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Note

High-efficiency *Agrobacterium*-mediated transformation of *Cryptomeria japonica* D. Don by co-cultivation on filter paper wicks followed by meropenem treatment to eliminate *Agrobacterium*

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Abstract To develop a more efficient genetic transformation system for *Cryptomeria japonica* (Japanese cedar), the present study evaluated the effects of culture support during co-cultivation and the use of β-lactam antibiotics (meropenem and carbenicillin) to eliminate *Agrobacterium* on the transformation efficiency. The co-cultivation of embryogenic tissues and bacterium on filter paper wicks prevented the excess growth of *Agrobacterium* compared with that observed on solid medium, leading to an increased number of gene-transferred cells. Meropenem successfully eliminated the bacterium at low concentrations (10 mg l^{-1}) and had no phytotoxic effect. The transformed tissues were screened based on kanamycin resistance and green fluorescent protein (GFP) fluorescence, and the integration of the transgene into the plantlet genome was confirmed by Southern blotting. We successfully generated a mean of 105.3±9.02 independent transgenic lines per gram of embryogenic tissues using a combination of filter paper wicks and meropenem. The transformation efficiency of the improved method was approximately 30-fold higher than that observed using the conventional method.

Key words: Conifer, *Cryptomeria japonica*, filter paper wick, meropenem, stable transformation.

Cryptomeria japonica D. Don (Japanese cedar or Sugi) is the most important commercial forest species in Japan, where it covers approximately 40% of the artificial forest area. *C. japonica* breeding projects such as plus tree selection for its growth, yield, stem straightness, and resistance to disease and insects have been performed for a long time. However, allergic reactions to *C. japonica* pollen have become a severe public health concern in Japan, and currently, more than 25% of the Japanese population is affected by the cedar pollinosis (Okamoto et al. 2009). Therefore, the production of *C. japonica* with a no-pollen trait is an important breeding objective, and the genetic transformation of plus trees is expected to be a powerful method for the rapid generation of these strains because it can induce desirable traits simply by the introduction of specific genes without unnecessary genetic transitions.

A research group in Japan published a database of 55,543 ESTs based on *C. japonica* cDNA libraries obtained from seedlings, inner barks, female strobili, male strobili, pollen, leaves, vegetative buds, and sapwood (ForestGEN; http://forestgen.ffpri.affrc.go.jp/en/info_cj.html). Furthermore, there have been recent reports on ESTs from male strobilus-specific suppression subtractive hybridization libraries and the xylem transition zone (Kurita et al. 2011; Yoshida et al. 2012). Genetic transformation is also an essential tool that facilitates the effective use of these extensive resources for reverse genetic studies and molecular breeding. The stable transformation of conifers has been reported in several genera, such as *Picea*, *Larix*, and *Pinus* (Malabadi and Nataraja 2007). However, there are only 2 reports on the genetic transformation of *C. japonica*, which used particle bombardment and an *Agrobacterium*-mediated method (Maruyama et al. 2000; Taniguchi et al. 2008), whereas the transformation efficiency levels were inadequate. Thus, we attempted to develop a more efficient *C. japonica* transformation method based on the *Agrobacterium*-mediated method reported by Taniguchi et al. (2008). Recently, it was reported that meropenem, a novel β-lactam antibiotic, is highly effective against *Agrobacterium*, and it improved transformation efficiencies in tobacco, tomato, rice, and apple compared with those observed by cefotaxime and carbenicillin

Abbreviations: EST, expressed sequence tag; GFP, green fluorescent protein; GUS, β-glucuronidase; MM, maintenance medium.
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(Li et al. 2011; Ogawa and Mii 2007). Furthermore, the use of filter paper wicks during co-cultivation contributed to the efficient transformation of cucumber, kabocha squash, and rice by controlling the growth of *Agrobacterium* (Nanasato et al. 2011; Nanasato et al. 2013; Ozawa 2009). In the present study, we focused on the infection efficiency and elimination of *Agrobacterium* and investigated the combined effects of co-cultivation on filter paper and meropenem treatment on *C. japonica* transformation.

