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

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Summary: Genetic transformation could be a new tree breeding method for conferring new and desirable traits to forest trees. However, little information is available on reliable transformation methods for Japanese forest tree species used in plantations. Reporter genes play an important role in establishing transformation methods. In this study, we examined the efficiency of a green fluorescent protein (GFP) gene as a reporter gene for forest tree species. We introduced the GFP gene by particle bombardment to the following targets: zygotic embryos of *Cryptomeria japonica,* somatic embryos of *Larix kaempferi and Acanthopanax* (= *Eleutherococcus*) *sciadophylloides,* shoot primordia of *Chamaecyparis obtusa* and embryogenic tissues of *Pinus densiflora, Pinus thunbergii* and *L. kaempferi*. Transient expression of the gene was observed in all targeted tissues tested. Moreover, the expressions were greatly enhanced by the pre-culture and osmotic treatments of targeted tissues before bombardment. These results indicate that the GFP gene would be useful for Japanese forest tree species.

1. Introduction

Tree improvement by genetic engineering could confer new and desirable traits, such as improved wood quality and growth characteristics, resistance to diseases and insects and tolerance to stress. A reliable and stable genetic transformation method to produce transgenic trees is required for the genetic engineering of forest trees. Since transgenic trees were first obtained for the poplar in 1987 (Fillatti et al., 1987), transformation techniques have been developed for many forest tree species (reviewed for conifers in Tang and Newton, 2003), such as *Picea abies* (Walter et al., 1999, Wenck et al., 1999), *Pinus radiata* (Walter et al., 1998), *Pinus taeda* (Tang et al., 2001), *Larix kaempferi* × *L. decidua* (Levée et al., 1997), *Eucalyptus camaldulensis* (Ho et al., 1998) and *Acacia mangium* (Xie and Hong, 2002). However, there is little information available on reliable transformation methods for Japanese forest trees used in plantations.

In developing a transformation method, reporter genes play an important role. A green fluorescent protein (GFP) gene of a jellyfish (*Aequorea victoria*)

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has an advantage over other reporter proteins, such as beta-glucuronidase or luciferase, because presence of GFP can be directly visualized by the emission of green light when excited with long UV or blue light. Moreover, an engineered synthetic GFP, sGFP(S65T) (replacement of the serine in position 65 with a threonine) has provided up to 100-fold brighter fluorescent signals than wild-type GFP (Chiu et al., 1996, Niwa, 2003). Transient or stable expression of beta-glucuronidase or luciferase has been reported for some Japanese forest trees (Mohri et al., 2000, Maruyama et al., 2000, Ishii, 2002). However, to our knowledge, the GFP expression in Japanese forest trees has not been reported.

The objective of this study was to examine the efficiency of using sGFP(S65T) as a reporter gene in six Japanese forest tree species. Five were coniferous tree species used in plantations, and the sixth was a broad-leaved tree species, *Acanthopanax* (= *Eleutherococcus*) *sciadophylloides*, which is useful for its edible young buds.

2. Materials and methods

2. 1 Plant materials

Seeds of *C. japonica* (Naka 6: Kanagawa and Ohno 2) were soaked in water for 3 d, surface sterilized with 70% ethanol for 2 min followed by 10% H_2O_2 for 10 min, and then rinsed three times with sterilized distilled water. Zygotic embryos were removed from the seeds and cultured on woody plant medium (Lloyd and McCown, 1980) that contained 30 g/l sucrose and 3 g/l gelrite for 4 d. The embryos were incubated for 4 h on high osmolality medium supplemented with an additional 0.4 M sucrose before bombardment. The number of embryos bombarded was 40 and 8 for Ohno 2 and Naka 6, respectively. The embryos without osmolality treatment (46 embryos of Ohno 2 and 8 embryos of Naka 6) were also bombarded.

Shoot primordia of *C. obtusa* (Ena 3) were induced from cotyledons of a seedling on CD medium (Campbell and Durzan, 1975), which contained 0.3 μM NAA and 10 μM BA, and subcultured monthly on CD medium containing 1.0 μM zeatin for propagation. Ten clumps (ca.1cm in diameter) of the shoot primordia were incubated for 4 h on high osmolality medium supplemented with an additional 0.4 M sucrose before bombardment. Ten clumps of shoot primordia without osmolality treatment were also bombarded.

