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Note

High-level gene expression in differentiating xylem of tobacco driven by a 2.0 kb *Poplar COMT2* promoter and a 4×35S enhancer

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Abstract Promoter constructs with high levels of xylem specific expression are needed to obtain efficient expression of candidate genes, microRNAs (miRNAs) and artificial microRNAs (amiRNAs) for the genetic modification of wood properties. The gene for caffeic acid *O*-methytransferase (*PtrCOMT2*) has the second most abundant transcript level of all the genes in monolignol biosynthesis in *Populus trichocarpa* and a high level of specificity in differentiating xylem. To characterize the *PtrCOMT2* promoter, we cloned a short (2.0 kb) and a long (3.3 kb) promoter segment and compared their expression using *GUS* as a reporter gene in the differentiating xylem of *Nicotiana tabacum*. Both the 2.0 kb and the 3.3 kb promoter segments showed high specificity for differentiating xylem in this heterologous system. GUS activity increased as much as 5 times when the 4×35S enhancer was inserted in front of the 2.0 kb promoter, but GUS activity was only increased 2 times when the enhancer was inserted behind the promoter. The enhancer inserted upstream reduced the expression of the 3.3 kb promoter. While expression of some of the enhancer-plus-promoter constructs increased expression, there was a loss of specificity.

Key words: *PtrCOMT2* promoter, xylem-specific, 4×35S enhancer, woody plants, genetic engineering.

The promoter of a gene is one of the most important factors affecting the abundance and tissue specificity of heterologous protein production (Streatfield 2007). Tissue-specific promoters are required when constitutive expression of proteins in plants is undesirable or when tissue specific regulation of gene expression is needed. For example, in wheat, constitutive expression of a *Bacillus subtilis* xylanase and an *Aspergillus niger* ferulic acid esterase, reduced growth and induced sterility, while endosperm-specific expression of these two enzymes did not (Harholt et al. 2010). Similarly, the expression of the same fungal ferulic acid esterase in tall fescue was improved with specific or inducible promoters compared to constitutive promoters (Buanafina et al. 2008).

High-level specific expression is important when miRNAs are used as dominant suppressors of gene activity to study gene-specific function. MiRNA suppressors act as leaky mutations because the level of suppression depends upon the level of miRNA produced. The higher the level of miRNA, the higher the level of suppression expected.

There are many genes involved in wood formation, and a substantial fraction are genes of unknown function. Inverted repeat RNA (RNAi) and amiRNA-mediated gene silencing have been used to study gene function in wood formation (Coleman et al. 2008; Shi et al. 2010a). Many genes related to wood formation are also essential for stress responses, disease resistance and other metabolic pathways (Chen et al. 2000; Shi et al. 2010b). For these reasons, strong xylem-specific promoters would advance studies of gene function in wood formation and would also advance the technology of engineering proteins to modify wood properties.

Shi et al. (2010b) identified 18 xylem-specific genes in *P. trichocarpa* encoding monolignol biosynthesis enzymes during wood formation. Among the 18 genes, *PtrCOMT2* encodes a single caffeic acid *O*-methyltransferase that catalyzes the 3′OH methylation of caffeate, 5-hydroxyferulate and 5-hydroxyconiferyl aldehyde for the biosynthesis of monolignols. *PtrCOMT2*

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encodes the second most abundant transcript of all the monolignol genes specific to differentiating xylem, slightly less abundant than the transcripts encoding cinnamyl alcohol dehydrogenase (PtrCAD1) (Shi et al. 2010b).

COMT promoters from maize, tobacco and birch were reported to be xylem-specific and responsive to diverse biotic and abiotic stresses (Capellades et al. 1996; Tiimonen et al. 2007; Toquin et al. 2003). Here, we report on the expression of two cloned promoter fragments derived from the *PtrCOMT2* gene and compare their activity using *GUS* as a reporter in the heterologous system of *N. tabacum* differentiating xylem. We have followed the expression of these constructs in tobacco because of the relatively short time needed to obtain the differentiating xylem and the relative conservation of tissue specificity previously observed between these two species (Tiimonen et al. 2007; Toquin et al. 2003). We have tested these promoter fragments with and without insertion of a 4×35S enhancer (Kay et al. 1987) and have found that the 2.0 kb *PtrCOMT2* promoter with the 4×35S enhancer inserted in front of the promoter conferred the highest GUS expression.

