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Research

Functional characterization of the switchgrass (*Panicum virgatum*) R2R3-MYB transcription factor *PvMYB4* for improvement of lignocellulosic feedstocks

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Summary

- The major obstacle for bioenergy production from switchgrass biomass is the low saccharification efficiency caused by cell wall recalcitrance. Saccharification efficiency is negatively correlated with both lignin content and cell wall ester-linked *p*-coumarate: ferulate (*p*-CA : FA) ratio. In this study, we cloned and functionally characterized an R2R3-MYB transcription factor from switchgrass and evaluated its potential for developing lignocellulosic feedstocks.
- The switchgrass PvMYB4 cDNAs were cloned and expressed in *Escherichia coli*, yeast, tobacco and switchgrass for functional characterization. Analyses included determination of phylogenetic relations, *in situ* hybridization, electrophoretic mobility shift assays to determine binding sites in target promoters, and protoplast transactivation assays to demonstrate domains active on target promoters.
- PvMYB4 binds to the AC-I, AC-II and AC-III elements of monolignol pathway genes and down-regulates these genes *in vivo*. Ectopic overexpression of PvMYB4 in transgenic switchgrass resulted in reduced lignin content and ester-linked *p*-CA : FA ratio, reduced plant stature, increased tillering and an approx. threefold increase in sugar release efficiency from cell wall residues.
- We describe an alternative strategy for reducing recalcitrance in switchgrass by manipulating the expression of a key transcription factor instead of a lignin biosynthetic gene. PvMYB4-OX transgenic switchgrass lines can be used as potential germplasm for improvement of lignocellulosic feedstocks and provide a platform for further understanding gene regulatory networks underlying switchgrass cell wall recalcitrance.

Introduction

Switchgrass is a C4 perennial forage grass native to most areas of the North American grasslands. Since switchgrass has high biomass yield and is well adapted to marginal lands, it has been selected as a dedicated lignocellulosic feedstock for bioenergy production in the United States (McLaughlin & Adams Kszos, 2005; Bouton, 2007; Schmer *et al.*, 2008). The biomass yield of switchgrass varies according to precipitation during the growing season, annual temperature, nitrogen fertilization, and the type of cultivar (Fuentes & Taliaferro, 2002; Wullschleger *et al.*, 2010). The estimated annual biomass yield of switchgrass in the United States is projected to average 12.9 metric tons per hectare for lowland ecotypes and 8.7 metric tons for upland ecotypes (Wullschleger *et al.*, 2010).

The major barrier to efficient conversion of lignocellulose to liquid transportation fuels is the recalcitrant nature of the cell wall. The cellulose microfibrils are embedded in a matrix of hemicelluloses that are covalently linked with lignin and other aromatic phenolic compounds. These linkages, and the masking effect of lignin itself, increase the pretreatment costs and block the access of enzymes to the polysaccharide chains (Akin, 2007; Himmel *et al.*, 2007; Pauly and Keegstra, 2008). Reduction of lignin content through transgenic approaches improves fermentable sugar yield and saccharification efficiency of alfalfa stems (Chen & Dixon, 2007; Jackson *et al.*, 2008), and chemical analysis of field-grown switchgrass stems indicated that the saccharification efficiency is negatively correlated with lignin content and ester-linked *p*-CA : FA ratio (Shen *et al.*, 2009a). Therefore, genetic modification of lignin and cell wall phenolic ester synthesis in switchgrass would be predicted to serve as an efficient method for reducing recalcitrance and thus improving bioenergy production efficiency (Hisano *et al.*, 2009; Keshwani & Cheng, 2009).

Lignin is formed by the oxidative polymerization of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols, to give the hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the mature lignin polymer. Although the monolignol biosynthetic pathway is now well understood (Supporting Information, Fig. S1), and a picture is beginning to emerge of its complex regulatory architecture, to date only a few of the lignin biosynthetic and regulatory genes from switchgrass have been functionally characterized or annotated by comparative bioinformatics analysis. This situation will soon be changed with the ongoing efforts in switchgrass genetics and genomics (Okada *et al.*, 2010).

Common regulatory *cis*-elements have been identified within the promoters of the genes in the lignin biosynthesis pathway (Hatton *et al.*, 1995; Raes *et al.*, 2003; Legay *et al.*, 2007). A detailed analysis of the bean *phenylalanine ammonia-lyase 2* (*PAL2*) promoter identified three AC elements together with a G-box involved in xylem-specific expression (Hatton *et al.*, 1995). Mutation of the AC-I (ACCTACC), AC-II (ACCAACC) and AC-III (ACCTAAC) elements resulted in a decrease in xylem-associated expression (Hatton *et al.*, 1995). Bioinformatic analysis of the promoters of all lignin biosynthetic genes in *Arabidopsis thaliana* indicated that such AC elements are present in the majority of the genes except for the promoters of ferulate 5-hydroxylase (*F5H*) and caffeic acid 3-*O*-methyltransferase (*COMT*) (Raes *et al.*, 2003). However, examination of 2 kb of a maize *COMT* gene promoter region identified a putative ACIII box (aACCTAAC) 200 bp upstream of the transcription start site (Fornalé *et al.*, 2006).

The lignin biosynthesis pathway is coregulated with the secondary cell wall biosynthesis program through a master switch system which includes a group of NAC and R2R3-MYB transcription factors (TFs) (Mitsuda *et al.*, 2007; Zhong *et al.*, 2010). The *Arabidopsis* genome contains at least 114 *NAC* genes (Olsen *et al.*, 2005; Shen *et al.*, 2009b) and 126 members of the R2R3-MYB family (Romero *et al.*, 1998; Stracke *et al.*, 2001). The R2R3-MYB family members are classified into at least 22 subfamilies based on their conserved C-terminal motifs (Romero *et al.*, 1998; Stracke *et al.*, 2001). A group of R2R3-MYB transcriptional activators have been shown to bind directly to AC elements *in vitro*. These activators include PtMYB1 and PtMYB4 from pine (Patzlaff *et al.*, 2003; Bomal *et al.*, 2008), EgMYB2 from *Eucalyptus* (Goicoechea *et al.*, 2005), VvMYB5a from *Vitis vinifera* (Deluc *et al.*, 2006), NtMYBJS1 from tobacco (Gális *et al.*, 2006), PtrMYB21a from poplar (Karpinska *et al.*, 2004; Bylesjo *et al.*, 2008) and AtMYB61, AtMYB68 and AtMYB63 from *Arabidopsis* (Zhou *et al.*, 2009; Wang *et al.*, 2010). It has been suggested that AtMYB68, AtMYB63 and their close homolog PtrMYB28 from poplar are lignin-specific TFs, whereas PtMYB4 and EgMYB2 also regulate the biosynthesis of cellulose and xylan and therefore serve as secondary cell wall master switches (Zhong *et al.*, 2010).

By contrast, members from subfamily 4 of the R2R3-MYB family have been shown to act as transcriptional repressors of monolignol biosynthetic genes. This was first demonstrated for AmMYB308 and AmMYB330 from *Antirrhinum majus* (Tamagnone *et al.*, 1998). Overexpression of AmMYB308 and AmMYB330 in tobacco causes reduced plant growth and a white lesion phenotype on leaves (Tamagnone *et al.*, 1998). Similar phenotypes were observed on overexpression of AtMYB4 in *Arabidopsis* (Jin *et al.*, 2000). AtMYB32 (Preston *et al.*, 2004), a close homolog of AtMYB4, *Eucalyptus gunnii* EgMYB1 (Legay *et al.*, 2007) and maize ZmMYB31 and ZmMYB42 have also been identified as lignin repressors, and ZmMYB31 was proposed to be a good candidate for biotechnological applications (Fornalé *et al.*, 2006, 2010; Sonbol *et al.*, 2009). However, no genetic manipulation of lignin repressors in monocot plants has yet been reported.

In the present study, we have identified and characterized the lignin repressor *PvMYB4* from switchgrass. Effects of overexpression of *PvMYB4* in transgenic tobacco and switchgrass suggest that the gene is functionally orthologous to *AtMYB4* and *ZmMYB31*. PvMYB4 binds directly to AC-I (preferred), AC-II and AC-III elements, both *in vitro* and in a yeast transcription system. Lignin biosynthetic genes are significantly downregulated in PvMYB4-overexpressing plants, associated with reductions in lignin content and ester-linked *p*-CA : FA ratio. Overexpression of *PvMYB4* in transgenic switchgrass increases saccharification efficiency threefold.

Materials and Methods

Plant materials and growth conditions

Tobacco (*Nicotiana tabacum* cv Xanthi NN) and switchgrass (*Panicum virgatum* L. cv Alamo) were grown in the glasshouse under standard conditions (temperature range 25–29°C with a 16 h day from 06:00 to 22:00 h facilitated by supplementary lighting (parabolic aluminized reflector, 125–55 μmol m^{-2}) and relative humidity 77–22%, average 51%). Plants were watered two to three times wk^{-1}, with fertilizer (Peters 20-10-20; J.R. Peters Inc., Allentown, PA, USA) added in the last watering. For tissue-specific gene expression pattern analysis, roots, leaves, leaf sheaths, internodes, and flowers were collected at the reproduction (R1) developmental stage (Moore *et al.*, 1991). Samples were immediately frozen in liquid nitrogen and kept at −80°C for storage, or were ground to powder in a freezer mill (SPEX SamplePrep, Metuchen, NJ, USA) under liquid nitrogen for further RNA isolation and analysis of lignin and soluble and wall-bound phenolics.

Analysis of lignin, phenolic compounds and sugar release efficiency

Cell wall residues (CWR) were prepared by sequentially extracting tobacco stems and switchgrass whole tillers with chloroform/methanol (1 : 1), 100% methanol, 50% methanol, and water (three times each). Fifteen milligrams of lyophilized sample was used for lignin analysis. The acetyl bromide method was employed to determine total lignin content, and thioacidolysis followed by GC-MS was used to identify and quantify lignin-derived monomers. Soluble phenolics were extracted from 30.0 mg freeze-dried tissue powder with 1.5 ml 50% methanol

plus 1.5% acetic acid for 12 h at room temperature. One hundred milligrams of extractive-free CWR were used for analysis of esterified cell wall-bound phenolics using low-temperature alkaline hydrolysis. Approx. 100 and 125 mg of CWR were used for determination of total sugar release and enzymatic saccharification without acid pretreatment, respectively. Full details of all analytical methods have been described previously (Shen *et al.*, 2009a).

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated with an RNeasy Mini Kit (Qiagen). RNA quality was analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Two micrograms (tobacco) and 3 μg (switchgrass) of total RNA were treated with DNase (Applied Biosystems, Ambion, Austin, TX, USA) for 1 h to remove genomic DNA contamination and then used for reverse transcription with SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The cDNA samples were diluted 20-fold and 2 μl diluted cDNA samples were used as the qRT-PCR templates. qRT-PCR and data analysis were as described previously (Karlen *et al.*, 2007; Shen *et al.*, 2009a). Primer pairs used for qRT-PCR are listed in Table S1.

Transcriptional repression and domain mapping in yeast

The pYES2 (Invitrogen) and pGBT-9 (Clontech Laboratories, Mountain View, CA, USA) vectors were used to construct the effector plasmids for transcriptional repression activity and motif mapping assays, respectively. The pLacZi-based vectors (3AC-I and 3AC-II) were a gift from Dr Malcolm Campbell (University of Toronto) (Patzlaff *et al.*, 2003). The six GAL4 DNA-binding motif was cloned from Addgene plasmid 14590 (Zhang *et al.*, 2003) and inserted into pLacZi vector in front of the Mini-pCYC1 promoter. The VP16 motif was cloned from Addgene plasmid 14594 (Kurosu & Peterlin, 2004) into pGBT-9 vector to fuse with the GAL4 binding domain and PvMYB4 C-terminal deletions. The reporter plasmids were first integrated into the genome of yeast strain YM4721 purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) as described by Rose *et al.* (1990) to make the reporter strains. Approx. 1 μg of effector plasmid was transformed into the yeast reporter strains with the EZ-Yeast Transformation Kit (MP Biomedicals, Solon, OH, USA). β-Galactosidase assays were performed as described in the yeast protocols handbook (Clontech).

