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Original Paper

Screening RAPD primers to assess clonal fidelity in somatic embryos of Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) and field performance of somatic embryo-derived trees

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Abstract In this study, 100 random amplified polymorphic DNA (RAPD) primers were screened to assess clonal fidelity in somatic embryos of Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.). Of the 100 primers tested, eight were selected based on their amplification products, which showed clear DNA fragmentation, generating 8.75 bands per primer in average. The amplification products showed no genetic variation among the tested somatic embryos. In addition, the performance of somatic embryo-derived trees was monitored in the field, and their mean height growth was measured until the age of 10 years. Although no phenotypic differences were observed, the somatic embryo-derived trees showed lower growth compared with seedlings.

Key words: *Chamaecyparis pisifera*, field performance, genetic stability, RAPD primers, Sawara cypress, somatic embryogenesis.

Sawara cypress (*Chamaecyparis pisifera*), a domestic tree species in Japan, is one of six species of *Chamaecyparis* found worldwide. It grows 30-m tall with 1-m diameter at breast height, and its lumber is resistant to degradation under wet conditions; therefore, it is mainly used for making barrels, buckets, and traditional bathtubs. In addition, it is prized for its ornamental uses such as garden hedges. Moreover, this species is an important genetic resource for hybridization with a related valuable Hinoki cypress species (*Chamaecyparis obtusa*) (Fukuhara 1989).

Somatic embryogenesis is more frequently applied for large-scale propagation of plants. Regeneration of Sawara cypress through somatic embryogenesis is important not only for the effective propagation of selected trees but also for genetic transformation and somatic hybridization to create disease-resistant hybrids. Several researchers have reported that tissue culture propagation can trigger somaclonal variation among in vitro-derived plants. Different responses of species to in vitro culture can be scored in the order of low to high genetic stability; hence, genetic stability of tissue culture-derived progeny is important and a key concern for any propagation systems. At present, different molecular analyses are used to assess the genetic fidelity of in vitro-derived plants. Of these, random amplified polymorphic DNA (RAPD) is the easiest, cheapest, and most efficient technique (Jayanthi and Mandal 2001).

We were the first to report somatic embryogenesis in Sawara cypress (Maruyama et al. 2002). Large-scale somatic embryo production and subsequent high plant conversion frequencies indicated the efficiency of the regeneration system and the high quality of the produced somatic embryos.

In this study, RAPD primers we screened to assess clonal fidelity in somatic embryos of Sawara cypress. In addition, field performance of somatic embryo-derived trees was monitored, and their mean height growth was measured until the age of 10 years.

Materials and methods

Somatic embryo production

In this study somatic embryogenesis was initiated from a single seed. Embryogenic cultures proliferated by 2–3-week-interval subcultures were transferred to maturation medium as previously described (Maruyama et al. 2002). Before the maturation step, approximately 10–20 mg of fresh weight (FW) embryogenic tissues from the solid medium were transferred to a 100-ml flask containing approximately 35 ml of maturation

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medium (composition equivalent to that used for maintenance and proliferation but lacking gellan gum) and were cultured for approximately 2 weeks in the dark on a rotary shaker (Model G25, New Brunswick Scientific Co., INC., New Jersey, USA) at 70 rpm at 25±1°C. Approximately 100 mg FW embryogenic tissues suspended in 2.5 ml of medium were placed on filter paper discs of 70-mm diameter in 90-mm diameter plates (SH90-20, Iwaki Glass Co., Ltd., Chiba, Japan) containing approximately 35 ml of maturation medium supplemented with basal salts of EM medium (Maruyama et al. 2000), 50 g l^{-1} maltose, 100 μM abscisic acid (ABA), 2 g l^{-1} activated charcoal (AC), 150 g l^{-1} polyethylene glycol (PEG) 4,000, and amino acids (7.3 g l^{-1} glutamine, 2.1 g l^{-1} asparagine, 0.7 g l^{-1} arginine, 0.079 g l^{-1} citrulline, 0.076 g l^{-1} ornithine, 0.055 g l^{-1} lysine, 0.04 g l^{-1} alanine, and 0.053 g l^{-1} proline; Smith 1996). The plates were sealed with Novix-II film (Iwaki Glass Co., Ltd., Chiba, Japan) and were incubated in the dark at 25±1°C for 6–8 weeks.