Embryogenic tissues of *P. densiflora* (Susaki a 27) and *P. thunbergii* (Ohseto ku 12) were induced from female gametophyte explants containing pre-cotyledonary embryos, and subcultured every three to four weeks on semisolid modified DCR medium (Taniguchi, 2001). Embryogenic tissue of *L. kaempferi* (Minamisaku 12) was also induced from female gametophyte explants and subcultured on semisolid modified CD medium (Taniguchi et al., 2001). These embryogenic tissues were suspended in liquid media and the suspension, which contained 500 mg cells, was poured over a 5.5 cm sterile filter paper in a funnel (Millipore, Bedford, Massachusetts). A short vacuum pulse was applied to drain the liquid, and the filter paper was placed on the same medium as used for the subculture. After 1 or 2 d of pre-culture, the embryogenic tissues on the filter paper were used for bombardment with plasmid DNA. Three or 4 filter papers with the embryogenic tissue of each species were bombarded for each treatment.

Larix kaempferi somatic embryos derived from

embryogenic tissue (Taniguchi et al., 2001) were incubated on modified CD medium supplemented with osmoticum (0.4 M sucrose, 0.4 M maltose or 0.2 M sorbitol + 0.2 M mannitol) for 5 h, followed by bombardment. Number of somatic embryos bombarded was 10 or 20 per treatment.

Somatic embryos of *A. sciadophylloides* were induced from mature seeds collected from a tree (Taniguchi et al., 1996) and subcultured every one to two months on MS medium (Murashige and Skoog, 1962) that contained 0.01 mg/l 2, 4-D, 30 g/l sucrose and 8 g/l agar. Before bombardment, the somatic embryos were cultured on high osmolality medium supplemented with an additional 0.5 M sucrose for 24 h. About 30 somatic embryos with or without the high osmolality treatment were bombarded.

2.2 Plasmid DNA

The pRT99 vector (Töpfer et al., 1993) was kindly donated by Dr R. Töpfer, Institute for Grapevine Breeding, Geilweilerhof, Germany. The plasmid CaMV35SΩ-sGFP (S65T)-nos3´ (Niwa, 2003) was kindly donated by Dr Y. Niwa, Laboratory for Plant

