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Downregulation of Cinnamyl Alcohol Dehydrogenase (CAD) Leads to Improved Saccharification Efficiency in Switchgrass

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Abstract The bioconversion of carbohydrates in the herbaceous bioenergy crop, switchgrass (*Panicum virgatum* L.), is limited by the associated lignins in the biomass. The cinnamyl alcohol dehydrogenase (*CAD*) gene encodes a key enzyme which catalyzes the last step of lignin monomer biosynthesis. Transgenic switchgrass plants were produced with a *CAD* RNAi gene construct under the control of the maize ubiquitin promoter. The transgenic lines showed reduced *CAD* expression levels, reduced enzyme activities, reduced lignin content, and altered lignin composition. The modification of lignin biosynthesis resulted in improved sugar release and forage digestibility. Significant increases of saccharification efficiency were obtained in most of the transgenic lines with or without acid pretreatment. A negative correlation between lignin content and sugar release was found among these transgenic switchgrass lines. The transgenic materials have the potential to allow for improved efficiency of cellulosic ethanol production.

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Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial C4 warm-season grass native throughout North America. Because of its high productivity, low nutrient and water requirements, adaptation to marginal soils, flexibility for multipurpose uses, low cost of production, and significant environmental benefits, switchgrass has been developed into a model herbaceous biofuel crop [1–3]. It has been shown that switchgrass managed as a biomass energy crop with moderate inputs can be as net energy efficient as low-input systems but produce significantly more energy per unit of land [4].

Switchgrass contains abundant sugars in the form of cellulose and hemicellulose, which can be converted to ethanol by hydrolysis and subsequent fermentation. The bioconversion of carbohydrates in this potential biomass resource, however, is limited by the associated lignins. It has been shown that glucose recovered from switchgrass was inversely correlated to maturity and lignin content [5]. The association of lignin with hemicellulose and cellulose has a negative impact in cellulosic ethanol production as it reduces the accessibility of polysaccharides during the pretreatment and enzymatic saccharification processes [6, 7]. The high cost incurred during these processing steps is the major limiting factor in cellulosic biofuel production and makes the price of cellulosic ethanol much higher than that of starch-based ethanol [8]. A direct and effective approach is to downregulate the enzymes involved in lignin

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biosynthesis to reduce lignin content or to modify its composition [6, 9, 10].

Lignins are complex phenolic polymers comprise of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol. Many enzymes are involved in lignin biosynthesis [11]. One of the key enzymes, cinnamyl alcohol dehydrogenase (CAD), catalyzes the last step in the biosynthesis of lignin precursors, which is the reduction of cinnamaldehydes to cinnamyl alcohols [12]. Transgenic manipulation of CAD activity has been reported in several dicot species, such as tobacco [13, 14], alfalfa [15, 16], poplar [17–19], eucalyptus [20], and flax [21]. In a recent study of CAD downregulated alfalfa, two transgenic lines had increased enzymatic saccharification efficiency, although the increase was less than observed for cinnamoyl CoA reductase downregulated alfalfa lines in an otherwise identical genetic background [16]. Because of the difficulties involved in generating transgenic monocots, there has been only one report on CAD downregulation in grasses, in which forage digestibility was improved in transgenic tall fescue [22]. In other monocot species, like maize and sorghum, certain natural or chemically induced mutants have been associated with changes in CAD activity. Recently, two independent studies revealed that the sorghum *bmr6* mutant is caused by a nonsense mutation in the *CAD* gene [23, 24]. The reduced CAD activity in maize *bm1* mutant was mapped to a genome region that contains a *CAD* gene, but a mutation was not identified in the gene [23, 25].

Like many other monocot species, switchgrass is considered recalcitrant for genetic transformation [26]. Transgenic modification of switchgrass offers an effective way of testing gene functions and developing new strategies for improvement of bioenergy crops. Here, we report the generation and characterization of transgenic switchgrass plants with downregulated expression of the *CAD* gene. Our results show that downregulation of CAD leads to reduced lignin content, altered S/G ratio, increased chlorogenic acid accumulation, increased forage digestibility and improved sugar release. The transgenic materials have the potential to allow for improved cellulosic ethanol production.

Materials and Methods

Plant Materials

A lowland switchgrass (*P. virgatum* L. cv. Alamo) was selected for genetic transformation and lignin modification. Switchgrass plants were grown in the greenhouse with16-h light (390 μE m^{-2} S^{-1}). The development of switchgrass was divided into five elongation stages (E1, E2, E3, E4, and E5) and three reproductive stages (R1, R2, and R3) according to the criteria described by Moore et al. [27].

Isolation and Expression of a Switchgrass *CAD* Gene

A high-quality cDNA library was constructed from leaves of 2-month-old switchgrass with a Creator™ SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, CA). A pair of polymerase chain reaction (PCR) primers (forward: CAAGGCCAACGC/TCGAGCAGTA, reverse: ACCGTGTCGATGATGTAGTCCAG) was designed in the conserved sequences of *CAD* genes based on EST information of switchgrass and other monocot species. A 438-bp fragment was amplified by PCR from the switchgrass cDNA library and sequenced. Based on the above sequence, a 1,413-bp *CAD* cDNA, including partial untranslated region sequences, was isolated by 5′-RACE and 3′ RACE following protocols from the manufacturer (Invitrogen, Carlsbad, CA). The GenBank accession number of our isolated *PvCAD* is GQ917176. A phylogenetic tree was generated with MEGA 3.1 software using the neighbor-joining method [28].

