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## ***Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue and transgenic plant regeneration in *Chamaecyparis obtusa* Sieb. et Zucc.**

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**Abstract** A genetic transformation procedure for *Chamaecyparis obtusa* was developed after co-cultivation of embryogenic tissues with disarmed *Agrobacterium tumefaciens* strain C58/pMP90, which harbours the *sgfp* (*synthetic green fluorescent protein*) visual reporter and *nptII* (*neomycin phosphotransferase II*) selectable marker genes. The highest transformation frequency was 22.5 independent transformed lines per dish (250 mg embryogenic tissue) following selection on kanamycin medium. Transgenic plantlets were regenerated through the maturation and germination of somatic embryos. The intensity of GFP fluorescence, observed under a fluorescence microscope, varied from very faint to relatively strong, depending on the transgenic line or part of the transgenic plant. The integration of the genes into the genome of regenerated plantlets was confirmed by Southern blot analysis.

**Keywords** *Agrobacterium tumefaciens* · *Chamaecyparis obtusa* · Green fluorescent protein · Somatic embryogenesis · Transgenic plant

### **Introduction**

*Chamaecyparis obtusa* Sieb. et Zucc., Hinoki cypress, is one of the most economically important conifers grown in plantations in Japan because its wood is of good quality and very suitable for timber. The current planting area of this species is the largest of all the commercially grown forests in Japan. Since the 1950s, plus-trees of this species have been selected in Japanese breeding programmes on the basis of their stem form and growth characteristics, and progeny testing has been undertaken. As a supplement to these traditional breeding programmes, im-

provement of *C. obtusa* by means of genetic engineering is expected to confer new and desirable traits, such as resistance to diseases and insects, improved growth characteristics and tolerance to stress.

Recently, transformation techniques have been developed for many conifers (reviewed by Tang and Newton 2003), and the regeneration of transgenic plants has been reported in many species—for example, *Picea abies* (Walter et al. 1999; Clapham et al. 2000), *P. glauca* (Ellis et al. 1993), *P. mariana* (Charest et al. 1996; Tian et al. 2000), *Larix laricina* (Klimaszewska et al. 1997) and *Pinus radiata* (Walter et al. 1998) by particle bombardment and *Larix kaempferi* × *L. decidua* (Levéé et al. 1997; Lelu and Pilate 2000), *Pinus strobus* (Levéé et al. 1999), *P. teada* (Tang et al. 2001; Gould et al. 2002), *P. radiata* (Charity et al. 2002), *Picea glauca* (Klimaszewska et al. 2001; Le et al. 2001), *P. mariana* (Klimaszewska et al. 2001) and *P. abies* (Klimaszewska et al. 2001) using the *Agrobacterium tumefaciens*-mediated method. The preferred method for genetic engineering of higher plants may be *Agrobacterium*-mediated transformation because it results in a simpler integration pattern and limited rearrangement in introduced DNA (Birch 1997). Moreover, the transformation frequency of conifers is generally higher using the *Agrobacterium*-mediated method than particle bombardment. To our knowledge, the highest transformation frequency obtained with the *Agrobacterium*-mediated method was over 1,000 transgenic events per gram material (embryogenic tissues) of *Picea mariana* (Klimaszewska et al. 2001). With respect to *C. obtusa*, only one report on genetic transformation has been published, and it describes gene delivery to shoot primordia by particle bombardment and the subsequent regeneration of the transformed plantlets (Ishii 2002). In an earlier investigation (Taniguchi et al. 2004b) we also attempted the genetic transformation of *C. obtusa* following this method, and although we obtained transiently transformed cells, we failed to produce transformed plants.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has an advantage over other reporter proteins, such as  $\beta$ -glucuronidase (GUS) or luciferase,