Germination of somatic embryos and plant conversion

Mature somatic embryos were transferred to the germination medium containing basal salts at the half concentration of the standard LP medium (Aitken-Christie and Thorpe 1984), 20 g l^{-1} sucrose, and 2 g l^{-1} AC and solidified with 10 g l^{-1} Wako agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cultures were incubated at 25±1°C under a photon flux density of approximately 65 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (100 V, 40 W; Toshiba Co., Tokyo, Japan) for 16 h daily. About 300 emblings that showed root and epicotyl growth were transferred to 300-ml Erlenmeyer flasks containing approximately 100 ml of growth medium (germination medium supplemented with 30 g l^{-1} sucrose and 5 g l^{-1} AC and solidified with 12.5 g l^{-1} Wako agar) and were cultured for approximately 2 months under the conditions described above before ex vitro acclimatization.

Ex vitro acclimatization and field performances of somatic embryo-derived trees

Developed emblings were transplanted into pots containing vermiculite and were acclimatized inside a growth chamber at 25°C in 80% relative humidity. During the first 2 weeks, the plants were kept in 420×330×140-mm height plastic boxes (Assist No.2, Shinkigosei Co., Ltd., Tokyo, Japan) covered with 405×315×105-mm height transparent covers (Assist cover No.2, Shinkigosei Co., Ltd.), and were irrigated with tap water one time per week. The covers were gradually opened during the next 2 weeks and were removed completely approximately one month after transplantation. The pots were fertilized using a modified nutrient solution (Nagao 1983), which included 143 mg l^{-1} NH_4NO_3, 55.1 mg l^{-1} $NaH_2PO_4 \cdot 2H_2O$, 47.1 mg l^{-1} KCl, 52.5 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 61 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 25 mg l^{-1} Fe(III) EDTA, 0.1 mg l^{-1} Cu EDTA, 0.1 mg l^{-1} Mn EDTA, 0.1 mg l^{-1} Zn EDTA, 1.5 mg l^{-1} H_3BO_3, 0.01 mg l^{-1} KI, 0.005 mg l^{-1} $CoCl_2 \cdot 6H_2O$, and 0.005 mg l^{-1} MoO_3. The acclimatized plants were transferred to a greenhouse and were grown at 25°C and 60% relative humidity for approximately 6 months before transplanting them to the field. One hundred somatic embryo-derived trees and ten seed-derived trees were planted in the field at a 1.0×1.0 m spacing. The field test was set in Chiyoda Experimental Station of the Forestry and Forest Products Research Institute, located at 36°11′N, 140°13′E, 35 m a.s.l., on the Kanto Plain in central Japan (Kasumigaura, Ibaraki, Japan). The main annual precipitation and air temperature from 1971 to 2000 around the study area were 1,186 mm and 13.5°C, respectively (Japan Meteorological Agency 2002). The soil in the area is of the type Andisol with Silty Loam texture and pH 5.0. The tree height was measured every 2 years during age of 4–10 years. The height of 30 somatic embryo-derived trees (somatic trees obtained from a single seed) and seven seed-derived trees (her sister seedlings obtained from other seeds of the same mother tree) was measured to calculate the mean growth after 4–10 years.

Determination of genetic stability of somatic embryos

DNA was extracted using a modified sodium dodecyl sulfate (SDS) method. Somatic embryos were placed in 200 μl of extraction buffer [0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.1 mg proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan)], homogenized with a Minitor homogenizer (Minitor Co., Ltd., Tokyo, Japan), and incubated at 37°C for 2 h. Thereafter, DNA was extracted using TE-saturated phenol. RNase was added to a final concentration of 50 μg ml^{-1}. The samples were incubated at 37°C for 1 h, and DNA was extracted twice using TE-saturated phenol. DNA was precipitated with one-tenth volume of 3 M sodium acetate and 2.5 volumes of cold ethanol, washed twice with 70% ethanol, and dissolved in 200 μl of TE.