The expression level of the *CAD* gene in different tissues was estimated by reverse transcription polymerase chain reaction (RT-PCR). Samples from root, stem, leaf, leaf sheath, spikelet at the R3 stage, and seedlings were collected and used to analyze the spatial pattern of *CAD* transcript level. Total RNA from the above samples was isolated using Trizol reagent (Promega, Madison, WI) and subjected to reverse transcription using an Omniscript® Reverse Transcription Kit (Qiagen, Valencia, CA) after incubation with RNase-free RQ1 DNase (Promega, Madison, WI) at 37°C for 10 min. The cDNA from reverse transcription was used as template to amplify the *CAD* product. The forward primer (AAC CAAACCCACCCGCTC) was located in the 5′-UTR, and the reverse primer (GGCCTGTGTTCCTGACGGT) was in the ORF region. A 180-bp amplification product was obtained using the following PCR conditions: 95°C for 2 min (one cycle); 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (27 cycles); 72°C extension for 10 min. The transcript level of the elongation factor 1a (*ELF1a*) gene was used as an internal control. A pair of PCR primers (forward: TCAGGATGTG TACAAGATTGGTG, reverse: GCCTGTCAATCTTGG TAATAAGC) were designed based on EST sequence of the switchgrass *ELF1a* gene (accession number FL728164). PCR conditions for amplifying *ELF1a* was: 95°C for 2 min (one cycle); 94°C for 30 s, 58°C for 30 s, 72°C for 30 s (23 cycles); 72°C extension for 10 min.

RNAi Vector Construction and Production of Transgenic Switchgrass Plants

To downregulate *CAD* gene expression in switchgrass, an RNAi binary vector was constructed based on the pANDA

gateway vector [29]. *CAD* cDNA fragments with introduced recombination reaction sites were amplified by PCR (forward: ggggacaagtttgtacaaaaaagcaggctGTCG GAGCTCACCAGGTACG; reverse: ggggaccactttgtacaa gaaagctgggtCTGAAGATGTGGTGGTCAAGGT) and placed in sense and antisense orientation in the pANDA vector. In the gene construct, hairpin RNA derived from the *CAD* gene is transcribed from the maize ubiquitin promoter. The pANDA-CAD construct was transferred into *Agrobacterium tumefaciens* strain EHA105.

The procedure described by Xi et al. [30] was followed for genetic transformation of switchgrass. Briefly, embryogenic calli were initiated from mature seeds of the switchgrass cultivar Alamo, and highly embryogenic calli were used for *Agrobacterium*-mediated transformation. These calli were infected and co-cultivated with *A. tumefaciens* strain EHA105 carrying the binary vector pANDA-CAD in the presence of 100-μM acetosyringone. Two days after co-cultivation, the calli were transferred onto M1 selection medium (Murashige–Skoog (MS) basal medium supplemented with 3% sucrose, 1.5 mg l^{-1} 2, 4-D, 75 mg l^{-1} hygromycin, 250 mg l^{-1} cefotaxime, and solidified with 0.75% agar). One week later, the calli were transferred onto fresh M1 selection medium and the selection process was continued. Resistant calli obtained after 6–7 weeks of selection were transferred to MSK regeneration medium (MS basal medium supplemented with 3% sucrose, 0.5 mg l^{-1} kinetin, 250 mg l^{-1} cefotaxime, and solidified with 0.75% agar). One month later, regenerated green shoots were transferred to MSO rooting medium (half-strength MS basal medium supplemented with 0.8% sucrose and solidified with 0.75% agar). After 4–5 weeks, the rooted plantlets were transferred to soil and grown in the greenhouse.

Molecular Analysis of Transgenic Switchgrass Plants

Total DNA was isolated from leaf tissues of each transgenic line using a plant DNA extraction kit (Qiagen, Valencia, CA). Positive transgenic switchgrass was identified by PCR with specific *GUS* linker primers (forward: AACAGTTCCTGAT TAACCACAAACC, reverse: GCCAGAAGTTCTTTTTC CAGTACC) using the following PCR conditions: 95°C for 2 min (one cycle); 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s (35 cycles); 72°C extension for 10 min. A 634-bp amplification product was obtained only in the transgenic lines.

RNA was isolated from internodes 2 and 3 at the E4 developmental stage. Semi-quantitative RT-PCR was performed to analyze the expression levels of *CAD* as well as the *GUS* linker. *CAD* mRNA levels were further analyzed by real-time quantitative RT-PCR. SYBR Green (Applied Biosystems, Foster City, CA) was used as the reporter dye. The cycle thresholds were determined using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The data were normalized using the levels of *ELF1a* transcripts. The primers used for real-time quantitative RT-PCR were the same as described above for RT-PCR. Reactions were pre-incubated at 50°C for 2 min for AmpErase® UNG activation, followed by denaturation/AmpliTaq Gold® DNA polymerase activation at 95°C for 10 min, and 40 amplification cycles, each of 15 s at 95°C plus 1 min at 60°C. The raw real-time RT-PCR data of each individual sample was collected and analyzed by the software of SDS 2.2.2 for 7900HT (Applied Biosystems, Foster City, CA) and the individual PCR efficiencies were checked by the LinRegPCR 7.0 software [31]. Relative transcript levels were calculated using the comparative Ct value according to the formula described by Ranasinghe et al. [32].

Assay of Extractable CAD Activity

Switchgrass internodes 2 and 3 collected at the E4 stage were homogenized in liquid nitrogen. Powdered tissue (about 500 mg) was extracted with 2-ml extraction buffer (100-mM Tris–HCl, pH 7.5, 2% polyethylene glycol 6000, 5-mM dithiothreitol, 2% polyvinylpolypyrrolidone) for 3 h at 4°C [33]. Protein concentration was measured by the Bradford method [34]. CAD activity assay essentially followed the procedures described by Dos Santos et al. [35]. Coniferaldehyde and sinapaldehyde (Sigma, St Louis, MO) were used as substrates with crude enzyme extracts and the formation of hydroxycinnamyl alcohol monitored as a measure of CAD activity. The assay mixture contained about 100-μg protein in 500 μl of 0.1-M NaH_2PO_4/Na_2HPO_4 buffer at pH 7.5, 0.2-mM NADPH and 0.02-mM aldehyde substrate. Reactions were incubated at 30°C for 30 min and then stopped by adding 50-μl 25% trichloroacetic acid (TCA). The mixtures were centrifuged at 13,000 rpm, and the supernatants analyzed using a Beckman programmable gradient high-performance liquid chromatography (HPLC) equipped with a Beckman 508 automated injector and a Beckman 168 diode-array detector (DAD; Beckman Coulter, Fullerton, CA). The mobile phase consisted of eluent A (0.1% (*v*/*v*) H_3PO_4/water) and eluent B (acetonitrile). Extracts were separated on a 250×4.6-mm C-18 column (Varian, Palo Alto, CA, USA) using a linear gradient of 8%–100% B (*v*/*v*) over 45 min, at a flow rate of 1.0 ml min^{-1}. Column effluent was monitored at 280 nm, and the product was identified by spectral scans using the diode-array detector followed by comparison of retention time and UV spectrum with those of authentic standards.

