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Research Article

Downregulation of caffeoyl-CoA O-methyltransferase (*CCoAOMT*) by RNA interference leads to reduced lignin production in maize straw

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Abstract

Lignin is a major cell wall component of vascular plants that provides mechanical strength and hydrophobicity to vascular vessels. However, the presence of lignin limits the effective use of crop straw in many agroindustrial processes. Here, we generated transgenic maize plants in which the expression of a lignin biosynthetic gene encoding CCoAOMT, a key enzyme involved in the lignin biosynthesis pathway was downregulated by RNA interference (RNAi). RNAi of *CCoAOMT* led to significantly downregulated expression of this gene in transgenic maize compared with WT plants. These transgenic plants exhibited a 22.4% decrease in Klason lignin content and a 23.3% increase in cellulose content compared with WT plants, which may reflect compensatory regulation of lignin and cellulose deposition. We also measured the lignin monomer composition of the RNAi plants by GC-MS and determined that transgenic plants had a 57.08% higher S/G ratio than WT plants. In addition, histological staining of lignin with Wiesner reagent produced slightly more coloration in the xylem and sclerenchyma than WT plants. These results provide a foundation for breeding maize with low-lignin content and reveal novel insights about lignin regulation via genetic manipulation of *CCoAOMT* expression.

Keywords: CCoAOMT, lignin biosynthesis, lignin and cellulose content, Maize.

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Introduction

Lignin is a major component of plant cell walls and accounts for approximately 30% of the organic carbon in the biosphere (Boerjan *et al.*, 2003). However, lignin in the plant cell walls hinders many agroindustrial processes, such as paper manufacturing and cellulosic biofuel production. Thus, many studies have focused on the repression or alteration of lignin biosynthesis to permit more efficient utilization of plant cell walls (Hu *et al.*, 1999; Blee *et al.*, 2001). Lignins from angiosperms are mainly polymerized from three cinnamyl alcohols (also called monolignols), including *p*-coumaryl, coniferyl and sinapyl alcohols, which form hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin, respectively. Accordingly, lignin biosynthesis in plants comprises two major steps, including monolignol biosynthesis and the subsequent crosslinking of lignin monomers to form different polymers (Boudet *et al.*, 2003). Considerable effort has been directed towards understanding the mechanisms of monolignol biosynthesis.

The biochemical pathways of monolignol biosynthesis are highly conserved throughout vascular plants, and enzymes involved in these biosynthetic pathways have been isolated and characterized. Recent studies have shown that at least 10 enzymes are required for monolignol biosynthesis, including phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), p-coumarate 3-hydroxylase (C3H), p-hydroxycinnamoyl-CoA (HCT), caffeoyl-CoA O-methyltransferase (CCoAOMT), hydroxycinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), ferulate 5-hydroxylase (F5H) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) (Boudet *et al.*, 2003). Lignin biosynthesis begins with the amino acid phenylalanine. PAL is one of the most intensively studied enzymes in plant secondary metabolism due to the key role that this enzyme plays in catalyzing the deamination reaction to produce cinnamic acid, which is then converted into *p*-coumaric acid by C4H. The downregulation of *PAL* and *C4H* gene expression in transgenic tobacco lead to a significant reduction in lignin content, which is consistent with the crucial roles of PAL and C4H in phenylpropanoid biosynthesis (Bate *et al.*, 1994; Elkind *et al.*, 1990; Sewalt, *et al.*, 1997). In addition, studies have also shown that PAL is mainly responsible for the biosynthesis of G-lignin, while the downregulation of *PAL* expression in plants mainly leads to a reduction in the G units of lignin, whereas the downregulation *C4H* expression mainly leads to a reduction in S units of lignin. In addition, many studies have shown that lignin content can by altered by modifying the

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expression of other key enzymes in the lignin biosynthesis pathway. For example, increasing evidence suggested that *CCoAOMT* is involved in a parallel pathway for lignin monomer formation (Ye *et al.*, 1994; Zhong *et al.*, 1998). The downregulation of *CCoAOMT* results in a reduction in lignin production, along with an increase in the S/G ratio due to a reduction in G units (Ye *et al.*, 1994; Guo *et al.*, 2001; Pincon *et al.*, 2001; Boerjan *et al.*, 2003). Studies from transgenic plants have shown that the downregulation of *COMT* expression leads the reduced production of S units, which suggests that COMT is mainly responsible for the biosynthesis of S monomers (Tsai *et al.*, 1998; Lapierre *et al.*, 1999; Doorsselaere *et al.*, 2003).