The embryogenic tissue used in the present study was an established cell line (Cj5-2-1), which was obtained and maintained according to the method described by Taniguchi et al. (2008). To investigate the effects of antibiotics on the elimination of *Agrobacterium* and the growth of embryogenic tissues, approximately 15 mg of embryogenic tissue was placed at 10 positions in the maintenance medium (MM; Taniguchi et al. 2008), which was supplemented with meropenem (meropenem trihydrate; Wako, Japan) at 5, 10, 20, or 40 mg l^{-1} or with carbenicillin sodium salt (Wako, Japan) at 250 mg l^{-1}, of the conventional method (Taniguchi et al. 2008). Each treatment comprised 3 replicate plates, which were incubated at 25°C for 2 weeks in the dark. The growing embryogenic tissues (approximately 0.2 g) were weighed and transferred to fresh medium. Two weeks after the transfer, all embryogenic tissues were weighed again, and the mean fresh weight increase was calculated for each embryogenic tissue.

Agrobacterium infection efficiency and transformation frequency were evaluated using binary vectors, i.e., pIG121-Hm (Ohta et al. 1990) and pUbiP-GFP/Hyg (Taniguchi et al. 2008), respectively. The pIG121-Hm vector contained 35S promoter-driven *intron-GUS*. The pUbiP-GFP/Hyg vector contained maize ubiquitin promoter-driven *intron-sGFP*(*S65T*) and kanamycin/hygromycin double-selective markers (Taniguchi et al. 2008). These vectors were transferred into *A. tumefaciens* strain GV3101/pMP90 (Koncz and Schell 1986) by electroporation using a MicroPulser Electroporator (Bio-Rad, USA).

The *Agrobacterium*-mediated transformation of *C. japonica* was performed according to the method of Taniguchi et al. (2008), with some modifications. The embryogenic tissues (1 g fresh weight) were grown on MM for 1 week and transferred to 20 ml of the bacterial suspension in a 50-ml test tube. The tube was horizontally placed on a rotating shaker at 100 rpm for 20 min in the dark at 25°C. Subsequently, 10 ml of the culture, which contained 0.5 g embryogenic tissue, was poured onto a 7-cm (diameter) sterile filter paper (No. 2, Whatman, Germany) in a Büchner funnel, and a vacuum pulse (5 s, 0.03 MPa) was used to filter the liquid completely. For co-cultivation, the filter paper with the embryogenic tissues was placed in a 90×15-mm Petri dish containing solid (4 g l^{-1} gelrite) MM supplemented with 50 μM acetosyringone, or on 3 sheets of 8.5-cm (diameter) sterile filter papers (No. 2, Advantec, Japan) containing 5.0, 5.5, or 6.0 ml of liquid MM supplemented with 50 μM acetosyringone. After co-cultivation for 2 days, the 2 sheets of filter papers with the tissues (1 g fresh weight) were soaked in 40 ml liquid MM in a 50 ml centrifuge tube. The tissues were then dislodged from the filter paper using a spatula, shaken several times to resuspend them in the liquid MM, and then centrifuged for 1 min at 150×*g* without brake. The supernatant was discarded, and the tissues were washed twice with fresh liquid MM and once with fresh liquid MM supplemented with 250 mg l^{-1} carbenicillin or 10 mg l^{-1} meropenem to eliminate *Agrobacterium*. Ten milliliters of the washed suspension, which contained 0.25 g of tissue, was poured onto a new filter paper in a Büchner funnel and filtered by applying a vacuum pulse. The tissues collected on the filter paper were then cultured in solid MM supplemented with 250 mg l^{-1} carbenicillin or 10 mg l^{-1} meropenem for 3 days, before they were transferred to fresh medium with the same composition every 2 weeks. After regrowth of the embryogenic tissues commenced, the filter paper with the tissues was transferred to selective medium (MM supplemented with 250 mg l^{-1} carbenicillin or 10 mg l^{-1} meropenem, and 25 mg l^{-1} kanamycin), and then transferred to fresh selection medium every 2 weeks. Somatic embryo induction from the embryogenic tissues grown on selection medium and the germination of the somatic embryos were conducted according to the methods of Taniguchi et al. (2008).

To compare *Agrobacterium* growth during co-cultivation on paper filter wicks with that on gelrite-solidified medium, the liquid MM supernatant from the first washing step was diluted 1000-fold. Twenty microliters of the liquid MM were plated onto solid Luria–Bertani (LB) medium, which was supplemented with 50 mg l^{-1} kanamycin, 25 mg l^{-1} gentamicin, and 25 mg l^{-1} rifampicin, respectively. The plates were incubated at 28°C for 2 days, and individual colonies were counted.

