RESEARCH PAPER

Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine

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Received 15 August 2006; Revised 5 October 2006; Accepted 10 October 2006

Abstract
An efficient transgenic eastern white pine (Pinus strobus L.) plant regeneration system has been established using Agrobacterium tumefaciens strain GV3850-mediated transformation and the green fluorescent protein (gfp) gene as a reporter in this investigation. Stable integration of transgenes in the plant genome of pine was confirmed by polymerase chain reaction (PCR), Southern blot, and northern blot analyses. Transgene expression was analysed in pine T-DNA transformants carrying different numbers of copies of T-DNA insertions. Post-transcriptional gene silencing (PTGS) was mostly obtained in transgenic lines with more than three copies of T-DNA, but not in transgenic lines with one copy of T-DNA. In situ hybridization chromosome analysis of transgenic lines demonstrated that silenced transgenic lines had two or more T-DNA insertions in the same chromosome. These results suggest that two or more T-DNA insertions in the same chromosome facilitate efficient gene silencing in transgenic pine cells expressing green fluorescent protein. There were no differences in shoot differentiation and development between transgenic lines with multiple T-DNA copies and transgenic lines with one or two T-DNA copies.

Key words: Agrobacterium tumefaciens, gene silencing, green fluorescent protein, Pinus strobus L., transgene.

Introduction
The green fluorescent protein gene (gfp) isolated from the jellyfish Aequorea victoria has been reported as a vital marker gene in bacteria, plants, and Caenorhabditis elegans (Chalfie et al., 1994; Rizzuto et al., 1996). The gfp reporter gene has been particularly useful because GFP fluorescence can be detected in a non-destructive manner and be screened in vivo at any growth stage (Heim et al., 1995; Chiu et al., 1996). GFP does not require a substrate and it is not toxic (Cubitt et al., 1995). Also, GFP allows monitoring of gene expression and protein localization at the subcellular, cellular, and plant level. The use of the GFP marker protein has been described in maize (Hu and Cheng, 1995; Sheen et al., 1995), sweet orange (Niedz et al., 1995), tobacco (Reichel et al., 1996), Arabidopsis (Sheen et al., 1995), and Pinus (Levee et al., 1999). Haseloff and Amos (1995) have demonstrated that the expression of the gfp cDNA is curtailed by aberrant mRNA splicing in Arabidopsis. Targeting of this GFP protein to the endoplasmic reticulum has improved its expression in stably transformed Arabidopsis (Haseloff et al., 1997). Genetic transformation of woody plants is a promising tool for their genetic improvement, since their breeding has limitations imposed in general by their high heterozygosity, long juvenile periods, and auto-incompatibility (Moore et al., 1992; Pena et al., 1997; Cervera et al., 1998). The gfp gene has been demonstrated to have value as a reporter marker gene for localizing transgenic events and for improving their selection and thus the recovery of woody transgenics. In conifers, transgenics have been produced from Larix decidua (Huang et al., 1991), Picea abies (Wenck et al., 1999; Klimaszewska et al., 2001), P. glauca (Klimaszewska et al., 2001; Le et al., 2001), P. mariana (Klimaszewska et al., 2001), Pinus strobus (Levee et al., 1999), P. taeda (Tang et al., 2001; Gould et al., 2002), and P. radiata (Cerda et al., 2002; Charity et al., 2002) via Agrobacterium-mediated transformation. However, the expression of gfp...
has been shown only in *Pinus strobus* (Levee et al., 1999).

Stable transgene expression is a critical parameter for the broad use of transgenic plants in plant biology and agricultural biotechnology. However, gene silencing is frequently observed in transgenic plants such as tobacco and *Arabidopsis* (Hobbs et al., 1990; Holtorf et al., 1995). Transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) are two different mechanisms that can be distinguished in transgensics (Vaucheret et al., 1998; Lechtenberg et al., 2003). TGS is classified as an abolished transcription of the introduced gene. It is associated with methylation of promoter sequences of the transgene and meiotic irreversibility (Meyer, 2000; Mette et al., 2000; Lechtenberg et al., 2003). PTGS is classified as when transcription is maintained, but the transgene mRNA is degraded. Small interfering RNAs (siRNAs) in sense and antisense orientations and specific for the transcribed transgene sequence are a hallmark of silenced lines (Hamilton and Baulcombe, 1999). TGS and/or PTGS has been found to be associated with multiple inserts of the introduced DNA. However, this phenomenon is not restricted to the repeat structures of the transgenes. Endogenous plant genes forming the repeat structures may also be subjected to gene silencing (Muskens et al., 2000; Lechtenberg et al., 2003).

