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Altered Lignin Biosynthesis Improves Cellulosic Bioethanol Production in Transgenic Maize Plants Down-Regulated for Cinnamyl Alcohol Dehydrogenase

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ABSTRACT Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme involved in the last step of monolignol biosynthesis. The effect of CAD down-regulation on lignin production was investigated through a transgenic approach in maize. Transgenic CAD-RNAi plants show a different degree of enzymatic reduction depending on the analyzed tissue and show alterations in cell wall composition. Cell walls of CAD-RNAi stems contain a lignin polymer with a slight reduction in the S-to-G ratio without affecting the total lignin content. In addition, these cell walls accumulate higher levels of cellulose and arabinoxylans. In contrast, cell walls of CAD-RNAi midribs present a reduction in the total lignin content and of cell wall polysaccharides. *In vitro* degradability assays showed that, although to a different extent, the changes induced by the repression of CAD activity produced midribs and stems more degradable than wild-type plants. CAD-RNAi plants grown in the field presented a wild-type phenotype and produced higher amounts of dry biomass. Cellulosic bioethanol assays revealed that CAD-RNAi biomass produced higher levels of ethanol compared to wild-type, making CAD a good target to improve both the nutritional and energetic values of maize lignocellulosic biomass.

Key words: Maize; lignification; lignocellulosic biomass; secondary cell wall.

INTRODUCTION

Lignin is a key polymer for vascular plants. This phenolic compound is localized in the secondary cell walls, improves the transport of water and solutes through the vascular system, and increases the stiffness and strength of the stem. In addition, its mere presence in the cell wall acts as a physical barrier against pathogens (Whetten et al., 1998; Mellerowicz et al., 2001; Boerjan et al., 2003; Boudet et al., 2003; Vanholme et al., 2010). Lignin is synthesized through the phenylpropanoid pathway (Davin et al., 2008; Vogt, 2010), starting with the deamination of the aminoacid phenylalanine and ending with the production of the three main monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. These alcohols are finally transported to the cell wall and polymerized to generate the final lignin polymer (Pomar et al., 2002; Ranocha et al., 2002; de Obeso et al., 2003; Caparrós-Ruiz et al., 2006; Berthet et al., 2011).

Nowadays, the maize (*Zea mays* L.) lignocellulosic biomass is considered as an agricultural waste due to the difficulty to access the polysaccharides stocked in the cell wall. As this

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hindrance is mainly attributed to its interaction with lignin, the improvement of maize lignocellulose quality is essential to exploit this huge amount of biomass as forage and/or as a source of cellulosic bioethanol (Godshall, 2005; Gray et al., 2006; Himmel et al., 2007; Torney et al., 2007). At present, very few functional studies have been addressed to understand how the different cell wall polymers are synthesized in maize. Only the spontaneous brown-midrib (*bm*) mutants have been associated with lignin metabolism, being the *bm1* defectives in cinnamyl-alcohol dehydrogenase (CAD) and the *bm3* mutant defectives in caffeic acid-*O*-methyltransferase (COMT) activity (Vignols et al., 1995; Halpin et al., 1998). Later on, a similar *bm3* phenotype was obtained by partial down-regulation of COMT in transgenic maize plants (Piquemal et al., 2002) and this work constitutes so far the only characterization of a maize transgenic plant directed to study lignification in this important monocotyledonous species.

CAD enzyme was one of the first enzymes studied in the lignin biosynthetic pathway and it catalyzes the final reduction in the hydroxyl-cinnamaldehydes to the corresponding alcohols (Mansell et al., 1974). In *Arabidopsis*, nine putative CAD genes have been identified (Raes et al., 2003; Kim et al., 2004) but only three of them have been demonstrated to be involved in monolignol synthesis (Kim et al., 2004; Sibout et al., 2005; Eudes et al., 2006; Thévenin et al., 2011). In maize, besides the CAD cDNA previously characterized as responsible for the *bm1* mutation, six other CAD genes were identified (Guillaumie et al., 2007).

Due to the important role of CAD in determining lignin content and composition, the involvement of this enzyme in lignin polymer biosynthesis has been studied in several plant species. To date, the effect of CAD activity reduction has been studied in dicot plants, such as tobacco (Bernard Vailhe et al., 1998; Yahiaoui et al., 1998; Chabannes et al., 2001; Ralph et al., 2001), alfalfa (Baucher et al., 1999; Jackson et al., 2008), poplar (Baucher et al., 1996; Lapierre et al., 1999; Lapierre et al., 2004; Pilate et al., 2002), eucalyptus (Valério et al., 2003), flax (Wrobel-Kwiatkowska et al., 2007), and *Arabidopsis* (Sibout et al., 2005). In monocots, besides the CAD-defective maize *bm1* mutants (Halpin et al., 1998), other CAD-defective monocot plants have been characterized, such as the sorghum *bmr6* (Saballos et al., 2009; Sattler et al., 2009), the rice golden hull2 (*gh2*) (Zhang et al., 2006), the transgenic switchgrass CAD-RNAi (Fu et al., 2011; Saathoff et al., 2011), and the tall fescue CAD-RNAi (Chen et al., 2003). These genetics approaches together with the knowledge provided by mutants demonstrated that CAD loss-of-function does not always drastically affect plant fitness. Thus, CAD-deficiency leads to a modified lignin through the incorporation of cinnamaldehyde subunits in CAD-transgenic and mutant plants (Ralph et al., 2001; Dauwe et al., 2007) or to brownish red internodes, leaf midribs, or rice-hulls (Halpin et al., 1998; Marita et al., 2003; Zhang et al., 2006; Sattler et al., 2009). In maize, the impact on lignin content and composition and general plant fitness depends on the variability of the residual CAD enzymatic activity of the different *bm1* mutants (Halpin et al., 1998; Vermerris et al., 2010). However, an increase in cell wall degradability and enhanced content in cinnamaldehydes in the final lignin polymer constitute common traits of all the maize *bm1* mutants (Halpin et al., 1998; Vermerris et al., 2010).

Here, we report the generation and characterization of transgenic maize plants in which CAD enzymatic activity was reduced by the RNAi approach. The results obtained show that maize CAD-RNAi plants present different levels of CAD activity reduction in different organs. Accordingly, the impact on lignin and polysaccharide biosynthesis differs in an organ-dependent manner. Despite these different responses, the availability of cell wall polysaccharides increases in transgenic plants, leading to an enhanced *in vitro* degradability and to a higher production of cellulosic bioethanol without visible effects on plant growth and development.

RESULTS

Reduction in CAD Enzymatic Activity Does Not Trigger the Brown Midrib Phenotype in Maize CAD-RNAi Plants

CAD-RNAi plants essentially present the same phenotype as wild-type plants (Figure 1A). Although their growth rate is initially delayed, plants achieve the same size as control plants at the flowering stage and only a slight pale-green pigmentation in all tissues of the transgenic plants is visible compared to wild-type (Figure 1A). None of the transgenic lines obtained presented the reddish to brown pigmentation typical of the spontaneous maize brown midrib (*bm1*) mutants (Halpin et al., 1998; Marita et al., 2003).

The initial analysis of CAD activity in four independent maize transgenic lines revealed that CAD enzymatic activity reduction ranges from 20 to 80% in roots, being always higher than the residual CAD activity of *bm1* roots (Supplemental Figure 1), and similar to what had already been reported (Halpin et al., 1998). Those having 20% CAD activity in roots were selected for further characterization.

Transgenic maize plants presented CAD activity reduction in all the tissues analyzed. In addition to 20% in roots, stems and leaves present 34 and 68% CAD activity compared to wild-type, respectively (Figure 1B). Thus, the absence of the brown midrib pigmentation in the CAD-RNAi plants indicates that, at least, a 68% CAD residual activity is sufficient to avoid the appearance of this phenotypical trait.

CAD-RNAi Plants Show Morphological Alterations in Lignified Tissues

The impact of CAD down-regulation on lignified tissues was studied microscopically and the presence of lignin visualized under UV light on hand-cross-sections of lignified tissues of stems and midribs of wild-type, CAD-RNAi, and *bm1* plants. CAD-RNAi internodes present an increased number of vascular bundles when compared to wild-type plants. However, this increase in number is accompanied by a general reduction in their diameter and a less-developed sclerenchyma (Figure 2).

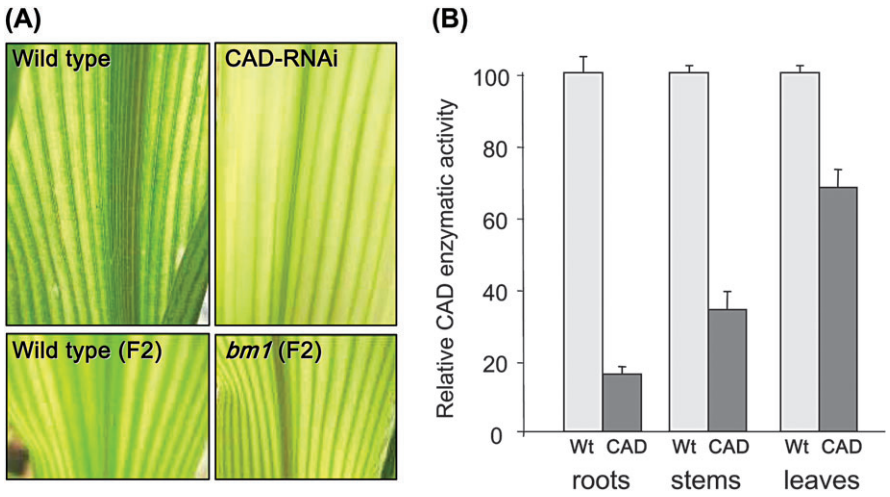


Figure 1. Macroscopic phenotype and enzymatic activity of maize CAD-RNAi plants.
(A) Leaf macroscopic phenotype of 6-week-old maize CAD-RNAi and *bm1* (F_2 genotype) plants and their corresponding wild-type controls.
(B) CAD enzymatic activity in different parts of maize wild-type and CAD-RNAi plants. Wt means wild-type plants and CAD means CAD-RNAi plants. Data correspond to the mean value ± SD of three independent assays.

