Somatic Embryogenesis and Genetic Transformation in Cupressaceae Trees

13

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Abstract

Coniferous trees belonging to the family Cupressaceae are distributed worldwide. Several species of these conifers possess high economic value and have been a source of important forest products and raw materials for the forest industry. Somatic embryogenesis is a potential tool for clonal propagation of superior lineages within these conifers and for molecular breeding by genetic transformation. In this chapter, the technologies developed to date for somatic embryogenesis in trees of the Cupressaceae family are introduced, and various factors affecting somatic embryogenesis are discussed. In addition, methods for improving the efficiency of stable transformation via somatic embryogenesis are described.

Keywords

Chamaecyparis obtusa • *Cryptomeria japonica* • Genetic transformation • Regeneration

13.1 Introduction

The tree family Cupressaceae sensu lato comprises 32 genera, with more than 130 species, and is the only family of conifers with a cosmopolitan distribution on all continents except Antarctica (Farjón 2005; Adams et al. 2009; Debreczy et al. 2009; Yang et al. 2012). In addition, it is the most diverse of all conifers and includes important ornamental trees and shrubs used in landscaping of gardens and parks, such as the genera *Chamaecyparis*, *Cupressus*, *Juniperus*, and *Thuja*. In particular, these genera as well as other genera, including *Calocedrus*, *Cryptomeria*, *Cunninghamia*, *Sequoia*, and *Taxodium*, collectively form the leading source of timber globally. Juniper (genus *Juniperus*) is widely used as ground cover in landscaping as well as for bonsai trees. Lawson's cypress [*Chamaecyparis lawsoniana* (A. Murray bis) Parl.] and Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc.) also provide a large number of dwarf and color cultivars for horticulture (Farjón 2008).

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Somatic embryogenesis is an effective procedure for not only mass propagation of breeding strains showing preferred traits but also for providing target tissue for genetic transformation. Konar and Oberoi (1965) reported the first embryo-like structures in conifers, termed "embryoids," which were obtained from the cotyledons of the Chinese arborvitae (*Thuja orientalis* L.). Somatic embryos capable of differentiating into normal plants from embryogenic tissues initiated from immature zygotic embryos were first reported in the Norway spruce (Chalupa 1985; Hakman and von Arnold 1985); since then, plant regeneration via somatic embryogenesis has been reported in many conifers (reviewed by Gupta and Grob 1995; Stasolla and Yeung 2003). In the Cupressaceae, somatic embryogenesis has additionally been reported in six genera, namely, *Chamaecyparis*, *Cryptomeria*, *Cunninghamia*, *Cupressus*, *Juniperus*, and *Sequoia* (Table 13.1). Although reports on stable and effective procedures for somatic embryogenesis and a plant regeneration system within Cupressaceae trees are limited, Hinoki cypress, Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.), and Sugi (*Cryptomeria japonica* D. Don) have been studied intensively.

Hinoki cypress, Sawara cypress, and Sugi are widely distributed in Japan (Farjón 2010). Hinoki cypress and Sugi are the two most important commercial forest species in Japan, collectively covering approximately 65 % of the artificial forest area. The wood quality of Sawara cypress is considered to be inferior to that of Hinoki cypress; however, Sawara cypress grows faster and is much more tolerant of cold conditions (Fukuhara 1978; Maruyama et al. 2002). Breeding projects for Hinoki cypress and Sugi, such as plus-tree selection for growth, yield, stem straightness, and resistance to disease and insects, have been conducted in the past for an extended time. However, allergic reactions to pollen of these species have become a severe public health concern in Japan. Therefore, the production of Hinoki cypress and Sugi with a *no-pollen* trait is an important breeding objective.

Genetic transformation is expected to be a powerful method for shortening the breeding period of trees that require many years for the production of progeny. Simply by the introduction of specific genes, genetic transformation can induce desirable traits without unnecessary genetic transitions. Stable transformation via somatic embryogenesis of conifers has been reported in several genera, including *Picea*, *Larix*, and *Pinus* (reviewed by Malabadi and Nataraja 2007). However, stable transformation of Cupressaceae has only been reported for Hinoki cypress and Sugi (Table 13.2).

In this chapter, we will review current approaches for somatic embryogenesis in Cupressaceae trees, focusing primarily on Hinoki cypress and Sugi. We will also review the application of somatic embryogenesis in genetic transformation.

13.2 Culture Initiation

The selection of the explant is a factor that determines success in establishing an embryogenic system. Most examples of somatic embryogenesis shown in Table 13.1 used immature zygotic embryos as explants. Although somatic embryos have not been successfully obtained from immature zygotic embryos of redwood (*Sequoia sempervirens*), they have been obtained from mature embryos, cotyledons, hypocotyls, and needles (Bourgkard and Favre 1989; Favre et al. 1995; Liu et al. 2006). To date, in the Cupressaceae family, somatic embryogenesis using somatic tissues other than zygotic embryos has been reported several times for Hinoki cypress (Ishii et al. 2003), China fir (*Cunninghamia lanceolata*; Xi and Shi 2005), prickly juniper (*Juniperus oxycedrus*; Gomez and Segura 1996), and redwood (Favre et al. 1995; Liu et al. 2006). The efficiency of somatic embryogenesis reported for the aforementioned studies was very low: less than five somatic embryos per callus. Moreover, only one report on the somatic embryogenesis and organogenesis from the in vitro needle leaves derived from mature trees has been published for redwood (Liu et al. 2006). However, utilizing needle explants to initiate embryogenic systems is effective in inducing somatic embryogenesis from the

Table 13.1 Somatic embryogenesis of Cupressaceae trees

Species	Initial explants	Culture response[a]	Established in soil[b]	References
Chamaecyparis obtusa	Seedling shoots	SE, PL	Yes	Ishii et al. (2003)
	Immature zygotic embryos	EMT, SE, PL	Yes	Taniguchi et al. (2004a)
				Maruyama et al. (2005)
Chamaecyparis pisifera	Immature zygotic embryos	EMT, SE, PL	Yes	Maruyama et al. (2002)
Cryptomeria japonica	Immature, mature zygotic embryos	EMT, SE	–	Ogita et al. (1999)
	Immature zygotic embryos	EMT, SE, PL	–	Igasaki et al. (2003a)
				Igasaki et al. (2003b)
				Nakagawa et al. (2006)
	Immature zygotic embryos	EMT, SE, PL	Yes	Maruyama et al. (2000)
				Igasaki et al. (2006)
				Maruyama and Hosoi (2007)
Cunninghamia lanceolata	Cotyledons, hypocotyls	SE, PL	–	Xi and Shi (2005)
	Mature zygotic embryos	SE, PL	–	Xi and Shi (2006)
Cupressus sempervirens	Immature zygotic embryos	EMT	–	Lambardi et al. (1995)
	Immature zygotic embryos	EMT, SE, PL	–	Sallandrouze et al. (1999)
				Lambardi (2000)
Juniperus communis	Immature zygotic embryos	EMT, SE, PL	–	von Arnold and Helmersson (2009)
Juniperus oxycedrus	Needles	EMT, SE	–	Gomez and Segura (1996)
Sequoia sempervirens	Mature zygotic embryos, cotyledons, hypocotyls	EMT, SE, PL	–	Bourgkard and Favre (1988)
				Bourgkard and Favre (1989)
				Favre et al. (1995)
	Needles	EMT, SE, PL	–	Liu et al. (2006)

[a]Abbreviation: *EMT* embryogenic tissues, *SE* somatic embryos, *PL* plantlets
[b]–, no data

clonal genotype that has revealed traits as a plus tree. In addition, when using needles from in vitro shoot cultures, the explants can be supplied all year round.