Materials and methods

Plant materials

Tobacco (*N. tabacum*) KY14 seeds were sterilized with 10% Clorox for 10 min and rinsed five times with sterile water. The sterile seeds were spread onto a solid 1/2 strength MS medium (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose, 1×B5 vitamins (Gamborg et al. 1968) and 100 mg l^{-1} myo-inositol. All plants were kept at 25°C under a 16/8 h light/dark cycle. *P. trichocarpa* (Nisqually-1) genomic DNA was isolated from fresh leaves with a DNeasy Plant Mini kit (Qiagen, Alameda, CA, USA). Vector constructs were maintained in *Escherichia coli* TOP10 and *Agrobacterium tumefaciens* C58.

Vector construction

Two fragments, a 2.0 kb proximal and a larger overlapping 3.3 kb fragment, of the *PtrCOMT2* (GeneBank accession no. EU603317.1) promoter were chosen, based on the genomic sequence of *P. trichocarpa* (Figure 1) (Tuskan et al. 2006). Genomic DNA was used to amplify both fragments. The primers for the 2.0 kb promoter fragment were 5′-AAGCTTCAT ATT CGA TCA AGG AAA TTA ACA CC-3′ (2.0kbF) and 5′-GGATCCTCT AGA AAC AAA AGG TTG AAG AAG GTG -3′ (2.0kbR), and the primers for the 3.3 kb promoter fragment were 5′-AAGCTTGTC TCA GTT TCC GTA TTA CGA CTT G-3′ (3.3kbF) and 5′-TGGATCCGGA AAC AAA AGG TTG AAG AAG GTG -3′ (3.3kbR). A *Hin*dIII restriction site (underlined) was added to the 5′ end of both promoter fragments and a *Bam*HI site (underlined) was added to the 3′ end of both fragments during amplification. Both

Figure 1. Schematic structure of the *PtrCOMT2* promoter (the upstream sequence of the *PtrCOMT2* gene in *P. trichocarpa*). The numeration starts with the translation initiation site. There is an *Eco*RI restriction site located at −2495. *Hin*dIII restriction sites were introduced by PCR at the 5′ ends of both promoter fragments and *Bam*HI sites were introduced at the 3′ ends.

promoter fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI, USA), with *E. coli* TOP10 as the host strain, and were confirmed by sequencing. The 4×35S enhancer fragment was excised with *Eco*RI from pTAG-8 (Hsing et al. 2007), cloned into pBluescript II Sk (+) (Stratagene, La Jolla, CA, USA), and was confirmed by sequencing, giving SK-35S. To introduce a *Hin*dIII restriction enzyme site at the 3′ flank of the 4×35S enhancer, we used another construct, pCR4-EuCesA2P, in which a 1.7kb eucalyptus cellulose synthase gene promoter was cloned in the pCR4 TOPO vector (with a *Hin*dIII site and a *Xba*I site at each end). The promoter was excised with *Pme*I/*Xba*I from pCR4-EuCesA2P and inserted in SK-35S, which was digested with *Sma*I/*Xba*I, resulting in SK-35S-CesA2P.

Constructs A and D were obtained by replacing the 35S promoter in pBI121 with the two promoter fragments excised from the pGEM-T easy vector at the *Hin*dIII/*Bam*HI sites. The 4×35S enhancer fragment was excised from SK-35S-CesA2P with *Hin*dIII and cloned in front of the *PtrCOMT2* promoter at the *Hin*dIII site of constructs A and D, resulting in constructs C and E. Similarly, the 4×35S enhancer fragments were excised from SK-35S with *Eco*RI and cloned behind the *PtrCOMT2* promoter at the *Eco*RI site of construct A, resulting in construct B. These five constructs were confirmed by sequencing (Figure 2).

