Evaluation of heterologous promoters in transgenic *Populus tremula* × *P. alba* plants

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Abstract

The pattern and expression level of β-glucuronidase (*gus*) reporter gene regulated by six heterologous promoters were studied in transgenic *Populus tremula* × *P. alba* plants obtained by *Agrobacterium*-mediated transformation. Binary vector constructs used contained the following promoter sequences: the CaMV35S from cauliflower mosaic virus; its duplicated version fused to the enhancer sequence from alfalfa mosaic virus; CsVMV from cassava vein mosaic virus; ubiquitin 3 from *Arabidopsis thaliana* (UBQ3); S-adenosyl-L-methionine synthetase (Sam-s) from soybean; and the *rolA* from *Agrobacterium rhizogenes*. Histochemical staining of root, stem and leaf tissues showed phloem and xylem-specific *gus* expression under *rolA* promoter, and constitutive expression with the other putative constitutive promoters. Quantitative GUS expression of 10 - 15 independently transformed *in vitro* grown plants, containing each promoter, was determined by fluorimetric GUS assays. The UBQ3-*gus* fusion induced the highest average expression level, although an extensive variation in expression levels was observed between independent transgenic lines for all the constructs tested.

Additional key words: GUS, poplar, transformation, transgene.

Introduction

Poplars (*Populus* spp.) are important forest species that are cultivated worldwide. High frequency of *in vitro* regeneration, relative small genome and high transformation efficiency make this genus a model system for studies in molecular biology of woody plants (Rishi *et al.* 2001).

Producing transgenic plants requires *in vitro* tissue culture procedures, an efficient DNA delivery system, and the ability to recover transformed cells. The latter can be obtained by the use of an efficient selection marker transgene regulated by promoter sequences recognised by the host plant transcription apparatus, which confers advantage to the transformed cells grown under selection pressure. The promoter that will drive the expression of the transgene of interest is also important. Its timing, location and extend expression are essential for an efficient recovery of elite events. Therefore, promoter choice is critical for achieving suitable pattern and level of transgenes expression in commercial transgenic lines (Atkinson *et al.* 2003).

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Abbreviations: 35S-D - duplicated version of CaMV35S promoter fused to the enhancer sequence from alfalfa mosaic virus; CaMV35S - 35S promoter from cauliflower mosaic virus; CsVMV - promoter from cassava vein mosaic virus; EDTA - ethylene diamine tetraacetic acid; GUS - β-glucuronidase; MU - 4-methylumbelliferone; MUG - 4-methylumbelliferyl-β-D-glucuronide; rolA - promoter from rolA gene from *Agrobacterium rhizogenes*; Sam-s - promoter from S-adenosyl-L-methionine synthetase gene from *Glycine max*; UBQ3 - promoter from ubiquitin 3 gene from *Arabidopsis thaliana*.

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The 35S promoter from cauliflower mosaic virus (CaMV35S) has been extensively used in constitutive transgene expression in plants, including gymnosperms, dicots and monocots. For the hybrid poplar *P. tremula* × *P. alba*, most of the reports concerning genetic transformation have also used the CaMV35S promoter (Brasileiro *et al.* 1992, Leplé *et al.* 1992, Han *et al.* 2000). Other promoters have been used for transgene expression in *Populus* species, such as those from ubiquitin 1 gene from *Zea mays* (Lindroth *et al.* 1999); from mannopine synthase (*mas*) (Fillatti *et al.* 1988) and nopaline synthase (*nos*) (Confalonieri *et al.* 1995) genes from *Agrobacterium tumefaciens*; and the cinnamyl alcohol dehydrogenase (*cad*) gene from *Eucalyptus gunnii* (Feuillet *et al.* 1995). However, using the CaMV35S promoter to regulate expression of more than one transgene in the same transformation vector can cause silencing of transgenes due to co-suppression phenomenon (Curtis *et al.* 2000). Furthermore, it may be advantageous to test constitutive promoters other than the CaMV35S promoter. Transgenic *Nicotiana tabacum* plants expressing the *CryIA(c*) gene under the control of a chimeric OM promoter and the omega factor showed an average expression level 2-fold higher that under the CaMV35S promoter (Chunlin *et al.* 1999). Bhattacharyya and co-workers obtained similar results showing that the figwort mosaic virus (FMV) Sgt promoter was about 2-fold stronger than the CaMV35S promoter in transgenic *N. tabacum* cells (Bhattacharyya *et al.* 2002). Thus, the utilisation of alternative promoters in a model plant, such as poplar, will contribute to better understanding of transgene expression and regulation in transgenic trees.