Genotype affects the ability to induce embryogenic tissues. In Hinoki cypress, the induction frequency of embryogenic tissues from open-pollinated immature zygotic embryos varied from 8 % to 48 % among ten plus-tree clones (Taniguchi et al. 2004a). The induction and maintenance rates in Sugi differed considerably among 20 plus-tree clones (Taniguchi and Kondo 2000), varying from 7.5 % to 78.5 % and from 1.2 % to 27.8 %, respectively. In addition, there was a positive correlation between the initiation and maintenance rates among these clones, thus indicating that clones that initiate embryogenic tissues at high frequency tend to maintain at a high frequency during subculture. Lambardi et al. (1994, 2000) showed that embryos from

Table 13.2 Genetic transformation of Cupressaceae trees

Species	Explants	Transformation method	Transgene[a]	Stable transformation[b]	References
Chamaecyparis nootkatensis	Pollens	Particle bombardment	*GUS*, *NPTII*	–	Hay et al. (1994)
Chamaecyparis obtusa	Shoot primordia	Particle bombardment	*GUS*, *BAR*	Yes	Ishii (2002)
	Shoot primordia	Particle bombardment	*GFP*, *NTPII*	–	Taniguchi et al. (2004b)
	Embryogenic tissues	Agrobacterium	*GFP*, *NTPII*	Yes	Taniguchi et al. (2005)
Cryptomeria japonica	Embryogenic tissues	Particle bombardment	*GUS*, *NPTII*	Yes	Maruyama et al. (2000)
	Zygotic embryos	Particle bombardment	*LUC*	–	Mohri et al. (2000)
	Zygotic embryos	Particle bombardment	*GFP*, *NTPII*	–	Taniguchi et al. (2004b)
	Embryogenic tissues	Agrobacterium	*GFP*, *HPT*, *NTPII*	Yes	Taniguchi et al. (2008)
	Embryogenic tissues	Agrobacterium	*GUS*	Yes	Kurita et al. (2013)
	Embryogenic tissues	Agrobacterium	*GFP*, *GUS*, *HPT*, *NTPII*	Yes	Konagaya et al. (2013)
Cupressus sempervirens	Embryogenic tissues	Particle bombardment	*GFP*, *GUS*, *NTPII*	–	Lambardi et al. (1998)
Sequoia sempervirens	Micropropagated shoots	Agrobacterium	pRi	Yes[c]	Mihaljević et al. (1999)

[a]Abbreviation: *BAR* bialaphos resistance, *GFP* green fluorescent protein, *GUS* β-glucuronidase, *HPT* hygromycin phosphotransferase, *LUC* luciferase, *NPTII* neomycin phosphotransferase II, *pRi* T-DNA in *Agrobacterium rhizogenes* pRi plasmid

[b]–, no data

[c]Gene transfer in adventitious roots only

only five of the 24 different clones of Italian cypress were able to initiate embryogenic tissue. Moreover, the optimal seed collection date to efficiently induce embryogenic tissues was different among the clones. Similar results have been reported for Sugi (Maruyama et al. 2000). These results suggest different timing of the developmental stages of embryo among the clones.

Several reports have suggested that seed collection dates are a critical factor for efficiently inducing embryogenic tissues from zygotic embryos (Ogita et al. 1999; Lambardi 2000; Maruyama et al. 2000; Igasaki et al. 2003a; Taniguchi et al. 2004a). In the Italian cypress (*Cupressus sempervirens*), embryogenic tissues have been reported to originate from embryos that were morphologically at the same stage of maturity, i.e., an early cotyledonary stage characterized by the two cotyledons that have just differentiated and are still tightly joined (Lambardi 2000). Taniguchi et al. (2004a) reported that embryogenic tissues of Hinoki cypress were induced from the immature seeds collected at the beginning of July containing pre-cotyledonary embryos (stages 6–11 as defined by Nagmani et al. 1995). During the middle of July, zygotic embryos had cotyledonary primordia (stage 12) or elongated cotyledons (stage 13), but megagametophytes containing such embryos could not induce embryogenic tissue. Similarly, in Sugi, the pre-cotyledonary embryo stage was also optimal (Igasaki et al. 2003a). Therefore, it is important to determine the optimal development stages in each tree species as well as genotypes.

13.2.1 Embryogenic Tissue Induction and Proliferation in Hinoki Cypress

At the beginning of July, we collected immature seeds of Hinoki cypress that contained precotyledonary embryos. Immature seeds were extracted from the sterilized cone, and the seeds were again surface sterilized with 70 % ethanol for 1 min followed by 6 % H_2O_2 for 5 min and then rinsed three times with sterilized distilled water. Explants (megagametophytes that contained intact immature zygotic embryos) were removed from the seeds using tweezers and a scalpel under a stereomicroscope. The explants were placed on Smith standard embryonic tissue capture medium (SM1; Smith 1996) containing sucrose (sugar source) and activated charcoal, but no plant growth regulator. Within 2–3 weeks of the explant being cultured on SM1, embryogenic tissue was induced from the micropylar end of the megagametophyte (Fig. 13.1a). After 4 weeks of culturing on SM1, embryogenic tissues that had been induced from the explants were transferred to Smith standard embryogenesis medium (SM2; Smith 1996) containing amino acids, but no activated charcoal or plant growth regulator, and cultured for 4 weeks. Following this 4-week culture period, the embryogenic tissues were transferred to Smith embryo development medium (SM3; Smith 1996), which had the same composition as SM2, but contained higher concentrations of amino acids and was solidified by Gelrite. The tissues were then subcultured at 2–3-week intervals on this medium. After 10 weeks of culture, the clumps of embryogenic tissue of each cell line varied greatly in size, with the largest being 2 cm in diameter. The embryogenic tissue was able to proliferate on SM3 (Fig. 13.1b), with an approximately threefold growth rate every 3 weeks (Taniguchi et al. 2004a).