The *Agrobacterium* infection efficiency was assessed using a histochemical β-glucuronidase (GUS) assay, as described by Konagaya et al. (2013), and a filter paper with the embryogenic tissues was incubated in 2 ml GUS assay solution (Konagaya et al. 2013).

Genomic DNA was isolated from fresh shoots with Nucleon PhytoPure (GE Healthcare, Japan) and used for Southern blotting. The purified genomic DNA (5 μg) was digested with *Hin*dIII or *Xba*I and fractionated on 1% agarose gel, blotted onto nylon membranes, positively charged (Roche, Germany), and probed with a digoxigenin-labeled *sGFP* fragment prepared by PCR using the primer set: 5′-CCTGAAGTTCATCTGCACCAC-3′ and 5′-GAACTCCAGCAGGACCATGT-3′.

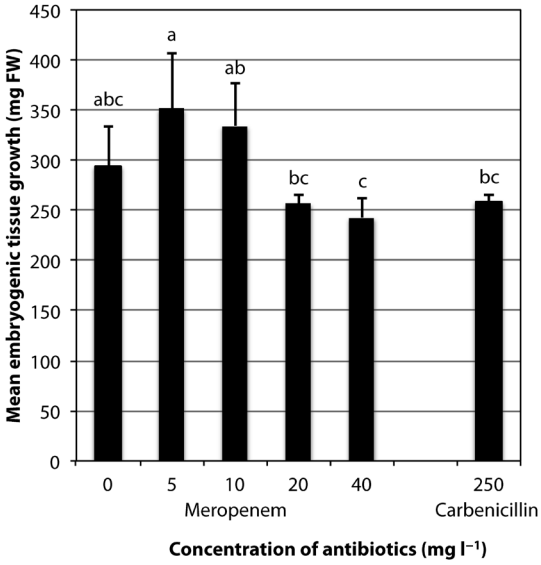

Figure 1. Effects of meropenem on embryogenic tissue growth compared with the control (0 mg l^{-1}) and carbenicillin. Embryogenic tissues weighing approximately 0.2 g were cultured on solidified MM, which was supplemented with each specified concentration of antibiotics. After 2 weeks, the increase in fresh weight was calculated for each embryogenic tissue. Each value represents the mean±SD based on 3 replicates. The data are the means of 10 samples from each replicate plate. Bars with the same letter are not significantly different according to Tukey's HSD test at $p<0.05$.

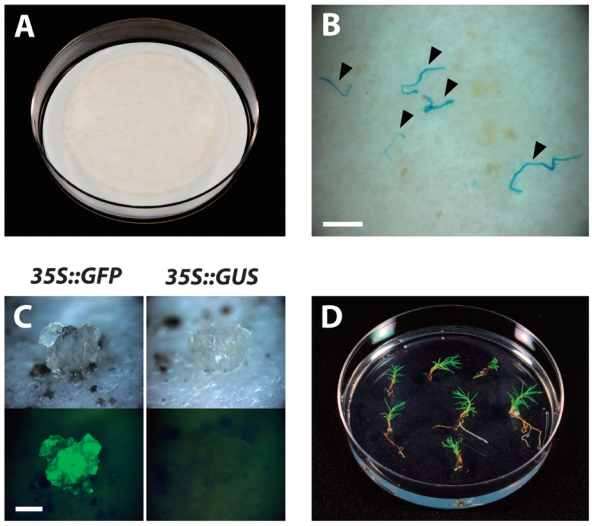

Figure 2. *Agrobacterium*-mediated transformation of *C. japonica* during co-cultivation using filter paper wicks. (A) Embryogenic tissues were co-cultured on 3 sheets of filter paper (8.5 cm in diameter, No. 2), which were moistened with 5.5 ml of MM supplemented with 50 μM acetosyringone. (B) The co-cultured embryogenic tissues on filter paper wicks stained with X-Gluc solution. Bar, 0.5 mm. The arrowheads indicate GUS-positive cells in the embryogenic tissue. (C) Colonies harboring pUbiP-GFP/Hyg (left) and pIG121-Hm (right) on selection medium, and a fluorescent image (lower). Bar, 1 mm. (D) Germination of transgenic somatic embryos at 8 weeks after culture on the germination medium.

