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Developmental Control of Lignification in Stems of Lowland Switchgrass Variety Alamo and the Effects on Saccharification Efficiency

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Abstract The switchgrass variety Alamo has been chosen for genome sequencing, genetic breeding, and genetic engineering by the US Department of Energy Joint Genome Institute (JGI) and the US Department of Energy BioEnergy Science Center. Lignin has been considered as a major obstacle for cellulosic biofuel production from switchgrass biomass. The purpose of this study was to provide baseline information on cell wall development in different parts of developing internodes of tillers of switchgrass cultivar Alamo and evaluate the effect of cell wall properties on biomass saccharification. Cell wall structure, soluble and wall-bound phenolics, and lignin content were analyzed from the top, middle, and bottom parts of internodes at different developmental stages using ultraviolet autofluorescence microscopy, histological staining methods, and high-performance liquid chromatography (HPLC). The examination of different parts of the developing internodes revealed differences in the stem structure during development, in the levels of free and well-bound phenolic compounds and lignin content, and in lignin pathway-related gene expression, indicating that the monolignol biosynthetic pathway in switchgrass is under complex spatial and temporal control. Our data clearly show that there was a strong negative correlation between overall lignin content and biomass saccharification efficiency. The ester-linked *p*-CA/FA ratio showed a positive correlation with lignin content and a negative correlation with sugar release. Our data provide baseline information to facilitate genetic modification of switchgrass recalcitrance traits for biofuel production.

Keywords Alamo switchgrass · Lignin · Cell wall · Biofuel · Cellulosic ethanol · Saccharification

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Introduction

Switchgrass is a C4 perennial forage grass native to most areas of the North American grasslands. Switchgrass has high biomass yield because it can reach 1.8–2.2 m in height and has been selected for development as a dedicated biomass/biofuel crop in the USA [1–3]. Different ecotypes of switchgrass have different genetic and physiological characteristics that help them grow better under different environmental conditions. The upland ecotypes, such as Blackwell and Trailblazer, favor drier soils and grow better in steppe climates. The lowland varieties, such as Alamo and Kanlow, prefer heavier soils and are found where water availability is more reliable. The lowland cultivars have

been found to have better genetic ability to produce high dry matter yields than the upland cultivars [4]. Alamo switchgrass was originally collected on the banks of the Frio River near George West, TX. It was evaluated, selected, and released in 1978 by the Texas Agricultural Experiment Station and NRCS as a superior ecotype for conservation performance, forage yield, and seed production (http://offices.sc.egov.usda.gov/locator/app?agency=nrcs). When compared with other cultivars such as Blackwell and Kanlow, Alamo is better adapted in southern areas and has less soil quality requirement, performing well from clays to fine sands [5].

During secondary cell wall synthesis, lignin is deposited within the carbohydrate matrix of the cell wall. The complex secondary cell well structures provide strong mechanical support for the grass to grow upward; however, they dramatically increase the energy cost for releasing the polysaccharide components from cell walls during biomass to biofuel conversion. One strategy to overcome the cell wall recalcitrance of lignocellulosic biomass for bioprocessing is to reduce lignin content and alter lignin composition [6, 7]. This strategy has been successfully used in the forage legume alfalfa. Transgenic alfalfa plants with down-regulated lignin biosynthetic genes have greater fermentable sugar release efficiency from lignocellulosic biomass compared to controls [6]. However, the lignin biosynthetic genes from switchgrass, although presumed to be similar to those from other species, have yet to be identified and characterized. The switchgrass variety Alamo has been chosen for genome sequencing by JGI (http://www.jgi.doe.gov/genome-projects/), and millions of expressed sequence tag (EST) sequences from different EST libraries (tissue or xylem cell-specific) are now available (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=switchgrass). Alamo has been shown to have higher transformation efficiency than other switchgrass genotypes (Z. Wang, unpublished data). Choosing the right candidate genes for transformation is critical, but it is also very important to develop standardized protocols for sampling and for performing basic anatomical, biochemical, and molecular analyses to compare transgenic with wild-type switchgrass plants.

The lignin content and composition of switchgrass varies significantly depending on ecotype, developmental stage, and environmental factors. Although several reports on genetic variability, trait relationships, and biomass production in switchgrass are now available, there is still limited information on cell wall structure and its effects on biomass saccharification efficiency. Recently, Sarath et al. [8, 9] reported the internode structure and cell wall composition in maturing tillers of switchgrass variety Kanlow [8] and cell wall composition and accessibility to hydrolytic enzymes in upland switchgrass genotypes [9]. In this study, we focus on cell wall biochemistry, lignin-related gene expression, and correlation between cell wall composition and saccharification efficiency of a quite different switchgrass cultivar, lowland switchgrass Alamo.

The development of the switchgrass stem can be divided into six elongation stages (E1, E2, E3, E4, E5, and E6) and three reproductive stages (R1, R2, and R3) according to the criteria described by Moore et al. [10]. In the present study, the collected stems were further dissected into different internodes ranging from I1 (basal, oldest internodes) to I6 (upper, youngest internodes), and the individual internodes collected at the E2 and E4 stages were dissected into different sections. The effects of cell wall composition on biomass saccharification were investigated. Our data show that switchgrass stem cellular structures, lignin properties, phenolic acid profiles, and lignin biosynthetic gene expression patterns are precisely controlled, not only in different internodes but also within different parts of a single internode at different developmental stages. The data presented here provide a baseline understanding of the lignification process and its effects on saccharification efficiency in an important biofuel crop variety.

Results

Lignin Deposition in Greenhouse-Grown Switchgrass Plants

Switchgrass often contains multiple tillers representing different developmental stages. The nomenclature of the different stages is indicated in Fig. 1, based on the system of growth stages of perennial forage grasses [10]. To assess carbon partitioning and allocation in greenhouse-grown Alamo switchgrass, dry matter and lignin contents of various tissues were measured. Samples were collected and separated into leaf, leaf sheath, stem, and inflorescence when plants reached the R1 stage. At this stage, the stem contributes more than 37% of the total biomass, followed by the leaf (32%; Fig. 2a). The stem also contains the highest lignin content compared to the other tissues analyzed (Fig. 2b).