13.2.2 Embryogenic Tissue Induction and Proliferation in Sugi

In Sugi, we collected immature seeds early during July, and explants were extracted from the seeds in the same manner as with Hinoki cypress. The explants were placed on initiation medium (IM), which consisted of half-strength MS medium (Murashige and Skoog 1962) supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose, and 8 g/l agar (Taniguchi and Kondo 2000). Within 2–3 weeks of the explant being cultured on IM, embryogenic tissue was induced from the micropylar end of the megagametophyte (Fig. 13.2a, b). After 4 weeks of culture on IM, embryogenic tissues were subcultured at 2-week intervals on maintenance medium (MM), which had the same composition as IM, except that it contained 1 g/l casein hydrolysate, 0.5 g/l L-glutamine, 2 μM 2, 4-D, and 50 % of the inorganic nitrate salt composition of IM and was gelled using 4.5 g/l Gelrite (Fig. 13.2c; Taniguchi and Kondo 2000; Taniguchi et al. 2008).

13.3 Somatic Embryo Maturation and Germination

In general, somatic embryo maturation is accelerated by adding abscisic acid (ABA) or a penetrating osmoticant (e.g., sugars, sugar alcohols) or nonpenetrating osmoticant [e.g., polyethylene glycol (PEG)] to the culture medium, which induces water stress (Lipavská and Konrádová 2004). Mature somatic embryos in Hinoki cypress were efficiently formed on the medium containing a combination of 100 μM ABA, 30 g/l maltose as sugars, and 150 g/l PEG (Taniguchi et al. 2004a). These results are consistent with those for other trees of Cupressaceae, such as Sawara cypress (Maruyama et al. 2002) and Sugi (Taniguchi 2007). Furthermore, the effect of various concentrations of Gelrite as a support medium on somatic embryo yield was investigated in the Hinoki cypress, which indicated that the most suitable concentration was 3.0 g/l (Taniguchi et al. 2004a). Although Maruyama et al. (2002) induced the somatic embryos of Sawara cypress on medium containing 5.0 g/l Gelrite, they did not test a lower concentration of Gelrite for somatic embryo induction. In *Pinus taeda*, somatic embryos were induced on a medium

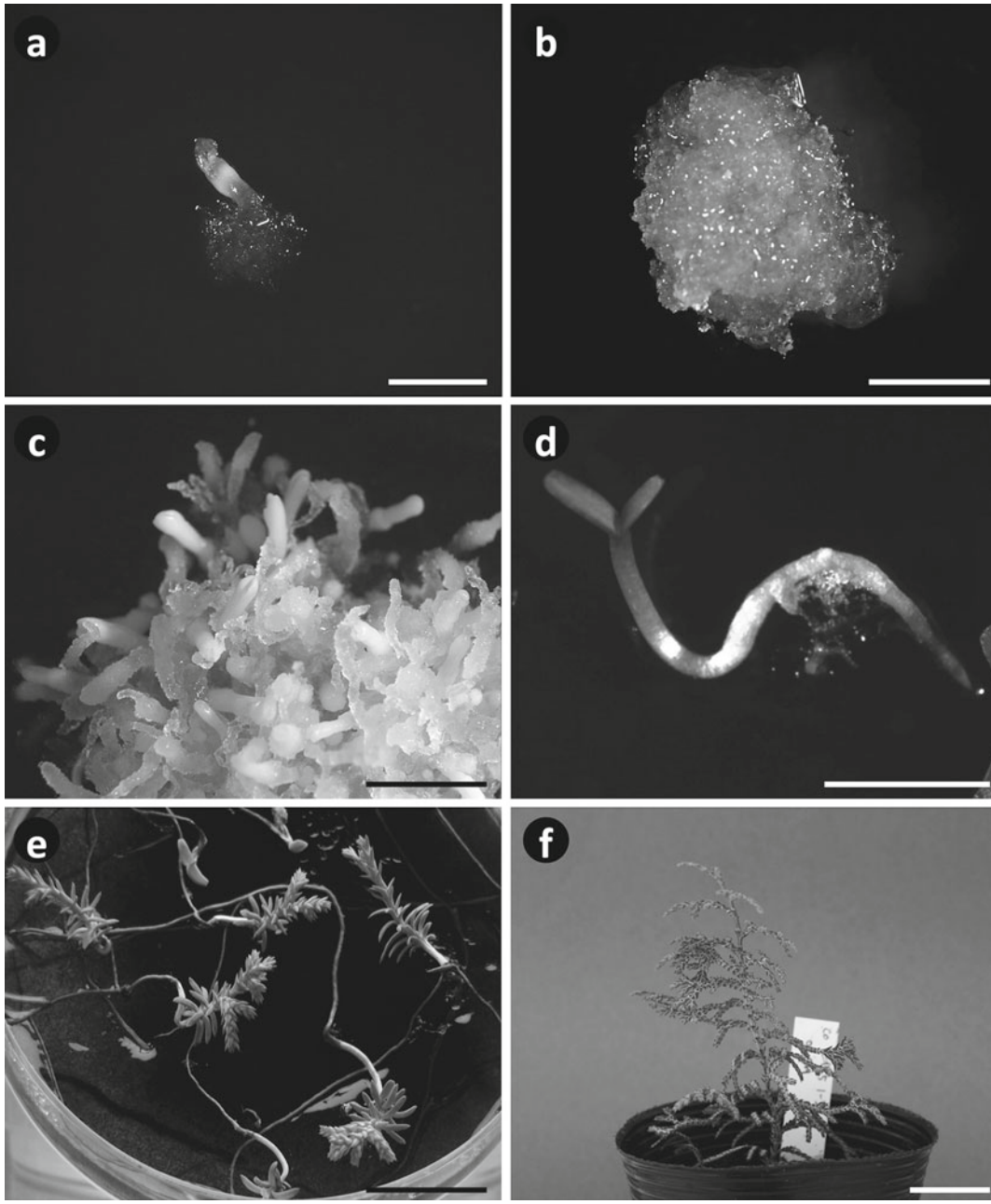

Fig. 13.1 Plant regeneration via somatic embryogenesis from immature embryo cultures in Hinoki cypress (*Chamaecyparis obtusa*; modified after Taniguchi et al. 2004a). (**a**) Embryogenic tissues induced from a megagametophyte explant. (**b**) Embryogenic tissue maintained on SM3 medium. (**c**) Cotyledonary somatic embryos after 8 weeks of culture on maturation medium. (**d**) Germination of a somatic embryo after 1 week of culture on germination medium. (**e**) Plantlets after 2 months of culture on germination medium. (**f**) Regenerated plant following acclimatization. *Bars*: 2 mm (**a**); 4 mm (**b**, **c**, **d**); 2 cm (**e**, **f**)

