Original Paper Plant Biotechnology, **17** (1), 49-54 (2000)

Expression of Genes for β-Glucuronidase and Luciferase in Three Species of Japanese Conifer (*Pinus thunbergii*, *P. densiflora* and *Cryptomeria japonica*) after Transfer of DNA by Microprojectile Bombardment

Takeshi MOHRI, Tomohiro IGASAKI, Toru SATO and Kenji SHINOHARA

Molecular and Cell Biology Section, Bio-Resources Technology Division, Forestry and Forest Products Research Institute, Ibaraki 305-8687, Japan

Received 24 August 1999; accepted 9 December 1999

Abstract

Transfer by particle bombardment and expression of genes for β-glucuronidase from *Escherichia coli* and luciferase from the firefly *Photinus pyralis* were examined in mature zygotic embryos of three species of Japanese conifer (*Pinus thunbergii* Parl., *Pinus densiflora* Sieb. and *Cryptomeria japonica* D. Don). Successful transfer of DNA was achieved with a Biolistic® particle gun delivery system. The efficient expression of the two genes was confirmed when gold particles of 1.6 μm in diameter were used to bombard zygotic embryos that had been cultured for 4 days, prior to bombardment, on medium that contained Murashige and Skoog's basal salts, Gamborg's B5 vitamins, 3% (w/v) sucrose and 0.3% (w/v) gellan gum. After transfer of DNA under these conditions, high-level expression of both reporter genes was detected in zygotic embryos of *P. thunbergii* and *P. densiflora*. However, only high-level expression of the gene for luciferase was detected in *C. japonica*. These results suggest that both genes can be used as reporter genes for the establishment of reliable procedures for the regeneration of transgenic *P. thunbergii* and *P. densiflora*, while only the gene for luciferase can be used as a reporter in the case of *C. japonica*.

1. Introduction

Genetic engineering has the potential to allow the selective improvement of a single trait in forest trees without the loss of any of the desired traits of the parental line. Using such techniques, we can overcome the difficulties associated with the breeding of long-lived perennials. Microprojectile bombardment for the delivery of DNA to plant cells is currently the most useful technique for the genetic engineering of plant species, in particular when species are not susceptible to infection by *Agrobacterium* (van Wordrangen and Dons, 1992). The microprojectile-mediated transfer of DNA to trees, which was first demonstrated in hybrid *Populus* (McCown *et al.*, 1991), offers an alternative to gene transfer using *Agrobacterium*. However, we have encountered many difficulties in the regeneration of transgenic woody plants, and efficient regeneration systems have not yet been established. To date, the microprojectile-mediated transformation of conifers has been limited to only few species, which include *Picea glauca* (Ellis *et al.*, 1993), *Picea mariana* (Charest *et al.*, 1996), *Larix laricina* (Klimaszewski *et al.*, 1997) and *Pinus radiata* (Walter *et al.*, 1998).

Pinus thunbergii (black pine), *Pinus densiflora* (red pine) and *Cryptomeria japonica* (sugi), as well as *Chamaecyparis obtusa* (hinoki), are the commercially important species of conifers in Japan. In the case of *P. thunbergii* and *P. densiflora*, the numbers of trees have decreased greatly as a result of infection by the pinewood nematode *Bursaphelenchus xylophilus* which causes pine wilt disease. In addition, *C. japonica* pollinosis is the most serious allergic disease in Japan. We are interested in the genetic engineering of transgenic *P. thunbergii* and *P. densiflora* to introduce resistance to the pinewood nematode, as well as of transgenic *C. japonica* with allergen-free pollen grains. We have already established a simple and reliable procedure for the regeneration of transgenic Japanese broad-leaved trees (Mohri *et al.*, 1996, 1997, 1999). However, to our knowledge, no studies of the transformation of Japanese coniferous species have been reported. In this report, we describe a simple method for the microprojectile-mediated

transfer of DNA and the transient expression of genes for β-glucuronidase (GUS) from *E. coli* and luciferase (LUC) from the firefly *Photinus pyralis* in zygotic embryos of three species of Japanese coniferous plants.