Maize (*Zea mays L.*) straw is one of the most important leading forage crops. However, maize straw contains considerable amounts of lignin, which seriously affects the digestion and nutrient absorption of maize straw by livestock. Moreover, high lignin content in plant cell walls has a negative impact on forage quality (Marita *et al.*, 2003). As mentioned above, many studies have examined the effect of enzymes at key positions in the monolignol biosynthesis pathway such as CCoAOMT and COMT. In this study, a 229 bp fragment corresponding to the fifth exon of maize *CCoAOMT* was generated by PCR amplification to construct RNA interference (RNAi) expression vector, which was transferred into maize by *Agrobacterium*-mediated transformation. The results showed that the repressed expression of *CCoAOMT* in maize largely reduces the lignin content of maize straw and significantly increases the S/G ratio and cellulose content. The results of this study reveal new details about the effects of the downregulation of *CCoAOMT* on lignin regulation and provide a basis for further studies aimed at breeding plants with low lignin content.

Materials and Methods

Constructs and maize transformation

The maize inbred line B73 was cultivated in a greenhouse at 28 °C with a 14:10 h light/dark photoperiod. Genomic DNA was extracted from the leaves of B73 using the hexadecyl trimethylammonium bromide (CTAB) method. This DNA was used in the amplification of the 229 bp fragment corresponding to the fifth exon of maize *CCoAOMT* (EU952463). To construct the RNAi expression vector, gene-specific primers 5'-TAAGGATCCAGAAGAACCACGGGTCGTTCGAC-3' (forward, *Bam*HI site underlined) and 5'-TAAGTCGACTCGTCGGCGGCGAGCGCCTTG-3' (reverse, *Sal*I site underlined) were used to amplify the 229 bp forward fragment. The products were cloned into the pMD18-T simple vector (TaKaRa, Japan), sequenced and then subcloned into the pUCCRNAi vector. The same fragment was inserted in the antisense orientation of the pUCCRNAi+1F vector digested with *Bgl*II and *Xho*I (compatible restriction endonucleases of *Bam*HI and *Sal*I) to construct the pUCCRNAi+2F vector. Subsequently, the 2F fragment (sense orientation fragment, antisense orientation fragment and an intron) was isolated from pUCCRNAi+2F vector and inserted into pCAMBIA1301 (*Bar*) vector under the control of a cauliflower mosaic virus (CaMV) 35S promoter. The construct was introduced into *Agrobacterium tumefaciens* strain Agl0 by the freeze-thaw method and then transformed into maize inbred line zheng58 by a previously described method (Li, *et al.*, 2008) with some modifications.

Polymerase chain reaction (PCR) amplification and Southern blotting

Phosphinothricin-resistant plants were confirmed by amplifying the 35S promoter from DNA isolated from these plants using the following primers: 5'-CCACAGATGGTTAGAGAGG-3' (forward) and 5'-GTCTTGCGAAGGATAGTGG-3' (reverse). For Southern blotting analysis, the DNA of PCR-positive transgenic plants was digested overnight with *Eco*RI, separated by electrophoresis on a 0.8% agarose gel and transferred onto a Hybond-N^+ nylon membrane (Amersham Pharmacia, UK). Southern blotting was performed with a digoxigenin (DIG)-labeled 35S promoter probe according to the manufacturer's instructions (Roche, Germany).

Expression of *CCoAOMT* in transgenic plants

Total RNA from untreated maize leaves was extracted with Trizol reagent (Invitrogen, USA) and treated with DNase I (Invitrogen, USA) for 20 min to remove possible contaminating genomic DNA. The first-strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA). Semi-quantitative RT-PCR (semiRT-PCR) was performed to detect the expression levels of maize *CCoAOMT* in different transgenic lines using the following thermal profile: 94 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The maize *Actin1* gene was amplified as an internal control using primers 5'-GTTGGGCGTCCTCGTCA-3' (forward) and 5'-TGGGTCATCTTCTCCCTGTT-3' (reverse).