In the case of midrib, the spatial distribution of vascular bundles is similar to that of the wild-type plants, although their size is significantly decreased. A general reduction in mechanical tissues is also observed (Figure 2).

The alterations observed in CAD-RNAi plants follows the same trend as those observed in stem and midrib sections of the spontaneous *bm1* maize in which the residual enzymatic activity of CAD is still lower than in CAD-RNAi plants (Figure 2).

The morphological alterations observed in the lignified tissues of CAD-RNAi plants were quantified by the Klason analyses and lignin composition determined by thioacidolysis. The results obtained indicate that lignin content does not differ between CAD-RNAi and wild-type stems (Table 1). However, the syringyl-to-guaiacyl (S/G) ratio of the lignin polymer is slightly decreased in the CAD-RNAi stems, by both a reduction in the syringyl (S) subunits and an increase of the guaiacyl (G) and *p*-hydroxyphenyl (H) subunits (Table 1).

The opposite situation occurs in the midribs. In this case, a 6.4% reduction in the total lignin content is observed in CAD-RNAi midribs compared to wild-type plants without affecting its monomeric composition (Table 1).

Down-Regulation of CAD Activity Mainly Affects the Development of Fibers Adjacent to Vessels of CAD-RNAi Stems

Ultrastructural studies were performed in transverse sections of vascular bundles and sclerenchyma of CAD-RNAi and wild-type stems. No significant changes were observed in the morphology of CAD-RNAi vessels, but the analysis of its lignin distribution revealed a slight perturbation of the sub-units' distribution (Figure 3). Thus, while, in wild-type, condensed units are essentially concentrated in the S1 and S3 layers of the secondary cell wall, in CAD-RNAi vessels, they are also present in the S2 layer, as shown by the immunolabeling with the anti-mixed guaiacyl/syringyl lignin antibodies (Figure 3C and 3D). In addition, the secondary cell walls of CAD-RNAi vessels accumulate higher levels of S subunits as shown by the immunolabeling with the anti-syringyl lignin antibodies (Figure 3E and 3F). Concerning the fibers adjacent to vessels, in addition to an enlargement in diameter, interruptions in secondary thickenings of transgenic adjacent fibers compared to wild-type can be observed, with more numerous and longer pit membranes, suggesting a delayed development of this type of secondary wall. In addition, these fibers contain slightly reduced levels of G and increased levels of S subunits, as shown by the immunolabeling with the anti-homoguaiacyl (Figure 3A and 3B) and anti-syringyl lignin antibodies (Figure 3E and 3F) and by the semi-quantitative counting of gold grains per μm^2 (Supplemental Table 1). With respect to the ultrastructure of the sclerenchymatic fibers, no significant changes occur in CAD-RNAi cell walls compared to wild-type plants (Figure 4 and Supplemental Table 1).

Down-Regulation of CAD Activity Slightly Affects Soluble Phenolics and Flavonoid Content in CAD-RNAi Plants

As lignin is synthesized through the phenylpropanoid pathway, we investigated the possible occurrence of changes in soluble phenolics and flavonoids that also belong to this metabolic route.

Thus, we determined the content of soluble phenolics, flavonoids, and anthocyanins in leaves and stems of transgenic and wild-type plants, and the results obtained (Table 2) show minor effects in the accumulation of these metabolites. CAD-RNAi stems display a slight increase in the endogenous level of soluble phenolics, while CAD-RNAi leaves accumulate higher levels of anthocyanins. The accumulation of flavonols does not vary in transgenic plants compared to wild-type.

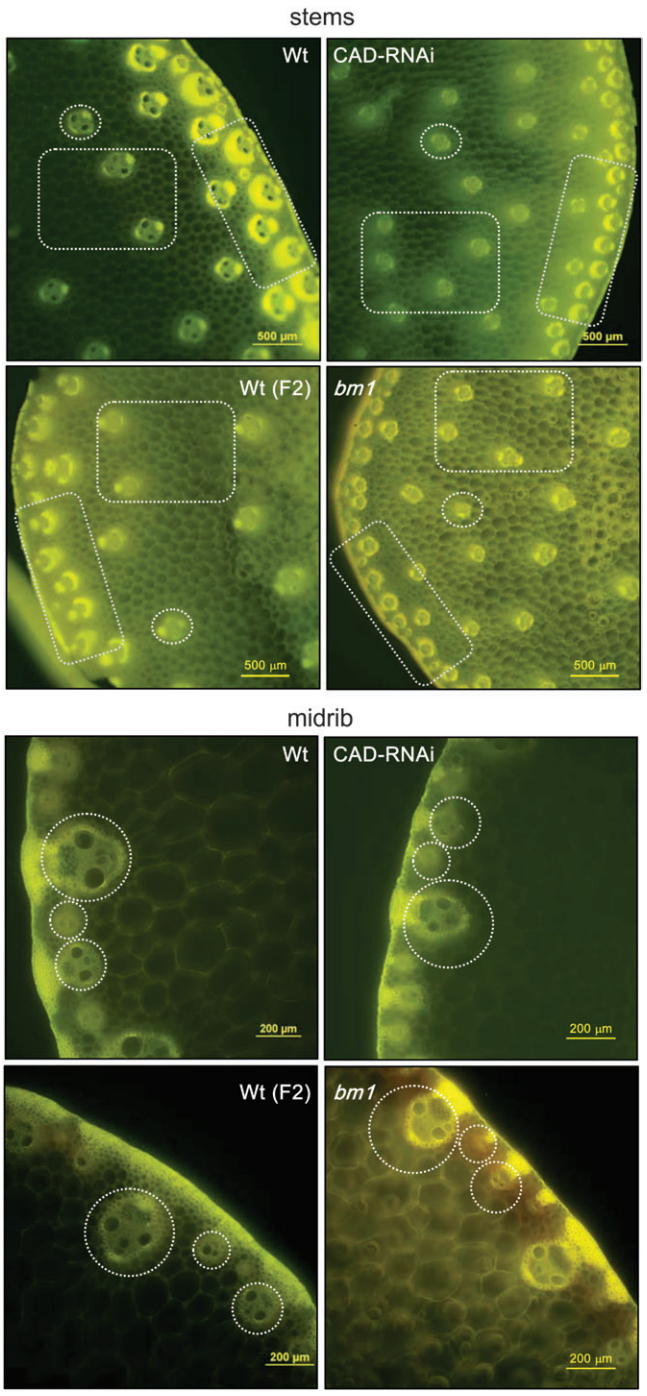


Figure 2. Lignin Auto-Fluorescence of Hand-Cross Sections of 6-Week-Old Maize Wild-Type, CAD-RNAi, and *bm1* Mutant Internodes and Midribs.
Identical size of dashed squares and dashed circles emphasized the density and size of vascular bundles, respectively.

Down-Regulation of CAD Activity Increases the Level of Free Sugars in CAD-RNAi Midribs

Lignin is a critical component of vascular plants and its alteration in CAD-RNAi plants may affect several metabolic pathways. Hence, we undertook a proteomic approach using transgenic and wild-type midribs to get a more complete overview. We identified 27 differentially expressed proteins, 11 of which were down-regulated and 16 up-regulated in CAD-RNAi plants with respect to wild-type controls. Of these 27 proteins, 19 were successfully identified (Supplemental Figure 2) and the majority of them catalyze different steps of the carbohydrate metabolism and cell detoxification, such as phosphoenolpyruvate decarboxylase, RuBisCO, fructose-bisphosphate aldolase, glyoxylase I, as well as ATP synthase, a protein participating in the oxygen evolving complex of PSII and a protein part of the cytochrome bf complex.

On the whole, these data suggest a possible perturbation of sugar metabolism in CAD-RNAi plants and, for this reason, we determined the endogenous levels of starch and free sugars in transgenic plants. The results show a 50% reduction in starch accumulation and a threefold increase in free sugars in CAD-RNAi midribs (Figure 5A and 5B). In contrast, no significant changes occur in CAD-RNAi stems.

Down-Regulation of CAD Activity Affects Cell Wall Polysaccharide Composition and Increases the *In Vitro* Degradability of CAD-RNAi Plants

To complete the study of the effect produced by the down-regulation of CAD on cell wall structure and composition, we quantified the main polysaccharidic components of cell walls in transgenic and wild-type stems and midribs. While the cell wall fraction of CAD-RNAi stems dry biomass is 26% higher than wild-type, the one corresponding to CAD-RNAi midribs is reduced by about 8% (Figure 5C).