RAPD reactions were based on a protocol modified from that of Williams et al. (1990). Polymerase chain reaction (PCR) was performed in 15 μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primer, 1 unit of Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), and 10 ng of genomic DNA in thermal cycler (PTC-200, MJ Research Inc., Quebec, Canada). In all, 100 primers were used for DNA amplifications, which were purchased in a kit (OPA-01 to OPC-20 and OPAA-01 to OPAB-20; Operon Technologies Inc., Alameda, California, USA). Twelve samples from somatic embryos were used for each primer. The PCR conditions were as follows: initial denaturation for 3 min at 94°C; 40 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 37°C, and extension for 120 s at 72°C; and final extension for 3 min at 72°C. PCR products of RAPD were analyzed on 2% agarose gel stained with ethidium bromide in 1×TAE buffer.

Results

Production of somatic trees and their field performance

Initial formation of cotyledonary embryos was observed approximately 4 weeks after the transfer of embryogenic tissues to the maturation medium and was clearly distinct after 6 weeks of culture (Figure 1A). Cotyledonary somatic embryos were collected and transferred to the germination medium. Initiation of germination (root emergence) was observed approximately a week after the transfer to the germination medium (Figure 1B). Subsequent formation of plantlets (emergence of both the root and epicotyl) was observed after 4–8 weeks of culture (Figure 1C). The results of germination and plantlet conversion from somatic embryos, and survival after acclimatization from regenerated emblings are shown in Table 1.

Acclimatized emblings showed 100% survival after being transplanted to the field. The mean height of 30 somatic trees and seven seedlings after 4–10 years of growth is shown in Figure 2. Although no significant differences were observed in the mean height until the age of 4 years, growth of the seedlings was markedly superior to that of somatic trees after 6 years of age.

Determination of genetic stability of somatic embryos

In all, 100 primers were screened against 12 randomly chosen cotyledonary somatic embryos. Of the 100 primers tested, 47% showed reproducible amplifications. Of these, OPAA-01, OPAA-11, OPAB-01, OPAB-04, OPAB-05, OPAB-07, OPAB-08, and OPAB-10 were selected based on their amplification products, which showed clear DNA fragmentation. These eight primers generated 70 scorable band classes with an average of 8.75 bands per primer (Table 2).

RAPD profiles of the sample somatic embryos with selected primers are shown in Figure 3. However, from these zymograms any aberration could not be detected in RAPD banding patterns among the tested somatic embryos.

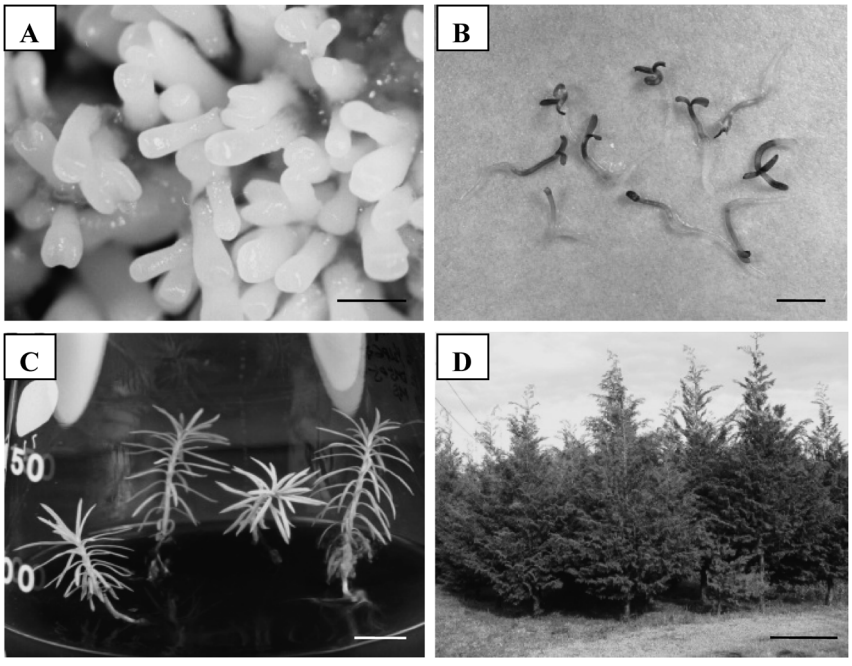

Figure 1. Somatic embryo production and plant regeneration of Sawara cypress. A: Maturation of somatic embryos. B: Germination of somatic embryos. C: Plant regeneration from somatic embryos. D: Somatic embryo-derived trees growing in the field. Bars 1 cm (A–C) and 1 m (D).