Owing to the controlled environmental conditions, the developmental stages of greenhouse-grown switchgrass tillers are more distinguishable than those of field-grown plants. Tillers or different internodes can be sampled by measuring different parameters (Table S1). To determine the best experimental and control tissues for investigating the lignification levels in stem tissues, the second internodes (I2) from the E2 and E4 stages were investigated. The individual internodes were collected and further divided into top (T), middle (M), and bottom (T) sections for detailed analysis (Fig. 1). The lignification process in I2 internodes at the E2 and E4 stages was studied by

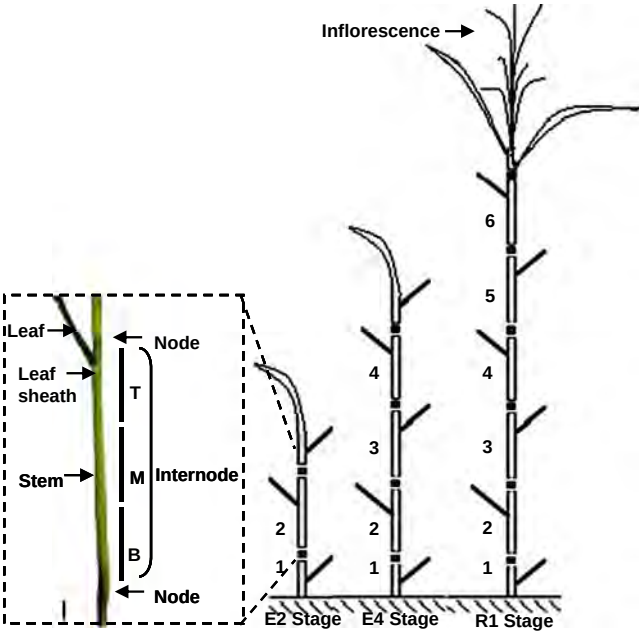

Fig. 1 Developmental stages of switchgrass, as described by Moore et al. [10]. *Black bar* 1 cm. Photo on the *left* shows the I2 internodes with leaf at E2 stage. *T* top part of individual internodes, *M* middle part of individual internodes, *B* bottom part of individual internodes

microscopy, utilizing both lignin autofluorescence under UV light and staining with phloroglucinol–HCl. At the E2 stage, although it is not clearly shown under UV microscopy, the phloroglucinol–HCL staining reveals that the top part of the stem is significantly more lignified compared to the bottom part. At the E4 stage, these differences were not visually discernable. Beside the vascular tissue, both parenchyma and interfascicular fiber cells were lignified at the E4 stage (Fig. 3). Acetyl bromide lignin analysis showed that lignin content decreased from the top to the bottom part of the internodes at the E2 stage, whereas there were no significant changes between the different parts at the E4 stage (Fig. 4a). Total lignin thioacidolysis monomer yields paralleled the acetyl bromide lignin measurements within the E2 internodes (Fig. 4b). The S/G ratios were lower in the top section than in other parts at both E2 and E4 stages (Fig. 4b, c), and on average, S/G ratio was higher in the E2-I2 internodes compared to the E4-I2 internodes. When comparing progressive internodes in the same tiller, the upper internodes had higher S/G ratios than the lower internodes (data not shown). However, there was no clear correlation between the lignin content and the S/G ratio at either the whole plant or single internode level (p=0.226; Figure S1).

Levels of Soluble and Wall-Bound Phenolic Compounds in Greenhouse-Grown Plants

Alamo switchgrass stems contain a number of soluble phenolic compounds, predominantly chlorogenic acid (CGA) and *p*-coumaric acid (*p*-CA; Fig. 5a). At the E2 stage, the bottom part of I2 internodes contained larger amounts of CGA and lower amounts of *p*-CA as compared to the top part. Interestingly, at the E4 stage, this pattern was reversed (Fig. 5b, c). The main ester-linked phenolics released by mild alkali treatment from the switchgrass stems are *p*-CA and ferulic acid (FA), with small amount of *p*-hydroxybenzaldehyde (*p*-HBA) and vanillin (Fig. 5d). The top part of the stem at the E2 stage exhibited a higher ester-linked *p*-CA content than the bottom part (p<0.05), but the FA content is similar to that in the bottom part, resulting in a decrease in *p*-CA/FA ratio from top to bottom (Fig. 5e, f). The *p*-CA contents were similar in all sections of E4-I2 internodes, close to the level in the top part of E2-I2 internodes, but the ester-linked FA content was lower in the E4 internodes (p<0.05; Fig. 5e). Compared to the E2 stage, stems at the E4 stage displayed a higher *p*-CA/FA ratio in all parts (with a mean of 2.5 and 5.5 at the E2 and E4 stages, respectively; Fig. 5e, f). Thus, the ester-linked *p*-CA/FA ratio seems to be an interesting variable correlated with lignin biosynthesis.

High temperature alkali hydrolysis treatment was applied to the wall debris after the low temperature hydrolysis. This releases ether-bound hydroxycinnamic acids from cell wall components. At the E2 stage, ether-linked ferulate (FA) decreased from the top to the bottom of the internodes (p<0.05); this was less apparent at the E4 stage (Fig. 5g). Higher levels of ether-bound FA were released from

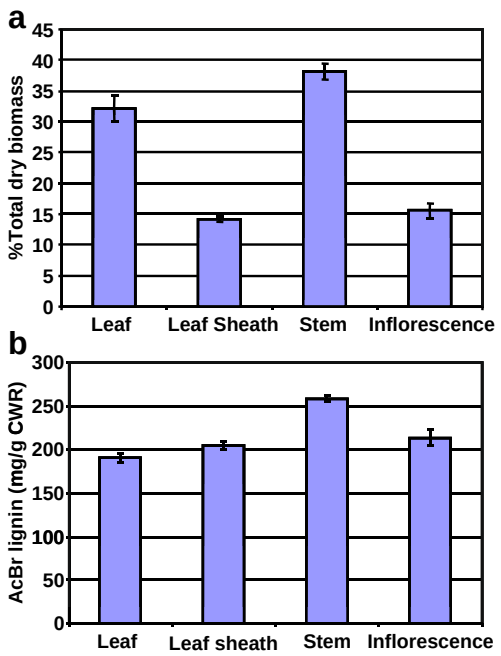

Fig. 2 Dry matter biomass and lignin content of various tissues of greenhouse-grown Alamo switchgrass. Data are means±SE (n=3) from pooled samples. **a** Dry matter biomass distribution. **b** Lignin content determined by the acetyl bromide method. *CWR* cell wall residual