In situ hybridization

Primers spanning 757 bp of the PvMYB4 open reading frame were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). After the cDNA template was obtained, separate reactions were performed for making the sense and antisense probes. The T7 promoter sequence was added in front of the reverse primer and the PCR reaction was conducted with the forward primers without T7 to make an antisense probe. In the same way, T7 promoter was added to the front of the forward primer in combination with the reverse primer without T7 to make the sense control probe. The specific primers used for *PvMYB4* are listed in Table S1. The probe was synthesized by *in vitro* transcription with a MAXIscript Kit (AM1308–AM1326) with 0.4–0.6 μg of template DNA obtained as described earlier. Digoxigenin-11-uridine-5′-triphosphate (DIG-11-UTP; Roche) was used for the labeling. The quality and quantity of the probes were checked with a Bioanalyzer.

The tissue preparation including fixation, dehydration, and paraffin embedding were as described previously (Jackson, 1991). In brief, the switchgrass stem sections were rehydrated with an ethanol to water series after the paraffin had been removed by two 10 min incubations in Histo-clear (National Diagnostics Inc., Charlotte, NC). After brief equilibration in 0.1 M triethanolamine, the tissue was acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Sections were then washed twice with 1× PBS buffer for 5 min before and after triethanolamine treatment. Prehybridization, *in situ* hybridization and imaging methods were as described previously (Zhou *et al.*, 2010).

Dual luciferase assay

Arabidopsis protoplasts were isolated according to a previously published protocol with minor modifications (Sheen, 2001; Asai *et al.*, 2002). In brief, leaves from healthy 30-d-old *Arabidopsis* were cut into 0.5–1 mm strips with fresh razor blades. The leaf strips were put into an enzyme solution composed of cellulase and macroenzyme, vacuum-infiltrated for 20 min and then digested for 3 h without shaking in the dark. After filtration, the protoplasts were collected and transformed by polyethylene glycol transfection. To make the effector constructs, coding sequences of PvMYB4 were inserted after the 35S promoter of the Gateway overexpression vector P2GW7 (http://gateway.psb.ugent.be/). Reporter constructs were prepared as reported (Wang *et al.*, 2010). Promoter activities were represented by Firefly Luciferase (LU)C/Renilla LUC activities, and normalized to the value obtained from protoplasts transformed with empty vector.

Cell imaging and histochemical staining

Switchgrass internode samples and tobacco leaf petioles were collected in the glasshouse and immediately frozen in liquid nitrogen. These samples were then cut with a Leica CM 1850 cryostat (Leica Microsystems Inc., Buffalo Grove, IL, USA) at −20°C and prepared for microscopy as described previously (Nakashima *et al.*, 2008). Phloroglucinol-HCl staining and Mäule staining were carried out as previously described (Fu *et al.*, 2011). Ultraviolet absorption microspectrophotometry was performed as previously described (Nakashima *et al.*, 2008). Photographs were taken using a Nikon DXM 1200 color camera attached to a

Nikon microphot-FX microscope system with ACT-1 software (Nikon Instruments Inc., Laguna Hills, CA, USA). Tobacco leaf infiltration was performed as described previously (Sparkes *et al.*, 2006). Imaging of GFP fluorescence by confocal microscopy was performed as described previously (Wang *et al.*, 2008).

Tissue culture and transformation

Tobacco (*Nicotiana tabacum* cv Xanthi NN) was transformed by the leaf-disc method (Horsch *et al.*, 1985). Leaf discs from a tobacco plant grown in a Magenta box were incubated with *Agrobacterium tumefaciens* (AGL1) harboring the MYB construct for 20 min. The leaf discs were then blotted dry on filter paper and plated on co-cultivation medium for 4 days' co-cultivation in the dark. Leaf discs were transferred to regeneration medium after co-cultivation. Leaf discs were subcultured every 2 wk in regeneration medium until plant regeneration. Transgenic plants were maintained in the glasshouse for analysis.

Agrobacterium-mediated switchgrass transformation was performed based on a previously published protocol (Xi *et al.*, 2009). The pANIC vector was used for switchgrass transformation (Mann *et al.*, 2011). Briefly, the binary vector contained the attR1-CmR-ccdB-attR2 Gateway-compatible cassette cloned downstream under the control of the *ZmUbi1* promoter. The *PvMYB4* coding sequence was cloned into the pCR8/GW/TOPO backbone, sequence-verified, and recombined into the expression vector using Gateway® LR Clonase® II enzyme mix (Invitrogen). The switchgrass ST2 line was used for stable transformation. The ST2 line was specifically selected for its high tissue culture response and provided by Dr Zeng-Yu Wang, Noble Foundation. Since the ST2 line is vegetatively propagated by tillers, all the lines are in the same genetic background, an important point for a highly heterozygous outcrossing species.

Electrophoretic mobility shift assays

PvMYB4a was cloned into the pDEST17 expression vector by the Gateway cloning method (Invitrogen). After sequencing, the plasmid was transformed into BL21 DE3 *Escherichia coli* competent cells. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression of PvMYB4. The recombinant protein was purified with the MagneHis™ Protein Purification System (Promega) then concentrated with an Amicon Ultra-15 Centrifugal Filter (Millipore) by washing with Tris buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 10 mM β-mercaptoethanol (β-ME)) to remove carried-over HEPES buffer and imidazole. Approx. 700–1000 ng of purified PvMYB4 protein was used for electrophoretic mobility shift assay (EMSA) in each reaction. The probes were labeled by annealing biotin-labeled oligonucleotides. Oligonucleotides used for EMSA are listed in Table S1. Binding conditions were 12 mM Tris–HCl (pH 7.5), 20 mM NaCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DL-dithiothreitol (DTT), 0.2 mM EDTA, 2.5% glycerol, 1 mM β-ME, 0.05% NP-40 and 2 fmol biotin-labeled probes. The samples were loaded and run in a 6% DNA retardant gel (Invitrogen) in the cold room after the reactions had been incubated at 4°C for 30 min. The DNA was transferred onto nylon membranes and signal detected with a LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) using standard protocols.

Statistical analysis

Different letters on the bars of figures indicate significant differences of values at the $P \leq 0.05$ level. The multiple comparison was done by Duncan grouping with SAS software (SAS Institute Inc., Cary, NC, USA). Asterisks on top bars indicate values that were determined by the Student's *t*-test (Microsoft Office Excel 2007) to be significantly different from the wild-type or its equivalent control ($P < 0.05$).

Results

Switchgrass PvMYB4 belongs to the R2-R3 MYB subfamily 4

To clone orthologs of the MYB subfamily 4 genes in switchgrass, we used the AtMYB4 amino acid sequence to blast against the switchgrass expressed sequence tag (EST) database (Tobias *et al.*, 2005). After identifying the most homologous gene sequence, we designed the primer pairs HS010 and HS011 (Table S1) to clone the open reading frame (ORF) from switchgrass (cv Alamo) using RT-PCR. An 836 bp full-length cDNA was cloned into the pENTR_D topo vector for sequencing. The sequence alignment of PvMYB4 with other MYB subfamily 4 members shows that PvMYB4 has a highly conserved R2–R3 domain at the N-terminal region, while the C-terminal domain is more divergent, both in sequence and in length (Fig. 1a). However, three typical protein motifs of the MYB subgroup 4 were identified at the C-terminal; 'LlsrGIDPxT/SHRxI/L', 'pdLNLD/ELxiG/S' and '$CX_{1-2}CX_{7-12}CX_2C$' (Stracke *et al.*, 2001; Fornalé *et al.*, 2006). We also identified a '$FLGLX_{4-7}V/LLD/GF/YR/SX_1LEMK$' motif using the ClustalW sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. 1a). These motifs were termed as the C1, C2, Zf and C4 motifs, respectively (Fig. 1b; Legay *et al.*, 2007).

We performed a phylogenetic analysis using the PhyML method and tools available at *Phylogeny.fr:* (http://www.phylogeny.fr/) to identify the most closely related R2R3-MYB factors within the subfamily 4 group (Dereeper *et al.*, 2008). Phylogenetic analysis also shows that PvMYB4 is more closely related to ZmMYB42, ZmMYB38 and ZmMYB31 than to the well-known dicot phenylpropanoid/lignin biosynthesis repressors such as AmMYB308, AtMYB4, and AtMYB32 (Fig. 1c).

Sequencing of the *PvMYB4* clones indicated the presence of at least five gene variants, namely *PvMYB4a*, *4b*, *4c*, *4d* and *4e*, in the tetraploid out-crossing switchgrass genome (Fig. S2). A similar phenomenon has been reported for the switchgrass lignin biosynthesis gene *PvCCR1* (Escamilla-Trevino *et al.*, 2009). All the constructs used in the present work were made based on the *PvMYB4a* sequence.

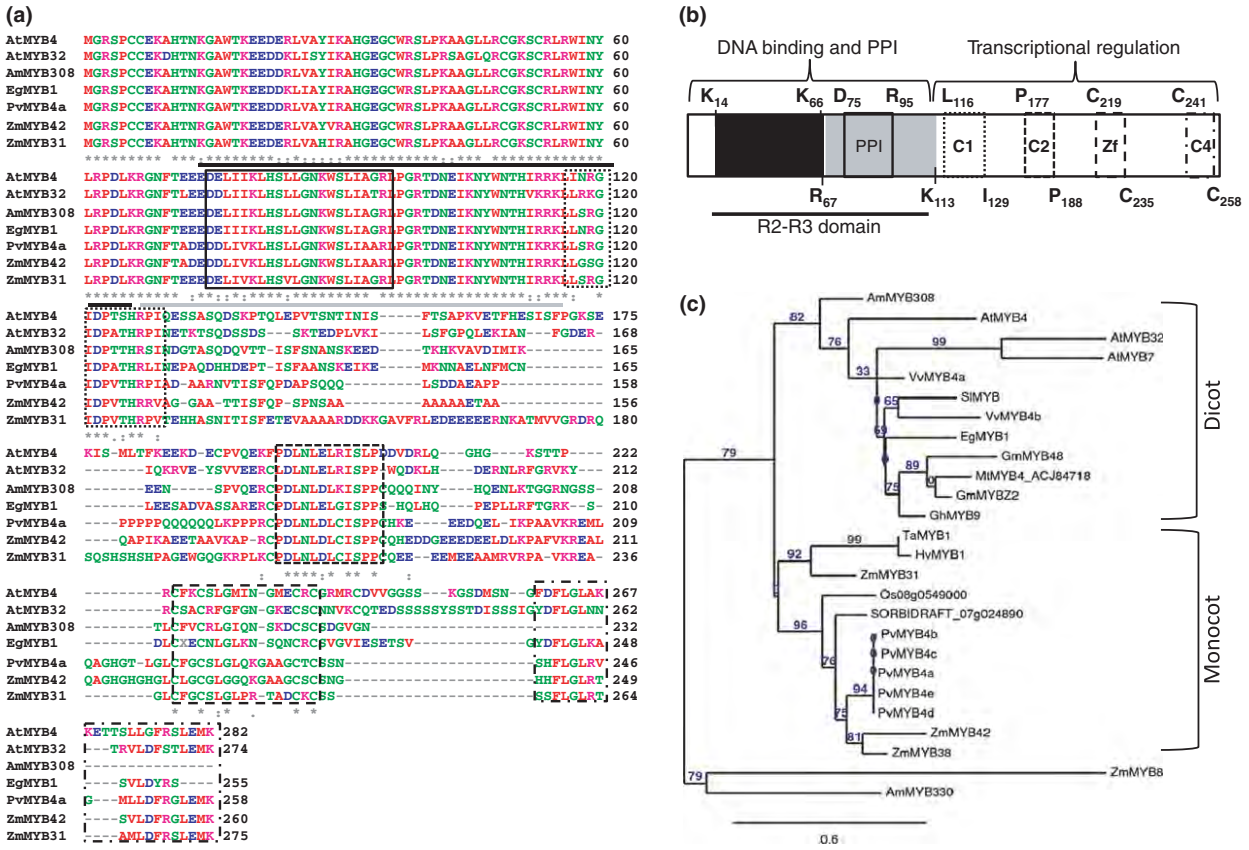

Fig. 1 PvMYB4 belongs to the R2R3-MYB subfamily 4. (a) ClustalW alignment of the amino acid sequences of PvMYB4 and other R2R3-MYB subfamily 4 proteins. The R2, R3 MYB domains are underlined. The boxed sequences are the potential functional motifs. (b) Structure of PvMYB4 protein domains and motifs. The boxed sequences are the potential functional motifs. Different types of lines indicate different motifs as shown in (b). PPI, protein–protein interaction motif for interaction with bHLH protein; C1, C2, Zf and C4, C-terminal motifs. Accession numbers for subgroup four sequences: PvMYB4a (JF299185), PvMYB4b (JF299186), PvMYB4c (JF299187), PvMYB4d (JF299188), PvMYB4e (JF299189), AtMYB32 (NM_119665), EgMYB1 (CAE09058), AmMYB308 (JQ0960), AtMYB4 (AY519615), AmMYB330 (JQ0957), ZmMYB42 (CAJ42204) and ZmMYB31 (CAJ42202). (c) Phylogenetic analysis of PvMYB4 with other subfamily 4 protein sequences by Phylogeny.fr program (http://www.phylogeny.fr/). PvMYB4a–e refers to different gene variants as shown in Supporting Information, Fig. S2.

