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Research

Silencing of 4-coumarate:coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production

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Summary

- The lignin content of feedstock has been proposed as one key agronomic trait impacting biofuel production from lignocellulosic biomass. 4-Coumarate:coenzyme A ligase (4CL) is one of the key enzymes involved in the monolignol biosynthethic pathway.
- Two homologous *4CL* genes, *Pv4CL1* and *Pv4CL2*, were identified in switchgrass (*Panicum virgatum*) through phylogenetic analysis. Gene expression patterns and enzymatic activity assays suggested that Pv4CL1 is involved in monolignol biosynthesis. Stable transgenic plants were obtained with *Pv4CL1* down-regulated.
- RNA interference of *Pv4CL1* reduced extractable 4CL activity by 80%, leading to a reduction in lignin content with decreased guaiacyl unit composition. Altered lignification patterns in the stems of RNAi transgenic plants were observed with phloroglucinol-HCl staining. The transgenic plants also had uncompromised biomass yields. After dilute acid pretreatment, the low lignin transgenic biomass had significantly increased cellulose hydrolysis (saccharification) efficiency.
- The results demonstrate that Pv4CL1, but not Pv4CL2, is the key 4CL isozyme involved in lignin biosynthesis, and reducing lignin content in switchgrass biomass by silencing *Pv4CL1* can remarkably increase the efficiency of fermentable sugar release for biofuel production.

Introduction

The production of biofuels from renewable biomass could alleviate the dependence on fossil fuels, and this concept has led to a strong interest in developing biofuel feedstock crops and new biofuel conversion technologies (Carroll & Somerville, 2009). Switchgrass (*Panicum virgatum*), a warm-season perennial C_4 grass, has been considered as one prime candidate for lignocellulose-based feedstock production in the US (McLaughlin & Adams Kszos, 2005). One major breeding objective is to improve switchgrass feedstock quality for 'transforming grass to gas' (Schubert, 2006). Feedstock quality essentially equates to the optimized cell wall composition of biomass, which impacts the efficiency of biofuel production through (bio)chemical conversion of sugars to fuels (Carroll & Somerville, 2009). Two major cell wall components, cellulose and hemicellulose, are the primary carbohydrate sources for lignocellulose-based bioethanol production through fermentation; while another cell wall component, lignin, adversely impacts bioconversion (Chen & Dixon, 2007). Lignin tightly binds to hemicellulose and cellulose, thereby blocking the access of hydrolytic enzymes, and also possibly inhibiting the activities of hydrolytic and fermentation enzymes during the bioconversion processes (Halpin, 2004; Keating *et al.*, 2006; Endo *et al.*, 2008; Abramson *et al.*, 2009).

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Therefore, it is hypothesized that switchgrass feedstock quality for bioethanol production can be improved by decreasing its lignin content (Carroll & Somerville, 2009). Comprehensive characterization of lignin biosynthesis pathways in switchgrass will enable us to manipulate the lignin content of switchgrass biomass through genetic engineering. Research on the molecular mechanisms regulating lignin biosynthesis in switchgrass has just started (Escamilla-Treviño *et al.*, 2009; Fu *et al.*, 2011a,b; Saathoff *et al.*, 2011a,b). One switchgrass lignin biosynthesis gene, *cinnamyl-alcohol dehydrogenase* (*CAD*), was recently identified, and the down-regulation of switchgrass *CAD1* resulted in a decreased lignin content of switchgrass biomass that potentially enhances biofuel production (Fu *et al.*, 2011b; Saathoff *et al.*, 2011a). The overall biomass production of the low-lignin switchgrass plants was not characterized in these reports (Fu *et al.*, 2011b; Saathoff *et al.*, 2011a), and therefore an argument could not be made for the advantages of growing *CAD* down-regulated switchgrass for feedstock production. By contrast, switchgrass plants down-regulated in the expression of another monolignol biosynthesis gene, *caffeic acid 3-O-methyltransferase* (*COMT*), were shown to have normal growth behavior and exhibit reduced recalcitrance for saccharification and fermentation to ethanol (Fu *et al.*, 2011a). In addition to providing proof of concept for lignin engineering in switchgrass, these results clearly confirm that the lignin biosynthesis pathways are evolutionarily conserved in different plant species, including switchgrass (Xu *et al.*, 2009; Weng & Chapple, 2010). In contrast to switchgrass, the monolignol biosynthetic pathways have been well studied in model plant species, such as Arabidopsis, alfalfa (*Medicago sativa*), and poplar (*Populus trichocarpa* × *Populus deltoids*, *P. tremuloides* or *P. tomentos*) (Smita & Nath, 2008; Carroll & Somerville, 2009). The knowldege of lignin synthesis from model plant species enables us to identify lignin-related genes in switchgrass, and therefore to manipulate the lignin content in switchgrass biomass at different stages in the pathway to optimize processing efficiency.

The phenolic polymer lignin is derived from ρ-hydroxycinnamic alcohols (monolignols) via combinatorial radical coupling reactions (Boudet, 2007; Umezawa, 2010). Approximately 10 key enzymes are involved in the monolignol biosynthesis pathway in model plant species (Hisano *et al.*, 2009), and most of these gene-homologs could be identified from the switchgrass expressed sequence tag (EST) database (Tobias *et al.*, 2008). Among the monolignol biosynthesis enzymes, 4-Coumarate:coenzyme A ligase (4CL) is a key enzyme involved in early steps of the monolignol biosynthesis pathway. 4CL catalyzes the formation of activated thioesters of hydroxycinnamic acids, which may act as substrates for entry into different branch pathways of phenylpropanoid metabolism (Lee *et al.*, 1997). *4CL* genes normally belong to a small gene family. In Arabidopsis, three 4CL isozymes, At4CL1, At4CL2, and At4CL3, with different substrate preferences and gene expression patterns, have been identified. At4CL1 and At4CL2 are involved in the monolignol biosynthesis pathway, while At4CL3 participates in flavonoid and other nonlignin biosynthesis pathways (Ehlting *et al.*, 1999; Cukovica *et al.*, 2001). In poplar, two functionally divergent 4CLs were identified. Ptr4CL1 is devoted to lignin biosynthesis in developing xylem tissues, whereas Ptr4CL2 is possibly involved in flavonoid biosynthesis in epidermal cells (Hu *et al.*, 1998). Down-regulaton of *At4CL1* in Arabidopsis or *Ptr4CL1* in poplar resulted in reduced lignin content (Hu *et al.*, 1999; Sanchez *et al.*, 2006; Voelker *et al.*, 2010) and little changed biomass production (Sanchez *et al.*, 2006), although *4CL* genes were not colocalized within the quantitative trait loci regulating biomass production in *Eucalyptus* (Kirst *et al.*, 2004). Based on the characterization of 4CLs in other plant species, we hypothesize that identifying the switchgrass 4CL isozyme involved in the monolignol biosynthetic pathway, and down-regulating this specific *4CL* gene, could reduce switchgrass lignin content without significantly adverse effects on biomass production.

In this report we identified two switchgrass *4CL* genes through phylogenetic analysis of different 4CL homologs. The enzyme activities and substrate preferences of the two switchgrass 4CL isoforms were determined. One gene, *Pv4CL1*, was silenced by RNA interference (RNAi). The phenotypes of the transgenic plants, including biomass yield, cell wall composition, and cellulose hydrolysis efficiency, were characterized in detail. Our results indicated that Pv4CL1, but not Pv4CL2, was the key 4CL isozyme involved the monolignol biosynthesis pathway, and reducing lignin content in switchgrass biomass by silencing *Pv4CL1* can significantly increase the efficiency of fermentable sugar release for biofuel production.

Materials and Methods

Cloning *Pv4CL1* and *Pv4CL2* cDNAs

4-Coumarate:coenzyme A ligase sequences of *Zea mays* were used as 'query' for BLAST searches against the available switchgrass sequences in public databases. Full-length consensus sequences from multiple cDNA alignments were used for primer design. TRIzol Reagent (Invitrogen) was used for RNA extraction. DNA contamination was eliminated by treating total RNA with UltraPure DNase I (Invitrogen). The integrity and quantity of total RNA were checked by running through a 0.8% agarose gel and through a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed using the SuperScript III First-Strand System for RT-PCR Kit (Invitrogen) with an oligo-dT primer. The full-length cDNA was amplified by

PCR using KOD DNA polymerase (EMD, San Diego, CA, USA), and cloned into the vector p-ENTR/D-TOPO. Sequences of all primers used in this study are listed in Supporting Information, Table S1. The primers used for *Pv4CL1* and *Pv4CL2* cloning were *Pv4CL1*_ORF_For and *Pv4CL1*_ORF_Rev, and *Pv4CL2*_ORF_For and *Pv4CL2*_ORF_Rev, respectively.