The study of transcribed inverted repeat structures has provided compelling evidence that silencing is mediated via RNA–RNA and RNA–DNA interactions (Waterhouse et al., 1998; Hamilton et al., 1999; Chuang and Meyerowitz, 2000). During the process of PTGS, RNA is processed to siRNAs, and siRNAs trigger the elimination of the homologous mRNA in the cytoplasm. This process of PTGS in plants is related to RNA interference in animals and quelling in fungi (Sijen et al., 2001; Zamore, 2002; Lechtenberg et al., 2003). To gain more insight into the role of the inverted repeat (IR) arrangements on gene expression, Lechtenberg et al. (2003) determined the influence of four IR T-DNA loci on the expression of different transgenes in *Arabidopsis thaliana*. Their results showed that arrangements of neither the tandemly repeated transgenes nor the inverted T-DNA structures were sufficient to trigger gene silencing. Instead, the study of the different transgenic lines suggested a correlation between silencing and high transgene doses (Lechtenberg et al., 2003).

Transgene silencing studies potentially contribute to molecular breeding with respect to the stability of transgene expression (Francis and Spike, 2005; Mishiba et al., 2005). In addition, transgene silencing might potentially affect the screening efficiency of transformants (Frommer et al., 1992; Francis and Spike, 2005). Although transgene silencing has been investigated in a number of angiosperms, the influence of multiple T-DNA copies on gene silencing and shoot development has not been investigated in gymnosperms. This unique transgene-silencing phenomenon has now been studied in eastern white pine (*Pinus strobus L*). The purposes of the study were to: (i) establish a high efficiency *Agrobacterium*-mediated genetic transformation system in eastern white pine; (ii) analyse multiple copies of T-DNA insertions induced by PTGS in a gymnosperm species; (iii) verify whether this silencing phenomenon can be explained by the existing transgene-silencing models established in model plant species such as tobacco and *Arabidopsis*; and (iv) explore the relationship between gene silencing and the location of transgenes in chromosomes. In this investigation, *Agrobacterium tumefaciens* strain GV3850 harbouring the expression vector pBINm-gfp5-ER was used to transform mature zygotic embryos of eastern white pine. The results demonstrate that multiple T-DNA copies induced efficient gene silencing in transgenic pine cells with at least two insertions in the same chromosome. However, there are no differences in shoot differentiation and development between transgenic lines with multiple T-DNA copies and transgenic lines with one or two T-DNA copies.

**Materials and methods**

**Production of transgenic pine**

Dry seeds of eastern white pine were purchased from F.W. Schumacher Co., Inc., Sandwich, MA, USA, and stored in plastic bags at 4 °C before they were used for tissue culture and transformation. Disinfection of seeds and isolation of mature zygotic embryos from the megagametophytes were conducted as described previously (Tang and Newton, 2005a). Mature embryos were used as explants in the transformation experiments. Embryos were cultured on a pretreatment PS medium (Tang and Newton, 2005a) supplemented with 10 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg l⁻¹ N₆-benzyladenine (BA), and 4 mg l⁻¹ kinetin for 3 d to ensure successful aseptic culture. The binary expression vector pBINm-gfp5-ER was kindly provided by CN Stewart and J Haseloff. pBINm-gfp5-ER contains the m-gfp5-ER gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the nopaline synthase polyadenylation region, and the selectable marker gene, *nptII*, which confers resistance to kanamycin. *Agrobacterium tumefaciens*-mediated transformation of eastern white pine was carried out as described before (Tang and Newton, 2005b) 3 d after mature zygotic embryos were cultured on a pretreatment PS medium (Tang and Newton, 2005a) used for selecting embryos not contaminated by natural bacteria. Kanamycin (5 mg l⁻¹) was added to the selection medium producing transformations with multiple copies of T-DNA (Francis and Spiker, 2005).

