Inducible Antisense-mediated Post-transcriptional Gene Silencing in Transgenic Pine Cells Using Green Fluorescent Protein as a Visual Marker

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An inducible post-transcriptional gene silencing (PTGS) system was established in Virginia pine (Pinus virginiana Mill.) cells. This system is based on the activation of an antisense gfp gene construct by a chimeric transcriptional activator GVG (Gal4-binding domain–VP16 activation domain–glucocorticoid receptor fusion) upon application of the inducer to gfp transgenic cell lines. A detailed characterization of the inducible PTGS system in transgenic cell lines demonstrated that this system is stringently controlled. The degree of silencing with this construct could be regulated by the concentration of inducer and the time of treatment. Such transgenic cell lines may provide a useful system to study signaling mechanisms of gene silencing in transgenic pine cells. The inducible system could be a useful tool for functional discovery of novel plant genes.

Keywords: Chimeric transcription factor — Conditional gene expression — Dexamethasone — Post-transcriptional gene silencing — RNA interference — Transgene.

Abbreviations: CaMV, cauliflower mosaic virus; DIG, digoxigenin; dsRNA, double-stranded RNA; GFP, green fluorescent protein (gfp, gene); GVG, Gal4-binding domain–VP16 activation domain–glucocorticoid receptor fusion; PTGS, post-transcriptional gene silencing; RdRP, RNA-dependent RNA polymerase; siRNA, small interfering RNA.

Introduction

Post-transcriptional gene silencing (PTGS) results in the specific degradation of a population of homologous RNAs and is a heritable change in gene expression that cannot be explained by changes in gene sequence (Baulcombe 2000, Vaucheret et al. 2001). It was first observed after introduction of an extra copy of an endogenous gene (or of the corresponding cDNA under the control of an exogenous promoter) into plants and originally called co-suppression (Napoli et al. 1990, Smith et al. 1990, van der Krol et al. 1990). Later, research from different groups demonstrated that the PTGS phenomenon is not a simple regulatory mechanism that controls the expression of endogenous genes (Dehio and Schell 1994, Ingelbrecht et al. 1994, Elmayan and Vaucheret 1996). PTGS greatly reduces endogenous mRNA accumulation in the cytoplasm in the presence of homologous double-stranded RNA (dsRNA) either locally injected or transcribed from an inverted repeat transgene (Tavernarakis et al. 2000); however, it does not affect transcription (van Blockland et al. 1994). Injected dsRNA, as well as transgenes expressing dsRNA, also triggers silencing of homologous transgenes in plants (Waterhouse et al. 1998, Chuang and Meyerowitz 2000, Schweizer et al. 2000, Vaucheret et al. 2001).

Significant accumulation of sense and antisense small interfering RNAs (siRNAs; approximately 20–25 nucleotides long) was observed in various PTGS systems in plants (Hamilton and Baulcombe 1999). The accumulation of both sense and antisense siRNAs suggests that dsRNA is produced prior to RNA degradation. How dsRNAs are produced is still not completely understood, but the finding that a gene encoding an RNA-dependent RNA polymerase (RdRP) is required for PTGS (Dalmay et al. 2000, Mourrain et al. 2000) suggests that this enzyme is involved in the process. The dsRNA is remarkably effective at suppressing specific gene expression in a number of organisms including plants (Waterhouse et al. 1998, Vaucheret et al. 2001). Gene silencing induced by long dsRNA constructs has been demonstrated in transgenic plants showing transient interference with endogenous gene expression (Schweizer et al. 2000), and with reporter gene expression in plant cells (Kanno et al. 2000, Akashi et al. 2001).