In this report we describe the expression pattern and level of the β-glucuronidase (*gus*) reporter gene under the control of six promoters in transgenic *P. tremula* × *P. alba*. We produced transgenic plants containing promoters derived from the following genes: *rolA* from *Agrobacterium rhizogenes* (Guivarc'h *et al.* 1996), the S-adenosyl-L-methionine synthetase from *Glycine max* (Sam-s) (Falco and Zhongsen 2000); the CaMV35S promoter and its duplicated version fused to a transcriptional enhancer from alfalfa mosaic virus (35S-D) (Datla *et al.* 1993); the CsVMV from the cassava vein mosaic virus (CsVMV) (Verdaguer *et al.* 1996); and the ubiquitin 3 from *Arabidopsis thaliana* (UBQ3) (Norris *et al.* 1993).

Materials and methods

Construction of expression vectors: All vectors utilised were based on the binary vector pCAMBIA 1391Z (kindly provided by Dr. Jefferson, Cambia, Australia). This vector contains the hygromycin phosphotransferase (*hpt*) gene as a selectable marker, a promoterless *gus* coding region, and the 3' polyadenylation signal of the *nos* gene. The *gus* coding region contains a castor bean catalase intron to prevent its expression in the prokaryotic systems (Tanaka *et al.* 1990). The CsVMV, Sam-s and 35S-D promoter sequences were cloned between the *Hind*III and *Nco*I sites of the polylinker located 5' of the *gus* coding region, whereas the UBQ3 promoter was inserted between the *Hind*III and *Bam*HI sites. The binary vector pCAMBIA 1301, which contains the CaMV35S-driven *gus* gene, has the same plasmid backbone as pCAMBIA 1391Z. *Agrobacterium* strain EHA105 containing the disarmed plasmid pEHA105 was then transformed with each obtained construct by the freeze-thaw method (Holsters *et al.* 1978)

The binary vector pBRA3GUS was utilised to study the expression of *gus* under the control of the tissue-specific *rolA* promoter (Guivarc'h *et al.* 1996). This vector contains the neomycin phosphotransferase II (*npt* II) gene as plant selectable marker conferring kanamycin resistance. The disarmed *Agrobacterium* strain LBA4404 harbouring pBRA3GUS was kindly provided by Dr. Carneiro (Embrapa, Brazil).

***P. tremula* × *P. alba* transformation:** Co-cultivation experiments with internode segments of *P. tremula* × *P. alba* were performed with *Agrobacterium* strains harbouring each construct, essentially as described by Leplé *et al.* (1992). The culture conditions for plant regeneration was 24 ± 2 °C with either a 16-h photoperiod or darkness, depending on the regeneration step of the protocol. A concentration of 10 mg dm^{-3} of hygromycin or 50 mg dm^{-3} of kanamycin was adopted for selection, based on preliminary experiments (data not shown). Independently transformed lines (15 - 20 plants of each construct) were transferred to micropropagation medium M1/2 (Leplé *et al.* 1992) supplemented with cefotaxime 150 mg dm^{-3}, and analysed for GUS activity (histochemical and fluorimetric assays) 35-40 d after transfer. Plants were then subcultivated in the M1/2 medium each 45 d.

GUS histochemical assays: Histochemical GUS activity was examined by incubating leaves, stems and roots of putative transformed and non-transformed plants at 37 °C for 14 - 16 h in reaction buffer containing X-Gluc (McCabe *et al.* 1988). For plants transformed with pBRA3GUS, stems and roots were sliced transversally before incubation to better visualise *gus* expression. Tissues were distained with ethanol 70 % before visual analysis.

Southern blot analysis: Genomic DNA was extracted from approximately 180 mg of leaves from 60-d-old putative transformed *P. tremula* × *P. alba* plants, essentially as described by Rogers and Bendich (1988).

DNA (10 μg) was digested with *Hind*III (plants transformed with pCsVMV/GUS and p35S-D/GUS) or with *Eco*RI (plants transformed with pCAMBIA 1301, pSAMS/GUS and non-transformed plants) using standard conditions. Digested DNA was electrophoresed on agarose gel 1 % overnight and then transferred to nylon *Hybond* N^+ membrane (*Amersham Biosciences*, Buckinghamshire, UK) by capillarity. The membrane was probed with a fragment of the *hpt* coding region (1 kpb) and labelled with α[-^{32}P] dCTP by random priming, using the *Ready-to-Go*™ Kit (*Amersham Biosciences*) following the manufacturer's instructions. The membrane hybridisation and washing were done under stringent conditions as previously described (Brasileiro *et al*. 1992).

