Gupta and Pullman, 1991; Jain *et al.*, 1995; Zoglauer *et al.*, 1995; Smith, 1996; Klimaszewska and Smith, 1997; Lelu *et al.*, 1999).

The results of experiments to determine the optimal liquid culture method for the proliferation of embryogenic cells are shown in **Fig. 4**. Fresh weight increased more than 100-fold and 80-fold after 18 days of culturing in a rotary shaker and rollacell, respectively. In contrast, a low proliferation rate was achieved with the culturing of cells in a jar fermentor (19-fold). Similar results were reported for suspension culture from nonembryogenic callus of sugi (Ishii and Sato, 1990).

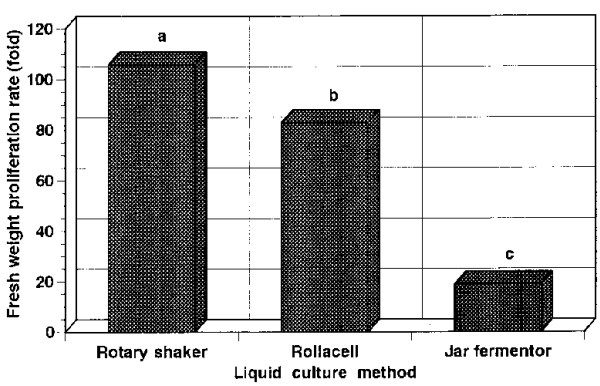

Fig. 4 Effect of liquid culture method on embryogenic cell proliferation in *Cryptomeria japonica*. Data with different letter are significantly different at $P<0.01$.

Further study is needed to elucidate the optimal conditions for culturing in a jar fermentor.

For further subcultures 1/2 EM, MSm, and LPm medium supplemented with 3-10 μM 2,4-D plus 1-3 μM BAP were used both in solid and liquid cultures (rotary shaker). Since proliferation is slow on solid media, these media supported cell growth longer than liquid media. Thus, embryogenic cells

Table 4. Variation in media tested to induce somatic embryo maturation in *Cryptomeria japonica*.

Media	Sucrose ($g\ l^{-1}$)	Maltose ($g\ l^{-1}$)	PEG ($g\ l^{-1}$)	Sorbitol ($g\ l^{-1}$)	Gelrite ($g\ l^{-1}$)	ABA (μM)
DM3	0	30	0	0	4.5	50
DM6	0	60	0	0	4.5	50
DM9	0	90	0	0	4.5	50
DM6P7.5	0	60	75	0	4.5	50
DS6P7.5	60	0	75	0	4.5	50
DM6So2	0	60	0	20	4.5	50
DM6P15	0	60	150	0	4.5	50
DS6P15	60	0	150	0	4.5	50

All media containing basal salts, vitamins and amino acids of Embryo Development Medium (Smith, 1996).

were subcultured at intervals of 4 and 2 weeks for solid and liquid media, respectively. In general, solid media were used for maintenance and liquid media for rapid proliferation of the cultures. For solid media, five embryonal masses (about 20 mg FW each) were subcultured per petri dish. For liquid culture, about 10 mg FW (or less) were subcultured per flask. We determined that the low density of subculture was beneficial for the proliferation and good condition and maintenance of embryogenic cells (densely embryonal head with distinct suspensor system) in suspension culture. When cells were subcultured at high density the development of the suspensor system was suppressed.

In other experiments, successful proliferation on a rotary shaker was achieved culturing 1-5 embryogenic cell clusters per flask (data not shown). Embryogenic cells of sugi showed a high proliferation capacity at very low culture density. This capacity was corroborated by the proliferation of embryonal masses from culturing of one isolated protoplast as described below.

Maintenance of embryogenic cells on media with no plant growth regulators has been reported for *Pinus radiata* by Smith (1996). He inferred that plant growth regulators such as 2,4-D and BAP may stimulate apparent growth of embryogenic tissue, in part due to formation of suspensor cells, but the use of plant growth regulators is not necessary, and that some embryogenic cell lines maintained on medium with 2,4-D and BAP lose their plant-forming potential much sooner than the cell lines which have been maintained on medium without plant growth regulators. Embryogenic cells of sugi maintained on media with no plant growth regulators led to embryo development (**Fig. 1D**).