Different strategies of gene silencing, such as inverted repeat transgene (Hamilton et al. 1998), microinjection of dsRNA (Fire et al. 1998, Cogni and Macino 2000, Cogni et al. 1996), and silencing vectors derived from either DNA or RNA viruses (Kjemtrup et al. 1998, Ruiz et al. 1998, Covey and Al-Kaff 2000, Peeler et al. 2001), have been used as a tool for inactivating gene expression. Constitutive expression of a hairpin RNA has been shown to silence target genes efficiently in transgenic plants (Waterhouse et al. 1998). The use of inducible RNAi systems has been described in Nicotiana species (Chen et al. 2003), and an inducible RNA interference (RNAi) approach has been reported in Arabidopsis thaliana (Guo et al. 2003). These two references describe the use of different systems providing spatial and temporal control of hairpin intron sequence expression and PTGS directed against transgenes and endogenous genes. However, no such a system has been reported in a tree species, particularly in pine species. In addition, the reported methods may not be the best choice to regulate GVG (Gal4-binding domain–VP16 activation domain–
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Results

Production of transgenic cell lines

Forty transgenic Virginia pine cell lines with insertion of the sense m-gfp5-ER reporter gene (Fig. 1A) were produced using Agrobacterium tumefaciens (strain GV3850)-mediated gene transfer as described in Tang et al. (2001a). After Southern blotting, Northern blotting, cell growth and green fluorescence analyses (data not shown), one transgenic cell line (PVt1) with one copy of sense m-gfp5-ER (confirmed by Southern blot analysis, data not shown), medium fluorescence (150–180 fluorescence intensity) and fast growth (1.2 mg DW l⁻¹ of cell cultures d⁻¹) was selected and transformed a second time using A. tumefaciens EHA105 containing the plasmid pINDEX3-antisense-m-gfp5-ER (Fig. 1B). Among 20 double-transgenic cell lines produced, four of them (P13, P17, P29 and P31) carrying one copy of sense m-gfp5-ER (Fig. 2A) and one
copy of the plasmid pINDEX3-antisense-m-gfp5-ER T-DNA [confirmed by PCR (Fig. 2B) and Southern blotting (data not shown)] were selected and used for the study of inducible PTGS.

Inducible PTGS and the accumulation of siRNA

We used a dexamethasone-inducible gene expression system to test the ability of antisense m-gfp5-ER mRNAs to trigger silencing. This system is based on the activation of a transcriptional factor by a chemical inducer to initiate transcription of the target gene. It is easy to detect gene silencing in microcalluses, cell masses and individual cells both with gfp as a reporter gene and with fluorescence and confocal microscopy. Virginia pine cell cultures were transformed sequentially with a modified gfp reporter gene encoding a jellyfish GFP with a targeting sequence to the endoplasmic reticulum (m-gfp5-ER) regulated by the cauliflower mosaic virus (CaMV) 35S RNA promoter (Haseloff et al. 1997), and then with an antisense m-gfp5-ER gene regulated by an inducible promoter (Aoyama and Chua 1997, Ouwerkerk et al. 2001). Without addition of the inducer, the constitutively expressed GVG transcription factor localizes in the cytoplasm, then transcription of the antisense m-gfp5-ER target gene is blocked and GFP fluorescence can be detected by fluorescence microscopy. In contrast, with the treatment by dexamethasone, GVG is transferred to the nucleus and interacts with the GVG recognition site, 4UAS, cloned upstream of the target gene antisense m-gfp5-ER, then activates transcription of the target gene. The antisense m-gfp5-ER mRNA accumulates, and the silenced cells will not exhibit green fluorescence coloration.