GUS fluorimetric assays: Plants (10 - 15) transformed with each construction and derived from independent transformation events (*e.g.* different calli) were chosen for GUS protein extraction. Approximately 10 mg of fresh tissue (leaf, stem and root) from plants grown on micropropagation medium (M1/2) for 35 - 40 d were macerated for protein extraction in 1 cm^3 of GUS extraction buffer (0.2 M sodium phosphate buffer, pH 7.0; 500 mM EDTA, pH 8.0; 0.1% Triton X-100, and 10 mM β-mercaptoethanol). GUS protein extracts were assayed using 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate and measuring the production of 4-methylumbelliferone (MU), as described by Jefferson *et al*. (1987). An aliquot of total protein extract (50 mm^3) was added to the assay buffer (1 mM MUG dissolved in GUS extraction buffer) before incubation at 37 °C. After 15, 30 and 45 min of incubation, 20 mm^3 aliquots of the reaction mixture were added to 0.2 M Na_2CO_3 stop buffer. Fluorescence was measured in the fluorometer *DyNA QUANT*™ *200* (*Hoefer Inc*., San Francisco, USA) with an excitation/emission of 460/365 nm. Non-transformed poplar tissues were used as negative control. Total protein concentration was measured using the Protein Assay Kit (*BioRad*, Hercules, USA). Each extract was measured at least twice. Data were statistically analysed by the Student *t*-test ($P = 0.05$).

Results and discussion

Transformation efficiency: An average transformation efficiency of 45 % was obtained for *P. tremula* × *P. alba*, 80 d after co-cultivation, independent of the binary vector used. The transformation efficiency was determined as the number of transformed shoots obtained per total number of co-cultivated explants (internode segments). The number of transformed shoots per callus was up to 20 in most cases, but only a single shoot was isolated from each callus for root induction to ensure that each shoot is an independent transformation event. Ten to 15 putative independent transformed lines for each construct were selected for further molecular and GUS analyses.

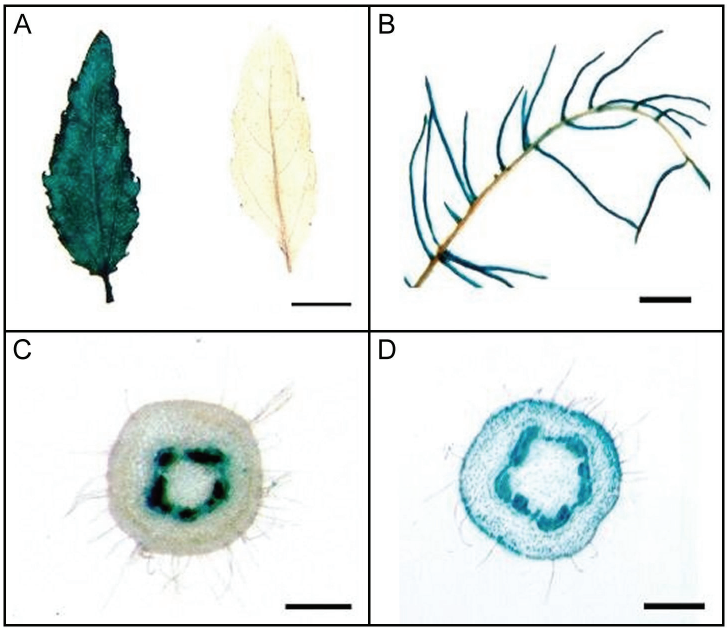

Fig. 1. *In situ* GUS staining of *P. tremula* × *P. alba* plants transformed with pSAMS/GUS (*A*, *B* and *D*) and with pBRA3GUS (*C*) binary vectors. *A* - leaves from transgenic (*left*) and non-transgenic (*right*) plants (*bar* = 1.5 mm); *B* - typical root staining of secondary roots and root tips, observed for all constitutive promoters tested (*bar* = 0.8 mm); *C* - GUS staining limited to the vascular root system; *D* - GUS staining in whole stem section, observed for all constitutive promoters tested (*bar* = 0.3 mm).

Expression patterns of *gus*: Histochemical assays showed a similar pattern of *gus* expression under the control of the CaMV35S, 35S-D, CsVMV and UBQ3 promoters in transgenic *P. tremula* × *P. alba*. GUS activity was detected throughout the leaf (Fig. 1*A*) and stem tissues analysed. However, in roots, *gus* expression was mainly observed in the tips and lateral roots (Fig. 1*B*). Similar results have been reported for constitutive promoters in roots of *Pinus contorta*, *Lycopersicon esculentum*, *N. tabacum*, and *A. thaliana* (Holtorf *et al*. 1995, Yibrah *et al*. 1996, Lindroth *et al*. 1999).