3.3 Maturation of somatic embryos

Embryogenic cells cultured for 10-15 days in liquid SM3 medium were collected on 100 μm nylon screen. Embryonal masses were then resuspended in liquid SM3 medium supplemented with 50 μM ABA and plated on maturation media. The results after 2 months of culturing are shown in **Fig. 5.** The addition of PEG stimulated the cotyledonary embryo production. The best results were obtained at 7.5% PEG in combination with 6% maltose (DM6P 7.5 medium) or 6% sucrose (DS6P 7.5 medium), with an average number of embryos collected per petri being 67 and 54, respectively (**Fig. 1E-1K**). However, the number of cotyledonary embryos decreased when 15% PEG was added to the media. This decrease was more obvious on medium supplemented with sucrose (DS6P15) rather than on maltose (DM6P15). In addition, the embryos produced did not germinate. The addition of maltose as carbohydrate source and PEG as osmotic agent into media was reported as an effective combination to enhance somatic embryo maturation in *Pinus taeda* (Li *et al.*, 1998).

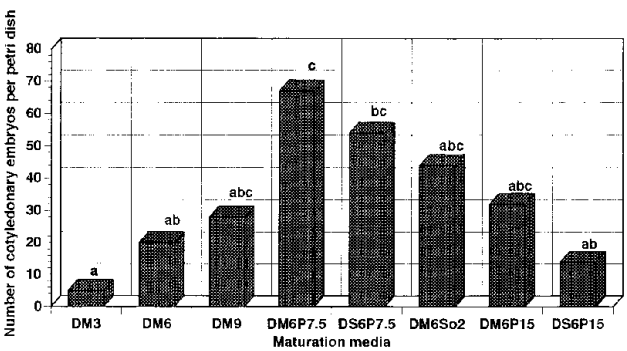

Fig. 5 Effect of madia on maturation of *Cryptomeria japonica* somatic embryos. Data with same letter are not significantly different at $P<0.01$.

In the absence of PEG, most of the proembryos did not develop into cotyledonary embryos on media containing 3% maltose. Embryogenic cell proliferation was evident and most of them devel-

oped into structures consisting of a small embryonal head with elongated suspensors extending from them (stage 1) as described elsewhere (von Arnold and Hakman, 1988). The production of cotyledonary embryos increased relative to the addition of 6-9% maltose (20-28 embryos/petri) but at a reduced level with regard to media containing PEG. In other experiments (data not shown) we used silver nitrate, activated charcoal, and potassium permanganate (as adsorption agents of head-space volatiles) in an attempt to induce maturation as reported for *Picea glauca* (Kong and Yeung, 1995) and *P. sitchenensis* (Selby *et al.*, 1996) without enhancing the production of cotyledonary embryos.

Addition of sorbitol in exchange for PEG as osmotic agent into medium was also effective to induce maturation. An average of 44 embryos per petri dish were collected after approximately 2 months of culturing. The use of sorbitol as osmotic agent in maturation media is described for several gymnosperms (Gupta and Pullman, 1990; 1991; Jain *et al.*, 1995).

In general, the best results for inducing somatic embryo maturation were achieved using PEG in the presence of ABA. Embryogenic cells on media without ABA did not develop beyond the embryo stage 1. Most of the studies on somatic embryogenesis in conifers have reported that ABA is a very important hormone in embryo development and that the number and quality of embryos produced was far inferior in absence of ABA (Durzan and Gupta, 1987; von Arnold and Hakman, 1998; Hakman and von Arnold, 1988; Attree and Fowke, 1993; Dunstan *et al.*, 1998). Somatic embryos of *Picea glauca - engelmannii* complex (Roberts *et al.*, 1990a) and *P. glauca* (Dunstan *et al.*, 1991) requires higher concentrations of ABA to promote normal development of plants. Lelu *et al.* (1999) have reported that much higher numbers of mature embryos of *Pinus sylvestris* and *P. pinaster* were produced and that the development of cotyledonary somatic embryos versus abnormal, shooty ones was enhanced with addition of 60 μM ABA in comparison with media without ABA. Somatic embryos of hybrid larch (*Larix* x *leptoeuropaea*) developed normally on medium supplemented with 60 μM ABA, but abnormally on medium with no ABA (Gutmann *et al.*, 1996). Higher concentrations of ABA resulted in higher numbers of mature somatic embryos in *Pinus strobus* (Klimaszewska and Smith, 1997). The use of ABA for somatic embryo maturation in gymnosperms is reported extensively in the compilation of Jain *et al.* (1995). However, in some of our experiments when cultures were kept for a long time on SM3 media with no ABA (4-6 months of culturing), spontaneous direct shoot formation from some embryogenic cultures was occasionally observed. Although this response is unclear, it may be related to the change of medium conditions due to a water content decrease in the medium over time. The effect of medium desiccation may be similar to the water stress induced by addition of PEG or a high concentration of gelrite into medium which have been reported to enhance somatic embryo maturation in conifer species. However in this case, the formation of shoots was direct from embryonal masses without distinct embryo formation, and in addition the characteristics of these shoots which have distinct bud scales (**Fig. 1O**) were markedly different from the typical shooty embryos produced. Further study is needed to clarify this response. After transferring of these shoots to the light condition most of them elongated and converted into plants (data not shown). Development of cotyledon-like structures and rhizogenesis from them were also observed on the same media (**Fig. 1P**). Reports concerning direct shoot formation from embryonal masses of sugi were not found, and information about organogenesis from callus is restricted to root formation from nonembryogenic callus (Sato, 1990).