PvMYB4 is expressed in the vascular bundles of switchgrass

To test the tissue-specific expression pattern of PvMYB4 in switchgrass, we isolated total RNA from different tissues at the R1 developmental stage and performed qRT-PCR analysis (Moore *et al.*, 1991; Sarath *et al.*, 2007). The *PvMYB4* gene was expressed in all the tissues tested, with highest expression in leaf and leaf sheath (Fig. 2a). *In-situ* hybridization analysis of young stem tissue sections indicated that *PvMYB4* is expressed throughout the stem, including the parenchyma cells, vascular tissues and epidermis. The highest expression level appeared to be in vascular bundles (Fig. 2b). To test whether PvMYB4 is localized to the nucleus, we performed infiltration assays of tobacco epidermal cells with a construct for expression of a PvMYB4: eGFP fusion protein. GFP fluorescence was shown to localize to the nucleus by confocal microscopy (Fig. 2c).

PvMYB4 is a transcriptional repressor and binds to the AC elements in the genes involved in the lignin biosynthesis pathway

We tested for transcriptional repression activity using a dual *Luciferase* reporter assay in *Arabidopsis* protoplasts. When *PvMYB4* was co-expressed with the *Arabidopsis PAL4* or *Arabidopsis CCoAOMT* (caffeoyl CoA 3-*O*-methyltransferase) promoters driving a *Luciferase* reporter gene, *Luciferase* expression was repressed by almost 80–90% compared with the values obtained from these lignin pathway promoters in the absence of PvMYB4 expression (Fig. 3a). Several R2-R3 MYB proteins from subfamily 4 have been reported to bind the AC elements in monolignol pathway gene promoters (Romero *et al.*, 1998; Legay *et al.*, 2007; Zhao *et al.*, 2007). Use of EMSA led to the conclusion that these proteins might bind to 'GKTWGGTR' elements *in vitro* (Romero *et al.*, 1998). The reverse complement sequence of 'GKTWGGTR' is 'YACCWAMC' with Y as T or C, W as T or A, and M as A or C. The possible elements can therefore be designated as 'T/CACCT/AAA/CC'. AtMYB4 can bind to the AC-I (ACCTACC), AC-II (ACCAACC) and AC-III (ACCTAAC) elements (Zhao *et al.*, 2007). It also binds to the 'ACCGCCC' elements (MYB elements) found in its own promoter region (Zhao *et al.*, 2007). EgMYB1 binds to a region of the promoter of the *EgCAD* (*cinnamyl alcohol dehydrogenase*) gene with the sequence 'TACCAAGACCCACCTACCGCATGTCGA' containing both the 'ACCCACC' and 'ACCTACC' elements (AC-I) (Legay *et al.*, 2007).

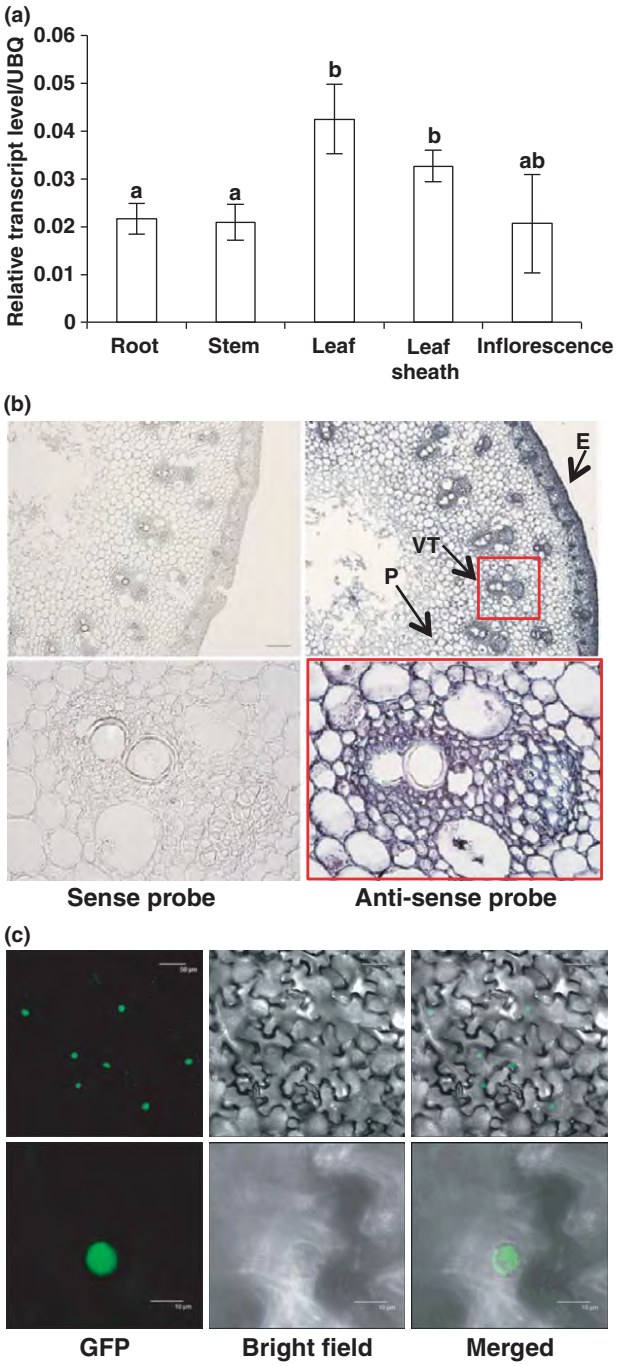

Fig. 2 Expression patterns of *PvMYB4*. (a) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of tissue expression pattern of PvMYB4 in switchgrass (*Panicum virgatum*). Different letters on the bars of figures indicate significant differences at the $P \leq 0.05$ level as tested by Duncan grouping with SAS software (SAS Institute Inc.). UBQ, ubiquitin. (b) *In situ* hybridization of *PvMYB4* in young stem sections. VT, vascular tissue; P, parenchyma; E, epidermis. Bar, 500 μm. (c) Confocal images of nuclear localization of PvMYB4:eGFP protein in tobacco (*Nicotiana tabacum*) epidermal cells. Bars, 50 μm (upper panel); 10 μm (lower panel).

To test whether PvMYB4 can bind to similar AC-rich elements, three repeats of the AC-I, AC-II, AC-III and AC-IV (a hypothetical AC-rich element 'ACCAAAC') elements (Fig. 3b) were cloned into the pLACZi vector immediately upstream of a pCYC1 minimal promoter driving the *LacZ* reporter gene, for expression in yeast (Fig. 3c). Since PvMYB4 has transcriptional repression activity, we cloned PvMYB4 as a fusion with the GAL4 activation domain (AD) to generate a chimeric effector (Fig. 3c). If PvMYB4 binds to the test AC-rich elements, it will block the expression of the *LacZ* reporter gene activated by the GAL4 AD. A R2R3-MYB transcriptional activator PtMYB4 (Bomal *et al.*, 2008) and a bZip transcription factor HY5 (ELONGATED HYPOCOTYL 5) (Oyama *et al.*, 1997) were used as positive and negative controls, respectively, along with the vector control containing only the GAL4-AD (Figs 3, S3). The positive control PtMYB4 increased the *lacz* reporter gene expression by *c.* 50–100-fold compared with the vector and negative controls, indicating that the system is functional in yeast (Fig. S3). By contrast, PvMYB4 repressed the expression from the three AC-I, AC-II and AC-III elements fused to the Mini-pCyC1 promoter driving the *lacZ* reporter gene by *c.* 37–82% compared with the vector and negative control (HY5) under the same conditions (Fig. 3d). However, PvMYB4 did not repress transcription driven by the three AC-IV elements in yeast (Fig. 3d), suggesting that it may not bind to the AC-IV element, or may do so only at very low affinity.

PvMYB4 was cloned into the pDEST17 vector with a His tag fusion at the N-terminal, and the purified protein from *E. coli* extracts was shown to bind to the AC-I, AC-II, AC-III and AC-IV elements in EMSA assays (Fig. 3e). Salmon sperm DNA was added as a nonspecific competitor (200×); it could not compete with the AC-I element but exhibited weak competition activity with the AC-II and AC-III elements, and stronger competition activity with the AC-IV element. The results indicate that PvMYB4 has the highest binding affinity to the AC-I element and the lowest affinity to the AC-IV element *in vitro*, consistent with the results of the repression activity assay in yeast (Fig. 3d).

It has been reported that the C1 motif (also called GIDP motif) has putative activation activity (Matsui *et al.*, 2008) and that C2 motifs in AtMYB4 function as the repression motifs (Jin *et al.*, 2000). To investigate the functions of the C-terminal motifs in PvMYB4 in more detail, we fused different deletions of PvMYB4 between the VP16 activation motif from herpes simplex virus protein VP16 (Sadowski *et al.*, 1988) and the GAL4 binding domain (Fig. 3f). The reporter construct was made by placing six GAL4 binding motifs in front of the pCYC1 minimal promoter driving the *LacZ* reporter gene. After expression of reporter and effector constructs in yeast, β-galactosidase assays indicated that the C2 and C4 motifs all have transcriptional repression activities, because the deletion of these motifs restored the transcriptional activation effects contributed to PvMYB4 by the VP16 activation motif. When the C1 motif was further deleted, the transcriptional activation was reduced slightly, consistent with a previous suggestion of transcriptional activation activity for the C1 motif (Matsui *et al.*, 2008). By contrast, the Zf motif (Fig. 1b) did not appear to have repression activity (Fig. 3g).