The *gus* expression pattern of *rolA* promoter in transgenic poplar plants showed specificity for vascular tissues (phloem and xylem; Fig. 1*C*). No GUS activity could be detected in leaves and roots tissues by histochemical assay. Guivarc'h *et al*. (1996) observed similar expression pattern in *N. tabacum* plants transformed with the same construct (pBRA3GUS). Phloem and xylem tissue-specific gene expression by *rolA* promoter in transgenic forest species could be of great importance for altering lignin biosynthesis or other wood-modifying genes. Down-regulation of lignin biosynthesis genes by a vascular-specific promoter such as *rolA*, *e.g.*, could avoid the putative problems associated with increased vulnerability to pathogens and undesirable impacts on other non-target tissues, when a strong constitutive promoter is used (Grima-Pettenati and Goffner 1999, Gittins *et al*. 2003).

Plants transformed with pSAMS/GUS showed GUS staining in whole leaves and stems (Fig. 1*D*) and in lateral root primordia and root apex, as observed for the other constitutive promoters tested. These results differ from previous reports using the Sam-s promoter isolated from *A. thaliana*. Peleman *et al.* (1989a,b) fused this promoter to the *gus* coding region and analysed its expression pattern in transgenic *A. thaliana* and *N. tabacum*. The chimeric construct directed *gus* expression mainly in the vascular tissues throughout the plant (root, stem and leaf veins). Moreover, Mijnsbrugge *et al.* (1996) using the same Sam-s promoter from *A. thaliana* showed a similar *gus* expression pattern in phloem and cortex tissues of transgenic *P. tremula* × *P. alba* plants, with intense staining in emerging lateral roots and root tips. Differences in *A. thaliana* and *G. max Sam-s* gene regulation could explain the variation in gene reporter expression patterns.

Southern blot analysis: Four putative transgenic plants transformed with each construct were analysed by Southern blot using an *hpt* probe. The copy number of the transferred *hpt* gene was determined by *Eco*RI or *Hind*III digestion of genomic DNA from transgenic plants. These digestions included the left border T-DNA junction, and resulted in fragments of different lengths, each indicating at least a separate integration event (Fig. 2).

The Southern blot analysis confirmed the integration of *hpt* gene into the genome of the putative transgenic poplar plants analysed. Each of the 4 analysed plants gave specific hybridisation patterns, confirming that these plants represent independent transformation events. The hybridisation pattern indicates that there were between one to four copies of the insert per poplar genome (Fig. 2). Rearrangements of the transgene constructs in plant cells may also contribute to the length variation. No bands were detected for the non-transformed control. In the plasmid DNA positive control, the probe detected a band of approximately 11 kbp, corresponding to the linear pCAMBIA 1391Z plasmid, digested with *Eco*RI.

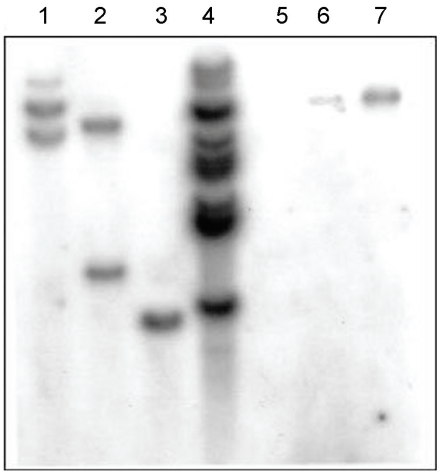

Fig. 2. Southern analysis of transformed *P. tremula* × *P. alba* plants. *Lanes 1-4*: DNA from plants transformed with the following constructs: p35S-D/GUS, pSAMS/GUS, pCAMBIA 1301, and pCsVMV/GUS, respectively; *lane 5*: DNA from non-transgenic plant (negative control); *lanes 6 - 7*: DNA from pCAMBIA 1391Z binary vector (45 and 90 pg, respectively) digested with *Eco*RI (positive control).

Promoter strength: Wide variation in *gus* expression was observed in all tissues analysed from independently transformed plants, even in plants containing the same promoter (Fig. 3). Although these values were not significantly different (P = 0.05), plants expressing *gus* regulated by UBQ3 promoter showed the highest average GUS activity in all tissues analysed [497.6, 799.6 and 907.9 mmol(MU) g^{-1}(protein) min^{-1} in leaves, stems and roots, respectively]. On the other hand, plants transformed with p35S-D/GUS showed the lowest average GUS activity values in all analysed tissues. The expression level obtained under the control of CsVMV, CaMV35S and Sam-s promoters showed intermediate levels. The GUS activity of pBRA3GUS derived plants was not analysed by the quantitative fluorimetric assay as it was shown to be tissue-specific.