Hybridization using DIG Easy Hyb buffer (Roche) and chemiluminescent detection with CDP-Star (Roche) were performed according to the manufacturer's instructions.

An efficient *Agrobacterium*-mediated transformation system requires the use of antibiotics to eliminate bacterium, which should have negligible effects on the growth potential of the transformed cells. Recently, it was reported that meropenem has a high antibacterial activity against *Agrobacterium* compared with other β-lactam antibiotics such as cefotaxime, carbenicillin, and cefbuperazone (Ogawa and Mii 2004). 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 (Malá et al. 2009; Mendes et al. 2009; Silva et al. 2009; Terakami et al. 2007). However, the effects of meropenem on *C. japonica* remain unknown. In the present study, we evaluated the effects of meropenem on the growth of *C. japonica* embryogenic tissues and its transformation efficiency compared with carbenicillin, which is the β-lactam antibiotic used to eliminate *Agrobacterium* after co-cultivation according to the conventional method (Taniguchi et al. 2008).

There were no significant differences in the effects of the antibiotics on embryogenic tissue growth for all media containing antibiotics and the control (MM without antibiotics; Figure 1). Meropenem had no inhibitory effects, even at higher concentrations (40 mg l^{-1}; Figure 1). 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^{-1} and 10 mg l^{-1}) using media containing meropenem (Figure 1). Based on these results, 10 mg l^{-1} was selected as the meropenem concentration in subsequent transformation experiments.

Unsuitable co-cultivation conditions may lead to unfavorable effects such as the 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, and the growth of *Agrobacterium* during co-cultivation can be controlled by adjusting the amount of liquid medium on the filter paper wicks used as culture supports (Ozawa 2009). Therefore, we aimed to determine the optimal co-cultivation conditions with filter paper wicks. After the embryogenic tissues and *Agrobacterium* were suspended in liquid medium, a filter paper with the embryogenic tissues was placed on a solid co-cultured medium, or on 3 sheets of filter paper moistened with 5–6 ml of liquid co-cultured medium (Figure 2A). Following co-cultivation, 1 g embryogenic tissues were resuspended in 40 ml of liquid MM, and the MM was plated onto LB medium to quantify the number of *Agrobacterium* cells (Table 1). The mean number of

Agrobacterium cells was significantly lower (>14-fold) in the 3 liquid media conditions compared with that on solid medium using the conventional method.

The infection efficiency was estimated based on the number of GUS-positive cells. GUS assay was performed using the embryogenic tissues at 3 days after the elimination of *Agrobacterium* using 10 mg l^{-1} meropenem (Figure 2B). Compared with co-cultivation on the solid media, the infection efficiency significantly improved (>16-fold) in the 3 liquid media conditions (Table 1). 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 co-cultivation procedures. The highest infection efficiency was observed with 5 ml of liquid medium (Table 1). However, washing the embryogenic tissues using this liquid medium condition was difficult because the embryogenic tissues floated after being suspended in MM. Thus, the filter paper wicks were moistened with 5.5 ml liquid medium in the subsequent co-cultivation tests.

To evaluate the improvement in the transformation efficiency, we attempted to produce *sGFP*-introduced transgenic *C. japonica* using a combination of co-cultivation on filter paper wicks moistened with 5.5 ml liquid MM supplemented with 50 μM acetosyringone and *Agrobacterium* elimination with 10 mg l^{-1} meropenem. *Agrobacterium* harboring pUbiP-GFP/Hyg was infected, and colonies of the embryogenic tissues were screened using 25 mg l^{-1} kanamycin and GFP fluorescence. Transformed colonies with GFP fluorescence were observed 2–4 months after *Agrobacterium* infection (Figure 2C), whereas no colonies that harbored pIG121-Hm (control) exhibited fluorescence (Figure 2C). Twelve lines of randomly selected GFP-positive colonies were cultured in somatic embryo maturation medium, and the mature somatic embryos were induced in all lines after 7–8 weeks of culture. The mature somatic embryos germinated in germination medium (Figure 2D), after which the transgenic plantlets acclimated and continued to grow normally in a containment glasshouse. The capacity for embryogenesis was 100–200 somatic embryos per plate containing approximately 250 mg embryogenic tissue, and the germination frequency of the somatic embryos was approximately 90%.