Table 1. Germination and plantlet conversion from somatic embryos, and survival after acclimatization from regenerated emblings of Sawara cypress.

Material	Germination (%)	Conversion (%)	Survival (%)
Somatic embryo	97 (12,125/12,500)	92 (11,500/12,500)	—
Embling	—	—	98 (196/200)

Values in parentheses represent (germinated or converted somatic embryos/total somatic embryos tested) and (successfully acclimatized emblings/total emblings tested).

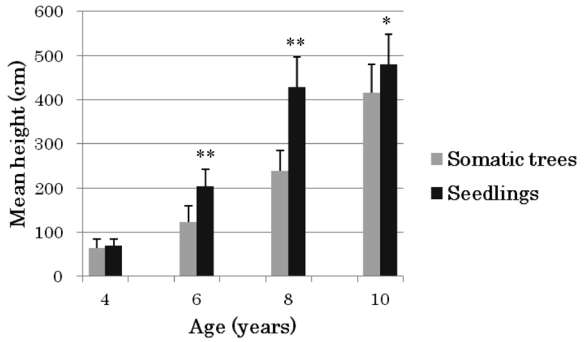

Figure 2. Increment in the mean height of somatic embryo-derived trees (somatic trees) versus seed-derived trees (seedlings) of Sawara cypress growing in the field during 4–10 years of growth. Significant at ** $p<0.01$, * $p<0.05$ *t*-test.

Table 2. Number of amplification products generated with eight selected primers in somatic embryos of Sawara cypress.

Primer	Sequence	No. of scorable bands	Reference
OPAA-01	AGACGGCTCC	6	Operon Tech. Inc., USA
OPAA-11	ACCCGACCTG	8	Operon Tech. Inc., USA
OPAB-01	CCGTCGGTAG	9	Operon Tech. Inc., USA
OPAB-04	GGCACGCGTT	10	Operon Tech. Inc., USA
OPAB-05	CCCGAAGCGA	10	Operon Tech. Inc., USA
OPAB-07	GTAAACCGCC	11	Operon Tech. Inc., USA
OPAB-08	GTTACGGACC	7	Operon Tech. Inc., USA
OPAB-10	TTCCCTCCCA	9	Operon Tech. Inc., USA

Discussion

The most important advantage of cloning conifers using somatic embryogenesis is the cryopreservation of the tissue without changing its genetic make-up and the loss of juvenility (Park et al. 1998). However, genotypic instability is commonly observed in plants derived from tissue culture, which is at least partly due to in vitro-induced stress (Burg et al. 2007). To establish an optimal protocol for the commercial production of high-quality somatic plants, it is important to demonstrate their genetic stability and comparable growth relative to that of conventional seedlings. Therefore, the use of somatic embryogenesis in clonal forestry requires an accurate assessment of genetic parameters and performance stability of clones for plantation (Wahid et al. 2012). Genetic stability of in vitro-propagated plants should be assessed as early as possible, especially in long-living trees such as conifers (Marum et al. 2009). At present, different molecular analytical techniques are used to highlight somaclonal variation in tissue culture and regenerants of several plants (Leva et al. 2012). Several researchers have used RAPD techniques to investigate genetic variability and have found it very efficient and reliable (Goto et al. 1998). In a study of the genetic integrity of black spruce somatic embryos, RAPD primers were screened, and no variation was observed among somatic embryos derived from individual zygotic embryos (Isabel et al. 1993). Similar results were reported using the RAPD technique in Norway spruce (Fourré et al. 1997), Japanese black pine (Goto et al. 1998), Japanese white oak (Thakur et al. 1999), loblolly pine (Tang 2001), almond (Martins et al. 2004), hazelnut (Nas et al. 2004), pedunculate oak (Valladares et al. 2006), cork oak (Fernandes et al. 2011), apricot (Soliman 2012), annatto (Siril and Joseph 2013), and seedless lemon (Goswami et al. 2013). In contrast, RAPD analysis reported genetic variation in poplar (Ostry et al. 1994; Rani et al. 1995), white spruce (Isabel et al. 1995, 1996), peach (Hashmi et al. 1997), teak (Gangopadhyay et al. 2003), Idaho locust (Ngezahayo et al. 2006), stone pine (Cuesta et al. 2010), and olive (Leva et al. 2012).