The use of ABA in combination with PEG has been reported as the most common method for stimulating somatic embryo maturation in many gymnosperms. Several authors have suggested that the role of ABA in somatic embryogenesis is the inhibition of cleavage polyembryony with consequent development of individual somatic embryos (Durzan and Gupta, 1987; Boulay *et al.*, 1988; Krogstrup *et al.*, 1988; Gupta *et al.*, 1991), to stimulate the accumulation of nutrients, lipids, proteins, and carbohydrates (Hakman and von Arnold, 1988), and to suppress precocious germination (Roberts *et al.*, 1990). The addition of PEG induces water stress similar to that generated by desiccation (Attree and Fowke, 1993), increases storage product deposition (Attree *et al.*, 1992; Misra *et al.*, 1993) and tolerance to water loss (Attree *et al.*, 1991). However, some authors have reported that PEG promotes maturation but inhibits further development of *Picea glauca* (Kong and Yeung, 1995) and *P. abies* somatic embryos (Bozhkov and von Arnold, 1998). In recent years, several studies have been reported that the maturation of somatic embryos is promoted by addition of ABA into media solidified with a high concentration of gellan gum (gelrite) in the absence of PEG (Klimaszewska and Smith, 1997; Lelu *et al.*, 1999). These results suggest that factors other than additives causing osmotic stress may be required for the

developmental progression of somatic embryos (Klimaszewska and Smith, 1997). In the present study, attempts at embryo maturation using several gelrite concentration (3, 4.5, 6, 7.5, 10, 15, and 20 g l^{-1}) were made. Production of cotyledonary embryos was achieved mostly in a range of 3 to 6 g l^{-1} gelrite. Almost all somatic embryos on media solidified with concentration more than 6 g l^{-1} gelrite (7.5, 10, 15 and 20 g l^{-1}) did not develop to the cotyledonary stage and mostly callused over (data not shown).

3.4 Germination and plant conversion

As shown in **Table 5**, the number of germinants (**Fig. 1L**) that then converted into emblings (**Fig. 1M**) was very low. The percentage of germination and plant conversion ranged from 0 to 12.5%, and the best results were achieved with somatic embryos developed on both maturation media containing 7.5% PEG. Most of the embryos collected from maturation media without PEG or with high concentration (15% PEG) did not develop into plants.

Partial drying treatment at high relative humidity following ABA maturation has been reported as beneficial to improve germination of some *Picea* and *Pinus* species (Roberts *et al.*, 1990b; Roberts *et al.*, 1991; Kong and Yeung, 1992; Jain *et al.*, 1995; Kong and Yeung, 1995). Attempts to enhance germination in sugi embryos using partial drying treatment based on that used by Roberts *et al.* (1990b), and cool drying treatment at 4 °C in empty petri dishes were tried but without a significant improvement in the number of germinants (data not shown). Most of the nongerminated embryos developed cotyledons which turned green under light conditions and some of them showed epicotyl growth and later plant conversion (data not shown) but with adventitious root formation (**Fig. 1Q** and **1R**). The cotyledonary embryos that did not develop epicotyls led to the formation of callus (secondary embryogenesis) or turned brown and then died. In some cases adventitious root formation was also observed.

Regenerated "emblings" were acclimatized successfully under conditions described above (**Fig. 1N**). New shoot growth started about 4 weeks after initiation of the acclimatization period which was approximately 6 weeks. Thereafter emblings were kept for 2-3 months more in a growth cabinet before transferring to a greenhouse.