We selected a low-background line P31 that contained only one copy of pBIN-m-gfp5-ER T-DNA and one copy of pINDEX-antisense-m-gfp5-ER T-DNA to test the inducible PTGS system. Without treatment with dexamethasone, expression of GFP was observed in single living cells (Fig. 3), microcalluses (Fig. 4), and cell cultures (Fig. 5) in double-transformed lines. The level of gfp expression in double-transformed tissues (Fig. 3B, 4B, 5B) was very close to that in the transgenic cell cultures transformed only with pBIN-m-gfp5-ER (Fig. 3A, 4A, 5A). Gene silencing was observed only after transgenic cells were treated with dexamethasone to activate the transcription of antisense-m-gfp5-ER. Silencing of gene expression was monitored by fluorescence and confocal microscopy. Gene silencing was observed in transgenic single living

Fig. 3 Detection of GFP fluorescence silenced by dexamethasone in P31 transgenic cells. (A) GFP fluorescence in a PVt1 transgenic cell. (B–I) GFP fluorescence in double-transformed P31 cells at 0 (B, 1 (C), 2 (D), 3 (E), 4 (F), 5 (G), 6 (H) and 7 d (I) after treatment with 10 mg l⁻¹ dexamethasone. GFP fluorescence was decreased after treatment with dexamethasone, but the same level of GFP fluorescence was observed in the control cell (A) and in a double-transgenic cell treated with dexamethasone at 0 d (B) (scale bars = 0.01 mm).

Fig. 4 Detection of GFP fluorescence silenced by dexamethasone in P31 transgenic microcalluses. (A) GFP fluorescence in a PVt1 transgenic microcallus. (B–I) GFP fluorescence in double-transformed P31 microcalluses at 0 (B, 1 (C), 2 (D), 3 (E), 4 (F), 5 (G), 6 (H) and 7 d (I) after treatment with 10 mg l⁻¹ dexamethasone. GFP fluorescence was decreased after treatment with dexamethasone, but the same level of GFP fluorescence was observed in the control microcallus (A) and in a double-transgenic microcallus treated with dexamethasone at 0 d (B) (scale bars = 0.1 mm).
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High-level silencing of GFP expression was observed 5–7 days after treatment with inducer (Fig. 3). Near complete gene silencing was observed 9 days after treatment with the inducer (Fig. 5K, L).

Fig. 5 Detection of GFP fluorescence silenced by dexamethasone in P31 transgenic cell masses. (A) GFP fluorescence in a PVt1 transgenic cell mass. (B–K) GFP fluorescence in double-transformed P31 cell masses at 0 (B), 1 (C), 2 (D), 3 (E), 4 (F), 5 (G), 6 (H), 7 (I), 8 (J) and 9 days (K) after treatment with 10 µg ml⁻¹ dexamethasone. (L) Non-transformed P31 cell masses. GFP fluorescence was decreased after treatment with dexamethasone, but the same level of GFP fluorescence was observed in the control cell mass (A) and in double-transformed cell masses treated with dexamethasone at 0 day (B) (scale bars = 0.05 mm).

RNA-blot hybridization was used to compare the degradation of gfp mRNAs in different double-transformed cell lines (P13, P17, P29 and P31) and at different times (24, 48 and 72 hours) after treatment with 10 µg ml⁻¹ dexamethasone. Fig. 6 shows that silencing of gfp expression was correlated with a dramatic decrease in gfp mRNA accumulation. We also assayed the double-transformed cell lines for siRNAs, which are a hallmark of PTGS (Hamilton and Baulcombe 1999, Vaucheret et al. 2001). Fractions enriched for small RNAs were hybridized with DNA probes representing the full-length gfp mRNA as indicated in Fig. 7. Fig. 7 shows that siRNAs of approximately 21 (cell lines P13 and P29) and 23 nucleotides (cell lines P17 and P31), fragments of gfp mRNA, accumulated in silent transgenic cells obtained from double-transformed tissues after treatment with 10 µg ml⁻¹ dexamethasone, but not in the double-transformed tissues without treatment with inducer. Together, these results confirm that the RNAs tested induce gene silencing at the post-transcriptional level.

