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Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging

OVE NILSSON[1,2], TORSTEN ALDÉN[1], FOLKE SITBON[1], C.H. ANTHONY LITTLE[1,3], VLADIMIR CHALUPA[4], GÖRAN SANDBERG[1] and OLOF OLSSON[1]*

[1]*Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden*
[2]*Department of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden*
[3]*Forestry Canada-Maritimes Region, P.O. Box 4000, Fredericton, New Brunswick, E3B 5P7, Canada*
[4]*Forestry and Game Management Research Institute, Praha 5, Zbraslav-Strnady, Czechoslovakia*

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A protocol has been developed for efficiently transforming and regenerating the hybrid aspen *Populus tremula* × *P. tremuloides*. Stem segments were co-cultivated with a strain of *Agrobacterium tumefaciens* carrying a disarmed binary vector conferring resistance to kanamycin or hygromycin. The respective vectors also carried a fused bacterial *luxF2* gene expressed from the cauliflower mosaic virus 35S promoter. All transformants had a normal phenotype. Genetic transformation and stable integration of the heterologous DNA was confirmed by Southern hybridization and luciferase expression. The latter was measured by destructive enzymatic assay throughout the transformant and by non-destructive image analysis in leaves left attached to intact plants. Both measurement techniques detected marked within- and between-organ variation in luciferase expression. However, the spatial patterns detected by each technique in the leaves were similar. The results indicate that *in vivo* imaging of light emission can be used to measure repeatedly the expression of a promoter–luciferase gene fusion in a particular leaf over an extended time period. It was also demonstrated that enzymatically assayed luciferase activity in leaves was notably lower in transgenic hybrid aspen plants than in tobacco plants transformed with the same vector. This was not due to a difference in luciferase enzyme activity between the two species, and therefore indicated that the 35S promoter is not as active in hybrid aspen as in tobacco.

Keywords: plant transformation; *Populus tremula* × *P. tremuloides*; bacterial luciferase; non-destructive assay; image analysis

Introduction

Although classical breeding techniques have been used to genetically improve many species of plants, they are inefficient for forest tree species because of their long generation period. Therefore, the improvement of forest trees might be accelerated significantly by genetic engineering (Ahuja, 1988; Cheliak and Rogers, 1990). Currently, a major problem associated with gene transfer into any heterologous system is regulating where and when the gene is expressed. To date, the regulatory elements for octopine synthase, mannopine synthase, 35S RNA of the cauliflower mosaic virus (CaMV), wound-inducible potato proteinase inhibitor, and the small subunit of ribulose bisphosphate carboxylase (RUBISCO) genes, have been shown to be functional in transformants of some deciduous tree species (Fillatti *et al.*, 1987; McGranahan *et al.*, 1988; James *et al.*, 1989; De Block, 1990; Klopfenstein *et al.*, 1991). However, the spatial and temporal expression of these heterologous promoters has been investigated only in plant species that live less than one year (Schell, 1987; Benfey and Chua, 1989).

Gene expression in perennial species, such as trees, is regulated not only within the year, during the annual cycle

*To whom correspondence should be addressed.

of activity and dormancy, but also between years, as the tree passes from the juvenile to the mature phase and, eventually, to a senescent condition (Kramer and Kozlowski, 1979). Consequently, to elucidate this regulation it will be necessary to characterize the activity of heterologous promoters in trees over the entire year, as well as to isolate novel, endogenous promoters whose activity varies seasonally and with increasing tree age. The non-destructive, luciferase reporter gene system (Ow *et al.*, 1986; Langridge *et al.*, 1989; Koncz *et al.*, 1990; Olsson *et al.*, 1990; Schneider *et al.*, 1990) would be an ideal tool for monitoring promoter activity over such an extended time period. To this end, we have refined the system based on the *luxAB* (*LuxF2*) fusion gene (Olsson *et al.*, 1989) encoding the light-emitting bacterial luciferase enzyme from *Vibrio harveyi.* This gene was fused behind the strong CaMV 35S promoter, which has been used to express chimaeric genes in a variety of monocotyledonous and dicotyledonous species (Fromm *et al.*, 1985; Odell *et al.*, 1985; Ou-lee *et al.*, 1986; Jefferson *et al.*, 1987; Lin *et al.*, 1987; Williamson *et al.*, 1989).

The 35S promoter–*luxF2* construct was used to transform a hybrid aspen, since *Populus* spp. not only can be transformed and regenerated (Parsons *et al.*, 1986; Ahuja, 1987; Fillatti *et al.*, 1987; Pythoud *et al.*, 1987; De Block, 1990; Klopfenstein *et al.*, 1991), but also have a small genome size, which facilitates genetic analysis, rescue cloning, mutagenesis and genomic library construction. In addition, many *Populus spp.* are important to the forest industry, being used to make paper, hardboard, plywood and packing material, and to produce biomass for energy conservation.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5α was used as the recipient for plasmid amplification. *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) was used as the receptor for the binary vectors and as the T-DNA donor. The hybrid *luxAB* gene, denoted *luxF2*, from plasmid pLX702-fab (Olsson *et al.*, 1989) was fused to the CaMV 35S RNA promoter in plasmids pPCV702.kana and pPCV702.hygro to create plasmids pPCV35SluxF2.kana and pPCV35SluxF2.hygro, respectively (Fig. 1), and as described previously (Olsson *et al.*, 1989).

Cloning methods

The preparation of the bacterial culture media and the procedures for purifying plasmids, isolating DNA fragments and using restriction endonucleases and other DNA enzymes were as recommended by the manufacturers, or as described (Olsson *et al.*, 1988; Sambrook *et al.*, 1989).