3.5 Cryopreservation of embryogenic cells

Embryogenic cells of sugi were cryopreserved successfully by the methods of (1) Hargreaves and Smith (1992), and (2) Sakai *et al.* (1991). Both methods are simple protocols that do not require complicated cryoprotective procedures or sophisticated and expensive controlled freezing equipment. Five replicates of two cell lines for each method were kept in LN for 1 day. Thawed cells transferred into growth recovery medium were observed weekly to determine their growth conditions. Slower growth of the cells was observed between 1-2 weeks of culturing. Almost all suspensor cells were killed or showed a damaged appearance. These observations were supported by results from staining cells with DAPI. Only embryonal heads (densely cytoplasmic cells) remained DAPI-reactive after freezing and thawing. Injury and death of vacuolated suspensor cells during freezing and thawing have been reported for embryogenic cells of Norway spruce and loblolly pine (Gupta *et al.*, 1987) and white spruce (Kartha *et al.*, 1988). About 2-3 weeks after thawing the initiation of suspensor cell formation from some embryonal heads was observed (**Fig. 1S**). Distinct proliferation of cryo-

Table 5. Germination and convertion for *Cryptomeria japonica* somatic embryos matured on different media.

Maturation media	Germination medium	Total No.	Germination No.	%	Conversion No.	%
DM3	LPmAC	10	0	0	0	0
DM6	LPmAC	30	1	3.3	0	0
DM9	LPmAC	30	1	3.3	1	3.3
DM6P7.5	LPmAC	40	5	12.5	5	12.5
DS6P7.5	LPmAC	40	4	10	4	10
DM6So2	LPmAC	40	3	5	2	5
DM6P15	LPmAC	30	1	3.3	1	3.3
DM6P15	LPmAC	10	0	0	0	0

Maturation media, see Table 4. LPmAC, half- strenght LP medium containing 20 g/*l* sucrose, 5 g/*l* activated charcoal and 12.5 g/*l* Wako agar.

preserved cells were observed after about 4 weeks of culturing. At this time the presence of many elongated suspensors from embryonal heads was observed and the appearance of embryogenic cells resembled that of unfrozen embryonal masses.

The slower growth of the cryopreserved cells, in comparison with other unfrozen cells, may be attributable to a decrease in the ability of cells to proliferate due to freezing stress. After about 3 to 4-weeks of post-thaw recovery the embryogenic cells proliferated rapidly and at the subsequent subculture no difference was observed when compared with the proliferation of unfrozen cells. Similar results concerning a slower growth of cryopreserved embryogenic cells was reported for *Picea abies* and *Pinus taeda* (Gupta *et al.*, 1987). They mentioned that from days 0 to 35, growth of frozen and thawed embryonal cells was not observed and that the cryopreserved cells reverted to normal embryonal growth rates after the third subculture. In contrast, Sakai *et al.* (1991) reported that frozen cells of *Citrus sinensis* resumed growth 3 days after plating and a rapid growth was observed after 12 days following a similar pattern to that of the unfrozen cells.

Cryopreservation offers the potential for reliable long-term storage of plant genetic resources without apparent risk of genetic instability using minimum space and with lower labor and maintenance costs.

The advantages of using somatic embryos in breeding programs would increase significantly if the embryos could be preserved during field evaluation and selection of different genotypes (von Arnold *et al.*, 1996). Subculturing embryogenic cells involved much handling, was time consuming, and increased the risk of loss of embryogenic potential of the cells over time. Cryopreservation is the best method for preservation of embryogenic cultures because it permits long-term storage and maintains the juvenility of donor tissue which is desirable for a stable plant regeneration system.

A recent review on cryopreservation of embryogenic cultures of conifers and its application to clonal forestry has been reported by Cyr (1999).

3.6 Isolation and culture of protoplasts

A reliable and simple method for isolation and culture of protoplasts from embryogenic cell cultures of sugi was developed. Isolation and culture conditions were determined by trial and error in preliminary experiments using at least 24 combinations of enzymes, different concentration of mannitol, and several combinations of plant growth regulators (data not shown). We succeeded in the isolation and culture of protoplasts from 3 embryogenic cell lines of sugi using the protocol described above.

Table 6. Effects of plant growth regulator combinations on the plating efficiency percentages of *Cryptomeria japonica* protoplasts.