RT-PCR and qRT-PCR

For quantitative reverse transcription polymerase chain reaction (qRT-PCR), total RNA was isolated from young switchgrass (*Panicum virgatum* L.) plants (E4 stage (elongation stage with four internodes) internodes, leaves, nodes, leaf sheaths, R1 (reproductive stage 1) inflorescences, and from fully elongated flower stalks, leaves, and internodes). For qRT-PCR, PRIMER EXPRESS_ software (version 3.0; Applied Biosystems, Foster City, CA, USA) was used to design primer sets for *Pv4CL1*, *Pv4CL2* and the reference genes (*Pv_UBIQUITIN* (FL955474.1) and *Pv_ACTIN2* (FL724919.1)) (Table S1). The qRT-PCR was performed with ABsolute Blue QPCR Sybr Green ROX mix (Thermo Scientific, Wilmington, USA) in the ABI 7500 Real-Time PCR System or ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc., Carlsbad, CA, USA) in a 25 or 10 μl reaction volume, respectively, according to the manufacturer's instructions. Each sample had three replicates, and the data were normalized against the reference genes. There was no amplification of the primer pairs without the cDNA templates. RT-PCR was also used to detect the transcript abundance of *Pv4CL1* in different transgenic lines using RNA isolated from the third internodes of each plant.

Expression of switchgrass Pv4CL1 and Pv4CL2 in *Escherichia coli*

Pv4CL1 and *Pv4CL2* were subcloned into the expression vector pDEST17 using Gateway technology (Invitrogen). *E. coli* strain Rosetta cells harboring the *Pv4CL1* or *Pv4CL2* constructs were cultured at 37°C until OD_{600} reached 0.6–0.7, and protein expression was then induced by adding isopropyl 1-thio β-galactopyranoside (IPTG) at a final concentration of 0.5 mM, followed by incubation at 16°C for 18–20 h. Frozen cell pellets from 25 ml of induced culture were thawed at room temperature and resuspended in 1.2 ml of extraction-washing buffer (10 mM imidazole, 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, and 10 mM β-mercaptoethanol). The extracts were sonicated three times for 20 s, and the supernatants recovered after centrifugation at 16 000 ***g*** were mixed with equilibrated Ni-NTA beads (Qiagen, Germantown, MD, USA) and incubated at 4°C for 30 min under constant inversion to allow the His-tag proteins to bind to the beads. After washing the beads three times with 1 ml of extraction-washing buffer, target proteins were eluted with 250 μl of elution solution (300 mM imidazole, 50 mM Tris-HCl buffer pH 8.0, 500 mM NaCl, 10% glycerol, and 10 mM β-mercaptoethanol). The purity of eluted target proteins was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentrations were determined using the Bio-Rad protein assay (BioRad).

Enzyme activity assays and kinetics

Pure recombinant enzymes (10–400 ng) were incubated at 30°C (10 or 30 min) with 50 mM Bis-Tris propane buffer (pH 7.5), 2.5 mM $MgCl_2$, 5 mM ATP, 1 mM coenzyme A (CoA), and 2–100 μM substrate (cinnamic acid, 4-coumaric acid, caffeic acid, ferulic acid or sinapic acid) in a final volume of 100 μl. The reactions were stopped by adding 10 μl of glacial acetic acid. Reaction products were analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column (Spherisorb 5 μ ODS2; Waters, Milford, MA, USA) in a step gradient using 1% phosphoric acid in water as solvent A and acetonitrile as solvent B. Calibration curves were constructed with authentic standards of each product. The 4CL test substrates cinnamic acid, 4-coumaric acid, caffeic acid, ferulic acid and sinapic acid were purchased from Sigma-Aldrich, while the 4CL products for calibration curves, 4-coumaroyl CoA, caffeoyl CoA, and feruloyl CoA, were synthesized as described previously (Stockigt & Zenk, 1975).

Construction of gateway compatible vectors

The pCAMBIA1305.2 vector was modified to be a Gateway-compatible binary vector for switchgrass transformation. The pUC19 vector was first digested with *Eco*RI and *Sph*I and blunt-ended with Klenow DNA polymerase. Re-ligation of the treated pUC19 vector led to the new plasmid pUC19-Δ*Eco*RI-*Sph*I that had a unique *Hin*dIII site. A *Hin*dIII DNA fragment from pAHC27 that carried the maize *Ubi* promoter and the *uid*A (*GUS*) gene was subcloned into pUC19-Δ*Eco*RI-*Sph*I. The *uid* A (*GUS*) gene was replaced with a *Bam*HI-*Eco*RV-HA-*Sac*I linker (5′-GGATCCGATATCTATCCATACGATGTGCCAGATTACGCATAGGAGCTC-3′) to generate pUC19-Ubi-HA-NosT. The ccdB(B) cassette frame A was then inserted into the *Eco*RV site of pUC19-Ubi-HA-NosT to generate pUC19-Ubi-DesA-HA-NosT. The *Hin*dIII fragment from pUC19-Ubi-DesA-HA-NosT was subcloned into the *Hin*dIII site of pCAMBIA1305.2, which resulted in pVT1629 (Fig. S2). This vector allowed us to either overexpress a target gene or silence a gene in the grass species.

An Entry vector, pEntry/D-Kannibal, for gene silencing was also constructed (Fig. S2b). The pEntry-1A vector (Invitrogen) was modified for cloning fragments of both

antisense and sense strands of *Pv4CL1*. In brief, a *Sal*I-*Xba*I DNA fragment carrying the PDK intron from pKannibal (Wesley *et al.*, 2001) was cloned into pEntry-1A to generate pEntry/D-Kannibal.

A 203 bp cDNA fragment of *Pv4CL1*, spanning part of the putative Box I domain (Stuible & Kombrink, 2001) (Fig. S1), was amplified from the cDNA of switchgrass cv Alamo using a nested RT-PCR method. The first pair of primers was Pv4CL_1st Round_For and Pv4CL_1st Round_Rev, and the nested PCR primers were Pv4CL_H3RI_For and Pv4CL SalXba_Rev. The *Pv4CL1* fragments from pEN-Pv4CL1 were sequentially cloned into the *Sal*I/*Eco*RI and *Hin*dIII/*Xba*I sites. This cloning step generated the RNAi entry vector pEntry/D-Kannibal-2x*Pv4CL1*. The Kannibal-2x*Pv4CL1* was cloned into pVT1629 by LR Gateway cloning reaction to generate pVT1629-2 × Pv4CL. The binary vector was transformed into *Agrobacterium tumefaciens* strain C58C1 by electroporation.

Switchgrass genetic transformation

Mature seeds of switchgrass line HR8 selected from cv Alamo were used for all tissue culture and genetic transformations in this study. A modified *Agrobacterium*-mediated transformation protocol was used to transform switchgrass with the RNAi binary vector (Somleva *et al.*, 2002). In brief, somatic embryogenic calluses were suspended in *Agrobacterium* solution (OD_{600} = 0.6) and vacuum-infiltrated for 10 min with occasional shaking. After *Agrobacterium* inoculation, the calluses were blotted on sterile paper towels and then transferred to the co-cultivation medium for 4 d at 23°C in the dark. After co-cultivation, the calluses were transferred onto callus and then regeneration media selected under 50 mg l^{-1} hygromycin B (Sigma). The regenerated plants were verified by PCR, Southern blot, and β-glucuronidase (GUS) staining.

The verified transgenic plants were grown in the horticulture glasshouse at Virginia Tech, with temperatures set at 22 : 28°C, night : day with a 12–14 h light regime. The plants were grown in Miracle-Gro Potting Mix (Miracle-Gro Lawn Products, Inc., Marysville, OH, USA) in 1.1×10^{-2} m^3 pots and watered about twice a week. Wild-type (WT) plants regenerated from nontransformed calluses were also grown in the same glasshouse under the same conditions. Each transgenic line was multiplied by splitting tillers and maintained in the glasshouse. Plant samples were harvested when 50% of the tillers had flowered.

Switchgrass is gametophytically self-incompatible. Therefore, we obtained T_1 plants by crossing the T_0 transgenic line-115 with WT plants. The T_1 plants segregated in a 1 : 1 ratio according to the presence of the *HPTII* gene detected by PCR (Table 1), and were grown and harvested under the same conditions as already mentioned.