2. Materials and Methods

2.1 Plant materials

Mature seeds collected from the several habitats of three species of Japanese conifer (*P. thunbergii* Parl., *P. densiflora* Sieb. and *C. japonica* D. Don), were generous gifts of the Nursery Management Office of the Forestry and Forest Products Research Institute (FFPRI). Zygotic embryos were prepared from the mature seeds as described previously (Sato, 1978). The embryos were maintained on medium that contained Murashige and Skoog's basal salts (Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg *et al.*, 1968) and 3% (w/v) sucrose (MSB5S medium) supplemented with 0.3% (w/v) gellan gum. They were incubated at 25 ℃ under cool white fluorescent light (30 μE · m^{-2} s^{-1}, 16-h photoperiod) for 4 days, unless otherwise indicated.

2.2 Microprojectile bombardment

A plasmid that carried a chimeric gene composed of the 35S promoter of cauliflower mosaic virus and the *GUS* gene (pBI221; Clontech Laboratories, Inc., Palo Alto, CA) and a plasmid that carried a chimeric gene composed of the 35S promoter and the *LUC* gene (Ow *et al.*, 1986) were used for microprojectile bombardment. Plasmid DNA was precipitated onto gold or tungsten particles (Bio-Rad Laboratories, Inc., Hercules, CA) by the $CaCl_2$ / spermidine method (Klein *et al.*, 1988). DNA was delivered with a Biolistic® particle gun delivery system (PDS-1000/He; Bio-Rad Laboratories). The parameters for bombardment were 1100 psi (7,584 kPa), a 1-cm distance between the rupture disc and the macrocarrier, a 1.1-cm macrocarrier flight distance, and a microcarrier flight distance of 9 cm (Kikkert, 1993). Eight to ten zygotic embryos of three species of Japanese conifer, in petri dishes of 9 cm in diameter that contained MSB5S medium (approximately 30 m*l*) supplemented with 0.3% (w/v) gellan gum, were bombarded with 833 ng of plasmid DNA. After bombardment, the zygotic embyos were incubated for 2 days at 25 ℃ under cool white fluorescent light and then examined for the expression of *GUS* and *LUC*.

2.3 Assay of GUS activity

The histochemical assay for detection of GUS activity was performed as described by Jefferson *et al.* (1987). Bombarded embryos were incubated overnight at 37 ℃ in a solution of 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) and 50 mM sodium phosphate buffer (pH 7.0). After extraction of chlorophyll with ethanol, GUS activity was estimated by counting the number of blue spots on the surface of bombarded embryos under a binocular microscope (SMZ-10; Nikon, Tokyo, Japan). Clumps of blue spots were each scored as a single spot.

2.4 Assay of LUC activity

Bombarded embryos were watered with a solution of 25 mM sodium citrate (pH 5.5) and 1 mM luciferin (Sigma, St. Louis, MO) and placed in a darkened chamber for observations of bioluminescence (Mohri and Shinohara, 1996). Bioluminescence of embryos was recorded with a low-light video microscope system (Argus-50/2D luminometer; Hamamatsu Photonics, Hamamatsu, Japan). Real-time image-processing techniques were used to aid in the detection and localization of luminescent cells (Wick, 1989). The intensity of bioluminescence was monitored using the area-intensity analysis system in the Argus-50 control program.

3. Results

3.1 Transfer of the GUS gene and expression in three species of Japanese conifer

The expression of GUS activity was clearly recognized in zygotic embryos of *P. thunbergii* and *P. densiflora* after microprojectile bombardment with gold particles that had been coated with plasmid pBI221 (**Fig. 1A and 1B**). However, no evidence of activity, namely, no blue spots were detected after bombardment with DNA-free gold particles. These results suggested that the *GUS* gene had been successfully transferred to the zygotic embryos of two species of pine by microprojectile bombardment.