Plant transformation

Transformation of the constructs into tobacco plants was performed using the *A. tumefaciens*-mediated leaf disc method of Horsch et al. (1988) and with our derivatives of the binary vector pBI121 (Chen et al. 2003; Figure 1). Transformed cells were selected on medium containing 50 mg l^{-1} kanamycin and were regenerated into plants (Horsch et al. 1988). The plants were moved to a greenhouse and were maintained for two months, when plant stems were collected for *GUS* gene expression analysis and enzyme assays.

qRT-PCR

Total RNA was extracted from the differentiating xylem of transgenic tobacco plants using RNeasy Plant Mini Kits (Qiagen, Alameda, CA, USA) and genomic DNA was removed with RNase-free DNase I (Promega, Madison, WI, USA). 150 ng of total RNA was reverse-transcribed to cDNA with TaqMan Reverse Transcription Reagents (Roche, Mannheim, Germany). Real-time PCR was conducted using an Applied

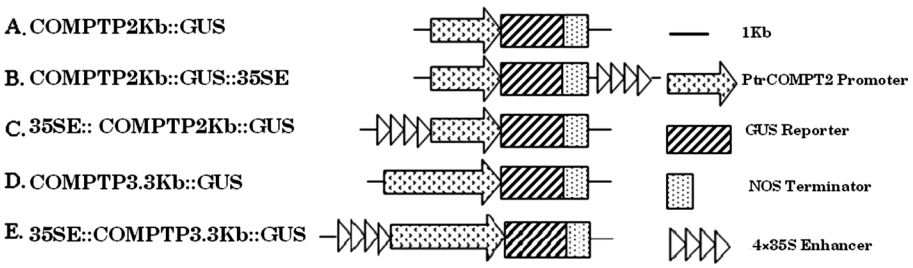

Figure 2. Schematic description of the constructs with selected fragments of the *PtrCOMT2* promoter and the 4×35S enhancer. *GUS* expression was driven by the 2.0 kb *PtrCOMT2* promoter in constructs A, B and C, while *GUS* was driven by the 3.3 kb *PtrCOMT2* promoter in constructs D and E. The 4×35S enhancer was inserted in front of *PtrCOMT2* promoter in constructs C and E and was inserted behind the *PtrCOMT2* promoter in constructs B. COMTP2kb, a 1967 bp long segment of the *PtrCOMT2* promoter; COMTP3.3kb, a 3341 bp long segment of the *PtrCOMT2* promoter; *GUS*, the *β-glucuronidase* gene; NOS terminator, the nopaline synthase terminator; 35SE, 4×35S Enhancer, containing four tandem repeats of the 35S enhancer.

Biosystems 7900HT sequence detection system. For each reaction, a 25 μl mixture contained the first-strand cDNA (equivalent to 1 ng of total RNA), 5 pmol *GUS* gene-specific primers GUS600F (5′-TCT GTT GAC TGG CAG GTG GT-3′) and GUS800R (5′-AGC GGG TAG ATA TCA CAC TC-3′), and 12.5 μl of the Fast Start Universe SYBR Green Master Mix (Roche, Mannheim, Germany). After PCR amplification (95°C, 10 min; 45 cycles at 95°C, 15 s; and 60°C, 1 min), a thermal denaturing cycle (95°C, 15 s; and 60°C, 15 s) was added to derive a dissociation curve of the PCR product to verify amplification specificity. Each reaction was repeated three times. PCR products were quantified using the pBI121 plasmid DNA as a standard to establish a quantitative correlation between the copy number of the *GUS* gene transcripts and the Ct values (Scheurer et al. 2007). The transcript copy numbers were calculated from the plasmid concentrations after serial dilution (10^0 to 10^{-5} pg ml^{-1}).

GUS assays

The histochemical localization of *GUS* expression in transgenic tobacco was conducted as described by Vitha et al. (1995). Hand-cut tobacco stem sections and petioles were immersed in a GUS staining solution containing 1 g l^{-1} 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), 50 mM sodium phosphate buffer (pH 7.4), 0.1% Triton X-100, 10 mM EDTA, and 2 mM potassium ferricyanide. After 30 min of vacuum infiltration, the sections were incubated in the dark at 37°C until the blue indigo color appeared. Then the samples were put in 70% (v/v) ethanol to remove chlorophyll and other pigments. Pictures were taken using bright field light microscopy (Zeiss Stemi DV 4) with a Zeiss AxioCam MRc.