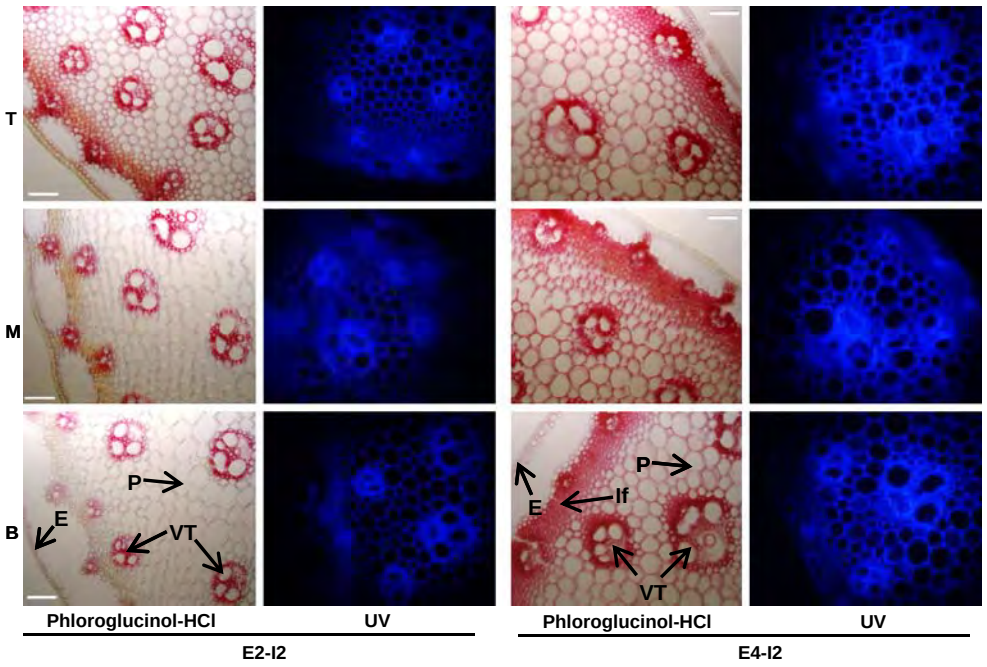

Fig. 3 UV microscopy and phloroglucinol–HCl staining of cross-sections of top, middle, and bottom regions of I2 internodes at different stages of development. *Red* indicates the staining of lignin. Plants were greenhouse-grown. Regions of the internodes and developmental stages are as shown in Fig. 1. *T* top part of individual internodes, *M* middle part of individual internodes, *B* bottom part of individual internodes, *E2-I2* internodes 2 at the E2 stage, *E4-I2* internodes 2 at E4 stage, *VT* vascular tissue, *P* parenchyma, *E* epidermis, *If* interfascicular fiber. *White bar* 100 μm

internodes at the E4 stage than at the E2 stage (with a mean of 3.1 and 2.3 mg/g cell wall residue (CWR) at the E4 and E2 stages, respectively; Fig. 5g). These data indicate that more phenolic compounds with ether linkages are integrated into the wall matrix as stems mature.

Overall, our data indicate that lignification and deposition of cell wall phenolics is processing actively within the I2 internodes at the E2 stage, whereas these processes are largely complete at the E4-I2 internode stage. Furthermore, there is a gradient of increasing lignification within the I2 internodes from bottom to top. A standard sampling protocol should be applied with these facts in mind when comparing transgenic and control plants in the future.

Monolignol Pathway Gene Expression in Greenhouse-Grown Plants

Little molecular genetic information is currently available for the developmental control of lignification in switchgrass. However, several candidate monolignol biosynthetic genes have recently been cloned at the Noble Foundation based on publicly available switchgrass EST data [11]. The cinnamyl alcohol dehydrogenase (*CAD*), caffeic acid *O*-methyltransferase (*COMT*), and cinnamoyl CoA reductase 1 (*CCR1*) genes have been functionally verified through expression of recombinant proteins in *E. coli* and demonstration of their *in vitro* catalytic activity [11] and were therefore selected here for the study of their expression patterns. First, we examined the expression of these genes in different tissues of greenhouse-grown plants using quantitative real-time PCR (Fig. 6a). The result shows that *CCR1*, *COMT*, and *CAD* were most highly expressed in stem tissue, which is the major site of lignification (Fig. 6a). On this basis, these gene candidates are likely involved in lignin biosynthesis and were thus selected to investigate gene expression within single internodes (Fig. 6b). Interestingly, these genes' expression patterns were different within the E2-I2 internodes. *CCR1* expression was higher in the top parts. In contrast, *COMT* and *CAD* had lower expression in the top part and higher expression in the middle and bottom parts. Thus, not all transcript profile of lignin-related genes exactly follows the pattern of lignin deposition within the individual stem internodes at the time point we collected.

Relationship Between Saccharification Efficiency and Lignin and Wall-Bound Phenolic Deposition in Field-Grown Plants

To assess the impact of development (maturity) on biomass saccharification, in field-grown Alamo switchgrass, whole stems and individual internodes, at various developmental stages were collected and subjected to enzymatic hydrolysis without pretreatment. Lignin content and composition and wall-bound phenolics were also analyzed using the same materials to investigate the relationships between saccharification and cell wall properties (Figs. 7 and 8). As shown in Fig. 7, lignin content increased with increasing plant maturity between the E1 and E4 stage and, in the case of a single tiller, increased from the top to the bottom internodes (Fig. 7a). The maturity stages of the stem inversely correlated with enzymatic hydrolysis efficiency, and this

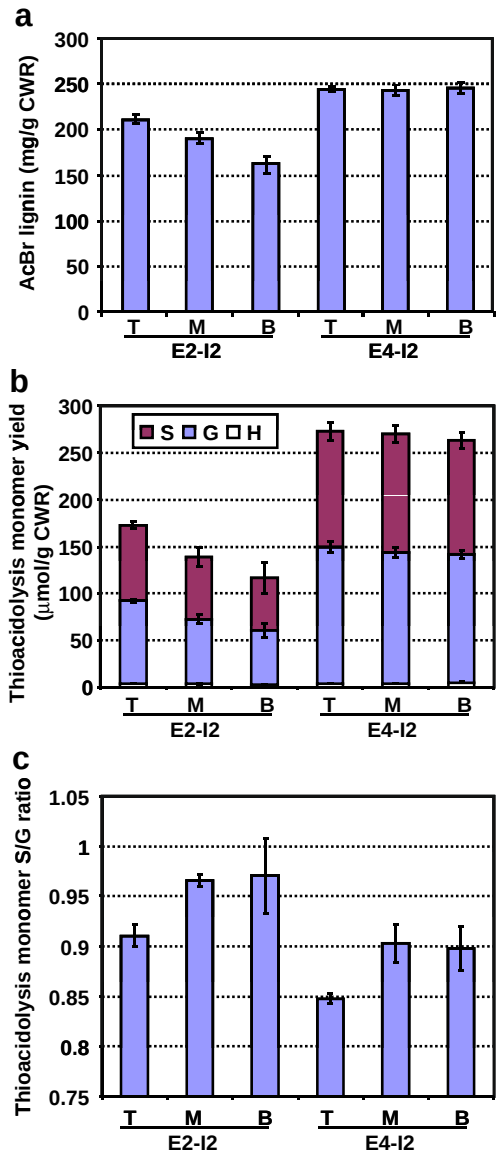

Fig. 4 Lignin content and composition of the top, middle, and bottom sections of I2 internodes at the E2 and E4 stages of development for greenhouse-grown plants. Regions of the internodes and developmental stages are as shown in Fig. 1. Data are means±SE (n=4). **a** AcBr lignin content of different parts of internodes. **b** lignin composition determined by thioacidolysis. **c** S/G ratio. *CWR* cell wall residue, *S* syringyl unit, G guaiacyl unit, *H* *p*-hydroxyphenyl unit