4CL activity assays in plant protein extracts

Ground stem tissue (1 g), harvested from stems at the same growth stage, was suspended in 2.7 ml of extraction buffer (100 mM Tris-Cl, pH = 7.5, 10% glycerol, 1 mM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride) and 0.5 mM of DTT), and 0.1 g of polyvinyl-polypyrrolidone was then added. The suspension was kept on ice for 45 min with occasional vortexing. The supernatant was recovered after centrifugation (12 000 ***g*** for 5 min), and desalted by passing it through a PD-10 column (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The crude protein extracts (3–4 μg) were incubated at 30°C for 10–30 min with 50 mM Bis-Tris propane buffer (pH 7.5), 2.5 mM $MgCl_2$, 5 mM ATP, 1 mM CoA and 60 μM 4-coumaric acid in a final volume of 100 μl. The reactions were stopped by adding 10 μl of glacial acetic acid. Reaction products were analyzed by reverse-phase HPLC on a C18 column (Spherisorb 5 μ ODS2; Waters) in a step gradient using 1% phosphoric acid in water as solvent A and acetonitrile as solvent B. Calibration curves were constructed with authentic standard of the product 4-coumaroyl CoA.

Carbohydrate and lignin assays

Whole stems (from the first internode and above) of RNAi transgenic and WT control plants were collected and dried for cell wall composition analysis. The structural carbohydrate compositions of switchgrass biomass were determined using a modified quantitative saccharification (QS) procedure (Moxley & Zhang, 2007). Monomeric sugars were measured with a Shimadzu HPLC equipped with a Bio-Rad Aminex HPX-87P column (Richmond,

Table 1 Growth performance of segregating switchgrass (*Panicum virgatum*) T_1 plants

T_1 plants	Number of plants	Mature root color	Basal stems color	Tiller number	Tiller height (cm)	Above-ground DW (g)	Below-ground DW (g)	Total DW (g)
Wild-type[a]	12	White	White	75.3[b] (14.1)	148.0[b] (9.3)	149.1[b] (29.9)	106.7[b] (27.3)	255.7[b] (50.3)
Transgenic[a]	13	Reddish brown	Reddish brown	83.9 (21.4)	147.8 (13.4)	141.2 (21.8)	118.8 (25.9)	259.4 (45.1)

[a]Detected by PCR with *HPTII* gene primers; standard error is in parenthesis.
[b]No statistically significant difference was detected between wild-type and transgenic plants.
DW, dry weight.

CA, USA). Lignin and ash were measured according to the standard National Renewable Energy Laboratory (NREL) biomass protocol (Sluiter *et al.*, 2004). The concentrations of glucose and xylose in the enzymatic hydrolysates were measured with a Shimadzu HPLC equipped with a Bio-Rad Aminex HPX-87H chromatography column. Furfural and Hydroxymethyl Furfurl (HMF) were not observed in the hydrolysates (< 0.001%, w/v).

Determination of monolignol composition by thioacidolysis/GC-MS

Whole stems of different plants were dried and treated for thioacidolysis followed by GC-MS to measure the monolignol composition. Extractive-free lignin was made by acetone extraction in a Soxhlet apparatus for 24 h (Rolando *et al.*, 1992). The dried lignin of each sample was processed through a recently revised thioacidolysis method (Robinson & Mansfield, 2009). The silylated sample was injected into the GC column (Restek RTX5-MS, 1 μM film thickness, 30 M × 3.2 mM i.d., Thames Restek UK Ltd., Windsor, UK). The GC-MS analysis was modified from a previous method (Rolando *et al.*, 1992) and performed on a VG 70SE double-focusing magnetic sector instrument, interfaced to a HP5790 GC.

Histology and microscopy

The internodes of the T_1 segregating plants were embedded in 2.5% agarose and cut with a Leica VT1200 vibrating blade microtome (Bannockburn, IL, USA) into 50-μm-thick sections. Phloroglucinol and Mäule staining of the 50-μm-thick stem sections were used to analyze the lignin deposition patterns by visualization under an Olympus SZXZ-RFL3 fluorescence microscope (Olympus America, Melville, NY, USA) (Pomar *et al.*, 2002; Coleman *et al.*, 2008).

Dilute acid (DA) pretreatment and enzymatic hydrolysis

The dried switchgrass materials were ground and sieved through a size 40–60 mesh. The switchgrass samples were pretreated with DA, using 1.3% (w/w) sulfuric acid at a solid loading of 10% (w/w) at 130°C, 15 psi (autoclave) for 40 min. After DA, the hydrolysates were separated by centrifugation. The switchgrass residues were washed with water before enzymatic hydrolysis. The DA-pretreated switchgrass samples were diluted to 20 g biomass l^{-1} in 50 mM sodium citrate buffer (pH 4.8) with supplementary addition of 0.1% (w/v) NaN_3, as described previously (Moxley & Zhang, 2007; Zhu *et al.*, 2009). All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50°C. The enzyme loadings were five filter paper units (FPUs) of cellulase (Novozymes Inc., Bagsvaerd, Denmark) and 10 units of β-glucosidase (Novozymes) per g of biomass. The cellulose and β-glucosidase enzyme activities were confirmed with standard protocols (Adney & Baker, 1996). The protein content was determined by bicinohoninic acid (BCA) assay using BSA as a protein standard. The estimated protein contents of cellulase and β-glucosidase were *c.* 143 mg ml^{-1}. After enzymatic hydrolysis, glucan digestibility was calculated as described previously (Zhang *et al.*, 2009). The mass balance of dilute acid pretreatment and enzymatic hydrolysis is shown in Fig. S3.

Results

Isolation and characterization of switchgrass *4CL* genes and proteins

Using both a full-length sequence and conserved domains of a maize (*Z. mays*) *4CL* gene, *Zm4CL* (AY566301), as a query to BLAST against the switchgrass nucleotide and EST databases, we identified six ESTs annotated as *4CL*-like genes. Only two genes were classified with other characterized *4CLs* by phylogenetic analysis (Fig. 1). We therefore named the switchgrass *4CL* gene EU491511.1 as *Pv4CL1*, and another *4CL* homolog (JF414903) as *Pv4CL2*.

Pv4CL1 has an open reading frame (ORF) of 1629 nucleotides encoding a 542-amino-acid protein with a predicted molecular mass of 58.35 kDa and a isoelectric point (pI) of 5.38. *Pv4CL2* has an ORF of 1728 nucleotides encoding a protein of 575 amino acids (61.07 kDa) with a calculated pI of 5.37. The protein sequences deduced from the *Pv4CL1* and *Pv4CL2* cDNAs show 60% identity (Fig. S1), which suggests they are homologs rather than two alleles, although, based on anlaysis of other monolignol gene families (Escamilla-Treviño *et al.*, 2009), the tetraploid switchgrass cv Alamo may have multiple alleles of *Pv4CL* genes. Both sequences have the AMP-binding domain (*PFSSGTTGLPKGV* for 4CL1 and *PYSSGTTGLPKGV* for 4CL2), the *GEICIRGR* motif (Stuible & Kombrink, 2001) and the conserved *VPP* and *PVL* domains (Schneider *et al.*, 2003), all characteristics of 4CL enzymes.

A phylogenetic tree of Pv4CL1, Pv4CL2 and most other 4CL proteins was constructed, and showed similar phylogenetic patterns to the ones reported previously (Fig. 1), in which all 4CLs could be classified into two major classes (Ehlting *et al.*, 1999; Cukovica *et al.*, 2001). Pv4CL1 was classified in the class I group, along with the characterized 4CL enzymes, such as Arabidopsis At4CL1, At4CL2, aspen Ptr4CL1, and pine Pt4CL1, that are devoted to the monolignol biosynthesis pathway (Hu *et al.*, 1998; Ehlting *et al.*, 1999; Wagner *et al.*, 2009). Pv4CL2 was classified in the class II group, in which the characterized 4CL enzymes,