Introduction of DNA into Agrobacterium *by electroporation*

Fresh cultures of *A. tumefaciens* cells were grown in yeast extract-beef (YEB) medium (0.1% yeast extract, 0.5% beef extract, 0.1% peptone, 0.5% sucrose and 2 mM $MgSO_4$) to an (optical density) OD_{595} of 0.5, washed three times in distilled water, and resuspended to about 10^9 cells ml^{-1} in 10% glycerol. Cells were stored at −70° C. A 50 µl aliquot was mixed with 50 ng of plasmid DNA. A single pulse (gene pulser, BioRad, Richmond, CA, USA)

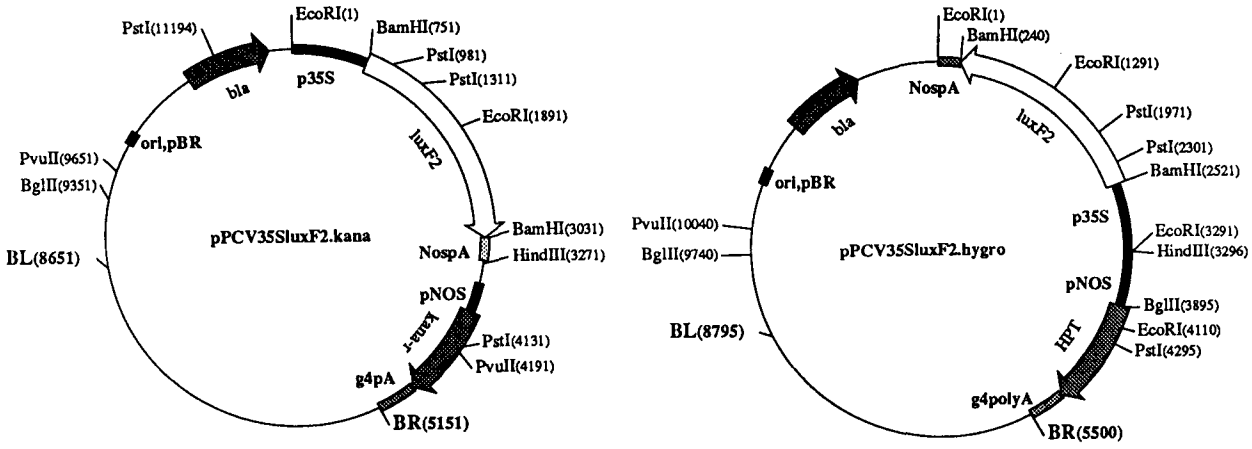


Fig. 1. Schematic maps of the binary vectors used in the study. *luxF2*, fused luciferase *luxA* and *luxB* gene; kana-r, gene coding for neomycin in phosphotransferase; p35S, promoter from the cauliflower mosaic virus; pNOS, promoter from the nopaline synthase gene; *HPT*, gene coding for hygromycin phospho transferase; *bla*, gene coding for TEM-1 β-lactamase; BR, right T-DNA border; BL, left T-DNA border; g4pA, DNA sequence including the poly-adenylation site from gene 4; NospA, DNA sequence including the poly-adenylation site from the nopaline synthase gene. Plasmid sizes are 11800 bp for the kana-vector, and 12750 bp for the hygro-vector.

was delivered to the mixture at 2 kV, 25 μF and 200 ohms. The electroporated cells were immediately transferred to recovery medium (YEB supplemented with 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$). After incubation at 28° C for 2 h, transformed cells were selected on solid YEB medium containing 100 μg ml^{-1} rifampicin, 100 μg ml^{-1} carbenicillin and 25 μg ml^{-1} kanamycin. Selected cells were spotted on a lawn of *Eschericha coli* strain DH5α on LB-plates containing 100 μg ml^{-1} ampicillin. The plates were incubated at 28° C for 6 h, then at 37° C over-night. Plasmids were isolated (Birnboin and Doly, 1979) from *E. coli* colonies that grew on the selective medium, and physically mapped by restriction endonuclease analysis.

In vitro *culture of hybrid aspen*

To obtain the starting material for sterile culture, root segments of four clones of the hybrid *P. tremula* × *P. tremuloides* were buried in moist peat, and sprouts were induced under greenhouse conditions (natural photoperiod extended to 18 h as required by metal halogen lamps giving a photon flux density of 150 μmol m^{-2} s^{-1}, a day/night temperature of 23° C/16° C, and a relative humidity of at least 50%). Shoots 100 to 200 mm-tall were surface-sterilized for 10 min in 0.1% $HgCl_2$ and rinsed three times in sterilized water. Segments 15 mm-long were excised from the stem, avoiding the nodes where possible, and placed on solid medium (Murashige and Skoog, 1962), hereafter refered to as MS1, which contained 0.1 μg ml^{-1} indole-3-butyric acid (IBA), 0.2 μg ml^{-1} 6-benzylaminopurine (BAP) and 0.001 μg ml^{-1} thidiazuron (TDZ; N-phenyl-N-1,2,3-thidiazol urea) to initiate shoots. The cultures were grown in a controlled environment room having a temperature of 25° C, a photoperiod of 16 h, and a light intensity of 40 μE m^{-2} s^{-1} from cool white fluorescent lamps. When the shoots were about 5 mm long, the cultures were transferred to MS2 medium (MS1 medium minus TDZ) to promote shoot elongation. After their length exceeded 6 cm, the shoots either were used for transformation (see below) or were induced to root by placing them on MS3 medium (half-strength MS medium without hormones). Rooted shoots were potted in peat, covered with a plastic bag, and placed in the greenhouse. The bag was ventilated one week later and removed after a second week.

Transformation of hybrid aspen and tobacco

The *A. tumefaciens* cells were grown in YEB medium supplemented with 50 μg ml^{-1} carbenicillin and 25 μg ml^{-1} kanamycin at 25° C for about 24 h. When OD_{595} reached 0.2–0.6, the cultures were centrifuged for 10 min at 3000 rpm, resuspended in MS medium containing 20 μM acetosyringone and grown at 28° C for 1 h on a gentle reciprocating shaker. Acetosyringone was applied to increase the efficacy of gene transfer between the *Agrobacterium* and the plant cell (Stachel and Zambryski, 1986; Stachel *et al.*, 1986a,b).

Stem segments of hybrid aspen were co-cultivated with *A. tumefaciens* cells in liquid MS medium for 0.5–2 h, then transferred to MS1 medium. After incubation for 48 h in the dark, the segments were washed twice in sterile 500 μg ml^{-1} cefotaxime and placed in the light on MS1 medium supplemented with 250 μg ml^{-1} cefotaxime and either 60 μg ml^{-1} kanamycin or 15 μg ml^{-1} hygromycin, depending on the vector used for gene transfer. After shoot initiation, the segments were transferred to MS2 medium, containing the same antibiotics, to promote elongation. Roots were initiated by transferring the cultures to MS3 medium. Rooted shoots were transferred to the greenhouse, and luciferase activity was measured when the plants were 0.5 to 3 m tall.

Tobacco (*Nicotiana tabacum* line W38) was transformed using the leaf disc method (Horsch *et al.*, 1985). Transformants were selected on solid K3 medium (Nagy and Maliga, 1976) containing 0.2 μg ml^{-1} kinetin, 0.6 μg ml^{-1} NAA, 500 μg ml^{-1} cefotaxime and either 100 μg ml^{-1} kanamycin or 40 μg ml^{-1} hygromycin. Greenhouse-grown plants were assayed at the stage of flower bud formation.