A region with a high percentage of proline (P) and glutamate (Q) residues is located between the C1 and C2 motifs of

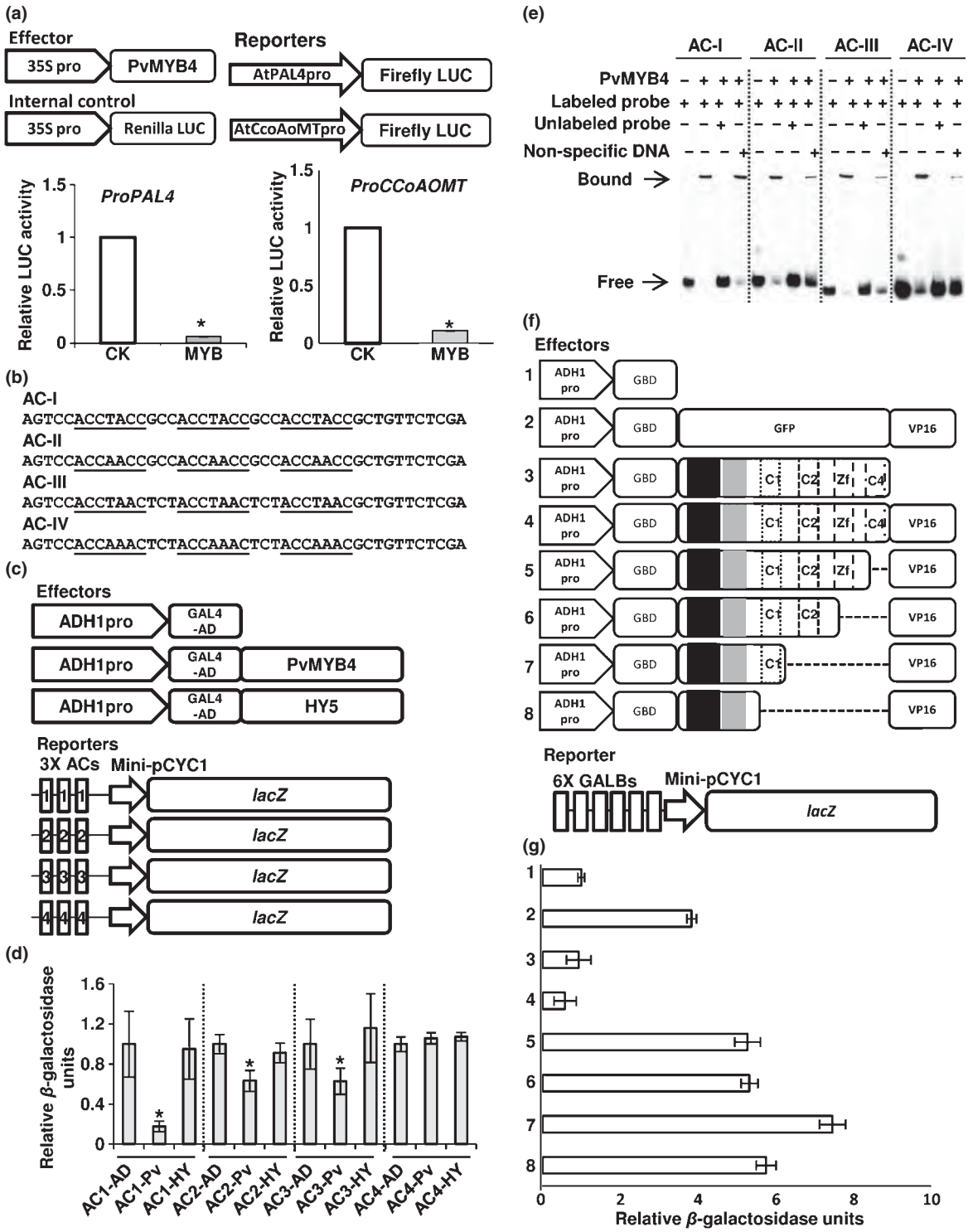

Fig. 3 PvMYB4 is a transcriptional repressor and binds to AC elements. (a) Dual *luciferase* (LUC) assay of 35S:PvMYB4 effector with firefly *luciferase* reporter constructs in *Arabidopsis thaliana* protoplasts. CK, vector control plasmid was used as effector; MYB, *35S:PvMYB4* plasmid was used as effector. (b) Synthetic AC elements tested in electrophoretic mobility shift assay (EMSA) and yeast assays. (c) Effector and reporter plasmids for testing the binding of PvMYB4 to AC elements in yeast. The transcription factor HY5 was used as negative control. (d) β-Galactosidase assays for lacZ expression driven by PvMYB4 binding to AC elements in yeast. AC(1,2,3,4)-AD, AC(1,2,3,4)-Pv and AC(1,2,3,4)-HY are the reporters containing three consecutive copies of the AC I, II, III, or IV elements fused to the pCYC1 minimal promoter and *lacZ* with either GAL4AD, GAL4AD-PvMYB4 or GAL4AD-HY5 as effectors. Data are means ± SE (n = 4). (e) EMSAs of PvMYB4 with AC elements. Free, free probe; bound, shifted band. Salmon sperm DNA (200×) was used as non-specific competitor. (f) Effector and reporter constructs for testing the PvMYB4 repression motif in yeast. GBD, GAL4 DNA binding domain; VP16, activation motif of the VP16 protein; GALBs, GAL4 protein binding sites. (g) β-Galactosidase assays showing activation of lacZ expression from the reporter 6GALBs-*LacZ* by the effectors with different PvMYB4 C-terminal deletions shown in Fig. 3(f). Data were normalized to the vector control pGBT-9, and are means ± SE (n = 4). Asterisks on top of the bars in (a) and (d) indicate values that were determined by Student's t-test to be significantly different from their equivalent control ($P < 0.05$).

PvMYB4, and most of the sequence variations were found in this area (Figs 1b, S2a). It has been reported that P-Q-rich and acidic-blob-type regions are associated with transcriptional activation domains (Ruden *et al.*, 1991). To test this hypothesis, we created a fusion of the PQ motif (green box, Fig. S2) with the GAL4 binding domain as an effector plasmid, and expressed it in yeast with the *6GAL4-Mini-pCYC1: lacZ* reporter construct; no transcriptional activation of β-galactosidase was detected (Fig. S4), indicating that the P-Q-rich region has no transcriptional activation activity.

Expression of *PvMYB4* in transgenic tobacco

To investigate the biological activity of PvMYB4 *in vivo*, we first overexpressed the gene under the control of the constitutive 35S promoter in tobacco. Several independent lines, such as #6, #9 and #16, showed high expression of *PvMYB4* transcripts (Fig. 4a). Overexpression of PvMYB4 gave rise to the same phenotypes as observed previously for AmMYB308 and AtMYB4 in transgenic tobacco (Tamagnone *et al.*, 1998; Jin *et al.*, 2000), namely a reduction in plant stature and appearance of numerous whitish lesions on the mature leaves (Fig. 4b). The latter phenotype has been linked to reduced concentrations of hydroxycinnamic acid derivatives (Elkind *et al.*, 1990; Tamagnone *et al.*, 1998).

The most abundant soluble phenolic compound in methanolic extracts of tobacco stems is chlorogenic acid (CGA, Rt 14.87 min), which exists with a minor isomer (Rt = 14.37 min) (Fig. 5a). Overexpression of PvMYB4 in tobacco caused an approx. 80% reduction in soluble CGA concentrations (Figs 5a, S5a). The most abundant ester-linked wall-bound phenolic compounds released from low-temperature hydrolysis of tobacco stem cell walls are vanillin (Rt = 19.63), syringyl aldehyde (Rt = 22.59) and *p*-coumaric acid (*p*-CA, Rt = 23.78). Overexpression of PvMYB4 significantly reduced the content of vanillin and *p*-CA compared with the wild-type, but syringyl aldehyde concentrations were not significantly changed (Figs 5b, S5b).

The lignin content and composition of PvMYB4-OX transgenic tobacco plants were also significantly changed. Leaf petiole sections of PvMYB4-OX plants showed reduced staining with phloroglucinol-HCl (Fig. S5c), suggesting an overall reduced concentration of lignin, confirmed by the reduction in acetyl bromide lignin content (Fig. 5c). Mäule staining showed a decrease in the

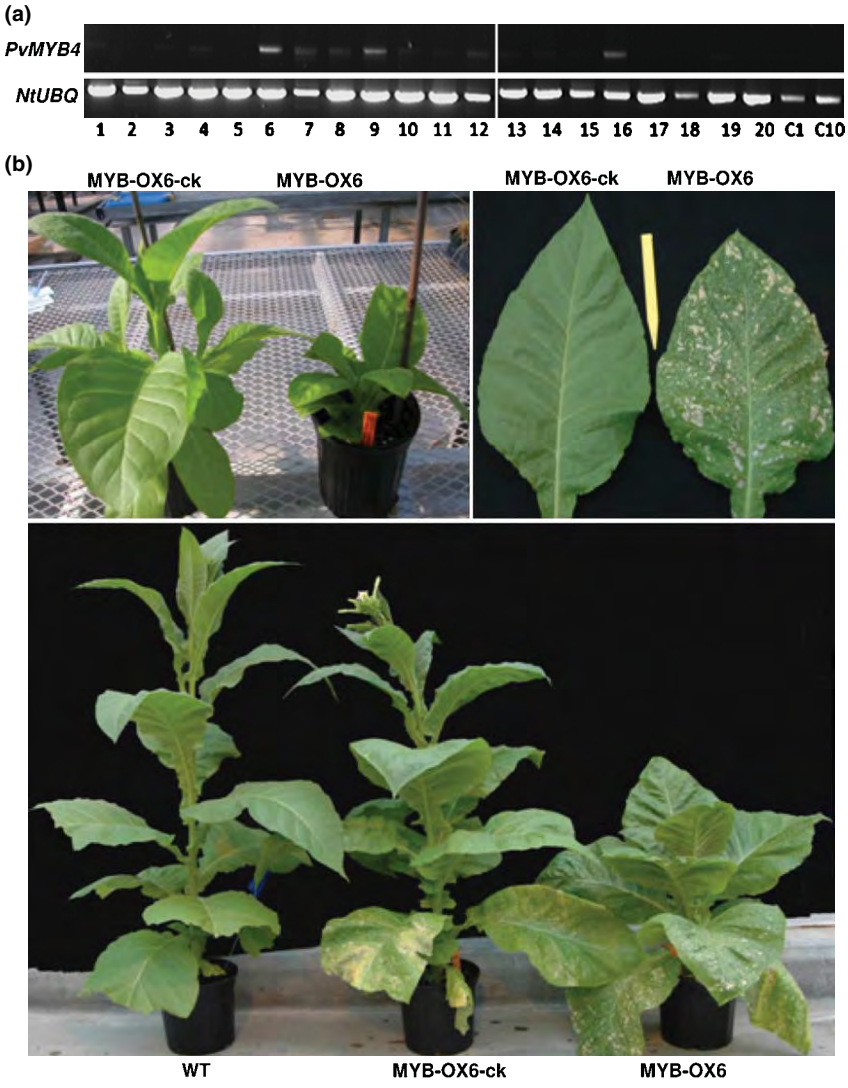

Fig. 4 Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing *PvMYB4* show reduced growth and lesion mimic phenotypes. (a) Semiquantitative reverse transcription polymerase chain reaction analysis of PvMYB4-OX transgenic tobacco plants. NtUBQ was used as reference gene.(b) Growth and leaf lesion phenotypes of PvMYB4-OX6 transgenic tobacco plants. MYB-OX6-ck indicates the T1 null-segregating line.