We examined the effects of particle characteristics and the duration of preculture of zygotic embryos on the efficency of DNA transfer and the expression of the *GUS* gene in embryos of *P. thunbergii* and *P. densiflora* (**Table 1 and 2**). We tested gold particles of 1.0 and 1.6 μm in diameter and tungsten particles of 0.7 and 1.1 μm in diameter, together with zygotic embryos that had been cultured for 0 to 5 days prior to bombardment. In each case, we detected the high-level expression of the *GUS* gene in embryos of both species. The efficiency of DNA transfer with gold particles was

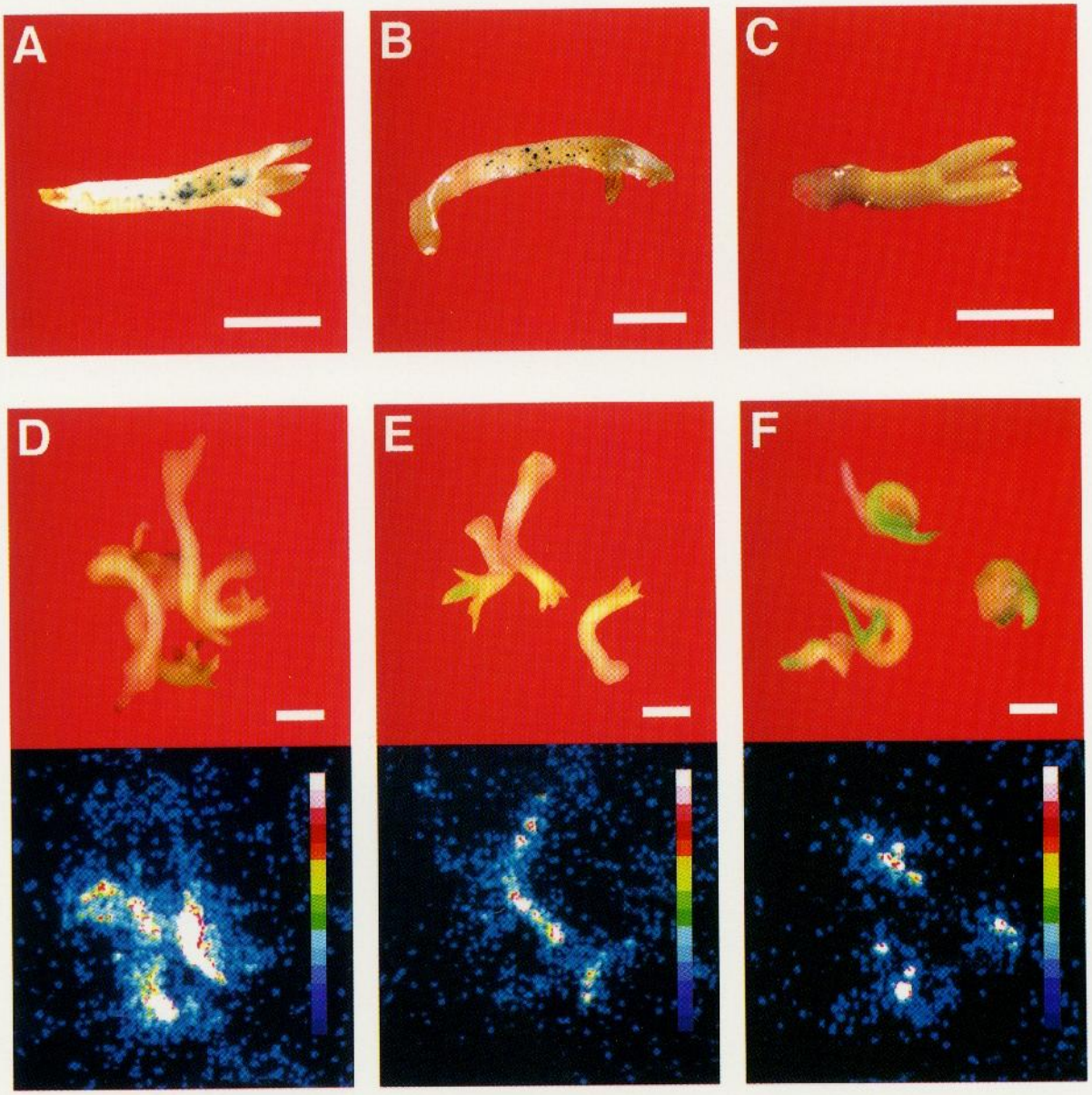

Fig. 1 Expression of GUS and LUC activities in zygotic embryos of three species of Japanese conifer after microprojectile bombardment. Photographs show zygotic embryos of *P. thunbergii* (A and upper ipanel in D), *P. densiflora* (B and upper panel in E) anad *C. japonica* (C and upper panel in F). GUS activity was detected by stainin g with x-gluc. Bioluminescence (lower panels in D, E and F) was examined after application of luciferin. The colored calibration bar indicates increasing numbers of photons from bottom to top (blue to white). Bars=2.5 mm.

Table 1. Effects of particle characteristics and the duration of culture prior to bombardment of zygotic embryos on the expression of GUS activity in *P. thunbergii* after microprojectile bombardment.

Duration of culture of zygotic embryos	Number of blue spots per zygotic embryo			
	Gold particles (diameter)		Tungsten particles (diameter)	
(days)	1.0 μ m	1.6 μ m	0.7 μ m	1.1 μ m
0	8.2 ± 1.7	12.3 ± 1.6	2.3 ± 0.4	5.2 ± 1.1
1	11.9 ± 2.3	8.9 ± 1.4	5.1 ± 0.5	4.1 ± 0.9
2	17.4 ± 2.6	16.1 ± 1.4	4.4 ± 1.1	11.3 ± 2.4
3	22.3 ± 3.0	23.8 ± 2.1	3.7 ± 0.5	14.6 ± 2.1
4	14.8 ± 1.6	32.4 ± 3.6	2.5 ± 0.5	12.0 ± 1.6