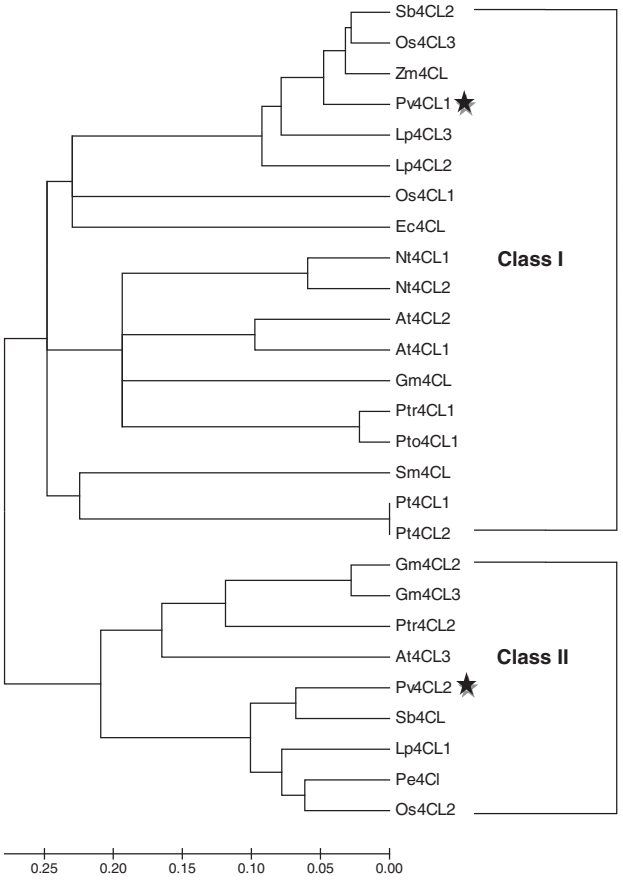

Fig. 1 Phylogenetic tree of Pv4CL1 and its homologs in some model plants. Pv4CL1 is classified in the class I group, whereas Pv4CL2 is in the class II group. The multiple alignments were done with ClustalW, and the Neighbor-Joining (NJ) tree was built using Mega4 software (Tamura *et al.*, 2007). The optimal tree with the sum of branch length = 3.656 351 42 is shown. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Scale indicates amino acid substitutions per position. The NCBI accession numbers of the protein sequences are as follows: Arabidopsis At4CL1 (NP_175579), At4CL2 (NP_188761), At4CL3 (NP_176686); Aspen (*Populus tremuloides*) Ptr4CL1 (AAC24503.1), Ptr4CL2, (AAC24504.1); Bamboo (*Phyllostachys edulis*) putative Pe4CL (FP101648.1); Eucalyptus (*Eucalyptus camaldulensis*) Ec4CL (ACX68559.1); Maize Zm4CL (NM_001111788); Poplar (*P. tomentosa*) Pto4CL1 (AAL02145.1); Pine Pt4CL1 (PTU12012), Pt4CL2 (PTU12013); Rice (*Oryza sativa*) Os4CL1 (BAD05189), Os4CL2 (Q42982), Os4CL3 (AB234050); Ryegrass (*Lolium perenne*) Lp4CL1 (AAF37732.1), Lp4CL2 (AAF37733.1), Lp4CL3 (AAAF37734.1); *Selaginella moellendorffii* Sm4CL (XP_002985214.1); Sorghum (*Sorghum bicolor*) Sb4CL (AAA64913.1), putative Sb4CL2 (XP_002451647.1); Soybean (*Glycine max*) Gm4CL (AAL98709), Gm4CL2 (P31687), Gm4CL3 (AAC97389); Tobacco (*Nicotiana tabacum*) Nt4CL1 (O24145), Nt4CL2 (O24146).

such as Arabidopsis At4CL3 and aspen Ptr4CL2, mainly participate in the flavonoid biosynthesis pathway (Hu *et al.*, 1998; Ehlting *et al.*, 1999).

The expression patterns of *Pv4CL1* and *Pv4CL2* were analyzed by real-time PCR (qRT-PCR). *Pv4CL1* transcripts were more abundant in the highly lignified internodes than in leaves and other tissues with relatively lower lignin contents (Fig. 2). In the internodes, the *Pv4CL1* transcript abundance is approx. seven times higher than that of *Pv4CL2* (Fig. 2). In switchgrass internodes, the lignin content increases with increasing distance from the peduncle (Sarath *et al.*, 2007; Shen *et al.*, 2009). The transcript abundance of *Pv4CL1* in different organs largely correlates with the cell wall lignification pattern. Based on the expression pattern and phylogenetic analysis, we hypothesize that *Pv4CL1* is the functional 4CL enzyme involved in the monolignol biosynthesis pathway in switchgrass.

Escherichia coli-expressed His-tagged Pv4CL1 and Pv4CL2 fusion proteins were purified to homogeneity (Fig. 3a). The enzymatic activities of the purified proteins were initially screened by determining their ability to ligate CoA to form the respective CoA esters. Both enzymes were active with 4-coumaric, caffeic, and ferulic acids, but cinnamic and sinapic acids were not substrates.

Kinetic parameters of both recombinant enzymes were determined for all three substrates using a fixed concentration of CoA (Table 2). Chromatograms and curves of reaction velocity vs substrate concentration for the three substrates are shown in Fig. 3(b–c). Kinetic parameters for Pv4CL2 using ferulic acid were not determined because the efficiency of the reaction was low in comparison with 4-coumaric acid or caffeic acid. The preferred substrate for both enzymes was 4-coumaric acid with similar efficiencies (K_{cat}/K_m), but the K_{cat} value for Pv4CL2 was lower than that for Pv4CL1 (Table 2).

Down-regulating *Pv4CL1* expression by RNAi

A 203 bp fragment of *Pv4CL1* (Fig. S1) that is specific to this gene was used to generate the RNAi construct pVT1629-2 × Pv4CL. One set of Gateway-compatible entry and destination vectors (Fig. S1) was constructed for generating the RNAi vector. *Agrobacterium*-mediated transformation of switchgrass with the RNAi vector yielded > 100 putative transgenic plants. The transgenic lines were verified by detecting the presence of the *HPTII* gene by PCR and Southern blot, and the *uidA* gene by GUS staining (data not shown).

We selected seven T_0-generation transgenic plants to monitor *Pv4CL1* transcript abundance by RT-PCR and qRT-PCR. The RT-PCR (Fig. 4a) and qRT-PCR results were consistent, showing that the transcript abundance of *Pv4CL1* in the transgenic lines ranged from 0.05- to 0.73-fold that of the WT control plants (Fig. 4b).

Segregated T_1 plants were further studied. In the transgenic T_1 plants, abundance of *Pv4CL1* transcripts, but not *Pv4CL2* transcripts, were greatly reduced (Fig. 4c), con-

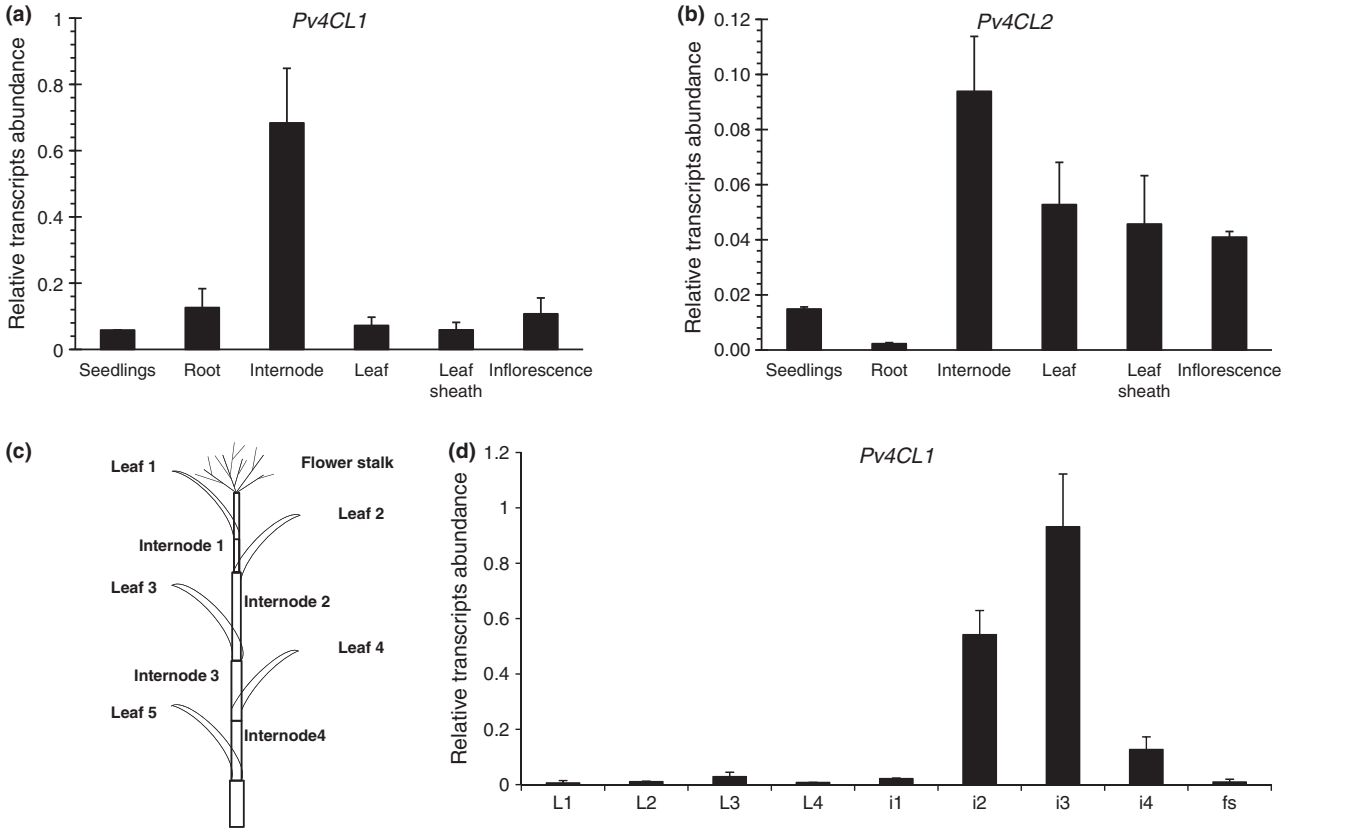

Fig. 2 Transcript abundance of *Pv4CL1* and *Pv4CL2* in different organs. Quantitative real-time PCR using *ACTIN* and *UBIQUITIN* as reference genes was used to determine transcript abundance of *Pv4CL1* (a) and *Pv4CL2* (b) in six switchgrass (*Panicum virgatum*) organs. (c) Graphical representation of a switchgrass plant. (d) Transcript abundance of *Pv4CL1* in different internodes and leaves. L1–4, leaf 1–leaf 4; i1–i4, internode 1 to internode 4; fs, flower stalk. Representative data are shown from two biological repeats. The dissociation curve for the quantitative reverse transcription polymerase chain reaction (qRT-PCR) products showed that the qRT-PCR primers were gene-specific. Error bars indicate +SE (standard error).