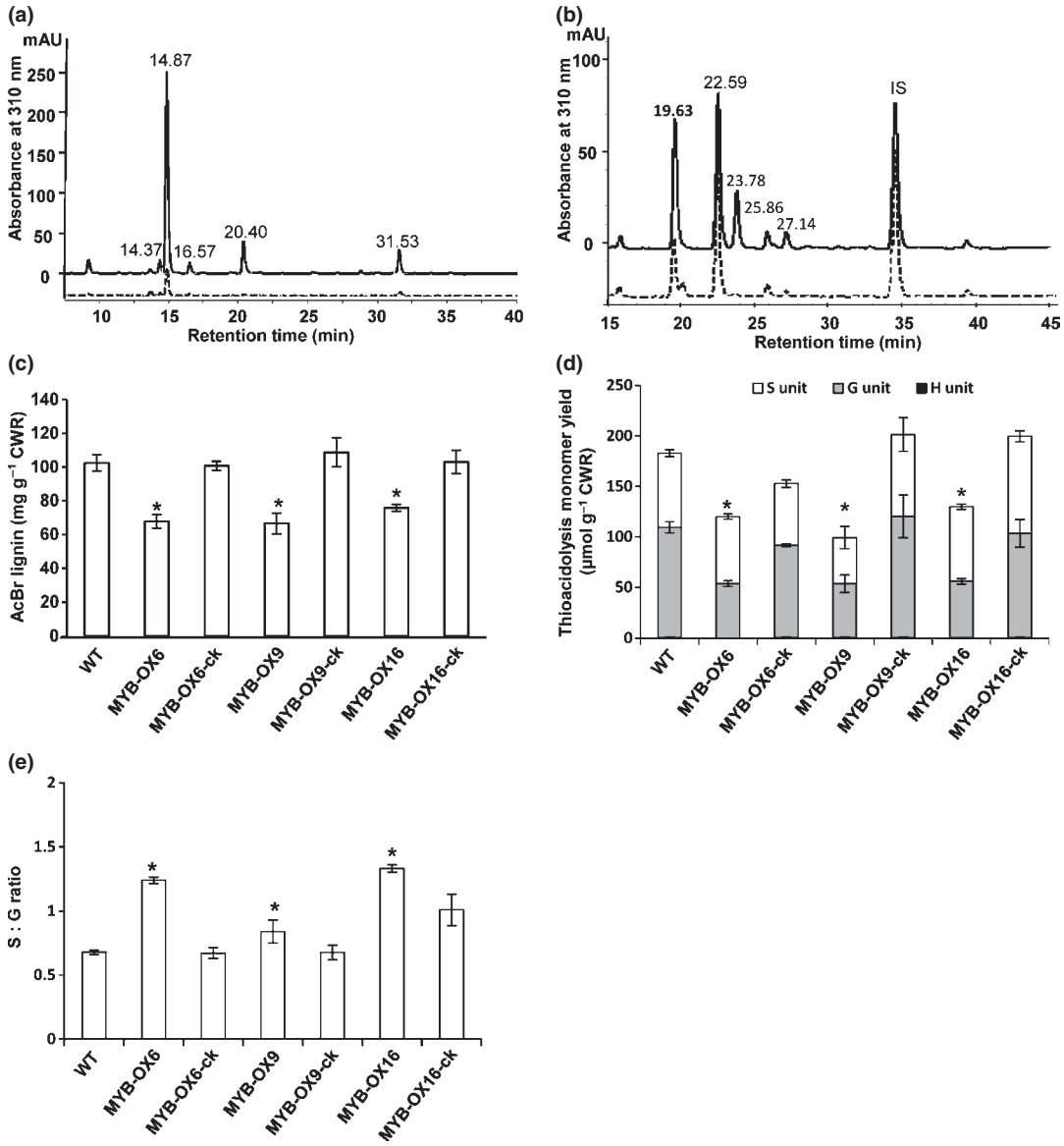

Fig. 5 Overexpression of PvMYB4 alters phenylpropanoid metabolism in transgenic tobacco (*Nicotiana tabacum*) plants. (a, b) Typical high-performance liquid chromatography (HPLC) chromatograms of methanol/water-soluble fractions (a) and ester-linked wall-bound phenolics from stems of wild-type (CK) and PvMYB4-ox tobacco lines (b). CK, solid line; MYB-OX, dashed line. The major soluble phenolic compound is chlorogenic acid (Rt = 14.87 min). Wall-bound ester-linked phenolic compounds: vanillin (Rt = 19.63); syringyl aldehyde (Rt = 22.59); *p*-coumaric acid (*p*-CA, Rt = 23.78) and ferulic acid (FA, Rt = 27.14). IS, internal standard (c) AcBr lignin content of stems of CK and PvMYB4-ox transgenic tobacco. MYB-OX-ck indicates the T1 null-segregating line. (d) Lignin composition of stems of CK and PvMYB4-ox transgenic tobacco as determined by thioacidolysis. S, syringyl unit (open bars); G, guaiacyl unit (gray bars). (e) S : G ratio (from thioacidolysis) of stems of CK and PvMYB4-ox transgenic tobacco. CWR, cell wall residue. All data are means ± SE ($n = 6$). Asterisks on top of the bars indicate values that were determined by the Student's *t*-test to be significantly different from their equivalent control ($P < 0.05$).

red coloration of the xylem vessels, consistent with a reduction in S lignin as was confirmed by thioacidolysis analysis of lignin monomer yield (Fig. 5d). The latter indicated a reduction in lignin content of from 40 to 60% in the PvMYB4-OX overexpressing lines compared with controls (Fig. 5d). However, overall the G units were reduced more than the S units, leading to an increased S : G ratio in the MYB4 overexpressors (Fig. 5e).

Overexpression of *PvMYB4* in switchgrass

To generate switchgrass PvMYB4-OX lines, the full-length *PvMYB4a* ORF was cloned into a binary vector (pANIC vector, Mann *et al.*, 2011) fused with an AcV5 epitope tag (Monsma & Blissard, 1995) at the C-terminal and driven by the maize *ZmUbi* promoter. The hygromycin B (*hph*) resistance gene was used as a selectable marker for transformation (Fig. S6a). Embryonic callus generated from immature inflorescences of switchgrass cv Alamo line ST2 was transformed by *Agrobacterium*-mediated transformation. The transformation process, based on modifications of previously published protocols (Somleva *et al.*, 2002; Xi *et al.*, 2009), is illustrated in Fig. 6(a).

Genomic DNA PCR analysis showed that transgenic lines 2A and 2B could serve as appropriate controls because only the hygromycin B resistance gene portion of the T-DNA was

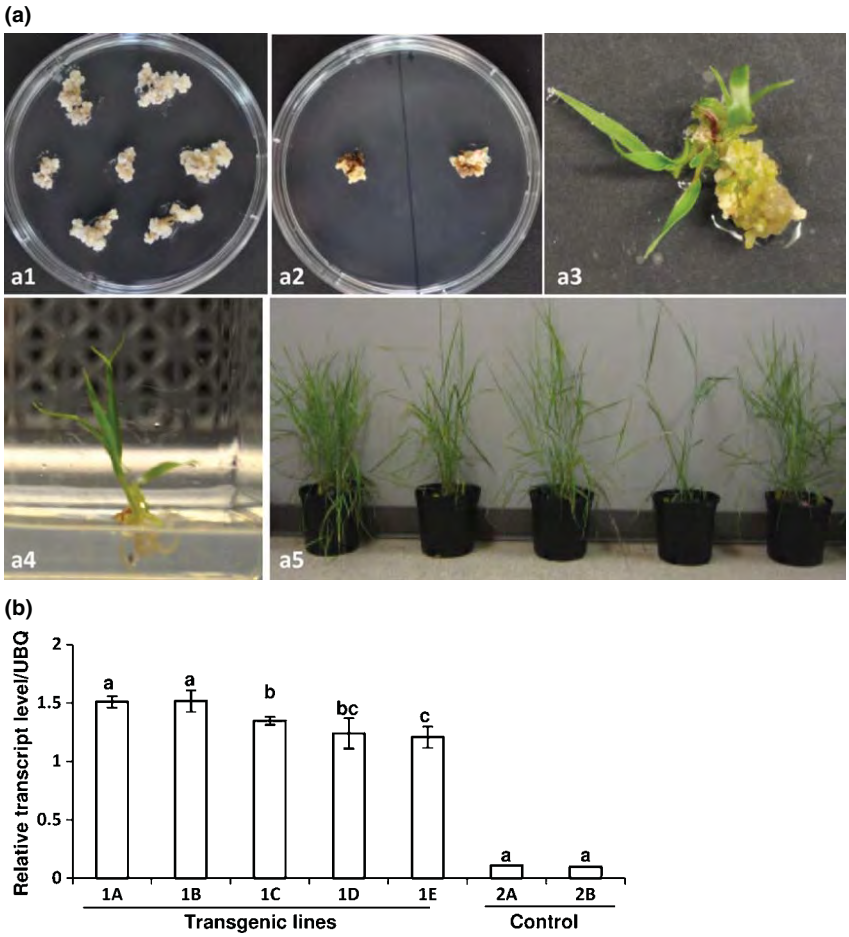

Fig. 6 Overexpression of *PvMYB4* in transgenic switchgrass (*Panicum virgatum*). (a) Representative pictures of the switchgrass transformation procedure. a1, embryonic callus used for transformation; a2, transformed callus on selection medium; a3, regenerated seedlings on regeneration medium; a4, regenerated seedling on rooting medium; a5, regenerated PvMYB4-OX plants in the glasshouse. (b) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *PvMYB4* transcripts in control and PvMYB4-OX transgenic switchgrass. Data are means ± SE (n = 3). UBQ, ubiquitin.

integrated into the genome, and the lines were phenotypically indistinguishable from untransformed ST2 lines. (Fig. S6b).

To confirm overexpression of the *PvMYB4* gene in the transgenic plants, two primer pairs, HS543 and HS544, which cover the PvMYB4 ORF and AcV5 tag sequence, were used for qRT-PCR analysis (Table S1). The selected PvMYB4-OX lines exhibited a 10–12-fold increase in *PvMYB4* expression (Fig. 6b). The HS360 and HS361 primer pairs were designed from the 3′-UTR region of the *PvMYB4* sequence to determine expression of the endogenous *PvMYB4* genes in switchgrass (Fig. S6c). The endogenous *PvMYB4* gene was repressed in the transgenic plants, indicating a similar self-repression feedback regulatory network as described for *AtMYB4* in *Arabidopsis* (Zhao *et al.*, 2007) (Fig. S6c).

Overexpression of *PvMYB4* in switchgrass had dramatic effects on plant morphology. The PvMYB4-OX transgenic plants showed a reduction in plant height (by *c.* 40% on average) but more tillers (up to a 2.5-fold increase) (Fig. 7a,b). By contrast to the PvMYB4-OX tobacco plants, no white lesions were observed on the leaves.

PvMYB4 overexpression reduces lignin content, alters cell wall phenolic content, and reduces recalcitrance in transgenic switchgrass

The lignin content in the middle parts of the different internodes of E4 stage stems of PvMYB4-OX and control switchgrass plants were first evaluated by staining with phloroglucinol-HCl and Mäule reagent. Clearly, total lignin content (phloroglucinol-HCl staining, red color) and S lignin (Mäule staining, reddish-brown color) were reduced in the stems of the transgenic plants, especially the mature I1 and I2 internodes (Fig. 7c). Furthermore, although the structure of the vascular bundles remained the same, their size was significantly reduced in the transgenic plants based on the measurement of vascular bundles from internode 2 (Figs 7c, S8a). We also found that the tillers of PvMYB4-OX lines were thinner than those of the controls (Fig. S8b).