Confocal imaging and quantitative analyses of PTGS

The efficiency of silencing was quantitatively determined from the confocal images taken by 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 and 520 nm in four double-transformed cell lines (P13, P17, P29 and P31) over the 7 day culture period. Fig. 8A–D shows the dynamics of gene silencing measured by green fluorescence intensities in transgenic cell lines P13 (Fig. 8A), P17 (Fig. 8B), P29 (Fig. 8C) and P31 (Fig. 8D) 1–7 days after treatment with 10 µg ml⁻¹ dexamethasone. Similar
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Changes in gene silencing were observed in all four transgenic lines. A higher incidence of silencing was detected 1–5 d after treatment with 1 and 10 mg l⁻¹ dexamethasone, respectively. No silencing was detected before the treatment with inducer. These results confirm that PTGS can be efficiently triggered by the inducible expressed full-length antisense mRNA and that the efficiency of gene silencing can be regulated by concentrations of inducers. Thus, it appears that the inducible PTGS system provides a reliable approach for analyses of gene silencing.

To assess the effectiveness of inducible PTGS in a plant cell system, we monitored gfp expression of four transgenic cell lines over 3 weeks after treatment with inducer. We found that the levels of gene silencing are similar between 7 and 21 d in all four transgenic cell lines (Fig. 9). These results demonstrated that antisense gfp RNA functions in isolated plant cells and specifically interfered with the gfp gene expression.

Discussion

Antisense expression is a frequently applied reverse genetics approach for analyzing the effects of genes of interest on processes such as metabolism, development and defense responses (Smith et al. 1990, Vanithatani et al. 2003). These functional studies would benefit from the availability of a system to induce gene expression at defined developmental stages or under particular conditions. Moreover, such a conditional gene expression system is an essential requirement for studying genes, the constitutive expression of which leads to severe growth defects, sterility or even lethality (Gatz and Lenk 1998, 2004).

Fig. 8 Quantitative analysis of GFP fluorescence silenced by 1 and 10 mg l⁻¹ dexamethasone in double-transgenic cells. GFP fluorescence was expressed as fluorescence intensity (arbitrary units). (A) Quantitative GFP fluorescence was measured from double-transformed cells of lines P13 (A), P17 (B), P29 (C) and P31 (D). No GFP fluorescence was detected in non-transgenic cells. Experiments were repeated three times, and each replicate consisted of 30–50 cells. Values represent the means ± SD.

Fig. 9 Quantitative analysis of GFP fluorescence silenced by dexamethasone 0, 7 and 21 d after treatment. The induction level of dexamethasone was 10 mg l⁻¹. GFP fluorescence was expressed as fluorescence intensity (arbitrary units). GFP fluorescence levels were not decreased in PVt1 transgenic cells (control) after treatment with dexamethasone, but nearly completely silenced fluorescence was obtained in double-transgenic cells at 7 and 21 d after treatment with dexamethasone. Experiments were repeated three times, and each replicate consisted of 30–50 cells. Values represent the means ± SD.
Many examples of PTGS of endogenous or reporter genes have been described in transgenic plants containing sense or antisense transgenes (Vance and Vaucheret 2001, Wesely et al. 2001, Vanithatani et al. 2003). In cases of either co-suppression or antisense suppression, there appears to be induction of a surveillance system within the plant that specifically degrades both the transgene and target RNAs. We use a dexamethasone-inducible transcription factor and a modified gfp reporter gene in our PTGS system. Our results show that transforming cells with reporter gene constructs that produce antisense mRNAs capable of dsRNA formation confers gene silencing on the double-transformed plant cells. This was accomplished by using transcripts from one sense gene and one antisense gene co-located in the plant genome, a single transcript that has sense and antisense transcripts from genes brought together by inducible gene expression.