**Visualization of GFP expression**

Transgenic callus cultures were examined with a stereo dissecting microscope equipped with a fluorescence module consisting of a 100 W mercury lamp and GFP Plus excitation and emission filters (Leica, Heerbrugg, Switzerland). This system (excitation filter 480/ 40 nm; dichroic mirror 505 nm LP; barrier filter 510 nm LP) permits visualization of GFP following excitation by blue light. The number of transient events was determined immediately by exposure to the blue light source for 3 min. The control embryos were monitored at the same intervals. The fluorescence images were
Gene expression was quantified in both control and targeted cells. GFP images were recorded with equal exposure time under non-saturated activity. For quantitative fluorescence determinations of m-gfp5-ER activity in transgenic cell lines, an LSM 510 laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using excitation with the 488 nm argon laser line and detection of emitted light between 500 nm and 520 nm was used to capture images. The confocal images of m-gfp5-ER expressing cells were created in the Expert Mode. Images were recorded with equal exposure time under non-saturated conditions for randomly chosen gfp-expressing transgenic cells. Gene expression was quantified in both control and targeted cells.

Fluorescence intensities (arbitrary units) of different samples were calculated from confocal images with the Zeiss LSM image Examiner software. The fluorescence level was quantified separately for the whole cell by circumscribing the respective area as a region of interest. Background correction was applied by adjusting fluorescence levels in a neighbouring non-transgenic cell to zero. Thirty to 50 cells were used for each sample.

Results

Detection of siRNA by RNase protection assay

Total RNA was extracted from cell cultures of transgenic lines with TRI reagent as described (Sambrook et al., 1989; Hamilton and Baulcombe, 1999). Total RNA was precipitated with yeast tRNA and ethanol for 30 min at −70 °C then dissolved in diethylpyrocarbonate (DEPC)-treated water. The small size RNA was enriched (Hamiton and Baulcombe, 1999) and it was used for the RNase protection assay (Sambrook et al., 1989). In brief, the enriched small RNA was hybridized to a DIG-labelled m-gfp5-ER DNA (816 bp) probe overnight at 35 °C. siRNA in the enriched fraction binds to the probe during this period. The unhybridized single-stranded RNA was digested with RNase T1 (Ambion, USA) in RNA digestion buffer (300 mM NaCl, 10 mM TRIS–HCl at pH 7.4 and 5 mM EDTA at pH 7.5) at 37 °C for 1 h. The reaction was stopped by adding 20 μl of 10% SDS and 10 μl of proteinase K, and incubated at 30 °C for 30 min. The mixture was purified followed by fractionation on a 15% denaturing polyacrylamide gel and exposure to X-Omat AR-2 film (Fisher Scientific) for 15 min. In vitro prepared small 21-nucleotide antisense RNA purchased from Qiagen was used as the marker.

Confocal laser scanning microscopy

For quantitative fluorescence determinations of m-gfp5-ER activity in transgenic cell lines, an LSM 510 laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using excitation with the 488 nm argon laser line and detection of emitted light between 500 nm and 520 nm was used to capture images. The confocal images of m-gfp5-ER-expressing cells were created in the Expert Mode. Images were recorded with equal exposure time under non-saturated conditions for randomly chosen gfp-expressing transgenic cells. Gene expression was quantified in both control and targeted cells.

Polymerase chain reaction (PCR) and Southern blot analyses

PCR and Southern blot analyses of putative transgenic cell lines were carried out as described before (Tang and Newton, 2005b). Genomic DNA was isolated from 500 μg of fresh tissue of control and putative transgenic callus using a Genomic DNA Isolation Kit (Sigma) following the manufacturer’s protocol. The PCR analysis was performed with a PTC-100TM Programmable Thermal Controller (MJ Research, San Francisco, CA, USA). For amplification of insert DNA, the nptII forward primer (npt) 5'-ACACAGA-CAATCGGCTGC-3' and the reverse primer (urp) 5'-AAGAACTCGTCAGAAGGCG-3' were used. A total of 300 ng of genomic DNA was used as a template in a 50 μl PCR mix. The PCR mixture consisted of 200 μM each of dATP, dCTP, dGTP, and dTTP, 35 pmol of each primer, 2.5 U of Taq DNA polymerase (Promega), 1.5 mM MgCl2, and 5 μl of 10× buffer (500 mM KCl, 100 mM TRIS–HCl pH 9.0 at 25 °C, 1% Triton X-100, 15 mM MgCl2). The PCR conditions were 95 °C for 5 min followed by 29 cycles at 95 °C for 60 s, 57 °C for 40 s, and 72 °C for 90 s. Cycling was followed by a final incubation of 72 °C for 10 min. PCR products were separated by electrophoresis on 1.0% agarose gels in 1× TAE buffer (Sambrook et al., 1989) and were detected by fluorescence under UV light (302 nm) after staining with 0.1% ethidium bromide. A molecular marker of 1 kb (Gibco-BRL) was used. Southern blot analysis was conducted as described previously (Tang et al., 2001). Genomic DNA was isolated from 5 g of fresh tissue of control and putative transgenic plants using a Genomic DNA Isolation Kit (Sigma). TAE electrophoresis buffer, a hybridization solution, and SSC solutions for final washes were prepared according to Sambrook et al. (1989). A 20 μg portion of DNA was digested overnight with the restriction enzyme HindIII (Boehringer Mannheim) at 37 °C. Probes (816 bp fragment of m-gfp5-ER DNA) were labelled with digoxigenin (DIG) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). Only a single HindIII restriction site exists in the inserted DNA.