The results obtained show a different behavior between these parts of the plant. In fact, CAD-RNAi stems accumulate more cellulose (20%), uronic acids (10%), and xylose (30%) in their cell walls, while glucose levels are reduced (60%) (Figure 6A). These cell wall polysaccharide modifications slightly increase the xylose-to-cellulose ratio and reduce the lignin-to-cell-wall polysaccharide ratio of CAD-RNAi stems compared to wild-type plants (Figure 6C). Contrarily to what is observed in stems, transgenic midribs present slightly reduced levels of the main cell wall components analyzed (Figure 6B) that lead to a slight reduction in the xylose-to-cellulose ratio (Figure 6C). As CAD-RNAi midribs also present a reduction in the total lignin content (Table 1), the final lignin-to-cell-wall polysaccharide ratio remains unchanged (Figure 6C). The decrease in both lignin and cell wall polysaccharides is in line with the 8% reduction in the cell wall fraction of the CAD-RNAi midrib dry biomass (Figure 5C).

The different effects of CAD down-regulation on the main cell wall polymers of CAD-RNAi plants prompted us to determine whether these transgenic cell walls were more susceptible to enzymatic degradation. Transgenic stems do not show significant differences in their enzymatic degradability when data are expressed per unit of cell wall. Nevertheless, CAD-RNAi stems result to be more degradable when data are expressed per unit of dry weight. In fact, transgenic stems contain a proportionally higher number of cell walls than wild-

Table 1. Lignin Content and Composition in 6-Week-Old Maize Wild-Type and CAD-RNAi Stems and Midribs.

	Stems		Midrib	
	Wt	CAD-RNAi	Wt	CAD-RNAi
% KL	16.61 ± 0.01	16.55 ± 0.12	17.04 ± 0.07	15.95 ± 0.01*
(H+G+S) μmol gKL^{-1}	675 ± 25	638 ± 20	603 ± 41	613 ± 10
S-to-G ratio	1.40 ± 0.0	1.25 ± 0.00*	0.60 ± 0.0	0.60 ± 0.0
% H	1.97 ± 0.0	2.69 ± 0.00*	4.38 ± 0.1	4.07 ± 0.00
% G	40.78 ± 0.1	43.33 ± 0.0*	59.61 ± 0.9	60.14 ± 0.2
% S	57.25 ± 0.1	53.98 ± 0.0*	36.01 ± 1.1	35.8 ± 0.2
μmol Coniferaldehyde gKL^{-1}	0.31 ± 0.04	0.35 ± 0.01	0.55 ± 0.01	0.46 ± 0.07

Lignin content is expressed as weigh percentage of Klason lignin (KL) in extract-free samples. Lignin composition is reflected by the total yield (in μmoles per gram of KL) and relative molar frequencies of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) thioacidolysis monomers. Data are mean values (and standard errors) of duplicate analyses. Asterisks refer to significant differences relative to the corresponding control.

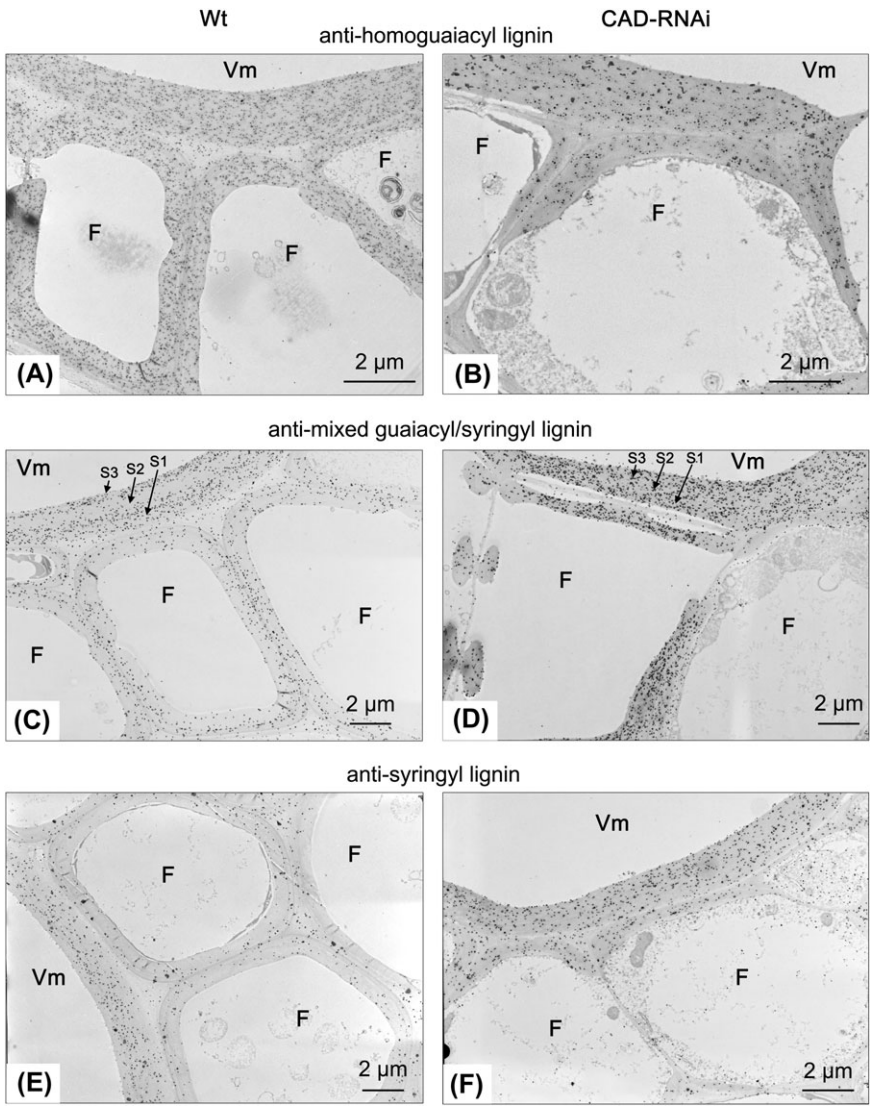


Figure 3. Ultrastructural Organization and Immunolocalization of the Homoguaiacyl (G), Guaiacyl/Syringyl (GS), and Syringyl (S) Lignin of 6-Week-Old Wild-Type (panels (**A, C, E**)) and CAD-RNAi (panels (**B, D, F**)) Metaxylematic Vessels (Vm) and Adjacent Fibers (F). S1, S2, and S3 represent the outer, middle, and innermost layers of the secondary cell wall.

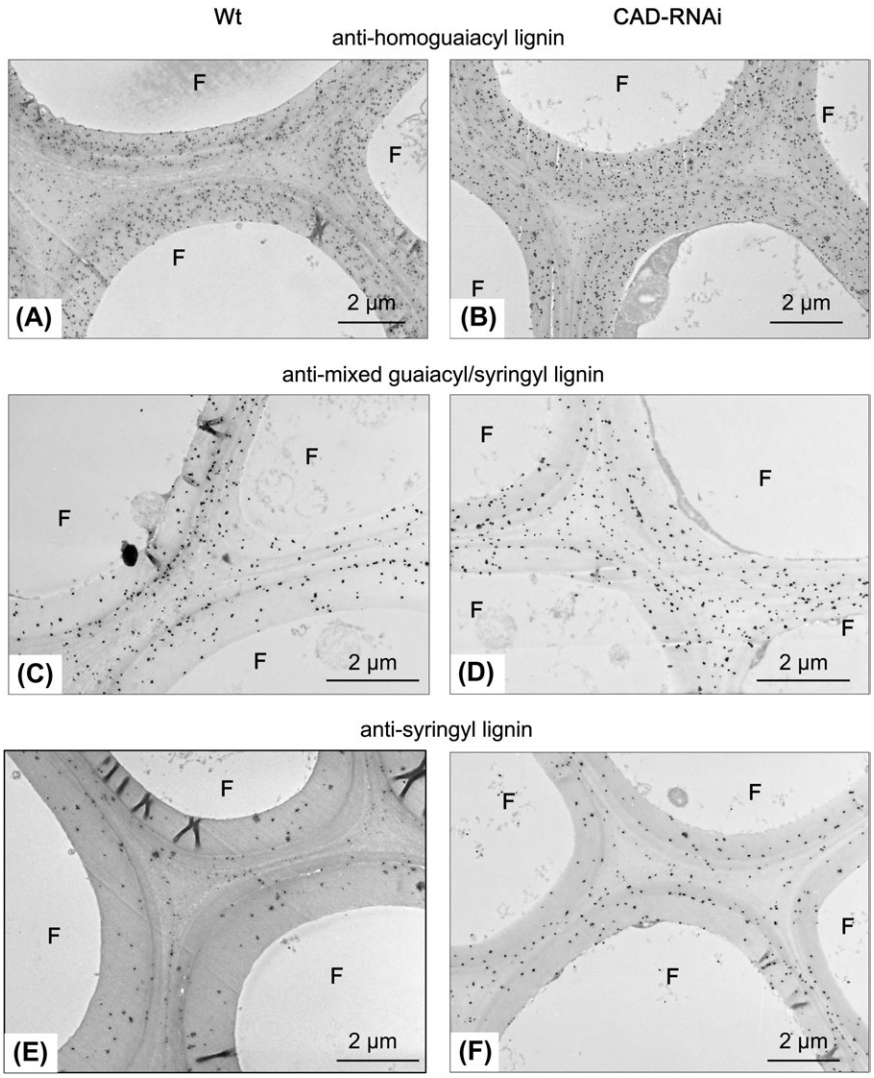


Figure 4. Ultrastructural Organization and Immunolocalization of the Homoguaiacyl (G), Guaiacyl/Syringyl (GS), and Syringyl (S) Lignin of 6-Week-Old Wild-Type (panels (**A, C, E**)) and CAD-RNAi (panels (**B, D, F**)) Stem Sclerenchymatic Fibers (F).