Determination of Klason lignin, cellulose and hemicelluloses contents in maize straw

The lignin content was analyzed as acid-insoluble Klason lignin according to the Chinese National Standards GB/T 747-2003. Cellulose content was determined by the nitric acid-ethanol method (Shi and He, 2003). Hemicelluloses content was detected as described previously (Xiao *et al.*, 2001).

Determination of Klason lignin monomer composition

Stems of transgenic and WT plants were dried and treated for thioacidolysis followed by gas chromatography

mass spectrometry (GC-MS), to measure the monolignol composition. GC-MS analysis was performed on a Thermo Scientific iCAP 6300 device as described previously, with some modification (González-Vila *et al.*, 1999; Mullen and Boateng, 2010).

Histological staining of lignin

Lignin histological staining was performed using Wiesner reagent (Pomar *et al.*, 2004). Stems of transgenic and WT plants were fixed in FAA solution, dehydrated through an alcohol series, embedded in paraffin and cut with a cryostat CM1900 microtome (Leica, Germany) into 40-µm-thick sections. Cross-sections of maize stems were incubated in a 2.5% (w/v) solution of phloroglucinol for 5 min, treated with 20% HCl for 5 min and observed under an Olympus FV1000 confocal microscope (Olympus, Japan).

Results

Expression level of maize *CCoAOMT* gene *(ZmCoA)* in RNAi transgenic plants

A 229 bp fragment was generated from the genomic DNA of maize inbred line B73 by PCR-amplification. Sequence alignment indicated that the 229 bp fragment shared 100% identity with the fifth exon of the *CCoAOMT* coding sequence (GRMZM2G127948_T01) from 485-713 bp (Supplementary material Figure S1A). This fragment was used to construct the RNA interference fragment (Figure S1B). Through *Agrobacterium tumefaciens* mediated transformation, a total of six PCR-positive plants were obtained (Figure S2A), and two single-copy transgenic plants, designated L1 and L2 (Figure S2B), were chosen for further experiments by Southern blotting. Both transgenic plants exhibited similar phenotypes with a characteristic of slightly delayed growth compared with WT plants (Figure 1).

To detect the expression level of *ZmCoA* in transgenic maize plants, total RNAs were extracted from L1 and L2 transgenic lines of T_1 plants. Three L1 (L1-1, L1-2 and L1-3) and two L2 lines (L2-1 and L2-2) were chosen randomly and subjected to semiRT-PCR analysis. The results showed that the expression of *ZmCoA* in transgenic plants was down-regulated in the transgenic plants compared with WT plants (Figure 2), suggested that the expression of *ZmCoA* in transgenic plants was significantly repressed due to the presence of the RNAi expression vector.

Effects of downregulated *CCoAOMT* expression on lignin, cellulose and hemicelluloses contents

To examine the effects of downregulation of *CCoAOMT* in maize, the Klason lignin content was measured in WT and transgenic plants. Compared with WT plants, the Klason lignin content was significantly reduced in transgenic plants. The average Klason lignin content of the transgenic plants was reduced to 10.35%, while the WT plants contained 13.34% Klason lignin (Figure 3A). The largest reductions in Klason lignin content was detected in the L1-1 line (9.94%).

Figure 1 - Phenotype of RNAi *CCoAOMT* transgenic plants and WT plants grown in soil.

The cellulose contents were also compared between WT and transgenic plants. In contrast to the Klason lignin content, the cellulose content of transgenic plants was significantly higher than that of WT plants. The cellulose content of transgenic plants ranged from 45.32% to 49.73%, while the WT plants contained only 38.4% cellulose (Figure 3B). Interestingly, the transgenic line L1-1 had the highest cellulose content among the transgenic plants, suggesting that cellulose biosynthesis is regulated by the down-regulation of *ZmCoA* in transgenic plants. The content of hemicelluloses was also determined in this study. As shown in Figure 3C, there were no significant differences between transgenic and WT plants, although some transgenic plants exhibited slightly increased levels. Indeed, the down-regulation of *CCoAOMT* in maize led to a 22.4% decrease in Klason lignin content and a 23.3% increase in cel-

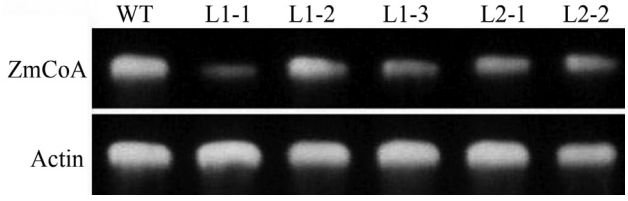

Figure 2 - Expression levels of *CCoAOMT* in WT plants and independent transgenic lines. The maize *Actin1* gene was used as internal control for PCR.

