

Video Article

Lignin Down-regulation of *Zea mays* via dsRNAi and Klason Lignin Analysis

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Abstract

To facilitate the use of lignocellulosic biomass as an alternative bioenergy resource, during biological conversion processes, a pretreatment step is needed to open up the structure of the plant cell wall, increasing the accessibility of the cell wall carbohydrates. Lignin, a polyphenolic material present in many cell wall types, is known to be a significant hindrance to enzyme access. Reduction in lignin content to a level that does not interfere with the structural integrity and defense system of the plant might be a valuable step to reduce the costs of bioethanol production. In this study, we have genetically down-regulated one of the lignin biosynthesis-related genes, cinnamoyl-CoA reductase (*ZmCCR1*) via a double stranded RNA interference technique. The *ZmCCR1_RNAi* construct was integrated into the maize genome using the particle bombardment method. Transgenic maize plants grew normally as compared to the wild-type control plants without interfering with biomass growth or defense mechanisms, with the exception of displaying of brown-coloration in transgenic plants leaf mid-ribs, husks, and stems. The microscopic analyses, in conjunction with the histological assay, revealed that the leaf sclerenchyma fibers were thinned but the structure and size of other major vascular system components was not altered. The lignin content in the transgenic maize was reduced by 7-8.7%, the crystalline cellulose content was increased in response to lignin reduction, and hemicelluloses remained unchanged. The analyses may indicate that carbon flow might have been shifted from lignin biosynthesis to cellulose biosynthesis. This article delineates the procedures used to down-regulate the lignin content in maize via RNAi technology, and the cell wall compositional analyses used to verify the effect of the modifications on the cell wall structure.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51340/>

Introduction

The production of biofuels from lignocellulosic biomass is highly desirable due to its present abundance in the U.S.¹, and in the case of the sustainable harvest of agricultural and forestry residues, the ability to not compete directly for cropland used for food and animal feed production. However, unlike maize grain, which is the main source of biofuel currently generated in the U.S., lignocellulosic materials are significantly more complex and difficult to break down. In addition to the long-chain carbohydrates, cellulose and hemicellulose, which are the main sources of sugars during fermentation of lignocellulosic materials, many types of plant cell walls also contain lignin, a phenylpropanoid polymer that provides strength, defense against pathogen attack, and hydrophobicity to cell walls. While necessary for plant growth and survival, lignin also presents a significant barrier to the successful enzymatic conversion of the cellulose and hemicellulose to soluble sugars. Materials with high lignin contents are generally less desirable materials for both the biofuel (through biological conversion pathways) and the pulp and paper industries due to the negative impacts on processing characteristics and product quality. Hence, genetic manipulation of plant materials for lignin reduction at a level that does not interfere with crop structural strength and defense systems might be important for the reduction of production costs for both the lignocellulosic biofuel and the pulp and paper industries.

In maize (*Zea mays*), lignin is covalently cross-linked to hemicellulose in the primary cell wall via ferulate and diferulate bridges². The lignin-hemicellulose complex binds to cellulose microfibrils through hydrogen bonds, forming a complex matrix that confers integrity and strength to the secondary cell wall. The mechanical strength of plant cell walls is largely determined by the type of lignin subunits³⁻⁵. In previous studies, altering the proportions of lignin subunits has shown no clear trend on enzymatic digestibility⁶⁻¹¹. However, reductions in lignin content generally show an improvement in conversions^{12,13} and may be a key to increasing the digestibility of plant material by hydrolytic enzymes including endocellulases, cellobiohydrolases, and β -glucosidases¹⁴.

Genetic engineering to regulate the expression level of transcripts has been extensively practiced to improve crop traits. Advanced techniques, including anti-sense¹⁵ and co-suppression¹⁶ technologies, enable effective down-regulation of target genes. Complete gene knock-out has also been achieved using gene constructs encoding intron-spliced RNA with a hairpin structure¹⁷. Furthermore, a double stranded RNA interference (dsRNAi) technique, *i.e.* a powerful and effective gene expression mediator that works by either targeting transcript degradation or translation

repression, provides a potent means to induce a wide range of suppression effects on the target mRNA¹⁸. Gene silencing techniques show several limitations. These techniques do not precisely regulate the level of transcription and it could cause unexpected silencing effects on other homologous genes.

In this method, we employed particle bombardment to carry the dsRNAi constructs into the maize genome. To date, a vast array of plant species have been successfully transformed using particle bombardment, *Agrobacterium* mediated transformation, electroporation, and microinjection methods. In maize genetic transformation, the particle bombardment method is advantageous over all the other methods because it is the most efficient. Particle bombardment is not dependent on bacteria, so the method is free of biological constraints such as the size of the gene, species of gene origin, or the plant genotype. The physical transgene delivery system enables high molecular weight DNA and multiple genes to be introduced into plant genomes and in certain cases into chloroplasts at high transformation efficiency¹⁹. The lignin reduction in the vascular system of the leaf mid-rib can be visualized via scanning electron microscopy (SEM) which is beneficial for examining the topography and composition of samples.