BAP (μM)	2,4-D (μM)				
	0.1	0.3	1.0	3.0	10.0
0.0	3.00	4.75	6.25	2.25	0.25
0.1	3.75	6.75	6.50	6.00	1.50
0.3	4.00	7.25	6.75	5.25	1.00
1.0	3.50	4.00	4.50	3.50	0.50

Protoplast viability as assayed by FDA staining was greater than 90%. This result indicated that the combination of Cellulase Onozuka RS (1%) and Pectolyase Y-23 (0.1%) in the presence of 0.6 M mannitol as osmotic agent was effective for the isolation of protoplasts from embryogenic cells of sugi.

Isolated protoplasts were cultured in media supplemented with different combinations of 2,4-D and BAP at a density of 2 x 10^3 ml^{-1} determined in another experiments as appropriate for sugi embryogenic cells (data not shown). The plating efficiency percentages are shown in **Table 6**. Colony formation was observed in all combinations of plant growth regulators, but with most efficiency at the concentration of 0.3-3 μM 2,4-D and decreasing considerably at 10 μM level. The best result was obtained in medium supplemented with 0.3 μM 2,4-D plus 0.3 μM BAP.

Additional success in colony formation and then proliferation of embryonal masses from individually cultured protoplasts of sugi was achieved in media supplemented with a combination of 1-3 μM 2,4-D plus 0-0.1 μM BAP. Isolated protoplasts (**Fig. 1T**) were picked up one by one using a micromanipulator (**Fig. 1J**) and cultured under conditions described. Cell division of individually cultured protoplasts was observed after 10-14 days of culturing (**Fig. 1V**). About 2 weeks after, the formation of colonies was evident and the initiation of elongated suspensor cells was observed (**Fig. 1W**). The formation of vacuolated suspensor cells increased over time and after about 6 weeks of culturing more than 50% of cluster cells showed distinct suspensor system resembling that of the original embryonal masses (**Fig. 1X**). Experiments on embryo maturation using embryonal masses proliferated from individually cultured protoplasts are in progress.

The technique of individual cell culture represents a powerful tool for studies on the physiology of

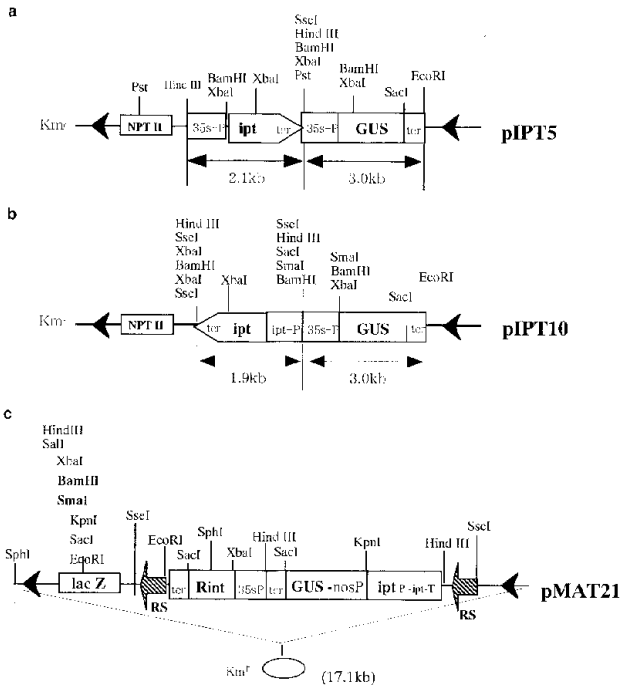

Fig. 6 Schematic of the plasmid vectors used in this study. a, plPT5 is a binary vector based on pBI121. The GUS reporter gene, the NPT II gene, and the ipt gene are controlled by the CaMV35S promoter. b, plPT10 is a binary vector based on pBI121. The GUS reporter gene and the NPT II gene are controlled by the CaMV35S promoter. The ipt gene is controlled by native promoter. c, pMAT21 is a binary vector based on pBI121. LacZ which has multi cloning site is a part of β - glucuronidase gene. R/RS (recombinase gene/recombination site) is the site-specific recombination system from *Zygosaccharomyces rouxii* is driven by the CaMV 35S promoter. The GUS gene is controlled by nopaline synthase promoter. The ipt gene is controlled by native promoter.

