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## Stability of transgene expression in poplar: A model forest tree species

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**Abstract** – We evaluated the stability of transgene expression in a hybrid poplar (*Populus tremula* × *P. alba*) clone transformed with constructs carrying a reporter gene (*uidA*) under the control of either a constitutive (35S) or a vascular-specific promoter. Analyses of transgene expression by GUS fluorometry and histochemistry was performed on several hundreds of trees, originating from 44 different transgenic lines, grown under in vitro, greenhouse and field conditions. While important variations in expression levels occurred, the transgene appeared to be stably expressed throughout a 6-year period. Only one silenced transgenic line was detected under in vitro conditions: molecular analyses indicated that this line contained an elevated number of transgene copies and was probably silenced from the beginning, at the post-transcriptional level. Overall, these results suggest that transgene expression in perennial species such as trees remains stable over an extended period.

**field trials / gene silencing / poplar / transgene stability / transgenic trees**

**Résumé – Stabilité d'expression de transgènes chez le peuplier : une espèce forestière modèle.** Notre étude a pour but d'évaluer la stabilité de l'expression d'un transgène chez un peuplier hybride (*Populus tremula* × *P. alba*) transformé avec le gène reporter *uidA* sous le contrôle soit du promoteur 35S, soit du promoteur du gène *cad2* de l'eucalyptus (EuCAD) à spécificité vasculaire. L'expression du transgène a été suivie quantitativement par fluorimétrie et qualitativement par histochimie à partir de matériel collecté in vitro, en serre ou en champ sur quelques centaines d'arbres, issus de 44 lignées transgéniques. Tandis qu'il existe d'importantes variations dans les niveaux d'expression du transgène selon les lignées, les arbres d'une même lignée, l'organe analysé ou la date de prélèvement, l'expression du transgène s'est révélée être stable sur une période de 6 ans. L'inactivation de l'expression d'un transgène n'a été observée que chez une seule lignée dès le stade in vitro. La caractérisation de cette lignée a permis de montrer qu'elle possédait un nombre élevé de copies du transgène, ce qui suggère qu'un phénomène de suppression post-transcriptionnel s'est produit dans cette lignée peu de temps après l'événement de transformation. Ainsi, dans l'ensemble, nos résultats suggèrent que l'expression des transgènes dans les arbres reste stable dans le temps.

**essai en champ / co-suppression / peuplier / stabilité d'expression / arbres transgéniques**

### 1. INTRODUCTION

Recently, a number of comprehensive reviews [27, 32, 48] have detailed our present knowledge of the mechanisms whereby an introduced gene, or 'transgene' is inactivated in transgenic plants with the result that the corresponding protein is no longer made. This phenomenon has also been identified in fungi, as well as in invertebrates and vertebrates [10]. Such gene inactivation has been termed 'gene silencing' or 'homology-dependant gene silencing' (HDGS) [33] since sequence homology appears to be a common aspect of transgene inactivation. HDGS can occur either at the transcriptional level (no mRNA is transcribed) in which case it is referred to as transcriptional gene silencing (TGS) [48], or the post-transcrip-

tional level (mRNA is transcribed, but then degraded) when it is known as 'post-transcriptional gene silencing' (PTGS) [33]. The over-expression of transgenes containing high sequence homology to endogenous genes can also result in the silencing of both the transgene and the endogenous gene; in this case the silencing event is referred to as 'cosuppression'. Cosuppression can occur at either the TGS- level or at the PTGS level.

Although the discovery of gene silencing was originally perceived as an obstacle to the use of genetic engineering for plant improvement, the study of this phenomenon has revealed that such epigenetic mechanisms are involved in a number of important plant processes such as plant development [18], plant defence against viruses and bacterial DNA [2], as well as in genome evolution involving transposable elements [27].

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Nevertheless, gene silencing remains a potential problem in the context of biotechnological programmes aimed at improving plants through a genetic engineering approach. While recent work [27, 33, 48] is starting to provide detailed information about the frequency and mechanisms of HDGS in annual herbaceous species, relatively little information is available about the occurrence of such phenomena in long-lived perennial species such as trees [12]. Such a question is particularly important in an applied context since genetic engineering has been proposed as a parallel strategy in tree improvement programmes [35, 41, 44, 47]. Indeed, a number of different potential applications including herbicide tolerance [4, 34], insect tolerance [16, 31, 42], flowering and sterility [12, 43] and modification of wood quality through alteration of lignin metabolism [1, 21, 29, 39] are currently being investigated.

The aim of such a strategy in forestry is to modify the phenotype of the tree by expressing a transgene to improve productivity and/or quality. It is, therefore, extremely important that the transgene is expressed stably and in a controlled fashion. For example, modifications (e.g. insect tolerance) targeted to all of the plant tissues through the use of transgenes under the control of a constitutive promoter must continue to be expressed until rotation age. The loss of transgene expression would reverse the modification and compromise the expected beneficial effects. Therefore, transgene expression must remain stable in *time*, which in the case of rapid-growing, short-rotation species such as *Eucalyptus*, Poplar, *Pinus radiata* and *P. taeda* can be of the order of 10–30 years or more. Over such a long time period, transgenic trees will be subjected to both abiotic and biotic stresses. Since abiotic stresses such as heat and drought have been shown to reduce the activities of transgene expression in herbaceous species [7, 9, 36], and, in some cases, to result in gene silencing, another important question concerns the long-term stability of transgene expression in trees under conditions of stress.

In the case of modifications aimed at particular tissues (e.g. wood, reproductive tissues) through the use of transgenes under the control of tissue-specific promoters, the situation is even more complex. Here, transgene expression must not only remain stable in *time*, but also in *space*. Similarly, transgenes under the control of inducible promoters should not be expressed until the promoter is activated.

Consequently, one of the crucial issues related to the use of genetic transformation in forest tree improvement is the stability of transgene expression. In this paper we try to address these issues by following transgene stability in hybrid poplar – a plant that has become a model species for molecular studies in woody plants [6].

## 2. MATERIALS AND METHODS

### 2.1. Plant material and experimental plan

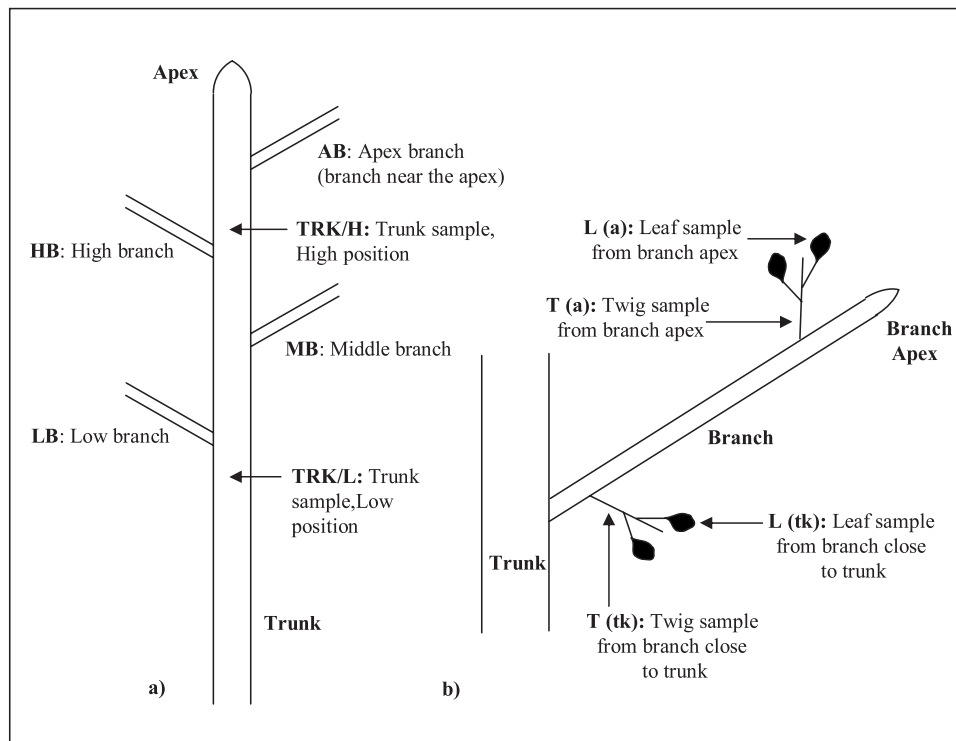
In order to assess the stability of transgene expression in poplar, 2 sets of plants were analysed. The first group (Group 1) involved hybrid poplar (*Populus tremula* × *P. alba*; INRA clone 717-1-B4) plants transformed by either cocultivation [30], or by co-inoculation [8] with a 35S-*uidA* construct. The trees were transferred to the field