DNA isolation and Southern blot analysis

DNA was extracted from leaves of transformed hybrid aspen plants, according to a modified CTAB procedure (Doyle and Doyle, 1990). *Eco* RI was used to digest 10 μg DNA, electrophoresed on an 0.7% agarose gel and blotted onto a nylon filter (Hybond-N, Amersham, Solna, Sweden). Filters were hybridized at 65° C with an oligo-primed ^{32}P-labelled 2251 bp *Bam HI luxF2* fragment (10^7cpm ml^{-1} hybridization solution), washed with 0.1 × standard saline citrate buffer and 0.1% sodium dodecyl sulphate at 65° C, and exposed to X-ray film (Hyperfilm^{tm}, Amersham) for three days (Sambrook *et al.*, 1989).

Enzymatic assay of luciferase activity

Leaf, stem and root samples were obtained as detailed in Results. Only healthy leaves were selected. Leaf number was counted basipetally from the shoot apex, number one being at least 1 cm long. The samples were ground in 500 μl of either lux-buffer (Koncz *et al.*, 1987; 50 mM Na–phosphate, pH 7, 50 mM β-mercaptoethanol) or modified lux buffer (50 mM Na–phosphate, pH 7, 4% (w/w) soluble PVP (MW 360000), 2 mM EDTA, 20 mM DTT). After 5 min of centrifugation, 200 μl of the supernatant were mixed with 300 μl lux buffer containing 2% BSA, and the mixture placed in the luminometer at room temperature. The luciferase reaction was started by adding 500 μl of light-reduced flavin monouncleotide in 200 mM tricine buffer, pH 7 and 20 μl of 0.1% (v/v) *n*-decanal sonicated in water. The height of the light peak produced during the first 10 s of the reaction was used as

the measure of luciferase activity, which was expressed as mV mg^{-1} total soluble protein in the supernatant. Protein was measured as described by Bradford (1976).

In vivo *image analysis of luciferase activity*

Luciferase activity was visualized in attached leaves 30–120 cm-tall transformed hybrid aspen plants, using a photon-counting video camera (CCD 3200, Astromed, Cambridge, UK). This camera is equipped with a P8303 Charge Coupled Device with 578 × 385 picture elements (pixels) installed in a liquid nitrogen cooler detector head giving a typical operating temperature of −140° C. The lower side of the leaf was laid upon moist black cloth in a petri-dish positioned to hold the leaf at its natural orientation in space. A total of 50 µl *n*-decanal (10% (v/v) sonicated solution in water, unless otherwise stated) was applied as small drops to the inside of the petridish, and the lid replaced. Following an initial photograph in the light, the photon emission was recorded for 30 min in absolute darkness. By means of an IBM PC/AT compatible host computer controlled by the UNIX operating system with the Astromed command language installed, a digitized pseudocolour image corresponding to the luciferase-catalyzed light emission pattern of the exposed leaf was constructed. The baseline of the colour calibration scale was set to eliminate photons of non-biological origin and inherent leaf bioluminescence.

Results

Establishment of hybrid aspen tissue cultures

Four independent clones of *P. tremula* × *P. tremuloides* were selected in the field, and the capacity for *in vitro* regeneration of root, leaf and stem segments compared. The stem was most responsive, hence only this organ was used in subsequent work. In the untransformed state, all four clones regenerated equally well. Shoots typically developed at both ends of the stem segment and, occasionally, also from a nodal bud if one was present. Adding thidiazuron (TDZ; N-phenyl-N-1,2,3-thidiazol urea) to the medium increased the frequency of shoot initiation from 10% to 90%. However, TDZ inhibited subsequent shoot elongation. Thus, segments with shoots were transferred from MS1 medium to MS2 medium when the shoots became visible. Recycling material several times through this part of the regeneration cycle increased both the rate of shoot production and the number of shoots produced. Practically all of the shoots rooted readily after placement on MS3 medium. Similarly, at least 95% of the rooted shoots survived the transfer from *in vitro* culture to the greenhouse, provided a high relative humidity was maintained around the plants for the first few days. The interval between placing the segment on shoot initiation medium and transferring the rooted shoot into the greenhouse was about 3 months.

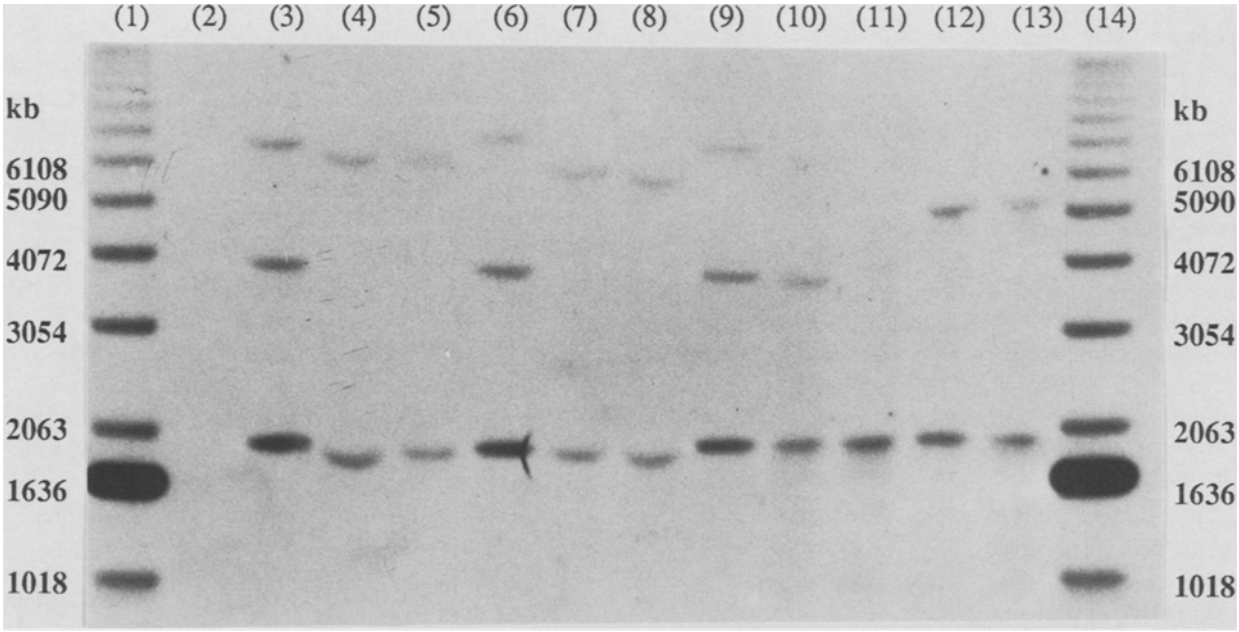


Fig. 2. Southern blot analysis of *P. tremula* × *P. tremuloides* genomic DNA. Chromosomal DNA from different plants was cut with *Eco* RI, and hybridized to a *lux* probe prepared from pPCV35S*luxF2*.kana as a 2280 *Bam* HI fragment (Fig. 1). (1) kb ladder, (2) untransformed control, (3) S1, (4) S3, (5) S4, (6) S5, (7) S7, (8) S8, (9) S9, (10) S23, (11) S28, (12) S29, (13) S32, (14) kb ladder.