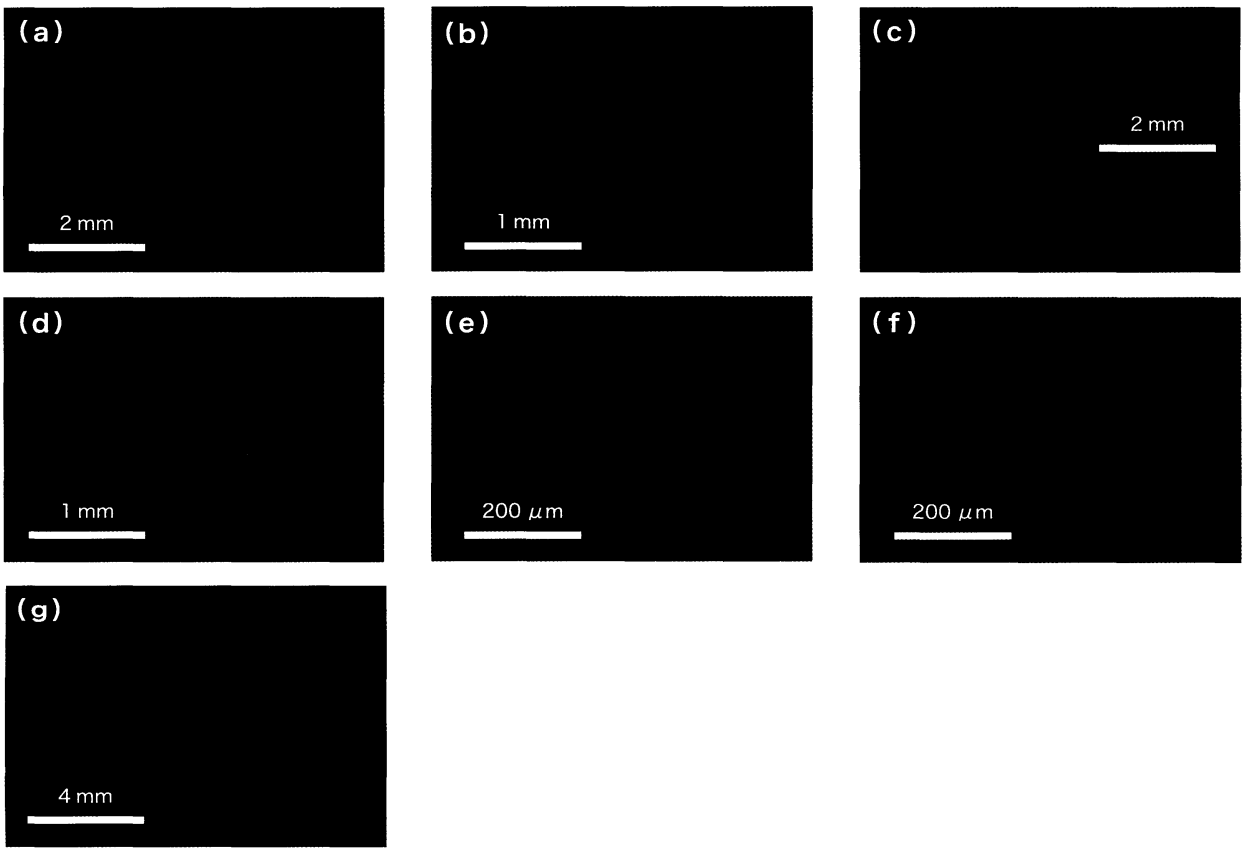

Fig. 1. Transient expression of the GFP observed under a fluorescence stereomicroscope with filter system (excitation filter: 480/40 nm, emission filter: 510 nm) in zygotic embryo of *Cryptomeria japonica* (a), somatic embryo of *Larix kaempferi* (b), somatic embryos of *Acanthopanaxs sciadophylloides* (c), shoot primodium of *Chamaecyparis obtusa* (d), a cell of *Larix kaempferi* embryogenic tissue (e), two cells of *Pinus densiflora* embryogenic tissue (f), and embryogenic tissue of *Pinus thunbergii* (g).

Cell Technology, University of Shizuoka, Shizuoka, Japan. A *Hin*dIII-*Eco*RI fragment that included a CaMV35S-sGFP (S65T)-nos3´ cassette was cut from a plasmid CaMV35Ω-sGFP (S65T)-nos3´ and inserted into the MCS of pRT99 to obtain pRT99sGFP.

2. 3 Particle bombardment

Gold particles were coated with plasmid DNA pRT99sGFP according to a standard protocol (Sanford et al., 1993) and plant materials bombarded using a Biolistic Particle Delivery System, PDS-1000/He (Bio-Rad, California). The bombardment condition was a vacuum of 28 inch mercury, a helium pressure of 1100, 1350 or 1500 psi, and a 6 or 9 cm target distance using 1.0 or 1.6 μm gold micro carriers.

2. 4 Visualization of GFP expression

GFP expression was observed in the bombarded tissues under a fluorescence stereomicroscope (MZ FLIII; Leica Microsystems, Heerbrugg, Switzerland) with a GFP Plus filter system (excitation filter: 480/40 nm, emission filter: 510 nm). Photographs of GFP expression were taken using a digital camera system (DC 300F; Leica). After 24 h of bombardment, the target tissues that expressed the GFP genes were observed and photographed.

3. Results and discussion

Transient expression of the GFP gene was observed in all species tested (Fig. 1), which indicates that this gene would be a very effective reporter gene for developing genetic transformation methods of these species. Differences in bombardment conditions (helium pressure, target distance and diameter of gold particles) had no obvious effect on the transient expression of the GFP gene (data not shown).

When embryogenic tissues without pre-culture were bombarded, the number of cells expressing the GFP gene were 67–78, 19–35 and 1–5 per dish for *P. densiflora*, *P. thunbergii* and *L. kaempferi*, respectively. Pre-culture before bombardment enhanced gene expression in embryogenic tissues of *L. kaempferi*. Although the number of cells expressing the genes was below 10 without pre-culture, it increased to 47–71 or 95–136 with pre-culture for 1 or 2 d, respectively. The gene expression in *P. thunbergii* was also enhanced by the pre-culture treatment (Fig. 1g). The cells in the pre-cultured embryogenic tissues were thought to have a high metabolic activity and the ability to rapidly divide. Genes introduced into such cells

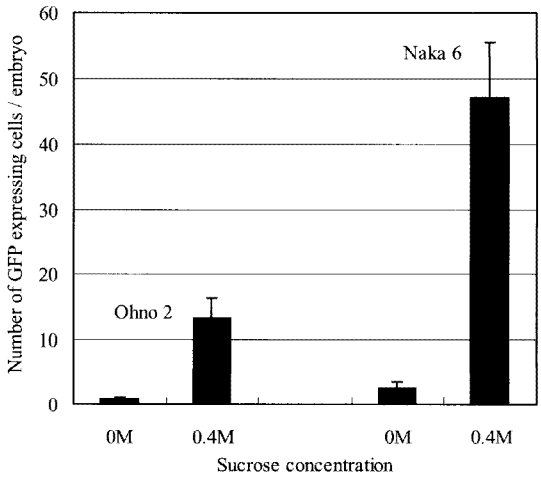

Fig. 2. Effect of osmoticum treatment with sucrose on transient expression of the GFP in zygotic embryos of *Cryptomeria japonica*. Forty-six or 40 embryos of Ohno 2, and 8 embryos of Naka 6 for each treatment were bombarded with GFP genes, and the number of cells expressing the GFP was counted after 1 d of bombardment. Error bars show standard error.

would have a higher probability of being expressed (Tang and Newton, 2003).