**Table I.** Summary of the sampling protocol to follow transgene expression in Group 1 plants. Numbers refer to the number of transgenic lines analysed, letters refer to the type of sample as indicated in Figure 1 except for R/B, R/M and R/S which represent samples taken from a big root, medium root and small root, respectively. N.D. = samples not taken.

Analysis	Sampling year and sample type				
	1992 (Oct)	1993 (Jul)	1995 (Jul)	1996 (Jul)	1997 (Aug)
	N.D.	N.D.	2 ×:	2 ×:	4 ×:
			HB: L (a,tk) HB: T(a,tk)	HB: L (a,tk) HB: T(a,tk)	AB: L (a,tk) AB: T (a,tk)
					1 ×:
Histochemistry					HB: L (a,tk) HB: T (a,tk)
					TRK/H TRK/L
					R/B R/M R/S
	1 ×:	4 ×:	N.D.	N.D.	4 ×:
Fluorometry	HB: L(a,tk)	AB: L (a,tk) HB: L (a,tk) MB: L (a,tk) LB: L (a,tk)			HB: L (a,tk)
		HB: T(a,tk)			

in spring 1991 following permission from the French “Commission du Génie Biomoléculaire” (authorization # 90.08.01). Three replicate plants each from four independent transgenic lines were planted together with three untransformed control plants of the same genotype. Transgene expression in plants was determined both quantitatively using GUS fluorometry in the years 1992, 1993 and 1997, and qualitatively by GUS histochemistry in the years 1995, 1996 and 1997 (summarised in Tab. I and Fig. 1).

The second group (Group 2) also involved hybrid poplar, however transformed with either a 35S-*uidA* construct, or a EuCAD-*uidA* construct [13], to assess the expression stability of the *uidA* coding sequence under the control of a tissue-specific promoter. All transgenic lines were obtained by cocultivation [30]. The 35S-*uidA* gene construct used was pBI-121 [3] and the EuCAD-*uidA* construct [13] was the kind gift of Dr Grima-Pettenati. Both constructs are shown in Figure 2. This second group was made-up of 20 independent transgenic lines containing and expressing the 35S-*uidA* cassette, and 20 independent transgenic lines containing and expressing the EuCAD-*uidA* construct. Transgene expression was assessed qualitatively in all 40 transgenic lines under *in vitro* conditions and using GUS histochemistry, and quantitatively in 9 selected 35S-*uidA* lines and 9 selected EuCAD-*uidA* lines using GUS fluorometry – also under *in vitro* conditions. For each line, 5 trees were then transferred to the greenhouse and subsequently planted in the field in autumn 1996, following permission from the French “Commission du Génie Biomoléculaire” (authorization #99/043; Ministère de l’Agriculture). The stability of transgene expression for the selected Group 2 plants (9 × 35S-*uidA* lines and 9 × EuCAD-*uidA* lines) was regularly monitored, qualitatively by GUS histochemistry under greenhouse and field conditions,



**Figure 1.** Sampling protocol used for group 1 plants, see also Table I. (a) Branch and trunk positions. (b) Position of leaf and twig samples on branch.

**Table II.** Summary of the sampling protocol to follow transgene expression in Group 2 plants. Figures indicate the number of different transgenic lines analysed; letters refer to the organs analysed (L = leaf, S = stem).

Analysis	Growth conditions and samples		
	In vitro	Greenhouse	Field
Histochemistry	20 (L) × 2 <sup>1</sup>	9 (S,L)	9 (S), monthly <sup>2</sup>
Fluorometry	6 (S) + 3 (S,L)	6 (S) + 3 (S,L)	N.D.

<sup>1</sup> The 20 *in vitro* lines were all analysed twice at an interval of 3 months; <sup>2</sup> field samples were harvested monthly from March 1997 to October 1997.

and quantitatively by fluorometry under greenhouse conditions as summarised in Table II.

## 2.2. Molecular characterization

Genomic DNA was extracted from the leaves of greenhouse grown plants according to [11]. Potential transgenic lines (Group 2 plants) growing on kanamycin-containing selection medium were characterized by PCR to confirm transformation and to determine the presence of any sequences from the binary vector located outside of the border sequences. Transformation and the presence of extra-border sequences in Group 1 plants were verified by hybridisation. The following sets of primers were designed using “C Primer” and “Amplify” software (freeware Molbio/mac, Indiana State University, USA): (1) *uidA* coding sequence: 5' primer: TAT ACG CCA TTT GAA GCC G; 3' primer: AAG CCA GTA AAG TAG AAC GGT; amplification product = 550 bp; (2) Right border sequence: 5' primer CCC ACT ATG GCA TTC TGC TG; 3' primer: GCG GTT CTG TCA GTT CCA AAC; amplification product = 389 bp; (3) Left

border sequence: 5' primer: ACG CTC TGT CAT CGT TAC AAT; 3' primer GCT GTT GCC CGT CTC AC; amplification product = 341 bp). After an initial denaturing step, all 3 PCR products were amplified by 30 cycles of the following programme: denaturing: 45 s, 94 °C; annealing: 60 s, 55 °C; extension: 45 s, 72 °C. The fragments amplified are indicated in Figure 2.