Transformation of hybrid aspen and regeneration of transformants

The transformation frequency of the four clones varied from 25% to 75%. The most responsive clone was used in all subsequent experiments. Similar between-genotype variation in *A. tumefaciens*-mediated transformation was also observed in experiments with *Salix* spp. (Vahala *et al.*, 1989) and other *Populus* hybrids (De Block, 1990). Transformation and subsequent selection on antibiotic-containing media did not affect the rate or frequency of shoot initiation. However, it did delay the initiation of roots, and decreased the percentage of roots produced from 95% to 40%. Both vector constructs used in this investigation (Fig. 1) similarly affected the frequency of transformation and regeneration, and kanamycin (for pPCV35SluxF2.kana) and hygromycin (for pPCV35SluxF2.hygro) were equally effective as selective agents. Dose–response experiments showed that the development of untransformed shoots was completely inhibited at 60 μg ml^{-1} kanamycin and 15 μg ml^{-1} hygromycin; hence these concentrations were used routinely.

Southern analysis of hybrid aspen transformants

Southern analysis of nuclear DNA from 11 kanamycin-resistant transformants showed that the *luxF2* gene was present and physically intact, as an *Eco* RI fragment having the expected size (1891 bp) (Fig. 1a) hybridized to the *luxF2* probe (Fig. 2). Because the probe also covered the right border sequence of the binary vector, additional fragments larger than 3260 bp were detected. Their number indicated the presence of one or two copies of the T-DNA in eight and three transformants, respectively (Fig. 2).

Enzymatic assay of luciferase activity in hybrid aspen and tobacco transformants

Purified luciferase added to hybrid aspen root and leaf extracts obtained with the lux buffer, was rapidly inactivated. Most likely, hybrid aspen cells contain quinones and phenolic compounds that inactivate the luciferase in this buffer. Therefore, an alternative extraction buffer was developed (denoted the modified *lux* buffer) in which luciferase activity was stable in the root and leaf extracts even after prolonged incubation. This buffer, which may stabilize the activity of any enzyme from *Populus* spp. tissues, was used in all our subsequent measurements of luciferase activity.

To investigate the spatial and short-term temporal expression of the 35S promoter in a woody plant species, three series of luciferase assays were performed with the hybrid aspen transformants. In the first, luciferase expression was monitored throughout the plant. Activity was detected in all surveyed organs and tissues, the level of expression generally following the order: young leaves

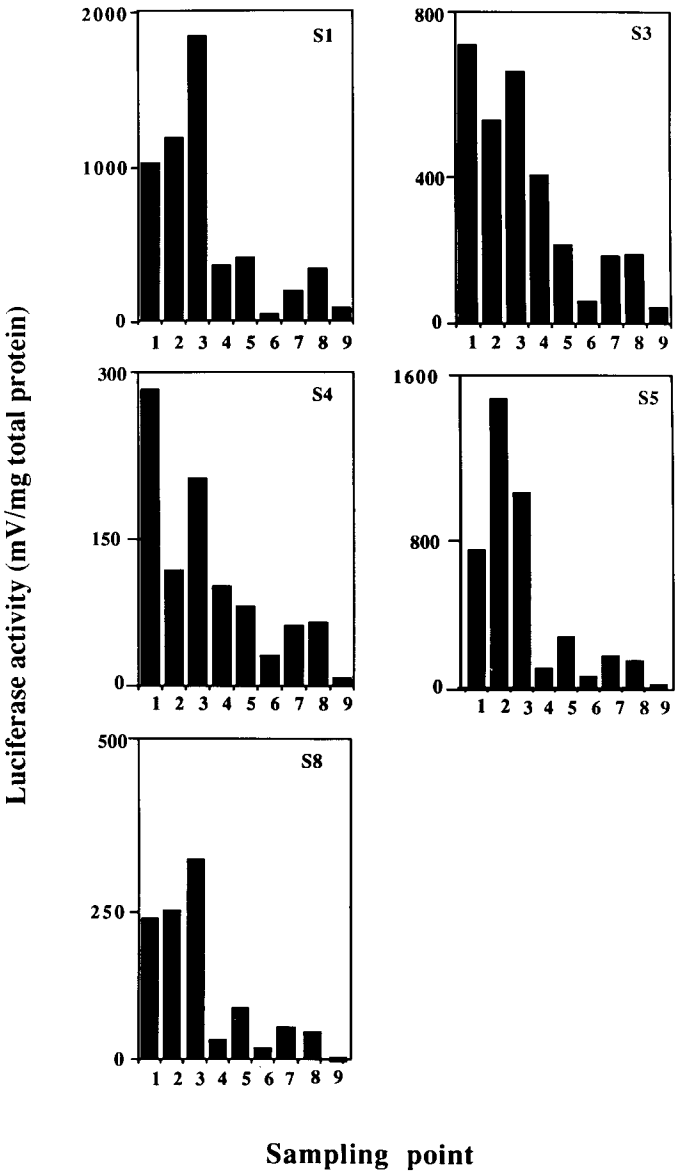


Fig. 3. Enzymatic assay of luciferase activity in different organs and tissues of individual hybrid aspen plants expressing a 35S promoter-*luxF2* gene fusion. Sampling point 1 to 5 = apical 20 mm of leaves of increasing age (1 and 2 were expanding and from nodes 6–10 and 8–13, respectively; 3 was almost fully expanded and from nodes 11–17; 4 and 5 were fully expanded and from nodes 20–34 and 30–46, respectively); 6 = apical 2 cm of large, elongating roots; 7 = differentiating xylem (scraped with a scalpel from the wood side of a stem segment, exposed by peeling the extraxylary tissues); 8 = cambium + phloem (scraped from the exposed side of the extraxylary peeling); 9 = cortex + epidermis (remainder of the extraxylary peeling). Note that the luciferase activity scale is different for each plant.

