A protocol for \textit{Agrobacterium}-mediated transformation of Japanese cedar, Sugi (\textit{Cryptomeria japonica} D. Don) using embryogenic tissue explants

Ken-ichi Konagaya*, Yoshihiko Nanasato, Toru Taniguchi

Forest Bio-Research Center, Forestry and Forest Products Research Institute (FFPRI), 3809-1 Ishi, Juo, Hitachi, Ibaraki 319-1301, Japan

*E-mail: konagaya@affrc.go.jp  Tel: +81-294-33-7348  Fax: +81-294-39-7306

Received October 31, 2019; accepted January 31, 2020 (Edited by Y. Tabei)

Abstract  Sugi (\textit{Cryptomeria japonica} D. Don) is the most important afforestation coniferous tree in Japan. Coniferous trees normally have a long juvenile period and require a long cultivation time for breeding. Through a traditional breeding project that began in the 1950s, first generation plus trees with excellent traits were selected primarily from artificial forests and used as seedlings. Recently, the second generation plus trees obtained by crossing between plus trees have been selected. In light of this situation, the improvement of Sugi by a transgenic approach is effective in terms of shortening the breeding period compared with conventional crossing-dependent approaches. There are three key points to an efficient \textit{Agrobacterium}-mediated transformation system: (1) establishment of explants with high regeneration ability, (2) optimal co-cultivation conditions for explants and \textit{Agrobacterium}, and (3) efficient elimination of \textit{Agrobacterium}. Here we describe a protocol for \textit{Agrobacterium}-mediated transformation of Sugi that meets the above criteria using embryogenic tissues as explants isolated from immature seeds obtained by crossing.

Key words:  \textit{Agrobacterium tumefaciens}, conifer, \textit{Cryptomeria japonica}, stable transformation.

Introduction

Widely distributed in Japan, \textit{Cryptomeria japonica} D. Don (Sugi, Japanese cedar) is one of the major afforestation coniferous species there. Sugi had been previously classified in the family Taxodiaceae, but as a result of recent molecular phylogenetic studies it has been integrated into the family Cupressaceae \textit{sensu lato} (Gadek et al. 2000; Kusumi et al. 2000). \textit{C. japonica} var. \textit{sinensis}, previously recognized as \textit{C. fortunei}, is distributed in eastern China (Fu et al. 1999), but there are no differences in chloroplast DNA sequences between \textit{C. japonica} and \textit{C. japonica} var. \textit{sinensis} (Kusumi et al. 2000; Tsumura et al. 1995). In Japan, there are two main lines locally known as Omote-sugi (\textit{C. japonica} distributed on the Pacific Ocean side) and Ura-sugi (\textit{C. japonica} var. \textit{radicans} distributed on the Sea of Japan side), which are distinguished by differences in morphological characteristics (Murai 1947), diterpene components (Yasue et al. 1987), reproductive system (Kimura et al. 2013), and DNA variation (Tsumura 2011; Tsumura et al. 2014), which may have contributed to their local adaptation. Since Sugi trees may exceed 50 m in height, and grow with a straight bole of soft wood making processing easy, it is popular for house construction, paneling, flooring, carpentry, joinery, and furniture making (Farjón 2010; Ohba 1993). Around 1950, due to a decline in demand for fuelwood and an increase in demand for timber associated with residential construction during the period of high economic growth, reforestation to convert natural forests into artificial forests was actively promoted and afforestation areas were enlarged. As a result, Sugi has been planted widely throughout Japan and now covers an area of 4.44 million ha, accounting for approximately 44% of all Japanese artificial forests (Forestry Agency 2017). However, allergic rhinitis caused by Sugi pollen (Sugi pollinosis) began to be reported in 1963 (Horiguchi and Saito 1964), since when the prevalence has continued to rise year by year, and now Sugi pollinosis has become a severe public health concern in Japan (Yamada et al. 2014). Therefore, the production of Sugi with a no-pollen trait has become...
an important breeding objective.

Genetic transformation, and genome editing technology based on it, can be a powerful tool for shortening the breeding period of trees whose generations are many years apart. A large number of expressed sequence tags obtained from vegetative buds, inner bark, female strobili, pollen, sapwood, xylem, cambium, and roots, have been identified (Fukuda et al. 2018; Futamura et al. 2006, 2008; Kurita et al. 2011; Mishima et al. 2014, 2018; Nose and Watanabe 2014; Tsumura et al. 1997; Ueno et al. 2019; Ujino-Ihara et al. 2000, 2003, 2005; Yoshida et al. 2007, 2012), and various genetic linkage maps and DNA markers have been developed (summarized by Tsumura 2011). Genetic transformation is also an essential tool for taking advantage of these abundant resources for molecular breeding and identification of gene function by reverse genetic analysis. Several stable transformation methods of coniferous trees have been reported in the family Pinaceae, including the genera Picea, Larix, and Pinus (summarized by Malabadi and Nataraja 2007). However, stable transformation in Cupressaceae has only been reported for Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc.; Ishii 2002; Taniguchi et al. 2005) and Sugi (Konagaya et al. 2013; Maruyama et al. 2000; Taniguchi et al. 2008).