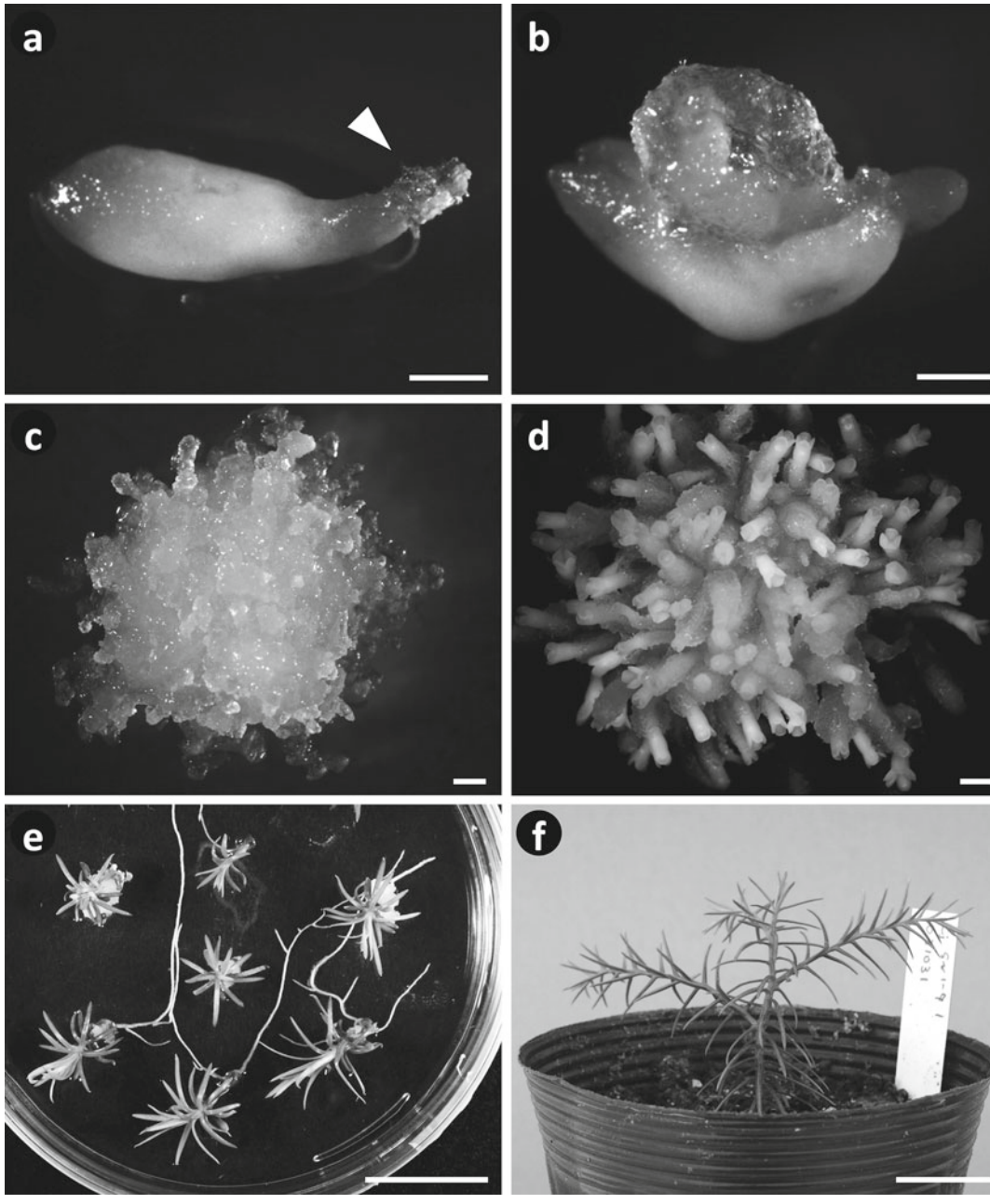

Fig. 13.2 Plant regeneration via somatic embryogenesis from immature embryo cultures in Sugi (*Cryptomeria japonica*). (**a**) Embryogenic tissues induced from the micropylar end of the megagametophyte. (**b**) Embryogenic tissues induced from an incision site in the megagametophyte (**c**) Embryogenic tissue maintained on MM medium. (**d**) Cotyledonary somatic embryos after 6 weeks of culture on maturation medium. (**e**) Plantlets after 2 months of culture on germination medium. (**f**) Regenerated plant following acclimatization. *Bars*: 1 mm (**a, b**, **c**, **d**); 2 cm (**e**, **f**)

containing 2.5 g/l Gelrite (Pullman et al. 2003). A high concentration of Gelrite is thought to be unsuitable for somatic embryogenesis, as it has been reported that Gelrite at high concentrations reduces the bioavailability of magnesium, calcium, zinc, and manganese in the medium (Van Winkle et al. 2003).

Recently, research has determined the peptide hormones that play an important role in plant cell growth and development (Ryan and Pearce 2001). An example of these hormones is phytosulfokine (PSK), which is a five-amino-acid sulfated peptide that is involved in the initial steps of cellular differentiation, proliferation, and redifferentiation (Igasaki et al. 2003b; Matsubayashi et al. 2004). PSK has also been shown to stimulate somatic embryogenesis in carrot, Sugi, Japanese larch, and *Daucus* species (Kobayashi et al. 1999; Igasaki et al. 2003b; Umehara et al. 2005; Mackowska et al. 2014) as well as induce cell division in suspension and protoplast cultures (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997; Grzebelus et al. 2012). It has also been reported to improve *Agrobacterium*-mediated transformation efficiency (Matsubayashi et al. 2004; Chen et al. 2005). Igasaki et al. (2003b) described how the addition of PSK to the medium results in not only the promotion of the growth and maintenance of embryogenic tissues in Sugi but also a dramatic stimulatory effect on the formation of somatic embryos. These results suggest the possibility of improving the efficiency of the embryogenic and transformation systems in conifers by utilization of PSK.

The ability of embryogenic tissues to produce somatic embryos is also affected by the genotype. We produced somatic embryos for nine clones among ten open-pollinated plus-tree clones of Hinoki cypress, and somatic embryo-forming frequency varied from 4.0 % to 24 % (number of somatic embryo forming cell lines per explants). Somatic embryos of Sugi were produced for all clones among six open-pollinated plus-tree clones, and the somatic embryo-forming frequency varied from 4.0 % to 20 % (Taniguchi 2007). Recently, we investigated the somatic embryo-forming frequency for immature zygotic embryos yielded from artificial crossing among plus trees. As a result, a certain family formed between the specific pollen and seed parent was shown to efficiently produce somatic embryos (unpublished data). This result suggests the possibility that this family harbors the genes to improve somatic embryogenesis.

13.3.1 Somatic Embryo Maturation in Hinoki Cypress and Sugi

To facilitate the maturation of somatic embryos in Hinoki cypress and Sugi, the embryogenic tissues subcultured on SM3 were transferred to a maturation medium (Taniguchi et al. 2004a) consisting of SM3 basal salts, vitamins, amino acids, 2 g/l activated charcoal, 100 μM abscisic acid, 150 g/l polyethylene glycol 4000, 3.0 g/l Gelrite, and 30 g/l maltose. The first cotyledonary embryos appeared at approximately 5 weeks of culture and presented in high numbers by 7–8 weeks of culture (Figs. 13.1c and 13.2d).