The transient expression of the GFP gene in zygotic embryos of *C. japonica* was greatly improved by pre-treatment with sucrose osmoticum (Fig. 1a). Gene expression was enhanced 16.8-fold in Ohno 2 embryos and 19.6-fold in Naka 6 embryos (Fig. 2). An increasing gene expression by the osmoticum treatment was also shown in somatic embryos of *A. sciadophylloides* (Fig. 1c) and in shoot primordia of *C. obtusa* (Fig. 1d). The effect of the kind of osmoticum (0.4 M sucrose, 0.4 M maltose or 0.2 M sorbitol + 0.2 M mannitol) on gene expression in somatic embryos of *L. kaempferi* was examined (Figs. 1b and 3). Although maltose was as effective as sucrose, the mixture of sorbitol and mannitol was less effective. The mixture of 0.2 M sorbitol and 0.2 M mannitol was used as the osmoticum in the bombardment of somatic embryos of *Picea mariana* (Tian et al., 2000), but was less effective than sucrose and maltose in *L. kaempferi*. For expression of transgenes in embryogenic tissues of *P. abies*, the mixture of sorbitol and mannitol was also less effective than sucrose and *myo*-inositol (Clapham et al., 1995).

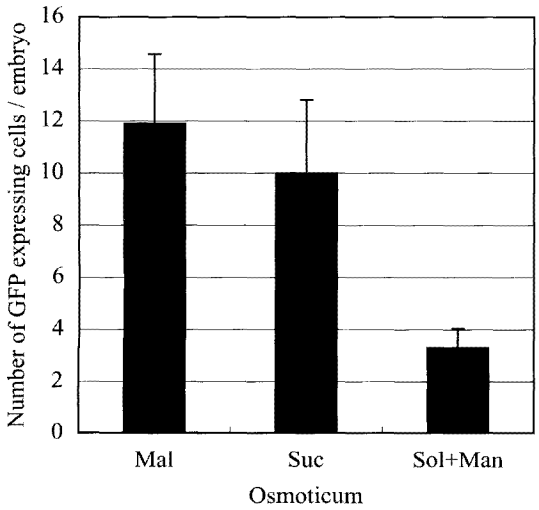

Fig. 3. Effect of osmotica on transient expression of the GFP in somatic embryos of *Larix kaempferi*. Somatic embryos treated on media supplemented with osmoticum (0.4 M sucrose, 0.4 M maltose, or 0.2 M solbitol+0.2 M mannitol) were bombarded with GFP genes, and the number of cells expressing the GFP was counted after 1 d of bombardment. Number of embryos was 10 or 20 per treatment. Error bars show standard error.

In conclusion, transient expression of the GFP gene was observed in all species tested: *C. japonica, C. obtusa, P. densiflora, P. thunbergii, L. kaempferi* and *A. sciadophylloides*. This suggests that the GFP gene would be a useful reporter gene for all of these Japanese forest tree species. Moreover, pre-culture and the osmoticum treatment of the targeted tissues enhanced the transient expression of the GFP gene.

Acknowledgements

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パーティクルガン法により導入された緑色蛍光タンパク質遺伝子のスギ，ヒノキ，アカマツ，クロマツ，カラマツ及びコシアブラでの一過性発現

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要旨：林木の遺伝子組換え技術は新しい育種方法として期待できるが，我が国の林木について遺伝子導入に関する知見は少ない。遺伝子導入法の開発の際にレポーター遺伝子は重要な役割を果たす。本研究では，緑色蛍光タンパク質（GFP）遺伝子のレポーター遺伝子としての有効性を調査した。GFP遺伝子をスギの種子胚，ヒノキの苗条原基，アカマツとクロマツ，カラマツのembryogenic tissueさらにカラマツとコシアブラの不定胚にパーティクルガン法で導入し，その一過性発現を確認した。また，その発現はターゲット組織の前培養と高浸透圧培地での処理により向上した。本研究の結果より，GFP遺伝子は，調査した樹種においてレポーター遺伝子として有効に使用できることが示唆された。

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