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because the presence of GFP can be directly visualized by the emission of green fluorescence when excited with long UV or blue light. However, the expression of the wild-type GFP gene is low in plants. Therefore, various GFP mutants such as mGFP4, mGFP5 and sGFP(S65T) have been produced by alterations in the cryptic intron in the wild-type gene and amino acid substitutions (Stewart 2001). sGFP(S65T), a synthetic GFP with threonine replacing serine at position 65, has been shown to provide fluorescent signals that are up to 100-fold brighter than wild-type GFP (Chiu et al. 1996; Niwa 2003). As such, GFP has become a very effective reporter for use in plant genetic transformation research. However, there are only a few reports of conifer transformation using GFP; GUS has more often been used as a reporter in conifer transformation. Using particle bombardment, Tian et al. (1997) detected transient expression of the mGFP4 gene in conifer tissues. We also observed transient expression of the GFP mutant sGFP(S65T) genes in some Japanese conifers following particle bombardment (Taniguchi et al. 2004b). A stable expression of mGFP5-ER and mGFP4 was observed in *Pinus strobus* and *Picea mariana* transformed embryonal cells, respectively, following *Agrobacterium*-mediated transformation (Tian et al. 1999). Levée et al. (1999) reported that in *Agrobacterium*-mediated transformed cells of *P. strobus*, the transient expression of mGFP5-ER could not be detected, but a stable expression was detected and the intensity of GFP varied from very faint to relatively strong.

We have recently developed a somatic embryogenesis and plant regeneration method from immature embryos of *C. obtusa* plus-trees (Taniguchi et al. 2004a). However, to our knowledge, *A. tumefaciens*-mediated genetic transformation of this species has not been reported. Therefore, the objective of the investigation reported here was to develop a transformation method of embryogenic tissues of *C. obtusa* plus-tree via the disarmed *A. tumefaciens* strain, C58/pMP90, using the synthetic GFP sGFP(S65T) gene. We obtained transformed lines at a high frequency and were able to regenerate transgenic plantlets. GFP expression was observed in transgenic cells or tissues using a fluorescence stereo microscope, and stable integration of the genes was confirmed by Southern blot analysis.

## Materials and methods

### Plant material

Open-pollinated immature seeds of a *Chamaecyparis obtusa* Sieb. et Zucc. plus-tree clone, Ena 3, were collected at a clone bank garden of the Forest Tree Breeding Center in Juo (Ibaraki, Japan) at the beginning of July 2002. Embryogenic tissues (cell line Ena 3 1-1) were initiated from an immature embryo on Smith standard embryonic tissue capture medium (Smith 1996), cultured for 4 weeks on Smith standard embryogenesis medium (Smith 1996) and sub-cultured at 2- or 3-week intervals on Smith embryo develop medium (SM3; Smith 1996). The cultures were maintained in the dark at 25°C. For details, see Taniguchi et al. (2004a).

### *Agrobacterium* strain and culture preparation

The disarmed *Agrobacterium tumefaciens* strain C58/pMP90 (=GV3101/pMP90; Koncz and Schell 1986) was kindly provided by Dr. C. Koncz, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany. The binary vector pBin19-sgfp was introduced into *A. tumefaciens* by tri-parental mating and subsequently used for transformation experiments. The plasmid pBin19-sgfp (kindly provided by Dr. N. Matuta, Fruit Science Institute, Tsukuba, Japan, with the permission of Dr. J. Sheen, Massachusetts General Hospital, Mass., USA) carries a reporter gene, the sGFP(S65T) gene (sgfp; Chiu et al. 1996; Niwa 2003), and a selectable marker gene, *nptII* (*neomycin phosphotransferase II*), which codes for kanamycin resistance. *sgfp* and *nptII* are driven by a CaMV 35S promoter and a NOS promoter, respectively.

*Agrobacterium* cells were grown overnight on a rotating shaker at 200 rpm and 28°C in a YEB medium (5 g/l Bacto beef extract, 5 g/l peptone, 1 g/l Bacto yeast extract, 2 mM MgSO<sub>4</sub>·H<sub>2</sub>O, 5 g/l sucrose, pH 7.2) supplemented with 50 mg/l kanamycin, 50 mg/l gentamycin and 50 mg/l rifampicin. Following this, freshly grown cells (OD<sub>600</sub> of bacterial suspension:1.1–1.3) were pelleted by centrifugation (5,000 rpm for 10 min) and resuspended in liquid SM3, which contained 50 µM acetosyringone, to an optical density (OD<sub>600</sub>) of 0.3.

### Transformation of embryogenic tissues

Embryogenic tissues (1-g aliquots, fresh weight) grown on SM3 for 2 weeks were transferred to 20 ml of the bacterial suspension in a 50-ml test tubes equipped with a screw cap. The tubes were placed horizontally on a rotating shaker at 100 rpm for either 20 min or 5 h in the dark at 25°C. Following this, we used a wide-mouthed pipette to pour 5 ml of a bacterial suspension that contained 0.25 g embryogenic tissue onto a 5.5-cm sterile filter paper (Whatman no. 2) lining the mouth of a funnel (Millipore, Bedford, Mass.) and applied a vacuum pulse to completely drain the liquid. For co-cultivation, the filter paper with the embryogenic tissues was placed on SM3, which contained 50 µM acetosyringone, in a 90×15-mm petri dish. Co-cultivation was carried out for 2 days in the dark at 25°C.