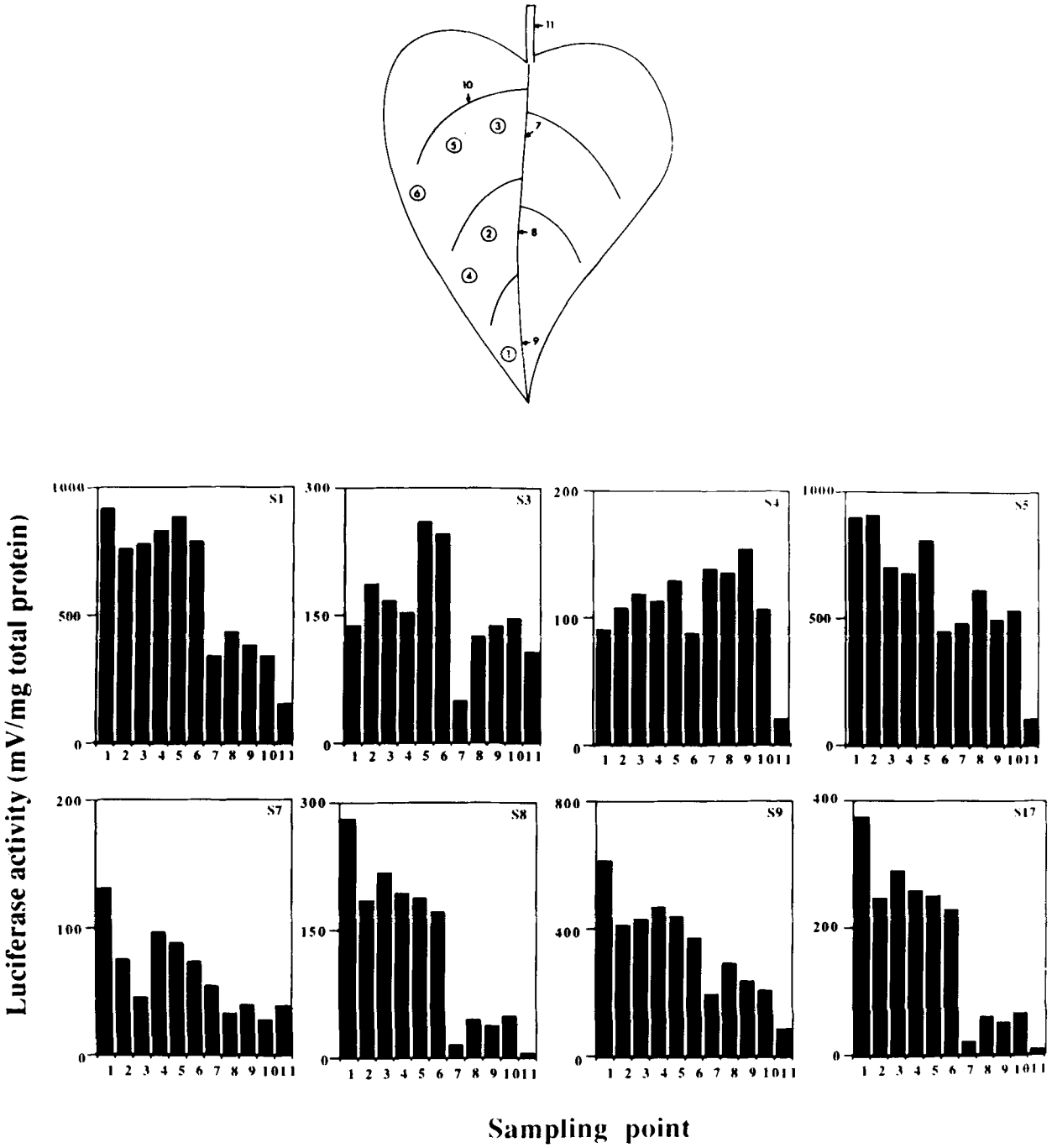


Fig. 4. Enzymatic assay of luciferase activity in a selected leaf borne on individual hybrid aspen plants expressing the 35S promoter-*luxF2* gene fusion. The sample from points 1 to 6 was obtained using a 7 mm-diameter corkborer, avoiding all major veins. Segments of the midvein (points 7–9) and a major secondary vein (point 10), and the petiole (point 11), were isolated with a scalpel. The selected leaf on each plant was fully expanded, 75 to 120 mm long, and located at nodes 8 to 17. Note the different scales of luciferase activity.

> old leaves ≥ cambium + phloem ≥ differentiating xylem > root tips > stem cortex + epidermis (Fig. 3). However, the actual between- and within-organ pattern of expression varied markedly among the plants. Moreover, there was up to 20-fold between-plant variation in the level of luciferase activity at any one sampling point, the highest levels being observed in plants S1 and S5.

The second series of assays measured the extent to which the pattern of luciferase activity in a particular organ varied between plants. A leaf of similar size and age was selected from each of eight transformants, and each leaf was assayed at 11 locations. Luciferase activity in all plants except S3, S4 and S7 was lowest in the vascular tissues, particularly in the petiole, and highest at the leaf