was most evident when comparing the progressive internodes from the top to the lower parts of the same tillers (Fig. 7b).

Our data from greenhouse-grown switchgrass tillers show anatomical, biochemical, and genetic features that may impact cell wall recalcitrance during different developmental stages. Exactly how cell wall composition affects biomass saccharification efficiency at the whole plant level was further analyzed using field-grown material. Our data clearly show that there was a strong negative correlation between overall lignin content and biomass saccharification efficiency (R^2=0.913, p<0.0001; Fig. 8a). The lignin monomer S/G ratio had no correlation with the sugar release efficiency (R^2=0.345, p=0.2269; Fig. 8b). The majority of the lignin subunits in switchgrass are of the S and G types (>90%) (Fig. 4b), and the saccharification efficiency correlated with both S and G lignin monomer levels with R^2= or >0.69 (Fig. 8c). The content of *p*-HBA showed a clear negative correlation (R^2=0.829) with sugar release efficiency (Fig. 8e), whereas the content of ester-linked FA showed a positive correlation (R^2=0.873; Fig. 7g). No correlations were found between the content of *p*-CA and the saccharification efficiency (R^2=0.495, p=0.07; Fig. 8f). As shown for greenhouse-grown material, the ester-linked *p*-CA/FA ratio may be used as one biochemical parameter to predict cell-wall recalcitrance; our data showed that a negative correlation exists between saccharification and ester linked *p*-CA/FA ratio in switchgrass stems (R^2=0.798, p=0.0006; Fig. 8h).

Discussion

Stem Cellular Structures Changes of Switchgrass Internodes

A mature grass plant is composed of leaves, roots, stems, and a seed head. The developmental growth process involves increasing the number and size of leaves and stems and changing from one growth stage to another. In switchgrass, the second node is apparent at the E2 stage (Fig. 1). At the E4 stage, the fourth node is visible. At the R1 stage, the switchgrass reproductive tiller contains 38% stem, 32% leaf, 14% leaf sheath, and 16% inflorescence (Fig. 2). Stems of most grass species have a greater fiber concentration than do leaf blades. In Alamo switchgrass, we found that the stem has the highest lignin content among all tissues (Fig. 2). Microscopy and histochemical staining showed that the parenchyma cells were largely unlignified at the bottom part of E2-I2 internodes, only the vascular tissues being lignified. At the top part of the stem, the parenchyma cell walls become increasingly lignified. A thin ring of interfascicular fibers is also apparent at this stage (Fig. 3). As the plant grows, cell wall lignification occurs in most cell types. The thickness of the interfascicular fiber ring was increased in E4-I2 internodes (Fig. 3). At this stage, all the cells are well developed and lignified in the bottom, middle, and top sections. This is similar to observations from tall fescue and Kanlow switchgrass [9, 12]. Sarath et al. [9] had found that parenchyma cells exhibited thin cell walls with limited secondary cell wall in the internodes closest to the peduncle, while significant cell wall thickening of the parenchyma cells was shown at the