13.3.2 Germination in Hinoki Cypress and Sugi

For germination in Hinoki cypress, the mature embryos were cultured on woody plant medium (Lloyd and McCown 1980), which contained 2 g/l activated charcoal, 20 g/l sucrose, and 5 g/l Gelrite. Within 1 week of the mature embryos being placed on germination medium, the embryos rooted and elongated their hypocotyls and cotyledons (Fig. 13.1d). Although the frequency of germination on the medium without activated charcoal was comparable to that on the medium containing charcoal, the germinant grew less vigorously. This result demonstrated that activated charcoal is effective in facilitating the germination of mature somatic embryos in Hinoki cypress (Taniguchi et al. 2004a). After 2 months of culture on germination medium, the germinants with elongated epicotyls (approx. 1 cm long, Fig. 13.1e) were transferred to pots containing soil for acclimatization. All plantlets successfully acclimated and continued growing in the greenhouse (Fig. 13.1f).

The mature embryos of Sugi were cultured on germination medium, which was a modified version of the Gresshoff and Doy medium (Okamura and Kondo 1995) containing 10 g/l sucrose and 5 g/l Gelrite. After 6 weeks of the mature embryos being placed on germination medium, the embryos rooted and elongated their hypocotyls and cotyledons (Fig. 13.2e). The germinants were subcultured every other month on this germination medium. After approximately 3 months, plantlets were transferred to half-strength woody plant medium, which contained 20 g/l sucrose and 2 g/l Gelrite. Elongated plantlets (approx. 10 cm long) were transferred to pots containing soil for acclimatization (Fig. 13.2f).

13.4 Genetic Transformation

Genetic transformation within Cupressaceae was first attempted by particle bombardment using yellow cypress (*Chamaecyparis nootkatensis*) pollen (Hay et al. 1994) and Italian cypress embryogenic tissues (Lambardi et al. 1998). However, only transient expression was observed. By employing *Agrobacterium rhizogenes* to introduce T-DNA derived from Ri plasmid, Mihaljević et al (1999) succeeded in inducing adventitious roots in micropropagated shoot explants of redwood. However, whole plant regeneration of stable transformants has only been reported in Hinoki cypress and Sugi.

Gene delivery to shoot primordia of Hinoki cypress by particle bombardment and the subsequent regeneration of the transformed plantlets were first reported by Ishii (2002). In an earlier investigation, Taniguchi et al. (2004b) similarly attempted the genetic transformation of Hinoki cypress following this method, and although transiently transformed cells were obtained, they failed to produce transformed plants (Taniguchi et al. 2005). With regard to Sugi, there are some reports of particle bombardment, for example, transient expression of the luciferase gene (Mohri et al. 2000) and green fluorescent protein (*GFP*) gene (Taniguchi et al. 2004b) in zygotic embryos. Maruyama et al. (2000) reported bud formation from embryos after introducing the β-glucuronidase gene. Generally, the *Agrobacterium*-mediated method obtains a higher transformation frequency of conifers than the particle bombardment method. Accordingly, we have developed *Agrobacterium tumefaciens*-mediated transformation methods of embryogenic tissue in Hinoki cypress (Taniguchi et al. 2005) and Sugi (Taniguchi et al. 2008, Konagaya et al. 2013). The highest transformation frequency obtained in Hinoki cypress was 22.5 independent transformed lines per dish (250 mg embryogenic tissue). Moreover, we attempted to improve the transformation efficiency in Sugi (Konagaya et al. 2013).

13.4.1 Culture Supports for Cocultivation

Unsuitable cocultivation conditions in *Agrobacterium*-mediated transformation may lead to unfavorable effects, such as bacterial overgrowth and/or tissue necrosis, thereby reducing the transformation efficiency. It has been suggested that an optimal concentration of *Agrobacterium* is required for transformation. Recently, it was reported that the use of filter paper wicks during cocultivation contributed to the efficient transformation of cucumber, kabocha squash, and rice by controlling the growth of *Agrobacterium* (Ozawa 2009; Nanasato et al. 2011, 2013). Therefore, we attempted to determine the optimal cocultivation conditions with filter paper wicks in Sugi. After the embryogenic tissues and *Agrobacterium* were suspended in liquid medium, a filter paper with the embryogenic tissues was placed on a solid cocultured medium or on three sheets of filter paper moistened with 5.5 mL of liquid cocultured medium. The mean number of *Agrobacterium* cells (>17-fold) obtained in the liquid medium conditions was significantly lower than that obtained on the solid medium using the conventional method (Taniguchi et al. 2008).

Compared with cocultivation on the solid medium, culturing in the liquid medium significantly improved the transformation efficiency (>16-fold). Ozawa (2009) showed that liquid

medium-moistened filter paper wicks regulated the growth rate of *Agrobacterium* in an effective manner, which improved the cell viability in the transformed callus obtained from rice. Previously, Nanasato et al. (2011, 2013) showed that filter paper wicks increased the *Agrobacterium* infection efficiency in cucumber and kabocha squash. These reports and our experimental results support the utility of filter paper wicks in cocultivation procedures.

13.4.2 Antibiotics to Eliminate *Agrobacterium*

An efficient *Agrobacterium*-mediated transformation system requires the use of antibiotics to eliminate bacteria; the antibiotics should have negligible effects on the growth potential of the transformed cells. Recently, it was reported that meropenem, a novel β-lactam antibiotic, is highly effective against *Agrobacterium*. Also, the transformation efficiencies observed in tobacco, tomato, rice, and apple were higher with meropenem than with other β-lactam antibiotics, such as cefotaxime, carbenicillin, and cefbuperazone (Ogawa and Mii 2004, 2007; Li et al. 2011). In contrast to meropenem, it has been observed that cefotaxime and carbenicillin have negative effects on the growth of embryonic tissue, somatic embryogenesis, and shoot regeneration in woody plants such as Norway spruce, cacao, orange, and pomegranate (Terakami et al. 2007; Malá et al. 2009; Mendes et al. 2009; Silva et al. 2009). However, meropenem had no inhibitory effects on the embryogenic tissue growth in Sugi, even at a higher concentration of 40 mg/l. Similar results with meropenem have been reported using Norway spruce embryogenic tissue (Malá et al. 2009). Moreover, embryogenic tissue growth was significantly higher at lower concentrations (5 mg/l and 10 mg/l) using medium containing meropenem. Based on these results, 10 mg/l was selected as the meropenem concentration in subsequent transformation experiments.