In maize plants, two of the cinnamoyl-CoA reductase (*ZmCCR1*: X98083 and *ZmCCR2*: Y15069) genes were found in the maize genome²⁰. Cinnamoyl-CoA reductase catalyzes the conversion of the hydroxycinnamoyl-CoA esters into cinnamyl aldehydes. We chose the *ZmCCR1* gene to down-regulate this enzyme because the gene is expressed in all lignifying tissues. The 523 nucleotides at the 3' terminus of the *ZmCCR1* gene were chosen for a dsRNAi construct because the sequences appeared to be more diverse as compared to those of *ZmCCR2*. Thus, the dsRNAi construct would precisely bind only to *ZmCCR1*, avoiding off-target silencing²¹. A *ZmCCR1_RNAi* construct was engineered into the cytoplasmic expression system ImpactVector1.1-tag (IV 1.1) containing the green tissue specific promoter, ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO).

To study the effects of the dsRNAi construct on transgenic plants, the lignin content was quantified. The Klason (acid-insoluble) lignin measurement is known to be more accurate compared to the acid detergent lignin quantification methods which solubilize some of the lignin²². Therefore, the Klason lignin was measured in transgenic maize stalks. This procedure consists of a two-step acid hydrolysis that converts polymeric carbohydrates into soluble monosaccharides²³. The hydrolyzed biomass was then fractionated into acid soluble and insoluble materials and the acid insoluble lignin was measured according to previous studies^{23,24}. Ideally, lignin analysis should include extractions with water and ethanol prior to the hydrolysis step, in order to remove soluble materials that can interfere with the results, and a post-hydrolysis combustion of the lignin residue to account for any ash present in the residue. Without these steps, the lignin content of the sample could be artificially inflated. The full method is presented here, however for our experiments we were unable to perform both of these steps due to the small volume of material available for testing.

Two other cell wall components, cellulose and hemicellulose were also analyzed in the lignin down-regulated transgenic maize lines. It has been reported that transgenic plants that have been down-regulated in either their phenylalanine ammonia-lyase (*PAL*)²⁵, 4-coumarate:CoA ligase (*4CL*)²⁶, or cinnamyl alcohol dehydrogenase (*CAD*)²⁷ show an increase in other cell wall structural components. As a first step in our studies, crystalline cellulose was measured using the Updegraff method²⁸. This method was originally devised for determination of cellulose in a large number of cellulolytic bacteria and fungi. Briefly, the milled maize stocks were treated with Updegraff reagent (acetic acid: nitric acid: water) to remove hemicellulose, lignin, and xylosans. The crystalline cellulose was completely hydrolyzed into glucose via Saeman hydrolysis by adding H₂SO₄. The crystalline cellulose was then assayed using the colorimetric anthrone method²⁹. To verify if the hemicellulose contents were changed, the monosaccharide extracts from milled stalks were hydrolyzed using trifluoroacetic acid, derivatized using the alditol acetate method and then analyzed by gas chromatography (GC)³⁰. The detailed procedures for crystalline cellulose content and matrix polysaccharides composition analyses are described in Foster *et al.* (2010)³¹.

Here, we describe the procedures used for lignin down-regulation in maize via a RNAi technology, particle bombardment transformation, and lignin analysis for accelerated deconstruction of maize lignocellulosic biomass into fermentable sugars for biofuels.

Protocol

1. Preparation of dsRNAi Constructs Used for the Down-regulation of *ZmCCR1*

1. Design gene specific primers including necessary restriction enzyme sites for making a dsRNAi construct to knock-out the *ZmCCR1* gene. Two primer sets were designed to amplify two fragment segments of *ZmCCR1* cDNA: a 523 bp fragment from nucleotide 748 to 1271, and a 285 bp fragment from nucleotide 986 to 1271. The *ZmCCR1* cDNA was provided from the Arizona Genome Institute (AGI). More details are described in **Figure 1**.
2. Amplify the large fragment by polymerase chain reaction (PCR) from the *ZmCCR1* cDNA template using the primers *ZmCCR1_748F_BglII* (5'-AGATCTACATCCTCAAGTACCTGGAC-3') and *ZmCCR1_1271R_NcoI* (5'-CCATGGTTTACACAGCAGGGGAAGGT-3'). Amplify the smaller fragment (285 bp) using the primers *ZmCCR1_986F_BglII* (5'-AGATCTGGAAGCAGCCGTACAAGTTC-3') and a *ZmCCR1_1271R_SacI* (5'-GAGCTCTTTACACAGCAGGGGAAGGT-3').
3. Individually ligate the fragments into pGEM-T Easy following the manufacturer's instructions.
4. Perform mini-prep plasmid DNA isolation from individual transformants, each containing the pGEM-T constructs using a commercial mini-prep plasmid kit.
5. Digest both the *pGEM-T::ZmCCR1* (523 bp) and ImpactVector (IV)-1.1 (cytoplasm expression vector) with both *BglII* and *NcoI*.
6. Ligate the large digested gel purified *ZmCCR1* fragment (523 bp) into the digested gel purified IV-1.1.
7. Digest the *pGEM-T::ZmCCR1*(285 bp) and *IV-1.1::ZmCCR1*(523 bp) with both *BglII* and *SacI* in order to insert the small fragment into the *IV-1.1::ZmCCR1*(523 bp).
8. Ligate the small digested gel purified *ZmCCR1* fragment (285 bp) into the digested gel purified *IV-1.1::ZmCCR1*(523 bp).
9. Clone both 523 bp and 285 bp fragments into IV-1.1 to make the *ZmCCR1* RNAi construct, which has a 285 bp inverted repeat sequence with a 238 bp spacer in the middle of the inverted repeat fragments (see **Figure 1**).

10. Transfer this construct into *Escherichia coli* (*E. coli*), grow them and perform a midi-prep size plasmid DNA isolation to obtain enough plasmid DNA for maize genetic transformation.