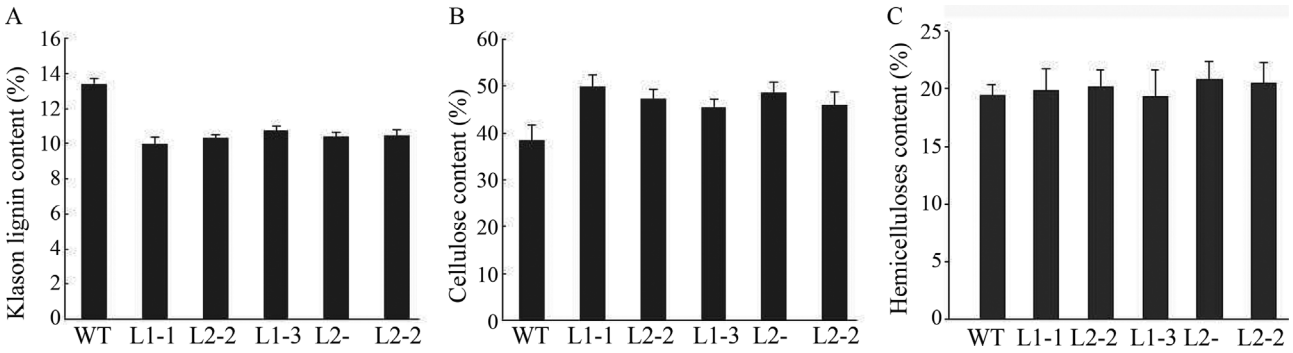

Figure 3 - Klason lignin content (A), cellulose content (B) and hemicelluloses content (C) in RNAi *CCoAOMT* transgenic plants and WT plants. Data are means ± SD of three replicates.

lulose content *vs.* the WT plants. Based on these results, we conclude that lignin and cellulose, important cell wall components in plants, may be regulated in a compensatory fashion, which is consistent with previous reports (Hu *et al.*, 1999).

Downregulation of *CCoAOMT* results in altered of S/G ratios

Many studies of other species have shown that the down-regulation of *CCoAOMT* can lead to a reduction in lignin content and an increase in the S/G ratio. Thioacidolysis is the most efficient method for degrading lignin polymers to reveal the lignin monomer composition (Lapierre *et al.*, 1995). Thus, in this study, maize stems of transgenic and WT plants were treated with thioacidolysis, followed by GC-MS, to measure the monolignol compositions of syringyl (S) and guaiacyl (G). As shown in Figure 4, the S/G ratio of the transgenic plants was 0.999, while the S/G ratio of WT plants was only 0.636. Therefore, the transgenic plants had a 57.08% higher S/G ratio than WT plants, which is consistent with previous reports.

Effects of downregulation *CCoAOMT* on lignin deposition

Lignin content is reflected by the intensity of phloroglucinol staining in the cell walls, while non-lignified cells are not clearly visible by this method. To examine lignin deposition in maize straw, cross-sections of stems of transgenic and WT plant were strained with the lignin-staining dye phloroglucinol–HCl (Wiesner reagent) to reveal lignin deposition. As shown in Figure 5, after Wiesner staining, lignin was barely visible in the transgenic plants, suggesting that down-regulation of *CCoAOMT* in maize led to a reduction in lignin staining (Figure 5B). By contrast, staining with Wiesner reagent produced a significant brown coloration in the xylem and sclerenchyma of WT plants (Figure 5A). These results suggest that down-regulation of *CCoAOMT* resulted in a dramatic reduction of lignin content in maize straw.