Application of meropenem treatment on Sugi transformation indicated that the transformation efficiency was increased approximately twofold with meropenem than with carbenicillin using the conventional method. In three replicate transformation experiments using our improved method (cocultivation on filter paper wicks and *Agrobacterium* elimination with meropenem), the mean transformation efficiency (the number of GFP-positive colonies per gram of cocultivated embryogenic tissues) was 105.3 ± 9.02. By contrast, the mean transformation efficiency achieved using the conventional method (cocultivation on Gelrite-solidified medium and *Agrobacterium* elimination by carbenicillin) was 3.5 ± 0.71. Thus, the transformation efficiency was increased approximately 30-fold by the improved method than with the conventional method. Moreover, the regrowth of *Agrobacterium* was occasionally observed after cocultivation when using the conventional method. However, no agrobacterial regrowth was observed with the improved method. Similar regrowth control using meropenem was reported after the transformation of *Phalaenopsis* (Sjahril and Mii 2006). These results suggest that the improved method prevents excess *Agrobacterium* growth during cocultivation, and transformed embryogenic tissues are efficiently selected using meropenem, even at low concentrations. High transformation efficiency values (colonies per gram of cocultivated embryogenic tissues) have been reported in conifer plants, such as 42 in *Larix* (Lelu and Pilate 2000), 67.3 in *Pinus* (Trontin et al. 2002), 60–1280 in *Picea* (Klimaszewska et al. 2001), and 90 in Hinoki cypress (Taniguchi et al. 2005). Thus, the transformation efficiency value that we achieved is one of the highest compared with those obtained using previously reported methods.

13.5 Conclusions and Future Prospects

Much research into somatic embryogenesis in Cupressaceae indicates that factors such as explant types and the developmental stage, genotypes, composition, and the supplements of the culture medium play an important role in improving the efficiency of embryogenic tissue induction and somatic embryogenesis. The *Agrobacterium*-

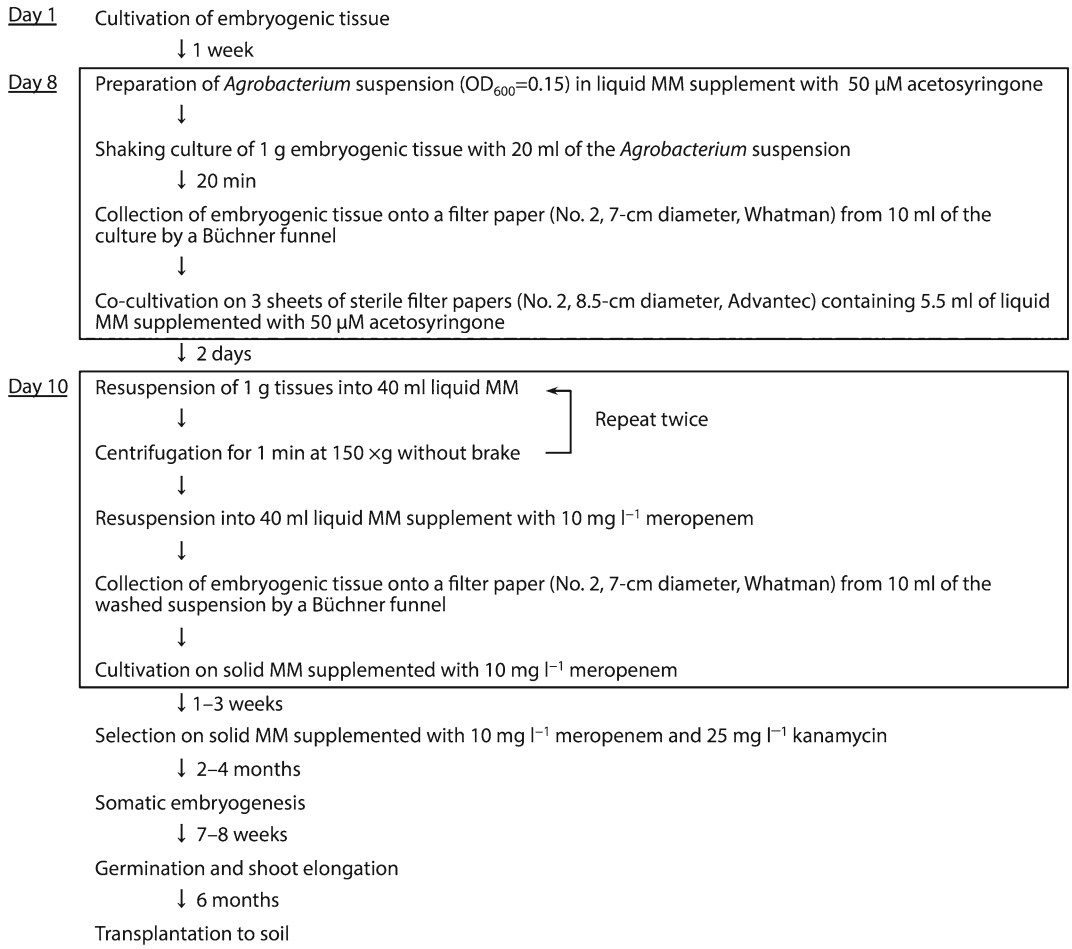

Fig. 13.3 Steps in the transformation of Sugi (Konagaya et al. 2013)

mediated transformation frequency of Sugi significantly increased using the improved method (Fig. 13.3). Currently, we have succeeded in producing the *no-pollen* Sugi by introducing a combination of the male gametophyte-specific promoter (Kurita et al. 2013) and the cytotoxic gene (manuscript in preparation). Although reports of a stable and effective procedure for somatic embryogenesis and genetic transformation of Cupressaceae are limited to *Chamaecyparis* trees and Sugi, these effective methods might be applied to other coniferous trees as well as other Cupressaceae trees. We hope that advances in clonal propagation and molecular breeding technology are accelerated in many coniferous trees.

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References

Adams PR, Bartel JA, Price RA (2009) A new genus, *Hesperocyparis*, for the cypresses of the Western Hemisphere (Cupressaceae). Phytologia 91:160–185

Bourgkard F, Favre JM (1988) Somatic embryos from callus of *Sequoia sempervirens*. Plant Cell Rep 7:445–448

Bourgkard F, Favre JM (1989) Somatic embryogenesis in *Sequoia sempervirens*: present possibilities and limits. Annales de Recherches Sylvicoles. Association Foret-Cellulose (AFOCEL), Paris, France, pp 83–95

Chalupa V (1985) Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.). Karst Commun Inst For 14:57–63

Chen D, Xu Z, Ma B, Li S (2005) Effect of phytosulfokine-α on *Agrobacterium*-mediated transformation in rice. Rice Sci 12:255–260

Debreczy Z, Musial K, Price RA, Rácz I (2009) Relationships and nomenclatural status of the nootka cypress (*Callitropsis nootkatensis*, Cupressaceae). Phytologia 91:140–158

Farjón A (2005) A monograph of Cupressaceae and Sciadopitys. Royal Botanic Gardens, Kew, Richmond, Surrey

Farjón A (2008) A natural history of conifers. Timber Press, Portland

Farjón A (2010) A handbook of the world conifers, vol 1. Brill Press, Leiden

Favre JM, Tranvan H, Arnaud Y, Bourgkard F (1995) Present state of somatic embryogenesis m *Sequoia sempervirens* (Lamb.) Endl. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 3., pp 357–370

Fukuhara N (1978) Meiotic observation in the pollen mother cell of interspecific hybrid between *Chamaecyparis obtusa* and *C. pisifera*. J Jpn For Soc 60:437–441