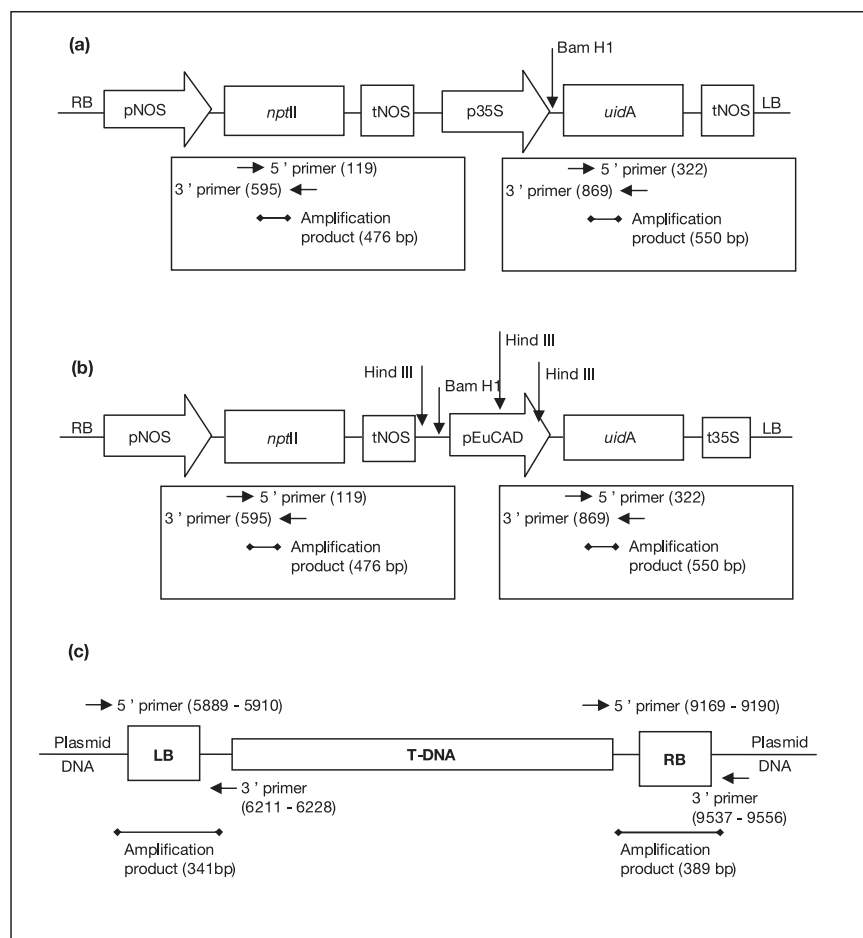
For Southern hybridisation analysis (Group 1 and 2 plants) genomic DNA was isolated from the leaves of *in vitro*-grown plants using the DNeasy Plant Kit (Qiagen, France) according to manufacturer's instructions. 2.5 µg DNA was digested separately by *Hind*III and *Bam*HI and separated on a 0.8% agarose TAE gel. DNA was transferred to positively charged nylon membranes (Roche, Germany) using a vacuum transfer apparatus (Appligene, France). Membranes were hybridised overnight at 42 °C with 15 ng·mL<sup>-1</sup> DIG-labelled DNA probe synthesized by PCR. Membranes were then washed and bound probe visualised by chemiluminescence (CPD-star), according to the manufacturer's instructions (Roche, Germany).

## 2.3. Determination of *uidA* expression

Expression of the *uidA* transgene was analysed by fluorometry to provide quantitative data and by histochemistry to evaluate the “spatial stability” (i.e. tissue-specific vs. constitutive expression) of transgene expression.

GUS fluorometry [25] was used in a microwell-based assay system to determine quantitative expression levels. For each individual tree, total soluble protein was extracted and quantified [5] from stems and leaves as indicated in Tables I and II. Three replicate plants were used per transgenic lines (Group 1 plants) and the results subjected to analysis of variance, and five replicate plants were used per transgenic line (Group 2 plants). The activity of each protein extract was measured four times.

X-Gluc histochemistry [24] was used to follow the spatial expression pattern of the *uidA* gene in the leaves and stems of plants grown



**Figure 2.** (a) 35S-*uidA* and (b) EuCAD-*uidA* constructs used for transformation. (c) Plasmid T-DNA region showing location of primers used to verify the correct functioning of the borders. Positions of 5' and 3' *uidA*, *nptII* and vector border PCR primers are indicated together with the size of the amplification products. Numbers in brackets indicate primer position within the gene (*uidA* and *nptII*) and the plasmid (vector border sequences – based on pBIN 19 sequence [3]). *HindIII* and *BamHI* restriction digest sites used for Southern hybridisation analysis are given.

under *in vitro*, greenhouse and field conditions. For analysis, stem and leaf samples were removed and incubated in 1 mL reaction buffer (100 mM sodium phosphate, pH 7; 10 mM EDTA; 0.5 mM potassium ferricyanide; 0.5 mM potassium ferrocyanide; 0.25% triton X-100; 0.05 mM X-Gluc (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide) at 37 °C overnight in the dark. Samples were then fixed in FAA, cleared in 70% EtOH and the expression pattern observed using a stereo microscope.

#### 2.4. RT-PCR

Total RNA was extracted from 100 mg ground leaves using the Qiagen RNeasy kit, according to the manufacturer's instructions. First strand cDNA was produced in a 20  $\mu$ L reverse transcriptase reaction using 1  $\mu$ g RNA, 100 ng oligo dT primer and 100 U of Superscript II (Gibco BRL) according to the manufacturer's instructions. Following completion of the reaction, water was added to a final volume of 60  $\mu$ L and 10  $\mu$ L of the resulting solution used in subsequent PCR reactions. PCR reactions were performed using (1) *uidA* primers detailed above and (2) *nptII* primers: 5' primer: TGTTCCG-GCTGTCAGCGCAG; 3' primer: TCGGCAAGCAGGCATCGCCA; amplification product 476 bp (Fig. 2). Water-channel protein primers were used as an internal control and were the kind gift of Dr Breton: 5' primer: GG(I)CAY(I)T(I)AAAYCC(I)GTN; 3' primer: GG(I)CCRAA-(I)SH(I)CK(I)GC(I)GGRTT, amplification product 390 bp. The *nptII* PCR product was amplified by 30 cycles of the following programme: denaturing: 45 s, 94 °C; annealing: 60 s, 60 °C; extension: 45 s, 72 °C; and the water-channel PCR product by 30 cycles of the

following programme: denaturing: 45 s, 94 °C; annealing: 50 s, 55 °C; extension: 45 s, 72 °C.