Analysis of Lignin Content and Composition

Internodes 2 and 3 harvested at E4 stage were ground in liquid nitrogen and lyophilized. Extractive-free cell wall

samples were prepared as described by Chen and Dixon [6]. Lyophilized extractive-free material was used for lignin analysis. The content and composition of lignin were determined according to described procedures [36, 37]. Thirty milligrams of lyophilized samples were used for AcBr lignin analysis. The acetyl bromide method described by Hatfield et al. [37] was employed to quantify lignin content. The thioacidolysis method [38] was used to determine lignin composition. Lignin-derived monomers (H, S, and G units) were identified and quantified by gas chromatography mass spectrometry (GC/MS) using a Hewlett–Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60 m× 0.25 mm×0.25-μm film thickness). Mass spectra were recorded in electron impact mode (70 eV) with 60–650 *m*/*z* scanning range.

Determination of Free and Wall-Bound Phenolics

Soluble phenolics were extracted twice from 30.0±0.06 mg ground lyophilized plant material with 1.5 ml 50% methanol plus 1.5% acetic acid for 12 h at room temperature [39, 40]. 7,8-Dihydroxy-6-methoxycumarin-8-β-D-glucopyranoside (Sigma, St Louis, MO) was added into the extract solution as an internal standard. Pooled supernatants were vacuum-dried, re-dissolved in 0.5 ml 50% methanol, and clarified by a brief centrifugation at 15,300×*g*, extracts were used for soluble phenolics profiling analysis. Cell wall-bound phenolics were released by high-temperature hydrolysis (4.0-M NaOH, 121°C, 4 h). After acidification with 6.0 M HCl, the aqueous phase (pH=2.0) was extracted three times with 0.5 ml of ethyl acetate. The pooled ethyl acetate extracts were vacuum dried and re-dissolved in 0.5-ml 50% methanol. After brief centrifugation at 15,300×*g*, extracts were profiled for wall-bound phenolics. Standard solutions of chlorogenic acid, *p*-coumaric acid, ferulic acid, and vanillin (Sigma, St Louis, MO) were prepared and analyzed together with the above samples by HPLC/DAD. The UV-absorbing metabolites were monitored at 280 and 320 nm.

Identification of phenylpropanoids was performed using liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS/MS) according to a previously described method [41]. In brief, an Agilent 1100 series II LC coupled to a Bruker Esquire Ion-trap Mass Spectrometer equipped with an electrospray-ionization source (ESI) system (Agilent Technologies, Palo Alto, CA) was employed. The methanol extracts analyzed previously were separated on a reverse phase, C18, 5 μm, 4.6×250 mm column at 28°C at a flow rate of 0.8 ml min^{-1}. The mobile phase consisted of eluent A (0.1% [v/v] CH_3COOH/water) and eluent B (acetonitrile), and separation was achieved using a linear gradient of 5–70% B (v/v) over 65 min. UV-visible spectra were obtained with a HP 1100 photodiode array detector. Mass determination was conducted by ESI in negative ion polarity. Mass spectra were recorded over the range 50–2,200 *m/z*.

In Vitro True Dry Matter Digestibility

Switchgrass samples were dried in an oven at 40°C and ground through a Wiley mill with 1-mm sieve. In vitro true dry matter digestibility (IVTDMD), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured using a near infrared reflectance spectroscopy (NIRS). NIRS analysis was performed using Foss NIRS 6500 monochromator with a scanning range of 1,100–2,500 nm (Foss NIR Systems Inc., Silver Spring, MD). Each sample was scanned eight times, and the average spectra were used for calibration. The existing commercial NIRS prediction equations (07GH50-2) developed by NIRS Forage and Feed Testing Consortium were employed to calculate NDF, ADF and IVTDMD (http://nirsconsortium.org/default.aspx). The precision of NIRS has been assessed by regression analysis of the predicted values and actual determined values.

Determination of Saccharification Efficiency

Cell wall residues generated for lignin analysis were also used to analyze total sugar and sugar components released by enzymatic hydrolysis according to previously described procedures [16]. The prepared cell wall residues were digested by the following treatments: 1) direct exposure to a cellulase and cellobiase mixture for 72 h (as untreated samples) or 2) pretreatment with dilute H_2SO_4 (1.5%) at 121°C for 40 min to remove most hemicellulose, and then exposure to the same enzyme mixture after first washing with water. Enzymatic saccharification of switchgrass samples was performed following the analytical procedure of the National Renewable Energy Laboratory (LAP-009) (http://www.nrel.gov/biomass/analytical_procedures.html). The enzyme cocktail was made by mixing equal volumes of Celluclat 1.5 L (cellulase from *Trichoderma reesei*) and Novozyme 188 (cellobiase from *Aspergillus niger*) (Sigma, St Louis, MO) and then diluting 500 fold with sodium citrate buffer (0.1 M, pH 4.8). The activity unit of above enzymes mixture was measured in term of filter paper units (FPU) according to the method described by Ghose [42]. The enzyme loadings were 21 FPU per g cell wall residue. Biomass samples (equal to 0.1 g equivalents of cellulose) were hydrolyzed in 10 ml of the above diluted enzyme mixture for 72 h. Enzyme blanks and Whatman #1 filter paper (0.1 g) were digested alongside the samples. Total sugars were analyzed spectrophotometrically using the phenol-sulfuric acid assay method [43]. Saccharification

efficiency was determined as the ratio of sugars released by enzymatic hydrolysis to the amount of sugars present in the cell wall material prior to enzymatic hydrolysis. Monomeric sugars (glucose, xylose, arabinose, galactose and mannose) in acid or enzymatic hydrolysates were also determined by HPLC (Agilent 1200 Series LC System with 1200 Series Refractive Index Detector) equipped with an Aminex HPX-87P column (Agilent Technologies, Palo Alto, CA).