Following co-cultivation, the tissues (1 g fresh weight) were collected from four sheets of the filter paper using a spatula, transferred to 40 ml of washing solution (liquid SM3 that contained 300 mg/l cefotaxime) in a 50-ml test tube and placed on a shaker at 150 rpm for 20 min. The tissues were then collected on a new filter paper lining a funnel following the application of a vacuum pulse. The washing treatment was carried out a total of three times. Following the final washing, 10 ml of the suspension, containing 0.25 g of tissue, was poured onto a new filter paper lining a funnel and subjected to a vacuum pulse; the collected tissues on the filter paper were then cultured first on SM3 containing 300 mg/l cefotaxime for 4 days and then transferred to SM3 supplemented with 300 mg/l cefotaxime and 25 mg/l or 50 mg/l kanamycin. The tissues and filter were transferred to fresh medium every 3 weeks. Colonies that formed on the filter paper were isolated 6 weeks later and proliferated on the same composition of medium. The wild-type embryogenic tissues could not grow on medium that contained 25 mg/l kanamycin.

### Maturation of somatic embryos and regeneration of transgenic plantlets

The embryogenic tissues sub-cultured on SM3 containing kanamycin and cefotaxime were transferred to a maturation medium (Taniguchi et al. 2004a) consisting of SM3 basal salts, vitamins and amino acids, 2 g/l activated charcoal (acid-washed with HCl; Sigma-Aldrich, St Louis, Mo.) and 100 µM (±) – abscisic acid (Sigma-Aldrich), 150 g/l polyethylene glycol 4000 (Wako Pure Chemical Industries, Osaka, Japan), 3.0 g/l gelrite (Wako) and 30 g/l maltose. The maturation medium also contained 25 mg/l kanamycin. The

cultures were kept in the dark at 25°C for 8 weeks without being sub-cultured.

For germination, the mature embryos were cultured on woody plant medium (Lloyd and McCown 1980) containing 2 g/l activated charcoal, 20 g/l sucrose, 5 g/l gelrite and 25 mg/l kanamycin and maintained at 25°C under a 16/8-h (day/night) photoperiod with light provided by cool-white fluorescent lamps at intensity of 100  $\mu\text{mol}/\text{m}^2$  per second.

Regenerated transgenic plantlets that developed roots and elongated epicotyls on the germination medium were transferred to pots containing a soil mixture of peat moss and vermiculite (1:1) and grown in a containment greenhouse. The pot was covered with a transparent polyethylene bag for the first 3 weeks to maintain high humidity; the polyethylene bag was subsequently removed gradually over a period of 1 week.

#### Observation of GFP expression

GFP expression in the co-cultivated embryogenic tissues, re-grown embryogenic tissues, somatic embryos and regenerated plantlets was visualized under a fluorescence stereo microscope (MZ FLIII; Leica Microsystems, Heerbrugg, Switzerland) with a GFP Plus filter system (excitation filter: 480/40 nm, emission filter: 510 nm) or a GFP Plant filter system (excitation filter: 470/40 nm, emission filter: 525/50 nm). The GFP Plant filter system can cut out the red colour of the auto-fluorescence of chlorophyll. Photographs of GFP expression were taken using a digital camera system (DC 300 F; Leica).

#### Southern blot analysis

Genomic DNA was extracted from fresh young shoots (200 mg) of transformed plantlets of independent lines or untransformed control plantlets using a DNeasy Plant Mini kit (Qiagen, Valencia, Calif.) according to manufacturer's instructions. DNA (10  $\mu\text{g}$ ) digested with *EcoRI* or *NheI* was fractionated on a 0.7% agarose gel, blotted onto a Hybond N<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) and probed with a digoxigenin-labeled *nptII-sgfp* fragment prepared by PCR amplification using the primer set (5'-CTTTACTTGTACAGCTCGTCCATG-3' and 5'-CCCCTCGGTATCCAATTAGAG-3'). Hybridization in the DIG Easy Hyb buffer (Roche, Mannheim, Germany) and chemiluminescent detection with CDP-star (Roche) were performed according to manufacturer's instructions.