firming that the RNAi construct specifically targeted *Pv4CL1*. Protein extracts from pooled stem tissues of three T_1 transgenic plants were assayed for 4CL activities with 4-coumaric acid and CoA as substrates under optimal conditions. The result showed that transgenic T_1 plants exhibited, on average, an 80% reduction in 4CL activity (Fig. 4d,e).

Suppression of *Pv4CL1* results in phenotypic alterations and reduced lignin content

Different T_0 transgenic switchgrass lines with low *Pv4CL1* transcript abundances showed browning on parts of the leaf midvein (Fig. 5a), and sporadically exhibited brown patches in stem internodes (Fig. 5b), similar to low lignin *brown-midrib* (*bm*) maize mutants (Cherney *et al.*, 1991). The inner sides of the basal stems (e.g. the stems below the fourth internodes) became reddish-brown (Fig. 5c). With decreasing distance from the flower stalks, the number of dark brown patches on the outside of the stems decreased, and the reddish-brown color on the inner side of the stems gradually reduced to that of WT plants. The mature roots of the transgenic lines turned reddish brown to various degrees. However, the newly elongated roots and the root tips were still white, similar to the WT plants (Fig. 5d).

The above-ground biomass yields of four independent T_0 lines and three tissue culture-regenerated WT plants were measured. As shown in Table 3, silencing *Pv4CL1* did not affect the biomass yields in T_0 transgenic lines. To confirm the effect of silencing *Pv4CL1* in switchgrass, the biomass yields and other phenotypes were further measured in T_1 plants. The reddish-brown color in mature roots and basal stems cosegregated with the RNAi transgene in T_1 plants. The biomass yield and other agronomic traits related to biomass production (e.g. tiller number and plant height) were not significantly different between T_1 plants with or without the transgene (Table 1), suggesting that silencing of *Pv4CL1* did not significantly affect the biomass yields of switchgrass plants grown under glasshouse conditions.

We measured the cell wall compositions of four individual T_0 plants (Tables 4, 5) and the pooled T_1 plants with or without the transgene (Table 6). The T_0 plants had 23–34% less acid-insoluble lignin or 17–32% less total lignin than WT plants, and varied amounts of cellulose (referred

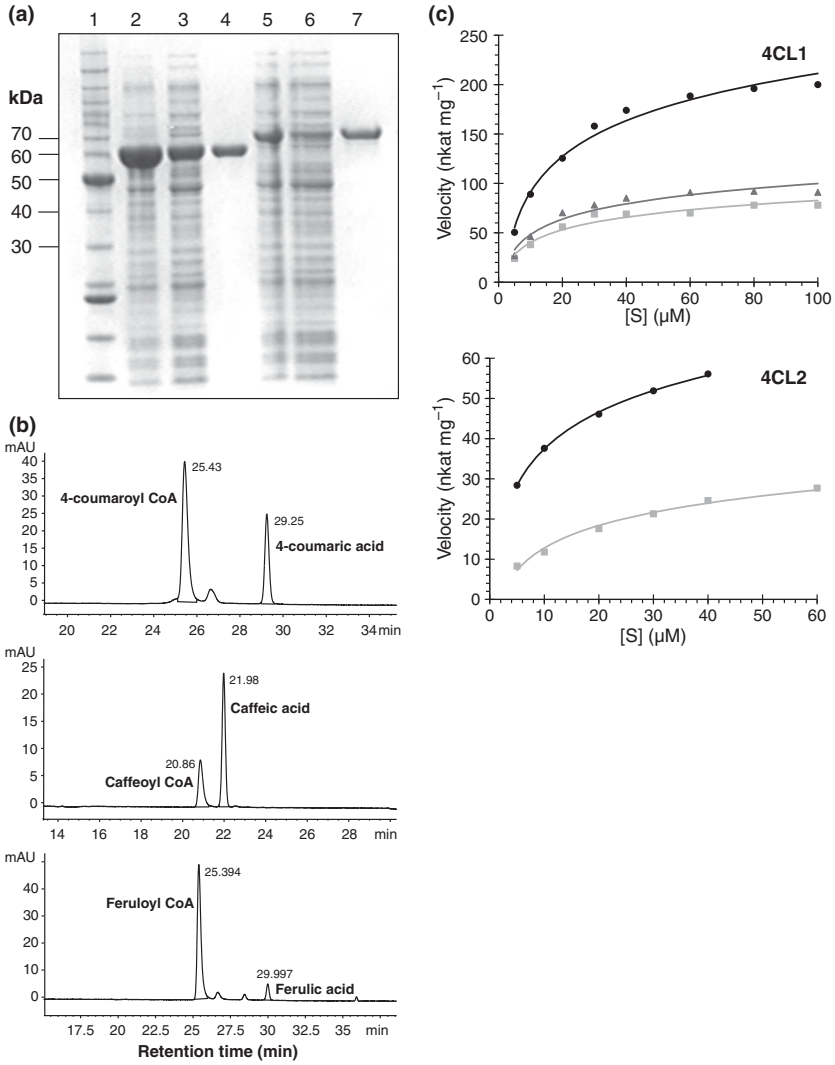

Fig. 3 Functional characterization of Pv4CL1 and Pv4CL2. (a) Protein gel blot analysis of recombinant enzymes expressed in *Escherichia coli*, 1; molecular weight markers, 2, 3, 4; Pv4CL1; 5, 6 and 7, Pv4CL2; 2 and 5, total proteins from induced cells; 3 and 6, soluble proteins from induced cells; 4 and 7, pure recombinant protein. (b) High-performance liquid chromatograms and retention times of substrates and products after 4-coumarate:coenzyme A ligase (4CL) reaction. (c) Curves of reaction velocity vs substrate concentration for Pv4CL1 and Pv4CL2 towards 4-coumaroyl coenzyme A (CoA, circles), caffeoyl CoA (squares) or feruloyl CoA (triangles).

Table 2 Kinetic parameters of Pv4CL1 and Pv4CL2

	K_m (μM)		K_{cat} (s^{-1})		K_{cat}/K_m (s^{-1} μM^{-1})	
Substrate	4CL1	4CL2	4CL1	4CL2	4CL1	4CL2
4-Coumaric acid	18.8	6.3	15.9	4.0	0.84	0.63
Caffeic acid	13.5	17.4	5.8	2.2	0.43	0.13
Ferulic acid	12.9	nd	7	nd	0.54	nd
Sinapic acid	No conversion					
Cinnamic acid	No conversion					

nd, not determined because of inefficient conversion.

to as glucan) and hemicellulose (referred to as xylan, the predominant component of hemicellulose in switchgrass) contents (Table 4). Monolignol compositions (hydroxyphenyl (H), guaiacyl (G), and syringyl (S)) of four T_0 transgenic lines and the WT control plants were also measured. As shown in Table 5, the T_0 transgenic lines had similar S, but less G and higher H contents than WT plants.