2. Maize Genetic Transformation

1. Preparation of Tungsten Particles
 1. Place 60 mg of tungsten beads (M10) in a 1.5 ml tube and wash with 1 ml of 70% ethanol by vortexing for 2 min. Incubate for 10 min at 23 °C then centrifuge at 18,894 x g for 2 min and discard the supernatant.
 2. Wash 3x with 1 ml of 100% ethanol, centrifuging for 2 min and discarding supernatant. Add 1 ml of sterile 50% glycerol to bring the microparticle concentration to 60 mg/ml.
2. Preparation of DNA for Bombardment
 1. Place the 50 µl (3 mg) of tungsten beads prepared in 50% glycerol into a 1.5 ml tube. Add 5 µl (1 µg) of *IV-1.1::ZmCCR1* RNAi plasmid DNA, 50 µl of 2.5 M CaCl₂ and 20 µl of 0.1 M spermidine. Vortex briefly between each addition of the above reagents.
 2. Vortex the tungsten bead-DNA mixture briefly and centrifuge at 18,894 x g for 30 sec. Pour off the supernatant and resuspend the pellets in 140 µl of 70% ethanol. Remove the liquid and discard. Add 140 µl of 100% ethanol. Remove the liquid and discard.
 3. Add 48 µl of 100% ethanol. Use immediately or store on ice for up to 4 hr prior to bombardment.
3. Bombardment
 1. Place a 3-5 cm diameter Hi-II embryogenic maize calli (provided from Maize transformation Center of Iowa State University) in the middle of 100 mm Petri dishes containing N6OSM media (as osmotium) at least 4 hr prior to the bombardment.
 2. Prepare the PSD-1000/He Particle Delivery device according to the manufacturer's instructions³².
 3. Sterilize chamber wall with 70% ethanol. Load sterile 650 psi rupture disk into sterile retaining cap. Spread 5-6 µl of the M10-DNA solution onto the surface of a macrocarrier, dry briefly. Load macrocarrier and stopping screen into microcarrier launch assembly.
 4. Place microcarrier launch assembly and maize calli in chamber at a selected distance from the stopping screen (L2 = 6 cm) and close door. Accelerate in a vacuum of 27 psi against a wire mesh screen.
 5. Press the fire button until rupture disk bursts and helium pressure gauge drops to zero. Release the fire button.
 6. Incubate the bombarded calli in a Petri dish containing N6OSM (osmotic medium) for 16 hr in the dark at 27 °C. Break the calli into about ten pieces and transfer to N6E (callus induction medium) in Petri dishes and incubate for 5 days in the dark at 27 °C.
4. Selection
 1. After 5 days on N6E, transfer the calli onto N6S medium (selection media). Subculture all calli on selection medium every 30 days for 8-12 weeks without disturbing the calli structure.
 2. After about 8-10 weeks, white fast-growing sectors will grow out of the non-proliferating and partially necrotic mother calli. Excise the white fast-growing tissues and subculture them to fresh selection medium (N6S) and continue to incubate as above.
5. Regeneration
 1. Transfer the white and fast growing embryonic calli onto regeneration medium and incubate as above for 1 week. Switch the regenerating embryogenic calli to a period of 16 hr daylight and 8 hr dark at 25-27 °C
 2. Transfer the regenerating shoots onto the rooting medium in a glass test tube after 3-4 weeks, continue to incubate as above. After substantial root development appears, wash roots carefully under water tap, then transplant the plantlets to 4" pots with soil. Cover the pots with plastic bags to keep moist. After 2 days make small holes the plastic bags. After 5-6 days remove the plastic bags. Continue to incubate as above for another 5-6 days.
6. Greenhouse
 1. Transfer the seedlings into 18" pots with soil and maintain in full summer sunlight or greenhouse light. The initial regenerated plants are called T₀ while the first seeds belong to the T₁ generation.

3. Histological Assay

1.
 1. Fix the maize leaf mid-ribs in 5 ml of 10% neutral buffered formalin.
 2. Process and vacuum infiltrate with paraffin on a tissue processor using a tissue processor.
 3. Embed the tissues in paraffin using a HistoCentre III embedding station.
 4. Remove the excess paraffin from the edges once blocks are cooled.
 5. Section sample at 4-5 µm with a microtome using a microtome.
 6. Place sections on microscope slides and dry in a 56 °C incubator for 2-24 hr. Make sure sections are fully adhered to the slide.
 7. Deparaffinize sections in two changes of xylene for 5 min at 23 °C.
 8. Hydrate slides through two changes of 100% ethanol for 2 min and two changes of 95% ethanol for 2 min at 23 °C.
 9. Rinse the sections in running tap water for 2 min.
 10. Stain with 0.05% toluidine blue O for 1-2 min and rinse briefly with ddH₂O.
 11. Place a coverslip on the samples with immersion oil and visualization with light microscopy.
2. Scanning Electron Microscopy (SEM)
 1. Fix the cross-sectioned maize leaf mid-ribs in 4% glutaraldehyde and 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 1-2 hr.
 2. Briefly rinse the samples in the buffer, dehydrated them in an ethanol series (25%, 50%, 75%, and 95%) for 10-15 min at each gradation and 100% ethanol for 10 min, 3x.
 3. Dry the dehydrated cross-sectioned maize leaf mid-ribs in a critical point dryer using liquid carbon dioxide as the transitional fluid.
 4. Mount the dried samples on aluminum stubs using high vacuum carbon tabs

5. Coat the maize leaf mid-ribs mounted on aluminum stubs with gold (approximately 20 nm thickness) in a sputter coater purged with argon gas.
6. Examine the coated samples in a JEOL JSM-6400V (lanthanum hexaboride electron emitter) scanning electron microscope.
7. Digital images were pictured using an analySIS Pro software (version 3.2).