## Results and discussion

### Co-cultivation of embryogenic tissue with *A. tumefaciens* and the recovery of transformed plants

Following the 2-day-long co-cultivation of embryogenic tissue with *A. tumefaciens*, the tissues, which were col-

lected using a spatula, became muddy from the growth of the bacteria, particularly in the case of longer periods (5 h) of co-cultivation in the liquid medium. A thorough washing of these tissues prevented subsequent bacterial growth on the decontamination medium, which contained cefotaxime. When the co-cultivated tissues were not washed or when some bacterial liquid remained on the filter paper following washing, the bacteria grew intensely on the medium and led to the death of all embryogenic tissue. Therefore, a thorough washing was a critical factor in the successful *A. tumefaciens*-mediated transformation of *C. obtusa*. In *Picea* spp. embryogenic tissue transformation, washing of the co-cultivated tissues also resulted in higher transformation frequencies than non-washing of the tissues (Klimaszewska et al. 2001).

After 3–4 weeks of co-cultivation of the embryogenic tissues with bacteria, the first signs of culture growth were determined visually, while the first colonies (about 1 mm diameter) formed on the selection medium, which contained kanamycin, after an additional 2–3 weeks. Overall 197 colonies were isolated from 12 dishes (total weight of the embryogenic tissues was 3 g), and these were transferred onto fresh selection medium. Two months after the colonies were isolated, 52 culture lines (26.4%), all of which were unable to grow on the kanamycin-supplemented medium or did not express GFP at all, were disposed off, and 145 lines of the transgenic embryogenic cultures were selected. Table 1 shows the effect of the transformation procedures on the transformation frequency of *C. obtusa* embryogenic tissues. The highest transformation frequency (22.5 lines per dish) was obtained using procedure A in which embryogenic tissues were co-cultivated in liquid medium for 5 h and the selection medium contained 25 mg/l kanamycin. When the co-cultivation period in liquid medium was shorter (procedure C), the transformation frequency was reduced by 50%. At the higher selection pressure of 50 mg/l kanamycin, transformation frequencies fell to the lowest level (5.5 lines per dish in procedures B and D).

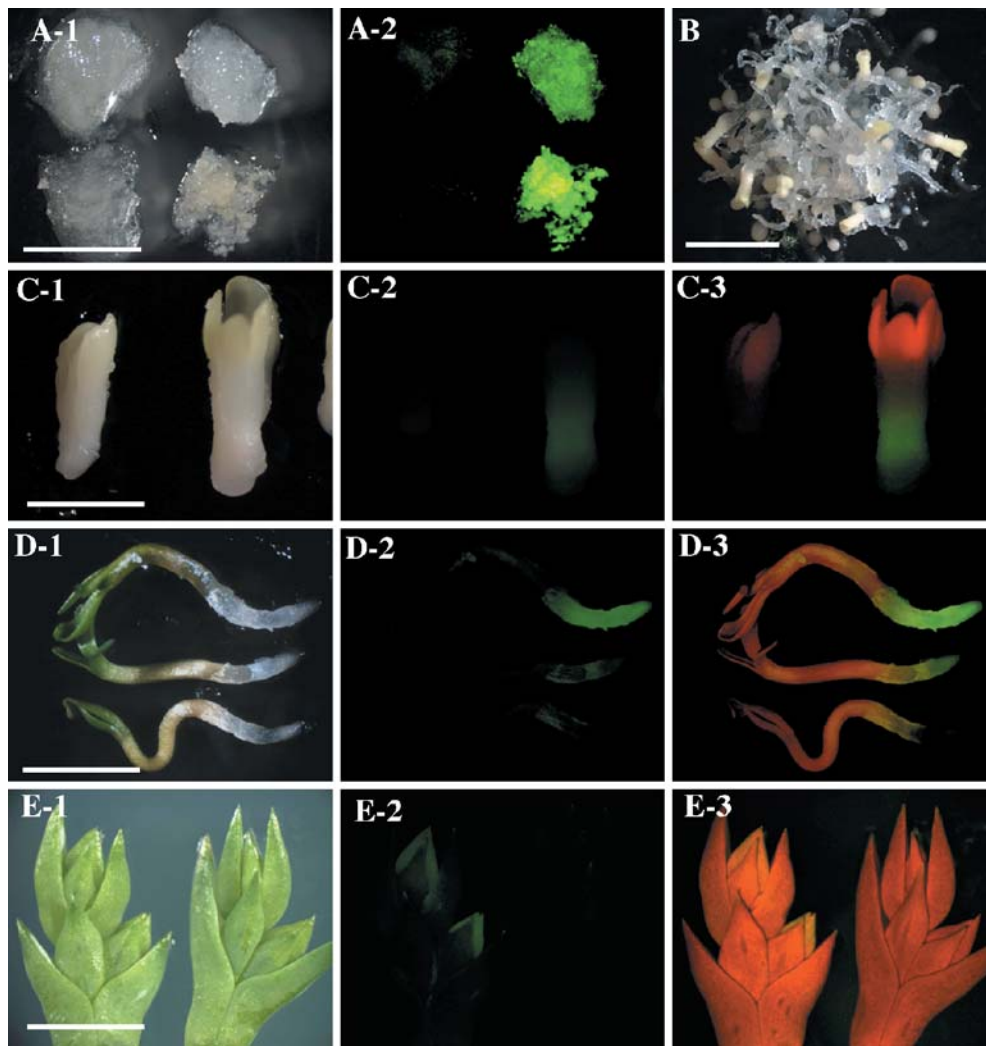
Twelve lines of the transformed embryogenic tissues were cultured on somatic embryo maturation medium, and after 8 weeks of culture, mature somatic embryos were induced in eight lines (Fig. 1B). The germination frequency of these mature embryos was 57% (Fig. 1D-1), and we obtained transformed plantlets of the eight independent lines. Both the ability to produce mature embryos