Statistical Analysis

Triplicate samples were collected for each transgenic line. Data from each trait were subjected to analysis of variance. The significance of treatments was tested at the $P<0.05$ level. Standard errors are provided in all tables and figures as appropriate. For relationships between X and Y, only linear model were considered. Correlations were calculated with Sigmaplot 2001 software (SPSS Inc., Chicago, IL, USA). All the statistical analyses were performed with the SPSS package (SPSS Inc., Chicago, IL, USA).

Results

Isolation of *CAD* cDNA Sequences from Switchgrass

A *CAD* cDNA, with an open reading frame of 1,095 bp, was isolated from a cDNA library constructed from 2-month-old switchgrass seedlings. A phylogenetic tree based on deduced amino acid sequences was built to evaluate the relationship of the CAD protein family (Eletctronic Supplementary Material, Fig. 1). Twelve CAD-like proteins from different plant species were classified into four different groups [44]. The switchgrass CAD (PvCAD) belongs to the first group which included other CAD proteins involved in lignin biosynthesis. At the amino acid level, our PvCAD shows high identity with predicted proteins of two recently isolated CAD sequences from switchgrass, PviCAD1 (98% identity) and PviCAD2 (99% identity) [45]. PvCAD also shares high amino acid sequence similarity to that of sorghum (93%) and tall fescue (86%). Semi-quantitative RT-PCR analysis indicated that the level of *CAD* transcripts was relatively higher in stems and roots than that in leaves and seedlings (Electronic Supplementary Material, Fig. 2).

Generation of Transgenic Switchgrass Plants with Altered Expression of *CAD*

The pANDA-CAD RNAi vector was constructed based on the pANDA gateway vector, which was designed to produce hairpin RNA molecules for gene suppression [29]. The maize ubiquitin promoter was placed upstream of the inverted repeats to trigger RNAi-induced degradation of *CAD* mRNA (Electronic Supplementary Material, Fig. 3). The RNAi construct was transferred into *A.*

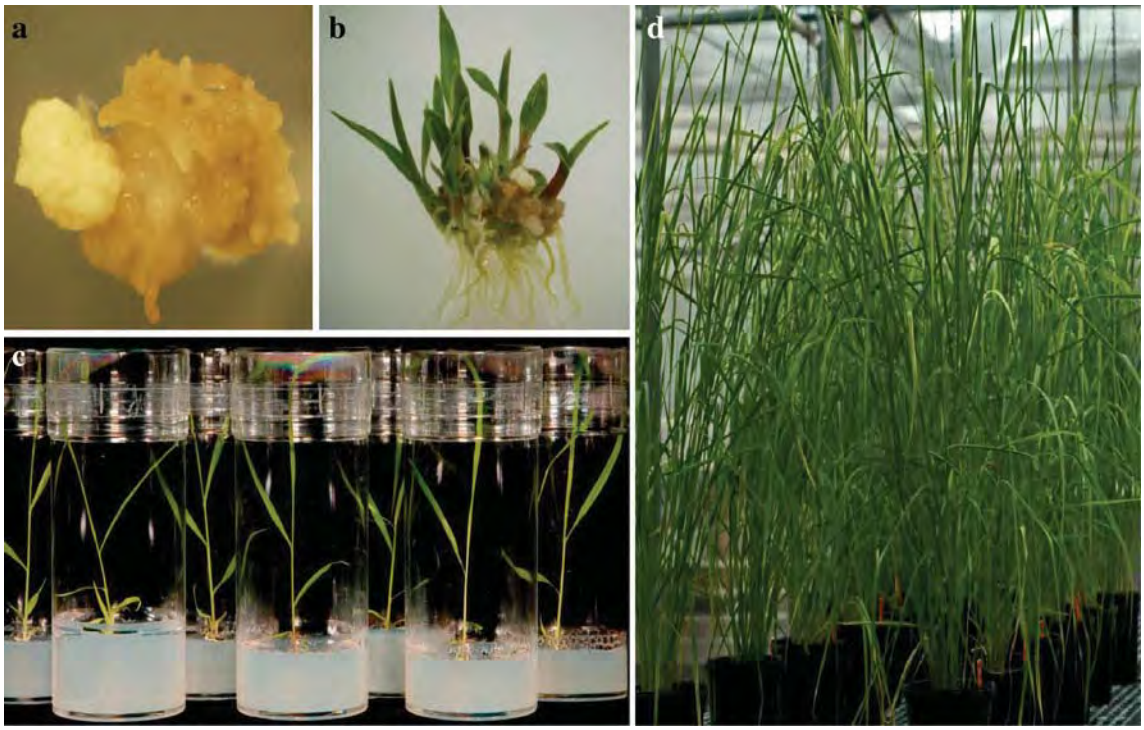

Fig. 1 Generation of transgenic switchgrass plants by *Agrobacterium*-mediated transformation. **a** Resistant callus obtained after hygromycin selection; **b**, **c** Regeneration of green shoots and plantlets from resistant calli; **d** Transgenic switchgrass plants growing in the greenhouse