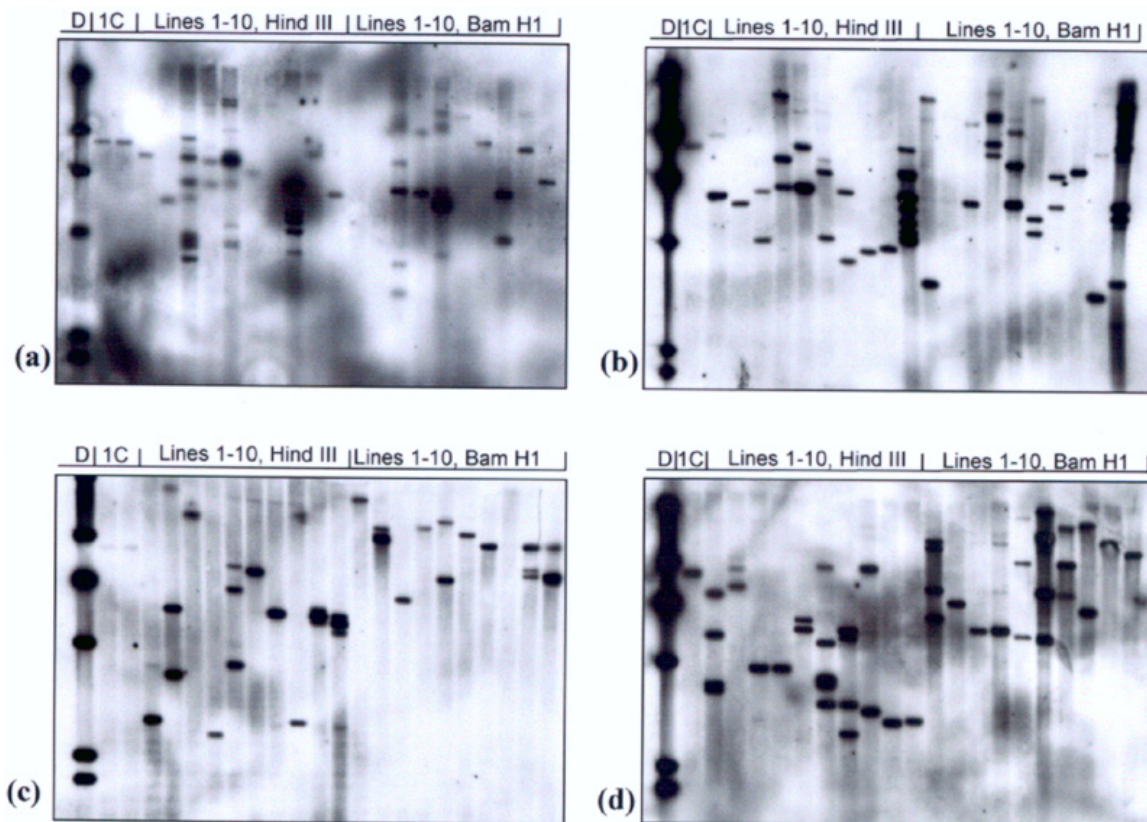
#### 2.5. Propionic acid treatments

Propionic acid was added to the culture medium of *in vitro* plants in an attempt to induce transgene methylation, thereby simulating the effect of changing environmental conditions as previously reported [46]. For each construct (35S-*uidA*, EuCAD-*uidA*), the effect of stress on transgene expression was assessed in two transformed lines which showed high expression levels. Five replicate plants per transgenic lines were multiplied on medium MS1/2 containing 0, 0.05, 0.5 and 1 mM of the stressing agent propionic acid [46]. After 3 months culture, plants were transferred to fresh medium containing the same concentration of propionic acid. Following 3 months further culture, plants were harvested and expression levels determined by fluorometry. Expression levels were determined in 3 individual plants per transformed line.

### 3. RESULTS

#### 3.1. Molecular characterization

Southern hybridisation analyses of locus numbers of transgene in the group 1 transgenic lines indicated that the lines A, B, C and D contained 1, 2, 5 and 1 loci of the *uidA* gene,



**Figure 3.** Southern hybridisation analysis of transformants. Genomic DNA from 20 35S-*uidA* transgenic lines and 20 EuCAD-*uidA* transgenic lines was digested separately by *Hind*III and *Bam*H1 and hybridized with a DIG-labelled *uidA* probe. (a) 35S-*uidA* lines: numbers 1–10; (b) 35S-*uidA* lines: numbers 10–20; (c) EuCAD-*uidA* lines: numbers 1–10; (d) EuCAD-*uidA* lines: numbers 10–20. DIG = dig-labelled molecular-weight markers; 1C = DNA from an estimated single-copy transformant.

respectively (data not shown). Important variations in the intensity of some of the bands suggest multiple integrations in several of these loci.

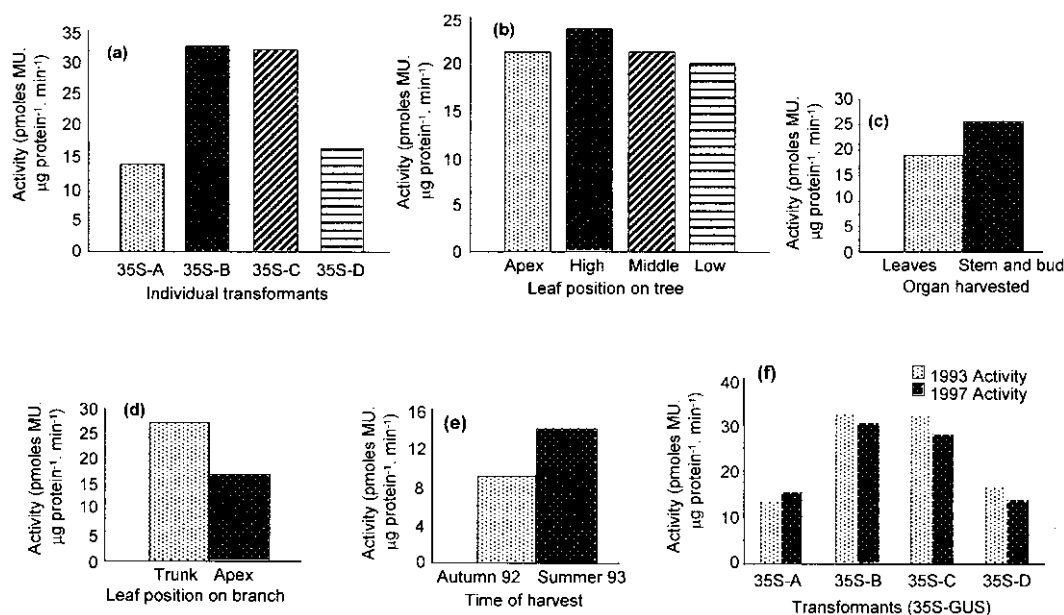
For the group 2 transformants, potential 35S-*uidA* and EuCAD-*uidA* positive lines were identified by PCR using *uidA* primers. In total, 34 × 35S-*uidA* transgenic lines and 31 × EuCAD-*uidA* transgenic lines were identified. It was decided to analyse and transfer to field conditions only those transgenic lines that did not contain vector sequences. When using *A. tumefaciens* as a vector for gene transfer, only the T-region is integrated in the host genome owing to the presence of two inverted repeats, named right and left borders, at each end of it. This particular feature allows to limit the DNA transfer to the T-DNA, that is the name of the T-region once it has been inserted into the plant host genome. However, it has been shown that, sometimes, one or both borders did not work properly, leading to unwanted integration of the binary vector backbone [26]. The proper functioning of the T-DNA borders was verified by PCR using 2 additional sets of primers covering the left and right borders, respectively. These analyses (data not shown) revealed that either the left or right borders, or both borders had not functioned in an elevated proportion of transgenic lines (14 lines for 35S-*uidA* (41.2%) and 11 lines for EuCAD-*uidA* (35.5%)).

Nevertheless, 20 × 35S-*uidA* transgenic lines and 20 × EuCAD-*uidA* transgenic lines containing no extra-border Ti plasmid sequences were identified, characterized further by Southern hybridisation analyses and transferred to greenhouse and field conditions.

Southern hybridisation analyses (Fig. 3) of the selected 40 lines (Group 2 transformants) show that the number of copies incorporated appears to vary from one to several copies, with some transgenic lines showing a high locus number. Approximately 50% of transgenic lines (both constructs) show only a single band on Southern autoradiograms, but the high intensity of some of these bands may indicate the insertion of several transgenes in tandem or other multiple single-site insertions, and care should be taken in determining locus number.

### 3.2. Transgene expression levels

Quantitative measurements of 35S-*uidA* transgene expression levels in field-grown trees planted in autumn 1991 (Group 1 plants) were made in October 1992, July 1993 and again in July 1997 according to the scheme in Table I. Figures 5a and 5b illustrate the appearance of the group 1 tree in 1997. Analyses of variance performed on samples collected in 1993 show that while significant differences ( $P < 0.01$ ) in the



**Figure 4.** Transgene (35S-*uidA*) expression levels of Group 1 plants. (a)  $\beta$ -glucuronidase activity levels of leaves [HB : L(a) – see Table I and Fig. 1] in the 4 individual Group 1 transformants. (b)  $\beta$ -glucuronidase activity levels [L(a)] collected from 4 different branch levels (AB, HB, MB, LB) in a single Group 1 transformant. (c)  $\beta$ -glucuronidase activity levels in leaves [HB : L(a)] and associated twig [stem and bud – HB : T(a)]. (d)  $\beta$ -glucuronidase activity levels in leaves collected from the apex of a branch [HB : L(a)] and close to the trunk [HB : L(tk)]. (e)  $\beta$ -glucuronidase activity levels in leaves [HB : L(a)] harvested in autumn 1992 and summer 1993. (f) Comparison of  $\beta$ -glucuronidase activity levels in leaves [HB : L(a)] harvested in summer 1993 and summer 1997.

transgene activity between different transgenic lines can be detected (Fig. 4a), the position of the leaf in the tree (Fig. 4b) has little effect ( $P = 0.24$ ). In contrast, significant differences ( $P = 0.05$ ) were observed between different organs (leaves and, stems and buds) (Fig. 4c), as well as for the position of the leaf on the branch (Fig. 4d;  $P < 0.01$ ). Comparison of the transgene activity levels for autumn (1992) and summer (1993) also indicated differences although not significant (Fig. 4e;  $P = 0.1$ ). Finally, the comparison of transgene activity levels in leaves from different transgenic lines for July 1993 and August 1997 (Fig. 4f), indicates that the trees continue to express the transgene after 6 years in the field and the 1997 transgene expression levels are not significantly different from those observed in 1993.