**Table 1** Transformation frequencies of embryogenic tissues of *Chamaecyparis obtusa* 2 months after co-cultivation with *Agrobacterium tumefaciens*

Procedure	Procedure A: 5 h in liquid medium and selected on 25 mg/l Kanamycin	Procedure B: 5 h in liquid medium and selected on 50 mg/l Km	Procedure C: 20 min in liquid medium and selected on 25 mg/l Km	Procedure D: 20 min in liquid medium and selected on 50 mg/l Km
Transgenic lines per dish	22.5 <sup>a</sup> (16–32) <sup>b</sup>	5.5 <sup>b</sup> (2–11)	11 <sup>a,b</sup> (10–12)	5.5 <sup>b</sup> (5–6)

<sup>a</sup> Average numbers of independent transgenic lines of embryogenic tissues which grew on kanamycin medium and expressed GFP. Each dish contained 250 mg fresh weight of embryogenic tissues co-cultivated with C58/pMP90 harbouring plasmid pBin19-sgfp. Values followed by different letters are significantly different ( $P < 0.05$ ) based on the non-parametric Kruskal-Wallis test

<sup>b</sup> The lowest and highest results are shown in parenthesis. Results of procedures A and B were obtained from four dishes; those of procedures C and D from two dishes



**Fig. 1** *Agrobacterium tumefaciens*-mediated genetic transformation of embryogenic tissue and the visualization of GFP expression in *Chamaecyparis obtusa*. **A-1** Embryogenic tissues of three independent transgenic lines and one non-transgenic line (*lower left*), **A-2** fluorescent image of **A-1** visualized with a GFP Plus filter (excitation filter: 480/40 nm, emission filter: 510 nm). **B** Maturation of somatic embryos of a transgenic line, after 8 weeks of culture on the maturation medium. **C-1** Cotyledonary somatic embryo of a transgenic line (*right*) and non-transgenic line (*left*), **C-2**

fluorescent image of **C-1** visualized with a GFP Plant filter (excitation filter: 470/40 nm, emission filter: 525/50 nm), **C-3** fluorescent image of **C-1** visualized with a GFP Plus filter. **D-1** Germination of somatic embryos (*lowest* is of a non-transgenic line), after 1 week of culture on the germination medium, **D-2**, **-3** the same images as **C-2**, **-3**, but for **D-1**. **E-1** Shoot tips of acclimated plants growing in a greenhouse. (*left* transgenic, *right* non-transgenic), **E-2**, **-3** the same images as **C-2**, **-3** but for **E-1**. Bars: 5 mm (**A,B,D**), 2 mm (**C,E**)

and the germination frequency of the transformed embryos was comparable with those of the non-transformed line (Taniguchi et al. 2004a). Some of the transgenic plantlets acclimated and continued to grow in a containment greenhouse (Fig. 2). There was no obvious difference between the transgenic and non-transgenic plants.