5	12.9 ± 2.4	29.0 ± 4.4	2.2 ± 0.4	10.1 ± 1.0

Eight or nine zygotic embryos, on MSB5S medium supplemented with 0.3% (w/v) gellan gum, were bombarded under the indicated conditions. The numbers of blue spots in the embryos were examined 2 days afer microprojectile bombardment. Numbers of blue spots per zygotic embryo are means ± S. E. of results from four replicate experiments.

Table 2. Effects of particle characteristics and the duration of culture prior to bombardment of zygotic embryos on the expression of GUS activity in *P. densiflora* after microprojectile bombardment

Duration of culture of zygotic embryos	Number of blue spots per zygotic embryo			
	Gold particles (diameter)		Tungsten particles (diameter)	
(days)	1.0 μm	1.6 μm	0.7 μm	1.1 μm
0	8.3 ± 1.4	5.1 ± 1.1	3.1 ± 0.4	3.1 ± 1.1
1	16.4 ± 2.1	10.8 ± 1.6	5.8 ± 0.5	4.9 ± 1.0
2	13.1 ± 1.8	16.2 ± 2.4	4.9 ± 1.1	2.2 ± 0.6
3	15.2 ± 2.6	23.7 ± 4.2	3.3 ± 0.4	5.3 ± 2.1
4	8.9 ± 1.5	29.2 ± 3.5	2.4 ± 0.4	7.1 ± 1.5
5	6.8 ± 1.0	20.8 ± 3.1	2.9 ± 0.5	4.0 ± 0.9

Values are means ± S. E. of results from four replicate experiments. For details, see footnote to Table 1.

much higher than that with tungsten particles. The duration of preculture of the embryos also had a remarkable effect on the expression of the *GUS* gene. Maximal *GUS* expression was achieved in embryos of *P. thunbergii* and *P. densiflora* when we used 1.6-μm gold particles and embryos that had been cultured for 4 days (**Table 1 and 2**). Bombardment with 1.0-μm gold particles shortened the necessary preculture period for maximal *GUS* expression. Thus, the developmental stage of the plant tissues was very important for the successful transfer of DNA by microprojectile bombardment and the subsequent efficient expression of the chimeric gene.