Between the T_1 plants with or without the RNAi transgene, changes of cell wall compositions were also observed. The pooled transgenic T_1 plants had 22% acid-insoluble lignin or 22% total lignin reduction compared with WT plants. The transgenic T_1 plants had similar S content, but 47% less G content and 45% more H content than non-transgenic control T_1 plants (Table 6). Compared with cell wall compositions of T_0 plants, segregated T_1 plants all had relatively high lignin content but low cellulose content at

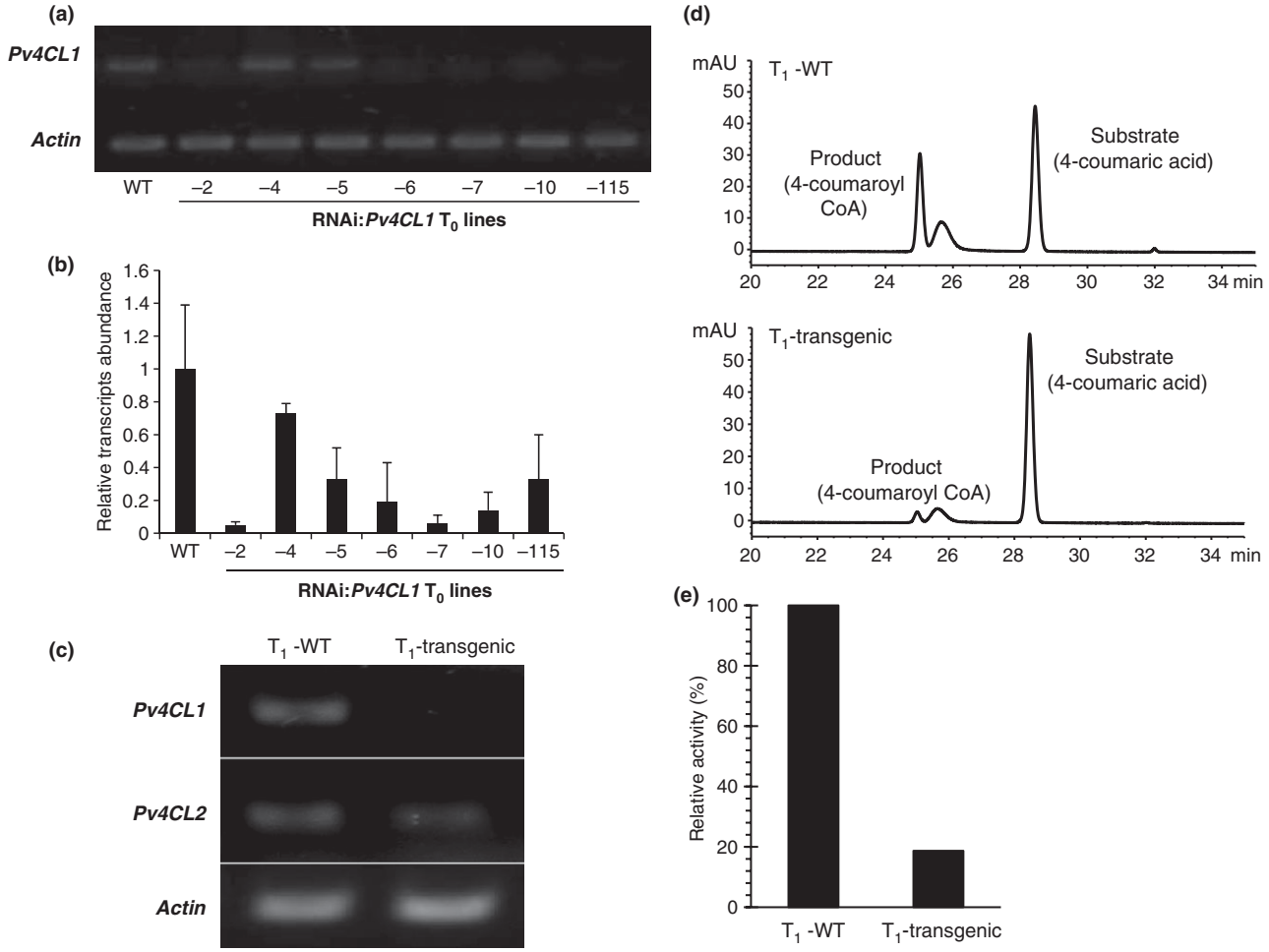

Fig. 4 4CL RNAi transgenic lines have reduced *Pv4CL1* transcript abundances and 4-coumarate:coenzyme A ligase (4CL) enzymatic activity. Reverse transcription polymerase chain reaction (RT-PCR) (a) and quantitative RT-PCR (b) showing decreased transcript abundance in individual switchgrass (*Panicum virgatum*) T_0 transgenic lines. In the T_1 lines, which segregated as transgenic or nontransgenic (WT), the pooled transgenic lines have decreased transcript abundance detected by RT-PCR (c), and lower 4CL enzymatic activity (d, e). All RNA was extracted from the third internode of switchgrass lines at the flowering stage. For RT-PCR, the reference gene was *Pv_ACTIN2* amplified with primers Pv_ACTIN_for and Pv_ACTIN_rev. A fragment of *Pv4CL1* was amplified with the primers Pv4CL_1st Round_For and Pv4CL SalXba_Rev; and a fragment of *Pv4CL2* was amplified with F_Pv4CL2 and R_Pv4CL2. The RT-PCRs were run with 28 cycles for *Pv4CL1*, *Pv4CL2* and *PvACTIN* with the same amount of cDNA loading in the PCR reaction mix. Two different sets of RNA of the samples were extracted to run two biological replicates of the qRT-PCR, and both *Pv_ACTIN2* and *Pv-UBIQUITIN* were used as reference genes.

harvesting time, possibly because T_0 and T_1 plants were grown and measured at different times. Therefore, cell wall compositions of T_0 and T_1 plants were only compared with their corresponding control plants that were grown under the same conditions. Nevertheless, consistent trends of low lignin content and altered monolignol compositions were observed in multiple T_0 and T_1 plants.

The lignin deposition patterns in T_0 and T_1 plants were characterized (Fig. 6). Phloroglucinol staining, which detects hydroxycinnamaldehyde end groups in native lignin (Pomar *et al.*, 2002), showed that there was reduced lignin deposition in the collenchyma, sclerenchyma, and even in the parenchyma cells of the transgenic T_1 plants. Mäule reagent, which specifically stains S lignin, showed no difference between transgenic lines and WT control plants (Fig. 6a,b) (Coleman *et al.*, 2008).

4CL-down-regulated switchgrass biomass has improved yields of fermentable sugars

Segregating T_1 plant material was subjected to enzymatic hydrolysis with or without acid pretreatment. All nonpretreated samples exhibited comparatively low enzymatic digestibility. The DA-pretreated samples exhibited enhanced enzymatic digestibility for glucan yield but not for xylan yield. The low lignin transgenic plant materials yielded 57.2% more fermentable sugar than the WT material with DA pretreatment, suggesting lignin content

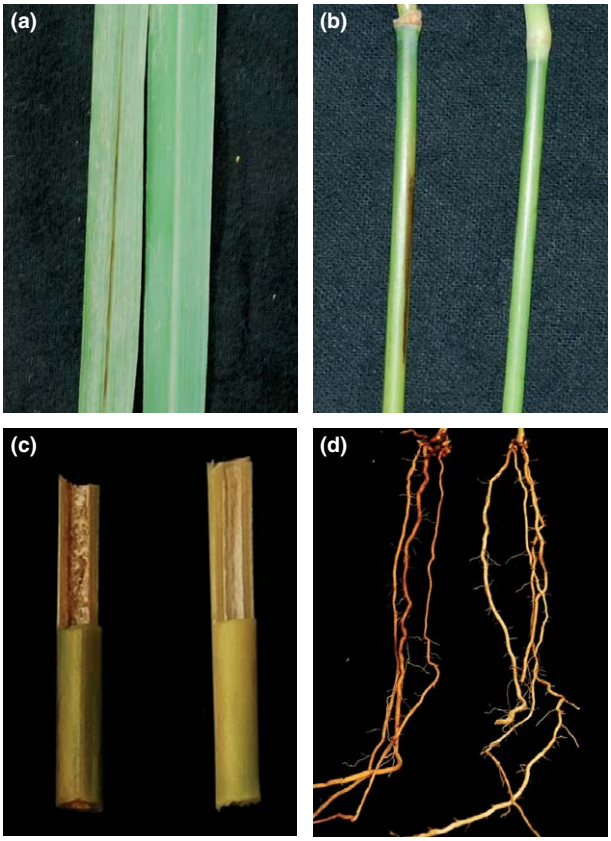

Fig. 5 Switchgrass (*Panicum virgatum*) RNAi:*Pv4CL1* lines have altered phenotypes. Some RNAi T_0 lines with low lignin content had brown coloration in parts of their leaf veins (a); brown patches in stems (b); reddish-brown coloration on the inner sides of basal stems (c); and brownish color in the mature roots (d). Note that in each picture, the plant materials on the left are from an RNAi transgenic plant, and those on the right are from the wild-type.

has a significant impact on biomass saccharification efficency in switchgrass (Fig. 7). Therefore, decreased lignin content in the RNAi:*Pv4CL1* transgenic plants may improve the economics of liquid biofuel production from switchgrass.