Most transformation methods in coniferous trees use embryogenic tissues (e.g., embryogenic cells, embryogenic callus, embryogenic mass, embryonal-suspensor mass, etc.) derived from immature seeds as explants. Somatic embryogenesis has long been studied in coniferous trees because it is an effective procedure not only for providing target tissue for genetic transformation but also for mass propagation of breeding strains showing preferred traits (summarized by Konagaya and Taniguchi 2016). One of the key factors for successful coniferous transformation is the use of embryogenic tissues of cell lines with high regeneration efficiency for somatic embryos, necessitating selection of many cell lines. It has been shown that the induction rate of embryogenic tissues from immature seeds in Sugi is affected by genotype and embryonic developmental stage (Igasaki et al. 2003; Maruyama et al. 2000; Taniguchi and Kondo 2000). Furthermore, it has been revealed that the frequency of somatic embryogenesis from embryogenic tissues is also affected by genotype (Taniguchi 2007; see the chapter by Taniguchi et al.). Somatic embryo maturation is generally promoted by abscisic acid, osmotic penetrants (sugars, sugar alcohols, etc.) or non-penetrating osmotica (e.g., polyethylene glycol), which induce water stress (Lipavská and Konrádová 2004). Mature somatic embryos in Cupressaceae trees have also been efficiently formed on a medium containing a combination of 100 µM abscisic acid, 30 g/l maltose as the sugar, and 150 g/l polyethylene glycol (Maruyama et al. 2002; Taniguchi 2007; Taniguchi et al. 2004a).

Genetic transformation of Sugi was first attempted by particle bombardment with, for example, gene transfer of the luciferase (Mohri et al. 2000) and green fluorescent protein (GFP; Taniguchi et al. 2004b) genes in zygotic embryos, but only transient expression was observed. Maruyama et al. (2000) reported bud formation from embryos after introducing the β-glucuronidase gene. The *Agrobacterium tumefaciens* (recently reclassified as *Rhizobium radiobacter*; Young et al. 2001; formerly, *A. tumefaciens*). We have used *A. tumefaciens* for convenience in this review because the majority of transgenic research has used this name)-mediated transformation method in Sugi was first successfully achieved by Taniguchi et al. (2008) and further improved by Konagaya et al. (2013). The improvements are described below.

Bacterial overgrowth during co-cultivation in *Agrobacterium*-mediated transformation may lead to undesirable effects, such as tissue necrosis, which can reduce transformation efficiency. Ozawa (2009) showed that liquid medium-moistened filter paper wicks regulated the growth rate of *Agrobacterium* in an effective manner, improving cell viability in the transformed callus obtained from rice. Thus, as a result of determining the optimal co-cultivation conditions with filter paper wicks in Sugi, transformation efficiency was greatly improved (>17-fold; Konagaya et al. 2013) despite the mean number of *Agrobacterium* cells obtained in liquid medium conditions being significantly less than that obtained on solid media using conventional methods (Taniguchi et al. 2008). Similar improvements have been reported in rice, cucumber, kabocha squash, *Jatropha*, and safflower transformation (Nanasato et al. 2011, 2013, 2015; Ozawa 2009; Rani et al. 2018). These results support the utility of filter paper wicks in co-cultivation procedures.

Recently, it has been reported that meropenem, a novel β-lactam antibiotic, eliminates *Agrobacterium* very effectively at low concentrations. Also, meropenem is more efficient for transformation in some crops than other β-lactam antibiotics, such as cefotaxime, carbenicillin, and cefubepazone (Li et al. 2011; Ogawa and Mii 2004, 2007). Meropenem treatment during Sugi transformation increased transformation efficiency approximately 2-fold compared with carbenicillin using the conventional method and did not affect growth. In transformation experiments using our improved method (co-cultivation on filter paper wicks and *Agrobacterium* elimination with meropenem), the mean transformation efficiency was approximately 105 GFP-positive colonies per gram of co-cultivated embryogenic tissues, approximately 30 times more than the conventional method (Konagaya et al. 2013). Currently, an independent transgenic Sugi tree of 10–50 lines has been obtained from 1 g of embryogenic tissues established from cross progeny between plus trees (Konagaya et al. 2013; Nanasato et al. unpublished data). In this review, we introduce our modified Sugi transformation protocol.
Materials

Stock solutions

1. Stock solution of antibiotics, hormones, and other compounds: See Table 1.

2. GK stock solution: Dissolve 5 g glutamine and 10 g casamino acid in 200 ml ddH\(_2\)O. Adjust pH to 5.7. Filter-sterilize and store at −20°C.

Table 1. Stock solutions of antibiotics, hormones, and other compounds.

| Compound          | Stock solution concentration | Amount (mg ml\(^{-1}\)) | Solvent    |
|-------------------|-----------------------------|--------------------------|------------|
| Antibiotic        |                             |                          |            |
| Rifampicin        | 25 mg ml\(^{-1}\)           | 25                       | DMSO       |
| Gentamycin        | 25 mg ml\(^{-1}\)           | 25                       | ddH\(_2\)O |
| Kanamycin         | 25 mg ml\(^{-1}\)           | 25                       | ddH\(_2\)O |
| Spectinomycin     | 100 mg ml\(^{-1}\)          | 100                      | ddH\(_2\)O |
| Hygromycin        | 5 mg ml\(^{-1}\)            | 5                        | ddH\(_2\)O |
| Meropenem         | 10 mg ml\(^{-1}\)           | 10                       | ddH\(_2\)O |
| Hormone           |                             |                          |            |
| 2,4-d            | 2 mM                        | 0.442                    | 0.1 N NaOH |
| ABA               | 100 mM                      | 26.4                     | DMSO       |
| Other compounds   |                             |                          |            |
| Acetosyringone    | 500 mM                      | 98                       | DMSO       |

Filter-sterilize all except 2,4-d and store at −20°C.