In this study, we used a disarmed strain of *A. tumefaciens*, C58/pMP90, for the genetic transformation of *C. obtusa* embryogenic tissues and were able to successfully transform the tissues at a high efficiency (Table 1) and regenerate transgenic plantlets. The C58/pMP90 strain should be very efficient for the genetic transformation of conifers since it has been successfully used for the production of transformed plantlets in many coniferous species by means of embryogenic tissue transformation:

*Larix kaempferi* × *L. decidua* (Levée et al. 1997; Lelu and Pilate 2000), *Pinus strobus* (Levée et al. 1999), *Picea glauca*, *P. mariana* and *P. abies* (Klimaszewska et al. 2001). Another disarmed *A. tumefaciens* strain, EHA 105 (pToK 47), which contains additional virulence genes to enhance the transformation efficiency, has been used for stably transforming embryogenic tissues of *Picea abies* (Wenck et al. 1999) and *P. glauca* (Le et al. 2001), and transformed plantlets have been regenerated in *P. glauca* (Le et al. 2001). In *Pinus radiata*, transformed embryogenic tissues were obtained using the LBA4404 strain, but transgenic plants were not regenerated (Cerdeña et al. 2002).

Unfortunately, mature embryo production greatly decreased in subsequent experiments because of a reduction in the ability of the embryos to mature in both the



**Fig. 2** Regenerated plants of the non-transgenic (left) and a transgenic (right) line. The width of the planter was 25 cm

transformed embryogenic tissues and untransformed tissues. This is quite a serious problem in embryogenesis and the transformation of *C. obtusa*.

#### Visual assays of GFP expression

After the 2-day co-cultivation period there were two or three single cells per dish that expressed GFP, but the intensity of GFP expression was faint. Following the culturing of the co-cultivated tissues on selection medium, we were able to distinguish more cell clusters that expressed GFP, and their fluorescent intensities varied from very faint to relatively strong. When we isolated the colonies formed on the filter papers, approximately one-half of the colonies did not express GFP. The number of colonies that did express GFP increased with the growth of the colonies, but the intensity of fluorescence was also faint in many cell lines. Figure 1A shows transgenic tissues having a very faint fluorescence (upper left in Fig. 1A-2) and relatively strong fluorescence (right in Fig. 1A-2). The non-transformed embryogenic tissues did not show any green fluorescence (lower left in Fig. 1A-2). When the embryos matured (Fig. 1C-1), a very faint green fluorescence was detected on the hypocotyl of the cotyledonary somatic embryo of the transgenic line (Fig. 1C-2). The red auto-fluorescence of chlorophyll was observed in the cotyledons of the embryo (Fig. 1C-3) using a GFP Plus filter. When the embryos germinated (Fig. 1D-1), fluorescence of the GFP was observed in the root using a GFP Plant filter, but a very faint green auto-fluorescence, which might be emitted from some unknown substance, was also detected in the upper part of the root of the non-transgenic line (the lowest one in Fig. 1D-2). Therefore, we could not distinguish the green fluorescence of GFP from the auto-fluorescence using this filter. When a GFP Plus filter was used, the auto-fluorescence in the upper part of the root was yellowish-green, and we could distinguish the green fluorescence of GFP from the auto-fluorescence (Fig. 1D-3). The intensity of the GFP fluo-

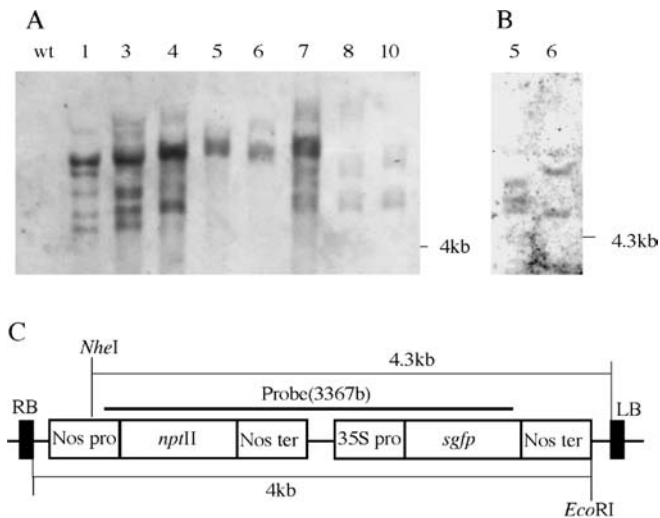
rescence in the root of a germinant shown in the centre of Fig. 1D-3 was very faint. In the youngest leaves of the shoot tips of acclimated plants grown in a containment greenhouse, a faint green fluorescence of GFP was visible using a GFP Plant filter (Fig. 1E-2); when these leaves were observed with a GFP Plus filter, the intensity of the red fluorescence of chlorophyll was so strong that it masked the green fluorescence, and the GFP fluorescence became yellowish-green (Fig. 1E-3).