Discussion

Plants with low lignin content can be obtained through breeding and selection, or by purposely altering lignin content through genetic engineering. Since lignin biosynthesis pathways are highly conserved in most plant species (Boerjan *et al.*, 2003; Umezawa, 2010), genetic engineering can be employed to directly manipulate the target genes involved in the lignin biosynthesis pathway by down-regulating the expression of these genes, or by manipulating transcription factors that regulate the expression of key lignin synthesis gene(s) (Sánchez *et al.*, 2005; Zhou *et al.*, 2009; Xu *et al.*, 2011;). To date, at least ten genes encoding the key enzymes in the lignin biosynthesis pathway have been isolated and characterized. Various enzymes in the lignin biosynthesis pathway have been shown to play important roles in regulating lignin content and quality. Amongst the genes encoding these enzymes, *CCoAOMT* was discovered only recently, and has mainly been studied in tobacco, poplar and *Medicago* (Martz *et al.*, 1998; Meyermans *et al.*, 2000; Guo *et al.*, 2001). Despite the importance of maize for forage and industrial materials, few studies have focused on the functional characterization of lignin synthesis-related genes in maize. In this study, a 229 bp fragment of maize *CCoAOMT* corresponding to the fifth exon of this gene was used to construct an RNAi vector, which was then transferred into maize. The results of this study confirmed many features of *CCoAOMT* that were previously reported, but also revealed some new aspects of this gene that differ from those observed in other species.

In transgenic poplar and alfalfa with repressed *CCoAOMT* activity, down-regulation of *CCoAOMT* leads to a general reduction in Klason lignin content but an increase in the S/G ratio (Meyermans *et al.*, 2000; Zhong *et al.*, 2000; Guo *et al.*, 2001). In addition, transgenic studies have indicated that the increase in the S/G ratio results mainly from a decrease in the amount of G units, with no reduction in S lignin. Our results showed that down-regulation of *CCoAOMT* in maize can largely reduce the Klason lignin content and significantly increase the S/G ratio,