The variation in copy number of the integrated T-DNA among the plants analysed by Southern blot (Fig. 2) could explain the wide variation range in GUS activity observed. Other reports analysing *gus* expression regulated by heterologous promoters in transgenic plants, also showed a wide range of variation in GUS activity, without apparent correlation between copy number and the levels of *gus* expression (Datla *et al.* 1993, Kamo *et al*. 2000). Thus, the wide variation in GUS activity

observed in our study may not be explained only by transgene copy number. Position effect, gene silencing and other complex events, perhaps related to the poplar transcription machinery, may also influence gene expression (Kooter *et al*. 1999, Kumar and Fladung 2001).

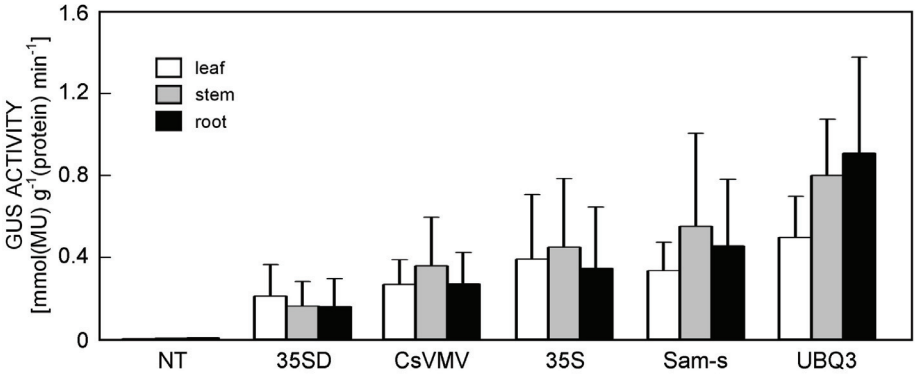

Fig. 3. Effect of promoter on level of GUS activity in leaves, stems and roots of transgenic *P. tremula* × *P. alba* plants grown *in vitro.* The specific GUS activity (mean ± SE) for each promoter was determined from 10 - 15 independent transformants by two fluorimetric assays. All plotted values are not significantly different as determined by the *t*-test ($P = 0.05$). NT = non-transformed plants.

Constitutive promoters: Several reports describe the positive effect on expression level of genes under control of 35S-D promoter placed in tandem and adjacent to the untranslated enhancer sequence from alfalfa mosaic virus (AMV) (Datla *et al*. 1993, Lacorte and Barros 2000). In our study, GUS activity under the 35S-D promoter in leaves, stems and roots was similar or even lower than for the single CaMV35S promoter (Fig. 3). In this case, some post-transcriptional factors in poplar could be influencing GUS activity. The lack of correlation between transcriptional level and enzyme activity was previously observed in *P. tremula* × *P. alba* transformed with acetolactate synthase mutant gene (*crs1-1*) under the control of its own promoter and a doubled CaMV35S promoter (Brasileiro *et al*. 1992).

Verdaguer *et al*. (1996) showed that the CsVMV promoter was as active as the CaMV35S in *N. tobacum* and *Oryza sativa*. In our study, poplar plants transformed with the pCsVMV/GUS also showed a similar GUS activity when compared to the CaMV35S (Fig. 3). Therefore this promoter represents an alternative for constitutive transgene expression in poplar.

Norris *et al*. (1993) observed that UBQ3 promoter lead to GUS activity in all organs and tissues of *A. thaliana*. These results indicated that this promoter could be used to constitutively express coding regions in transgenic plants in general. Indeed, similar results were obtained for poplar, *e.g.*, plants transformed with pUBQ3/GUS showed constitutive GUS activity in leaves, stems and roots (Fig. 3).

In summary, we have investigated the expression level and pattern of *gus* expression driven by the *rolA*, Sam-s, 35S-D, UBQ3, CsVMV and CaMV35S promoters in stably transformed *P. tremula* × *P. alba* plants. Tissue-specific *gus* expression in the vascular system was obtained using *rolA* promoter, which could be exploited for down regulation of lignin in transgenic trees or other wood-modifying genes. The Sam-s, CsVMV and UBQ3 promoters could be used in experiments with *P. tremula* × *P. alba* where a high constitutive level of the transgene expression is required, and possibly also in other woody species. These promoters represent an alternative for the CaMV35S promoter for regulating transgene expression, perhaps avoiding or minimizing problems associated with silencing and hence, assuring high constitutive and stable expression of foreign genes in transgenic trees.

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