In this study, sGFP(S65T) was shown to be available as a reporter in *C. obtusa*. However, the fluorescence was often not clear in many tissues of the transgenic lines, which indicated a low expression of GFP. Levée et al. (1999) found that the expression of mGFP5-ER was also not strong in *Agrobacterium*-mediated transformed embryogenic tissues of *P. strobus*. However, the transient expression of GFP after particle bombardment was clearer in cotyledonary somatic embryos of *Larix kaempferi* and zygotic embryos of *Cryptomeria japonica* (Taniguchi et al. 2004b). In the present case, many copies of *sgfp* might be introduced to single cells by particle bombardment, resulting in a strong intensity of fluorescence.

The low expression of *sgfp* in *C. obtusa* might be due to the low activity of the 35S promoter. More active promoters than the 35S promoter might express *sgfp* more actively in transformed *C. obtusa*. Clapham et al. (2000) found that in *Picea abies* embryogenic tissues the *Zea* ubiquitin promoter was 12- to 16-fold more active with respect to GUS expression than the 35S promoter. GUS expression driven by the polyubiquitin promoter of *Helianthus* was also more than twice that when driven by an enhanced 35S promoter with SARs and 20-fold that when driven by a 35S promoter in *P. abies* (Clapham et al. 1995). Moreover, the fluorescence of GFP could not be visualized in leaves, except for the youngest leaves of the shoot tips, and was detected in the roots of all transgenic *C. obtusa*. It might depend on a very low activity of the 35S promoter in leaves. In *Betula pendula*, activity of the 35S promoter in the leaves was one seventeenth of that in the roots (Lemmetyinen et al. 1998).

#### Southern blot analysis

Shoots of the eight transgenic and one non-transgenic line were analysed by Southern hybridization following the digestion of the genomic DNA with *EcoRI* (Fig. 3A). As expected, no hybridization signal was detected in the non-transgenic line. Genomic DNA of all the transgenic lines hybridized with a *nptII-sgfp* probe, which confirmed that the transgenes were integrated into the genome of the *C. obtusa* plants. Each transgenic line showed different hybridization signal patterns, which indicated that all of the transgenic lines analysed were derived from independent transformation events. Other than line 5, all transgenic lines had multiple hybridizing signals. The genomic DNA of line 5 digested with *NheI* showed three hybridizing signals (Fig. 3B). Therefore, all of the transgenic lines tested contained multiple gene copies.



**Fig. 3** Southern blot analysis of transgenic plantlets of *C. obtusa*. **A** Genomic DNA of the shoot of the non-transgenic line (*wt*) and eight transgenic lines (*1, 3, 4, 5, 6, 7, 8, 10*) were digested with *EcoRI* and hybridized with a *nptII-sgfp* probe. **B** Genomic DNA of the shoot of two transgenic lines (*5, 6*) were digested with *NheI* and hybridized with a *nptII-sgfp* probe. **C** T-DNA construct used in this study that showed the *EcoRI* and *NheI* restriction site and the position of the probe used for the Southern blot analysis

## Conclusions

We stably transformed the embryogenic tissues of the *C. obtusa* plus-tree by means of *A. tumefaciens* at a high frequency and regenerated transgenic plantlets. It took approximately 10 months from the initiation of co-cultivation of embryogenic tissues with *Agrobacterium* to regenerate transgenic plantlets. To our knowledge, this is the first report on *Agrobacterium*-mediated genetic transformation and transgenic plant regeneration of *C. obtusa*. The introduced gene, *sgfp*, was available as a reporter gene in *C. obtusa*, although gene expression driven by the 35S promoter was not strong. The method developed here should enable us to generate genetically engineered *C. obtusa* trees, such as trees resistant to a serious disease caused by the filamentous fungi, *Seiridium unicorne*, or to insect damage caused by *Epinotia grani-talis*.

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