Fluorometric GUS assays were conducted following Jefferson et al. (1987). One hundred milligrams of transgenic tobacco stem tissue without bark or pith was ground to a fine powder in liquid nitrogen using a mortar and pestle. The total protein was extracted from the powder with 800 μl of GUS extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% (w/v) sodium lauroyl sarcosine, 0.1% (v/v) Triton X-100, 10 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, USA). Extracts were clarified by centrifugation at 14,000×*g* at room temperature. The supernatants were used for enzyme assays and protein determinations. Total protein concentration was determined using the Bio-Rad Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA). Five microliters of each extract was added to a 245 μl reaction containing 1 mM of 4-methylumbelliferyl-β-D-glucuronide trihydrate (4-MUG). The reactions were incubated at 37°C for 2 h. Five microliters of the reaction was transferred to an eppendorf tube, and 995 μl of 0.2 M Na_2CO_3 was added to stop the reaction. The concentration of 4-methylumbelliferone (4-MU) released was measured by fluorescence (excitation at 365 nm, emission at 460 nm) in a TD-700 fluorometer calibrated with a series of 4-MU standards. GUS activity was calculated in units of *pmoles* 4-MU produced min^{-1} g^{-1} protein.

Results

Production of transgenic tobacco plants

Using *A. tumefaciens*-mediated transformation, we produced transgenic tobacco plants containing the constructs A, B, C, D and E individually (Figure 2). Fourteen transgenic lines from each construct were planted in the greenhouse and maintained for two months. All the transgenic plants had normal growth and indistinguishable phenotypes compared to wild-type plants, as expression of *GUS* does not affect the development or growth of transformants (Jefferson et al. 1987).

The 4×35S enhancer increased the expression of the 2.0 kb PtrCOMT2 promoter

QRT-PCR was performed with total RNA from transgenic tobacco xylem tissue toobtain absolute copy number estimates of the *GUS* gene transcripts under the control of different promoter constructs (Figure 3). The objective of these experiments was to obtain quantitative estimates of the activity of the promoter derivatives and the relative efficiency of different insertions. COMTP2kb::GUS and COMTP3.3kb::GUS had similar

GUS gene transcript levels, suggesting that the 2.0 kb promoter contains all of the *cis*-elements for normal *PtrCOMT2* expression. Addition of the 4×35S enhancer segment increased the activity of the 2.0 kb promoter. The *GUS* expression in 35SE::COMTP2kb::GUS with the 4×35S enhancer inserted in front of the *PtrCOMT2* promoter (Construct C) was as much as 4 times higher than that in COMTP2kb::GUS without the 4×35S enhancer (Construct A). In COMTP2kb::GUS::35SE with the 4×35S enhancer inserted behind the promoter (Construct B), the *GUS* expression was increased up to one and a half-fold compared to the equivalent transgenic plants without the enhancer. Unexpectedly, the *GUS* transcripts were decreased when the 4×35S enhancer was inserted in front of the 3.3 kb promoter (Construct E) compared to that of the 3.3 kb promoter only (Construct D). The different effects of the 4×35S enhancer on the expression activity of *PtrCOMT2* promoter derivatives may be attributed to the location and its different interactions (Zhao et al.1999). We cannot rule out whether DNA rearrangement may have interrupted the function of COMTP3.3kb and resulted in decreased expression in 35SE::COMTP3.3kb::GUS.

Fluorometric assays were also conducted to examine the GUS activity in different constructs using total crude protein extracted from tobacco xylem tissues. The highest GUS activity came from the 35SE::COMTP2kb::GUS (Construct C) with up to 356.3 units (*pmoles* 4-MU $min^{-1} \mu g^{-1}$ protein), followed by COMTP2kb::GUS::35SE (Construct B), COMTP3.3kb::GUS (Construct D), COMTP2kb::GUS (Construct A) and 35SE::COMTP3.3kb::GUS (Construct E) respectively (Figure 4). The GUS activity was increased by as much as 5 fold in 35SE::COMTP2kb::GUS and as much as 2 fold in COMTP2kb::GUS::35SE compared with COMTP2kb::GUS; while GUS activity was decreased by 50% when the 4×35S enhancer was inserted in front of the 3.3 kb promoter.

PtrCOMT2 is regulated at the transcriptional level

The expression pattern of the tobacco *COMT* II gene is determined in considerable part at the transcriptional level because the changes of *COMT* II promoter activity and endogenous COMT II enzyme activity are correlated (Toquin et al. 2003). Our study showed that the expression of the heterologous *PtrCOMT2* promoter is also regulated at the transcriptional level, because the GUS enzyme activity in the GUS fluorometric assays for all constructs conducted using tobacco xylem tissues was strongly correlated (R^2=0.885) with the *GUS* transcript level obtained by qRT-PCR (Figure 5).