Gomez MP, Segura J (1996) Morphogenesis in leaf and single-cell cultures of mature Juniperus oxycedrus. Tree Physiol 16:681–686

Grzebelus E, Szklarczyk M, Gren J, Sniegowska K, Jopek M, Kacinska I, Mrozek K (2012) Phytosulfokine stimulates cell divisions in sugar beet (*Beta vulgaris* L.) mesophyll protoplast cultures. Plant Growth Regul 67:93–100

Gupta PK, Grob JA (1995) Somatic embryogenesis in conifers. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 1. Kluwer, Dordrecht, pp 81–98

Hakman I, von Arnold S (1985) Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J Plant Physiol 121:149–158

Hay I, Lachance DA, von Aderkas P, Charest PJ (1994) Transient chimeric gene expression in pollen of five conifer species following microparticle bombardment. Can J For Res 24:2417–2423

Igasaki T, Sato T, Akashi T, Mohri T, Maruyama E, Kinoshita I, Walter C, Shinohara K (2003a) Somatic embryogenesis and plant regeneration from immature zygotic embryos of *Cryptomeria japonica* D. Don. Plant Cell Rep 22:239–243

Igasaki T, Akashi T, Ujino-Ihara T, Matsubayashi M, Sakagami Y, Shinohara K (2003b) Phytosulfokine stimulates somatic embryogenesis in *Cryptomeria japonica*. Plant Cell Physiol 44:1412–1416

Igasaki T, Akashi T, Shinohara K (2006) Somatic embryogenesis in *Cryptomeria japonica* D. Don: gene for phytosulfokine (PSK) precursor. Somatic Embryogenesis. Plant Cell Monogr 2:201–213

Ishii K (2002) Liquid culture and transformation of Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc.). J For Res 7:99–104

Ishii K, Maruyama E, Hosoi Y (2003) Plant regeneration by somatic embryogenesis from *in vitro*–cultured shoots of Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc). Propag Ornam Plant 3:19–22

Klimaszewska K, Lachance D, Pelletier G, Lelu M-A, Séguin A (2001) Regeneration of transgenic *Picea glauca*, *P. mariana*, and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol Plant 37:748–755

Kobayashi T, Eun CH, Hanai H, Matsubayashi Y, Sakagami Y, Kamada H (1999) Phytosulphokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. J Exp Bot 50:1123–1128

Konagaya K, Kurita M, Taniguchi T (2013) High-efficiency *Agrobacterium*-mediated transformation of *Cryptomeria japonica* D. Don by co-cultivation on filter paper wicks followed by meropenem treatment to eliminate *Agrobacterium*. Plant Biotechnol 30:523–528

Konar RN, Oberoi YP (1965) *In vitro* development of embryoids on the cotyledons of *Biota orientalis*. Phytomorph 15:137–140

Kurita M, Konagaya K, Watanabe A, Kondo T, Ishii K, Taniguchi T (2013) The promoter of an *A9* homolog from the conifer *Cryptomeria japonica* imparts male strobilus-dominant expression in transgenic trees. Plant Cell Rep 32:319–328

Lambardi M (2000) Somatic embryogenesis in cypress (*Cupressus sempervirens* L.). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 6. Kluwer, Dordrecht, pp 553–567

Lambardi M, Menabeni D, Wilson SM (1994) Sviluppo di embrioni somatici ed isolamento di protoplasti da tessuto sospensoriale embriogenico di cipresso (*Cupressus sempervirens* L.). Monti Boschi 45:53–59

Lambardi M, Harry IS, Menabeni D, Thorpe TA (1995) Organogenesis and somatic embryogenesis in *Cupressus sempervirens*. Plant Cell Tiss Org Cult 40:179–182

Lambardi M, Lachance D, Séguin A, Charest PJ (1998) Evaluation of microprojectile-mediated DNA delivery and reporter genes for genetic transformation of the Mediterranean cypress (*Cupressus sempervirens* L.). Plant Cell Rep 18:198–202

Lelu MA, Pilate G (2000) Transgenic in *Larix*. In: Jain SM, Minocha SC (eds) Molecular biology of woody plants, vol 2. Kluwer, Dordrecht, pp 119–134

Li J, Komori S, Sasaki K, Mimida N, Matsumoto S, Wada M, Soejima J, Ito Y, Masuda T, Tanaka N, Shigeta N (2011) Pre-culture before *Agrobacterium* infection to leaf segments and meropenem improves the transformation efficiency of apple (*Malus*×*domestica* Borkh.). J Jpn Soc Hortic Sci 80:244–254

Lipavská H, Konrádová H (2004) Somatic embryogenesis in conifers: the role of carbohydrate metabolism. In Vitro Cell Dev Biol Plant 40:23–30

Liu C, Xia X, Yin W, Huang L, Zhou J (2006) Shoot regeneration and somatic embryogenesis from needles of redwood (*Sequoia sempervirens* (D.Don.) Endl.). Plant Cell Rep 256:21–628

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot tip culture. Proc Int Plant Propag Soc 30:421–427

Mackowska K, Jarosz A, Grzebelus E (2014) Plant regeneration from leaf-derived protoplasts within the *Daucus* genus: effect of different conditions in alginate embedding and phytosulfokine application. Plant Cell Tiss Org Cult 117:241–252

Malá J, Pavingerová D, Cvrčková H, Bříza J, Dostál J, Šíma P (2009) Tolerance of Norway spruce (*Picea abies* [L.] Karst.) embryogenic tissue to penicillin, carbapenem and aminoglycoside antibiotics. J For Sci 55:156–161

Malabadi RB, Nataraja K (2007) Genetic transformation of conifers: applications in and impacts on commercially forestry. Transgenic Plant J 1:289–313

Maruyama E, Hosoi Y (2007) Polyethylene glycol enhance somatic embryo production in Japanese cedar (*Cryptomeria japonica* d. Don). Propag Ornam Plant 7:57–61

Maruyama E, Tanaka T, Hosoi Y, Ishii K, Morohoshi N (2000) Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese Cedar (*Cryptomeria japonica* D. Don). Plant Biotechnol 17:281–296

Maruyama E, Hosoi Y, Ishii K (2002) Somatic embryogenesis in Sawara Cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) for stable and efficient plant regeneration, propagation and protoplast culture. J For Res 7:23–34

Maruyama E, Ishii K, Hosoi Y (2005) Efficient plant regeneration of Hinoki cypress (*Chamaecyparis obtusa*) via somatic embryogenesis. J For Res 10:73–77

Matsubayashi Y, Sakagami Y (1996) Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. Proc Natl Acad Sci U S A 93:7623–7627

Matsubayashi Y, Takagi L, Sakagami Y (1997) Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high-and low-affinity binding sites. Proc Natl Acad Sci U S A 94:13357–13362