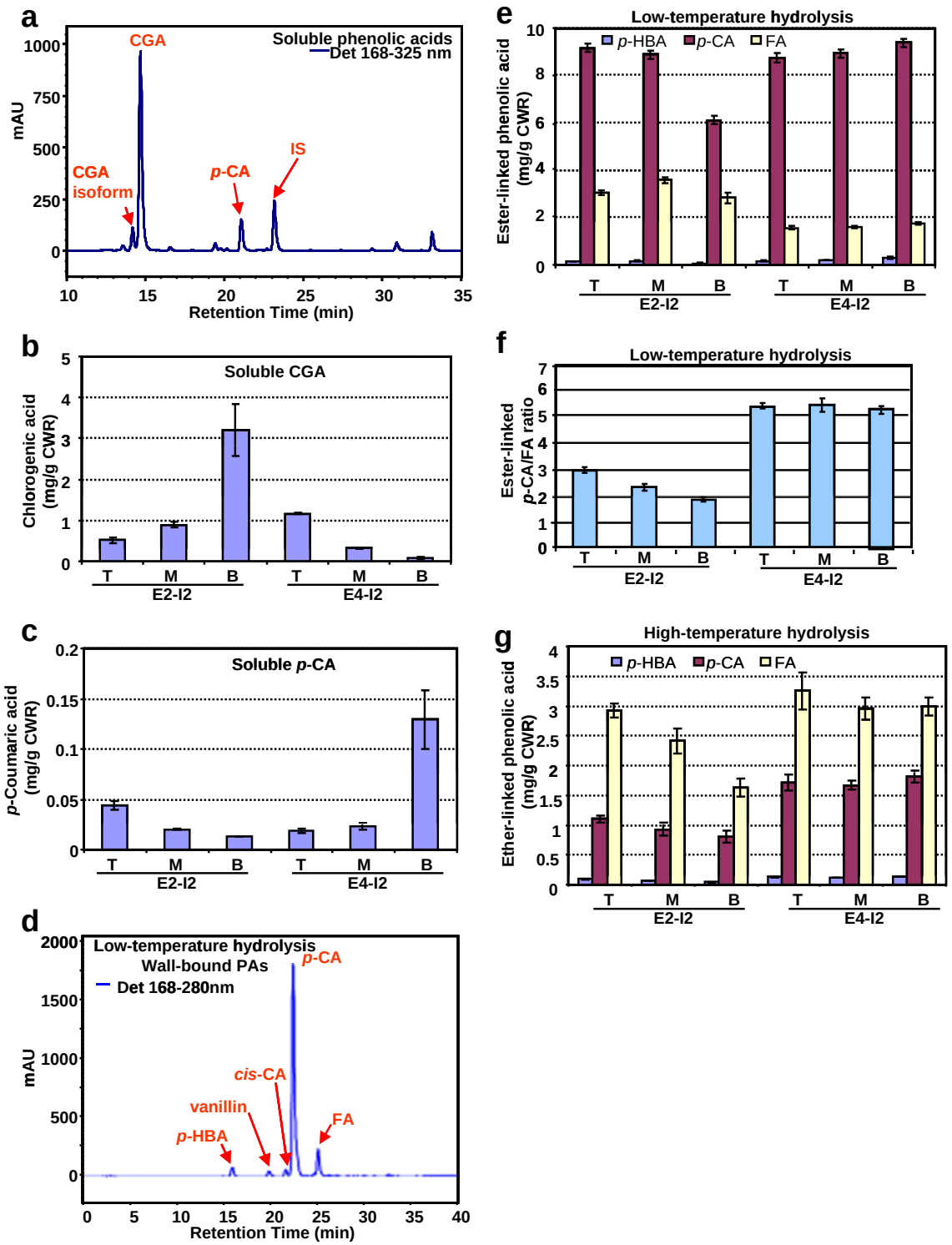

Fig. 5 Levels of soluble and wall-bound phenolic compounds in greenhouse-grown switchgrass internodes. Regions of the internodes and developmental stages are as shown in Fig. 1. Data are means±SE (n=4). **a** A typical HPLC chromatogram of methanol/water soluble fractions from Alamo internodes. **b** Chlorogenic acid content in different parts of internodes. **c** *p*-Coumaric acid content in different parts of internodes. **d** A typical HPLC chromatogram of ester-linked wall bound phenolics from Alamo internodes. **e** Levels of ester-linked wall-bound phenolics. **f** *p*-Coumaric acid/ferulic acid ratios of the ester-linked wall-bound phenolics. **g** Levels of ether-linked wall bound phenolics. *IS* internal standard

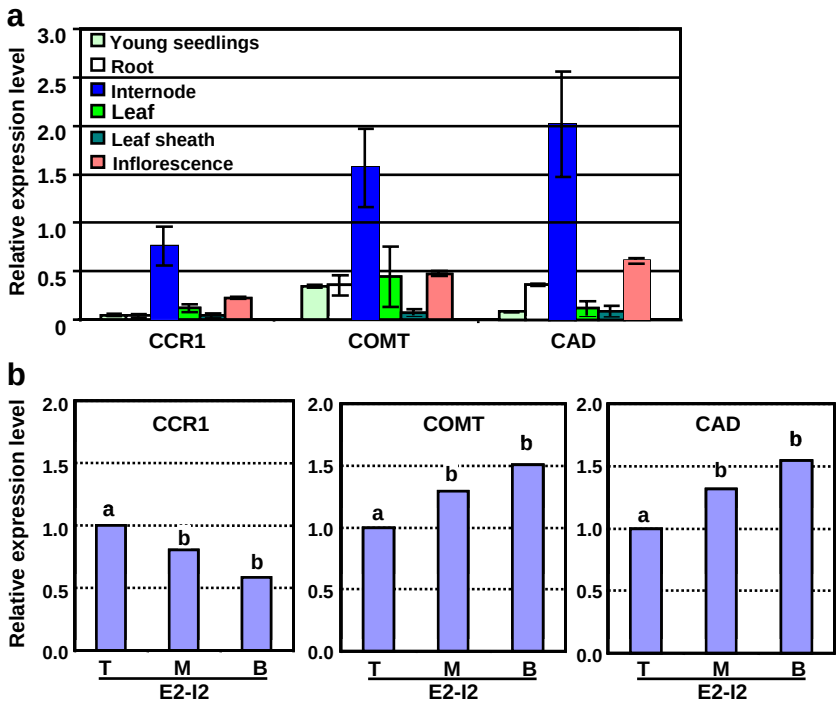

Fig. 6 Expression profiles of monolignol biosynthetic candidate gene of greenhouse switchgrass. **a** Characterized lignin gene expression profiles in different tissues. All the candidate genes have the highest expression level in the internodes compared to root, leaf, leaf sheath, and inflorescences. The gene expression level was compared to the expression of the housekeeping gene *ubiquitin 10*. Data are means± SE (n=3). **b** characterized lignin gene expression profiles in the top, middle, and bottom parts of I2 internodes at E2 stage. The gene expression level is set as 1 in the top part of the internodes. *Different letters on the bar* indicate significant different of expression level at α=0.05 level. *CCR* cinnamoyl Coenzyme A reductase, *COMT* caffeic acid 3-*O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase. Data are means±SE (n=5)

lowest internode (sixth from the peduncle). In maize, after internode elongation, the metaxylem becomes lignified, and sclerenchyma associated with vascular bundles also thickens and lignifies. The parenchyma in the rind region develops a thick, lignified secondary wall during post-elongation development [13]. It was estimated that the wall content of the thick-walled cells accounts for 80% of the dry weight of the internodes in sorghum [14]. The parenchyma and sclerenchyma cell walls of switchgrass and maize differ widely in the concentration of lignin and *p*-CA and in the ratios of xylose to arabinose and *p*-CA to FA [15, 16]. Our results show, even within one internode, that the stem cellular structures from different sections are quite different at certain developmental stages.

Developmental Changes in Lignin and Cell Wall-Associated Phenolic Compounds

Lignin content increased during the progressive elongation and reproductive stages and showed a clear trend of increase along a tiller from the top to bottom internodes (Fig. 7). Changes of lignification were also observed in sections within single internodes (Figs. 3 and 4). Since mature tissues contain a greater proportion of lignified cortical interfascicular fiber ring and lignified parenchyma cell walls, it is not unexpected that lignin content would be higher in these tissues at later developmental stages. At the early elongation stage, lignin content increased rapidly from the bottom to the top part of the basal internodes. The variation of lignin content within one internode is much less in the later developmental stage (E4) as compared to the earlier stage (E2) (Fig. 3). In addition to lignin, the warm-season grasses corn (stover), sugarcane, sorghum, and switchgrass have especially high levels of ester-linked phenolic acids [17]. *p*-CA and FA are the main ester-linked wall-bound phenolics in Alamo switchgrass. In grasses, *p*-CA and FA are covalently bound to lignin and other cell wall components [18, 19]. FA is hypothesized to provide a nucleation site where the lignin polymers can grow [20, 21]. When compared to E4-I2 internodes, E2-I2 internodes contain higher levels of ester-linked FA (p<0.05), while the ether-bound phenolic levels were higher in more mature tissues (p<0.05) in parallel with the higher lignin content.