Total lignin content in whole stems of E4 stage plants was reduced by *c.* 40–50% (determined as AcBr lignin) or 60–70% (determined by thioacidolysis) as a result of overexpression of PvMYB4 (Fig. 8a,b). However, in contrast to the PvMYB4-OX tobacco lines, the S : G ratio remains unchanged (Fig. S7). The ester-linked *p*-CA : FA ratio was reduced by *c.* 50% compared with the control lines (Fig. 8d) and this was mainly caused by the significant reduction of the ester-linked *p*-CA content in the cell wall residue (Fig. 8c). Since both the lignin content and the ester-linked *p*-CA : FA ratio are reduced in the PvMYB4-OX transgenic switchgrass lines, we predicted that these lines would exhibit significantly higher saccharification efficiency than the control lines based on our previous observations of natural variation (Shen *et al.*, 2009a). To test this, we measured sugar release from CWR by enzymatic saccharification without acid pretreatment. The total sugar released from the PvMYB4-OX transgenic

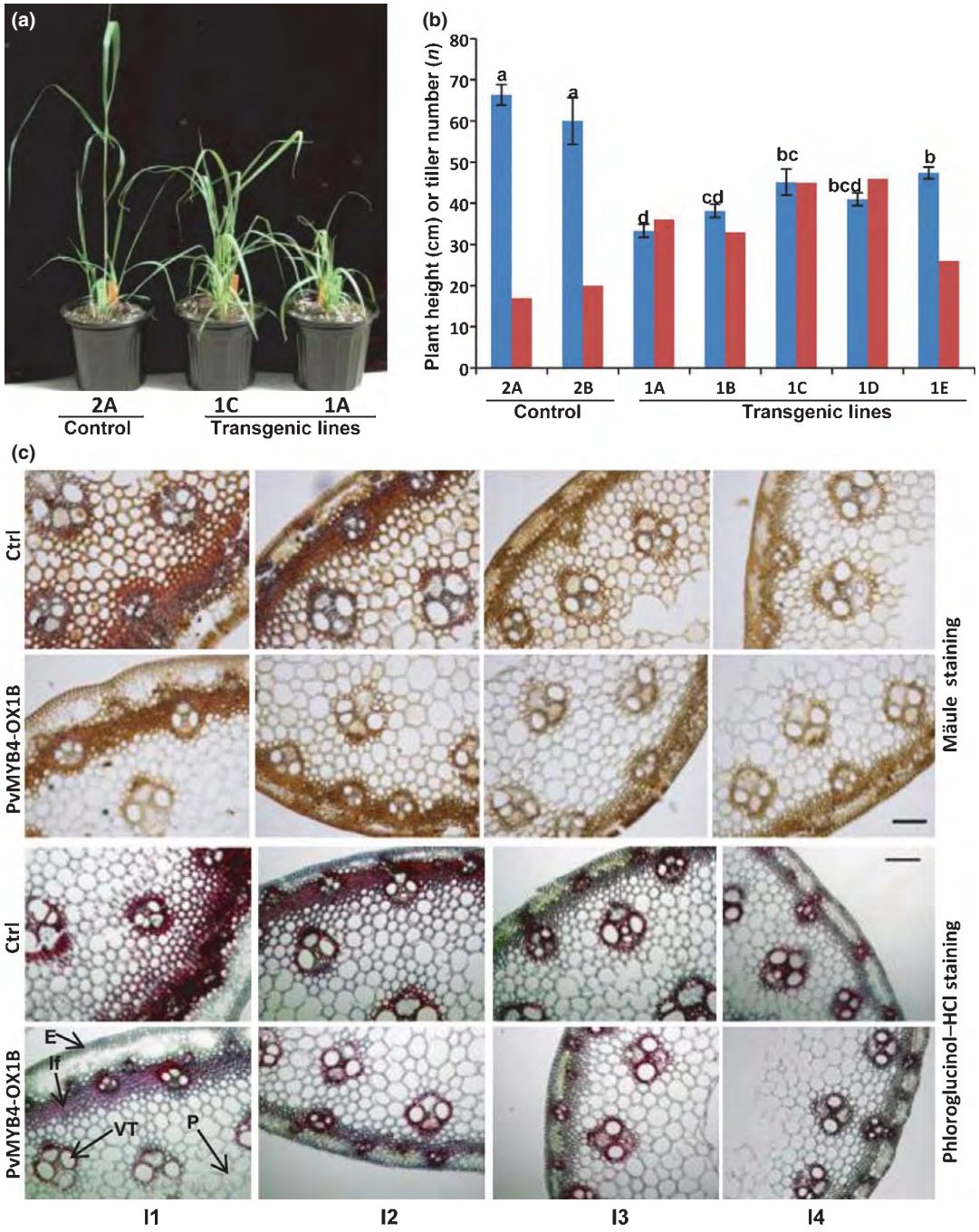

Fig. 7 PvMYB4-OX transgenic switchgrass (*Panicum virgatum*) has reduced lignin and altered growth morphology.(a) Visible phenotypes of control (2A) and PvMYB4-OX transgenic switchgrass (1A, C). The overexpressing lines have reduced stature but increased tillering. (b) Plant height (blue bars) and tiller number (red bars) for control and PvMYB4-OX transgenic switchgrass. Data are means ± SE ($n \geq 4$). The letters indicates significant differences at the $P \leq 0.05$ level. (c) Phloroglucinol-HCl staining (lower panel) and Mäule staining (upper panel) of cross-sections of different internodes of PvMYB4-OX transgenic switchgrass. I1–I4, internode 1 (lower) to internode 4 (upper) of E4 tillers (Shen *et al.*, 2009a); VT, vascular tissue; P, parenchyma, E, epidermis; If, interfascicular fiber. All the pictures were taken at 100× magnification. Bars, 100 μm.

switchgrass lines as a function of total available cell wall sugar content was approx. threefold higher from the PvMYB4 overexpressing lines than from the controls (Fig. 8e,f). We also estimated the dry biomass production under glasshouse conditions; the transgenic PvMYB4-OX lines had less (1A and 1B lines), similar (line 1C and 1E lines) or *c.* 20% increased (line 1D) biomass depending on the line (Fig. S9).

Downstream target genes of *PvMYB4*

Given that PvMYB4 represses phenylpropanoid metabolism and lignin content in both switchgrass and tobacco, and represses transcription of the *Arabidopsis PAL4* and *CCoAOMT7* promoters *in vitro*, we used the transgenic PvMYB4-OX lines to gain a broader picture of the downstream

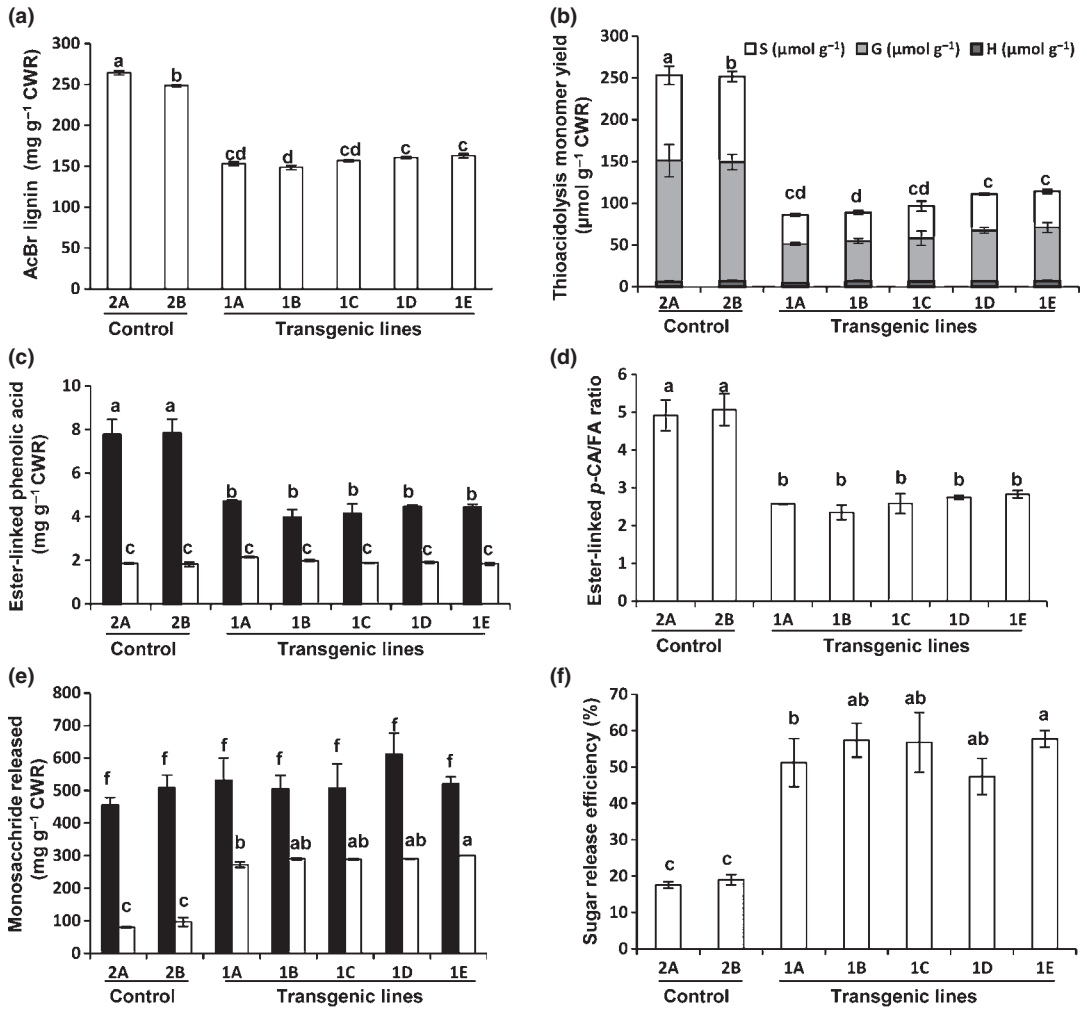

Fig. 8 Overexpression of PvMYB4 alters phenylpropanoid metabolism in transgenic switchgrass (*Panicum virgatum*) plants. (a) AcBr lignin content of whole E4 stems. (b) Lignin composition determined by thioacidolysis. (c) The content of ester-linked *p*-coumaric acid (*p*-CA, closed bars) and ferulic acid (FA, open bars) in switchgrass stems. CWR, cell wall residue. (d) *p*-CA : FA ratios of the ester-linked wall-bound phenolics. (e) Total monosaccharide released (closed bars) from cell wall residues and monosaccharide released from enzymatic saccharification without acid pretreatment (open bars). (f) Sugar release efficiency of enzymatic saccharification without acid pretreatment. Data are means ± SE (n = 3).

gene targets within the phenylpropanoid and monolignol biosynthesis pathways.

Total RNA was extracted from the leaves of tobacco lines and young seedlings of switchgrass lines. First-strand cDNA was synthesized and qRT-PCR primer pairs (Table S1) were designed for each of the genes encoding the enzymes of phenylpropanoid and monolignol biosynthesis. In the transgenic control lines, *F5H* (*ferulate 5-hydroxylase*), *CCoAOMT* and *HCT* (*hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase*) genes showed overall lower transcript abundance than other genes (Fig. 9a,b). The transcript abundance of *PAL*, *C4H*, *C3′H* (*coumaroyl shikimate 3′-hydroxylase*), *4CL* (*4-coumarate: CoA ligase*), *COMT* (*caffeic acid 3-O-methyltransferase*), *CCR* (*cinnamoyl CoA reductase*) and *CAD* genes was reduced by at least 70% in the PvMYB4-OX tobacco lines and 50% in the switchgrass lines (Fig. 9a,b). Because of potential cross-talk and/or spillover between lignin and flavonoid pathways in plants down-regulated in lignin biosynthesis (Besseau *et al.*, 2007), we also analyzed genes involved in flavonoid biosynthesis (*chalcone synthase*, *chalcone isomerase* and *flavonol synthase*); none of these was affected by overexpression of PvMYB4 (Fig. S10). Combining the results from biochemical and target gene expression analyses, we conclude that PvMYB4 functions as a repressor of the phenylpropanoid and lignin pathways.