4. Klason Lignin Measurement

1. Mill the samples through a 2 mm screen.
2. Use a moisture analyzer to determine the moisture content of each sample and record the value.
3. Weigh out ~1.5 g of each sample and record the mass. Extract the samples using water for the first extraction, followed by ethanol for the second extraction using either an automated solvent extractor (3 cycles per extraction, ~14 min per cycle) or soxhlet apparatus (8 hr per extraction). (Note: This step removes extractives that can condense during acid hydrolysis and interfere with accurate lignin measurement, increasing the apparent Klason lignin content.)
4. Dry the extracted samples at 45 °C overnight, then allow them to cool in the desiccator, and weigh again.
5. Set an incubator to 30 °C. Measure 0.3 g of each dry, extracted sample into screw-top high pressure tubes (triplicates per sample is recommended) and record the weights to the nearest 0.1 mg. Add 3 ml of 72% H₂SO₄ to each pressure tube.
6. Mix the sample using a glass or Teflon stir rod. Leave the stir rod in the tube until water is added following incubation.
7. Place vials in an incubator set to 30 °C and 150 rpm for 60 min. After 1 hr add 84 ml of deionized water to dilute the acid concentration to 4% and mix with the stir rod. Be careful not to leave large amounts of sample on the sides of the vial above the water line.
8. Tightly seal the stoppers on all vials and place them into a metal rack or large beakers. Autoclave at 121 °C using a liquid sterilization cycle for 1 hr. Allow them to cool to room temperature before opening.
9. Pre-ash the filtering crucibles in a furnace at 575 °C for at least 4 hr. Allow crucibles to cool in a desiccator for at least an hour.
10. Vacuum filter the solution from each tube through a separate crucible, using a rubber adaptor to secure the crucible. Use deionized water to rinse any remaining particles from the tube.
11. Dry the lignin residue at 105 °C for a minimum of 4 hr. Record the weight of the dry crucible and residue.
12. If using a 575 °C, pre-fire the samples over a Bunsen burner until there is no smoke or ash and then place in the furnace for 24 hr, or if using a programmable furnace, do not pre-ash and use the following program:
13. Ramp from room temperature to 105 °C and hold for 12 min.
14. Ramp to 250 °C at 10 °C/min and hold for 30 min.
15. Ramp to 575 °C at 20 °C/min and hold for at least 180 min.
16. Remove the crucibles from the furnace and cool in a desiccator. Weigh the crucible and ash.
17. Calculate the acid insoluble residue using the following equation:

$$\text{Acid insoluble lignin (\%)} = \frac{(M_{\text{RESIDUE}} - M_{\text{ASH}})(M_{\text{POST}})}{(M_{\text{VIAL}})(M_{\text{PRE}})(1 - \text{MC})} * 100\%$$

M_{PRE} = Mass of pre-extracted biomass

M_{POST} = Mass of post-extracted biomass

M_{VIAL} = Mass of extracted biomass added to the vial

M_{RESIDUE} = Mass of crucible and lignin residue

M_{ASH} = Mass of crucible and ash

MC = Moisture content of pre-extracted biomass, total weight basis

5. Carbohydrate Analysis

1. Perform cell wall carbohydrate analyses based on the Foster *et al.* (2010) protocol³¹. In brief, Prepare alcohol insoluble residue from freeze dried plant material. Then hydrolyze the material with trifluoroacetic acid and match the solubilized monosaccharide derivatives to their corresponding alditol acetates. Analyze these volatile derivatives by gas chromatography (GC) connected to a quadruple mass spectrometer.

Representative Results

We have demonstrated a reduction in the lignin content in maize plants via RNAi. The particle bombardment transformation method yielded around 30% transformation efficiency. The gene silencing of *ZmCCR1* was consistently observed in T0-T2 generations. The lignin reduced transgenics grew similarly to wildtype maize plants except for displaying brown coloration in the leaf mid-rib, husk, and stem. The histological assay has shown that the mutant lines exhibit a significant reduction in the cell wall thickness of the sclerenchyma fibers in the maize leaf mid-rib¹⁸. Despite the reduction of cell wall thickness, the structure of other major vascular systems including the xylem vessel, phloem, and sheath cells did not reveal any differences compared to the wild-type control (**Figure 2A**)¹⁸. This implies that there are no detrimental effects for either water transport, nutrient transfer, or mechanical strength to the stems in the *ZmCCR1_RNAi* mutant lines.

The lignin content was quantified using a Klason lignin measurement. **Figure 3** shows the amount of acid-insoluble Klason lignin (g/kg con stover) in wildtype maize and *ZmCCR1_RNAi* transgenic lines (T1). Three transgenic lines (1c-4, -5, and -6) showed a statistically significant lignin reduction (8.1%, 7.0%, and 8.7% respectively) compared to that of the wild-type maize plants¹⁸. To determine whether carbon flow was shifted from the lignin biosynthetic pathway to cell wall carbohydrate biosynthesis pathways, cellulose was analyzed via the Updegraff method. **Figure 3A** shows that two *ZmCCR1_RNAi* mutant lines (1b-6 and 1c-6) contained significantly increased levels of crystalline cellulose (1.5 and

1.8 fold respectively)¹⁸. The hemicellulose content was also analyzed. **Figure 4B** shows the amount of four main hemicellulose components (arabionose, xylose, galactose, and glucose). None of the four carbohydrate groups revealed any changes in the mutant lines¹⁸.