p-CA is ester-linked to syringyl lignin subunits in grasses [22]. The small amount of *p*-CA recovered from high temperature hydrolysis is likely due to the incomplete

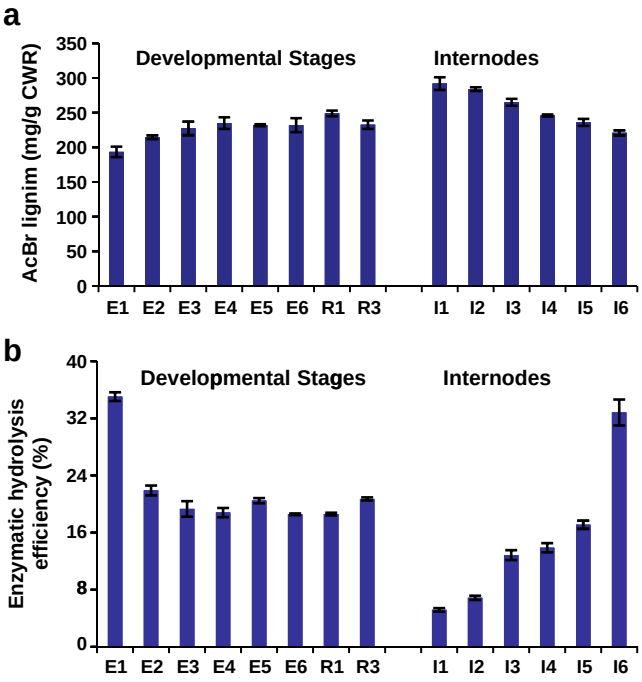

Fig. 7 Lignin content and saccharification of field-grown Alamo switchgrass material at different developmental stages (E1 to E6 represent elongation stages and R1 to R3 represent reproductive stage; I1 to I6 represent individual internodes). Data are ±SE (n=3) from pooled samples. **a** Saccharification efficiency of switchgrass at different developmental stage with a cellulase mixture. **b** Lignin content of switchgrass samples used in the saccharification experiment

hydrolysis of ester-linked *p*-CA in stems. At the E2 stage, *p*-CA increased with increasing tissue maturity. At the E4 stage, the level of *p*-CA remained roughly constant. Together, our data suggest that, at the E2-I2 stage, phenolic compounds were rapidly incorporated into the cell wall in an esterified form, and at the later stage, these ester linked-phenolics further cross-link with lignin or are used as a starting point for lignification. The increase in wall-bound phenolic levels may have ended by the E4-I2 stage, supporting the hypothesis that wall-bound FA is a nucleation site for lignification. Therefore, down-regulation of wall-bound FA content may be one strategy to overcome recalcitrance of biomass to biofuel conversion in switchgrass.

Developmental Changes in Soluble Phenolic Compounds

Alamo switchgrass stems have a high CGA content. More than 3.0 mg CGA per gram dry CWR was detected in the bottom part of E2 internodes under our experimental conditions. At the E4 stage, the CGA content is less than 1 mg/g CWR. Sarath et al. had reported the decrease of three major phenolic peaks; however, they did not identify these peaks in the soluble fractions [9]. Switchgrass stems also contain *p*-CA in their methanol/water soluble fraction. I2 internodes at the E4 stage have higher free *p*-CA content than E2-I2 internodes. In the switchgrass variety, Kanlow, the soluble phenolics dramatically decrease from the top to the bottom internodes, while the levels of *p*-CA remained relatively constant for the top three internodes and then increased in the more mature internodes [9]. CGA has antifungal and anti-insect activity [23, 24]. It is currently not clear how these soluble phenolic acids affect biomass pretreatment and downstream fermentation processing. On the other hand, the high levels of soluble and wall-bound phenolics in switchgrass means it could provide a feedstock for value-added co-products from future integrated biorefining.

Relationships Between Cell Wall Composition and Saccharification

It had been demonstrated that lignin is one of the determining factors affecting biomass saccharification and cell wall degradability [6, 25, 26]. Our results on the sugar release efficiency and lignin analyses from the field-grown samples indicate that saccharification efficiency has a strong negative correlation with the lignin content (R^2=0.913) in switchgrass, while the lignin S/G ratio shows no significant correlation with enzymatic saccharification (p=0.2269). FA is proposed to be among the factors most inhibitory to the biodegradability of cell wall polysaccharides [26–28]. In the Kanlow switchgrass, the esterified wall-bound ferulate levels did not exhibit a substantial change with increasing distance of the internodes from the top of the plant [9]. In this study, we find that there are no significant changes in ester-linked FA levels within one internode but that ester-linked FA is lower in E4-I2 internodes than that in E2-I2 internodes. The ester-linked FA level correlated negatively with cell wall lignification (p<0.0001; Figure S1g) and positively with saccharification efficiency (p<0.0001; Fig. 8g). The other major cell wall phenolic compound, *p*-CA, is mainly esterified to lignin syringyl units in plant cell walls and may not directly affect plant cell wall digestibility [29]. Ester linked *p*-CA did not correlate well with lignin content or cell wall saccharification (Fig. 8f and Figure S1f). The *p*-CA/FA ratio is another indicator of lignification, as the *p*-CA/FA ratio increased with increasing plant maturity [9]. In our analyses, it showed a positive correlation with lignin content (R^2=0.845; p=0.0001; Figure S1h) and a negative correlation with sugar release (R^2=0.798; p=0.0006; Fig. 8h). These results agree with the suggestion that factors affecting cell wall degradation are complex and not related to a single linkage type of wall-bound phenolic [8].