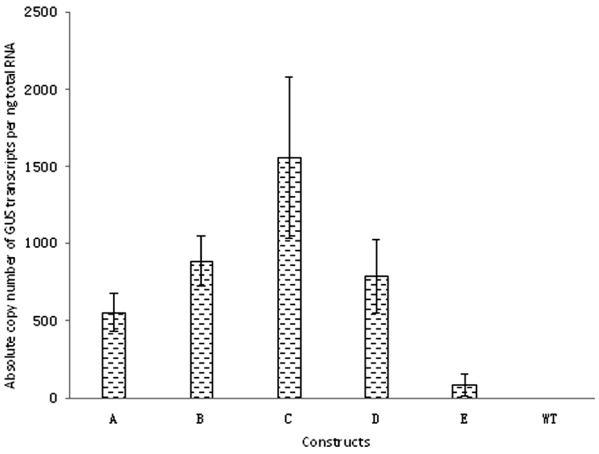

Figure 3. The average (bars added for one SEM) of the transcript abundance of *GUS* in the differentiating xylem tissue of transgenic tobacco of each construct. Fourteen lines for each construct have been analyzed. Each reaction was repeated 3 times. A, construct COMTP2kb::GUS; B, construct COMTP2kb::GUS::35SE; C, construct 35SE::COMTP2kb::GUS; D, construct COMTP3.3kb::GUS; E, construct 35SE::COMTP3.3kb::GUS; WT, wild-type tobacco plants.

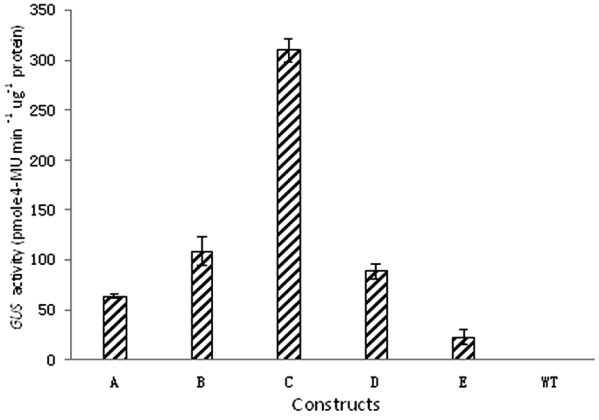

Figure 4. The average (bars added for one SEM) GUS fluorometric activity of the transgenic plants. For each construct, 14 lines were analyzed. The crude protein extracts from differentiating xylem were used for assays, and each assay was repeated 3 times. A, construct COMTP2kb::GUS; B, construct COMTP2kb::GUS::35SE; C, construct 35SE::COMTP2kb::GUS; D, construct COMTP3.3kb::GUS; E, construct 35SE::COMTP3.3kb::GUS; WT, wild-type tobacco plants.

The 4×35S enhancer affected the specificity of the PtrCOMT2 promoter

Promoter specificity is a primary criterion of the utility of a promoter in genetic engineering. For example, the non-floral expression of a cytotoxin-containing construct intended to induce sterility in poplar displayed by floral promoters can cause poor growth of transgenic trees (Wei et al. 2006). Many lignin biosynthetic genes expressed during wood formation may also be expressed during the defense response (Bhuiyan et al. 2009) or in other developmental or metabolic pathways (Savidge 1987). The non-xylem "background" expression of xylem specific promoters used for silencing (or overexpressing)

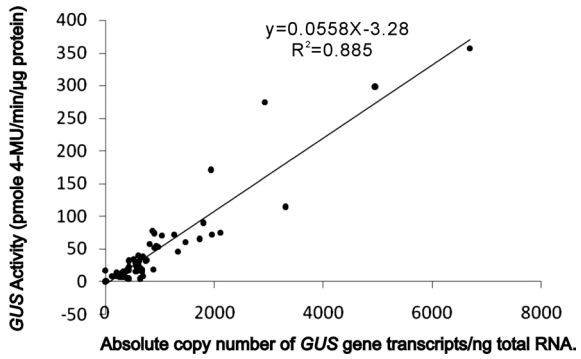

Figure 5. The relationship of GUS activity and the gene transcript copy number of *GUS* gene transcripts in the differentiating xylem in all the constructs. Separately, R^2 values are 0.768, 0.629, 0.926, 0.926 and 0.978 for constructs A, B, C, D, and E, respectively.