PTGS of plant genes using antisense or co-suppression constructs usually results in only a modest proportion of silenced individuals (Wesely et al. 2001, Van Houdt et al. 2003, Vanithatani et al. 2003). In this study, an inducible gene construct containing the inducible transcription factor GVG (Aoyama and Chua 1997, Ouwerkerk et al. 2001) and antisense m-gfp5-ER was used, and an efficient silencing was obtained in a wide range of transgenic cell lines. The degree of silencing with this inducible construct was similar among different cell lines. The inducible construct we used should facilitate the cloning of gene libraries or large numbers of defined genes, as those in expressed sequence tag (EST) collections. This system may facilitate the large-scale determination and discovery of plant gene functions. GFP expression in transgenic cells was also monitored using fluorescence microscopy. To quantify the effect of siRNAs on GFP expression, images of transgenic cells expressing GFP were recorded with equal exposure time under non-saturating conditions with further monitoring of green fluorescence. For comparison, at least 120 randomly chosen control and targeted cells were quantified in each sample. The results revealed that silencing of gfp expression was highly controlled. Low molecular weight RNA was isolated 72 h after treatment with inducer. RNA gel blot analysis with a specific probe designed to hybridize with the non-targeted region of gfp revealed the accumulation of siRNAs (Fig. 8). This result indicates synthesis of siRNAs in transgenic cells and demonstrates the spreading along the targeted region as proposed for RNA silencing. Such molecules were not detected in transgenic cells with sense gfp plasmid DNA alone.

It is generally recognized that a silencing-inducing locus can efficiently reduce the expression of genes that give rise to transcripts partially homologous to those produced by the silencing-inducing locus (Wesely et al. 2001, Klahre et al. 2002, Van Houdt et al. 2003). Sequences upstream from the region homologous to the silencing inducer in the primary target transcripts give rise to approximately 22 nucleotide small RNAs, coinciding with the region homologous to the secondary target (Hamilton and Baulcombe 1999, Vaucheret et al. 2001, Klahre et al. 2002). The presence of these small RNAs corresponds with reduced expression of the target. Although it had been established that high molecular weight dsRNAs can trigger silencing confined to bombarded cereal cells (Jeddeloh et al. 1999, Schweizer et al. 2000, Klahre et al. 2002) and systemic silencing in Caenorhabditis elegans (Fire et al. 1998), it had not been shown previously that high molecular weight single-stranded mRNAs produced by inducer can trigger silencing in transgenic pine cell cultures. The present study bears on the mechanisms underlying inducible PTGS. Indirect evidence supports a branched model in which sense, antisense and defined aberrant RNAs feed at the dsRNA step into a common pathway similar to RNAi in animals (Fire et al. 1998, Vaucheret et al. 2001). Several current models hold that the silent state is maintained by a self-sustaining cycle involving RNA intermediates such as dsRNA and siRNA (Wassenegger and Pelissier 1998, Meins 2000, Klahre et al. 2002). We found that dsRNA can trigger formation of siRNAs in silent tissues in transgenic pine cells. Similar conclusions have been drawn from recent studies of silencing induced by virus vectors carrying partial transgenes (Vaistij et al. 2002). Studies with Drosophila embryo extracts (Lipardi et al. 2001) and C. elegans (Sijen et al. 2001) suggest that siRNAs can serve as primers for generating dsRNAs from the target RNA mediated by RdRP, and that new siRNAs are generated in a cycle of dsRNA synthesis and degradation. We speculate that a similar mechanism capable of amplifying siRNA signals operates in transgenic pine cells.

PTGS is important for protecting plants against infection with viruses and has been proposed as a surveillance system for recognizing potentially deleterious foreign nucleic acid sequences (Meins 2000, Vance and Vaucheret 2001). Recent studies of PTGS mutants and viral suppressors of PTGS suggest that PTGS-like mechanisms may also play a role in downregulated plant genes during development (Meins 2000, Vaucheret et al. 2001, Klahre et al. 2002). Although the GVG system and other conditional expression systems have been characterized extensively in dicotyledonous plants (Aoyama and Chua 1997, Ouwerkerk et al. 2001), none of these was tested in gymnosperms, especially in pine. We adapted the GVG system for specific use in Virginia pine and created an inducible antisense transgene expression construct. The system was tested using a constitutive CaMV 35S promoter to drive GVG expression, and the gfp reporter gene for qualitative and quantitative analysis of induction levels of gene silencing. Experiments carried out in the laboratory demonstrated the usefulness of the system for pine PTGS research.