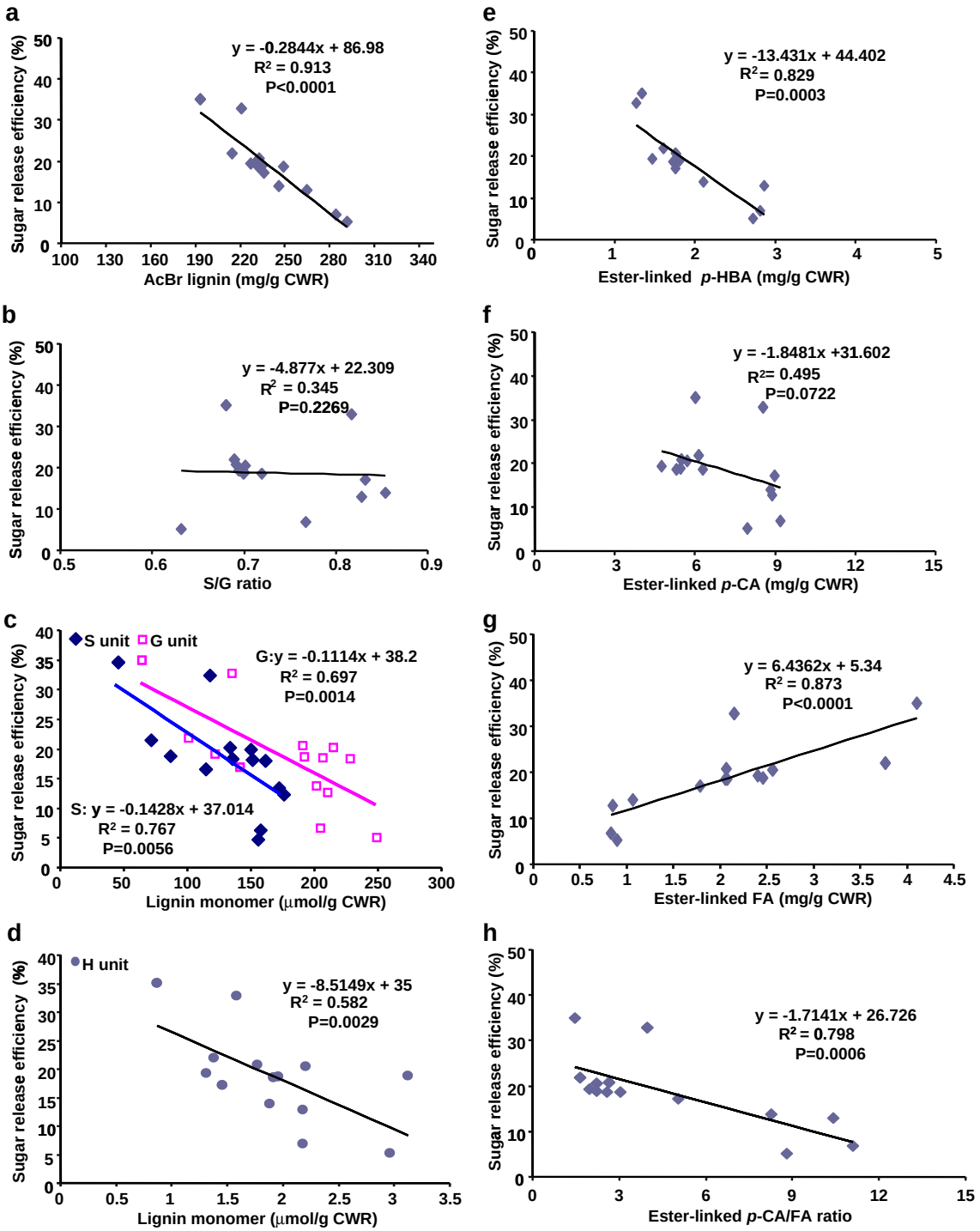

Fig. 8 Relationships between saccharification efficiency and lignin and wall-bound phenolic deposition. Data for field-grown switchgrass materials were used for correlation analysis. **a**–**d** Correlation of saccharification efficiency and lignin content and composition. **e**–**h** Correlation of saccharification efficiency and wall-bound phenolics

Target Genes for Lignin Engineering in Switchgrass

The monolignol and wall-bound phenolic biosynthesis pathways share the common enzymes phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate coenzyme A ligase, hydroxycinnamoyl-CoA/shikimate/quinate hydroxycinnamoyltransferase, coumarate 3-hydroxylase, and caffeoyl CoA 3-*O*-methyltransferase, whereas cinnamoyl coenzyme A reductase (*CCR*), ferulate 5-hydroxylase, caffeic acid 3-*O*-methyltransferase (*COMT*), and cinnamyl

alcohol dehydeogenase (*CAD*) are thought to be monolignol-specific. CCR is the first reaction specific for the monolignol pathway. Down-regulation of *CCR* in poplar and alfalfa has resulted in 50% and 32% reductions in lignin content, respectively [30, 31]. Our data show that *CCR1* gene expression follows lignin deposition within I2-E2 internodes. In maize stems, when comparing basal and younger internodes, all genes known to be involved in constitutive monolignol biosynthesis had a higher expression in younger internodes [32]. *CCR1* may have a critical role in controlling monolignol synthesis in switchgrass. The tissue expression analysis of other selected monolignol synthetic gene candidates (*CAD* and *COMT*) showed that they have high expression in tissues undergoing active lignification, indicating their possible roles in monolignol biosynthesis.

Transcript profiling in the E2-I2 internodes can provide a sensitive measurement to detect changes in gene expression and to characterize genetically engineered switchgrass in the future. We were able to obtain reproducible results from multiple biological replicates of these internodes collected by measuring a set of parameters in Table S1. When comparing transgenic plants with non-transgenic plants, the same portion of the internode samples (top, middle, and bottom) should be dissected in order to obtain accurate and reliable results. To minimize the sampling variation, we recommend using the E4-I2 internodes for lignin analysis.

Materials and Methods

Plant Materials and Sample Collection

Switchgrass (*Panicum virgatum* L. cv. Alamo) was planted in the greenhouse under standard conditions (temperature range, 25–29°C) with a 16-h day from 0600 to 2200 hours facilitated by supplementary lighting (inside light, parabolic aluminized reflector, 125–55 μmol) and relative humidity 77–22%, average 51%. Plants were watered three times per week, and the fertilizer (Peter's Fert 20-10-20, 100 ppm) was added in the last watering. For tissue-specific gene expression pattern analysis, roots, leaves, leaf sheaths, internodes, and flowers were collected at the R1 development stage [10]. These samples were also used for lignin determination, except the seedlings and roots. For analysis of individual internodes, the internodes at the E2 and E4 stages were excised in order of position (one at the bottom and four at the top), and the leaf, leaf sheaths, and nodes were carefully removed. Similar length internodes were pooled and cut into three equal sections representing the bottom, middle, and top parts (parameters of internodes collected were shown in Table S1). Samples were immediately frozen in liquid nitrogen and kept at −80°C. Then, the samples were ground to powder with freeze mill under liquid nitrogen for further RNA isolation and chemical analysis including soluble and wall-bound phenolics and lignin analysis as described below.

Field-grown switchgrass plants were grown from seed in a field nursery in 2004 on the campus of the Noble Foundation in Ardmore, OK. The stands received 50–100 lb/Ac fertilizer as urea each year, all applied in the spring (by the first week of May), soon after initiation of growth. Plants were harvested in the summer of 2006 at different developmental stages. Whole tillers containing leaves or individual internodes from stems at the R1 stage were oven-dried at 60°C for 72 h and ground to pass through 1-mm screens. Ground plant materials were used for saccharification analyses. The oven-dried materials were subjected to wall-bound phenolics and lignin analyses to investigate the correlation of biomass saccharification with cell wall components.