Further evidence of the stability of transgene expression in the field-grown plants (Group 1 plants) was provided by the positive results of the GUS histochemistry performed in 1995 and 1996 (data not shown), and in 1997 (Fig. 5). Figures 5c–5h indicate that in 1997 the 35S-*uidA* transgene was still expressed constitutively in the leaves, branches, the trunk and roots of these 7-year-old trees that had been grown in the field for 6 years. Figures 5c–5h show that the blue GUS coloration, due to  $\beta$ -glucuronidase activity, in these organs is limited to the living tissues (bark and xylem parenchyma), while dead cells (xylem vessels and fibres) do not show any coloration. The incomplete blue coloration, observed in the leaf sample (Fig. 5c), is presumably the result of substrate penetration problems due to the presence of the leaf's impermeable cuticle.

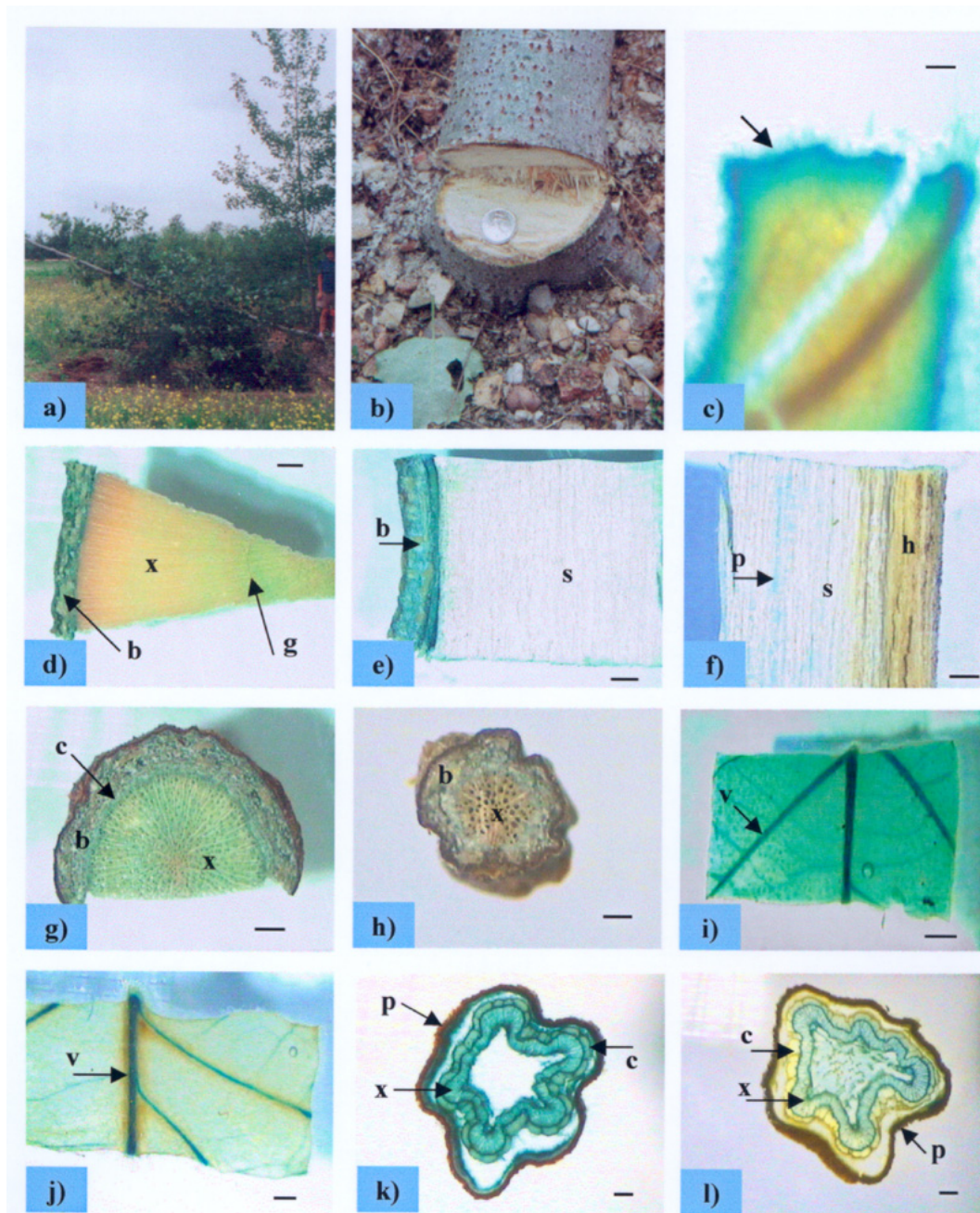
For the 20  $\times$  35S-*uidA* lines and the 20  $\times$  EuCAD-*uidA* lines (Group 2 transformants), qualitative analyses by GUS

histochemistry (data not shown) of leaves from in vitro plants also gave indications for a stable transgene expression. Indeed, all transgenic lines continued to express the transgene 3 months after the initial analyses, in a constitutive fashion for the 35S-*uidA* lines, and in a tissue-specific fashion for the EuCAD-*uidA* lines.

Comparison of the 35S-*uidA* transgene expression levels, as determined by fluorometry, between stems and leaves of 3 individual in vitro transgenic lines (Fig. 6a) revealed that while differences in activity levels could be detected between different transformants, no significant differences could be detected between these two organs. In contrast, similar analyses of the EuCAD-*uidA* lines (Fig. 6b) revealed that the transgene activity was significantly higher in the stems than in the leaves for all 3 lines examined.