Discussion

PvMYB4 is a lignin biosynthetic pathway repressor

R2R3-MYB4 subfamily 4 members have been shown to function as repressors of lignin biosynthesis in several species (Tamagnone *et al.*, 1998; Jin *et al.*, 2000; Fornalé *et al.*, 2006, 2010; Legay *et al.*, 2007; Zhao & Dixon, 2011). The *PvMYB4* gene is expressed in all tissues of switchgrass including the roots, stems, leaves, leaf sheaths and inflorescences at the R1 development stages. *In situ* hybridization of young stem sections showed that

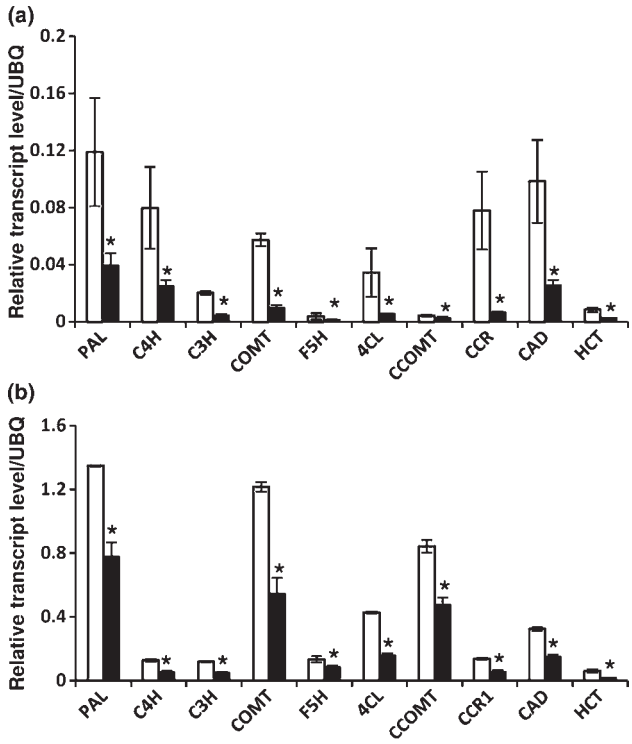

Fig. 9 Identification of genes down-regulated by PvMYB4 in tobacco (*Nicotiana tabacum*) and switchgrass (*Panicum virgatum*). (a) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of the expression levels of lignin synthetic genes in PvMYB4-OX transgenic tobacco (closed bars). (b) qRT-PCR analysis of the expression levels of lignin synthetic genes in PvMYB4-OX transgenic switchgrass (closed bars). The CK value (open bars) represents the combined/average result from both control (2A and 2B) lines. PAL, phenylalanine ammonia-lyase; C4H, coumaroyl shikimate 4-hydroxylase; C3H, coumaroyl shikimate 3-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase. UBQ, ubiquitin.

PvMYB4 is highly expressed in the vascular bundles, consistent with its binding activity for the xylem expression-associated AC elements. The strong expression level of *PvMYB4* in the vascular bundles and parenchyma cells at earlier developmental stages is consistent with lower degrees of lignification in young compared with mature stems.

Protein motif analysis showed that PvMYB4 has four conserved motifs, C1, C2, Zf and C4, in the C-terminal regulatory region. The C1 motif may function as an activation domain, confirming a previous report (Matsui *et al.*, 2008), and the C2 motif functions as the repression domain, consistent with results from similar analysis of AtMYB4 (Jin *et al.*, 2000). The C4 motif also acts as a repression motif although AmMYB308, another lignin repressor, does not have this motif. The coexistence of the activation and repression motifs at the C-terminal of PvMYB4 reflects the complexity of the protein structures and potential gene regulatory network. The function of the Zf motif within the C-terminal regulatory region remains unknown for the R2R3-MYB subfamily 4 proteins.

Five *PvMYB4* gene variants were identified by EST cloning in switchgrass. Allelic variation in a polyploid genome has been suggested as being responsible for heterosis (Springer & Stupar, 2007). The lignin biosynthetic gene *PvCCR* has at least four gene variants (Escamilla-Trevino *et al.*, 2009). These natural gene variants provide a basis for molecular marker discovery. It will be interesting to determine whether these variations in lignin pathway synthetic and regulatory genes and alleles are linked to recalcitrance phenotypes using marker-assisted approaches.

Overexpression of transcriptional repressors of the lignin synthetic pathway reduces lignin content in a range of dicot plants (Tamagnone *et al.*, 1998; Jin *et al.*, 2000; Fornalé *et al.*, 2006, 2010; Legay *et al.*, 2007). However, this reduction had not yet been shown in monocots. The basal internodes of switchgrass have high lignin content during development (Sarath *et al.*, 2007; Shen *et al.*, 2009a). Chemical staining and wet chemistry analysis of the stems of PvMYB4-OX transgenic plants showed that lignin content was significantly reduced. The amounts of both S and G subunits were reduced in both tobacco and switchgrass overexpressing PvMYB4; the reduction in S and G units was similar in switchgrass, whereas the greater reduction in G units resulted in an increased S : G ratio in tobacco. Phylogenetic analysis shows that PvMYB4 is closely related to ZmMYB42 and ZmMYB31, and overexpression of ZmMYB42 in transgenic *Arabidopsis* decreased S : G ratio through the relative enrichment of H and G subunits and reduction of S subunits (Sonbol *et al.*, 2009). However, overexpression of ZmMYB31 in transgenic *Arabidopsis* reduced lignin content but led to an increased amount of H subunits with no effect on the S : G ratio (Fornalé *et al.*, 2010). Thus, qualitative regulation of monolignol composition by MYB repressors appears to be both repressor- and species-specific, and not simply a result of whether the *MYB* gene or target species is monocot or dicot.

Although all the phenylpropanoid/monoligol biosynthetic genes were repressed by PvMYB4 in transgenic tobacco and switchgrass, whether this effect is directly or indirectly regulated through the binding of PvMYB4 to AC elements is not clear. ZmMYB31 can bind directly to the *ZmF5H* gene promoter region containing an ACII-like element (tCCAACC), as shown by quantitative reverse transcription chromatin immunoprecipitation (qRT-ChIP) assays in maize (Fornalé *et al.*, 2010). The potential presence of AC elements in (all) the switchgrass monolignol pathway genes, and direct binding of PvMYB4 to these putative elements, can be evaluated once the switchgrass genome sequence becomes available.

Biotechnological applications of PvMYB4 for development of switchgrass as a dedicated bioenergy crop

Bioenergy is commonly defined as the energy generated from renewable biological products (such as plant biomass) and is regarded as a clean and sustainable alternative to fossil sources of energy due to the potential contribution to reduced carbon dioxide emissions (McLaughlin *et al.*, 2002; McLaughlin & Adams Kszos, 2005; Williams *et al.*, 2009). Switchgrass feedstock is highly lignified plant biomass, and the high lignin content dramatically increases the energy cost for releasing the polysaccharide components from cell walls during biomass to biofuel conversion.

The molecular, genomic and biotechnological resources for switchgrass are limited but increasing exponentially (Tobias *et al.*, 2005, 2008; Okada *et al.*, 2010). Reliable genetic transformation of switchgrass using *Agrobacterium*-mediated methods has recently been developed (Burris *et al.*, 2009; Xi *et al.*, 2009), paving the way for increased biotechnological applications for biofuel and bioproduct improvements. For example, successful engineering of a functional metabolic pathway for the production of polyhydroxybutyrate (PHB) in transgenic switchgrass has been reported (Somleva *et al.*, 2008), suggesting that complex traits can be engineered in this dedicated bioenergy crop. One obvious strategy to overcome the cell wall recalcitrance of switchgrass biomass is to reduce the lignin content (Hisano *et al.*, 2009), based on the effectiveness of this approach in alfalfa and other species (Bell *et al.*, 2004; Chen & Dixon, 2007). This has recently been achieved in switchgrass through down-regulation of the monolignol biosynthetic genes *CAD* (Saathoff *et al.*, 2011) and *COMT* (Fu *et al.*, 2011), leading to increased sugar release and, in the latter case, improved ethanol yield at lower cellulase loadings.

The present report describes an alternative strategy for reducing recalcitrance in switchgrass by manipulating the expression of a key transcription factor. The lignin content, wall-bound ester-linked *p*-CA content and *p*-CA : FA ratio were reduced in the transgenic switchgrass, both features that could result in significantly improved saccharification efficiency based on our previous studies of natural variation in these cell wall parameters (Shen *et al.*, 2009a) and the phenotypes of the *PvCOMT* down-regulated transgenic switchgrass (Fu *et al.*, 2011). Indeed, the PvMYB4-OX transgenic switchgrass lines have approx. threefold increased saccharification efficiency compared with the control lines. Down-regulation of lignin pathway genes can result in negative growth impacts, possibly as a result of metabolic spill-over (Besseau *et al.*, 2007; Li *et al.*, 2010; Gallego-Giraldo *et al.*, 2011). The PvCOMT down-regulated switchgrass plants have a normal growth phenotype under glasshouse conditions, whereas the PvMYB4-OX plants have reduced stature but increased tillering. More detailed analysis of gene expression in these plants utilizing the recently available switchgrass Affymetrix Gene Chip may help explain the basis of this growth phenotype, which is reminiscent of the reduced stature but increased branching observed in alfalfa with down-regulated HCT expression (Gallego-Giraldo *et al.*, 2011). In these alfalfa plants, alterations in cytokinin and gibberellin concentrations and/or signaling may contribute to the phenotype (Gallego-Giraldo *et al.*, 2011).

Based on an estimation of dry biomass production under glasshouse conditions, some of the transgenic PvMYB4-OX lines have similar (line 1C) or *c.* 20% increased (line 1D) biomass production. Evaluation of these lines is therefore necessary under field conditions, with the potential for selection of lines retaining both improved biomass characteristics and good growth phenotypes.

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References

Akin DE. 2007. Grass lignocellulose: strategies to overcome recalcitrance. *Applied Biochemistry and Biotechnology* **137**: 3–15.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. 2002. Map kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415**: 977–983.

Bell J, Lehmann D, Wang ZY, Chen L, Auh CK, Dowling P. 2004. Transgenic down-regulation of caffeic acid *o*-methyltransferase (comt) led to improved digestibility in tall fescue (*Festuca arundinacea*). *Functional Plant Biology* **31**: 235–245.

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M. 2007. Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects Auxin transport and plant growth. *Plant Cell* **19**: 148–162.

Bomal C, Bedon F, Caron S, Mansfield SD, Levasseur C, Cooke JEK, Blais S, Tremblay L, Morency MJ, Pavy N. 2008. Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative in planta analysis. *Journal of Experimental Botany* **59**: 3925–3939.

Bouton JH. 2007. Molecular breeding of switchgrass for use as a biofuel crop. *Current Opinion in Genetics & Development* **17**: 553–558.

Bylesjo M, Nilsson R, Srivastava V, Gronlund A, Johansson AI, Jansson S, Karlsson J, Moritz T, Wingsle G, Trygg J. 2008. Integrated analysis of transcript, protein and metabolite data to study lignin biosynthesis in hybrid aspen. *Journal of Proteome Research* **8**: 199–210.

Chen F, Dixon RA. 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology* **25**: 759–761.

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde J-P, Merillon J-M, Hamdi S. 2006. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiology* **140**: 499–511.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M. 2008. Phylogeny. Fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* **36**: W465–W469.

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ. 1990. Abnormal plant development and down regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proceedings of the National Academy of Sciences, USA* **87**: 9057–9061.

Escamilla-Trevino LL, Shen H, Uppalapati SR, Ray T, Tang Y, Hernandez T, Yin Y, Xu Y, Dixon RA. 2009. Switchgrass (*Panicum virgatum*) possesses a divergent family of cinnamoyl CoA reductases with distinct biochemical properties. *New Phytologist* **185**: 143–155.

Fornalé S, Shi X, Chai C, Encina A, Irar S, Capellades M, Fuguet E, Torres JL, Rovira P, Puigdomènech P. 2010. ZmMYB31 directly represses maize lignin genes and redirects the phenylpropanoid metabolic flux. *Plant Journal* **64**: 633–644.

Fornalé S, Sonbol F-M, Maes T, Capellades M, Puigdomènech P, Rigau J, Caparrós-Ruiz D. 2006. Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid *o*-methyl-transferase genes by two new maize R2R3-MYB transcription factors. *Plant Molecular Biology* **62**: 809–823.

Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Chen F, Bouton J, Foston M, Dixon RA, Wang Z-Y. 2011. Genetic manipulation of lignin biosynthesis in switchgrass significantly reduces recalcitrance and improves biomass ethanol production. *Proceedings of the National Academy of Sciences, USA* **108**: 3803–3808.

Fuentes RG, Taliaferro CM. 2002. Biomass yield stability of switchgrass cultivars. In: Janick J, Whipkey A, eds. *Trends in new crops and new uses.* Alexandria, VA, USA: ASHS Press, 276–282.

Gális I, Šimek P, Narisawa T, Sasaki M, Horiguchi T, Fukuda H, Matsuoka K. 2006. A novel R2R3 MYB transcription factor NtMYBJS1 is a methyl jasmonate-dependent regulator of phenylpropanoid-conjugate biosynthesis in tobacco. *Plant Journal* **46**: 573–592.