Isolation of RNA and northern blot analysis

Cell cultures were collected from the control and Southern-positive transgenic lines and were immediately frozen in liquid nitrogen and stored at −80 °C. Samples of RNA were extracted from 1.5 g of fresh tissue of each line using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer’s protocol. A 10 μl aliquot of total RNA was separated by agarose gel electrophoresis. Electrophoresis and northern blotting of RNAs were performed as described by Tang and Newton (2005b). Probes (816 bp fragment of m-gfp5-ER DNA) were labelled with DIG. Conditions for hybridization and washing of blots were those recommended for use (Sambrook et al., 1989). Hybridization of the probe DNAs to the blots was recorded on blue-sensitive X-ray film. Equal loading of RNA samples was verified on the control tobacco 25S rRNA.

In situ hybridization chromosome analysis

Cell cultures were grown on filter paper moistened with liquid TE medium (Tang et al., 2001) in Petri dishes at room temperature and subcultured weekly. Cultured cells were collected after three subcultures and pretreated with ice water for 24 h, then were fixed in Carnoy’s I (1 part glacial acetic acid + 3 parts 95% or absolute ethanol) for 2 d. Cells were stained in 1% aceticarmine (10 g of carmine (Fisher C579-25) was dissolved in 1.0 l of 45% glacial acetic acid, then boileezers were added, and cells were refluxed for 24 h). Cells were filtered into dark bottles and stored at 4 °C for 30 min to 1 h. Squash preparations were made in 1% aceticarmine. In situ hybridization chromosome analysis using DIG-labelled probes was conducted as described by Araki et al. (1997) and Rebay (1996; http://www.fruitfly.org/about/methods/cytogenetics.html).

Detection of siRNA by RNase protection assay

Total RNA was extracted from cell cultures of transgenic lines with TRI reagent as described (Sambrook et al., 1989; Hamilton and Baulcombe, 1999). Total RNA was precipitated with yeast tRNA and ethanol for 30 min at −70 °C then dissolved in diethylpyrocarbonate (DEPC)-treated water. The small size RNA was enriched (Hamilton and Baulcombe, 1999) and it was used for the RNase protection assay (Sambrook et al., 1989). In brief, the enriched small RNA was hybridized to a DIG-labelled m-gfp5-ER DNA (816 bp) probe overnight at 35 °C. siRNA in the enriched fraction binds to the probe during this period. The unhybridized single-stranded RNA was digested with RNase T1 (Ambion, USA) in RNA digestion buffer (300 mM NaCl, 10 mM TRIS–HCl at pH 7.4 and 5 mM EDTA at pH 7.5) at 37 °C for 1 h. The reaction was stopped by adding 20 μl of 10% SDS and 10 μl of proteinase K, and incubated at 30 °C for 30 min. The mixture was purified followed by fractionation on a 15% denaturing polyacrylamide gel and exposure to X-Omat AR-2 film (Fisher Scientific) for 15 min. In vitro prepared small 21-nucleotide antisense RNA purchased from Qiagen was used as the marker.
were transferred onto regeneration medium for shoot formation. Transgenic shoots 3–5 cm in height were obtained on regeneration medium in 6 weeks (Fig. 1D–F). Callus cultures and shoots of each transgenic line were further used for T-DNA insertion analysis by Southern blotting.