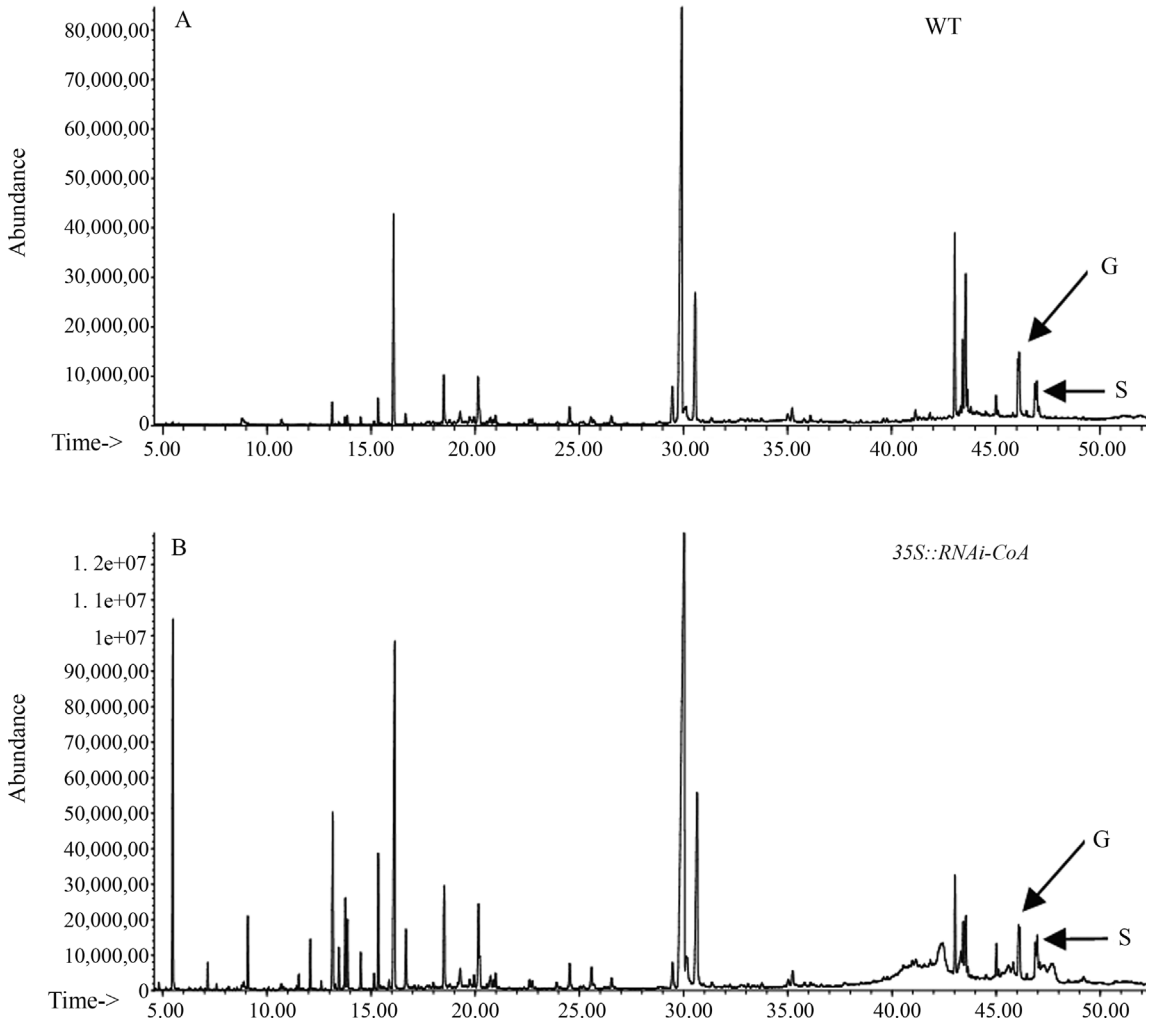

Figure 4 - GC-MS chromatogram of syringyl (S) and guaiacyl (G) units isolated from WT (A) and *CCoAOMT* downregulated transgenic plants (B).

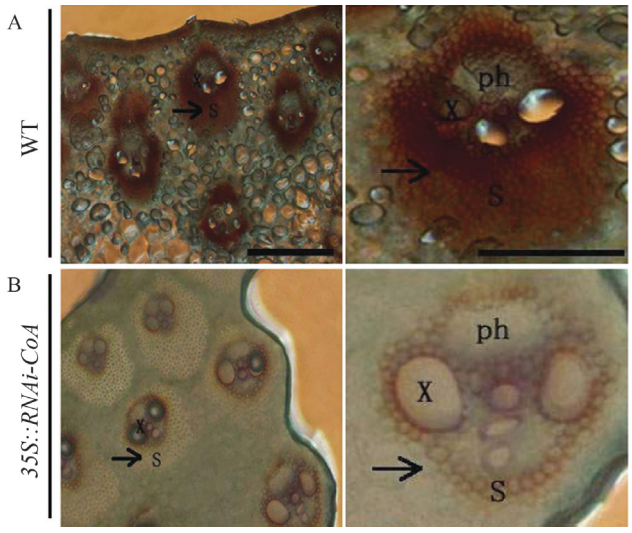

Figure 5 - Histological staining for lignin deposition in stem cross-sections of WT (A) and RNAi *CCoAOMT* transgenic maize plants using Wiesner reagent (B). *X* xylem, *S* sclerenchyma, *ph* phloem.

which are consistent with the previous studies. Meyermans *et al.* (2000) showed that introducing the antisense of *CCoAOMT* into transgenic poplar resulted in an 11% increase in the S/G ratio. By contrast, our results showed that the S/G ratio in plants with downregulated *CCoAOMT* expression was 57.08% higher than that of WT plant, possibly due to the different plant species or methods used to repress gene expression in these two studies. More interestingly, transgenic plants showed a concomitant increase in cellulose content in the lignin-reduced transgenic plants, suggesting that lignin and cellulose deposition may be regulated in a compensatory fashion in maize, which may contribute to metabolic flexibility and a growth advantage to help sustain mechanical strength in lignin-deficient straw. However, the mechanism by which this compensatory phenomenon in lignin and cellulose deposition occurs is still unknown.

To obtain plants with reduced lignin, studies should focus not only on lowing lignin content and composition, but also on the selection of transgenic plants with normal development. Many studies have focused on altering the