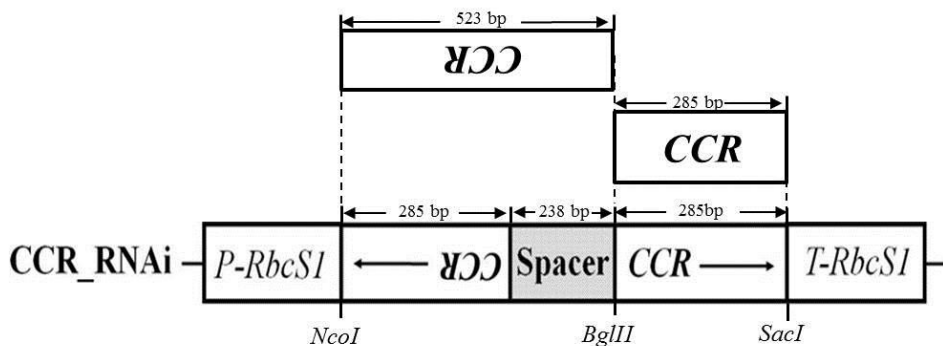


Figure 1. Cloning strategy for dsRNAi plasmid constructs for the down-regulation of the *ZmCCR1*. PRbcS1: Ribulose biphosphate carboxylase promoter from *Chrysanthemum morifolium* Ramat. T-RbcS1: Ribulose biphosphate carboxylase small unit terminator from *Asteraceous chrysanthemum*. This figure has been modified from Park et al (2012)¹⁸.

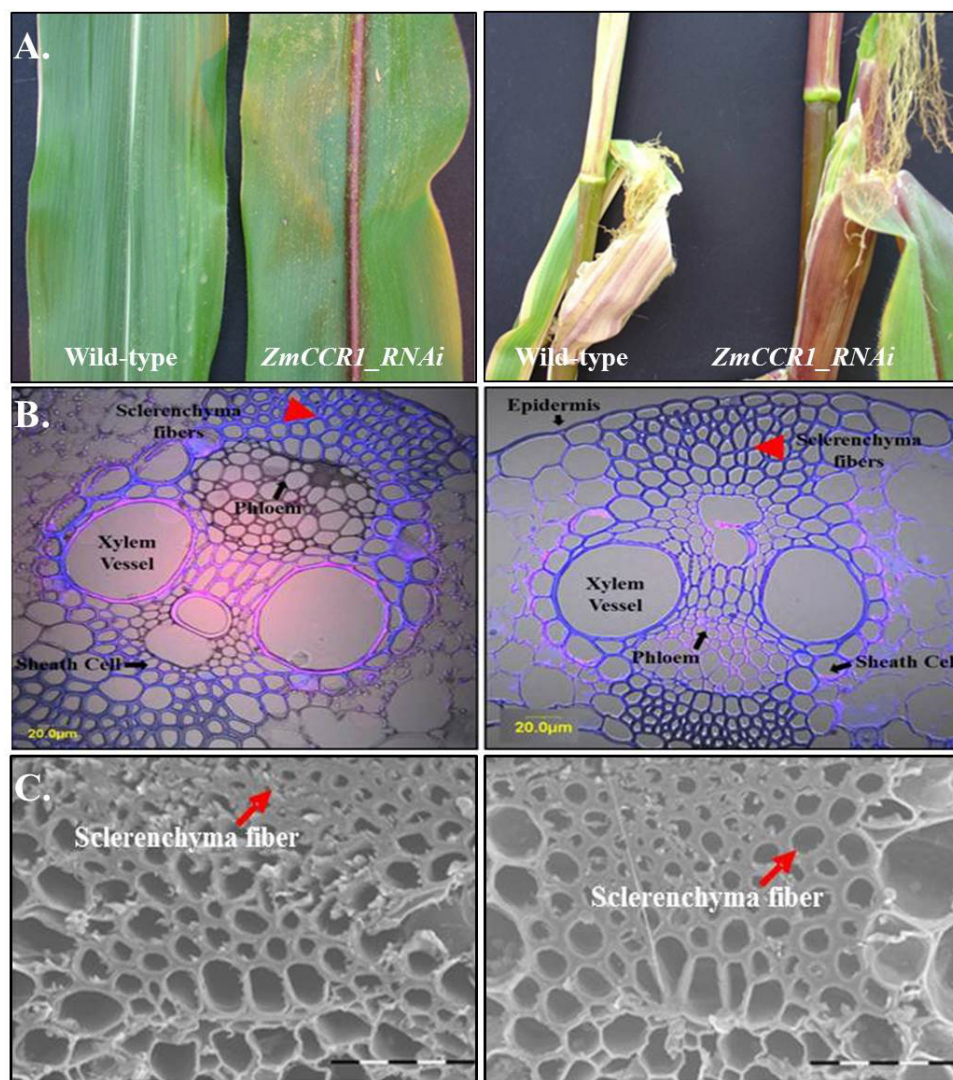


Figure 2. Phenotypic analyses of wild-type corn (Hi-II) and *ZmCCR1_RNAi* mutant leaf midrib. **A)** Brown coloration was seen in *ZmCCR1* down-regulated corn leaves, stems and corn husks. **B)** The cross-sectioned maize leaf midribs of wild-type HI-II (left) and *ZmCCR1_RNAi* mutant line (right) were stained with 0.05 % toluidine blue O for 1 min to visualize secondary xylem tissues. The red arrowhead indicates the cell walls of sclerenchyma fibers of the leaf midrib. **(C)** Scanning electron microscopy (SEM) of *ZmCCR1* down-regulated transgenic maize leaf midrib (right) as compared to that of wild-type non-transgenic control plant (left). The red arrow indicates sclerenchyma fibers. This figure has been modified from Park *et al.* (2012)¹⁸.