Materials and Methods

Plasmid constructs

After the pINDEX3 binary vector (Ouwerkerk et al. 2001), provided by Dr P. B. F. Ouwerkerk and Dr A. H. Meijer (Institute of
Molecular Plant Sciences, Leiden University, Clusius Laboratory, The Netherlands), was isolated and purified by SNAP MiniPrep Kit (Invitrogen Life Technologies, Carlsbad, CA, USA), the plasmid pINDX3 was digested by Spe1 and Xho1 (Promega Corporation, Madison, WI, USA) at 37°C. Gel bands containing digested plasmid DNA were purified by SNAP Gel Purification Kit (Invitrogen). The m-gfp5-ER fragments (Haseloff et al. 1997, Stewart 2001) were amplified from plasmid pBIN-m-gfp5-ER. The restriction enzymes Spe1 and Xho1 sites were introduced by PCR with Stratagene’s Pfu DNA polymerase (Stratagene, Cedar Creek, TX, USA). For amplification of m-gfp5-ER fragments, the gfp forward primer 5′-aaacatgtagcttaactactgctc-3′ and the reverse primer 5′-cttcattgtatatcaccttgcat-3′ (Sigma Chemical, St Louis, MO, USA) were used. A total of 200 ng of plasmid 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, 250 ng of each primer, 2.5 U of Stratagene’s Pfu DNA polymerase, 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 with a final incubation of 72°C for 10 min. PCR products were cloned into TOPO TA Cloning vector using a TOPO TA Cloning Kit (Invitrogen). Plasmids were purified by SNAP MiniPrep Kit (Invitrogen) and digested by Spe1 and Xho1 (Promega) at 37°C. Gel bands were purified by SNAP Gel Purification Kit. Ligation of the purified insert (30 ng) and vector (90 ng) was conducted in a 20 µl volume with 2.5 U of T4 DNA ligase (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, IN, USA) at 16°C for 16 h. A 1 µl aliquot of ligation products was used to transform 20 µl of NovaBlue competent cells (Novagen, Madison, WI, USA). Overnight cultures from single clones were used to isolate plasmid pINDX3-antisense-m-gfp5-ER with the SNAP MiniPrep Kit. The purified plasmid was introduced into A. tumefaciens EHA105 competent cells by electroporation (Bio-Rad Laboratories, Hercules, CA, USA).