Gallego-Giraldo L, Jikumaru Y, Kamiya Y, Tang Y, Dixon RA. 2011. Selective lignin down-regulation leads to constitutive defense response expression in alfalfa (*Medicago sativa* L.). *New Phytologist* **190**: 627–639.

Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N *et al.* 2005. EgMYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis. *Plant Journal* **43**: 553–567.

Hatton D, Sablowski R, Yung MH, Smith C, Schuch W, Bevan M. 1995. Two classes of cis sequences contribute to tissue-specific expression of a *PAL2* promoter in transgenic tobacco. *Plant Journal* **7**: 859–876.

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315**: 804–807.

Hisano H, Nandakumar R, Wang Z-Y. 2009. Genetic modification of lignin biosynthesis for improved biofuel production. *In Vitro Cellular & Developmental Biology – Plant* **45**: 306–313.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. 1985. A simple and general method for transferring genes into plants. *Science* **227**: 1229–1231.

Jackson D. 1991. *In-situ* hybridization in plants. In: Bowles DJ, Gurr SJ, McPherson M, eds. *Molecular plant pathology: a practical approach*. New York, USA: Oxford University Press, 163–174.

Jackson LA, Shadle GL, Zhou R, Nakashima J, Chen F, Dixon RA. 2008. Improving saccharification efficiency of alfalfa stems through modification of the terminal stages of monolignol biosynthesis. *BioEnergy Research* **1**: 180–192.

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C. 2000. Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *The EMBO Journal* **19**: 6150–6161.

Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N. 2007. Statistical significance of quantitative PCR. *BMC Bioinformatics* **8**: 131.

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G. 2004. MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. *Plant Molecular Biology* **56**: 255–270.

Keshwani DR, Cheng JJ. 2009. Switchgrass for bioethanol and other value-added applications: a review. *Bioresource Technology* **100**: 1515–1523.

Kurosu T, Peterlin BM. 2004. VP16 and ubiquitin; binding of P-TEFb via its activation domain and ubiquitin facilitates elongation of transcription of target genes. *Current Biology* **14**: 1112–1116.

Legay S, Lacombe E, Goicoechea M, Briere C, Séguin A, Mackay J, Grima-Pettenati J. 2007. Molecular characterization of EgMYB1, a putative transcriptional repressor of the lignin biosynthetic pathway. *Plant Science* **173**: 542–549.

Li X, Bonawitz ND, Weng JK, Chapple C. 2010. The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. *Plant Cell* **22**: 1620–1632.

Mann DGJ, LaFayette PR, Abercrombie LL, King ZR, Mazarei M, Halter MC, Poovaiah CR, Baxter H, Shen H, Dixon RA *et al.* 2011. Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass and other monocot species. *Plant Biotechnology Journal*. doi: 10.1111/j.1467-7652.2011.00658.x.

Matsui K, Umemura Y, Ohme-Takagi M. 2008. Atmybl2, a protein with a single myb domain, acts as a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. *Plant Journal* **55**: 954–967.

McLaughlin SB, Adams Kszos L. 2005. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass and Bioenergy* **28**: 515–535.

McLaughlin SB, De La Torre Ugarte DG, Garten CT Jr, Lynd LR, Sanderson MA, Tolbert VR, Wolf DD. 2002. High-value renewable energy from prairie grasses. *Environmental Science & Technology* **36**: 2122–2129.

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M. 2007. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. *Plant Cell* **19**: 270–280.

Monsma SA, Blissard GW. 1995. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *Journal of Virology* **69**: 2583.

Moore KJ, Moser LE, Vogel KP, Waller SS, Johnson BE, Pedersen JF. 1991. Describing and quantifying growth stages of perennial forage grasses. *Agronomy Journal* **83**: 1073–1077.

Nakashima J, Chen F, Jackson L, Shadle G, Dixon RA. 2008. Multi-site genetic modification of monolignol bioynthesis in alfalfa (*Medicago sativa* L.)- effects on lignin composition in specific cell types. *New Phytologist* **179**: 738–750.

Okada M, Lanzatella C, Saha MC, Bouton J, Wu R, Tobias CM. 2010. Complete switchgrass genetic maps reveal subgenome collinearity, preferential pairing, and multilocus interactions. *Genetics* **185**: 745–760.

Olsen AN, Ernst HA, Lo Leggio L, Skriver K. 2005. NAC transcription factors: structurally distinct, functionally diverse. *Trends in Plant Science* **10**: 79–87.

Oyama T, Shimura Y, Okada K. 1997. The *Arabidopsis* hy5 gene encodes a bZip protein that regulates stimulus-induced development of root and hypocotyl. *Genes & Development* **11**: 2983.

Patzlaff A, McInnis S, Courtenay A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten RW, Sederoff RR *et al.* 2003. Characterisation of a pine MYB that regulates lignification. *Plant Journal* **36**: 743–754.

Pauly M, Keegstra K. 2008. Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant Journal* **54**: 559–568.

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW. 2004. AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. *Plant Journal* **40**: 979–995.

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W. 2003. Genome-wide characterization of the lignification toolbox in *Arabidopsis*. *Plant Physiology* **133**: 1051–1071.

Romero I, Fuertes A, Benito MJ, Malpica JM, Leyva A, Paz-Ares J. 1998. More than 80 R2R3-MYB regulatory genes in the genome of *Arabidopsis thaliana*. *Plant Journal* **14**: 273–284.

Rose MD, Winston FM, Heiter P. 1990. *Methods in yeast genetics: a laboratory course manual*, Vol. 19. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Ruden DM, Ma J, Li Y, Wood K, Ptashne M. 1991. Generating yeast transcriptional activators containing no yeast protein sequences. *Nature* **350**: 250–252.

Saathoff AJ, Sarath G, Chow EK, Dien BS, Tobias CM, Herrera-Estrella A. 2011. Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PLoS ONE* **6**: 804–807.

Sadowski I, Ma J, Triezenberg S, Ptashne M. 1988. Gal4-VP16 is an unusually potent transcriptional activator. *Nature* **335**: 563–564.

Sarath G, Baird LM, Vogel KP, Mitchell RB. 2007. Internode structure and cell wall composition in maturing tillers of switchgrass (*Panicum virgatum*. L). *Bioresource Technology* **98**: 2985–2992.

Schmer MR, Vogel KP, Mitchell RB, Perrin RK. 2008. Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences, USA* **105**: 464–469.

Sheen J. 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiology* **127**: 1466–1475.

Shen H, Fu C, Xiao X, Ray T, Tang Y, Wang Z, Chen F. 2009a. Developmental control of lignification in stems of lowland switchgrass variety Alamo and the effects on saccharification efficiency. *BioEnergy Research* **2**: 233–245.

Shen H, Yin Y, Chen F, Xu Y, Dixon R. 2009b. A bioinformatic analysis of NAC genes for plant cell wall development in relation to lignocellulosic bioenergy production. *BioEnergy Research* **2**: 217–232.

Somleva MN, Snell KD, Beaulieu JJ, Peoples OP, Garrison BR, Patterson NA. 2008. Production of polyhydroxybutyrate in switchgrass, a value-added co-product in an important lignocellulosic biomass crop. *Plant Biotechnology Journal* **6**: 663–678.

Somleva MN, Tomaszewski Z, Conger BV. 2002. Agrobacterium-mediated genetic transformation of switchgrass. *Crop Science* **42**: 2080–2087.

Sonbol F-M, Fornalé S, Capellades M, Encina A, Touriño S, Torres J-L, Rovira P, Ruel K, Puigdomènech P, Rigau J *et al.* 2009. The maize ZmMYB42

represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*. *Plant Molecular Biology* **70**: 283–296.

Sparkes IA, Runions J, Kearns A, Hawes C. 2006. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protocols* **1**: 2019–2025.

Springer NM, Stupar RM. 2007. Allelic variation and heterosis in maize: how do two halves make more than a whole? *Genome Research* **17**: 264.

Stracke R, Werber M, Weisshaar B. 2001. The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**: 447–456.

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C. 1998. The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell* **10**: 135–154.

Tobias CM, Sarath G, Twigg P, Lindquist E, Pangilinan J, Penning BW, Barry K, McCann MC, Carpita NC, Lazo GR. 2008. Comparative genomics in switchgrass using 61,585 high-quality expressed sequence tags. *The Plant Genome* **1**: 111–124.

Tobias CM, Twigg P, Hayden DM, Vogel KP, Mitchell RM, Lazo GR, Chow EK, Sarath G. 2005. Analysis of expressed sequence tags and the identification of associated short tandem repeats in switchgrass. *Theoretical and Applied Genetics* **111**: 956–964.

Wang H, Avci U, Nakashima J, Hahn MG, Chen F, Dixon RA. 2010. Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants. *Proceedings of the National Academy of Sciences, USA* **107**: 22338–22343.

Wang YS, Yoo CM, Blancaflor EB. 2008. Improved imaging of actin filaments in transgenic *Arabidopsis* plants expressing a green fluorescent protein fusion to the C and N termini of the fimbrin actin binding domain 2. *New Phytologist* **177**: 525–536.

Williams PRD, Inman D, Aden A, Heath GA. 2009. Environmental and sustainability factors associated with next-generation biofuels in the US: what do we really know? *Environmental Science & Technology* **43**: 4763–4775.

Wullschleger SD, Davis EB, Borsuk ME, Gunderson CA, Lynd LR. 2010. Biomass production in switchgrass across the United States: database description and determinants of yield. *Agronomy Journal* **102**: 1158–1168.

Xi Y, Ge Y, Wang ZY. 2009. Genetic transformation of switchgrass. *Methods in Molecular Biology* **581**: 53–59.

Zhang F, Barboric M, Blackwell TK, Peterlin BM. 2003. A model of repression: CTD analogs and PIE-1 inhibit transcriptional elongation by P-TEFb. *Genes & Development* **17**: 748–758.

Zhao Q, Dixon RA. 2011. Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends in Plant Science* **16**: 227–233.

Zhao J, Zhang W, Zhao Y, Gong X, Guo L, Zhu G, Wang X, Gong Z, Schumaker KS, Guo Y. 2007. SAD2, an importin -like protein, is required for UV-B response in *Arabidopsis* by mediating MYB4 nuclear trafficking. *Plant Cell* **19**: 3805–3818.

Zhong R, Lee C, Ye Z-H. 2010. Evolutionary conservation of the transcriptional network regulating secondary cell wall biosynthesis. *Trends in Plant Science* **15**: 625–632.

Zhou J, Lee C, Zhong R, Ye ZH. 2009. MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. *Plant Cell* **21**: 248–266.

Zhou R, Jackson L, Shadle G, Nakashima J, Temple S, Chen F, Dixon RA. 2010. Distinct cinnamoyl CoA reductases involved in parallel routes to lignin in *Medicago truncatula*. *Proceedings of the National Academy of Sciences, USA* **107**: 17803–17808.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The phenylpropanoid and monolignol biosynthesis pathways.

Fig. S2 *PvMYB4* gene variants in switchgrass.

Fig. S3 PtMYB4 binds to AC elements in a yeast transcription system.

Fig. S4 The PQ-rich motif does not possess transcriptional activation activity.

Fig. S5 Overexpression of PvMYB4 alters phenylpropanoid metabolism in transgenic tobacco plants.

Fig. S6 Genomic DNA PCR and qRT-PCR analysis of PvMYB4-OX transgenic switchgrass.

Fig. S7 The S : G ratio of PvMYB4-OX transgenic switchgrass.

Fig. S8 PvMYB4-OX transgenic switchgrass has smaller vascular bundles and thinner tillers.

Fig. S9 Estimation of total dry biomass yield for PvMYB4-OX transgenic switchgrass.

Fig. S10 qRT-PCR analysis of flavonoid biosynthetic gene transcripts in PvMYB4-OX transgenic tobacco.

Table S1 Sequences and references for the gene-specific primers used in this work

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