Table 2. Quantification of Soluble Phenolics, Flavonols, and Anthocyanins from 6-Week-Old Wild-Type and CAD-RNAi Transgenic Plants.

	Stems		Leaves	
	Wt	CAD-RNAi	Wt	CAD-RNAi
Total phenolics (mg gallic acid eq./gFW)	0.22 ± 0.01	0.31 ± 0.03	0.61 ± 0.03	0.65 ± 0.01
Flavonols (mg rutin eq./gFW)	0.23 ± 0.01	0.24 ± 0.02	1.03 ± 0.10	1.15 ± 0.10
Anthocyanins (mg cyaniding chloride eq./gFW)	4.3 ± 1.3	2.9 ± 0.3	11.6 ± 1.4	18.0 ± 3.4

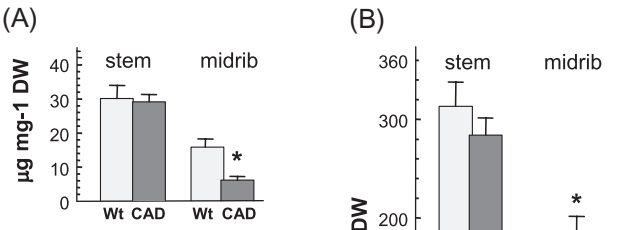
Data correspond to the mean value ± SD of three independent assays.

type plants (Figure 5C), making the amount of sugars released 25% higher compared to controls (Table 3).

Despite the lower content of cell walls per unit of dry mass, CAD-RNAi midribs show a higher degradability when referred to unit of cell wall (19%) or to unit of dry weight (16%) compared to wild-type (Table 3).

CAD-RNAi Biomass Produces More Cellulosic Bioethanol

The results obtained from the enzymatic degradability assays indicated that CAD-RNAi plants are more degradable than wild-type. This feature leads to hypothesize a higher potential of these plants as a source of cellulosic bioethanol. To test this possibility, we field-grew in Navarra (Spain) transgenic and



(C)

	mg CW / g DW	
	Stem	**Midrib**
Wild type	253± 3	563± 6
CAD-RNAi	320± 3	517± 5
	+26%	-8%

Figure 5. Quantification of free sugars, starch and cell wall yield in CAD-RNAi plants.

(A) Quantification of total starch content in 6-week-old wt and CAD-RNAi stems and midribs.

(B) Quantification of free sugar content in 6-week-old wt and CAD-RNAi stems and midribs. Asterisks indicate significant differences by Student's *t*-test ($P < 0.05$).

(C) Cell wall yield of wild-type and CAD-RNAi dry matter. CW refers to cell wall. Data are expressed as mean value of four biological repeats ± SD.

control plants. For each line, three plots were sown with 24 plants each. Transgenic plants did not show changes in growth or plant development with respect to controls, except for the slight chlorotic appearance of the leaves (Figure 7). Whole plants were harvested at the kernel milk-stage, dried at 70°C and then used for cellulosic bioethanol assays, and each experiment was repeated three times. The assays performed revealed that the production of cellulosic bioethanol from CAD-RNAi plants is 8% higher than in wild-type when expressed per dry biomass unit. As the CAD-RNAi plants' dry-biomass production is 40% higher than wild-type, the increase in bioethanol rises up to 51% when data are expressed per unit of cultured area (Figure 7).

DISCUSSION

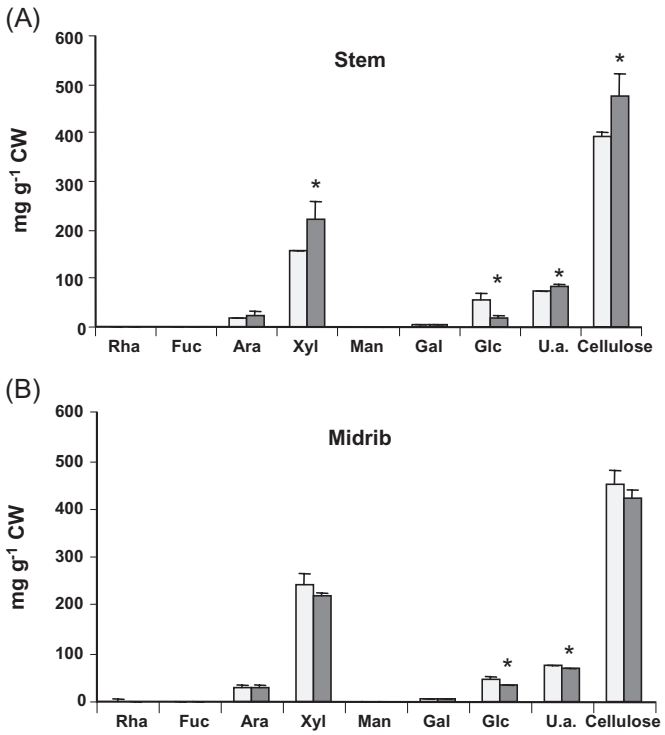
CAD catalyzes the conversion of cinnamyl aldehydes to cynnamyl alcohols, and this step is the last one before the polymerization of monolignols in the cell walls. Thus, this enzyme is considered of key importance in the synthesis of lignin and, in the last few years, many studies have been devoted to its characterization in different plant species, but little knowledge has been acquired in monocots so far. In maize, the residual CAD activity (from 14 to 60%) in the *bm1* mutant differs, depending on tissue, genotype, and developmental stage, and differently affects lignin accumulation and composition and plant growth and development (Halpin et al., 1998; Marita et al., 2003). However, an increase in cell wall degradability constitutes a common trait in all CAD-deficient plants studied so far (O'Connell et al., 2002; Marita et al., 2003; Barrière et al., 2004; Dien et al., 2009). Due to the great importance of maize as a forage crop and as a potential source of cellulosic bioethanol, in this work, we produced transgenic maize plants in which CAD activity is less severely down-regulated than the *bm1* mutant, to avoid the appearance of undesired effects on plant growth and to increase both the degradability and the potential as a source of cellulosic bioethanol.

The *CAD* gene region used to trigger the gene silencing (Zm*CAD2*, Y13733) was also identical to another *CAD* gene, annotated as 2405118.2.1 (Guillaumie et al., 2007). The highest level of the joint expression of these two genes is found in roots, followed by shoots and then leaves (Guillaumie et al., 2007). The maize CAD-RNAi lines that were obtained retained a 20% CAD activity in roots, 35% in stems, and 68% in leaves, in agreement with the gene expression pattern of these two *CAD* genes. This result suggests that the enzymatic reduction in these two CAD enzymes can not be fully compensated for by the rest of the enzymes belonging to this family, in agreement with the lignin-specific role assigned at least to one of them (Zm*CAD2*, Halpin et al., 1998) and with a functional diversification of the *CAD* gene family (Saathoff et al., 2011).

The maize CAD-RNAi plants do not show macroscopic effects when grown in a greenhouse or in the field, analogously to what has been observed with other species in which CAD activity was repressed, such as poplar (Pilate et al., 2002) or tobacco (Chabannes et al., 2001). In addition, these maize CAD-RNAi plants do not present the brown pigmentation of the midrib, typically observed in the maize brown-midrib mutants (Barrière et al., 2004). The absence of the brown-midrib phenotype has also been observed in other monocotyledonous CAD-repressed plants in which the CAD enzymatic activity is less severely reduced than that of the existing CAD mutants. This is the case for switchgrass (Saathoff et al., 2011), rice (Zhang et al., 2006), and Tall Fescue (Chen et al., 2003). These data, together with the phenotype of the maize CAD-RNAi plants obtained in this study, reinforce the idea already proposed that CAD enzymatic activity needs to drop below a minimum threshold before becoming rate-limiting (Anterola and Lewis, 2002; Saathoff et al., 2011).

The microscopic observations and the analysis of lignin content and composition show the occurrence of alterations in the distribution and structure of the vascular bundles of transgenic plants that seem to respond in an organ-dependent manner to the repression of CAD activity. Thus, CAD-RNAi shows a general reduction in the size of their vascular bundles but an increase in their number in the stems. This pattern resembles that observed in *bm1* grown in the same conditions (Figure 2).

The maize *bm1* mutant presents a reduction in the total lignin content without changes in its monomeric composition (Halpin et al., 1998). The same pattern of lignin perturbations is observed in the midrib of the maize CAD-RNAi plants but, in this case, the higher residual CAD activity of the transgenic midribs leads to a lower reduction in the total lignin content (6.4%) compared to *bm1* plants (20%).



(C)

	mg / g CW			
	Stem		Midrib	
	X/C	L/CWP	X/C	L/CWP
Wild type	0.40	0.23	0.54	0.20
CAD-RNAi	0.47	0.20	0.52	0.20

Figure 6. Quantification of cell wall polysaccharides in CAD-RNAi plants.