Although somaclonal variation has been extensively studied, its trigger mechanisms remain largely unknown or are under theoretical speculation (Skirvin et al. 1993, 1994). With respect to the origin and causes of somaclonal variation in micropropagated plants, the system of regeneration induction, explant sources, culture media, culture conditions, culture cycles, and genotype effect are some of the factors that induce variation during in vitro culture (Leva et al. 2012; Pierik 1987). In the micropropagation industry, the occurrence of uncontrolled variation during the culture process is an undesirable phenomenon (Karp 1994). In contrast, somaclonal variation is a valuable source of genetic variation to improve crops by selecting novel variants (Karp 1995).

In Sawara cypress, high efficiency of embryo maturation was obtained when embryogenic cells were cultured on the media supplemented with a combination of maltose, PEG, ABA, and AC. More than 1000 mature cotyledonary embryos were produced from approximately 100 mg (FW) inoculum on a maturation medium supplemented with 150 g l^{-1} PEG (Maruyama et al. 2002). In addition to a highly synchronized and high-yielding maturation procedure, subsequent high germination and plant conversion frequencies demonstrated the high quality of the produced somatic embryos. Somatic embryos readily germinated after transferring it to a plant growth regulator-free medium and subsequently formed plantlets. Thus, somatic embryo production efficiency, quality of embryos produced, and their genetic stability are the key criteria to optimize an efficient plant regeneration system via somatic embryogenesis.

The results of RAPD analysis showed absence of somaclonal variation in our plant propagation system. Somatic embryogenesis is less prone to genetic alterations because it involves the expression of many genes (Vasil 1995). Similarly, Jain et al. (1995) emphasized how plants regenerated via somatic