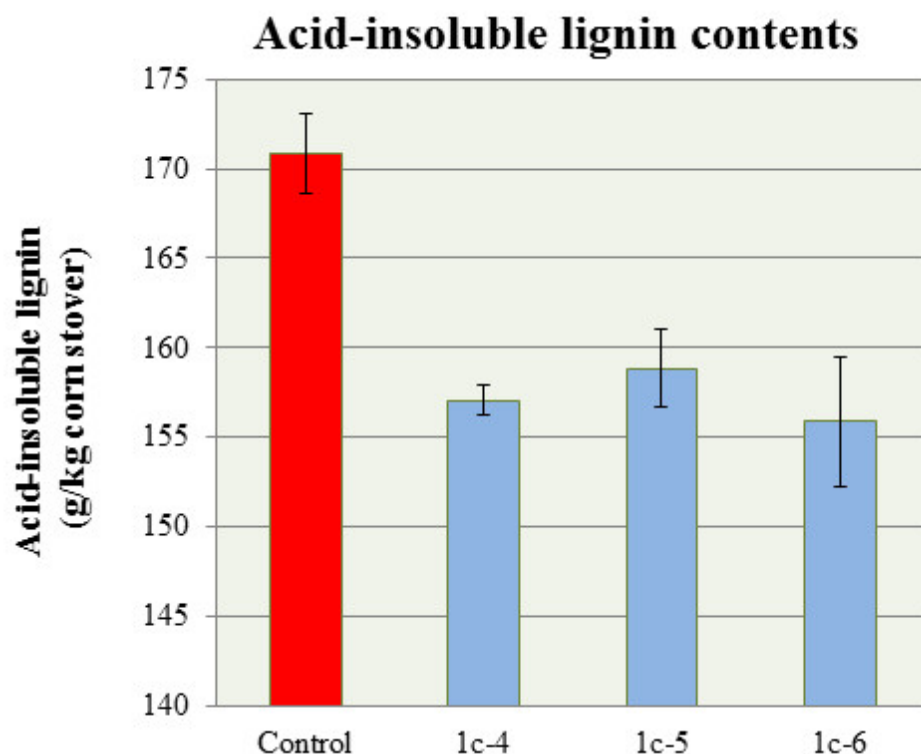


Figure 3. Klason lignin measurements (acid-insoluble lignin contents) of wild-type HI-II and *ZmCCR1_RNAi* mutant. The three mutant lines 1c, 1c-5, and 1c-6 had statistically lower lignin content, 8.5%, 7.5%, and 9.2% respectively, as percent of dry matter compared with the wild-type control plants. Mean \pm standard deviation ($P < 0.05$, $n = 3$). This figure has been modified from Park *et al.* (2012)¹⁸.