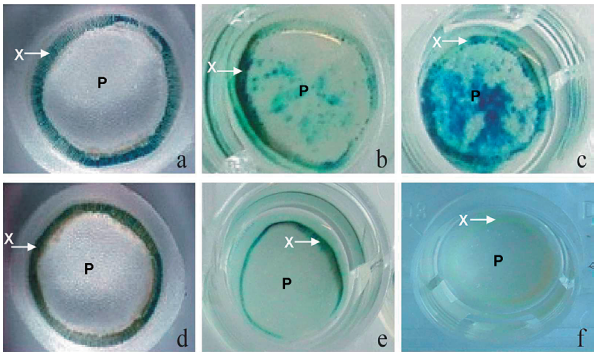

Figure 6. GUS histochemical assays of stem cross sections from transgenic tobacco. a, Construct A (COMTP2kb::GUS); b, Construct B (COMTP2kb::GUS::35SE); c, Construct C (35SE::COMTP2kb::GUS); d, Construct D (COMTP3.3kb::GUS); e, Construct E (35SE::COMTP3.3kb::GUS); f, WT(wild type). X: xylem. P: pith.

of other wood biosynthesis genes may affect the stress response or change the development or adaptation of trees. When introducing the 2.0 kb and 3.3 kb *PtrCOMT2* promoter-driving *GUS* gene into tobacco, the transgenic tobacco displayed xylem-specific expression (Figures 6a and 6d). However, after insertion of the 4×35S enhancer, the 2.0 kb *PtrCOMT2* promoter reduced its specificity with increased expression in pith and phloem (Figures 6b and 6c).

Discussion

It may be argued that a heterologous promoter may have species-specific effects on gene expression and such effects have been observed (Nilsson et al. 1996). However, it is important to study multiple promoters in heterologous systems because of the need to use heterologous promoters in "gene stacking" constructs to avoid gene silencing. In other studies we have tested five promoters of *P. trichocarpa* xylem-specific genes, *PtrGT8D1*, *PtrGT8D2*, *Ptr4CL3*, *PtrCAD1*, and *PtrCOMT2*, in tobacco and GUS expression driven by these five promoters all displayed a xylem-specific pattern (Li et al. 2011, and unpublished). We have used these five promoters to drive expression of 20 candidate genes in *P. trichocarpa*. The transgene expression is xylem-specific as predicted by the results in tobacco (unpublished). In these cases, at least, tobacco has been a reliable and rapid screen for xylem-specific expression.

Table 1. The sequences and positions of motifs in the *PtrCOMT2* promoter.

Motif	Position	Sequence	Strand
X_3 (1)	−1801	GGTGGGGGGT	Negative strand
X_3 (2)	−207	GGTAGGGAGT	Positive strand
XL_12	−156	GGGACCAACC	Positive strand
XL_13	−154	GTGGTTGGTC	Negative strand

The *PtrCOMT2* promoter has the potential to regulate specific high-level gene expression in transgenic woody plants, for genetic engineering of wood properties. In previous work Shi et al. (2010b) identified four motifs, including one XL-13, one XL-12, and two X-3 core motifs, in the *PtrCOMT2* promoter involved in abundant xylem-specific expression. The sequences and position of the four motifs are shown in Table 1. These four motifs are among the five motifs identified in the promoters of all the key monolignol biosynthesis genes in *P. trichocarpa* (Shi et al. 2010b). These motifs are similar in sequence to *cis*-regulatory elements previously identified in lignin genes or vascular gene expression in poplar, Arabidopsis and other plant species (PLACE: http://www.dna.affrc.go.jp/PLACE/; Higo et al. 1999) and are usually located within the proximal 600 bp of the translation start site (Shi et al. 2010b).