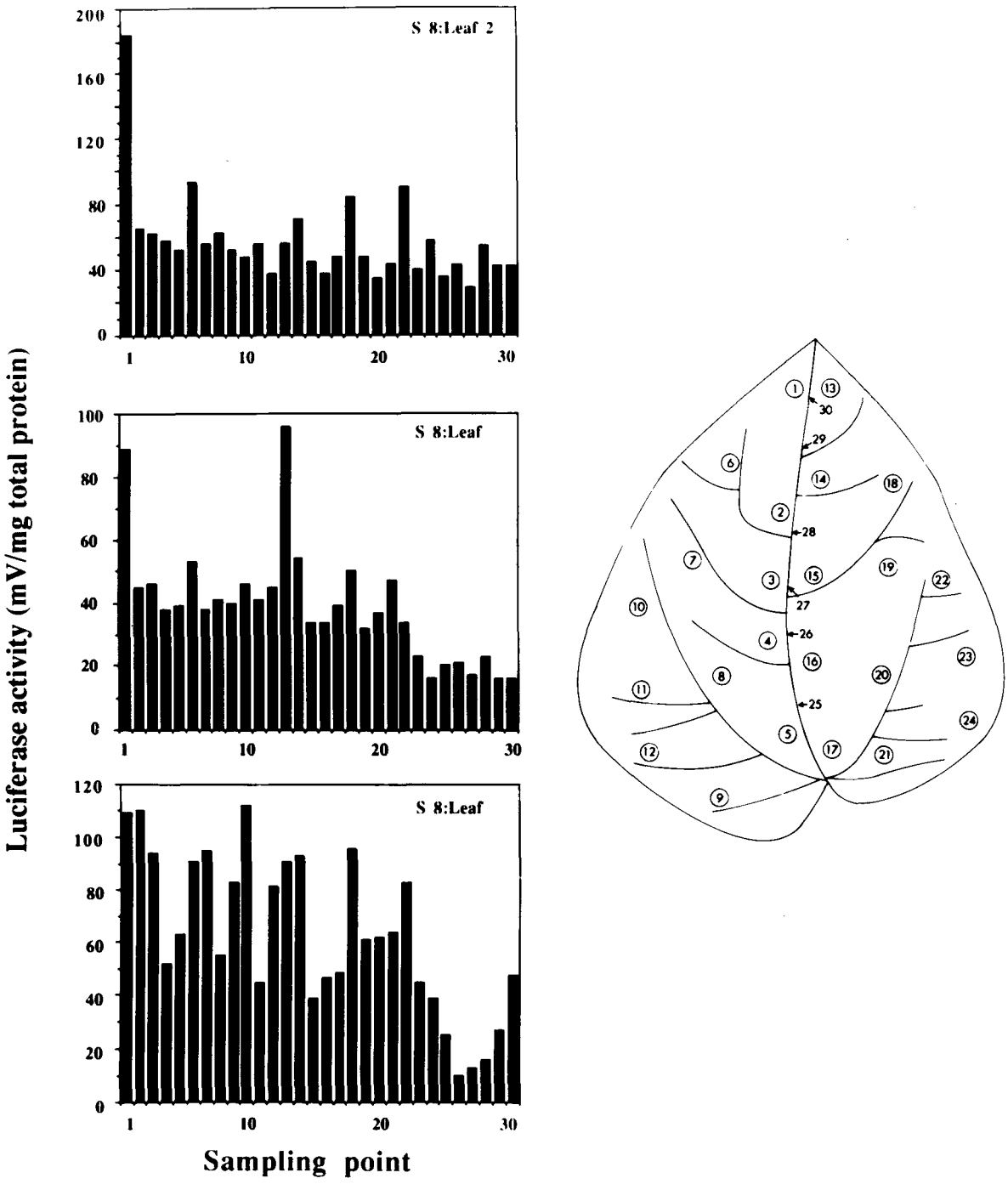


Fig. 5. Enzymatic assay of luciferase activity in three similar leaves borne on hybrid aspen plant S8, which expressed the 35S promoter-*luxF2* gene fusion. The sample from points 1 to 24 (see drawing) was obtained using a 7 mm-diameter corkborer, avoiding all major veins. The midvein was isolated and divided into six equal lengths (points 25–30). The leaves were fully expanded, about 160 mm long, and located at nodes 25, 26 and 27. Note that the scale of luciferase activity is different for each leaf.

apex (Fig. 4). The specific spatial pattern in each leaf, however, was different. The overall level of luciferase activity varied several-fold between plants, being highest in S1, S5 and S9.

The third series of assays investigated the extent of variation in luciferase activity between visually similar leaves borne on the same plant. Three large, relatively old leaves of similar size and age were measured at 30 different points. Luciferase expression was generally lower in the vascular tissues than elsewhere in the leaf and greatest towards the margin near the apex (Fig. 5). Nevertheless, even in these leaves there was notable

variation in the actual spatial pattern and absolute amount of expression.

The average luciferase activity in all hybrid aspen leaves measured was 630 mV mg^{-1} total protein (range 24 to 1833; n=11). This average was 12 times lower than the value calculated for leaves of tobacco plants expressing the same 35S promoter-*luxF2* gene fusion (average 7514 mV mg^{-1}; range 282 to 20500; n=25).

In vivo *imaging of luciferase activity in attached leaves of hybrid aspen transformants*

The photon-trapping coupled charge devise (CCD) chip in our imaging equipment is cooled in liquid N_2. Therefore, no random photons are generated from dark current during the exposure period. However, a constant, inherent current in the chip gives a background value of ca 10 photons per pixel, which corresponds to about 850 on the colour scale shown in Fig. 6. Thus, a baseline setting above 850 was initially used to eliminate photons of non-biological origin.

To minimize any possible effect of environmentally induced stress on light emission, measurements were performed on plants that received adequate water, fertilizer, temperature and light in the greenhouse. A plant was transferred to the imager only during the day and immediately before the measurement. In wildtype plants, and in transformed plants prior to decanal addition, a very weak light emission above the initial baseline setting of 850 was routinely detected at the end of the 30 min exposure period. This light was evenly distributed over the leaf surface (Fig. 6b). To eliminate this natural bioluminescence, the initial baseline setting was raised to 890, and all subsequent measurements were performed using this setting. In dose–response experiments involving decanal, a volatile substrate, the intensity of emitted light was observed to increase with increasing concentration, up to 10% (Fig. 6c–e). Higher concentrations did not increase the light emission, and concentrations approaching 100% caused necrosis.

When leaves of increasing age were imaged in the same transformant, light emission was higher in young leaves than in old leaves, with the third leaf below the shoot apex emitting the most light (Fig. 7). The intensity of emitted light varied within the leaf as well, being greatest at the apex in leaf number 5 and in the major veins of leaf number 7.

To investigate the extent of within-leaf variation between plants, an equivalent leaf borne on eight different plants was imaged. Luciferase expression was greatest in the leaf from plants S1, S5 and S9, higher in the mid-vein than anywhere else in the leaf from plants S3, S4 and S7, and varied inconsistently across the lamina in all plants (Fig. 8).

Finally, we investigated if stressing a transformed leaf immediately prior to imaging affected the light emission pattern. It was observed that light emission did not occur from the part of the leaf that was either punctured with a needle (Fig. 9b) or crushed with forceps (data not shown). In contrast, gentle rubbing of the same, punctured leaf increased the intensity of emitted light (Fig. 9c), although the same treatment did not stimulate light emission from an untransformed leaf (data not shown). Exposing a transformed leaf to an extended dark period decreased the amount of emitted light (Fig. 9d–f).