Table 2. Composition of the COM medium.

| Component      | Stock solution (g l\(^{-1}\)) | Final concentration (mg l\(^{-1}\)) |
|----------------|-------------------------------|-------------------------------------|
| COM-I stock solution (25×)\(a\) |                                |                                    |
| KNO\(_3\)      | 35.775                        | 1,431                               |
| MgSO\(_4\)·7H\(_2\)O | 10                            | 400                                 |
| CaCl\(_2\)·2H\(_2\)O | 0.625                         | 25                                  |
| NaNO\(_3\)     | 7.75                          | 310                                 |
| NH\(_4\)H\(_2\)PO\(_4\) | 5.625                         | 225                                 |
| COM-II stock solution (50×)\(a\) |                                |                                    |
| MnSO\(_4\)·4H\(_2\)O | 0.18                          | 3.6                                 |
| H\(_2\)BO\(_3\) | 0.4                           | 8                                   |
| ZnSO\(_4\)·7H\(_2\)O | 1.25                          | 25                                  |
| KI             | 0.05                          | 1                                   |
| CuSO\(_4\)·5H\(_2\)O | 0.12                          | 2.4                                 |
| Na\(_2\)MoO\(_4\)·2H\(_2\)O | 0.01                          | 0.2                                 |
| CoCl\(_2\)·6H\(_2\)O | 0.01                          | 0.2                                 |
| COM-III stock solution (50×)\(a,b\) |                                |                                    |
| Na\(_2\)-EDTA | 2                             | 40                                  |
| FeSO\(_4\)·7H\(_2\)O | 1.5                           | 30                                  |
| COM-IV stock solution (100×)\(a\) |                                |                                    |
| Thiamine–HCl   | 0.5                           | 5                                   |
| Nicotinic acids | 0.5                           | 5                                   |
| Pyridoxine–HCl | 0.05                          | 0.5                                 |
| COM-AA stock solution (20×)\(a,\(b\) |                                |                                    |
| Citrulline     | 0.395                         | 19.75                               |
| Ornithine      | 0.38                          | 19                                  |
| Lysine         | 0.275                         | 13.75                               |
| Alanine        | 0.2                           | 10                                  |
| Proline        | 0.175                         | 8.75                                |
| Glutamine      | 11                            | 550                                 |
| Asparagine     | 10.2                          | 510                                 |
| Arginine       | 3.5                           | 175                                 |
| Other components |                                 |                                    |
| Myo-inositol   | 1,000                         | 1                                   |
| Maltose        | 30,000                        | 30                                  |
| Polyethylene glycol 4000 | 150,000                      | 150                                 |
| Activated charcoal | 2,000                         | 20                                  |
| Gelzan CM      | 3,000                         | 30                                  |
| Abscisic acid  | 26.4                          | 100 µM                               |

Filter-sterilize and store at 4°C. \(a\) Dissolve each compound individually in about 200 ml of ddH\(_2\)O, add the FeSO\(_4\)·7H\(_2\)O solution little by little to the Na\(_2\)-EDTA solution, and then dilute to 1 l. Store at −20°C. \(b\) Adjust stock solution to pH 5.7, filter-sterilize, and store at −20°C. \(b\) Add after adjusting the medium to pH 5.7 and autoclaving. Final concentration is 100 µM.

Table 3. Composition of the mGD medium.

| Component     | Stock solution (g l\(^{-1}\)) | Final concentration (mg l\(^{-1}\)) |
|---------------|-------------------------------|-------------------------------------|
| mGD-I stock solution (25×)\(a\) |                                |                                    |
| (NH\(_4\))\(_2\)SO\(_4\) | 5                             | 200                                 |
| KNO\(_3\)      | 25                            | 1,000                               |
| KCl            | 7.5                           | 300                                 |
| MgSO\(_4\)·7H\(_2\)O | 6.25                          | 250                                 |
| Na\(_2\)PO\(_4\)·H\(_2\)O | 2.25                          | 90                                  |
| Na\(_2\)HPO\(_4\) | 0.75                          | 30                                  |
| MnSO\(_4\)·5H\(_2\)O | 0.3565                        | 14.26                               |
| mGD-II stock solution (100×)\(a\) |                                |                                    |
| H\(_2\)BO\(_3\) | 0.3                           | 3                                   |
| ZnSO\(_4\)·7H\(_2\)O | 0.3                           | 3                                   |
| KI            | 0.075                         | 0.75                                 |
| Na\(_2\)MoO\(_4\)·2H\(_2\)O | 0.025                         | 0.25                                 |
| CuSO\(_4\)·5H\(_2\)O | 0.025                         | 0.25                                 |
| CoCl\(_2·6H\(_2\)O | 0.025                          | 0.25                                 |
| mGD-III stock solution (100×)\(a\) |                                |                                    |
| CaCl\(_2·2H\(_2\)O | 15                            | 150                                 |
| mGD-IV stock solution (100×)\(a\) |                                |                                    |
| Na\(_2\)-EDTA | 3.73                          | 37.3                                |
| FeSO\(_4·7H\(_2\)O | 2.78                          | 27.8                                |
| mGD-V stock solution (100×)\(b\) |                                |                                    |
| Myo-inositol | 1                             | 10                                  |
| Nicotinic acids | 0.01                         | 0.1                                 |
| Pyridoxine–HCl | 0.01                          | 0.1                                 |
| Thiamine–HCl  | 0.1                           | 1                                   |
| Other components |                                 |                                    |
| Sucrose        | 10,000                        | 1                                   |
| Gelzan CM      | 5,000                         | 5                                   |

Adjust the medium to pH 5.7. \(a\) Filter-sterilize and store at 4°C. \(b\) Dissolve each compound individually in about 200 ml of ddH\(_2\)O, add the FeSO\(_4\)·7H\(_2\)O solution little by little to the Na\(_2\)-EDTA solution, and then dilute to 1 l. Store at −20°C.
3. COM medium (modified Smith Embryo Development medium; Smith 1996; Taniguchi et al. 2004a, 2008) stock solutions; See Table 2.
4. mGD medium (modified Gresshoff and Doy medium; Okamura and Kondo 1995) stock solutions; See Table 3.