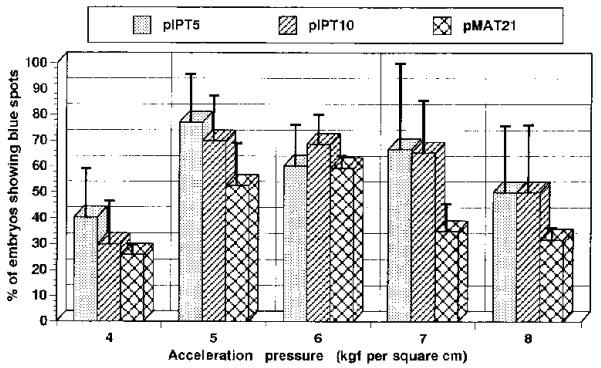

Fig. 7 Effects of acceleration pressure on the expression of GUS activity in *Cryptomeria japonica* embryos after microprojectile bombardment. Bars indicate standard error from three replicate experiments.

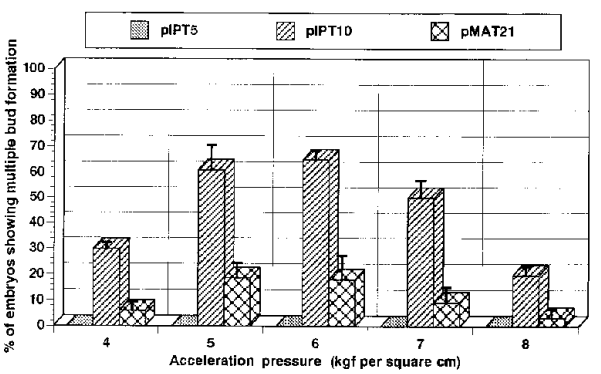

Fig. 8 Effects of acceleration pressure on the multiple bud formation in microprojectile bombarded *Cryptomeria japonica* embryos after culturing on medium without plant growth regulators. Bars indicate standard error from three replicate experiments.

different cell types, analysis of differentiation programs, genetic manipulation of plant cells, and cell-cell interactions (Schweiger *et al.*, 1987). Individual protoplast culture has often been described for *Nicotiana tabacum, Brassica napus* and *Hordeum vulgare* (Koop and Schweiger, 1995; Spangenberg *et al.*, 1986; Schweiger *et al.*, 1987; Eigel and Koop, 1989; Schaffler and Koop, 1990). In recent years, plants have been regenerated from protoplasts isolated from embryogenic suspension of some conifer trees (Tautorus *et al.*, 1991), however, to our knowledge, there is no information concerning individual protoplast culture of these species.

3.7 Biolistic approach for plant transformation

The microprojectile-mediated DNA delivery system was used to attempt plant transformation in sugi. Preliminary experiments to determine the appropriate bombardment conditions indicated that the most number of particles and explants showing GUS expression was achieved using a target distance of 30 mm. Particle bombardment at target distances more than 30 mm (60 , 90 mm) resulted in very low or no GUS expression in explants (data not shown). Thus, for further experiments the target distance was fixed at 30 mm.

Fig. 7 and **8** shows the effects of acceleration pressure as a function of the number of embryos showing GUS expression and multiple bud formation after transferring to medium with no plant growth regulators. In general, the best results were obtained using an acceleration pressure between 5-6 kg cm^{-2}. The GUS expression in bombarded sugi embryos indicated that the pIPT5 vector was introduced, however, after culturing on plant growth regulator-free medium all of them developed deformity and became brown and died (**Fig. 9D**). None of