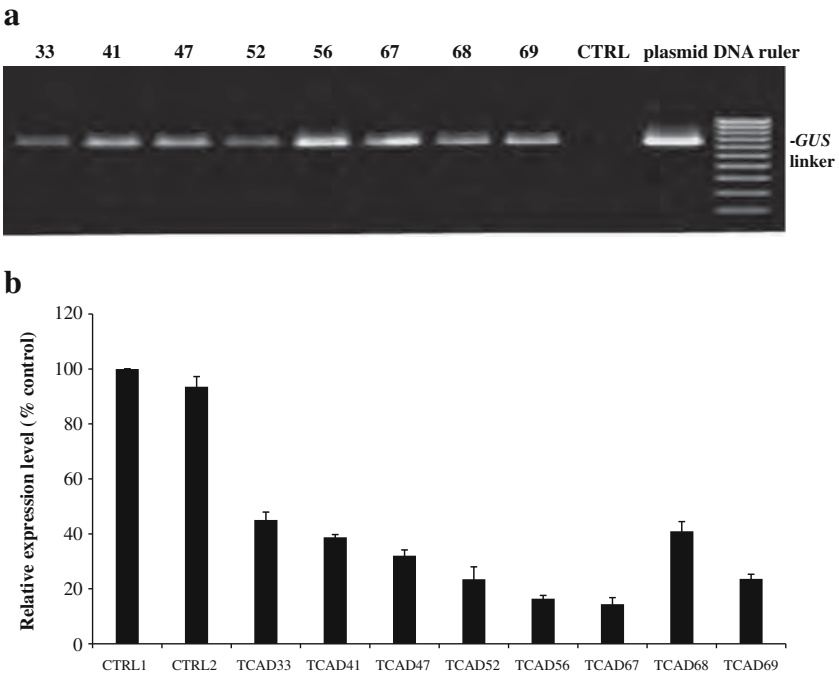

Fig. 2 Molecular analysis of transgenic switchgrass plants. **a** PCR amplification of genomic DNA isolated from transgenic and control switchgrass; **b** Real-time RT-PCR analysis of *CAD* transcript levels in transgenic switchgrass. *CAD* transcript levels relative to CTRL1 are presented

tumefaciens strain EHA105. Embryogenic calli were infected with *Agrobacteria* and resistant calli were obtained after hygromycin selection (Fig. 1a). Green shoots and plantlets were regenerated after transferring the resistant calli onto regeneration medium (Fig. 1b, c). Fifty-two putative transgenic plants derived from approximately 1,000 embryogenic calli were transplanted to soil and grown in the greenhouse (Fig. 1d). PCR analysis revealed that forty transgenic plants contained the CAD RNAi construct.

The transgenic plants showed normal growth in the greenhouse (Fig. 1d). Eight independent transgenic lines with similar developmental stage were used for further studies. Two controls were used in the analyses. Control 1 is a wild-type switchgrass plant while control 2 is an escape lacking T-DNA. To achieve uniform and comparable materials for molecular and biochemical analyses, the transgenic and control plants were cut back and allowed to regrow for about 3 months before sampling. Internodes 2 and 3 at the E4 stage were collected and used for PCR (Fig. 2a), semi-quantitative RT-PCR (Electronic Supplementary Material, Fig. 4) and real-time quantitative RT-PCR (Fig. 2b) analyses. Compared with the control plants, the transcript levels of the target gene were reduced by 55–86% in the transgenic plants (Fig. 2b).

Extractable CAD Activity

To determine whether reduced *CAD* transcript levels resulted in reduction of enzyme activity, CAD activity was analyzed in extracts from internodes of transgenic and control plants using coniferaldehyde and sinapaldehyde as substrates. Significantly reduced CAD activities were found in the transgenic plants with reduced *CAD* transcript levels

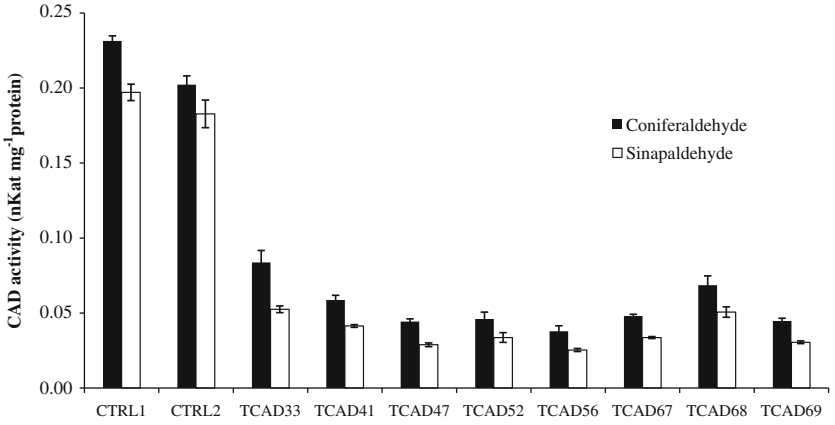

Fig. 3 Extractable CAD activity in transgenic switchgrass. Coniferaldehyde and sinapaldehyde were used as substrates

(Fig. 3). CAD activity of the transgenics was 17–39% of that of the controls when coniferaldehyde was used as substrate (Fig. 3). When sinapaldehyde was used as substrate, CAD activity of the transgenics was only 12–24% of that of the controls.

Effects of *CAD* Suppression on Lignin Content and Composition

Lignin content and composition of switchgrass internodes at the same developmental stage were analyzed (Table 1). AcBr lignin levels of control plants averaged 239 mg g^{-1} cell wall residue (CWR) while corresponding levels in the transgenics varied from 185 to 206 mg g^{-1} CWR (Table 1). The reduction of AcBr lignin content in the transgenic lines was in the range of 14–22%. Lignin composition of transgenic plants was also changed. Compared with the control plants, S lignin and G lignin in the transgenic plants were reduced 13–42% and 18–33%, respectively (Table 1). Ratios of S/G in the control plants averaged 0.91; this value was reduced in most of the transgenics.