expression of various enzymes in the lignin biosynthesis pathway, some of which have had important effects on plant growth and development. For example, down-regulation of *4CL* in transgenic aspen leads to reduced lignin content, but also to the production of plants with enhanced growth phenotypes such as thicker stems, longer internodes, and larger leaves than control plants (Hu *et al.*, 1999). On the contrary, down-regulation of *CCR* in *Leucaena leucocephala* produced plants with significant changes in phenotypes, such as stunted growth and development, wrinkled leaves, and delayed senescence, with a 24.7% reduction of Klason lignin *vs.* WT plants (Prashant *et al.*, 2011). In the current study, transgenic maize plants with downregulated *CCoAOMT* expression exhibited significant reductions in Klason lignin, but displayed a normal phenotype compared with control plants, besides a slightly delayed growth, suggesting that *CCoAOMT* is an effective and ideal target gene for the regulation of the lignin biosynthesis pathway. However, there is an inherent relationship between lignin content and plant lodging resistance. Although our results show that plants with down-regulated *CCoAOMT* expression had almost normal phenotypes, except the slightly delayed growth, we did not adequately address the effect of reduced lignin content on lodging resistance in these lines, due to the small number of independent transgenic lines obtained in this study. Thus, more transgenic lines will be required to examine the effects of reduced lignin content on lodging resistance in maize in a future study. In addition, lignin deposition is influenced by abiotic and biotic stresses (Halpin, 2004; Boudet, 2007), it will be interesting to compare the lignin content of *CCoAOMT* down-regulated plants under field and greenhouse conditions.

Acknowledgments

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Supplementary Material

The following online material is available for this article:

Figure S1 - Molecular characterization of *CCoAOMT* down-regulated transgenic plants.

Figure S2 - PCR confirmation of *CCoAOMT* downregulated transgenic lines by amplifying the 35S promoter.

This material is available as part of the online article from http://www.scielo.br/gmb.

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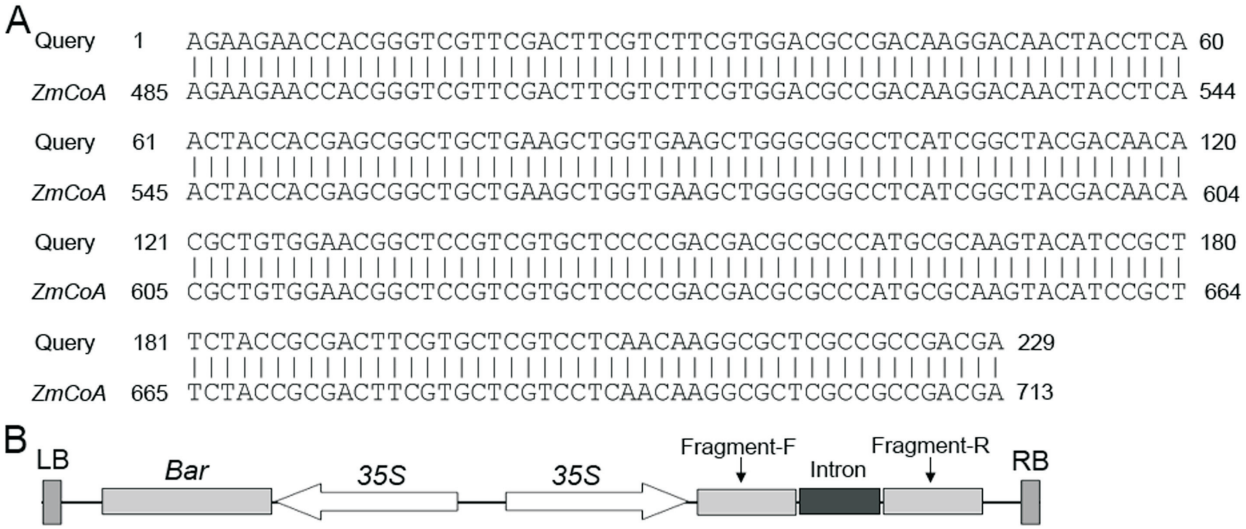

Figure S1 - Molecular characterization of *CCoAOMT* down-regulated transgenic plants.

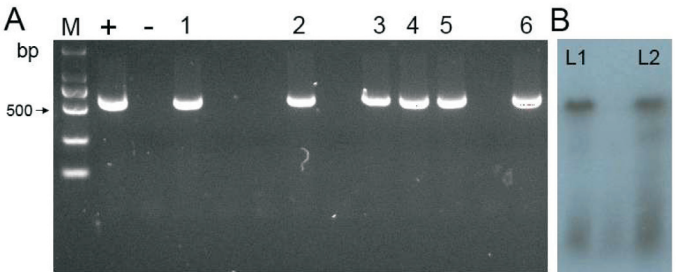

Figure S2 - PCR confirmation of *CCoAOMT* downregulated transgenic lines by amplifying the 35S promoter.