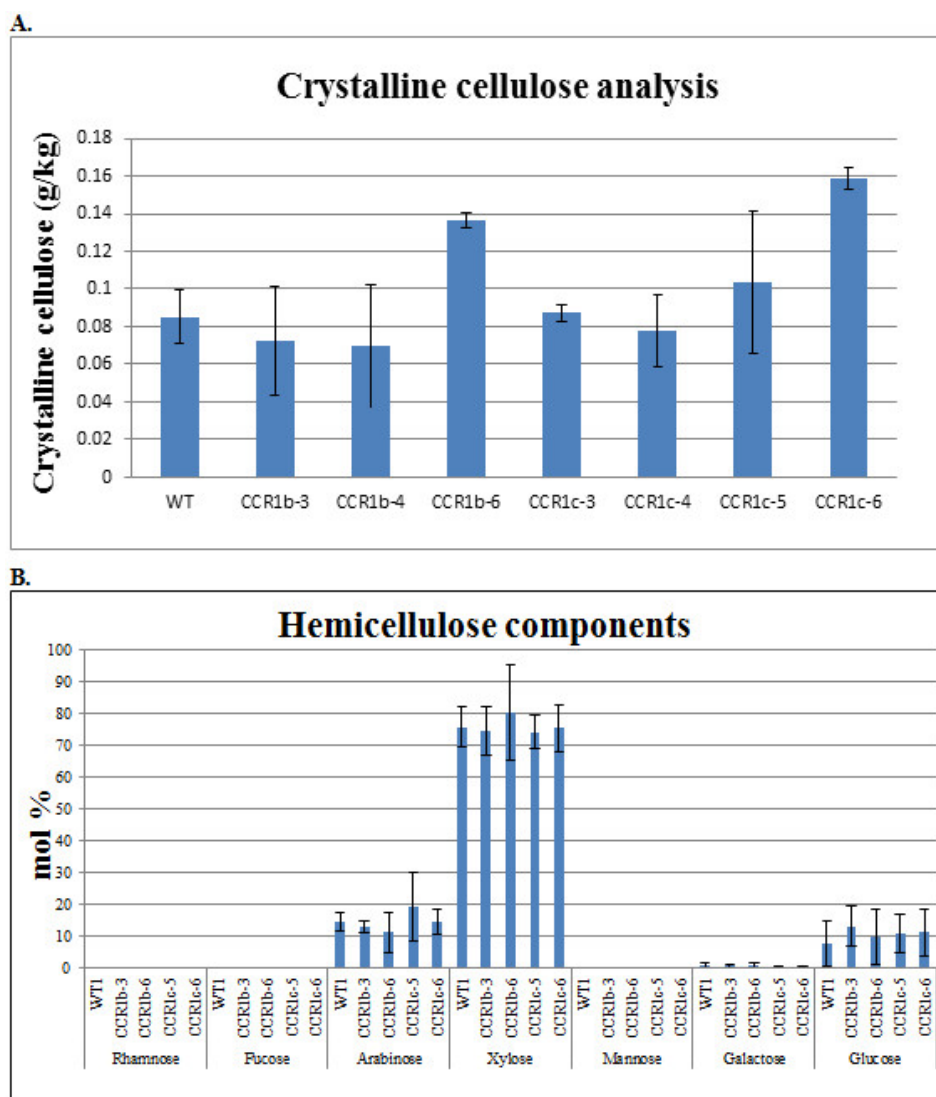
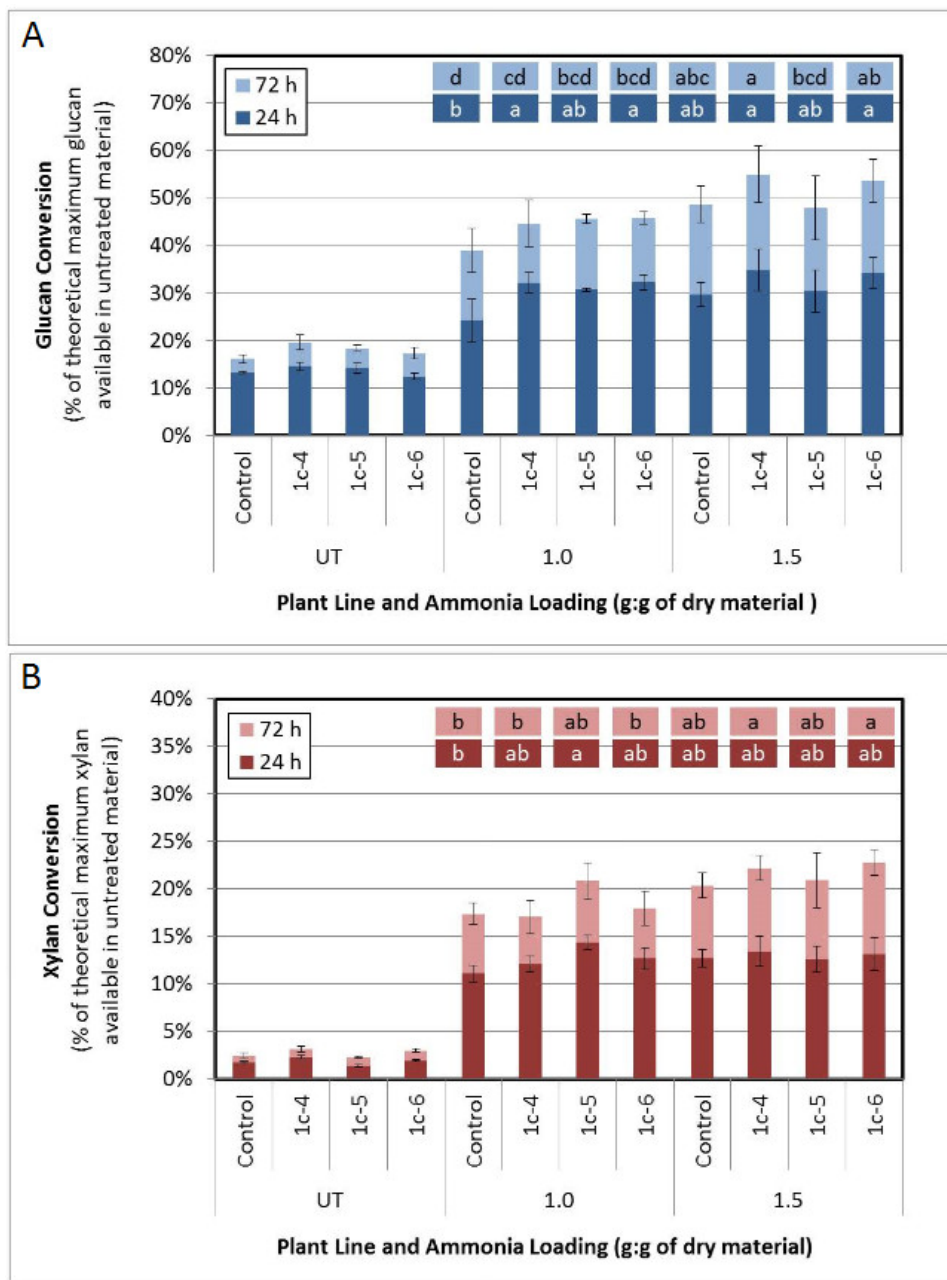


Figure 4. Cell wall compositional analyses. A) Crystalline cellulose analysis of *ZmCCR1_RNAi* lines (Tukey's pairwise comparisons, * $P < 0.05$, $n=3$). **B)** Hemicellulose compositional analysis of wild-type maize and *ZmCCR_RNAi* transgenic maize lines (T1) via gas chromatography (GC). The main peaks from the chromatograms were integrated, identified based on retention times and fragment ion signatures, and expressed as mol percentage ($P > 0.05$, $n = 3$) (Tukey's pairwise comparisons, $P > 0.05$; $n = 3$). This figure has been reused from Park *et al.* (2012)¹⁸.