Effects of Suppression of *CAD* on Hydroxycinnamic Acid Levels

To determine whether downregulation of *CAD* expression had quantitative and/or qualitative effects on wall-bound and soluble phenolic compounds produced via the monolignol pathway, reverse-phase HPLC analysis was used to characterize UV-absorbent metabolites in extracts from internodes of the transgenic plants. HPLC analyses revealed that ester and ether-linked *p*-coumaric acid (9.68 mg g^{-1} dry matter) and ferulic acid (5.12 mg g^{-1} dry matter) were the major phenolic acids that accumulated in the cell walls of switchgrass internodes. In addition, vanillin (0.69 mg g^{-1} dry matter) was also detected (Electronic Supplementary Material, Table 1). No significant differences were detected in the levels of wall-bound *p*-coumaric acid, ferulic acid or vanillin between control and transgenic lines (Electronic Supplementary Material, Table 1).

In contrast, changes of *CAD* expression in transgenic plants had an impact on the levels of soluble phenolic compounds. Preliminary analyses of free phenolics in methanol extracts from internodes revealed that one UV-absorbent peak exhibited a large increase in the transgenic lines with reduced CAD activity. To obtain better insight into the level and complexity of soluble metabolites, a LC-MS/MS protocol was utilized to identify this compound. Based on chromatographic behavior, UV light absorption, MS data and comparison to an authentic compound, this compound was identified as chlorogenic acid (caffeoyl quinic acid). Most of the *CAD* suppressed transgenic switchgrass lines (TCAD41, 47, 52, 56, 67, and 69) had significantly increased levels of chlorogenic acid, with the levels of increase in the range of 40–170%. No significant difference was found in the level of *p*-coumaric acid in the extracts (Fig. 4).

Effects of *CAD* Suppression on Cell Wall Polysaccharides and Forage Digestibility

To determine whether cell wall polysaccharides are affected in the transgenic plants, the accumulation of cellulose and hemicellulose in cell walls was evaluated. Cellulose and hemicellulose made up approximately 46% and 28% of the dry mass of internodes in the control switchgrass, respectively. No consistent difference was observed between control and transgenic plants regarding the content of cell wall polysaccharides (Electronic Supplementary Material, Table 2). The samples were also analyzed for their in vitro true dry matter digestibility (IVTDMD). IVTDMD of the controls averaged 58.6%, while values of the transgenics

Table 1 Lignin content and composition of transgenic switchgrass with downregulated expression of *CAD*

Plant line	Acetyl bromide lignin (mg g^{-1} CWR)	Thioacidolysis yield (μmol g^{-1} CWR)	S lignin (μmol g^{-1} CWR)	G lignin (μmol g^{-1} CWR)	S/G
CTRL1	234.0±9.2	241.20±1.00	114.38±0.69	126.82±0.31	0.90
CTRL2	244.8±2.3	248.93±9.29	120.05±5.38	128.93±3.91	0.93
TCAD33	206.0±1.5	204.45±3.81	101.38±0.37	103.06±3.44	0.98
TCAD41	185.6±3.1	163.89±2.54	70.34±0.14	93.55±2.40	0.75
TCAD47	196.0±2.3	160.63±13.01	67.61±4.49	93.02±8.53	0.73
TCAD52	195.2±1.2	160.88±4.14	75.02±2.00	85.86±2.14	0.87
TCAD56	205.5±4.8	172.63±7.76	76.57±3.59	96.06±4.16	0.80
TCAD67	193.0±3.2	164.03±2.60	75.73±1.89	88.30±0.71	0.86
TCAD68	202.2±0.6	206.79±5.38	101.89±2.40	104.90±2.98	0.97
TCAD69	190.0±6.8	169.70±11.61	84.54±3.51	85.16±8.10	1.00

Values are means±SE (three replications). Samples were collected from internodes 2 and 3 at the E4 stage

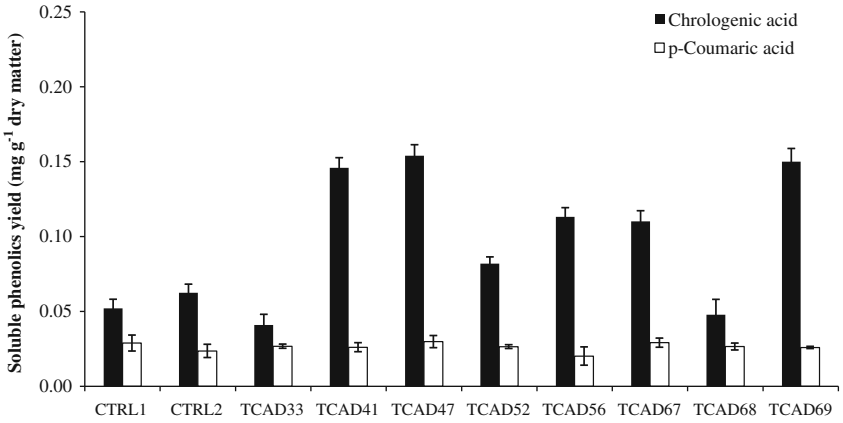

Fig. 4 Content of chlorogenic acid and *p*-coumaric acid in internodes of transgenic switchgrass

varied from 60.7% to 71.1%. The increase in digestibility was statistically significant for most of the transgenic lines (TCAD41, 47, 52, 56, and 67; Electronic Supplementary Material, Table 2).

Effects of *CAD* Suppression on Sugar Release and Enzymatic Saccharification

Sugars present in the direct enzymatic hydrolysates from control plants comprised glucose (~59.1 mg g^{-1} CWR), xylose (~25.0 mg g^{-1} CWR), and trace amounts of arabinose, galactose, and mannose. Without acid pretreatment, most of the transgenic lines (TCAD41, 47, 52, 56, 67, and 69) released 28–59% more glucose than did the controls (Fig. 5). With pretreatment, all the transgenic lines released 15–35% more glucose than did the controls (Fig. 5).