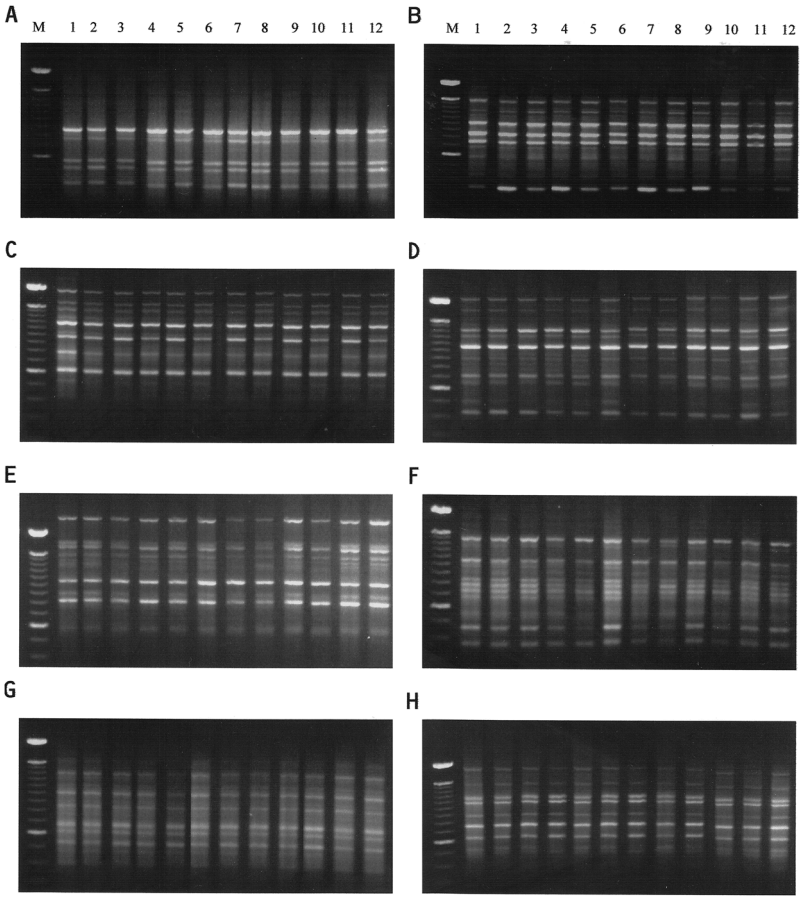

Figure 3. DNA amplification product patterns of the 12 samples of Sawara cypress somatic embryos obtained using RAPD primers. M is the molecular size marker (100-bp ladder from BRL). RAPD primers are as follows: A: OPAA-01, B: OPAA-11, C: OPAB-01, D: OPAB-04, E: OPAB-05, F: OPAB-07, G: OPAB-08, and H: OPAB-10.

embryogenesis produced true-to-type progeny and showed minimized variation. In several cases, embryogenic cell lines are highly stable and do not exhibit somaclonal variation (Isabel et al. 1993; Thakur et al. 1999). Somatic embryos derived from pre-embryogenic determined cells (zygotic embryo cells) tend to be genetically stable (Sharp et al. 1980). However, depending on the plant species and genotype, somaclonal variation can also occur in embryogenic cultures, if the embryos are kept in cultures for an extended period. Generally, the longer the culture phase, the greater the potential to generate somaclonal variation (Brar and Jain 1998).

In this study, about 12,000 emblings were produced in vitro from a single immature zygotic embryo of Sawara cypress within a short time, and 200 regenerated emblings were successfully acclimatized to undergo field testing (Figure 1D). Although no morphological differences were observed until the age of 10 years, the growth of somatic trees was lower than that of seedlings. The reason for that was not explored in this study; however, we speculate that the difference in growth can be attributed to the culture conditions of somatic plants. Similarly, Bozhkov and von Arnold (1998) and Högberg et al. (2001) reported inferior growth increment in somatic plants versus that in seedlings of *Picea abies*. For the same species, Cyr et al. (1991) reported that the differences between somatic plants and seedlings occurred early during plant development. Although somatic plants were consistently shorter than seedlings after the first growth period, the differences in height decreased by the end of the second growth period (Högberg et al. 2003). In contrast, for coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii)*, survival rate and tree height of 37 somatic clones after 7.5 years were inferior to those of zygotic trees across the five tests studied (Dean et al. 2008). Similarly, Niskanen et al. (2008) reported that the growth of Scots pine (*Pinus sylvestris)* clones was lower than that of seedlings of the same origin throughout the 10-year field trial.

Evidences that culture conditions during somatic embryo development may reduce the growth of somatic plants were reported for several conifers (Högberg et al. 2003). Use of PEG and long-term contact with ABA during maturation substantially reduced the growth of regenerated Norway spruce plants (Bozhkov and von Arnold 1998, Högberg et al. 2001). Grossnickle et al. (1994) found that somatic plants of interior spruce

were significantly shorter than seedlings at the time of ex vitro transfer and that these differences remained after 2 years in the field. However, height increments were similar for both plant types (Grossnickle and Major 1994a, 1994b). Högberg et al. (2001) found that the after effects of culture conditions could be avoided by modifying the procedure of somatic embryogenesis. In addition, epicotyl length and presence of lateral roots as combined selection criteria at ex vitro transfer could be used to identify somatic plants having height growth characteristics comparable to those of seedlings. Early selection according to these criteria improves clonal performance and reduces intraclonal variation in somatic plants of Norway spruce (Högberg et al. 2003).

To our knowledge, this is the first report to assess genetic stability in somatic embryos of Sawara cypress and growth performance of the corresponding somatic plants in the field. The results obtained in this study suggest that selected primers could be used to assess the genetic fidelity in Japanese cypresses. On the other hand, the achieved differences in growth performance between somatic trees and seedlings represent an important parameter for improving the culture protocol. Moreover, to facilitate the understanding of growth dynamics of somatic trees of Sawara cypress, growth increments should be monitored for further several years.

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