The transformation efficiency of mature embryos was analysed in a time-course experiment. Transient gfp expression was examined daily for 1 week. The highest frequency (85% of Agrobacterium-inoculated embryos) of transient expression was obtained 3–4 d after inoculation. The highest frequency (35% of Agrobacterium-inoculated embryos) of callus formation was obtained 6–7 weeks after inoculation. After transgenic calli were transferred onto regeneration medium, the highest frequency (37% of callus cultures) of shoot formation was obtained in 6–7 weeks. The regeneration efficiency of transgenic callus cultures was examined by analysing the number of shoots per gram of callus. About 25 shoots were obtained from 1 g of callus cultures in 6–7 weeks.

Molecular analyses of transgenic cell lines
Transgenic callus lines were screened by PCR (Fig. 2A) for the presence of T-DNA sequences. In ~300 putative transgenic callus lines, 288 callus lines were identified (Table 1). The PCR-positive callus lines were further analysed by Southern blotting (Fig. 2B) for the T-DNA insertions. Southern blot analysis showed that T-DNA was introduced into all transgenic callus lines. Among 288 transgenic lines, 80 transgenic lines had one copy of the T-DNA insertion (27.8% of total transgenic lines) (Table 1). Transgenic callus lines with positive Southern blots were further analysed by northern blot to monitor gfp expression at the transcriptional level. A representative northern blot of transgenic callus lines with one copy of the T-DNA insertion is shown in Fig. 2C.

Stable transformation and transgenic plants established in soil
Transgenic shoots regenerated from transgenic callus lines with one copy of T-DNA were transferred onto rooting
medium to induce root formation (Fig. 3A). One to three roots were obtained from single shoots in 6 weeks. The rooting frequency of transgenic shoots was similar to the non-transgenic shoot control, and the highest rooting frequency was obtained in the sixth week. After acclimatization for 1 week, plantlets 2–4 cm in height were established in soil (Fig. 3B, C). The survival rate of transgenic plantlets was examined weekly for 7 weeks after transgenic plantlets were established in soil in the greenhouse. The survival rate of transgenic plantlets was similar to that of the non-transgenic control, and the highest survival was 79% in the sixth week.

**Laser scanning microscopy of transgenic cell lines with different T-DNA insertions**

To establish suspension cell cultures, five transgenic lines were sampled from each of the copy-number classes of lines (Fig. 4A) with 1–7 copies of T-DNA insertions. After cell cultures were transferred into fresh liquid culture medium for 3 d, gfp expression in cells of different transgenic lines was examined by laser scanning microscopy. Similar green fluorescence was observed in transgenic cells with 1–3 copies of T-DNA insertions (Fig. 4B–D). However, green fluorescence was lower in transgenic cells with 4–7 copies of T-DNA insertions. Green fluorescence of transgenic cells with 5–7 copies of T-DNA insertions is shown in Fig. 4E–G. According to the difference in green fluorescence of different transgenic cell lines, it is speculated that gene silencing may occur in transgenic cells with multiple copies of T-DNA insertions.

**Multiple copies of T-DNA insertions do not influence shoot formation and development**

To study the influence of multiple copies of T-DNA insertions on shoot formation and development, five transgenic lines were sampled from each of the copy-number classes of lines. Transgenic shoots were induced from all transgenic callus cultures with 1–7 copies of T-DNA insertions on regeneration media. Examples of regenerated shoots from transgenic callus cultures with one, two, three, six, and seven copies of T-DNA insertions are shown in Fig. 5A–D, respectively. Non-transgenic control callus did not survive in selection media with kanamycin. The frequency of shoot formation was examined in the sixth week on regeneration media and there were no differences among the transgenic cell lines. The results demonstrate that insert copy number affected neither shoot production nor rooting.

**Gene silencing in transgenic cell lines**

Because differences were observed in green fluorescence among different transgenic cell lines with different numbers of copies of T-DNA insertions, the same transgenic cell lines used for laser scanning microscopy were used for mRNA accumulation analysis by northern blot (Fig. 5A), for quantitative analysis of green fluorescence.
Less gfp mRNA accumulation was obtained in transgenic cell lines with >3 copies of T-DNA insertions, compared with transgenic cell lines with 1–3 copies of T-DNA insertions (Fig. 5B). An example of northern blotting analysis is shown in Fig. 5A. PTGS in these transgenic lines was further confirmed by quantitative analysis of green fluorescence (Fig. 5C). Compared with transgenic cell lines with 1–3 copies of T-DNA insertions, green fluorescence intensity (arbitrary units) was decreased 30–40% (Fig. 5C). Based on the results from northern blot and quantitative analysis of green fluorescence obtained from transgenic lines, the existence of siRNA in these transgenic lines was examined by RNase protection assays. siRNA was not detected from transgenic cell lines with 1–3 copies of T-DNA insertions (Fig. 5D), but was obtained from transgenic cell lines with 4–7 copies of T-DNA insertions (Fig. 5D).

**In situ hybridization chromosome analysis**

To study further gene silencing in transgenic lines with multiple copies of T-DNA insertions, cultured cells of all transgenic lines were examined by *in situ* hybridization chromosome analysis (Table 1). No gene silencing was observed from transgenic lines with one or two copies of T-DNA insertions, and only one gene-silenced line (3%) was obtained from 33 transgenic lines with three copies of T-DNA insertions (Table 1). To explore further the probable cause of gene silencing in transgenic lines with multiple copies of T-DNA insertions, the location of transgenes in all transgenic lines was examined by *in situ* hybridization.

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**Table 1. In situ hybridization chromosome analysis of transgenic cell lines**

<table>
<thead>
<tr>
<th>Copies of T-DNA</th>
<th>No. of transgenic lines</th>
<th>No. of transgenic lines with two T-DNAs in the same chromosome</th>
<th>No. of transgenic lines with three T-DNAs in the same chromosome</th>
<th>No. of transgenic lines with gene silencing (%)</th>
<th>No. of transgenic lines without gene silencing</th>
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<td>1</td>
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<td>6</td>
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</table>

**Fig. 3.** Transgenic plants regenerated from kanamycin-resistant callus cultures in pine. (A) Kanamycin-resistant plantlet derived from transgenic callus (bar = 0.8 cm). (B) Transgenic plants (right) and non-transgenic control (left) established in soil in the greenhouse for 3 weeks (bar = 2.1 cm). (C) Transgenic plants (right) and non-transgenic control (left) established in soil in the greenhouse for 12 weeks (bar = 5 cm).
The results demonstrate that gene silencing was obtained in transgenic lines with two or three T-DNA insertions (76.5% of transgenic lines with four copies of T-DNA, 95.2% of transgenic lines with five copies of T-DNA, 96.6% of transgenic lines with six copies of T-DNA, and 94.3% of transgenic lines with seven copies of T-DNA) in the same chromosome (Table 1).

Fig. 4. Transgene expression analysis. (A) Total number of transgenic cell lines with different copies of T-DNA insertions. (B–D) Laser scanning microscopy of transgenic cell lines with one copy (B), two copies (C), and three copies (D) of T-DNA insertions, where gfp expression was not silenced. (E–G) Laser scanning microscopy of transgenic cell lines with five copies (E), six copies (F), and seven copies (G) of T-DNA insertions, where gfp expression was silenced (bars in B–G = 0.1 mm). (H) Chromosomes of transgenic cell line with one copy of T-DNA insertion. (I) Chromosomes of transgenic cell line with four copies of T-DNA insertion and three copies of T-DNA insertion in the same chromosome (H–I arrows indicate the transgene, bars = 0.005 mm).
Production of transgenic lines with multiple copies of T-DNA insertions

Plant transformation is a key methodology that has fostered diverse forms of plant biology and biotechnology (Birch, 1997). Multiple copies of transgene loci have been reported to induce transgene silencing at a higher rate than a single copy in many species (Muskens et al., 2000). Recently, it was reported that a high frequency of transgenic lines with silenced T-DNA integrations can be obtained from transgenic *A. thaliana* lines on medium without kanamycin selection (Francis and Spike, 2005). However, pine as an economically important group of coniferous species has not been studied relative to transgene silencing. In producing eastern white pine transformants with multiple copies of T-DNA, the concentration of kanamycin was decreased from 15 mg l⁻¹ to 5 mg l⁻¹ in the selection medium. Kanamycin at 15 mg l⁻¹ is normally used in pine transformation for efficiently selecting transgenic lines (Tang and Newton, 2005b). In this case, some transgenic lines may not be identified with kanamycin because the T-DNAs in those lines may have integrated into genomic regions that repress transgene expression (Francis and Spike, 2005). With a decreased concentration of kanamycin in the selection medium, 288 transgenic lines have been produced from 7500 *Agrobacterium*-inoculated mature embryos in eastern white pine which were used in this study of transgene silencing.

Laser scanning microscopy enhances selection of transgenic lines expressing gfp

GFP of jellyfish (*A. victoria*) is a potentially useful reporter in heterologous systems (Chalfie et al., 1994). Compared with the widely used β-glucuronidase (GUS) reporter system which requires an exogenous substrate for histochemical visualization, GFP expression can be monitored directly in live tissue (Heim et al., 1995). This property of GFP holds great promise in the optimization of gfp expression in transgenic cells. GFP fluorescence was expressed as fluorescence intensity (arbitrary units). Experiments were repeated three times, and each replicate consisted of 30–50 cells. Values represent the means ± SE. (D) Detection of small RNAs. Low molecular weight RNA fractions were isolated from transgenic cells, separated on polyacrylamide gels, blotted onto Hybond N+ membranes, and hybridized with 816 bp gfp-coding sequences. The 21 nucleotide siRNA oligomers were used as size controls (size indicated in nucleotides). Each numbered lane contains the low molecular weight RNA fraction of transgenic cells with 4–7 copies of T-DNA insertion. No specific signal could be detected in transgenic cells with one copy of T-DNA insertion with the probes. Lanes 1–3: no siRNA fraction was detected from transgenic cells with 1–3 copies of T-DNA insertion. Lane C: non-transgenic cells are presented as a control. Lane M: the 21 nucleotide small gfp-specific RNAs were used as a marker.
of transformation protocols for various plant species. Since GFP is not native to plants, it is suitable for introduction into various plant species for the examination of the mechanism of gene silencing. In this study, green fluorescence was used as a visual reporter to select transgenic pine lines. The ability to detect this protein in living cells non-destructively may permit the production of an amount of transgenic lines by early evaluation of transgene expression in putative clones obtained under laser scanning microscopy. In total, 300 transgenic cell lines were screened by laser scanning microscopy for the presence of T-DNA sequences, and 288 transgenic cell lines were confirmed by PCR and Southern blot analysis (96% screening efficiency). This method allows the identification of silenced transgene lines with low green fluorescence intensity due to multiple copies of T-DNA insertion.

**Multiple copies of T-DNA insertions result in gene silencing in transgenic cells**

Transgene silencing in plants has been found to be associated with multiple inserts of the introduced DNA, which is often associated with DNA methylation in many plant species (Waterhouse et al., 1998; Hamilton et al., 1999; Lechtenberg et al., 2003; Mishiba et al., 2005). A total of 288 transgenic cell lines of white pine with 1–7 copies of T-DNA insertions have been produced (Table 1). Of these, 95 silenced transgene lines, comprising one line with three copies of T-DNA insertions, 13 lines with four copies of T-DNA insertions, 20 lines with five copies of T-DNA insertions, 28 lines with six copies of T-DNA insertions, and 33 lines with seven copies of T-DNA insertions, have been identified (Table 1). Laser scanning microscopy (Figs 4B, 5C) and northern blot analysis (Fig. 5A) demonstrated that transgene silencing was obtained from transgenic lines with >3 copies of T-DNA insertions. Detection by RNase protection assays demonstrates that increased numbers of T-DNA copies may increase the probability of RNA duplex formation, which is processed to siRNA and triggers the targeted elimination of the homologous mRNA in the cytoplasm. When transgenes were located in different chromosomes, the frequency of gene silencing was decreased because non-synchronous transcription may occur among different chromosomes and this may reduce the opportunity for siRNA formation in the cytoplasm of the cell. According to the present results (Table 1), there is a break between three T-DNA copies and four T-DNA copies with regard to the effect on transgene silencing. However, it is currently not known why such a break exists. It is speculated that increased numbers of T-DNA copies may increase the possibility of two T-DNA inserts located in the same chromosome.

**Acknowledgements**

We are grateful to CN Stewart and J Haseloff for providing us with the m-gfp5-ER constructs, and to research assistants Adaeez Okoye, Nicki Whitley, and Tinya DeLaGarza for their work in isolating mature embryos for callus induction. This work was supported by the East Carolina Christmas Tree Program.

**References**