Southern blotting was performed using young shoots to confirm the stable integration of the transgene into the plant genome (Figure 3). A DIG-labeled *sGFP* probe hybridized with the digested DNA from transgenic plants but not with that of the wild-type. All selected plants exhibited different patterns of insertion and the number of signals was 1–9, indicating that single or multiple copies of foreign genes were integrated into

Table 1. Effects of the co-cultivation condition on *Agrobacterium* growth during co-cultivation, and the infection frequency.

Co-cultivation condition[a]	Mean No. of *Agrobacterium* cells ($\times 10^7$ cfu ml^{-1})[b]	Mean No. of GUS-positive cells per plate (0.25 g tissues)[c]
Filter paper wicks with 5 ml MM	1.66±0.1	48.44±25.5
Filter paper wicks with 5.5 ml MM	1.68±0.2	30.75±26.3
Filter paper wicks with 6 ml MM	2.01±0.3	10.83±7.8
Gelrite-solidified MM	29.15±5.4	0.67±0.6

[a] A filter paper (7 cm in diameter) with embryogenic tissues was co-cultured on 3 sheets of filter paper (8.5 cm in diameter, No. 2), which were moistened with 5–6 ml of liquid MM or gelrite-solidified MM supplemented with 50 μM acetosyringone at 25°C for 2 days. [b] Co-cultured 1 g embryogenic tissues were resuspended in 40 ml of liquid MM, and the MM was plated onto LB medium to quantify the number of *Agrobacterium* cells. [c] Washed embryogenic tissues were incubated for 3 days in MM supplemented with 10 mg l^{-1} meropenem, and the GUS-positive cells were quantified. Each value represents the mean±SD of 3 replicates.

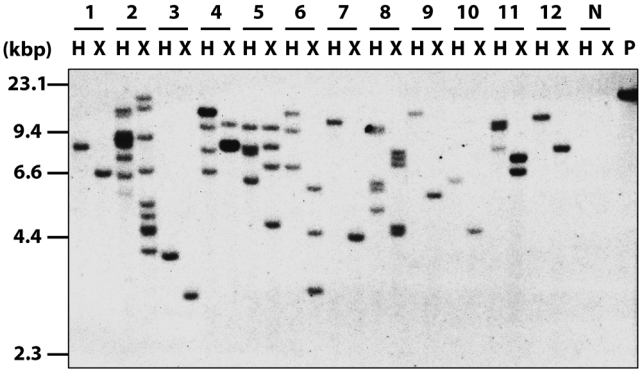

Figure 3. Southern blotting of *C. japonica* transgenic plants. Genomic DNA (5 μg) was digested using *Hin*dIII (H) or *Xba*I (X) and hybridized with an *sGFP* probe. Lane N, non-transgenic line. Lane P, *Hin*dIII-digested pUbiP-GFP/Hyg (30 pg). Lanes 1–12, independent transgenic lines.

the *C. japonica* genome. In 3 replicate transformation experiments using our improved method (co-cultivation on filter paper wicks and *Agrobacterium* elimination with meropenem), the mean transformation efficiency (the number of GFP-positive colonies per gram of co-cultivated embryogenic tissues) was 105.3±9.02, whereas the method where carbenicillin was used to eliminate *Agrobacterium* had a lower transformation efficiency of 56.5±7.78 (Table 2). This result indicates that the transformation efficiency was increased approximately 2-fold with meropenem than that observed with carbenicillin. Because the number of GFP-minus colonies was 1–3 in all experiments (data not shown), it is considered that this method had a very low escape rate. By contrast, the mean transformation efficiency with the conventional method (co-cultivation on gelrite-solidified medium and *Agrobacterium* elimination by carbenicillin) was 3.5±0.71 (Table 2). Thus, the transformation efficiency was increased approximately 30-fold by the improved method than that observed using the conventional method. Moreover, the re-growth of *Agrobacterium* was occasionally observed after co-cultivation when using the conventional method (data not shown). However, no agrobacterial re-growth was observed with the improved method. Similar re-growth 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 co-cultivation and transformed embryogenic tissues are efficiently selected using meropenem, even at low concentrations. A high transformation efficiency (colonies per gram of co-cultivated embryogenic tissues) has 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 *Chamaecyparis*

Table 2. Effects of culture support on co-cultivation and β-lactam antibiotics on the *Agrobacterium*-mediated transformation of *C. japonica*.