Discussion

This report describe an *in vitro* protocol for transforming and regenerating a forest tree species, hybrid aspen *P. tremula* × *P. tremuloides*, at high frequency. The *A. tumefaciens* strain used for transformation carried a disarmed binary vector that contained an antibiotic resistance gene and a 35S promoter-*luxF2* gene fusion. It was demonstrated that both kanamycin and hygromycin could be used as the selectable marker, and that adding TDZ to the shoot initiation medium markedly increased shoot production. The efficacy of this compound for inducing shoot formation has also been demonstrated in experiments with other tree species (Kerns and Meyer, 1986; Nieuwkirk *et al.*, 1986; Chalupa and Aldén, 1988;

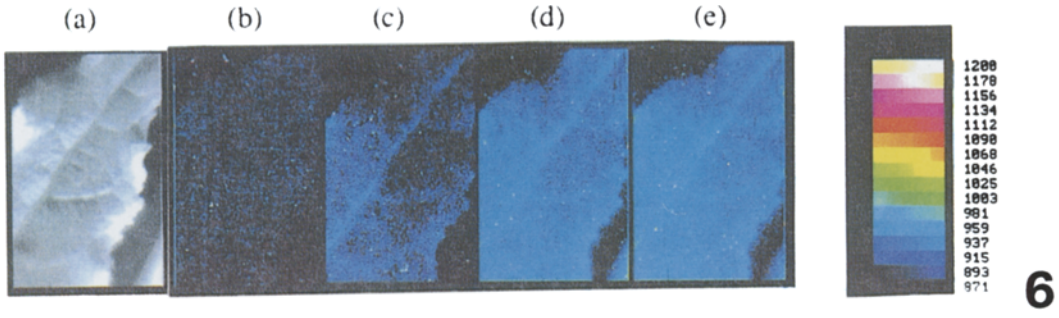
Figs 6–9. *In vivo* visualization of luciferase activity in attached leaves borne on hybrid aspen plants expressing a 35S promoter-*luxF2* gene fusion. The colour calibration bar at the right of each figure indicates the relative number of photons counted by the imaging system during the 30-minute exposure period.

Fig. 6. (a) Photograph in the light of leaf 5 on transformant S7; (b)–(e) light emission image in the dark of the same leaf in the presence of 0, 1, 5 and 10% decanal, respectively.

Fig. 7. (a)–(d) Photograph in the light of the shoot apex + leaves 1 and 2, and of leaves 3, 5 and 7, respectively, on transformant S5; (e)–(h) light emission image in the dark of (a)–(d). The length of leaves 3, 5 and 7 was 60, 70 and 120 mm, respectively.

Fig. 8. (a)–(h) Light emission image in the dark of leaf 5 on transformant S1, S3, S4, S5, S7, S8, S9 and S17, respectively. Each leaf was fully expanded and about 70 mm long.

Fig. 9. (a), (d) Photograph in the light of leaf 7 (about 90 mm long) on transformant S4 and S5, respectively; (b) light emission image of (a) immediately after it had been repeatedly punctured towards the margin with a needle; (c) image of the punctured leaf shown in (b) after it had been rubbed gently between the fingers; (e) image of (d) directly after transfer from the greenhouse; (f) image of the same leaf as in (e) after it had been enclosed in aluminium foil for 48 h.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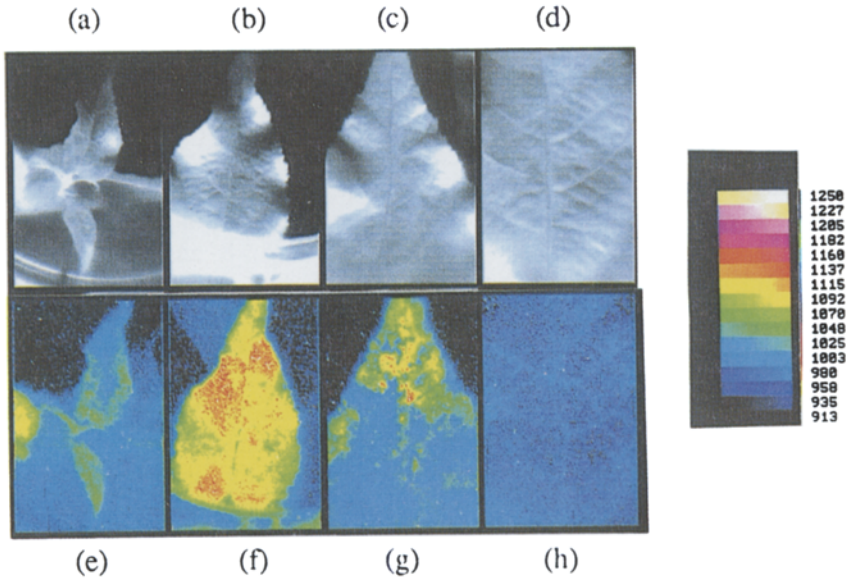
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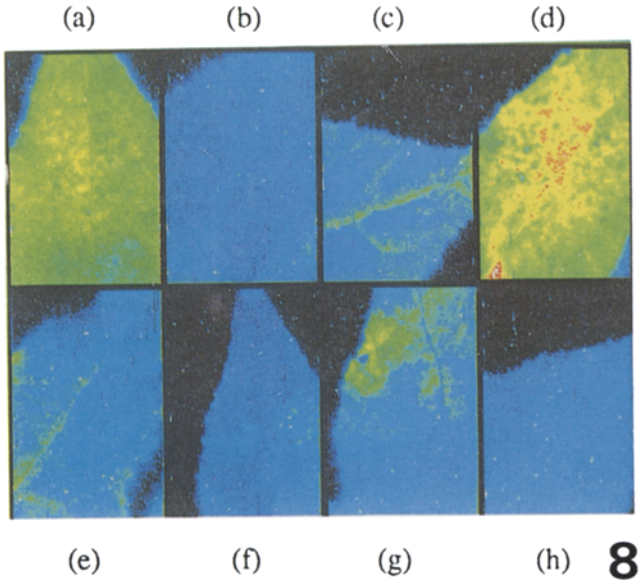
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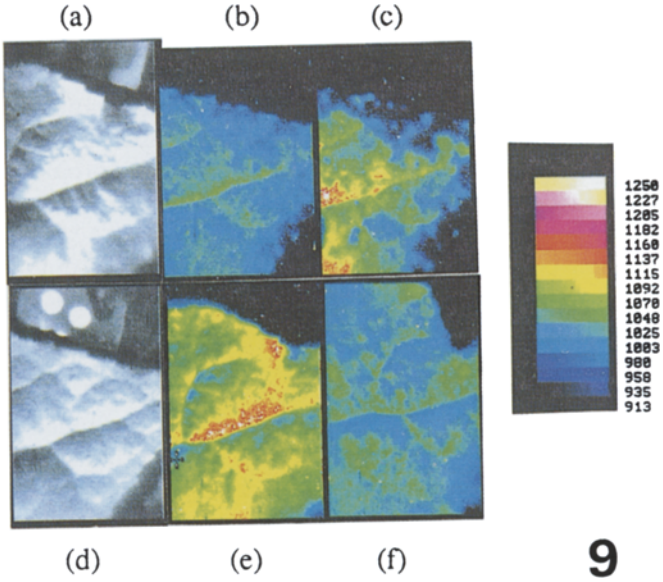
(a)
(b)
(c)
(d)
(e)
(f)
1250
1227
1205
1182
1160
1137
1115
1092
1070
1048
1025
1003
980
958
935
913

9

Russel and McCown, 1988; De Block, 1990; Fiola *et al.*, 1990). Stable integration of the T-DNA in the plant genome was confirmed by Southern hybridization (Fig. 2) and by expression of the luciferase gene (Figs 3–9). All transformants had a normal phenotype.