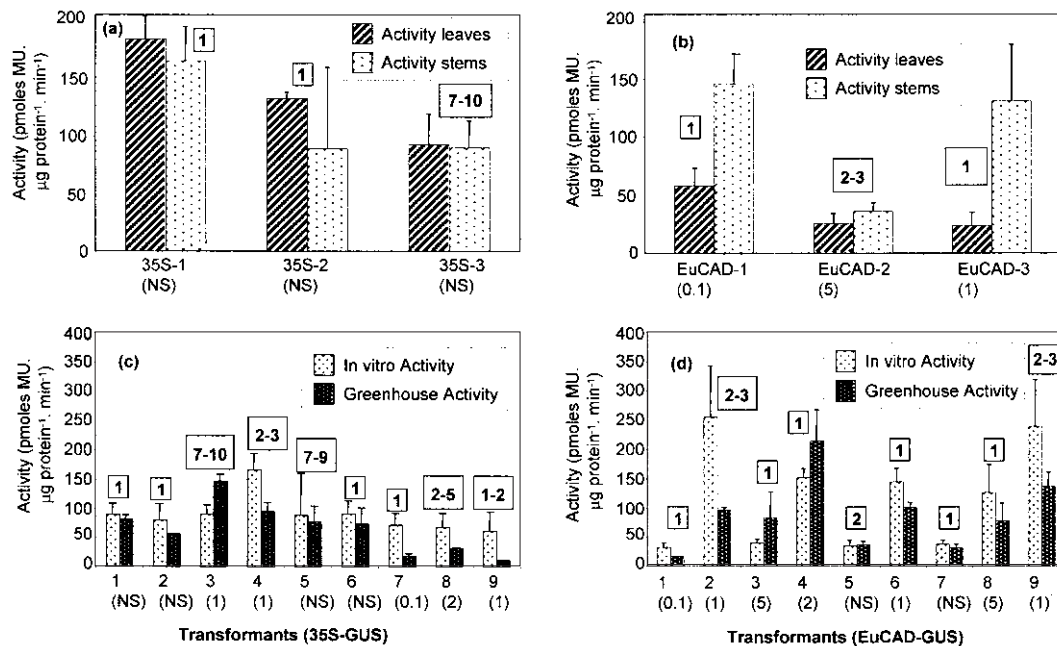
For the 9 selected 35S-*uidA* lines and the 9 selected EuCAD-*uidA* lines, qualitative analysis by GUS histochemistry of leaves (Figs. 5i and 5j) and stems (data not shown) from greenhouse plants gave indications that transgene expression remained stable under these conditions. All transformed lines analysed continued to express the transgene (constitutively for the 35S-*uidA* lines, and in a tissue-specific fashion for the EuCAD-*uidA* lines).

Comparison of the 35S-*uidA* transgene activity by fluorometry, in stems of in vitro and greenhouse plants (Fig. 6c) showed that in 4 out of the 9 lines examined (lines 1, 2, 5, 6) no significant differences in activity could be detected between in vitro and greenhouse plants. For 4 transgenic lines (4, 7, 8, 9), transgene activity was significantly higher in in vitro plants, while in one line (line 3) transgene activity was significantly higher in the greenhouse plants.



**Figure 5.** GUS histochemistry of Group 1 and Group 2 plants. **(a–h)** : Group 1 plants. **(i–l)** : Group 2 plants. **(a)** General view of the transgenic plantation in September 1997 with seven-year-old Group 1 transformant selected for analyses. **(b)** View of the trunk base of the Group 1 transformant, diameter of coin = 2.4 cm. **(c)** Leaf sample [HB : L(a)] of transformant, blue coloration (arrow) indicates  $\beta$ -glucuronidase activity. Bar = 0.2 mm. **(d)** Transversal section of branch sample [HB: T(tk)] of transformant, blue coloration indicates  $\beta$ -glucuronidase activity. b = bark, x = xylem, g = first year growth-ring. Bar = 0.5 mm. **(e)** Longitudinal section of trunk sample (TRK/H: bark to sapwood), blue coloration indicates  $\beta$ -glucuronidase activity. b = bark, s = sapwood. Bar = 1 mm. **(f)** Longitudinal section of trunk sample (TRK/H: sapwood to heartwood), blue coloration indicates  $\beta$ -glucuronidase activity. s = sapwood, h = heartwood, p = axial parenchyma. Bar = 1 mm. **(g)** Transversal section of root (R/M), blue coloration indicates  $\beta$ -glucuronidase activity. b = bark, x = xylem, c = vascular cambium. Bar = 0.5 mm. **(h)** Transversal section of root (R/S), blue coloration indicates  $\beta$ -glucuronidase activity. b = bark, x = xylem. Bar = 0.2 mm. **(i)** Leaf sample (Group 2 plant) of greenhouse plant transformed with the 35S-uidA construct, blue coloration indicates  $\beta$ -glucuronidase activity. v = leaf vein. Bar = 1 mm. **(j)** Leaf sample (Group 2 plant) of greenhouse plant transformed with the EuCAD-uidA construct, blue coloration indicates  $\beta$ -glucuronidase activity. v = leaf vein. Bar = 0.5 mm. **(k)** Transversal section of young branch from field-grown plant transformed with the 35S-uidA construct and harvested at the end of March 1997, blue coloration indicates  $\beta$ -glucuronidase activity. x = xylem, c = cortex (with sclerenchyma), p = periderm. Bar = 0.2 mm. **(l)** Transversal section of young branch from field-grown plant transformed with the EuCAD-uidA construct and harvested at the end of March 1997, blue coloration indicates  $\beta$ -glucuronidase activity. x = xylem, c = cortex (with sclerenchyma), p = periderm. Bar = 0.2 mm.





**Figure 6.**  $\beta$ -glucuronidase (GUS) activity in Group 2 plants with the *uidA* gene under the control of the 35S promoter (a and c) and the EuCAD promoter (b and d). (a) Comparison of transgene activity in leaves and stems of 35S-*uidA* transformants, in vitro plants. (b) Comparison of transgene activity in leaves and stems of EuCAD-*uidA* transformants, in vitro plants. (c) Comparison of stem transgene activity in 35S-*uidA* transformants, in vitro and greenhouse plants. (d) Comparison of stem transgene activity in EuCAD-*uidA* transformants, in vitro and greenhouse plants. Error bars = 95% confidence limits; numbers in brackets = *P* value (%) for significant differences as determined by student's *t*-test (NS = differences not significant). Numbers in boxes = estimated transgene locus number.

Similar analyses for the EuCAD-*uidA* plants (Fig. 6d) revealed that in 5 out of the 9 lines examined (lines 1, 2, 6, 8, 9) the transgene activity was significantly higher in in vitro plants as compared to the greenhouse plants. In 2 lines (lines 3, 4), transgene activity was significantly higher in the greenhouse plants as compared to in vitro plants, while in the remaining 2 lines (lines 5, 7) no significant differences in transgene activity could be determined between in vitro and greenhouse plants. The maximum activity (approximately 250 pmoles  $\text{Mu. } \mu\text{g protein}^{-1} \cdot \text{min}^{-1}$ ) was observed, when the *uidA* gene was driven by the EuCAD promoter (line 2).

Comparison of transgene activity with estimated locus number (Fig. 7) indicated that activity was not related to the number of 35S-*GUS* transgenes for in vitro plants ( $R^2 = 0.004 - 0.005$ , Figs. 7a and 7b) and only weakly correlated ( $R^2 = 0.329 - 0.409$ , Fig. 7) for greenhouse plants. In contrast, for plants transformed with the EuCAD-*GUS* transgene, activity was not correlated with estimated transgene number in greenhouse plants ( $R^2 = 0.0004 - 0.02$ , Figs. 7c and 7d) and only weakly correlated in in vitro plants ( $R^2 = 0.246 - 0.485$ , Figs. 7c and 7d).