**Bacterial media**
1. YEB medium (Vervliet et al. 1975): 5 g l$^{-1}$ beef extract, 1 g l$^{-1}$ yeast extract, 5 g l$^{-1}$ peptone, 5 g l$^{-1}$ sucrose, and 2 mM MgSO$_4$$\cdot$7H$_2$O, adjust pH to 7.2. The medium is supplemented with bacterial selection antibiotics, 1 ml l$^{-1}$ rifampicin stock solution (final concentration is 25 mg l$^{-1}$), and 1 ml l$^{-1}$ gentamycin stock solution (final concentration is 25 mg l$^{-1}$) for the selection of A. tumefaciens strain GV3101/A. tumefaciens
2. mGD medium (modified Gresshoff and Doy medium; Okamura and Kondo 1995) stock solutions: See Table 3.

**Plant media**
1. 1/2MD’ medium: 2.203 g l$^{-1}$ (half-strength) Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962; ready-to-use product, Duchefa, Netherlands, cat. no. M0222), 5 ml l$^{-1}$ 2,4-D stock solution (final concentration is 10 µM), 30 g l$^{-1}$ sucrose, 8 g l$^{-1}$ agar, adjust pH to 5.7, and autoclave the medium. Cool to <60°C, pour 40 ml aliquots into petri dishes (inner size 90×15 mm; AS ONE, Japan, cat. no. 1-9467-02), and store at 4°C.
2. 1/2MD medium: Half-strength and 50% of the inorganic nitrate salts of MS salts and vitamins (1.315 g l$^{-1}$, ready-to-use product, Duchefa, USA, cat. no. M0237), 1 ml l$^{-1}$ 2,4-D stock solution (final concentration is 2µM), 30 g l$^{-1}$ sucrose, 4 g l$^{-1}$ Gelzan CM (for solid medium; Sigma-Aldrich, USA, cat. no. G1910), adjust pH to 5.7. Autoclave the medium, cool to <60°C, and add 20 ml of GK stock solution (final concentration is 0.5 g l$^{-1}$ glutamine and 1 g l$^{-1}$ casamino acid). Pour 40 ml aliquots of solid medium into petri dishes (inner size 90×15 mm), and store at 4°C.
3. Co-cultivation medium: Liquid 1/2MD medium supplemented with 100 µl l$^{-1}$ acetylsyringone stock solution (final concentration is 50 µM) after autoclaving.
4. Decontamination medium: Solid/liquid 1/2MD medium supplemented with 1 ml l$^{-1}$ meropenem stock solution (final concentration is 10 mg l$^{-1}$) after autoclaving.
5. Selection medium: Solid 1/2MD medium supplemented with 1 ml l$^{-1}$ meropenem stock solution (final concentration is 10 mg l$^{-1}$), and 1 ml l$^{-1}$ kanamycin stock solution (when using neomycin phosphotransferase gene, NPT II, as a selection marker; final concentration is 25 mg l$^{-1}$) or 1 ml l$^{-1}$ hygromycin stock solution (when using hygromycin phosphotransferase gene, HPT, as a selection marker; final concentration is 5 mg l$^{-1}$) after autoclaving.
6. COM medium: 40 ml l$^{-1}$ COM-I stock solution, 20 ml l$^{-1}$ COM-II stock solution, 20 ml l$^{-1}$ COM-III stock solution, 10 ml l$^{-1}$ COM-IV stock solution, 1 g l$^{-1}$ myo-inositol, 30 g l$^{-1}$ maltose, 150 g l$^{-1}$ polyethylene glycol 4000, 2 g l$^{-1}$ activated charcoal (Sigma-Aldrich, cat. no. C9157), 3 g l$^{-1}$ Gelzan CM, and adjust pH to 5.7. Autoclave the medium with a stir bar, cool to <60°C with sufficient stirring, and add 1 ml of ABA stock solution, and 50 ml l$^{-1}$ COM-AA stock solution (see Table 2 for final concentration). Pour 40 ml aliquots into petri dishes (inner size 90×15 mm), and store at 4°C.
7. mGD medium: 40 ml l$^{-1}$ mGD-I stock solution, 10 ml l$^{-1}$ mGD-II stock solution, 10 ml l$^{-1}$ mGD-III stock solution, 10 ml l$^{-1}$ mGD-IV stock solution, 10 ml l$^{-1}$ mGD-V stock solution (see Table 3 for final concentration), 10 g l$^{-1}$ sucrose, 5 g l$^{-1}$ Gelzan CM, adjust pH to 5.7, and autoclave the medium. Cool to <60°C, pour 50 ml aliquots into petri dishes (inner size 90×20 mm; Eiken Chemical, Japan, cat. no. AR2000), and store at 4°C.
8. 1/2WPM-f medium: Half-strength McCown Woody Plant medium including vitamins (1.231 g l$^{-1}$, ready-to-use product, Duchefa, cat. no. M0220; Lloyd and McCown 1980), 20 g l$^{-1}$ sucrose, 2 g l$^{-1}$ Gelzan CM, adjust pH to 5.7. Dissolve in a hot water bath, pour 80 ml aliquots into 450-ml culture bottles (AS ONE, cat. no. 2-085-04) and autoclave.

**Protocols**

**Step 1: Establishment of embryogenic tissues**
1. From late June to mid-July of the previous year, soak or spray branches in 100 ppm gibberellic acid (GA$_3$; Kyowa-Hakko, Japan) aqueous solution to promote setting of male strobili. From late July to early August of the same year, treat with GA$_3$ in the same way to promote setting of female strobili. In March of the following year, perform artificial crossing or open pollination and collect immature female cones from late June to early July.