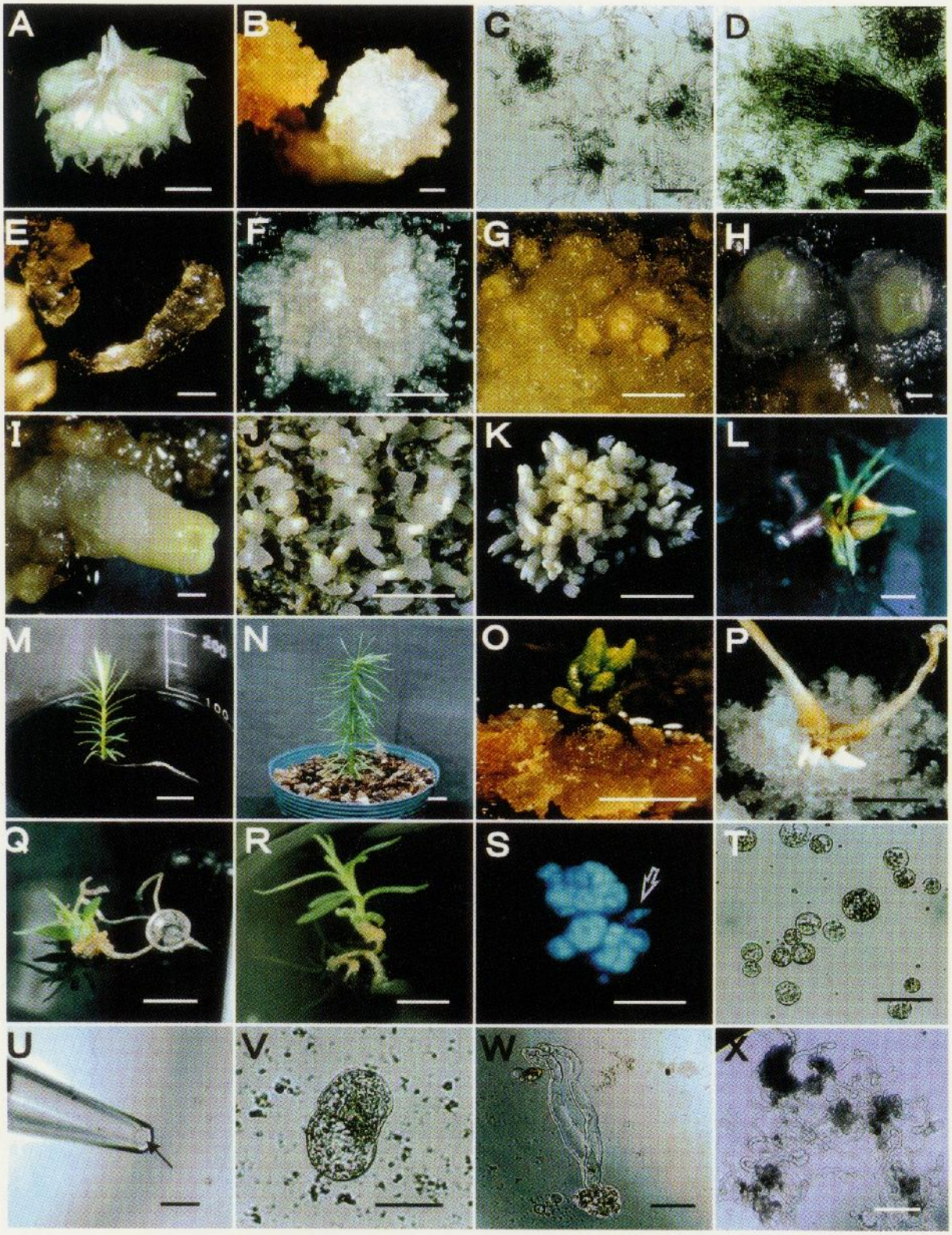

Fig. 1 Somatic embryogenesis in *Cryptomeria japonica*. **A** : Opened cone showing immature seeds (bar = 1 cm). **B** : Embryogenic cell induction (bar = 1 mm). **C** : Embryogenic cells proliferating in liquid medium (bar = 1 mm). **D** : Development of embryogenic cell in medium with no plant growth regulators (bar = 1 mm). **E-K** : Different developmental maturation stage of somatic embryos on medium containing **ABA** and **PEG** (bars : **E, H** and **I** = 1 mm; **F, G, J** and **K** = 1 cm). **L** : Germination (bar = 5 mm). **M** : Embling growth *in vitro* (bar = 1 cm). **N** : Acclimatizated embling (bar = 1 cm). **O** : Direct shoot formation on embryonal masses (bar = 1 cm). **P** : Cotyledon -like structure formation on embryonal masses and rhizogenesis from them (ber = 1 cm). **Q** and **R** : Adventitious root formation and plant development from somatic ematic embryos (bar = 1 cm). **S** : DAPI staining of cryopreserved cells showing dense embryonal heads and initiation of a suspensor (arrow) (bar = 1 mm). **T** : Isolated protoplasts (bar = 100 μm). **U** : Pick up of a protoplast using a micromanipulator (bar = 100 μm). **V** : Cell division of an individually cultured protoplast (bar = 100 μm). **W** : Formation of suspensor cells (bar = 500 μm). **X** : Embryonal proliferation from an individually cultured protoplast (bar = 1 mm).

them showed multiple bud formation. This phenomenon can be explained by the theory that CaMV35S promoter controls the expression of *ipt* gene which synthesizes the surplus cytokinin *in vivo*. This morphological shape resembled that of embryos cultured on medium with high concentration of exogenous cytokinins.