(A) Sugar analyses of cell walls of 6-week-old CAD-RNAi and wild-type stems.

(B) Sugar analyses of cell walls of 6-week-old CAD-RNAi and wild-type midrib.

(C) Ratios between the main cell wall components of wild-type and CAD-RNAi stems and midrib. Data are expressed as mean value of six independent assays ± SD. Asterisks represent significant differences ($p < 0.05$). White bars refer to wild-type plants and gray bars refer to CAD-RNAi plants. u.a., uronic acids; Glc, glucose; Gal, galactose; Man, mannose; Xyl, xylose; Ara, arabinose; Fuc, fucose; Rha, rhamnose; X/C, xylose/cellulose; L/CWP, lignin/cell wall polysaccharides.

The analysis of the monomeric composition of lignin in CAD-RNAi stems shows a slight reduction in the S-to-G ratio but without increasing its cinnamaldehyde content. However, in the maize *bm1* mutant, there is a strong increase in cinnamaldehydes in the final lignin content (Halpin et al., 1998; Barrière et al., 2004). This difference between our transgenic CAD-RNAi and the maize *bm1* mutant is likely related once more to the fact that CAD activity is not reduced enough in these maize plants to induce the accumulation of cinnamaldehydes and their incorporation into lignin polymer. Thus, the extent of the repression of CAD enzymatic activity seems to account for the quite variable effects on lignin content (from unchanged to reduced) and composition (from unchanged to lower S-to-G ratio) found in several grasses, as well as on the accumulation of cinnamaldehydes (Halpin et al., 1998; Chen et al., 2003; Barrière et al., 2004; Chen et al., 2004; Zhang et al., 2006; Saballos et al., 2009; Sattler et al., 2009; Fu et al., 2011; Saathoff et al., 2011).

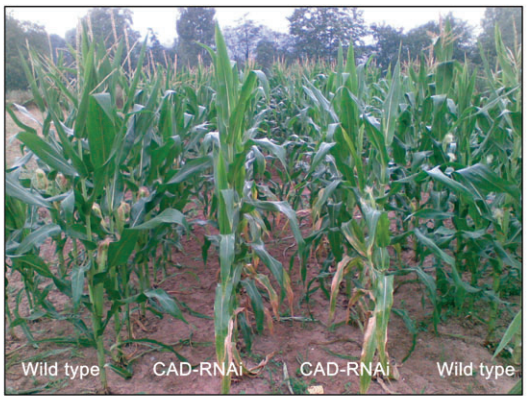
The ultrastructure analyses revealed that the down-regulation of CAD activity in maize CAD-RNAi plants mainly impacts on the morphology of the fibers adjacent to vessels, while the structure of vessels and sclerenchyma remain unaffected in CAD-RNAi stems, although an increase in condensed lignin is observed in the S2 layer of the secondary cell walls of CAD-RNAi vessels. These effects suggest that, despite the structural weakening of fibers adjacent to vessels, maize CAD-RNAi stems preserve their vascular integrity to ensure a normal plant development. This finding is in accordance with similar studies performed on various *bm* mutants in which vessels were the only lignified elements that remained unaffected by the mutation (Vermerris et al., 2010).

The down-regulation of CAD enzyme increases the levels of cellulose and xylans and the corresponding ratio hemicelluloses/cellulose (accounted as xylose/cellulose) in transgenic stems. This increase in cell wall polysaccharides, together with the unaltered lignin content, also makes the lignin-to-cell-wall polysaccharide ratio lower than that of wild-type stems. These alterations, together with the decreased S-to-G ratio of stem lignin would make stem cell walls more degradable, as previous studies showed that hemicelluloses are more susceptible to enzymatic degradation than cellulose (Riboulet et al., 2008). Nevertheless, no differences in the *in vitro* degradability are observed when comparing the cell walls of the transgenic and wild-type stems.

Table 3. Resistance of Cell Walls to Digestion with Polysaccharide Hydrolases.

	Stems		Midrib	
	mg g^{-1} CW	mg g^{-1} DW	mg g^{-1} CW	mg g^{-1} DW
Wild-type	235.8 ± 4.9	59.0 ± 1.2	326.1 ± 24.6	178.1 ± 12.7
CAD-RNAi	229.8 ± 10.6	73.5 ± 3.4	389.4 ± 25.9	206.4 ± 13.7
Degradability ratio CAD-RNAi / Wt	0.97	1.25	1.19	1.16

Cell wall (CW) degradability assay was performed using stems and midribs of 6-week-old maize wild-type and CAD-RNAi plants. Data correspond to the mean value ± SD of three independent assays.



	g / kg DW		g / m^2	
	Wild type	CAD-RNAi	Wild type	CAD-RNAi
Fermentable sugars	264.9 ± 3.0	286.0 ± 1.7	108.6 ± 1.2	164.6 ± 1.0
Not Fermentable sugars	67.4 ± 0.9	68.7 ± 0.6	27.6 ± 0.4	39.5 ± 0.4
bioethanol	158.8 ± 2.5	171.4 ± 2.9	65.1 ± 1.0	98.6 ± 1.7

Figure 7. Cellulosic bioethanol production from field-grown CAD-RNAi plants.

Macroscopic phenotype of field-grown maize wild-type and CAD-RNAi plants. Fermentable and not fermentable sugar contents and cellulosic bioethanol production from field-grown maize wild-type and CAD-RNAi dry matter. Data are referred to unit of biomass and unit of cultured field plot.

However, the higher amount of total sugars released observed when comparing transgenic and wild-type dry matters suggests that the increase in cell wall production is the determinant of the higher CAD-RNAi stem degradability, rather than any other perturbation produced in the cell walls.

An opposite situation is found in CAD-RNAi midribs in which the total lignin content is reduced by 6.4% without altering its monomeric composition. The decrease in the levels of cellulose and cell wall polysaccharides also leads to a slight decrease in the hemicellulose-to-cellulose ratio. However, in this case, the *in vitro* degradability of transgenic cell walls increases, indicating that, in this situation, the reduction in the total lignin content is the main factor affecting the availability of the cell wall polysaccharides in CAD-RNAi midribs.

The changes observed in the cell wall polysaccharide composition of CAD-RNAi midribs are accompanied by a huge increase in free sugars and a reduced level of starch compared to wild-type. The proteomic data showed that RuBisCO and FBP-Aldolase are induced in CAD-RNAi midribs, suggesting an enhancement of sugar synthesis in midribs that may be committed to supplying the observed increase in cell wall polysaccharides in transgenic CAD-RNAi stems. Alterations in carbohydrate metabolism were also observed in CAD-deficient tobacco plants (Dauwe et al., 2007). Altogether, it seems that CAD-RNAi plants counteract the alteration of lignin metabolism in a tissue-specific fashion, depending on the different residual enzymatic activity and as already suggested by previous authors (Marita et al., 2003). The physiological response relies on a functional compensation accomplished by polysaccharides and leads to a significant increase in plant dry weight of CAD-RNAi plants compared to controls.

As a result of CAD down-regulation, the extractability of plant polysaccharides increases in the whole transgenic plant without affecting plant growth performance. Thus, an 8% increase in the production of cellulosic bioethanol is obtained with respect to wild-type plants when the same unit of biomass is compared. As CAD-RNAi plants have increased levels of dry matter, the increase in bioethanol rises to 51%. Similar results have been obtained in a sorghum *bmr* mutant impaired in CAD activity (Dien et al., 2009).

In sum, we show that a relatively slight modification in plant cell wall composition can be translated into a relevant increase in the lignocellulosic biomass potential as a source of cellulosic bioethanol without compromising maize plant growth and development. These effects make CAD a good target to improve both the nutritional (forage) and energetic values of maize lignocellulosic biomass.

METHODS

Production of the Maize CAD-RNAi Plants

A genomic *CAD* fragment spanning the first exon, the first intron, and part of the second exon of the gene described as responsible for the *bm1* mutation (Zm*CAD2*, accession no. Y13733, Halpin et al., 1998) was cloned and used to generate the RNAi construct following the scheme of Supplemental Figure 3. This *CAD* gene region is also identical to the 2405118.2.1 contig previously identified as a maize *CAD* (Guillaumie et al., 2007).

The insert was then cloned downstream of the maize ubiquitin gene promoter and upstream of the nopaline synthase gene terminator of the pAHC25 vector (Christensen and Quail, 1996) for maize transformation.

Maize embryos or calli transformation and regeneration of transgenic plants were performed following the protocols of the Plant Transformation Facility, Iowa State University (www.agron.iastate.edu/ptf/#) and the scheme shown in Supplemental Figure 3. Maize transgenic and wild-type plants were grown for 6 weeks under standard greenhouse conditions (25°C day and 22°C night with 60% humidity) and a 16/8-h photoperiod. *bm1* mutant (F_2 genotype) plants were grown and treated under identical conditions.

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) and PCR assays were run to determine the presence of the full CAD-RNAi construct, using specific primers— primer forward: 5′-CAACCAGATCTCCCCCAAATC-3′ and primer reverse 5′-TGCAACGGAAGATGACTAG-3′ (to amplify a fragment comprising the ubiquitin gene promoter and the sense-oriented exon); primer forward 5′-TGCAGTGTACGCTCGCC-3′ and primer reverse 5′-CCAAATGTTTGAACGATCGG-3′ to amplify a fragment comprising the antisense exon and the nopaline synthase terminator.