*Tip: Although the above-mentioned period for GA$_3$ treatment is effective for the main Sugi planting areas in Japan (Honsyu, Sikoku, and Kyushu islands), optimization of the treatment date in advance depending on other areas may be required because it is affected by environmental conditions (Nagao et al. 1989). In order to prevent insect damage, wrap the cones...*
2. Wrap the female cones in a net and wash with running tap water with 0.1% neutral detergent (Contaminon N; FUJIFILM Wako, Japan, cat. no. 037-10361) for 30 min (Figure 1A). Then soak the cones in 70% ethanol for 10 min and rinse three times with distilled water.

3. Make a cut in the proximal region of each female cone with a scalpel and split it in half lengthwise (Figure 1B, C), release the immature seeds from separated individual ovuliferous scales, and drop them in a small beaker.

4. Rinse the immature seeds with 70% ethanol for 1 min, sterilize with 6% hydrogen peroxide for 5 min, and rinse 3 times with sterile water.

5. Under a stereomicroscope, aseptically remove the megagametophytes containing immature precotyledonary zygotic embryos from the sterilized immature seeds (Figure 1D, E) and place them on initiation medium (1/2MD medium; Figure 1F). Tip: Cut the seed coat vertically with a scalpel, and lightly press it from above with tweezers to expose the megagametophyte (Figure 1E). Peel off the nucellus coating the megagametophyte.

6. After incubation in the dark at 25°C for 1 month, transfer onto maintenance medium (solid 1/2MD medium), and then subculture every 2 weeks in the same medium (Figure 1G).

7. Transfer embryogenic tissues from megagametophytes onto solid 1/2MD medium. After proliferation, divide into pieces ~5 mm in diameter and repeat the subculture to obtain embryogenic tissues for transformation (Figure 1H, I).
Step 2: Preparation of A. tumefaciens
1. Transfer a binary vector into A. tumefaciens strain GV3101/pMP90 by electroporation or the freeze-thaw method. Store A. tumefaciens cells harboring a binary vector in 30% glycerol at −80°C prior to use. *Tip: The NPTII or the HPT gene can be used as a selection marker of transgenic plants. We generally use binary vectors derived from pIG121-Hm or pPZP backbone.*
2. Transfer 10–50 µl of glycerol stocks to 20 ml of YEB medium supplemented with antibiotics. Incubate with shaking (180 rpm) at 28°C until the optical density (OD₆₀₀) reaches 0.2–0.6. *Tip: Excessive incubation (OD₆₀₀>0.8) results in significantly reduced infection efficiency. Use YEB medium as the blank for OD₆₀₀ measurement.*
3. Pellet by centrifugation (10,000 × g for 3 min) and resuspend in co-cultivation medium to obtain a final OD₆₀₀=0.15.

Step 3: Co-cultivation of embryogenic tissues
1. Grow the embryogenic tissue on solid 1/2MD medium for 1 week and transfer 1 g of fresh weight (approximately 10 masses of 8 mm in diameter; Figure 1H and I) to 20 ml of the bacterial suspension in a 50 ml tube (Figure 2A).
2. Resuspend the embryogenic tissues by vigorously shaking 10–20 times.
3. Incubate the tube horizontally on an orbital shaker at 100 rpm for 20 min at 25°C.
4. Place a sterile filter paper (7-cm diameter, No. 7, Advantech, Japan, cat. no. 01701070) in a Büchner funnel on a suction bottle, pour in a little co-cultivation medium to moisten, and aspirate briefly.
5. Pour approximately 10 ml (containing 0.5 g of embryogenic tissues) of the culture in a Büchner funnel (Figure 2B), and filter the liquid completely using a vacuum pump (3–5 s, 0.03 MPa).
6. For co-cultivation, lay the filter paper with the embryogenic tissue on a stack of three pieces of sterile filter paper (8.5-cm diameter, No. 2, Advantech, cat. no. 00021085) containing 5.5 ml co-cultivation medium in a 90 × 20 mm petri dish (Figure 2C). Repeat step 5 and 6 for the remaining culture.
7. Co-culture for 2 days at 25°C in the dark.

Step 4: Eliminating Agrobacterium contamination
1. Immerse the upper filter paper (from the stack of filter papers) with co-cultured tissue (0.5 g fresh weight) in 40 ml liquid decontamination medium in a 50 ml tube (Figure 2D).
2. Resuspend the tissue by vigorously shaking 10–15 times and then discard the filter paper. *Tip: If embryogenic tissues remain on the filter paper, dislodge them with a spatula (Figure 2E).*
3. Combine the embryogenic tissues from two pieces of...
filter paper (total 1g fresh weight) into 40 ml liquid decontamination medium.

4. Wash the tissues using one of the following methods:

(i) **Vacuum method**

1-1. Pour 40 ml of the culture containing 1 g of embryogenic tissues onto a sterile filter paper (7-cm diameter) in a Büchner funnel, and filter the liquid completely using a vacuum pump.

1-2. Pour 50 ml liquid decontamination medium and gently swirl until the tissues are resuspended.

1-3. Filter the liquid completely using a vacuum pump. Repeat step i-2 three times. Finally resuspend in 40 ml of liquid decontamination medium.

1-4. Collect the resuspended tissue into a 50 ml tube and fill up to 40 ml with liquid decontamination medium.

1-5. Resuspend tissues by vigorously shaking 5–10 times.