Media	Chemical compositions
N6OSM	4 g/L N6 salts
(Osmotic medium)	1 ml/L N6 vitamin stock
	2 mg/L 2,4-D
	100 mg/L myo-inositol
	0.69 g/L proline
	30 g/L sucrose
	100 mg/L casein hydrolysate

	36.4 g/L sorbitol
	36.4 g/L mannitol
	2.5 g/L gelrite, pH 5.8
	Add filter sterilized silver nitrate (25 µM) after autoclaving
N6E	4 g/L N6 salts
(Callus induction)	1 ml/L (1,000x) N6 vitamin stock
	2 mg/L 2,4-D
	100 mg/L myo-inositol
	2.76 g/L proline
	30 g/L sucrose
	100 mg/L casein hydrolysate
	2.5 g/L gelrite, pH 5.8
	Add filter sterilized silver nitrate (25 µM) after autoclaving
N6S media	4 g/L N6 salts
(Selection media)	1 ml/L N6 vitamin stock
	2 mg/L 2,4-D
	100 mg/L myo-inositol
	0.69 g/L proline
	30 g/L sucrose
	100 mg/L casein hydrolysate
	36.4 g/L sorbitol
	36.4 g/L mannitol
	2.5 g/L gelrite, pH 5.8
	Add filter sterilized silver nitrate (25 µM) after autoclaving
Regeneration medium	4.3 g/L MS salts
	1 ml/L (1000x) MS vitamin stock
	100 mg/L myo-inositol
	60 g/L sucrose
	3 g/L gelrite, pH 5.8 (100 x 25 mm Petri plates)
	Add filter sterilized bialaphos (3 mg/L) added after autoclaving
Rooting medium	4.3 g/L MS salts
	1 ml/L MS vitamin stock
	100 mg/L myo-inositol
	30 g/L sucrose
	3 g/L gelrite, pH 5.8 (100 x 25 mm Petri plates)
10% Neutral buffered formalin	100 ml of formalin
(1 liter)	900 ml of ddH ₂ O
	4.0 g of Sodium dihydrogen phosphate, monohydrate (NaH ₂ PO ₄ ·H ₂ O)

Table 1. Table of specific reagents.

Discussion

The accessibility of microbial cellulases to plant cell wall polysaccharides is largely dependent on the degree to which they are associated with phenolic polymers²³. The conversion rate from lignocellulosic biomass to fermentable sugar is negatively correlated with lignin content deposited in plant secondary cell walls. This correlation is ascribed to the physical properties of lignin such as hydrophobicity²⁴, chemical heterogeneity, and the absence of regular hydrolysable intermonomeric linkages²⁵.

In this study, a dsRNAi technique induced various levels of gene down-regulation on genetic targets. Lignin down-regulation, mediated by a *ZmCCR1_RNAi* construct, has resulted in the brown-coloration in T1 transgenic lines. Brown-midrib (*bm*) coloration is a naturally occurring

phenomenon that is caused by reduced lignin content and altered lignin composition. Unlike other naturally occurred *bm* mutants, which show the brown coloration only in leaf mid-ribs, the *ZmCCR1_RNAi* mutant lines revealed the phenotype in other parts of the plant, including the stems and husks. The histological assay also indicated that the sclerenchyma cell wall thickness of *ZmCCR1* down-regulated leaves was much less than those of the wild-type control plants (**Figure 2A**). However, the structure and cell wall thickness of the main vascular systems including xylem vessels, phloem, or sheath cells was not changed. This could explain the normal growth of the *ZmCCR1_RNAi* transgenic lines which grew normally in terms of plant height and stem diameter.

A reduction of more than 20% in the lignin content has generally caused a loss of biomass and made the plants more vulnerable against microbial pathogens and pests^{27,28}. However, the mutant lines produced in this research, expressing less than a 10% lignin reduction, did not compromise the plant biomass and defensive mechanism against abiotic and biotic stresses.

Previous studies have shown that transgenic tobacco lines with significantly reduced CCR expression also showed an increase of other cell wall constituents such as glucose, xylose, and wall-bound phenolic compounds (e.g., sinapic and ferulic acids). In this study, the mild lignin reduction increased the level of crystalline cellulose in some of the *ZmCCR1* down-regulated maize plants. Conversely, a cellulose compensation mechanism was also observed in *Arabidopsis* mutants which exhibited ectopic lignification when cellulose synthesis genes were defective³⁴. The quantitative or qualitative changes of one cell wall carbohydrate component induces the alternation of other components³⁵. Such compensation mechanisms are important to maintain the homeostasis of plant vascular systems. However, in this study, hemicelluloses showed no statistically significant changes in *ZmCCR1* down-regulated mutant lines. This result may be because the observed lignin reduction was not sufficient to trigger additional hemicellulose synthesis.

The decreased level of lignin and the increased crystalline cellulose level would be doubly beneficial for biofuel production. The lower lignin contents would require fewer inputs (e.g., H₂SO₄, cellulases, etc.) during processing and facilitate the biomass conversion process. The extra cellulose may increase the yield of fermentable sugars. The genetic manipulation of *ZmCCR1* detailed in this study can be implemented to help make lignocellulosic biomass derived bioethanol more commercially competitive.

Disclosures

No conflicts of interest declared.

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