PCR analyses showed that 32 out of 59 independent transformation events contained at least one full copy of the CAD-RNAi sequence integrated into the genome (data not shown) and, among them, 19 independent events were able to regenerate plants (R0 generation).

All the transformation events containing the full-length insert were then subjected to qPCR assays to quantify the number of transgene insertions in CAD-RNAi plants. Assays were performed from two aliquots of DNA preparations and from two separate extractions, totaling four biological replicates and three technical replicates for each of them. qPCR reactions were performed using the AbiPrism 7000 (Applied Biosystems) and TaqMan probes. Gene-specific primers for the CAD-RNAi construct were designed using the Primer Express software (Applied Biosystems). Primer forward: 5′-TGTTACTTCTGCAGGTCGACTCTAGA-3′ (within the maize ubiquitin intron of the pAHC25 construct), reverse: TGGCGGGCGATGGA (within the CAD exon) and the TaqMan probe: ATCCCGGGCTACACCACCTTGTGC (overlapping the ubiquitin intron and the CAD exon sequences). The maize invertase primers and TaqMan probe used for data normalization have been already described (Hernandez et al., 2004). PCR conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of a denaturation step at 95°C for 10 s and an annealing/extension step at 60°C for 30 s. These analyses allowed six independent lines to be classified as low copy lines (up to three CAD-RNAi copies) that were then backcrossed to obtain the R1 generation and the expression of *CAD* gene was analyzed in the offspring of the hemizygous CAD-RNAi lines by RT–PCR. Total RNA was extracted with Trizol Reagent according to the manufacturer's instructions (Invitrogen). RT–PCR was performed using oligo dT and a no RT negative control. CAD-specific primers were used to analyze the transcript levels (forward: 5′-CTAGCTACACCACCTTGTGCGGGC-3′; reverse: 5′-GTGGCAGATCCCGCAGTAGAGC-3′). The maize ubiquitin primers used for data normalization have already been described (Fornalé et al., 2006). Several transgenic maize CAD-RNAi lines showed a significant decrease om CAD mRNA accumulation in roots (Supplemental Figure 4). Two of these lines showing a 15–20% reduction in CAD activity in roots at the hemizygous (Supplemental Figure 1) stage were selected to produce the homozygous stage by auto-pollination (R2 generation) and were classified as one-copy lines according to the 1:3 segregation of the CAD-RNAi construct of the offspring derived from the auto-pollination.

CAD Enzyme Activity

Enzyme activity assays were performed in different plant tissues (leaf, stem, and root) of transgenic and wild-type plants according to Chabannes et al. (2001) with minor modifications. Frozen tissues were homogenized at 4°C in five volumes of extraction buffer (100 mM Tris-HCl pH 7.5, 2% (w/v) PEG 6000, 5 mM DTT, 2% (w/v) PVPP) and centrifuged three times at 10 000 *g* for 10 min at 4°C to remove cell debris. Protein content was determined by the method of Bradford (1976) and 300 μg of protein extract were used for the enzymatic assay. Triplicates of each sample were incubated at 30°C in the presence of 100 mM Tris-HCl pH 8.8, 100 μM coniferyl alcohol and 200 μM NADP. Increase in absorbance at 400 nm (indicative of the conversion of coniferyl alcohol to coniferyl aldehyde) was recorded.

Klason, Thioacidolysis, and Histology

Maize wild-type and CAD-RNAi midribs and stems were ground to a fine powder. Lignin analyses were performed on extract-free cell wall residue and lignin content was determined by the Klason procedure as already described (Dence, 1992). Lignin monomeric composition was determined by thioacidolysis followed by GC–MS of lignin-derived monomer trimethylsilyl derivatives (Lapierre et al., 1986).

Hand-cross-sections of leaf midrib and stems from wild-type, CAD-RNAi, and *bm1* plants were mounted onto microscope slides for observation under light microscopy. Lignin auto-florescence was detected using UV-excitation under DAPI-filter.

Soluble Phenolics Determination

Total phenolics were determined using a modified Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Fresh leaf tissue (100 mg) was extracted in 1 ml ethanol (80%), incubated for 2 h at 4°C in the dark, and then centrifuged to remove cell debris. Aliquots of supernatant were made up to a volume of 3 ml with distilled water. Then, 0.5 ml Folin Ciocalteau reagent (1:1 with water) and 2 ml of Na_2CO_3 (20%) were added. The solution was warmed for 15 min at 45°C, cooled to room temperature, and the absorbance was measured at 650 nm. Data were expressed as mg g^{-1} FW using gallic acid as a standard.

Total flavonols were determined according to Chang et al. (2002). Leaf tissues were extracted in 80% methanol at 4°C for 2 h. After centrifugation, aliquots of supernatant were taken to 2 ml with methanol and sequentially mixed with 0.1 ml aluminum chloride (10% water solution), 0.1 ml K-acetate 1 M and 2.8 ml distilled water. After 30-min incubation at room temperature, absorbance at 415 nm was recorded. Flavonol content was expressed as mg g^{-1} FW on the basis of a calibration curve using rutin as a standard.

Total anthocyanins were determined according to Laitinen et al. (2008). Leaf tissues (100 mg) were extracted with 1 ml of extraction solvent (methanol, water, hydrochloric acid, 7:2:1) at 4°C for 20 h and centrifuged (20 min, 10 000 rpm, 4°C). The absorbance of the supernatants was measured at 530 nm and the anthocyanin content was expressed as mg g^{-1} FW on the basis of a calibration curve using cyanidine chloride as a standard.

Determination of Starch and Free Sugars

Intraprotoplasmic soluble material was extracted from powder-dried material by treatment with 70% ethanol (10 ml g^{-1} DW) at room temperature for 24 h (twice). Suspension was clarified by centrifugation and the pellet (alcohol-insoluble residue)

washed with 70% ethanol (1 ml g^{-1} DW; twice). Supernatants and washings were mixed, concentrated, and partitioned against ethyl acetate (three times). Intraprotoplasmic soluble sugars were assayed in the aqueous phase by the phenol sulfuric acid method (Dubois et al., 1956) and expressed as the glucose equivalent. Starch content was estimated by quantification of glucose released after the α-amilase digestion of the alcohol-insoluble residue. This was washed twice with cold 10 mM K-phosphate buffer, pH 7.0, and treated with α-amylase (EC 3.2.1.1) type VI from hog pancreas (10 ml g^{-1} DW; 2.5 U ml^{-1} in 10 mM K-phosphate buffer, pH 7.0) (Sigma) for 24 h at 37°C. Suspension was acidified, clarified by centrifugation, and glucose released was assayed from the supernatant by the antrone assay (Dische, 1962).

Cell Wall Analysis

Dried plant material (1 g) was added to Poly-Prep tubes and extracted with 10 volumes of 70% EtOH for 5 d at room temperature with wheel-shaking, then washed six times with 70% EtOH, six times with acetone, and air-dried to obtain the alcohol-insoluble residue (AIR). AIR was then de-starched, treated with acidified phenol, and washed with organic solvents to obtain the cell wall residue as previously described (Encina et al., 2002; Mélida et al., 2010).

Neutral sugar analysis was performed according to Albersheim et al. (1967). Dried cell walls were hydrolyzed with 2 M TFA (trifluoroacetic acid) for 1 h at 121°C and the resulting sugars were derivatized to alditol acetates and analyzed by gas chromatography (GC) on a Supelco SP-2330 column. Uronic acid contents were determined by the m-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973), with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff, 1969) with the hydrolytic conditions described by Saeman et al. (1963) and quantification of the glucose released by the anthrone method (Dische, 1962) with glucose as a standard.

For the cell wall degradability assays, cell walls were hydrolyzed (20 mg/1.5 ml) in a mixture of Cellulase R10 (1%), Macerozyme R-10 (0.5%), and purified Driselase (0.1%) dissolved in sodium acetate 20 mM (pH 4.8). Aliquots were taken at 6, 48, and 72 h, clarified by centrifugation, and assayed for total sugars (Dubois et al., 1956).

Electron Microscopy and Immunolocalization Assays

Hand-cut transverse sections of the basal region of stems of 5-week-old plants were processed for TEM as previously described (Day et al., 2005). Wall polysaccharides of ultra-thin transverse sections (50 nm) were PATAg (Periodic acid-thiocarbohydrazide-silver proteinate) stained according to Ruel et al. (1977). Briefly, the periodate oxidation was with a 5% solution of periodic acid in water for 90 min and soaked in thiocarbohydrazide for 48 h before silver proteinate (30 min). Three polyclonal antibodies directed against specific lignin structures were used: S/C_{Zt} directed against non-condensed mixed guaiacyl–syringyl lignin structures; G_{ZI} directed against condensed lignin homo-guaiacyl structures were prepared and characterized as described by Joseleau and Ruel (1997); and S_{Zt} directed against non-condensed homo-syringyl lignin structures (S) (Joseleau et al., 2004). The samples were immunolabeled on ultrathin transverse sections (50 nm) floating downwards on plastic rings as previously described (Joseleau and Ruel, 1997). Briefly, the sections were incubated on 50-μl droplets of the diluted antisera (1:50 to 1:120). The secondary marker was Protein A-gold (pA G5, BioCell). The gold particles were further enhanced using a silver-enhancement kit (SPI-Mark™). Finally, the thin sections were transferred to carbon-coated copper grids, post-stained with 2.5% aqueous uranyl acetate, and examined with a Philips CM 200 Cryo-TEM at an accelerating voltage of 80 kV.