The cell walls of transgenics and the controls had similar total sugar content (Fig. 6a). Compared with control plants, significant increases in saccharification efficiency were achieved in most of the transgenic lines with or without acid pretreatment (Fig. 6b). Without acid pretreatment, an increase of 19–89% in saccharification efficiency was achieved in the transgenic lines, with highest efficiency at 23% (Fig. 6b). With pretreatment, saccharification efficiency of the transgenics was increased by 19–44%, and the best transgenic line reached 43% overall efficiency (Fig. 6b).

The amount of sugars released from non-pretreated or pretreated switchgrass biomass was negatively correlated with lignin content (r^2=0.74 and 0.82, respectively) (Fig. 7a, c). However, no correlation was found between lignin composition (S/G ratio) and enzymatic sugar release (Fig. 7b, d). Taken together with the above data, it appears that lignin level, rather than lignin composition (S/G ratio), is the major factor affecting sugar release of switchgrass.

Discussion

The *CAD* cDNA isolated from switchgrass showed high sequence identity and similar transcript accumulation pattern to those of other monocot CADs such as tall fescue [22] and rice [33], indicating that the gene we isolated is likely involved in lignin biosynthesis. An RNAi vector was constructed using partial sequences of the *CAD* coding region and introduced into switchgrass by *Agrobacterium*-

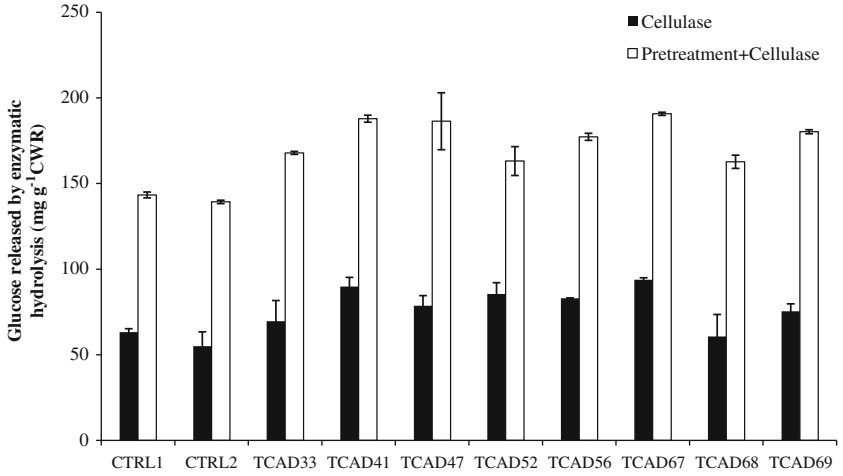

Fig. 5 Glucose release from switchgrass biomass by enzymatic digestion with or without acid pretreatment

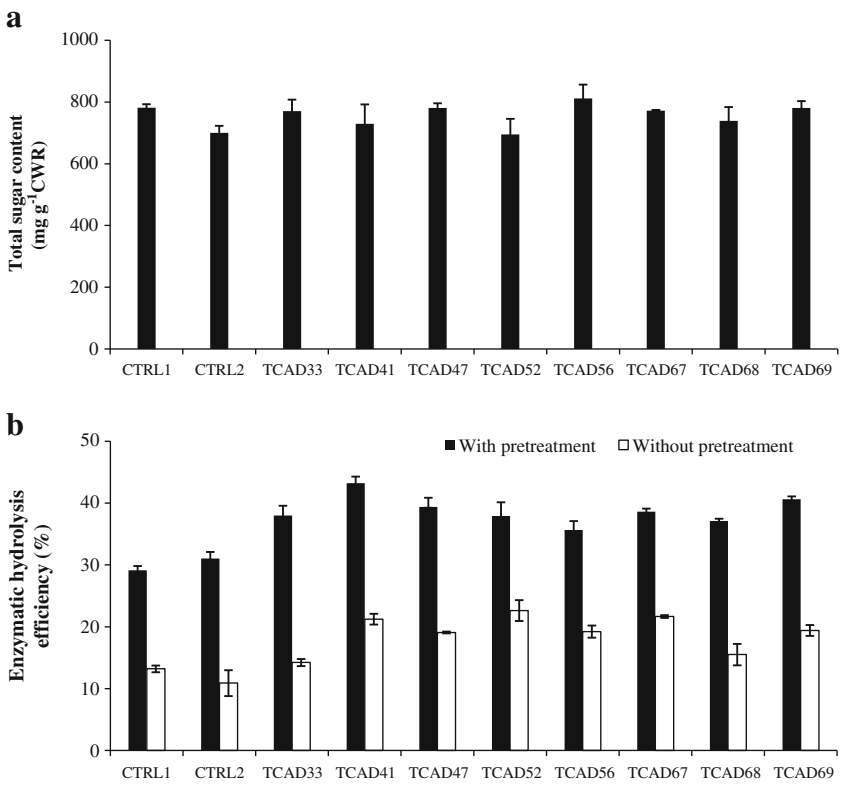

Fig. 6 Saccharification efficiency of switchgrass biomass. Internodes 2 and 3 at the E4 stage were collected from control and transgenic plants. **a** The amount of total sugars in cell wall preparations from internodes; **b** enzymatic saccharification efficiency with or without acid pretreatment

mediated transformation. The transgenics showed substantially reduced levels of transcripts and significantly reduced CAD enzymatic activities, indicating that RNAi technology is efficient in knocking down gene expression in switchgrass. Because of the presence of different CAD homologs in switchgrass, it may be difficult to further reduce gene expression in such a tetraploid outcrossing species. The transgenics showed decreased lignin content, altered lignin