Microscopy and Histochemical Staining of Lignin

Switchgrass stem samples were collected in the greenhouse and immediately frozen in liquid nitrogen. About 0.5-cm tissue sections from the top, middle, and bottom parts of the internodes were cut with a Leica CM 1850 cryostat at −20°C and prepared for microscopy as described previously [33]. Phloroglucinol–HCl staining was carried on as described [34]. Ultraviolet absorption microspectrophotometry was performed according to Nakashima et al. [33]. Photographs were taken using a Nikon DXM 1200 color camera attached to a Nikon microphot-FX microscope system with ACT-1 software (Nikon, Japan).

Determination of Lignin Content and Composition

CWR were prepared by extracting the samples (greenhouse- and field-grown) with chloroform/methanol (1:1), 100% methanol, 50% methanol, and water (three times each). Fifteen milligram of lyophilized samples was used for lignin analysis. The acetyl bromide method [35] was employed to determine total lignin content. Thioacidolysis followed by gas chromatography–mass spectrometry was used to identify and quantify lignin-derived monomers [36, 37]. The numbers of replications are shown in figure legends as appropriate.

Analysis of Soluble and Wall-Bound Phenolic Compounds

Soluble phenolics were extracted from 30.0 mg freeze dried internode powder with 1.5 mL 50% methanol plus 1.5% acetic acid for 12 h at room temperature [9]. The 7-hydroxy coumarin (0.05 mmol/L) was added to the extract solution as internal standard. After centrifugation at 12, 000×*g* for

30 min, the supernatant was taken for profiling soluble phenolics. One hundred milligrams of extractive-free CWR were used for analysis of esterified cell wall-bound phenolics using low-temperature alkaline hydrolysis (2.0 M NaOH, 30°C, 24 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 for HPLC analysis. HPLC was carried out on a Beckman System Gold HPLC system consisting of a programmable solvent module 126, a System Gold 508 autosampler, and a System Gold 168 diode array detector. A Waters Spherisorb ODS-2 5 μ reverse phase column (5 μm particle, 250×4.6 mm) was used (solvent: A, 0.1% phosphoric acid in water; B acetonitrile; gradient: for soluble phenolics, 8% B to 44% B in 55 min; for wall bound phenolics, 5% B to 25% B in 50 min). The residue after low temperature hydrolysis was thoroughly washed with methanol and water and then freeze dried. The dried samples were weighed and subjected to high temperature hydrolysis (4.0 M NaOH, 130°C, 4 h) to release ether-linked phenolic compounds. The phenolic compounds were recovered and analyzed by HPLC as described above. Serial dilutions of standard solutions of chlorogenic, *p*-coumaric, and FAs (Sigma, St Louis, MO) were prepared and analyzed together with the above samples by HPLC/diode array detector. The UV absorption of the metabolites was monitored at 280 and 320 nm. The numbers of replications are shown in figure legends as appropriate.

Enzymatic Hydrolysis and Determination of Saccharification Efficiency

Switchgrass plants were grown in the field and oven-dried after harvest. CWR were prepared by removing soluble extractives from the collected samples ground and lyophilized as above. The extractive-free material was lyophilized and analyzed for total sugar and sugar components released by enzymatic hydrolysis with a mixture of cellulase and cellobiase based on the previously described procedures according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (LAP-009). Biomass samples equal to 0.1 g equivalents of cellulose were hydrolyzed with a mixture of Celluclast 1.5 L (cellulase from *Trichoderma reesei*) and Novozyme 188 (cellobiase from *Aspergillus niger*; Sigma, St Louis, MO; 1:1 *v*/*v*) in 10 mL sodium citrate buffer (0.1 M, pH4.8) for 72 h. The enzyme loadings were in excess (~20 FPU per gram CWR). Enzyme blanks and Whatman number 1 filter paper were digested alongside the samples. Hydrolysis of filter paper was always more than 95% [6]. Total sugars of cell wall preparations were analyzed spectrophotometrically using the phenol-sulfuric acid assay method [38]. 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.

Quantitative RT-PCR

Switchgrass samples were collected from the greenhouse. Measurement parameters were applied when sampling the top, middle, and basal parts of internodes as described in Table S1. Total RNA was isolated with TRI Reagent® RT (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. The RNA quality was analyzed with an Agilent 2100 Bioanalyzer. Three micrograms of total RNA were digested with DNase (Applied Biosystems, Ambion, Austin, TX) then reverse transcribed with reverse transcript III kit following the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). Quantitative RT-PCR was performed in real-time PCR HT 7900 sequence detection system (Applied Biosystems, Ambion, Austin, TX) with SYBR green dye. The quantitative RT-PCR (qRT-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 PCR data of each individual sample was collected and exported by the software of SDS 2.2.2 for 7900HT (Applied Biosystems, Foster City, CA), and the PCR efficiencies and ct values for each sample were checked by the LinRegPCR 7.0 software [39]. Relative transcript levels were calculated using the comparative Ct value according to the formula described by Ranasinghe et al. [40]. The gene expression level was calculated by comparison to the housekeeping gene ubiquitin 10 (UBQ10). For comparing the gene expression levels in the op, middle, and bottom parts, the top samples were normalized to 1. The qRT-PCR primers are as follows: UBQ10-F, 5′- TTCGTGGTGGCCAGTAAG -3′; UBQ10-R, 5′- GAAGGGTGGCGTCTTTAAGTATCT-3′; CCR1-F, 5′- GCGTCGTGGCTCGTCAA -3′; CCR1-R, 5′- TCGGG TCATCTGGGTTCCT -3′; COMT-F, 5′- CAACC GCGTGTTCAACGA -3′; COMT-R, 5′- GTTCTC GTGTCCGAGGTGTGT -3′; CAD-F, 5′- TCACATCAAG CATCCACCATCT -3′; CAD-R, 5′- GTTCTC GTGTCCGAGGTGTGT -3′.

Statistical Analysis

Data from each analysis were subjected to ANOVA. 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 models were considered. Correlations were calculated with SAS software (SAS Institute Inc., Cary, NC, USA).

Statistically significant ($P<0.01$) differences are indicated in the presentation of results (Fig. 8 and Figure S1). Spearman correlation coefficients were determined among the response traits for wall-bound phenolics, lignin content, and composition with sugar release efficiency or lignin content. All correlations with p value smaller than 0.01 were treated as correlated.

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