In zygotic embryos of *C. japonica*, we found no GUS activity after bombardment with gold and with tungsten particles coated with pBI221 (**Fig. 1C**). We examined many sets of conditions in attempts to induce the *GUS* expression in *C. japonica* by changing bombardment parameters, such as pressure and the microcarrier flight distance, and using more than 1,000 embryos. We also bombarded cotyledons, hypocotyls and roots of seedlings, as well as calli and suspension-cultured cells derived from mature seed. However, no blue spots were detected in any of the tissues of *C. japonica* examined. Therefore, we used a plasmid that included the *LUC* gene as a reporter, instead of the *GUS* gene.

3.2 Transfer of the LUC gene and expression in three species of Japanese conifer

Zygotic embryos of *P. thunbergii* and P. *densiflora* that had been cultured for 4 days, were

bombarded with gold particles of 1.6 μm in diameter that carried a plasmid including the *LUC* gene, under the previously established optimal conditions for transfer of the *GUS* gene. High-level expression of LUC activity was recognized in the embryos of the two species of pine (**Fig. 1D and 1E**). The *LUC* gene was also efficently transferred to and strongly expressed in zygotic embryos of *C. japonica* under the same conditions (**Fig. 1F**).

The expression of LUC activity in *C. japonica* was studied in relation to the developmental stage of zygotic embryos before bombardment. Embryos were cultured for 3 to 7 days on MSB5S medium supplemented with 0.3 % (w/v) gellan gum. The bioluminescence from groups of ten embryos increased with the progress of development, reaching approximately 2.8 times the initial value after 7 days of preculture (data not shown). Considering the increase in size of zygotic embryos during preculture, we calculated that the relative LUC activity per average area of a horizontal section of an embryo was greatest after 4 days of preculture (**Fig. 2**). Thus, the efficiency of transfer and the expression of the *LUC* gene in *C. japonica* were also affected by the developmental stage of zygotic embryos.

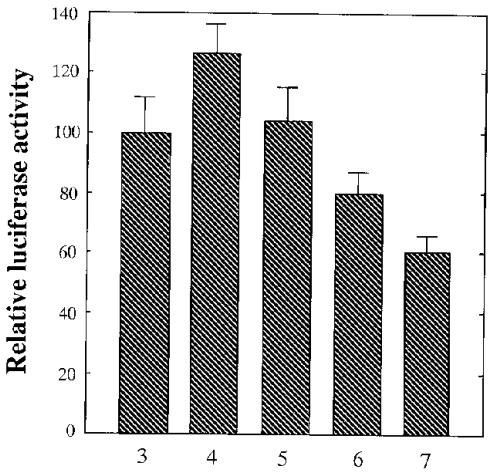

Fig. 2. Effects of the duration of culture prior to bombardment of zygotic embryos on the LUC activity in *C. japonica* after microprojectile bombardment. In each case, ten zygotic embryos on MSB5S medium supplemented with 0.3% (w/v) gellan gum were bombarded with gold particles of 1.6 μm in diameter. The intensity of bioluminescence derived from ten embryos was measured with a low-light video microscope system. The average intensity per plate was calculated from the results obtained from two similar plates. Relative LUC activity was calculated by dividing the average intensity by the average area of the horizontal section of the visualized embryo, taking the value determined after preculture for 3 days as 100. Values are means $\pm$S.E. of results from four replicate experiments.