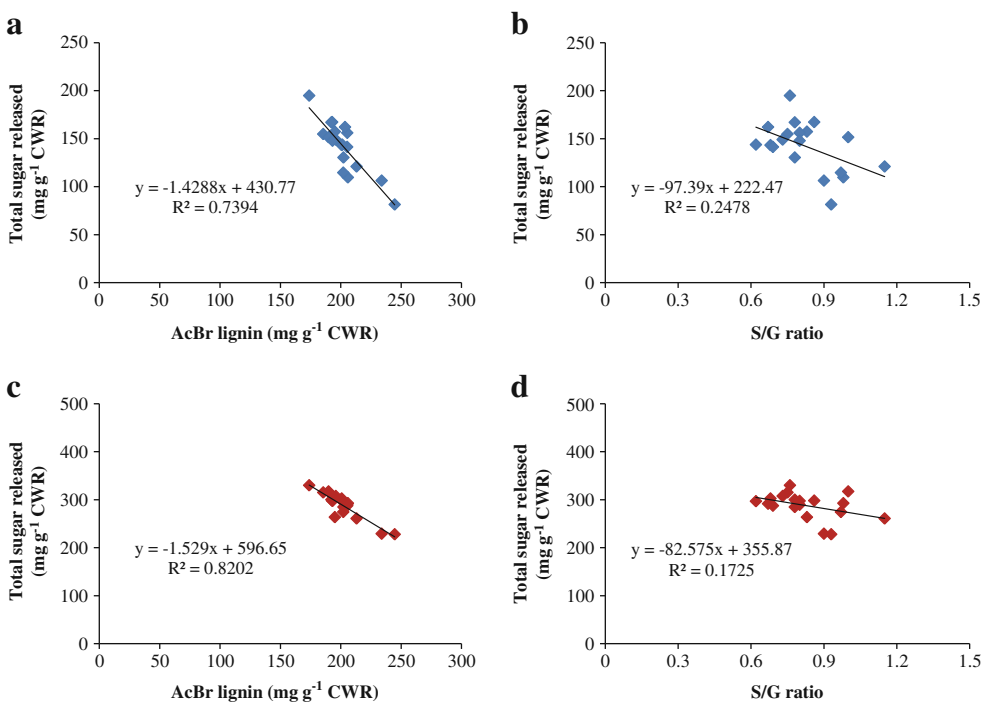

Fig. 7 Correlation between lignin content/composition and sugar release in transgenic switchgrass. Total sugar released is shown as a function of lignin content **(a)** and S/G ratio **(b)** of samples without acid treatment, or as a function of lignin content **(c)** and S/G ratio **(d)** of acid pretreated samples

composition, increased soluble phenolics content and elevated digestibility. Therefore, consistent and closely related molecular and biochemical data demonstrated that the transgenic switchgrass lines were downregulated in their lignin biosynthesis.

Although a recent study in alfalfa [16] reported that CAD downregulation resulted in both reduced lignin content and altered lignin composition, most of the previous work on CAD modification in dicot species such as tobacco [14, 46], alfalfa [15], and poplar [17–19] showed that reduction of CAD activity only led to changes in lignin composition or in the levels of phenolic compounds. In the transgenic switchgrass, the amount of total lignin determined by the acetyl bromide method was reduced significantly and the reduction in G and S units determined by thioacidolysis reflected the decrease in AcBr lignin content. In addition to the reduction in lignin content, the S/G ratio of the transgenic switchgrass was also decreased in most of the lines. Reduction in lignin content and alteration in S/G have been observed in transgenic tall fescue [22] and sorghum *bmr6* mutant [47]. Even though both lignin content and composition were changed, the transgenic switchgrass showed normal development and architecture.

Changes in lignin composition of CAD downregulated tobacco, alfalfa and poplar resulted in increased lignin extractability or degradability [13–15, 17]. However, studies in alfalfa lines downregulated independently in six enzymes upstream of CAD showed that lignin content, rather than lignin composition, was the major factor affecting digestibility and saccharification efficiency [6, 48]. In transgenic switchgrass, although the S/G ratio was changed, correlation analysis revealed that lignin content was likewise the major factor affecting saccharification efficiency.

Downregulation of CAD in switchgrass did not affect the accumulation of wall-bound phenolics. The levels of ferulic acid, *p*-coumaric acid and vanillin were similar in control and transgenic plants. In contrast, a soluble phenolic compound, chlorogenic acid, was significantly increased in most transgenics, indicating that precursors left unused by the lignin pathway had been reallocated. Chlorogenic acid is an important antioxidant for both plants and animals and functions as a performed protectant against biotic challenges [49]. Increasing the levels of chlorogenic acid has been pursued in food crops for their potential health benefit [49]. CAD downregulation is a potential new strategy to achieve such results.

The transgenic switchgrass plants showed a large increase in saccharification efficiency of cell wall materials either with or without acid pretreatment. Overall saccharification efficiency was much higher when the materials were pretreated. Optimization of acid pretreatment and enzymatic hydrolysis conditions could further increase the saccharification efficiency of switchgrass. Nevertheless, the transgenics showed a signification improvement of saccharification efficiency over the controls under the conditions used in this study. Notably, this large improvement in the release of fermentable sugars was achieved in a widely grown commercial switchgrass cultivar, Alamo. It has been recognized that a major obstacle for ethanol production from lignocellulosic feedstocks is the relatively high cost of obtaining sugars for fermentation [50]. Thus, transgenic switchgrass with improved processing properties has the potential to greatly improve the energy and cost efficiencies of bioethanol production. For perennial species like switchgrass, reduction of lignin by genetic methods is probably one of the most effective ways in reducing costs associated with pretreatment and hydrolysis of the material. Because switchgrass is an outcrossing species, transgene flow is a major concern in adopting the transgenic approach. The challenge is how to mitigate gene flow and produce new transgenic materials that satisfy regulatory requirements.

Reduction of CAD activity also led to increased in vitro dry matter digestibility in most of the transgenic switchgrass plants. Increases in dry matter digestibility were observed in transgenic tall fescue carrying sense and antisense *CAD* transgenes [22]. However, the percentage of transgenics showing reduced lignin and increased digestibility was much smaller than that of switchgrass, suggesting the RNAi approach is more efficient in gene downregulation in grasses. Because digestibility is one of the most important traits for forage grasses, the more digestible transgenic switchgrass lines have the potential to be used for animal grazing. Considering the complications involved in biomass transportation and other aspects of bioethanol production, the development of multiuse switchgrass will be beneficial to farmers.

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