Matsubayashi Y, Goto T, Sakagami Y (2004) Chemical nursing: phytosulfokine improves genetic transformation efficiency by promoting the proliferation of surviving cells on selective media. Plant Cell Rep 23:155–158

Mendes AF da S, Cidade LC, Oliveira MLP de, Otoni WC, Soares-Filho W dos S, Costa MGC (2009) Evaluation of novel beta-lactam antibiotics in comparison to cefotaxime on plant regeneration of *Citrus sinensis* L. Osb. Plant Cell Tiss Org Cult 97:331–336

Mihaljević S, Katavić V, Jelaska S (1999) Root formation in micropropagated shoots of *Sequoia sempervirens* using *Agrobacterium*. Plant Sci 141:73–80

Mohri T, Igasaki T, Sato T, Shinohara K (2000) Expression of genes for β-glucuronidase and luciferase in three species of Japanese conifer (*Pinus thunbergii*, *P. densiflora* and *Cryptomeria japonica*) after transfer of DNA by microprojectile bombardment. Plant Biotechnol 17:49–54

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

Nagmani R, Diner AM, Carton S, Zipf AE (1995) Anatomical comparison of somatic and zygotic embryogeny in conifers. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 1. Kluwer, Dordrecht, pp 23–48

Nakagawa R, Ogita S, Kubo T, Funada R (2006) Effect of polyamines and L-ornithine on the development of proembryogenic masses of *Cryptomeria japonica*. Plant Cell Tiss Org Cult 85:229–234

Nanasato Y, Konagaya K, Okuzaki A, Tsuda M, Tabei Y (2011) *Agrobacterium*-mediated transformation of kabocha squash (*Cucurbita moschata* Duch) induced by wounding with aluminum borate whiskers. Plant Cell Rep 30:1455–1464

Nanasato Y, Konagaya K, Okuzaki A, Tsuda M, Tabei Y (2013) Improvement of *Agrobacterium*-mediated transformation of cucumber (*Cucumis sativus* L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. Plant Biotechnol Rep 7:267–276

Ogawa Y, Mii M (2004) Screening for highly active β-lactam antibiotics against *Agrobacterium tumefaciens*. Arch Microbiol 181:331–336

Ogawa Y, Mii M (2007) Meropenem and moxalactam: novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. Plant Sci 172:564–572

Ogita S, Ishikawa H, Kubo T, Sasamoto H (1999) Somatic embryogenesis from immature and mature zygotic embryos of *Cryptomeria japonica* I: embryogenic cell induction and its morphological characteristics. J Wood Sci 45:87–91

Okamura M, Kondo T (1995) Manual for tissue culture of pine. Bull For Tree Breed Center 13:139–143

Ozawa K (2009) Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). Plant Sci 176:522–527

Pullman GS, Johnson S, Peter G, Cairney J, Xu N (2003) Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. Plant Cell Rep 21:747–758

Ryan CA, Pearce G (2001) Polypeptide hormones. Plant Physiol 125:65–68

Sallandrouze A, Faurobert M, Maataoui MEI, Espagnac H (1999) Two-dimensional electrophoretic analysis of proteins associated with somatic embryogenesis

development in *Cupressus sempervirens* L. Electrophoresis 20:1109–1119

Silva TER, Cidade LC, Alvim FC, Cascardo JCM, Costa MGC (2009) Studies on genetic transformation of *Theobroma cacao* L.: evaluation of different polyamines and antibiotics on somatic embryogenesis and the efficiency of *uidA* gene transfer by *Agrobacterium tumefaciens*. Plant Cell Tiss Org Cult 99:287–298

Sjahril R, Mii M (2006) High-efficiency *Agrobacterium*-mediated transformation of *Phalaenopsis* using meropenem, a novel antibiotic to eliminate *Agrobacterium*. J Hortic Sci Biotechnol 81:458–464

Smith DR (1996) Growth medium. US Patent 5,565,355

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Org Cult 74:15–35

Taniguchi T (2007) Somatic embryogenesis and genetic transformation in forest tree species. Bull For Tree Breed Center 23:63–119

Taniguchi T, Kondo T (2000) Difference in the ability of initiation and maintenance of embryogenic cultures among Sugi (*Cryptomeria japonica* D. Don) seed families. Plant Biotechnol 17:159–162

Taniguchi T, Kurita M, Itahana N, Kondo T (2004a) Somatic embryogenesis and plant regeneration from immature zygotic embryos of Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc.). Plant Cell Rep 23:26–31

Taniguchi T, Ohmiya Y, Kurita M, Kondo T (2004b) Transient expression of the green fluorescent protein gene in *Cryptomeria japonica*, *Chamaecyparis obtusa*, *Pinus densiflora*, *Pinus thunbergii*, *Larix kaempferi* and *Acanthopanax sciadophylloides* following particle bombardment. Bull For Tree Breed Center 20:1–8

Taniguchi T, Kurita M, Ohmiya Y, Kondo T (2005) *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue and transgenic plant regeneration in *Chamaecyparis obtusa* Sieb. et Zucc. Plant Cell Rep 23:796–802

Taniguchi T, Ohmiya Y, Kurita M, Tsubomura M, Kondo T (2008) Regeneration of transgenic *Cryptomeria japonica* D. Don after *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue. Plant Cell Rep 27:1461–1466

Terakami S, Matsuta N, Yamamoto T, Sugaya S, Gemma H, Soejima J (2007) Agrobacterium-mediated transformation of the dwarf pomegranate (*Punica granatum* L. var. *nana*). Plant Cell Rep 26:1243–1251

Trontin JF, Harvengt L, Garin E, Lopez-Vernaza M, Arancio L, Hoebeke J, Canlet F, Pâques M (2002) Towards genetic engineering of maritime pine (*Pinus pinaster* Ait.). Ann For Sci 59:687–697

Umehara M, Ogita S, Sasamoto H, Eun CH, Matsubayashi Y, Sakagami Y, Kamada H (2005) Two stimulatory effects of the peptidyl growth factor phytosulfokine during somatic embryogenesis in Japanese larch (*Larix leptolepis* Gordon). Plant Sci 169:901–907

Van Winkle SC, Johnson S, Pullman GS (2003) The impact of gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. Plant Cell Rep 21:1175–1182

von Arnold S, Helmersson A (2009) Embryogenic cell lines of *Juniperus communis*; easy establishment and embryo maturation, limited germination. Plant Cell Tiss Org Cult 96:211–217

Xi M, Shi J (2005) Organogenesis and somatic embryogenesis from cotyledons and hypocotyls of *Cunninghamia lanceolata* Hook. Mol Plant Breed 3:846–852

Xi M, Shi J (2006) Organogenesis and somatic embryogenesis from mature zygotic embryos of *Cunninghamia lanceolata*. Scientia Silvae Sinicae 42:29–35

Yang ZY, Ran JH, Wang XQ (2012) Three genome-based phylogeny of Cupressaceae s.l.: further evidence for the evolution of gymnosperms and Southern Hemisphere biogeography. Mol Phylogenet Evol 64:452–470