4. Discussion

In this study, we established a simple and reliable procedure for introducing DNA into mature zygotic embryos of three species of Japanese conifer. To our knowledge, this is the first report of the microprojectile-mediated transfer of DNA to Japanese conifers. The *GUS* gene was strongly expressed in *P. thunbergii* and *P. densiflora*, however, the expression was not detected in *C. japonica* (**Fig. 1A to 1C**). There are at least three plausible explanations for the absence of GUS activity in *C. japonica*. First, the plasmid DNA might not have been successfully transferred to *C. japonica*. Second, the 35S promoter of cauliflower mosaic virus might not have functioned in *C. japonica* in spite of the successful transfer of the *GUS* gene. Third, expression of GUS activity might have been inhibited post-transcriptionally or post-translationally in *C. japonica*. Therefore, we used a plasmid that included the *LUC* gene as a reporter, instead of the *GUS* gene, to examine these possibilities. The *LUC* gene was strongly expressed in *C. japonica* as well as in *P. thunbergii* and *P. densiflora* (**Fig. 1D to 1F**). These results suggest that the 35S promoter can function as a promoter for expression of foreign genes in all three Japanese conifers and, moreover, that GUS activity is inhibited in all tissues of *C. japonica* after the introduction of the *GUS* gene. Further studies are needed to identify the factor(s) that inhibits GUS activity in *C. japonica*. A similar result has been reported by Campbell *et al.* (1992), who found that extracts from *P. radiata* suspension-cultured cells inhibit GUS activity in *E. coli* extracts. Thus, both *GUS* and *LUC* genes can be used as reporter genes for the establishment of reliable procedures for the regeneration of transgenic *P. thunbergii* and *P. densiflora*, while only the *LUC* gene can be used as a reporter in the case of *C. japonica*.

Genetic transformation of conifers by microprojectile bombardment (Ellis *et al.*, 1993; Charest *et al.*, 1996; Klimaszewski *et al.*, 1997; Walter *et al.*, 1998) and by *Agrobacterium* (Shin *et al.*, 1994; Tzfira *et al.*, 1996; Levée *et al.*, 1997) has been reported. For those plant species that are not susceptible to infection by *Agrobacterium*, the biolistic method for delivery of DNA into cells is currently the most useful technique. The advantage

of this type of transformation system, in particular when the *LUC* gene is used, is that bombarded plant materials can be cultured directly to regenerate transgenic plants after a non-destructive assay for the presence of the introduced gene. A reproducible system for regeneration of plants from embryogenic callus is essential for the genetic transformation of Japanese conifers. We have tried to establish such a system but we have encountered many difficulties. To date, to our knowledge, no system exists for regeneration of plants from embryogenic callus of these Japanese conifers. Microprojectile bombardment-mediated transformation of Japanese conifers will be possible when we have established a reliable and reproducible regeneration system. In the future, the present simple and reliable technique for the transfer of DNA should be useful for the genetic engineering of transgenic *P. thunbergii* and *P. densiflora* with resistance to the pinewood nematode, as well as of transgenic *C. japonica* with allergen-free pollen grains. Furthermore, this method should allow the development of assays for the transient and homogeneous expression of the promoters of various genes in Japanese conifers (Kojima *et al.* 1994).

Acknowledgments

The authors express their gratitude to the Nursery Management Office of the FFPRI for generous gifts of mature seeds used in this study. This work was supported in part by Grants-in-Aid (Integrated Research Program for the Use of Biotechnological Procedures for Plant Breeding and Integrated Research Program for the Effective Use of Biological Activities to Create New Demand) from the Ministry of Agriculture, Forestry and Fisheries of Japan, and in part by a Grant-in-Aid (Basic Research for Life and Society) from the Science and Technology Agency of Japan.

References

Campbell, M.A., Kinlaw, C.S., Neale, D.B., 1992. Expression of luciferase and β-glucuronidase in *Pinus radiata* suspension cells using electroporation and particle bombardment. Can. J. For. Res., **22:** 2014-2018.