Culture support	Experiment No.	No. of GFP-positive embryogenic tissue colonies[c]	
		Meropenem	Carbenicillin
Filter paper wicks[a]	#1	114	51
	#2	96	62
	#3	106	NT
	Mean	105.3±9.02	56.5±7.78
Gelrite[b]	#1	NT	3
	#2	NT	4
	Mean	NT	3.5±0.71

All experiments used 1 g of embryogenic tissues for co-cultivation, 10 mg l^{-1} meropenem or 250 mg l^{-1} carbenicillin as β-lactam antibiotics to eliminate *Agrobacterium*, and 25 mg l^{-1} kanamycin for selection. [a] Three sheets of filter paper (8.5 cm in diameter, No. 2) were moistened with 5.5 ml of MM supplemented with 50 μM acetosyringone. [b] Gelrite-solidified MM supplemented with 50 μM acetosyringone. [c] The GFP fluorescence of kanamycin-resistant colonies obtained after selective cultivation for 4 months were observed by fluorescence microscopy. NT, not tested.

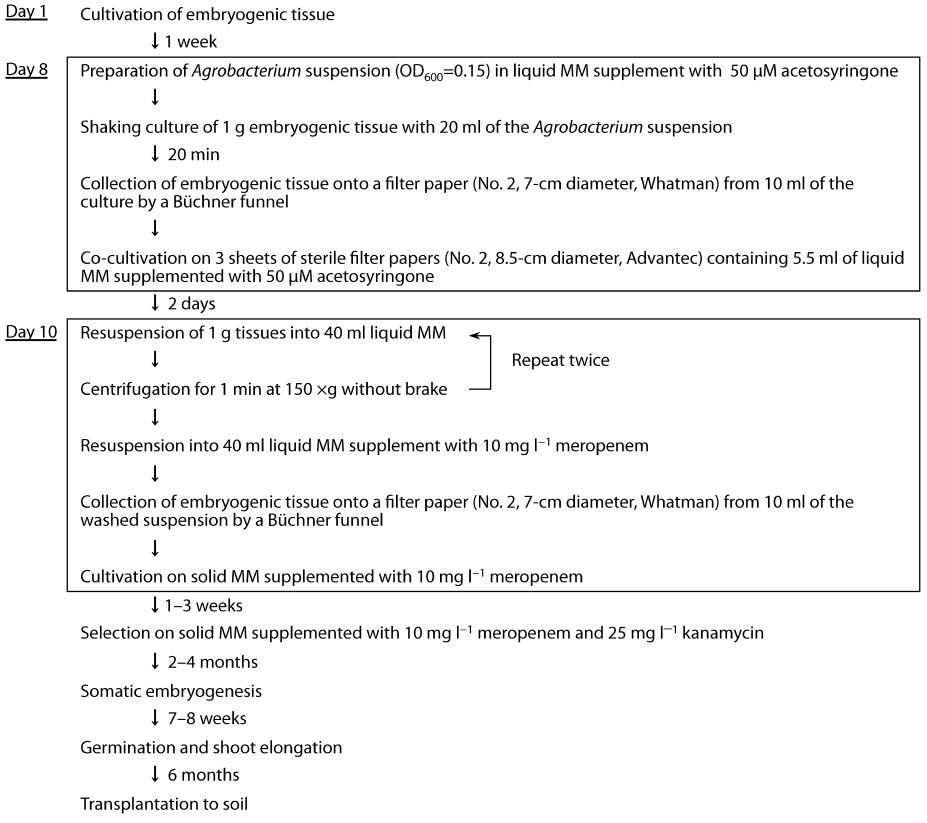

Figure 4. Steps in the transformation of *C. japonica*.

(Taniguchi et al. 2004). Thus, our transformation efficiency value is one of the highest compared with previously reported methods.

In conclusion, the transformation frequency of *C. japonica* significantly increased using the method developed in the present study (Figure 4). We have also efficiently produced transgenic *C. japonica* using several other cell lines of the embryogenic tissues. The improvements reported in this study might help to increase the transformation frequency during other conifer transformations. We hope that this improved transformation method will contribute to future studies of the molecular genetics of *C. japonica*.

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