(ii) **Strainer method**

ii-1. Pour 40 ml of the culture containing 1 g of embryogenic tissue into a 200-mesh tea

Figure 3. Transformant selection, regeneration and acclimatization. (A) GFP fluorescent spots in embryogenic tissues introduced with pUbiP-GFP/Hyg (Taniguchi et al. 2008) cultured on decontamination medium for 3 days. Tissues are observed under a MZ FLIII stereo fluorescence microscope (Leica Microsystems, Germany). (B) Embryogenic tissues after culturing on a selection medium (supplemented with 25 mg l⁻¹ kanamycin) for 45 days. Arrowheads indicate white kanamycin-resistant colonies. (C, D) Bright field image (C) and fluorescence image (D) of kanamycin-resistant colonies. GFP fluorescent colonies, indicated by arrowheads, often show differences in fluorescence intensity between transgenic strains. (E, F) Bright field image (E) and fluorescent image (F) of somatic embryos regenerated from the embryogenic tissue cultured for 45 days on COM medium. The inset shows a 3× magnified image of a single somatic embryo. (G) Germination of transgenic somatic embryos at 8 weeks after culture on mGD medium. (H) Plantlets (~8 cm high) grown in a culture bottle (8-cm diameter) about 5 months after germination. (I) Plantlets transplanted to pots (10.5-cm diameter) in a container for acclimatization. (J) Transgenic Sugi plants (1 year after acclimatization) grown in a semi-closed greenhouse. The pot size is 15 cm in diameter. The scale bars represent 5 mm.
strainer (Total Kitchen Goods, Japan, cat. no. 5-0706-0703) resting on a 200–300 ml beaker or a 450 ml culture bottle (Figure 2F) and allow the liquid to strain through completely.

ii-2. Gradually pour 50 ml liquid decontamination medium and allow the liquid to strain through completely. Repeat three times.

ii-3. Pour approximately 10 ml liquid decontamination medium and quickly transfer the resuspended tissues to a new 200 ml beaker. Repeat four times and collect total 40 ml (Figure 2G).

Tip: Transformation efficiency between the vacuum method and the strainer method is equivalent. The strainer method is simpler than the vacuum method, but if it is difficult to get a high-density mesh strainer, use the vacuum method.

5. Pour 10 ml of the washed suspension containing 0.25 g of tissues onto a new filter paper (7-cm diameter) in a Büchner funnel and filter completely using a vacuum pump.

6. Place the filter paper with the tissues on solid decontamination medium, incubate in the dark at 25°C for 3 days, then transfer to new medium.

Tip: When a binary vector containing a visualization marker (GFP, β-glucuronidase gene, etc.) is used, transient expression can be clearly observed by culturing for 3 days on a solid decontamination medium (Figure 3A; Konagaya et al. 2013).

Step 5: Selection of transgenic embryogenic tissues

1. Transfer the filter paper with the embryogenic tissues to the selection medium approximately 2 weeks after infection (counted from the day when co-cultivation was started).

Tip: Slightly protruded cell masses (approximately 1–2 mm in diameter) observed by regrowth of the tissues is used as a criterion for transfer timing.

2. Incubate in the dark at 25°C and then subculture every 2 weeks in the same medium.

3. Transgenic colonies are formed about 1 to 3 months after infection (Figure 3B, C, D). Pick up these colonies with tweezers and grow them on a new selection medium. Subculture every 2 weeks in the same medium.

Tip: By repeating the subculture, Agrobacterium is almost completely removed. At this stage, insertion of transgenes and removal of Agrobacterium can be confirmed by PCR using genomic DNA extracted from the embryogenic tissue and primers specific to the transgene and Agrobacterium genome (e.g., HrC gene; Nanasato et al. 2011).

Step 6: Somatic embryogenesis and germination

1. Transfer ~5 mm (diameter) of embryogenic tissues to somatic embryo maturation medium (COM medium; five masses per petri dish) and culture in the dark at 25°C.

2. Somatic embryos are formed after 6–8 weeks (Figure 3E, F). Pick up somatic embryos with tweezers, transfer to germination medium (mGD medium), and culture at 25°C with a regime of 16 h light (70 µmol m⁻² s⁻¹ light intensity) and 8 h darkness (Figure 3G).

3. Subculture every 1 month in the same medium.

Step 7: Shoot elongation and acclimatization

1. After 2–3 months, transfer plantlets (now ~2–3 cm high) to 1/2WPM-f medium in a culture bottle (Figure 3H).

Tip: To promote growth, make a hole in the lid of the culture bottle and seal it with the Milliseal (Millipore, Germany, cat. no. FWMS01800) to improve ventilation.

2. Subculture every 1 month in the same medium. After 3–4 months, carefully wash the roots of the elongated plantlets (now ~10–15 cm high) and transfer to pots containing soil mixture consisting of peat moss-based soil (Jiffy Mix; Sakata Seed, Japan), akadama soil (granules of volcanic ash soil; Tachikawa Heiwa Nouen, Japan, cat. size middle, 7–14 mm diameter) and vermiculite in the ratio of 1:1:1 (Figure 3I).

3. For acclimatization, cover the container containing the plantlets with a polythene sheet to maintain high humidity. Place in a closed glasshouse at 25°C and grow in the shade for the first few weeks.

4. After 5–7 days, gradually reduce the humidity by punching holes in the polythene sheet, increasing the number of holes daily.

5. Approximately 3 months later, transfer the Sugi plants to a semi-closed greenhouse and grow under natural conditions (Figure 3J).

Tip: It is possible to easily induce setting of male strobili by GA3 treatment even for current year plants of about 15–20 cm height.