All comparative immunolabeling experiments were carried out in parallel in order to keep the same experimental conditions. Pre-immune serum for each antibody was assayed as for the immuno-gold labeling. Observations were performed at 80 kV with a Philips CM 200 Cryo-electron microscope.

2-D Electrophoresis

Tissues from wild-type and CAD-RNAi midribs at the same growth stage were collected, frozen in liquid nitrogen, and stored at –80°C until analyzed. Protein extraction was performed as previously described (Irar et al., 2006).

700 μg of total proteins were loaded onto non-linear pH 4–7, 18-cm immobilized pH gradient (IPG) strips (Immobiline DryStrips, Amersham Biosciences) for the first dimension. Bi-dimensional electrophoresis was performed according to Campo et al. (2004) and gels were stained with CBB G-250 (Bio-Rad). The experiment was repeated with two biological replicates and three experimental replicates for biological sample. To evaluate protein expression differences among gels, relative spot volume (% vol.) was used as a normalized value, representing the ratio of a given spot volume to the sum of all spot volumes detected in the gel. Those spots showing a quantitative variation $\geqslant$ Ratio 1 were selected as differentially expressed. Statistically significant protein abundance variation was validated by Student's *t*-test ($p < 0.05$). The selected differential spots were excized from the CBB G-250-stained gels and identified either by PMF using MALDI–TOF MS or by peptide sequencing at the Proteomics Platform (Barcelona Science Park, Barcelona, Spain). The MALDI–TOF MS analysis was performed according to Oliveira et al. (2007).

Cellulosic Bioethanol Assays

Transgenic and wild-type maize were grown at the Iden Biotechnology facilities (Navarra, Spain). For each line, three plots of 24 plants each were harvested at the kernel milk-stage. The whole aerial part was dried at 70°C and the dry weight was recorded. For each plot, three aliquots of 50 g (DW) of ground tissues were subjected to alkaline hydrolysis in the presence of 100 mg of NaOH in 250 ml distilled water and autoclaved for 3 h at 130°C. After adjusting the pH to 5, samples were subjected to enzymatic digestion at 55°C during 24 h using

a commercial kit (Novozymes). Samples were finally centrifuged for 20 min at 4000 rpm and the supernatant was concentrated to a final volume of 300 ml.

The supernatant was supplemented with the reagents required for yeast (*Saccharomyces cerevisiae*) growth (ammonium sulfate, K-phosphate, ammonium phosphate, magnesium sulfate, yeast extract, malt extract) and the pH was adjusted to 5. Finally, 620 mg of yeast were added and the samples were incubated for 48 h at 32°C with 150 rpm shaking and the obtained ethanol was quantified using an alcoholmeter.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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REFERENCES

Albersheim, P., Nevins, D.S., and English, P.D. (1967). A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr. Res. **5,** 340–345.

Anterola, A.M., and Lewis, N.G. (2002). Trends in lignin modifications: a comprehensive analysis of the effects of genetics manipulations/mutations on lignification and vascular integrity. Phytochemistry. **61,** 221–294.

Barrière, Y., Ralph, J., Méchin, V., Guillaumie, S., Grabber, J.H., Argillier, O., Chabbert, B., and Lapierre, C. (2004). Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. C. R. Biol. **327,** 847–860.

Baucher, M., Bernard-Vailhe, M.A., Chabbert, B., Besle, J.M., Opsomer, C., Van Montagu, M., and Botterman, J. (1999). Down-regulation of Cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. Plant Mol. Biol. **39,** 437–447.

Baucher, M., et al. (1996). Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiol. **112,** 1479–1490.

Bernard Vailhe, M.A., Besle, J.M., Maillot, M.P., Cornu, A., Halpin, C., and Knight, M. (1998). Effect of down-regulation of Cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. J. Sci. Food Agric. **76,** 505–514.

Berthet, S., et al. (2011). Disruption of LACCASE4 and 17 results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. Plant Cell. **23,** 1124–1137.

Blumenkrantz, N., and Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. Anal. Biochem. **5,** 484–489.

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. Annu. Rev. Plant Biol. **54,** 519–546.

Boudet, A.M., Kajita, S., Grima-Pettenati, J., and Goffner, D. (2003). Lignin and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci. **8,** 576–581.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Campo, S., Carrascal, M., Coca, M., Abián, J., and San Segundo, B. (2004). The defense response of germinating maize embryos against fungal infection: a proteomics approach. Proteomics. **4,** 383–396.

Caparrós-Ruiz, D., Fornalé, S., Civardi, L., Puigdomenech, P., and Rigau, J. (2006). Isolation and characterisation of a family of laccases in maize. Plant Sci. **171,** 217–225.

Chabannes, M., Barakate, A., Lapierre, C., Marita, J.M., Ralph, J., Pean, M., Danoun, S., Halpin, C., Grima-Pettenati, J., and Boudet, A.M. (2001). Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant J. **28,** 257–70.

Chang, C.C., Yang, M.H., Wen, H.M., and Chern, J.C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. **10,** 178–182.

Chen, L., Auh, C., Dowling, P., Bell, J., and Wang, Z.Y. (2004). Improving forage quality of tall fescue (*Festuca arundinacea*) by genetic manipulation of lignin biosynthesis. In Molecular Breeding of Forage and Turf, Hopkins, A., Wang, Z.Y., Mian, R., Sledge, M., Barker, R.E., eds, (Dordrecht: Kluwer Academic Publishers), pp. 181–188.

Chen, L., Auh, C., Dowling, P., Bell, J., Chen, F., and Hopkins, A. (2003). Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down regulation of cinnamyl alcohol dehydrogenase. Plant Biotechnol. J. **1,** 437–449.

Christensen, A.H., and Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. **5,** 213–218.

Dauwe, R., et al. (2007). Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell-wall metabolism, primary metabolism, stress metabolism and photorespiration. Plant J. **52,** 263–285.

Davin, L.B., Jourdes, M., Patten, A.M., Kim, K.W., Vassão, D.G., and Lewis, N.G. (2008). Dissection of lignin macromolecular configuration and assembly: comparison to related biochemical processes in allyl/propenyl phenol and lignan biosynthesis. Nat. Prod. Rep. **25,** 1015–1090.

Day, A., Ruel, K., Neutelings, G., Crônier, D., David, H., Hawkins, S., and Chabbert, B. (2005). Lignification in the flax stem: evidence for an unusual lignin in bast fibers. Planta. **222,** 234–245.

de Obeso, M., Caparrós-Ruiz, D., Vignols, F., Puigdomènech, P., and Rigau, J. (2003). Characterisation of maize peroxidases having differential patterns of mRNA accumulation in relation to lignifying tissues. Gene. **309,** 23–33.

Dence, C. (1992). Lignin determination. In Methods in Lignin Biochemistry, Dence, C., Lin, C., eds, (Berlin: Springer Verlag), pp. 33–61.

Dien, B., Sarath, G., Pedersen, J., Sattler, S., Chen, H., Funnell-Harris, D., Nichols, N., and Cotta, M. (2009). Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolour* L. Moench) lines with reduced lignin contents. Bioenerg. Res. **2,** 153–164.

Dische, Z. (1962). Color reactions of carbohydrates. In Methods in Carbohydrate Chemistry, V0l. 1, Whistler R.L., Wolfrom, M.L., eds, (New York: Academic Press), pp. 475–514.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, D.A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. **28,** 350–356.

Encina, A., Sevillano, J.M., Acebes, J.L., and Alvarez, J. (2002). Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plant. **114,** 182–191.

Eudes, A., Pollet, B., Sibout, R., Do, C.T., Séguin, A., Lapierre, C., and Jouanin, L. (2006). Evidence for a role of AtCAD 1 in lignification of elongating stems of *Arabidopsis thaliana*. Planta. **225,** 23–39.

Fornalé, S., Sonbol, F.M., Maes, T., Capellades, M., Puigdomènech, P., Rigau, J., and Caparrós-Ruiz, D. (2006). Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid O-methyltransferase genes by two new maize R2R3-MYB transcription factors. Plant Mol. Biol. **62,** 809–823.

Fu, C., Xiao, X., Xi, Y., Ge, Y., Chen, F., Bouton, J., Dixon, R.A., and Wang, Z.Y. (2011). Down regulation of Cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. Bioenerg. Res. doi: 10.1007/s12155–010–9109-z.

Godshall, M.A. (2005). Enhancing the agro-industrial value of the cellulosic residues of sugarcane. Int. Sugar. J. **107,** 53–60.

Gray, K.A., Zhao, L., and Emptage, M. (2006). Bioethanol. Curr. Opin. Chem. Biol. **10,** 141–146.

Guillaumie, S., San-Clemente, H., Deswarte, C., Martinez, Y., Lapierre, C., Murigneux, A., Barrière, Y., Pichon, M., and Goffner, D. (2007). MAIZEWALL: database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol. **143,** 339–363.