Discussion

Plants with reduced lignin content can be identified through breeding and selection (such as the maize *bm* and sorghum *bmr* mutants (Vignols *et al.*, 1995; Halpin *et al.*, 1998) or purposely altered through genetic engineering (Hisano *et al.*, 2009). Genetic engineering can directly manipulate the genes involved in the lignin biosynthetic pathway by silencing (e.g. RNAi), blocking the expression of the genes by artificial zinc finger chimeras (Sanchez *et al.*, 2006), or by manipulating transcription factors that regulate the expression of single to multiple lignin synthesis gene(s) (Zhou *et al.*, 2009; Zhao *et al.*, 2010; Zhong *et al.*, 2010). Since limited information about lignin synthesis and its regulatory mechanism is available for grass species including switchgrass (Carroll & Somerville, 2009; Escamilla-Treviño *et al.*, 2009; Saathoff *et al.*, 2011b), silencing the gene(s) involved in the monolignol biosynthetic pathway is currently the most straightforward way to reduce lignin content.

Despite the importance of grass species for forage and future biofuel production, only a few reports are available on the functional characterization of lignin-synthesis-related genes in grasses (besides the maize *bm* and sorghum *bmr* mutants) (Fu *et al.*, 2011a,b; Chen *et al.*, 2003; Bell *et al.*, 2004; Escamilla-Treviño *et al.*, 2009; Tu *et al.*, 2010; Saathoff *et al.*, 2011b), possibly because of the difficulty in identifying the potential target gene(s) and the time-consuming genetic transformation processes.

Pv4CL1 is a key functional 4CL isozyme in the monolignol biosynthesis pathway

Lignin biosynthesis pathways are conserved in most plant species (Boerjan *et al.*, 2003; Umezawa, 2010). Both the recombinant Pv4CL1 and Pv4CL2 proteins showed 4CL enzyme activity *in vitro*, and both proteins possess the representative domains of 4CL enzymes (Stuible & Kombrink, 2001; Schneider *et al.*, 2003). Both enzymes had similar

Table 3 Growth performance of switchgrass (*Panicum virgatum*) wild-type (WT) and T_0 lines

			Second-year yield			
Switchgrass lines	Mature root color	Basal stems color	Tiller number	DW (g)	Averaged dry biomass (g)	
Wild-type 1	White	White	19	76.5	WT	96.6 ± 27.6[a]
Wild-type 2	White	White	17	96.0		
Wild-type 3	White	White	23	131.0		
T_0-4	Light brown	Light brown	17	84.6	T_0 transgenic plants	93.2 ± 15.8
T_0-6	Reddish brown	Reddish brown	18	86.5		
T_0-7	Reddish brown	Reddish brown	20	84.8		
T_0-115	Reddish brown	Reddish brown	24	117.0		

Second-year biomass production of four T_0 lines and three WT plants was measured.
[a]No statistically significant difference was detected between wild-type and T_0 transgenic plants.

Table 4 T_0 Transgenic switchgrass (*Panicum virgatum*) plants have reduced lignin content

	Carbohydrates (mg per 100 mg)					Lignin (mg per 100 mg)		
Switchgrass lines	Glucan	Xylan	Galactan	Arabinan	Mannan	Acid-insoluble	Acid-soluble	Total lignin
Wild type	34.8 (0.4)	17.6 (0.3)	1.6 (0.0)	2.2 (0.0)	0.1 (0.2)	18.5 (0.0)	0.7 (0.2)	19.2 (0.2)
Line 4	35.4 (1.2)	17.6 (0.9)	2.0 (0.2)	2.4 (0.3)	0.2 (0.0)	14.2** (0.4)	1.7** (0.3)	15.9** (0.5)
Line 6	36.9 (0.5)	19.3 (0.0)	1.9 (0.2)	2.5 (0.2)	0.0 (0.0)	14.2** (0.9)	1.4** (0.2)	15.6** (0.9)
Line 7	34.7 (0.8)	18.8 (0.6)	1.7 (0.1)	2.0 (0.2)	0.2 (0.2)	13.8** (0.2)	1.3** (0.4)	15.1** (0.4)
Line 115	39.2 (0.1)	19.5 (0.3)	1.2 (0.3)	1.8 (0.3)	0.0 (0.3)	12.2** (0.2)	0.9 (0.3)	13.1** (0.4)

**, $P < 0.01$.
Three wild-type plants were pooled together as control for the analysis. The carbohydrate data were averaged from the results of two separate experiments. Standard error is in parenthesis.

Table 5 T_0 transgenic switchgrass (*Panicum virgatum*) plants have altered monolignol compositions

	Monolignol compositions (mg per 100 mg)			
Switchgrass lines	H	G	S	G : S
Wild-type	0.23 (0.09)	11.60 (0.18)	7.37 (0.27)	1.57
T_0-4	0.30 (0.23)	8.58 (1.04)**	7.01 (0.95)	1.22**
T_0-6	1.48 (0.23)**	7.20 (0.69)**	6.92 (0.92)	1.04**
T_0-7	1.41 (0.35)**	7.08 (0.08)**	6.61 (0.39)	1.07**
T_0-115	1.11 (0.19)**	3.60 (0.22)**	8.39 (0.03)	0.43**

**, $P < 0.01$.
Three wild-type plants were pooled together as controls for the analysis. Standard error is in parenthesis.

efficiencies (K_{cat}/K_m), but Pv4CL1 had the higher K_m and K_{cat} values. It is possible that the substrate availability or concentration in a specific cell or tissue type could be a factor in determining which of the two 4CL forms is responsible for 4-coumaroyl CoA formation. Furthermore, our transgenic plants down-regulated for Pv4CL1, but not for Pv4CL2, showed > 80% reduction of 4CL activity in stems, indicating that Pv4CL1 provides most of the activity in a tissue with active lignification.

Correlation between *Pv4CL1* transcript abundance and cell wall composition change

The T_0-generation transgenic plants had different *Pv4CL1* transcript abundances, which is a rather common phenomenon for RNAi transgenic plants. The lignin contents of different T_0 transgenic lines largely correlated with their *Pv4CL1* transcript abundances, although inconsistencies were sometimes observed. The inconsistencies might be caused by the heterozygous genetic background, even though all the T_0 plants are half-siblings, and by the independent T-DNA inserted loci. Therefore, it is important both to analyze the T_1 segregating plants with pooled samples to minimize the effects of differences in genetic background, and also to analyze several independent T_0 RNAi transgenic lines to minimize the effects of the T-DNA insertion sites in the genome.

RNAi:*Pv4CL1* transgenic plants exhibited a substantial decrease in G units, and slightly increased H units in the lignin polymer, as consistently illustrated in T_0 and segregating T_1 plants (Tables 5, 6). Similar results were observed following down-regulation of 4CL orthologs in Arabidopsis (Lee *et al.*, 1997), tobacco (Kajita *et al.*, 1997), and pine (Wagner *et al.*, 2009). In the monolignol biosynthesis pathway, Arabidopsis ferulate 5-hydroxylase (F5H), has a K_m of 3.06 μM for catalyzing conversion of coniferaldehyde to 5-OH-coniferaldehyde, or a K_m of 1.76 μM for catalyzing conversion of coniferyl alcohol to 5-OH-coniferyl alcohol, the latter of which is a precursor of sinapyl alcohol (S monolignol) (Weng *et al.*, 2010b). However, the most efficient Arabidopsis cinnamyl alcohol dehydrogenase (CAD), AtCAD5, which can catalyze conversion of coniferaldehyde to coniferyl alcohol, has a Km of 35 μM (Kim *et al.*, 2004). In switchgrass, F5H homologs have not yet been character-

Table 6 Cell wall compositions of segregating switchgrass (*Panicum virgatum*) T_1 plants

	Carbohydrates (mg per 100 mg)		Lignin (mg per 100 mg)			Monolignol composition (mg per 100 mg)			
Switchgrass T_1 lines	Glucan	Xylan	Acid-insoluble	Acid-soluble	Total lignin	H	G	S	G : S
Wild-type	27.7 (1.0)	15.5 (0.5)	22.4 (0.5)	1.2 (0.5)	23.6 (0.7)	0.39 (0.03)	11.27 (0.43)	10.74 (0.46)	1.05
Transgenic	29.9 (1.6)	17.4 (1.1)	17.4** (1.2)	1.0 (0.4)	18.4** (1.3)	0.71** (0.04)	5.98** (0.11)	10.70 (0.15)	0.56**

**, $P < 0.01$.
Stems of the segregating plants were independently pooled (transgenic and wild-type) together, and used for cell wall composition analysis. Three experimental repeats were conducted with the pooled plant material. Standard error is in parenthesis.