Charest, P.J., Devantier, Y., Lachance, D., 1996. Stable genetic transformation of *Picea mariana* (black spruce) via particle bombardment. *In Vitro* Cell. Dev. Biol. - Plant, **32:** 91-99.

Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J., Robert, D.R., Raffa, K.F., McCown, B.H., 1993. Stable transformation of *Picea glauca* by particle acceleration. Bio/Technology, **11:** 84-89.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., **50:** 151-158.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6:** 3901-3907.

Kikkert, J.R., 1993. The Biolistic® PDS-1000/He device. Plant Cell, Tissue and Organ Culture, **33:** 221-226.

Klein, T.M., Gradziel, T., Fromm, M., Sanford, J.C., 1988. Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Bio/Technology, **6:** 559-563.

Klimaszewska, K., Devantier, Y., Lachance, D., Lelu, M.A., Charest, P.J., 1997. *Larix laricina* (tamarack): somatic embryogenesis and genetic transformation. Can. J. For. Res., **27:** 538-550.

Kojima, K., Sasaki, S., Yamamoto, N., 1994. Light-independent and tissue-specific expression of a reporter gene mediated by the pine *cab-6* promoter in transgenic tobacco. Plant J., **6:** 591-596.

Levée, V., Lelu, M.-A., Jouanin, L., Cornu, D., Pilate, G., 1997. *Agrobacterium tumefaciens*-mediated transformation of hybrid larch (*Larix kaempferi* × *L. decidua*) and transgenic plant regeneration. Plant Cell Rep., **16:** 680-685.

McCown, B.H., McCabe, D.E., Russell, D.R., Robinson, D.J., Barton, K.A., Raffa, K.F., 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep., **9:** 590-594.

Mohri, T., Yamamoto, N., Shinohara, K., 1996. *Agrobacterium*-mediated transformation of lombardy poplar (*Populus nigra* L. var. *italica* Koehne) using stem segments. J. For. Res., **1:** 13-16.

Mohri, T., Shinohara, K., 1996. A biolistic approach for the transfer and expression of a luciferase gene in paulownia (*Paulownia fortunei*). J. For. Res., **1:** 65-66.

Mohri, T., Mukai, Y., Shinohara, K., 1997. *Agrobacterium tumefaciens*-mediated transformation of Japanese white birch (*Betula platyphylla* var. *japonica*). Plant Sci., **123:** 53-60.

Mohri, T., Igasaki, T., Futamura, N., Shinohara, K., 1999. Morphological changes in transgenic poplar by expression of the rice homeobox gene *OSH1*. Plant Cell Rep., **18:** 816-819.

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

Ow, D.W., Wood, K.V., Deluca, M., de Wet, J.R., Helinski, D.R., Howell, S.H., 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science, **234:** 856-859.

Sato, T., 1978. Search for the synthetic media suitable for embryo culture of *Cryptomeria* and *P. thunbergii*. J. Jap. For. Soc., **60:** 81-86 (in Japanese with English summary).

Shin, D.-I., Podila, G.K., Huang, Y.H., Karnosky, D.F., 1994. Transgenic larch expressing genes for herbicide and insect resistance. Can. J. For. Res., **24:** 2059-2067.

Tzfira, T., Yarnitzky, O., Vainstein, A., Altman, A., 1996. *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill.. Plant Cell Rep., **16:** 26-31.

van Wordragen, M.F., Dons, H.J.M., 1992. *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. Plant Mol. Biol. Rep., **10:** 187-190.

Walter, C., Grace, L.J., Wagner, A., White, D.W.R., Walden, A.R., Donaldson, S.S., Hinson, H., Gardner, R.C., Smith, D.R., 1998. Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. Plant Cell Rep., **17:** 460-468.

Wick, R.A., 1989. Photon counting imaging: applications in biomedical research. Biotechniques, **7:** 262-268.