Concluding remarks

In order to enhance the efficiency of Agrobacterium-mediated transformation of Sugi and simplify the procedure, we have improved the co-cultivation, antibiotics for eliminating Agrobacterium contamination, and washing method of embryogenic tissues. These effective methods could be applied to transformation of embryogenic tissues in other coniferous trees. Currently, we are developing no-pollen Sugi by genome editing via transformation. Using the present method,
it is possible to acclimatize the seedlings within about 1 year of transformation (Nanasato et al., manuscript in preparation), and it is possible to determine pollen-forming ability approximately 6 months after GA₃ treatment. Although it requires time to establish useful cell lines as explants and to establish a stable supply, cryopreservation technology ameliorates this problem. We hope that the advancement of molecular breeding and molecular genetics will be accelerated in many coniferous trees by the wide utilization of genetic transformation technology.

Acknowledgements

The authors would like to thank Ms. Kimie Ohbu, Ms. Tomoko Okuyama, and Ms. Maki Konnai for technical assistance. This work was partly supported by Grant-in-Aid (Development of Technologies for Control of Pollen Production by Genetic Engineering) from the Forest Agency of Japan. The authors would like to thank Enago (www.enago.jp) for the English language review.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Farjón A (2010) A Handbook of the World Conifers, Vol. 1. Brill, Press, Leiden
Forestry Agency (2017) Statistics of Forest and Forestry 2017. Forest Agency, Japan (in Japanese)
Fu LG, Yu YF, Mill RR (1999) Taxodiaceae. In: Wu ZY, Raven P (eds) Flora of China, Vol. 4. Science Press, Beijing, pp 54–61
Fukuda Y, Hirao T, Mishima K, Ohira M, Hiroaka Y, Takahashi M, Watanabe A (2018) Transcriptome dynamics of rooting zone and aboveground parts of cuttings during adventitious root formation in Cryptomeria japonica D. Don. BMC Plant Biol 18: 201
Futamura N, Totoki Y, Toyoda A, Igasaki T, Nonjo T, Seki M, Sakaki Y, Mari A, Shinozaki K, Shinohara K (2008) Characterization of expressed sequence tags from a full-length enriched cDNA library of Cryptomeria japonica male strobili. BMC Genomics 9: 383
Futamura N, Ujino-Ihara T, Nishiguchi M, Kanamori H, Yoshimura K, Sakaguchi M, Shinohara K (2006) Analysis of expressed sequence tags from Cryptomeria japonica pollen reveals novel pollen-specific transcripts. Tree Physiol 26: 1517–1528
Gadek PA, Alpers DL, Heslewood MM, Quinn CJ (2000) Relationships within Cupressaceae sensu lato: A combined morphological and molecular approach. Am J Bot 87: 1044–1057
Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPTZ family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25: 989–994
Horiguchi S, Saito Y (1964) The cases of Japanese cedar pollinosis in Nikko Tochigi prefecture. Jpn J Allergol 13: 16–18 (in Japanese)
Igasaki T, Sato T, Akashi T, Mohri T, Maruyama E, Kinosita H, Walter C, Shinohara K (2003) Somatic embryogenesis and plant regeneration from immature zygotic embryos of Cryptomeria japonica D. Don. Plant Cell Rep 22: 239–243
Ishii K (2002) Liquid culture and transformation of Hinoki cypress (Chamaecyparis obtusa Sieb. et Zucc.). J For Res 7: 99–104
Kimura M, Kabeya D, Saito T, Moriguchi Y, Uchiyama K, Migita C, Chiba Y, Tsumura Y (2013) Effects of genetic and environmental factors on clonal reproduction in old-growth natural populations of Cryptomeria japonica. For Ecol Manage 304: 10–19
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
Konagaya K, Taniguchi T (2016) Somatic embryogenesis and genetic transformation in Cupressaceae trees. In: Mujib A (ed) Somatic Embryogenesis in Ornaments and Its Applications. Springer, Berlin, pp 203–216
Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol Gen Genet 204: 383–396
Kurita M, Taniguchi T, Nakada R, Kondo T, Watanabe A (2011) Spatiotemporal gene expression profiles associated with male strobilus development in Cryptomeria japonica by suppression subtractive hybridization. Breed Sci 61: 174–182
Kusumi J, Tsumura Y, Yoshimaru H, Tachida H (2000) Phylogenetic relationships in Taxodiaceae and Cupressaceae sensu stricto based on matK gene, chlL gene, trnL–trnF IGS region, and trnL intron sequences. Am J Bot 87: 1480–1488
Li J, Komori S, Sasaki K, Mimida N, Matsumoto S, Wada M, Soejima J, Ito Y, Masuda T, Tanaka N, et al. (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 Plant 40: 23–30
Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Proc Int Plant Prop Soc 30: 421–427
Malabadi RB, Nataraka K (2007) Genetic transformation of conifers: Applications in and impacts on commercially forestry. Transgenic Plant J 1: 289–313
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, 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
Mishima K, Fujiwara T, Iki T, Kuroda K, Yamashita K, Tamura M, Fujisawa Y, Watanabe A (2014) Transcriptome sequencing and profiling of expressed genes in cambial zone and differentiating xylem of Japanese cedar (Cryptomeria japonica). BMC Genomics 15: 219
Mishima K, Hirao T, Tsukomura M, Tamura M, Kurita M, Nose M, Hanaoka S, Takahashi M, Watanabe A (2018) Identification of novel putative causative genes and genetic marker for male sterility in Japanese cedar (Cryptomeria japonica D. Don). BMC Genomics 19: 277
Mohri T, Igasaki T, Sato T, Shinozaka K, Seki M, Mohri T, Maruyama E, Kinosita H, Walter C, Shinohara K (2003) Somatic embryogenesis and plant regeneration from immature zygotic embryos of Cryptomeria japonica D. Don. Plant Cell Rep 22: 239–243
Murai S (1947) Major forestry tree species in the Tohoku region