Therefore, pIPT10 and pMAT21 vectors, having the native promoter of the ipt gene were constructed and introduced into sugi embryos under the same conditions described for pIPT5 vector. The embryos with introduced pIPT10 and pMAT21 showed GUS expression (**Fig. 9B** and **9C**) and resumed normal shape producing multiple buds (**Fig. 9F**) and then rooted shoots (**Fig. 9G**). On the other hand, the formation of multiple buds from embryos (control) cultured on medium with exogenous cytokinin (**Fig. 9I**) was obviously different from that observed in bombarded embryos cultured on medium without plant growth regulators. The former produced multiple buds mostly from the apical meristem whereas the latter from the vector introduced-site. These results suggest that the endogenous cytokinin levels were increased by the expression of *ipt* gene into the putatively transformed embryos stimulating the formation of multiple buds on medium with no plant growth regulators. In contrast, nonbombarded embryos (control) when cultured on medium without plant growth regulators never produced multiple buds and led to germination and later growth (**Fig. 9H**).

The amplification of the marker sequence in several different putatively transformed embryos were analyzed by PCR (**Fig. 9E**). From this result, the presence of the GUS gene was confirmed in all transgenics, and not detected in non-transformed tissue. This result shows that the inserting vector was introduced into sugi embryos.

In a recent report on transformation of Japanese conifer trees by microprojectile bombardment (Mohri *et al.*, 2000), the authors indicated that the GUS gene was strongly expressed in *Pinus thumbergii* and *P. densiflora* but not detected in *Cryptomeria japonica*. However, under the same conditions the Luciferase gene was strongly expressed in the three species. These results suggest that factors other than physical bombardment and explant conditions may be influential for GUS expression in *C. japonica*.

4. Discussion

The induction of embryogenic cells from explants of immature seeds of sugi occurred at a relatively high frequency when material was collected from about the end of June to mid-July. These results indicated that during period the zygotic embryos are highly responsive to induced somatic embryogenesis.

Great differences among maternal sources and seed collection years were not observed. However, it is important to note that these results were obtained from a small number of trees (5 trees of one clone source, and another 3 trees from different sources)(all data not shown) and only for 3 years of seed collection. Likewise, the negative results regarding embryonal mass induction from mature seeds were achieved from only 2 lots of stored seeds. More research is needed to clarify the potential of mature stored seeds to induce somatic embryogenesis in sugi. This is important because the use of mature zygotic embryos from stored seeds provides plant material for use throughout the year and from year to year (Tautorus *et al.*, 1991).

Embryogenic cultures of sugi have been maintained and proliferated for more than 2 years. However, some cell lines that were initiated could not be maintained for a long time. Differences among cell lines with regard to proliferation rate, to morphological structure (size of embryonal heads, size, number or distribution of suspensors), as well as to plant conversion capacity were also observed. More than 50 cell lines were proliferated and of these at least 20 cell lines were tested in embryo maturation experiments obtaining cotyledonary embryos in 5 cell lines and plant conversion in only one of them (data not shown). In addition, a decrease in capacity to develop cotyledonary embryos was observed after the first year of culturing. Actually, embryogenic cells on maturation media do not develop beyond developmental stage 1 or 2. This result suggests that the capacity to develop cotyledonary embryos decreased over time or that the improvement of culture condition is needed due to the changing characteristics of embryogenic cells cultured over time. Further studies including identification and selection of individual cells are required to improve the culture system in order to be used for stable large-scale production.

In this study, a simple culture procedure for the individual culture of single protoplast of sugi was established. This procedure offers significant advantages for improvement of the tissue culture system by the identification and selection of desirable cells, as well as for genetic manipulation through direct gene transfer by such techniques as microinjection and electroporation.

On the other hand, preliminary results achieved here indicated that the microprojectile bombardment-mediated transformation of sugi will be en-

hanced together with the establishment of an stable and effective plant regeneration system.

Although the plant conversion rate obtained here was low, to our knowledge this is the first report on plant regeneration of sugi *via* somatic embryogenesis, as well as the first one with regard to the use of cryopreservation of embryogenic cells to culture individual protoplast, and the first to study the biolistic approach using pIPT and pMAT vectors. Consequently the results of this study may be considered as a first step for the genetic transformation of sugi.

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