The utility of a promoter depends not only on its specific activity, but also on the extent that it has been characterized in different genetic environments. In previous studies directed to the genetic modification of wood properties, the most widely used promoter constructs have been driven by the constitutive CaMV 35S promoter (Baucher et al. 1998; Coleman et al. 2008; Hu et al. 1999; Zhong et al. 2000). Xylem-specific promoters such as that of cinnamyl alcohol dehydrogenase (CAD) (Feuillet et al. 1995; Jouanin et al. 2000; Wagner et al. 2009), phenylalanine ammonia-lyase (PAL) (Gray-Mitsumune et al. 1999; Guo et al. 2001), 4-coumarate: CoA ligase (4CL) (Li et al. 2003), caffeoyl CoA-3-*O*-methyltransferase (CCoAOMT) (Chen et al. 2000), and caffeate/5 hydroxyferulate *O*-methyltransferase (COMT) (Tiimonen et al. 2007) were studied and applied to modifying wood properties. However, not all these promoters were ideal for genetic engineering of woody plants. Reduced growth was observed when a constitutive CaMV 35S promoter was used to down-regulate *CCR* and when the poplar *4CL1* gene was used to drive antisense *4CL1* in a poplar

hybrid (*P. tremula*×*alba*) (Leple et al. 2007; Voelker et al. 2010). Only a 2-fold increase of COMT activity was obtained when either a construct with a double CaMV 35S promoter or a eucalyptus *CAD* promoter was used to overexpress *COMT* in a hybrid poplar (*P. tremula*×*alba*) (Jouanin et al. 2000). *CAD* promoters may have expression in other tissues such as leaves (Feuillet et al. 1995) and therefore ectopic expression of the *CAD* promoter may affect the normal growth of transgenic trees.

We have used the Cauliflower Mosaic Virus (CaMV) 35S enhancer in our experiments because it is well known to increase expression in many chimeric promoters (Kay et al. 1987; Mitsuhara et al. 1996). Its high level of expression made possible T-DNA activation tagging to overexpress and identify regulators of metabolic pathways and to identify critical genes controlling plant growth and development (Weigel et al. 2000). The 35S enhancer activates transcription of adjacent genes both upstream and downstream of its insertion site (Borevitz et al. 2000; van der Fits et al. 2001). In Arabidopsis, activation was detected at distances up to 3.6 kb by a tetrameric 35S enhancer (4×35S) and within 12.5 kb in rice by a 35S enhancer octamer (Hsing et al. 2007; Weigel et al. 2000). Borevitz et al. (2000) isolated a bright-purple Arabidopsis mutant, in which an adjacent CaMV 35S enhancer activated the PAP1 MYB transcription factor. This mutant displayed broad transcriptional activation of the pathway for biosynthesis of phenylpropanoid natural products in all vegetative organs throughout plant development Busov et al. (2003) transformed a hybrid poplar (*P. tremula*×*alba*) with a 4×35S construct to create a population of trees to screen for mutant phenotypes by activation tagging. They identified a gene for a gibberellin oxidase that regulates tree height (Busov et al. 2003).

In most cases, the timing and specificity of expression of genes in activation tagged mutants is not changed compared to the wild type (Jeong et al. 2002; Weigel et al. 2000). Beilmann et al. (1992) and Zhao et al. (1999) found that the CaMV 35S enhancer can have synergistic interactions with the truncated PR-1 promoter and the plasma membrane H^+-ATPase *pma*4 transcription promoter from *Nicotiana plumbaginifolia* and that it may have brought some characteristics of the CaMV 35S promoter to the chimeric promoters. Although the expression specificity of the *PtrCOMT2* promoter was affected by a 4×35S enhancer in our study, the insertion of the enhancer in front of the 2.0 kb *PtrCOMT2* promoter greatly increased gene expression, expanding the potential for the engineering of candidate genes in trees.

While many enhancers direct very precise tissue-specific expression, enhancer–promoter crosstalk is known to alter promoter specificity (Singer et al. 2010a, b; Singer and Cox 2013; Stacy et al. 2013; Zheng et al. 2007). The CaMV 35S enhancer can change the transcription initiation site in the promoter of the reporter gene and also its specificity (Singer et al. 2010a). In our experiments the *PtrCOMT2* promoter specificity was observed to change when the 35S enhancer was inserted either in front or behind the 2 kb *PtrCOMT2* promoter, but did not change the specificity of the 3.3 kb promoter. It is possible that in the former case the 35S enhancer is closer to the initiation site and therefore capable of activating GUS expression. More work needs to be done to increase our understanding of the mechanisms that underlie enhancer–promoter interactions in plants.

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