Copyright © 2020 The Japanese Society for Plant Cell and Molecular Biology
and their varietal problems. In: Aomori-ринюкай (ed) Kokudo Saiken Zourin Gijutsu Koushii. pp 131–151 (in Japanese) Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Nagao A, Sasaki S, Pharis RP (1989) *Cryptomeria japonica*. In: Halevy AH (ed) *Handbook of Flowering*, Vol. 6. CRC Press, Boca Raton, Florida, pp 247–269

Nanasato Y, Kido M, Kato A, Ueda T, Suharsono S, Widyastuti U, Tsuchimoto H, Akashi K (2015) Efficient genetic transformation of *Jacarpha curcas* L. by means of vacuum infiltration combined with filter-paper wicks. *In Vitro Cell Dev Biol Plant* 51: 399–406

Nanasato Y, Konagaya K, Okuzaki A, Tsuda M, Tabei Y (2011) *Agrobacterium*-mediated transformation of kabocho squash (*Cucurbita moschata* Duch) induced by wounding with aluminum borate whiskeys. *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

Nose M, Watanabe A (2014) Clock genes and circadian rhythms in the leaves of Sugi (Cryptomeria japonica). *J Plant Biochem Biotechnol* 23: 63–119

Nouguichui K, Yoshimi 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

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and maintenance of embryogenic cultures among Sugi (*Cryptomeria japonica* D. Don) induced by wounding with aluminum borate whiskeys. *Plant Cell Rep* 30: 1455–1464

Ogawa Y, Mii M (2007) Meropenem and moxalactam: Novel β-lactam antibiotics against *Agrobacterium tumefaciens* transformation. *Microbiol Arch* 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

Ohba K (1993) Clonal forestry with Sugi (*Cryptomeria japonica*). In: Ahuja MR, Libby WJ (eds) *Clonal forestry II*. Springer, Berlin, pp 66–90

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813

Okamor M, Kondo T (1995) Manual for tissue culture of pine. *Bull Natl 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

Rani A, Panwar A, Sathe M, Kush A (2018) A modified in planta method of *Agrobacterium*-mediated transformation enhances the transformation efficiency in safflower (*Carthamus tinctorius* L.). *J Plant Biochem Biotechnol* 27: 272–283

Smith DR (1996) Growth medium. US Patent, no. 5,565,355

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, 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

Taniguchi T, Ohmiya Y, Kurita M, Tsuobmura 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

Tsumura Y (2011) Cryptomeria. In: Kole C (ed) *Wild crop relatives: Genomic and breeding resources, forest trees*. Springer, Berlin, pp 49–63

Tsumura Y, Suyama Y, Yoshimura K, Shirato N, Mukai Y (1997) Sequence-tagged-sites (STSs) of cDNA clones in *Cryptomeria japonica* and their evaluation as molecular markers in conifers. *Theor Appl Genet* 94: 764–772

Tsumura Y, Uchiyama K, Moriguchi Y, Kimura MK, Ueno S, Ujino-Ihara T (2014) Genetic differentiation and evolutionary adaptation in *Cryptomeria japonica*. *G3 (Bethesda)* 4: 2389–2402

Tsumura Y, Yoshimura K, Tomaru N, Ohba K (1995) Molecular phytyogen of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. *Theor Appl Genet* 91: 1222–1236

Ueno S, Uchiyama K, Moriguchi Y, Ujino-Ihara T, Matsumoto A, Wei FJ, Saito M, Higuchi Y, Futamura N, Kanamor H, et al. (2019) Scanning RNA-Seq and RAD-Seq approach to develop SNP markers closely linked to MALE STERILITY 1 (MS1) in *Cryptomeria japonica* D. Don. *Breed Sci* 69: 19–29

Ujino-Ihara T, Kanamori H, Yamane H, Taguchi Y, Namiki N, Mukai Y, Yoshimura K, Tsumura Y (2005) Comparative analysis of expressed sequence tags of conifers and angiosperms reveals sequences specifically conserved in conifers. *Plant Mol Biol* 59: 895–907

Ujino-Ihara T, Taguchi Y, Yoshimura K, Tsumura Y (2003) Analysis of expressed sequence tags derived from developing seed and pollen cones of *Cryptomeria japonica*. *Plant Biol* 5: 600–607

Ujino-Ihara T, Yoshimura K, Ugawa Y, Yoshimura H, Nagasaka K, Tsumura Y (2000) Expression analysis of ESTs derived from the inner bark of *Cryptomeria japonica*. *Plant Mol Biol* 43: 451–457

Yamada T, Saito H, Fujieda S (2014) Present state of Japanese cedar pollinosis: The national affliction. *J Allergy Clin Immunol* 133: 632–639

Yasue M, Ogijyama K, Suto S, Tsukahara H, Miyahara Y, R. vitis. (1987) Geographical differentiation of natural Cryptomeria stands analyzed by diterpene hydrocarbon constituents of individual trees. *Nihon Shinrin Gakkaishi* 69: 152–156

Yoshida K, Futamura N, Nishiguchi M (2012) Collection of expressed genes from the transition zone of *Cryptomeria japonica* in the dormant season. *J Wood Sci* 58: 89–103

Yoshida K, Nishiguchi M, Futamura N, Nanjo T (2007) Expressed sequence tags from *Cryptomeria japonica* sapwood during the drying process. *Tree Physiol* 27: 1–9

Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* *Conn* 1942 and *Alchorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes, R. rubi, R. undicola and R. vitis*. *Int J Syst Evol Microbiol* 51: 89–103

Vrvelje G, Holsters M, Teuchy H, Van Montagu M, Schell J (1975) Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. *J Gen Virol* 26: 33–48