Halpin, C., Holt, K., Chojecki, J., Oliver, D., Chabbert, B., Monties, B., Edwards, K., Barakate, A., and Foxon, G.A. (1998). Brown-midrib maize (bm1): a mutation affecting the Cinnamyl alcohol dehydrogenase gene. Plant J. **14,** 545–553.

Hernandez, M., Esteve, T., Prat, S., and Pla, M. (2004). Development of real-time PCR systems based on SYBR®Green I, Amplifluor™ and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. J. Cereal Sci. **39,** 99–107.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., and Foust, T.D. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. **315,** 804–807.

Irar, S., Oliveira, E., Pagès, M., and Goday, A. (2006). Towards the identification of late-embryogenic-abundant phosphoproteome in *Arabidopsis* by 2-DE and MS. Proteomics. **6,** S175–S185.

Jackson, L., Shadle, G., Zhou, R., Nakashima, J., Chen, F., and Dixon, R. (2008). Improving saccharification efficiency of alfalfa stems through modification of the terminal stages of monolignol biosynthesis. Bioenerg. Res. **1,** 180–192.

Joseleau, J.-P., and Ruel, K. (1997). Study of lignification by non invasive techniques in growing maize internodes: an investigation by Fourier transform infrared, cross-polarisation-magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy. Plant Physiol. **114,** 1123–1133.

Joseleau, J.-P., Faix, O., Kuroda, K.-I., and Ruel, K. (2004). A polyclonal antibody directed against syringylpropane epitopes of native lignins. C. R. Biologies. **327,** 809–815.

Kim, S.J., Kim, M.R., Bedgar, D.L., Moinuddin, S.G.A., Cardenas, C.L., Davin, L.B., Kang, C.L., and Lewis, N.G. (2004). Functional reclassification of the putative Cinnamyl alcohol dehydrogenase multigene family in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **101,** 1455–1460.

Laitinen, R.A.E., Ainasoja, M., Broholm, S.K., Teeri, T.H., and Elomaa, P. (2008). Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. J. Exp. Bot. **59,** 3691–3703.

Lapierre, C., et al. (1999). Structural alterations of lignin in transgenic poplar with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant Physiol. **119,** 153–163.

Lapierre, C., Pilate, G., Pollet, B., Mila, I., Leplé, J.C., Jouanin, L., Kim, H., and Ralph, J. (2004). Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. Phytochemistry. **65,** 313–321.

Lapierre, C., Rolando, C., and Monties, B. (1986). Thioacidolysis of poplar lignins: identification of monomeric syringyl products and characterization of guaiacyl syringyl fractions. Holzforschung. **40,** 113–118.

Mansell, R.L., Gross, C., Stockigt, J., Franke, H., and Zenk, M.H. (1974). Purification and properties of cinnamyl alcohol dehydrogenase from higher plants involved in lignin biosynthesis. Phytochemistry. **13,** 2427–2437.

Marita, J.M., Vermerris, W., Ralph, J., and Hatfield, R.D. (2003). Variations in the cell wall composition of maize midrib mutants. J. Agric. Food Chem. **51,** 1313–1321.

Mélida, H., Encina, A., Álvarez, J., Acebes, J.L., and Caparrós-Ruiz, D. (2010). Unraveling the biochemical and molecular

networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil. Mol. Plant. **3,** 842–853.

Mellerowicz, E.J., Baucher, M., Sundberg, B., and Boerjan, W. (2001). Unravelling cell wall formation in the woody dicot stem. Plant Mol. Biol. **47,** 239–274.

O'Connell, A., Holt, K., Piquemal, J., Grima-Pettenati, J., Boudet, A., Pollet, B., Lapierre, C., Petit-Conil, M., Schuch, W., and Halpin, C. (2002). Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. Transgenic Res. **11,** 495–503.

Oliveira, E., Amara, I., Bellido, D., Odena, M.A., Dominguez, E., Pagès, M., and Goday, A. (2007). LC-MSMS identification of *Arabidopsis thaliana* heat-stable seed proteins: enriching for LEA-type proteins by acid treatment. J. Mass. Spectrom. **42,** 1485–1495.

Pilate, G., et al. (2002). Field and pulping performances of transgenic trees with altered lignification. Nat. Biotechnol. **20,** 607–612.

Piquemal, J., et al. (2002). Down-regulation of caffeic acid o-methyltransferase in maize revisited using a transgenic approach. Plant Physiol. **130,** 1675–1685.

Pomar, F., Caballero, N., Pedreño, M., and Ros Barceló, A. (2002). H(2)O(2) generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. FEBS Lett. **529,** 198–202.

Raes, J., Rohde, A., Christensen, J.H., van der Peer, Y., and Boerjan, W. (2003). Genome-wide characterization of the lignification toolbox in *Arabidopsis.* Plant Physiol. **133,** 1051–1071.

Ralph, J., et al. (2001). Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. Phytochemistry. **57,** 993–1003.

Ranocha, P., Chabannes, M., Chamayou, S., Danoun, S., Jauneau, A., Boudet, A.M., and Goffner, D. (2002). Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. **129,** 145–155.

Riboulet, C., Lefevre, B., Denoue, D., and Barriere, Y. (2008). Genetic variation in maize cell wall for lignin content, lignin structure, p-hydroxycinnamic acid content, and digestibility in set of 19 lines at silage harvest maturity. Maydica. **53,** 11–19.

Ruel, K., Comtat, J., and Barnoud, F. (1977). Localisation histologique et ultrastructurale des xylanes dans les parois primaires des tissus d'Arundo donax. C. R. Acad. Sci. **284,** 1421–1424.

Saathoff, A.J., Sarath, G., Chow, E.K., Dien, B.S., and Tobias, C.M. (2011). Downregulation of Cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulose treatment. PLoS One 6. doi: 10.1371/journal.pone.0016416.

Saballos, A., Ejeta, G., Sanchez, E., Kang, C., and Vermerris, W. (2009). A genomewide analysis of the cunnamyl alcohol dehydrogenase family in sorghum (*Sorghum bicolor* (L.) Moench) identifies *SbCAD2* as the *brown midrib6* gene. Genetics. **181,** 783–795.

Saeman, J.F., Moore, W.E., and Millett, M.A. (1963). In Methods in Carbohydrate Chemistry, Vol. 3, Whistler, R.L., ed. (New York: Academic Press), pp. 54–69.

Sattler, S.E., Saathoff, A.J., Haas, E.J., Palmer, N.A., Funnel-Harris, D.L., and Sarath, G. (2009). A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib 6 phenotype. Plant Physiol. **150,** 584–595.

Sibout, R., Eudes, A., Mouille, G., Pollet, B., Lapierre, C., Jouanin, L., and Séguin, A. (2005). *CINNAMYL ALCOHOL DEHYDROGENASE-C* and *-D* are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis*. Plant Cell. **17,** 2059–2076.

Singleton, V.L., and Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. **16,** 144–158.

Thévenin, J., Pollet, B., Letarnec, B., Saulnier, L., Gissot, L., Maia-Grondard, A., Lapierre, C., and Jouanin, J. (2011). The simultaneous repression of CCR and CAD, two enzymes of the lignin biosynthetic pathway: results in sterility and dwarfism in *Arabidopsis thaliana*. Mol. Plant. **4,** 70–82.

Torney, F., Moeller, L., Scarpa, A., and Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from maize. Curr. Opin. Biotechnol. **18,** 193–199.

Updegraff, D.M. (1969). Semi-micro determination of cellulose in biological materials. Anal. Biochem. **32,** 420–424.

Valério, L., Carter, D., Rodrigues, J.C., Tournier, V., Gominho, J., Marque, C., Boudet, A.M., Maunders, M., Pereira, H., and Teulières, C. (2003). Down-regulation of cinnamyl alcohol dehydrogenase, a lignification enzyme, in *Eucalyptus camaldulensis*. Mol. Breed. **12,** 157–167.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., and Boerjan, W. (2010). Lignin biosynthesis and structure. Plant Physiol. **153,** 895–905.

Vermerris, W., Sherman, D.M., and McIntryre, L.M. (2010). Phenotypic plasticity in cell walls of maize *brown midrib* mutants is limited by lignin composition. J. Exp. Bot. **61,** 2479–2490.

Vignols, F., Rigau, J., Torres, M.A., Capellades, M., and Puigdomenech, P. (1995). The *brown midrib3 (bm3)* mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. Plant Cell. **7,** 407–416.

Vogt, T. (2010). Phenylpropanoid biosynthesis. Mol. Plant. **3,** 2–20.

Whetten, R.W., MacKay, J.J., and Sederoff, R.R. (1998). Recent advances in understanding lignin biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49,** 585–609.

Wrobel-Kwiatkowska, M., Starzycki, M., Zebrowski, J., Oszmianski, J., and Szopa, J. (2007). Lignin deficiency in transgenic flax resulted in plants with improved mechanical properties. J. Biotechnol. **128,** 919–934.

Yahiaoui, N., Marque, C., Myton, K.E., Negrel, J., and Boudet, A.M. (1998). Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. Planta. **204,** 8–15.

Zhang, K., Qian, Q., Huang, Z., Wang, Y., Li, M., Hong, L., Zeng, D., Gu, M., Chu, C., and Cheng, Z. (2006). Gold Hull and Internode2 encodes a primarily multifunctional Cinnamyl alcohol dehydrogenase in rice. Plant Physiol. **140,** 972–983.