For the 9  $\times$  35S-*uidA* lines and the 9  $\times$  EuCAD-*uidA* lines, qualitative analyses by GUS histochemistry (Figs. 5k and 5l, and data not shown) of field plants indicated that transgene expression remained rather stable throughout the harvesting period (March–October 1997). During this period, all transgenic lines continued to express the transgene in a constitutive fashion for the 35S-*uidA* lines, and in a tissue-specific fashion

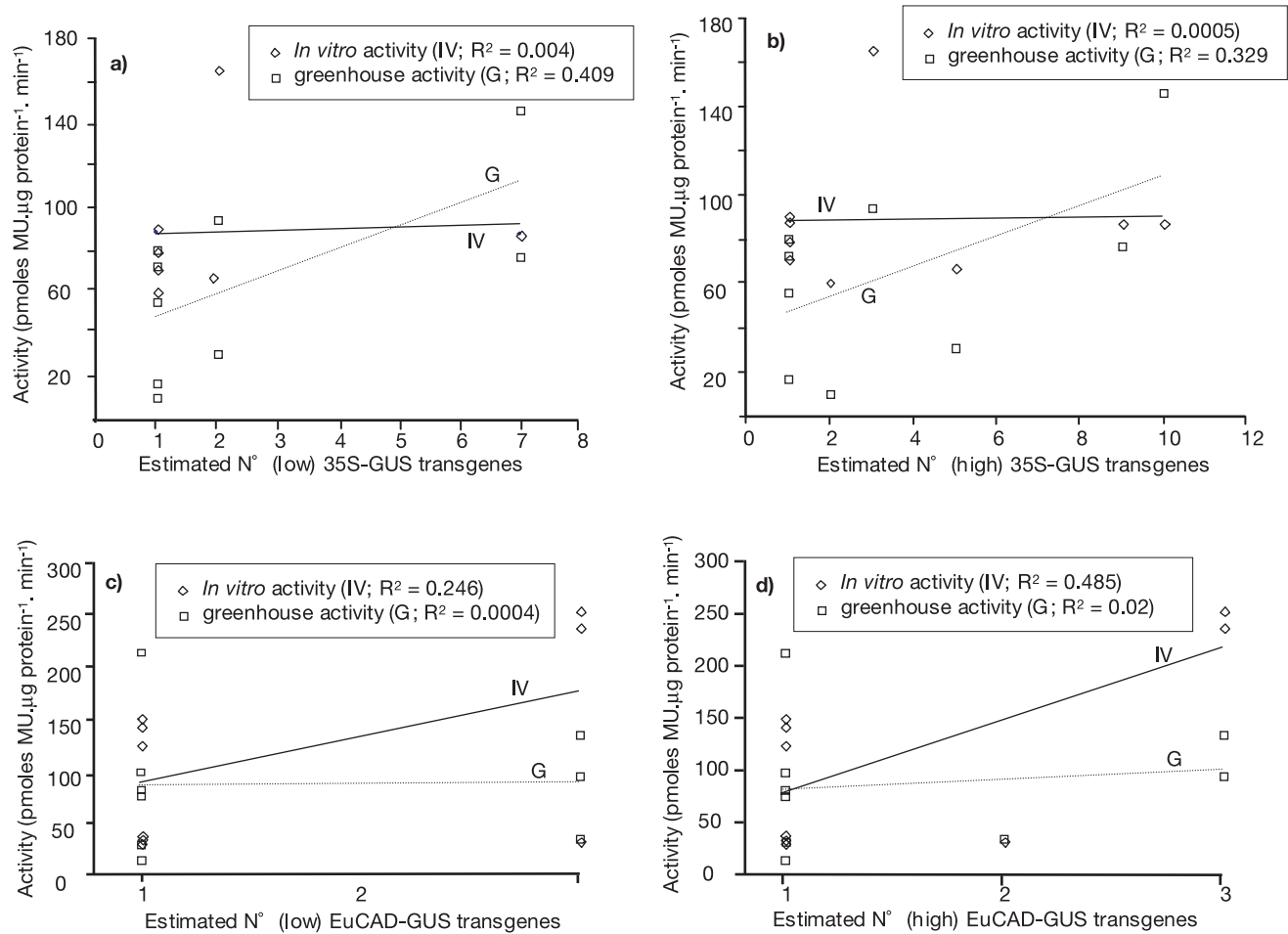
for the EuCAD-*uidA* lines. No cases of gene-silencing were observed.

### 3.3. Effect of stress on transgene expression

In order to assess any effect of changing environmental conditions (stress) on transgene expression, 2  $\times$  35S-*uidA* lines and 2  $\times$  EuCAD-*uidA* lines were grown for a period of 6 months on medium containing 0, 0.05 mM, 0.5 mM, and 1 mM propionic acid. The concentration of 1 mM proved to be toxic and plants grown on this concentration died. Analyses of transgene activity levels in plants grown on medium containing 0.05 mM and 0.5 mM propionic acid indicated that this treatment had little effect on transgene activity.

### 3.4. Analysis of silenced line

GUS histochemistry of the positive transgenic lines (as confirmed by PCR) revealed that one of the 35S-*uidA* transgenic lines (pBI-121-4) did apparently not express the *uidA* transgene. This line also contained extra-border Ti plasmid sequences and so was not used in the greenhouse and field trials. Southern hybridisation analysis of this line (Fig. 8) revealed that this line contained an elevated number of transgene copies. In order to determine whether the expression was blocked at the transcriptional or post-transcriptional levels RT-PCR for the *uidA* gene and the *nptII* selection gene were performed. Both the *uidA* gene (Fig. 9) and the *nptII* gene (data not



**Figure 7.** Regression analyses of in vitro- and greenhouse-transgene activity with estimated locus number for 35S-GUS transformants (a, b) and EuCAD-GUS transformants (c, d). Low- (a, c) and high- (b, d) estimates of transgene number (see Fig. 6) were used for the analyses.

shown) are transcribed in the silenced line suggesting that the “silencing” occurs at the post-transcriptional level.

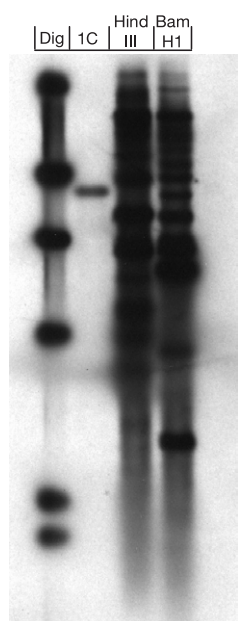
#### 4. DISCUSSION

Genetic engineering is potentially a powerful technique for improving woody species since it allows the introduction of new characteristics into already selected “elite” genotypes [35, 41]. However, the future utilisation of transgenic trees on a commercial basis will depend upon a thorough evaluation of the environmental risks, modified phenotypes and transgene stability over extended time periods [12]. In this paper we have addressed the stability aspects by following transgene expression in in vitro-, greenhouse- and field-grown poplar, which has become a model forest tree species [6, 17]. Since for most biotechnological uses, the transgenes will need to be regulated by inducible or tissue-specific promoters, so as to control both the location and the time of expression in the plant [15, 45, 49], we decided to evaluate the expression stability of the

*uidA* reporter gene under the control of both a tissue-specific promoter (EuCAD) and a constitutive promoter (35S).

Two groups of plants were evaluated. Group 1 plants were transformed with a 35S-*uidA* construct and 4 individual transgenic lines were transferred to the field in 1991. Analyses of transgene expression by both GUS histochemistry and fluorometry in 1992, 1993, 1995, 1996 and 1997 was used to evaluate both variation of expression level in planta, as well as the stability of this gene cassette under field conditions over a 6-year period, thereby addressing the issue of long-term stability. Group 2 plants were transformed with the *uidA* coding sequence under the control of either a 35S constitutive promoter or the EuCAD tissue-specific promoter, and 20 individual transgenic lines for each construct were transferred to the field in 1996. Analyses of transgene expression in a rather large number of different transgenic lines under in vitro, greenhouse and field conditions enabled us to evaluate the stability of expression with a tissue specific promoter.