Transformation of cultured cells and glucocorticoid hormone treatments

Cell cultures of Virginia pine were prepared as described in Tang et al. (2001b). Cell cultures were maintained by weekly subculture of a 2.5 ml suspension into 25 ml of liquid culture medium [1× TE Salt Base (Tang et al. 2001a), 30 g 1⁻¹ sucrose, 500 mg 1⁻¹ myo-inositol, 2 mg 1⁻¹ 2,4-D, 0.5 mg 1⁻¹ BA, pH 5.8] and incubated at 25°C, with orbital shaking at 150 rpm, in the dark. Cell cultures were transformed using A. tumefaciens strain GV3850 carrying pBIN-m-gfp5-ER as described in Tang et al. (2001a). Stable transformed plants containing one copy of the pBIN-m-gfp5-ER T-DNA were transformed a second time with A. tumefaciens EHA105 containing the binary vector plasmid pINDX3-antisense-m-gfp5-ER that contains, between the left and right T-DNA borders, a plant hygromycin-selectable marker, the GVG chimeric transcription factor-coding sequence under control of the CaMV 35S promoter, and the antisense-m-gfp5-ER reporter gene. Agrobacterium, grown for 24 h to an optical density (OD₅₆₀ nm = 0.8–1.0) of bacteria in 3 ml of YEPP broth (Sambrook et al. 1989), were centrifuged and re-suspended in liquid medium. Cell suspension cultures were infected with bacteria cultures for 15 min. The infected cell suspension cultures were harvested with 42.5 µm filter papers, transferred to 125 ml Erlenmeyer flasks, and co-cultivation was carried out for 1–2 d. Agrobacterium was removed by washing the cell suspension cultures in 50 ml sterile tubes with 500 mg 1⁻¹ timentin (ticarcillin : clavulanic acid 3 : 0.1, SmithKline Beecham, Philadelphia, PA, USA) solution for 3 min. The wash was repeated five times. Transgenic cell cultures were suspended in 25 ml of liquid medium containing 5 mg 1⁻¹ hygromycin and 500 mg 1⁻¹ timetin and were incubated for 7 d at 25°C, with orbital shaking at 150 rpm in darkness. Cells grown from these cultures were transferred to 25 ml of fresh liquid medium containing 5 mg 1⁻¹ hygromycin. Further subculturing was performed weekly on media containing only hygromycin. To obtain large quantities of transformed cell cultures for further analysis, selected cell suspension cultures were again introduced on a liquid proliferation medium. After 6 weeks, the cultures were actively producing 50–70 mg of tissue in 1 liter of cultures each week, and they were then used to prepare DNA for PCR and Southern blot analysis.

Stable transgenic cell lines confirmed by PCR and Southern blot analysis were used for inducible gene expression experiments. For treatments with demethasone, transgenic cell lines were grown in liquid medium for 3 d then transferred into fresh liquid medium supplemented with demethasone at different concentrations (1 and 10 µg l⁻¹) for 24 h. Cells were washed three times, transferred to fresh liquid medium, and were observed under the fluorescence microscope at different times after transfer. At the same time, cell cultures were harvested for quantitative fluorescent microscopy and for Northern blot analysis. At different times after treatment with demethasone, cell cultures were harvested for quantitative fluorescent microscopy. Glucocorticoids (Sigma Chemical Co., St Louis, MO, USA) were stored as 100 mM solution in dimethylsulfoxide (DMSO) at –20°C. Experiments were replicated at least three times.

PCR of transgenic cultures

Genomic DNA was extracted from 500 mg cell cultures of control and putative transgenic cell lines using an Easy-DNA Kit (Invitrogen) following the manufacturer’s protocol. The PCR products were used with a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., San Francisco, CA, USA) using DNA extracted from putative transformed and control cell cultures. The primers used for PCR were the gfp forward primer (gfpF) 5′-aaacatgtagcttaactactgctc-3′ and the reverse primer (gfpR) 5′-cttcattgtatatcaccttgcat-3′, the hpt forward primer (hptF) 5′-cttcagctgtatggggtc-3′ and the reverse primer (hptR) 5′-aagaaagatggtggccagc-3′, and the npt forward primer (npF) 5′-aaacacacactgctgctgctc-3′ and the reverse primer (npr) 5′-aagacacagcagagggc-3′. A total of 200 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 4 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 with 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, Gaithersburg, MD, USA) was used.

RNA isolation and Northern blot analysis

Total RNA was isolated from 1.5 g of transgenic cell cultures harvested through 42.5 µm filter papers and ground in liquid nitrogen using a Micro-tmidi total RNA Purification System (Invitrogen) following the manufacturer’s protocol. A 10 µg aliquot of RNA was separated by agarose gel electrophoresis. Electrophoresis and Northern blotting of RNAs were performed as described by Tang and Tian (2003). Baked blots were pre-hybridized in 1 M NaCl, 1% SDS, 10% dextran sulfate and 50 mg ml⁻¹ denatured herring sperm DNA at 64°C, washed with 0.1× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 6.5), 0.5% SDS at 45°C, and autoradiographed. DIG-oxigenin (DIG)-labeled m-gfp5-ER DNA (816 bp) (Roche Diagnos-
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**References**


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