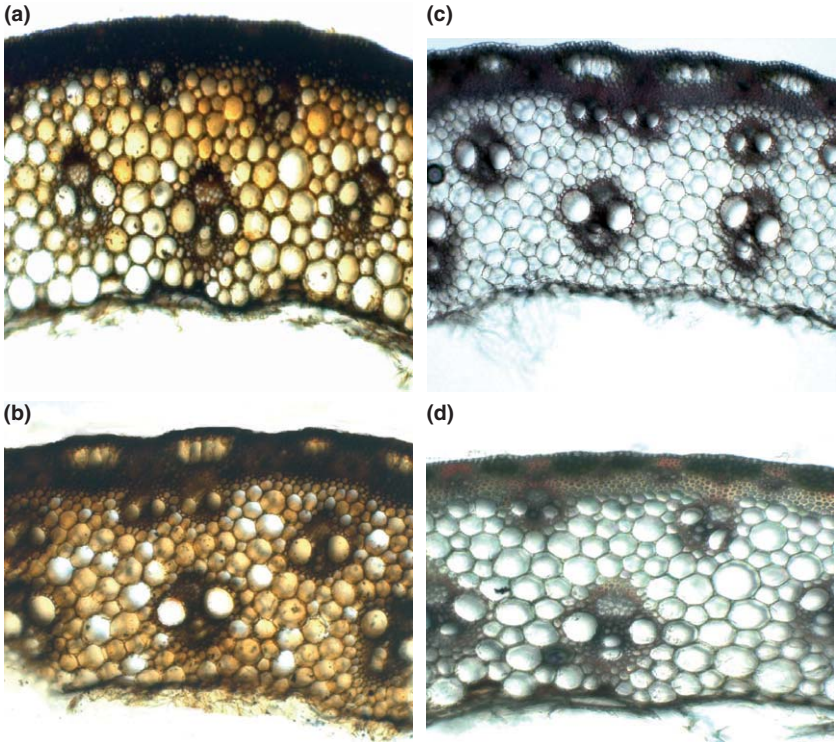

Fig. 6 Lignin histological staining for lignin deposition. Compared with the switchgrass (*Panicum virgatum*) wild-type plants (a, c), the transgenic plants did not show altered staining by Mäule reagent (b). However, lignin staining by phloroglucinol-HCl reagent was clearly reduced (d). The third internodes were used to make the 50-μm-thick sections. Five tillers from the transgenic or non-transgenic (wild-type) lines were taken for the sections, and consistent observations were obtained.

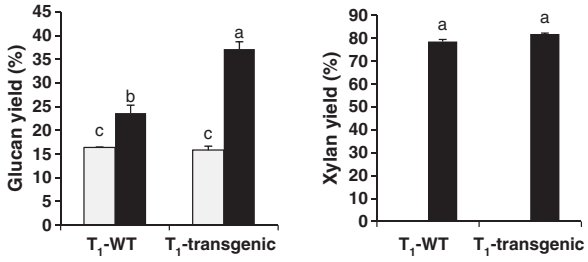

Fig. 7 4-Coumarate:coenzyme A ligase (4CL) down-regulated switchgrass (*Panicum virgatum*) plants have improved saccharification efficiency. Biomasses of segregating T_1 plants were either treated with diluted acid (DA treated, black bars) or analyzed without pretreatment (untreated, gray bars). Without DA treatment, the xylan yields of both wild-type (WT) and transgenic lines were zero. The bars are standard errors. Different letters above the bars indicate statistically significant difference at the level of $\alpha = 0.01$.

ized. Switchgrass has at least two *CAD* genes. The expression level of *PvCAD1* is > 10 times higher than *PvCAD2*, and PvCAD1 has a relatively high K_m (compared with that of F5H) of 10.9 μM for catalyzing conversion of coniferaldehyde (Saathoff *et al.*, 2011a,b).Therefore, lower amounts of coniferaldehyde resulting from the diminished substrate pool caused by the down-regulation of 4CL1 may favor F5H rather than CAD because of its 10-fold lower K_m, and this might explain the decreased formation of G but not S lignin.

Growth performance of RNAi:*Pv4CL1* transgenic lines

4-Coumarate:coenzyme A ligase participates in an early step of the general phenylpropanoid pathway by producing the monolignol precursor ρ-coumaroyl-CoA. This metabolic intermediate is also a precursor for the production of many secondary metabolites, such as stilbenes and flavonoids (Boudet, 2007). Therefore, the down-regulation of *4CL* could have pleiotropic effects, such as color changes in leaf midribs, mature stems and roots. These color changes are common phenomena when down-regulating gene(s) in the monolignol biosynthesis pathway (Kajita *et al.*, 1996; Wagner *et al.*, 2009; Voelker *et al.*, 2010), which often leads to ectopic accumulation of flavonoids (Besseau *et al.*, 2007). However, the ectopic accumulation of flavonoids caused by silencing monolignol biosynthesis pathway genes does not in itself directly impact plant growth, at least in Arabidopsis (Li *et al.*, 2010).

We measured biomass yields and cell wall compositions in both T_0 and T_1 transgenic lines, and did not see any significant change in plant growth among four T_0 and T_1 segregating plants (Tables 1, 3). Down-regulation of *At4CL1* in Arabidopsis did not result in compromised biomass production (Sanchez *et al.*, 2006). However, the silencing of 4CLs in tobacco (Kajita *et al.*, 1997), pine (Wagner *et al.*, 2009) and poplar (Voelker *et al.*, 2010) resulted in stunted plant growth in some transgenic lines, primarily caused by deformation of xylem tissue, and deposition of tyloses and phenolics in xylem vessels of

poplar, thus blocking water transport (Kitin *et al.*, 2010). Possibly because of the anatomical difference between grasses and trees, or because of differences in their tolerance to lignin modification, the present RNAi:*Pv4CL1* switchgrass plants did not show any obvious growth abnormalities under glasshouse conditions. Likewise, RNAi:*CCR1* and RNAi:*COMT1* transgenic ryegrass (Tu *et al.*, 2010) and RNAi:*COMT* transgenic switchgrass (Fu *et al.*, 2011a) did not show compromised biomass production.

Because lignin deposition is influenced by abiotic and biotic stresses (Halpin, 2004; Boudet, 2007), it will be interesting to further confirm if RNAi:*Pv4CL1* transgenic switchgrass plants have low lignin content under both glasshouse and field conditions. A recent study showed that low lignin transgenic poplar and WT poplar differed in their field performance possibly because of reduced wood strength and stiffness in transgenic plants (Voelker *et al.*, 2011). It will also be interesting to observe the stand integrity (e.g. lodging) of the low lignin switchgrass under field conditions.

Reducing biofuel production costs by down-regulating feedstock lignin content

Reduced lignin content of biomass could improve saccharification efficiency through enzyme hydrolysis, and therefore reduce the cost of biofuel production (Fu *et al.*, 2011a), although in some bioenergy processes such as pyrolysis and combustion, a higher lignin feedstock could be desirable because of its high energy contents (Boateng *et al.*, 2008). In this study, the T_0 transgenic plants have varied S : G ratios (Table 5). Therefore, it is still inconclusive whether the increased S : G ratio together with lower lignin contents contribute to the increased cellulose hydrolysis efficiency. F5H overexpressing transgenic poplar has a higher S lignin ratio with unchanged lignin content, and this leads to a significant improvement in pulping and bleaching efficiencies (Huntley *et al.*, 2003), presumably because the S units form fewer crosslink bonds (Huntley *et al.*, 2003; Grabber *et al.*, 2004). However, no major correlations between sugar-release efficiency and monolignol unit compositions have been shown from studies on biomass derived from *Medicago truncatula* (Chen & Dixon, 2007).

Reducing lignin content has increased cellulose hydrolysis efficiency (saccharification efficiency) in model plants such as *M. truncatula* and Arabidopsis (Chen & Dixon, 2007; Weng *et al.*, 2010a). The same is true in switchgrass when comparing the processing ability of tissues at different developmental stages (Shen *et al.*, 2009), and more recently by the analysis of genetically modified switchgrass with reduced expression of *CCOMT* or *CAD* (Fu *et al.*, 2011a; b; Saathoff *et al.*, 2011a). Similar results with the present low-lignin plant materials were observed (Fig. 7). Further improvements of switchgrass feedstock quality by genetic engineering along with efficient bioprocessing and conversion technologies will lead to economical biofuel production in the future.

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Supporting Information

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

Fig. S1 Alignment of two switchgrass 4CL amino acid sequences with their maize and rice orthologs (S1a) and alignment of two switchgrass *4CL* nucleotide sequences (S1b).

Fig. S2 Key features of the vectors used in this study.

Fig. S3 Mass balance of dilute acid pretreatment and enzymatic hydrolysis of T_1 transgenic (a) and wild-type (b) biomass.

Table S1 Primers used in this study

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