Although *A. tumefaciens*-mediated gene transfer has been demonstrated for a wide variety of tree species (Parsons *et al.*, 1986; Sederoff *et al.*, 1986; Dandekar *et al.*, 1987; Fillatti *et al.*, 1987; Pythoud, 1987; Ahuja, 1988; Choi *et al.*, 1988; Mackay *et al.*, 1988; McGranahan *et al.*, 1988, 1990; Pang and Sanford, 1988; Ellis *et al.*, 1989; James *et al.*, 1989; Vahala *et al.*, 1989; De Block, 1990; Hood *et al.*, 1990; Loopstra *et al.*, 1990; Stomp *et al.*, 1990), to date transgenic, intact plants have been recovered only for species of *Populus* (Fillatti *et al.*, 1987; De Block, 1990; Klopfenstein *et al.*, 1991), *Juglans* (McGranahan *et al.*, 1990) and *Malus* (James *et al.*, 1989). The development of efficient transformation-regeneration protocols for forest tree species, combined with the use of disarmed binary vectors will be crucial for the practical exploitation of genetic engineering in forestry.

Both enzymatic assay and non-destructive imaging indicated that luciferase activity varied between and within organs borne on the same transformant, as well as between equivalent organs borne on different transformants. In general, luciferase was more active in leaves than in stem or roots (Fig. 3), in young leaves than in old leaves (Figs 3 and 7), in the cambial region than in cortex + epidermis of stems (Fig. 3), in leaf parts containing only minor veins than in the major veins (Figs 4, 5, 7 and 8), and at the apex than in the remainder of expanded leaves (Figs 4, 5 and 7), although some notable exceptions were detected in plants S3, S4 and S7. Spatial and temporal variation has also been observed in tobacco plants transformed with other reporter genes fused to the 35S promoter (Jefferson *et al.*, 1987; Williamson *et al.*, 1989; Schneider *et al.*, 1990), indicating that the activity of this promoter is not expressed equally either in all cells or over time. This notion is further supported by the finding that luciferase expression in a particular leaf could be decreased by etiolation (Fig. 9). Thus, 35S promoter–luciferase expression crudely follows the metabolic status of the cell, with a higher luciferase activity in more rapidly dividing cells, although an exception to this is the root tips, where we found low luciferase activity. The molecular mechanisms of a possible 'metabolic gene regulation' are unknown.

It is difficult to compare directly the spatial pattern deduced by enzymatic assay with that determined by histochemical analysis (e.g. *E. coli* β-glucuronidase) or imaging (bacterial and firefly luciferase). The distribution deduced from enzymatic measurements varies with the basis used to express the activity (Jefferson *et al.*, 1987; Schneider *et al.*, 1990, O. Olsson, unpublished results). The pattern revealed by histochemical analysis can be affected by such factors as method of fixation, localized occurrence of degradative enzymes, *in situ* reaction conditions, and diffusion of reaction products. Finally, light reflection or quenching and uneven substrate distribution can alter the light emission pattern detected by imaging. Ideally, the promoter under study should be fused to different reporter genes, and the expression of all the fusions monitored in parallel.

The between- and within-leaf patterns of luciferase activity obtained by enzymatic assay and imaging, described herein, were similar. Specifically, the data obtained by both techniques indicated that luciferase expression was higher: (1) in plants S1, S5 and S9 than in any other plant (Figs 3, 4 and 8); (2) in young leaves than in old ones (Figs 3 and 7); (3) at the apex than at the base of an expanded leaf borne on plant S5 (Figs 4 and 7g); (4) in the main veins than in most other parts of an expanded leaf borne on plants S3, S4 and S7 (Figs 4, 8b,c,e and 9b); and (5) in contrast to (4), in parts containing only minor veins than in the main veins of most expanded leaves (Figs 4, 5, 8 and 9e). We interpret the similarity in distribution patterns as indicating that our visualization technique accurately depicts the *in vivo* pattern of luciferase expression, at least at the macroscopic level of resolution.

The finding that luciferase activity varied markedly in time and space (Figs 3–9) emphasizes the importance of standardizing the premeasurement physiological and environmental conditions of the plant and organ when studying gene expression. Biological variation can be further reduced by limiting the study to a specific organ and measuring it repeatedly. This approach necessitates that the organ not be damaged by the assay. Previously published protocols that exploited luciferase reporter enzymes (Koncz *et al.*, 1990; Schneider *et al.*, 1990) were not truly non-destructive, as the organ to be assayed typically was detached from the plant to facilitate either the performing of the measurement or the feeding of the substrate. In the case of the firefly luciferase, it is possible to assay an intact plant by supplying the luficerin substrate to the roots, but this requires growing the plants in sand or agarose, because all of the supporting medium must be removed from the roots prior to the assay. This procedure is relatively time-consuming, involves extensive physical handling of the plant, biases the light emission pattern, and limits the size of the plant that can be measured. In contrast, our assay protocol can be performed without destroying or manipulating the plant or organ. The measured leaf receives minimal handling and is left attached to an unstressed, intact plant, while the decanal substrate, which is taken up directly and non-preferentially by all tissues (Figs 6–9), is applied in such a way that it does not injure the leaf. The importance of minimizing physical contact prior to the measurement is

emphasized by the finding that gentle massage increased the intensity of light emission (Fig. 9c).

Thus, our imaging technique can be used to follow the expression of a promoter-*luxF2* gene fusion in a particular leaf over an extended time period, *e.g.* during ontogeny or while an environmental stress is being imposed and relieved.

Our finding that enzymatically assayed luciferase activity in leaves was on average 12 times lower in transformed plants of hybrid aspen than in tobacco transformants can be attributed to between-species variation in the activity of either the enzyme or the 35S promoter. Experiments with purified enzyme indicated that *Populus* spp. tissues contain compounds that inhibit enzymatic activity (Fillatti *et al.*, 1987). Even, however, when this inhibition was prevented by extracting luciferase with a modified buffer (data not shown), we found less luciferase activity in hybrid aspen than in tobacco. The cause of this difference is unknown, but could be due to a lower activity of the 35S promoter in hybrid aspen.

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