Detailed assessments in 1992 and 1993 by GUS fluorometry of transgene activity in group 1 plants indicated that while

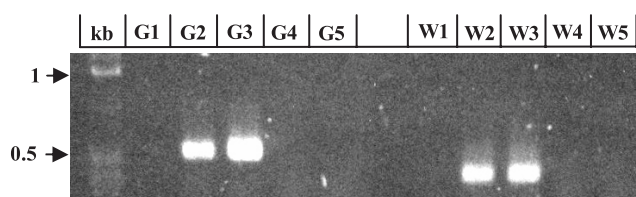


**Figure 8.** Southern hybridisation analysis of silenced line pBI-121-4 (35S-*uidA*). Genomic DNA digested separately with Hind III and Bam H1. Dig = dig-labelled molecular weight markers, 1C = genomic DNA from an estimated single-copy transformant.

differences in expression levels between different organs, and between leaves at different stages of maturity could be detected, the total protein content of these samples also varied accordingly, thereby suggesting that the observed differences most probably reflect differences in general metabolic levels. Nevertheless, such observations serve to underline the inherent sampling difficulties when working with large trees growing under field conditions as opposed to small, in vitro plants growing under controlled conditions.

Analyses of these plants by histochemistry in 1995, 1996 and 1997, and by fluorometry in 1997 indicated that all transgenic lines continued to express the transgene (in all organs investigated) during the 6 years after field plantation. Further, the expression levels determined in 1997 were not significantly different from those observed in 1993. These observations would seem to suggest that the transgene expression of the 35S-*uidA* construct is stable over an extended time period under field conditions with no cases of gene silencing occurring.

The frequency of gene silencing was evaluated by investigation of the expression stability in a larger sample of 65 PCR-positive transgenic lines. Of these 65 lines, 64 expressed the *uidA* transgene, as determined by histochemistry. Only a single 35S-*uidA* transformant (pBI-121-4) did not express the transgene under in vitro conditions and this line probably never expressed the transgene, or it was very rapidly silenced following transformation. Molecular analyses revealed that this line contained an elevated number of transgene copies (> 10) and extra-border vector sequences. While both of these conditions are often associated with gene-silencing events [22, 23, 27], the fact that 25 lines (out of the 65 transformed lines) contained extra-border sequences but still expressed the transgene would suggest that, in poplar, the presence of Ti-plasmid



**Figure 9.** RT-PCR of silenced line pBI-121-4 (35S-*uidA*). PCR with *uidA* primers (G1–G5); water channel primers (W1–W5). G1,W1 = negative control 1 (no RNA used for the RT step); G2,W2 = silenced line; G3,W3 = positive control (transgene-expressing line, 35S-*uidA* 1, one copy), G4,W4 = negative control 2 (PCR performed with RNA extract, no RT step, silenced line); G5,W5 = negative control 3 (PCR performed with RNA extract, no RT step, transgene-expressing line).

sequences is not a major factor in influencing transgene expression. RT-PCR experiments performed on the silent line revealed that both the *uidA* gene and the selection gene *nptII* were transcribed suggesting that silencing occurs at the post-transcriptional level. Treatment of the silenced line with the de-methylating agent 5-Azacytidine [28, 38] had no effect on the expression of this line which remained silent. This is in agreement with our hypothesis of PTGS since the positive effect of 5-Azacytidine is generally attributed to its role in demethylating promoter regions in lines blocked at the TGS level [20, 27, 32, 38, 48]. However, these are preliminary results and further molecular analyses are obviously necessary to confirm the nature of the silencing mechanism involved.

Twenty 35S-*uidA* lines and 20 EuCAD-*uidA* lines were selected from among the 64 transgene-expressing lines for transfer to greenhouse and field conditions (group 2 plants). Analyses by GUS histochemistry of these lines under in vitro conditions revealed that transgene expression appeared to be stable with regards to both absolute expression and tissue-specific expression. More detailed analyses by GUS fluorometry of 9 selected 35S-*uidA* lines and 9 EuCAD-*uidA* lines revealed differences in transgene expression between different transformants. Although high transgene copy number has often been associated with gene-silencing events and a reduction in transgene expression [20, 32, 48], we observed either a lack of correlation, or only a weak positive correlation between locus number and activity. Although the one line that was silenced contained an elevated number of copies, other transgenic lines containing up to 10 copies (35S-*uidA*) showed expression levels comparable to those of apparently single copy transformants. However, despite the fact that we saw little evidence for any gene-dose effect on silencing, it would probably be advisable to use single copy transgenic lines so as to minimize genome disturbance in the context of a commercial programme.

For the 35S-*uidA* constructs, no differences in expression levels could be detected between the leaves and stems of in vitro plants, while in all 3 EuCAD-*uidA* lines tested, the transgene expression level was higher in the stems than in the leaves. Such differences are, perhaps, to be expected since the activity of the EuCAD promoter has been shown [13, 19] to be spatially and temporally associated with the process of lignification which is more developed in the woody tissues of the stem.

Analyses of transgene expression in the 9 selected 35S-*uidA* lines and the 9 selected EuCAD-*uidA* lines transferred to the greenhouse revealed no cases of gene silencing. Nevertheless, it is interesting to note that - for both constructs - approximately half of the transgenic lines showed a reduction in transgene expression levels following transfer to greenhouse conditions. Such an observation is in agreement with previous studies [7, 9, 36, 40] showing that stress and other changes in environmental conditions, as well as the developmental stage of the plant can affect the expression level of transgenes and, in consequence, the characteristic targeted. Although the level of transgene expression was reduced in some transgenic lines upon transfer to greenhouse conditions, the transgene continued to be expressed in them when grown in field conditions. In these lines, transferred to the field in autumn 1996, GUS histochemistry indicated that transgene expression was stable from March 1997 to October 1997 with no cases of gene silencing. The constitutive 35S promoter continued to control expression in a constitutive manner while the EuCAD promoter controlled transgene expression in a tissue-specific fashion as previously reported [13, 19].

Changing environmental conditions ("stress") experienced in the field have been shown to be associated with changes in transgene expression levels - often accompanied by changes in DNA methylation levels [32, 42]. Fatty acids such as propionic acid and butyric acid have been previously used to simulate the effects of changing environmental conditions on transgene expression since they also induce transgene methylation and a reduction in transgene expression levels in petunia and tobacco [32, 42]. However, our preliminary experiments with propionic acid gave little indication of any reduction in transgene expression.

In conclusion, our results suggest that transgene expression in woody plants appears to be stable under in vitro, greenhouse and field conditions. We observed only one case of gene silencing, but this line was probably silenced from the beginning and, in the context of the commercial utilisation of transgenic trees, would have been detected in a standard screening programme. Other researchers [14, 37] have also suggested that gene silencing is relatively rare in woody trees and our results indicate that transgene expression is stable over an extended period under field conditions. Nevertheless, it is important to note that in this study reporter genes were used and that additional studies of this type involving other coding sequences (e.g. lignin metabolism), older trees and more elaborate stressing experiments are necessary. In addition, gene silencing events in herbaceous plants are often associated with second generation plants which are homozygous for the transgene whereas the plants analysed in this study were first generation transgenic lines and hence heterozygous for the transgene. While we demonstrated in this study that gain of function transgenes appear to be stably expressed, studies on the stability of gene suppression are also critically needed as these methods are likely to be important for commercial traits such as lignin modification, and bio-safety traits such as floral sterility.

While further studies are needed, the work presented in this paper contributes to provide the information necessary for making decisions concerning the utilisation or not of transgenic trees in forestry improvement programmes.

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## ABBREVIATIONS

DIG: digoxigenine; EuCAD: promoter of the cinnamyl alcohol dehydrogenase gene from *Eucalyptus gunnii* Hook.; FAA: Formol-acetic-alcohol; RT-PCR: Reverse transcription – polymerase chain reaction; TAE: Tris acetate ethylenediamine-tetra-acetic acid; X-Gluc: 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide.