

Journal of Visualized Experiments

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

--Manuscript Draft--

Manuscript Number:	JoVE51340R2
Full Title:	Lignin Down-regulation of Zea mays via dsRNAi and Klason Lignin Analysis
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Zea mays, cinnamoyl-CoA reductase (CCR), dsRNAi, Klason lignin measurement, cell wall carbohydrate analysis, gas chromatography (GC)
Manuscript Classifications:	5.5.393.420: Genetic Engineering; 8.1.158.201.85: Carbohydrate Biochemistry
Corresponding Author:	Sang-Hyuck Park University of Arizona Tucson, Arizona UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	parksa31@msu.edu
Corresponding Author's Institution:	University of Arizona
Corresponding Author's Secondary Institution:	
First Author:	Sang-Hyuck Park
First Author Secondary Information:	
Other Authors:	Sang-Hyuck Park, Ph.D. Rebecca Garlock Ong Chuansheng Mei Mariam Sticklen, Ph.D.
Order of Authors Secondary Information:	
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.
Author Comments:	Dear Editor,

	<p>Thank you so much for your invitation to the journal. Please let me know if you have any question regarding the manuscript. We are looking forward to the opportunity to publish our research results.</p> <p>Sincerely,</p> <p>Sang-Hyuck Park</p>
Additional Information:	
Question	Response



The School of Plant Sciences
College of Agriculture and Life Sciences
1145E. South Campus Drive, P.O. Box 210036
Tucson, Arizona 85721-0036

Date: June 25, 2013

Dear Editor-in-Chief,

I am enclosing herewith a manuscript entitled “Lignin Down-regulation of *Zea mays* and Cell Wall Compositional Analyses” for publication in the *Journal of Visualized Experiments*.

The corresponding author of this manuscript is Dr. Sang-Hyuck Park. Three co-authors (Chuansheng Mei, Rebecca Garlock Ong, and Mariam Sticklen) provided contributions (dsRNAi gene construct, lignin measurement, and as a primary investigator, respectively) to this research.

Ms. Elizabeth Sheeley has invited and assisted us in the submission process. Six peer reviewers have been submitted online.

We appreciate your invitation for publication of this manuscript and look forward to hearing from you.

Sincerely,

A handwritten signature in black ink, reading 'shpark'.

Sang-Hyuck Park, PhD
Postdoctoral Research Associate
The School of Plant Sciences
University of Arizona

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

Sang-Hyuck Park*, Rebecca Garlock Ong, Chuansheng Mei, and Mariam Sticklen

Sang-Hyuck Park
The School of Plant Sciences
University of Arizona, Tucson, AZ 85721
shpark@email.arizona.edu

Rebecca Garlock Ong
Department of Chemical Engineering and Materials Science
DOE Great Lakes Bioenergy Research Center
Michigan State University, Lansing MI 48910
garlock1@msu.edu

Chuansheng Mei
The Institute for Sustainable and Renewable Resources
The Institute for Advanced Learning and Research, Danville, VA 24540
chuansheng.mei@ialr.org

Mariam Sticklen
Department of Plant, Soil and Microbial Sciences
Michigan State University, East Lansing, MI 48824
stickle1@msu.edu

*Corresponding author: Sang-Hyuck Park, Ph.D.

Keywords: *Zea mays*, cinnamoyl-CoA reductase (CCR), dsRNAi, Klason lignin measurement, cell wall carbohydrate analysis, gas chromatography (GC)

Short Abstract

A double stranded RNA interference (dsRNAi) technique is employed to down-regulate the maize cinnamoyl coenzyme A reductase (*ZmCCR1*) gene to lower plant lignin content. Lignin down-regulation from the cell wall is visualized by microscopic analyses and quantified by the Klason method. Compositional changes in hemicellulose and crystalline cellulose are analyzed.

Long 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.

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 subunits^{3,4,5}. 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}.

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 Text

1. Preparation of dsRNAi constructs used for the down-regulation of *ZmCCR1*

1.1 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.

1.2 Amplify the large fragment by polymerase chain reaction (PCR) from the *ZmCCR1* cDNA template using the primers ZmCCR1_748F_BglIII (5'-AGATCTACATCCTCAAGTACCTGGAC-3') and ZmCCR1_1271R_NcoI (5'-CCATGGTTTACACAGCAGGGGAAGGT-3'). Amplify the smaller fragment (285 bp) using the primers ZmCCR1_986F_BglIII (5'-AGATCTGGAAGCAGCCGTACAAGTTC-3') and a ZmCCR1_1271R_SacI (5'-GAGCTCTTTACACAGCAGGGGAAGGT-3').

1.3 Individually ligate the fragments into pGEM-T Easy following the manufacturer's instructions.

1.4 Perform mini-prep plasmid DNA isolation from individual transformants, each containing the pGEM-T constructs using a commercial mini-prep plasmid kit.

1.5 Digest both the *pGEM-T::ZmCCR1* (523 bp) and ImpactVector (IV)-1.1 (cytoplasm expression vector) with both *BglIII* and *NcoI*.

1.6 Ligate the large digested gel purified *ZmCCR1* fragment (523 bp) into the digested gel purified IV-1.1.

1.7 Digest the *pGEM-T::ZmCCR1*(285 bp) and *IV-1.1::ZmCCR1*(523 bp) with both *BglIII* and *SacI* in order to insert the small fragment into the *IV-1.1::ZmCCR1*(523 bp).

1.8 Ligate the small digested gel purified *ZmCCR1* fragment (285 bp) into the digested gel purified *IV-1.1::ZmCCR1*(523 bp).

1.9 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).

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

Preparation of tungsten particles

2.1 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.

2.2 Wash three times 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.

Preparation of DNA for bombardment

2.3 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::ZmCCRI* 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.

2.4 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.

2.5 Add 48 µl of 100 % ethanol. Use immediately or store on ice for up to 4 hours prior to bombardment.

Bombardment

2.6 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 media³² (as osmotium) at least 4 hours prior to the bombardment.

2.7 Prepare the PSD-1000/He Particle Delivery device according to the manufacturer's instructions³³.

2.8 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.

2.9 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.

2.10 Press the fire button until rupture disk bursts and helium pressure gauge drops to zero. Release the fire button.

2.11 Incubate the bombarded calli in a petri dish containing N6OSM (osmotic medium)³⁴ for 16 hours in the dark at 27 °C. Break the calli into about ten pieces and transfer to N6E (callus induction medium)³⁴ in petri dishes and incubate for 5 days in the dark at 27 °C.

Selection

2.12 After 5 days on N6E, transfer the calli onto N6S medium (selection media)³². Subculture all calli on selection medium every 30 days for 8-12 weeks without disturbing the calli structure.

2.13 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)³² and continue to incubate as above.

Regeneration

2.14 Transfer the white and fast growing embryonic calli onto regeneration medium³² and incubate as above for 1 week. Switch the regenerating embryogenic calli to a period of 16 hours daylight and 8 hours dark at 25-27 °C

2.15 Transfer the regenerating shoots onto the rooting medium³² in a glass test tube after 3-4 wks, 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.

Greenhouse

2.16 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

3.1 Fix the maize leaf mid-ribs in 5 ml of 10 % neutral buffered formalin.

3.2 Process and vacuum infiltrate with paraffin on a tissue processor using a tissue processor.

3.3 Embed the tissues in paraffin using a HistoCentre III embedding station.

3.4 Remove the excess paraffin from the edges once blocks are cooled.

3.5 Section sample at 4-5 microns with a microtome using a microtome.

3.6 Place sections on microscope slides and dry in a 56 °C incubator for 2-24 hours. Make sure sections are fully adhered to the slide.

3.7 Deparaffinize sections in two changes of xylene for 5 min at 23 °C.

3.8 Hydrate slides through two changes of 100 % ethanol for 2 min and two changes of 95 %

ethanol for 2 min at 23 °C.

3.9 Rinse the sections in running tap water for 2 min.

3.10 Stain with 0.05 % toluidine blue O for 1-2 min and rinse briefly with ddH₂O.

3.11 Place a coverslip on the samples with immersion oil and visualization with light microscopy.

Scanning electron microscopy (SEM)

3.12 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 hours.

3.13 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, 3 times.

3.14 Dry the dehydrated cross-sectioned maize leaf mid-ribs in a critical point dryer using liquid carbon dioxide as the transitional fluid.

3.15 Mount the dried samples on aluminum stubs using high vacuum carbon tabs

3.16 Coat the maize leaf mid-ribs mounted on aluminum stubs with gold (approximately 20 nm thickness) in a sputter coater purged with argon gas.

3.17 Examine the coated samples in a JEOL JSM-6400V (lanthanum hexaboride electron emitter) scanning electron microscope.

3.18 Digital images were pictured using an analySIS Pro software (version 3.2).

4. Klason lignin measurement

4.1 Mill samples through a 2 mm (5/64") screen. Determine the moisture content of each sample using a moisture analyzer and record the value.

Note: Two-stage acid hydrolysis was performed based on National Renewable Energy Laboratory Technical Report³⁵.

4.2 Turn on incubator to 30 °C. Perform three replicates of each sample. Measure 0.3 g biomass into a screw-top high pressure tubes and record the weight to the nearest 0.1 mg. Add 3 ml of 72 % H₂SO₄ to each pressure tube.

4.3 Screw the stoppers onto the tubes and swirl a little bit to mix the contents. If the sample does not mix well, mix the sample using a glass stir rod. Leave the stir rod in the tube until water is added following incubation.

4.4 Place vials in the incubator for 60 min. Remove tubes from the incubator after 1 hour hydrolysis. Remove the stopper and add 84 ml of deionized water using an accurate pipette to dilute the acid concentration to 4%.

4.5 Tightly seal the stoppers on all vials. Place vials into a metal rack or large beakers and place in the autoclave. Set the autoclave to 121°C using a liquid sterilization cycle, and autoclave for 1 hour.

4.6 Carefully remove the tubes from the autoclave after 1 hr autoclave and allow them to cool to room temperature before opening.

Note: Acid insoluble lignin measurement was also performed based on National Renewable Energy Laboratory Technical Report ³⁵, with slight modifications.

4.7 Weigh one sheet of 47 mm, 0.22 µm pore-size, mixed-cellulose ester filter discs for each sample and record the weight.³²

4.8 Vacuum filter the solution from each tube through a separate filter disc. Rinse the particles from the tube onto the disc using deionized water.

4.9 Carefully remove the filter paper and residue from the unit and place inside of a desiccator overnight. Following drying, weigh the filter paper and residue for each replicate and record the weight.

4.10 Calculate the acid insoluble residue using the following equation:

$$\text{Acid insoluble lignin (\%)} = \frac{(\text{Mass of paper and residue} - \text{Mass of paper})}{\{\text{Total mass of biomass added} * (1 - \text{Moisture content})\}}$$

5. Carbohydrate analysis

5.1 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

Results Text

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 wild-type 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 (Fig 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 wild-type 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 legends

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.

Figure 5. Percent sugar (glucan and xylan) conversions for untreated (UT) and AFEXTM-pretreated (90 °C, 5min) maize stover at different concentrations of ammonia (1.0: 1.0g NH₃:g dry biomass 1.5: 1.5g NH₃:g dry biomass). Error bars represent the standard deviation of the mean and are based on two replicates for the untreated samples and four replicates (two pretreatment replicates with two hydrolysis replicates each) for the pretreated samples. Pretreated sugar conversions (24 h or 72 h) labeled with different letters are statistically different based on Tukey's pairwise comparisons ($P < 0.05$). This figure has been reused from Park et al (2012)¹⁸.

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 defective³⁶. The quantitative or qualitative changes of one cell wall carbohydrate component induces the alternation of other components³⁷. 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.

Acknowledgements

The microscopic imaging was conducted via the services of the Michigan State University Center for Advanced Microscopy. Maize callus was purchased from the Maize Transformation Center of Iowa State University. The authors would like to thank Jeffrey R. Weatherhead of the MSU Plant Research Laboratory for his technical assistance on the carbohydrate analysis. This research was generously funded by the Corn Marketing Program of Michigan (CMPM) and the Consortium for Plant Biotechnology Research (CPBR).

Disclosures

No conflicts of interest declared.

Table of Specific Reagents / Equipment

For brightfield microscopy, the images were recorded using a Zeiss (Jena, Germany) PASCAL confocal laser scanning microscope with a 488-nm excitation mirror, a 560-nm emission filter, and a 505 to 530-nm emission filter. Image analysis was performed using Laser scanning microscope PASCAL LSM version 3.0 SP3 software.

References

- 1 Perlack, R. D. *et al.* (ed USDA-DOE) (USDA-DOE, http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf, 2005).
- 2 Ralph, J., Grabber, J. H. & Hatfield, R. D. Lignin-Ferulate Cross-Links in Grasses - Active Incorporation of Ferulate Polysaccharide Esters into Ryegrass Lignins. *Carbohydrate research* **275**, 167-178, doi:Doi 10.1016/0008-6215(95)00237-N (1995).
- 3 Park, S.-H. *Expediting Cellulosic Biofuels Agenda: Production of High Value-Low Volume Co-Products and Lignin Down-Regulation of Bioenergy Crops* Ph.D. thesis, Michigan State University, (2011).
- 4 Boerjan, W., Ralph, J. & Baucher, M. Lignin biosynthesis. *Annual review of plant biology* **54**, 519-546, doi:10.1146/annurev.arplant.54.031902.134938 (2003).
- 5 Gibson, L. J. The hierarchical structure and mechanics of plant materials. *Journal of the Royal Society, Interface / the Royal Society* **9**, 2749-2766, doi:10.1098/rsif.2012.0341 (2012).
- 6 Dien, B. S. *et al.* Enhancing alfalfa conversion efficiencies for sugar recovery and ethanol production by altering lignin composition. *Bioresource technology* **102**, 6479-6486, doi:DOI 10.1016/j.biortech.2011.03.022 (2011).
- 7 Fu, C. X. *et al.* Downregulation of Cinnamyl Alcohol Dehydrogenase (CAD) Leads to Improved Saccharification Efficiency in Switchgrass. *Bioenerg Res* **4**, 153-164, doi:DOI 10.1007/s12155-010-9109-z (2011).
- 8 Grabber, J. H., Ralph, J., Hatfield, R. D. & Quideau, S. p-hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *Journal of agricultural and food chemistry* **45**, 2530-2532, doi:Doi 10.1021/Jf970029v (1997).
- 9 Li, X. *et al.* Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. *Biotechnology for biofuels* **3**, doi:Artn 27Doi 10.1186/1754-6834-3-27 (2010).
- 10 Mansfield, S. D., Kang, K. Y. & Chapple, C. Designed for deconstruction--poplar trees altered in cell wall lignification improve the efficacy of bioethanol production. *The New phytologist* **194**, 91-101, doi:10.1111/j.1469-8137.2011.04031.x (2012).
- 11 Studer, M. H. *et al.* Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 6300-6305, doi:DOI 10.1073/pnas.1009252108 (2011).
- 12 Chen, F. & Dixon, R. A. Lignin modification improves fermentable sugar yields for biofuel production. *Nature biotechnology* **25**, 759-761, doi:10.1038/nbt1316 (2007).
- 13 Ziebell, A. *et al.* Increase in 4-coumaryl alcohol units during lignification in alfalfa (*Medicago sativa*) alters the extractability and molecular weight of lignin. *The Journal of biological chemistry* **285**, 38961-38968 (2010).
- 14 Park, S.-H. *et al.* The quest for alternatives to microbial cellulase mix production: corn stover-produced heterologous multi-cellulases readily deconstruct lignocellulosic biomass into fermentable sugars. *Journal of Chemical Technology & Biotechnology* **86**, 633-641, doi:10.1002/jctb.2584 (2011).
- 15 Mol, J. N. *et al.* Regulation of plant gene expression by antisense RNA. *FEBS letters* **268**, 427-430 (1990).
- 16 Adamo, A. *et al.* Transgene-mediated cosuppression and RNA interference enhance germ-line apoptosis in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3440-3445, doi:10.1073/pnas.1107390109 (2012).

558 17 Smith, N. A. *et al.* Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320,
559 doi:10.1038/35030305 (2000).

560 18 Park, S.-H. *et al.* Downregulation of Maize Cinnamoyl-Coenzyme A Reductase via
561 RNA Interference Technology Causes Brown Midrib and Improves Ammonia Fiber Expansion-
562 Pretreated Conversion into Fermentable Sugars for Biofuels. *Crop Sci.* **52**, 2687-2701,
563 doi:10.2135/cropsci2012.04.0253 (2012).

564 19 Altpeter, F. *et al.* Particle bombardment and the genetic enhancement of crops: myths
565 and realities. *Mol Breeding* **15**, 305-327, doi:DOI 10.1007/s11032-004-8001-y (2005).

566 20 Pichon, M., Courbou, I., Beckert, M., Boudet, A. M. & Grima-Pettenati, J. Cloning and
567 characterization of two maize cDNAs encoding cinnamoyl-CoA reductase (CCR) and
568 differential expression of the corresponding genes. *Plant molecular biology* **38**, 671-676 (1998).

569 21 Mansoor, S., Amin, I., Hussain, M., Zafar, Y. & Briddon, R. W. Engineering novel traits
570 in plants through RNA interference. *Trends in plant science* **11**, 559-565, doi:DOI
571 10.1016/j.tplants.2006.09.010 (2006).

572 22 Hatfield, R. D., Jung, H.-J. G., Ralph, J., Buxton, D. R. & Weimer, P. J. A comparison
573 of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. *J*
574 *Sci Food Agr* **65**, 51-58, doi:10.1002/jsfa.2740650109 (1994).

575 23 Sluiter, J. B., Ruiz, R. O., Scarlata, C. J., Sluiter, A. D. & Templeton, D. W.
576 Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods.
577 *Journal of agricultural and food chemistry* **58**, 9043-9053, doi:10.1021/jf1008023 (2010).

578 24 NREL. Determination of structural carbohydrates and lignin in biomass : laboratory
579 analytical procedure (LAP). (2008).

580 25 Bate, N. J. *et al.* Quantitative Relationship between Phenylalanine Ammonia-Lyase
581 Levels and Phenylpropanoid Accumulation in Transgenic Tobacco Identifies a Rate-Determining
582 Step in Natural Product Synthesis. *Proceedings of the National Academy of Sciences of the*
583 *United States of America* **91**, 7608-7612, doi:DOI 10.1073/pnas.91.16.7608 (1994).

584 26 Hu, W. J. *et al.* Repression of lignin biosynthesis promotes cellulose accumulation and
585 growth in transgenic trees. *Nature biotechnology* **17**, 808-812, doi:10.1038/11758 (1999).

586 27 Lapierre, C. *et al.* Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar
587 lignins. *Phytochemistry* **65**, 313-321 (2004).

588 28 Updegraff, D. M. Semimicro determination of cellulose in biological materials. *Anal*
589 *Biochem* **32**, 420-424 (1969).

590 29 Yemm, E. W. & Willis, A. J. The estimation of carbohydrates in plant extracts by
591 anthrone. *The Biochemical journal* **57**, 508-514 (1954).

592 30 Filomena, A. P., Cherie, W., Geoffrey, B. F. & Antony, B. Determining the
593 polysaccharide composition of plant cell walls. *Nature protocols* **7**, 1590-1607,
594 doi:10.1038/nprot.2012.081 (2012).

595 31 Foster, C. E., Martin, T. M. & Pauly, M. Comprehensive Compositional Analysis of
596 Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *Journal of visualized*
597 *experiments : JoVE*, e1837, doi:doi:10.3791/1837 (2010).

598 32 Department of Agronomy, I. S. U. *Particle bombardment of Hi II immature zygotic*
599 *embryos and recovery of transgenic maize plants*, (2005).

600 33 BIO-RAD. *Biolistic® PDS-1000/He Particle Delivery System*, <[http://www.bio-](http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf)
601 [rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf](http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf)> (

602 34 *Particle bombardment of Hi II immature zygotic embryos and recovery of transgenic*
603 *maize plants*, (2005).

- 35 Sluiter, A. *et al.* Determination of Structural Carbohydrates and Lignin in Biomass;
Laboratory Analytical Procedure (LAP). (National Renewable Energy Laboratory, 2008).
- 36 Cano-Delgado, A., Penfield, S., Smith, C., Catley, M. & Bevan, M. Reduced cellulose
synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *The Plant journal*
: *for cell and molecular biology* **34**, 351-362 (2003).
- 37 Boudet, A. M., Kajita, S., Grima-Pettenati, J. & Goffner, D. Lignins and
lignocellulosics: a better control of synthesis for new and improved uses. *Trends in plant science*
8, 576-581, doi:10.1016/j.tplants.2003.10.001 (2003).

Figure
[Click here to download high resolution image](#)

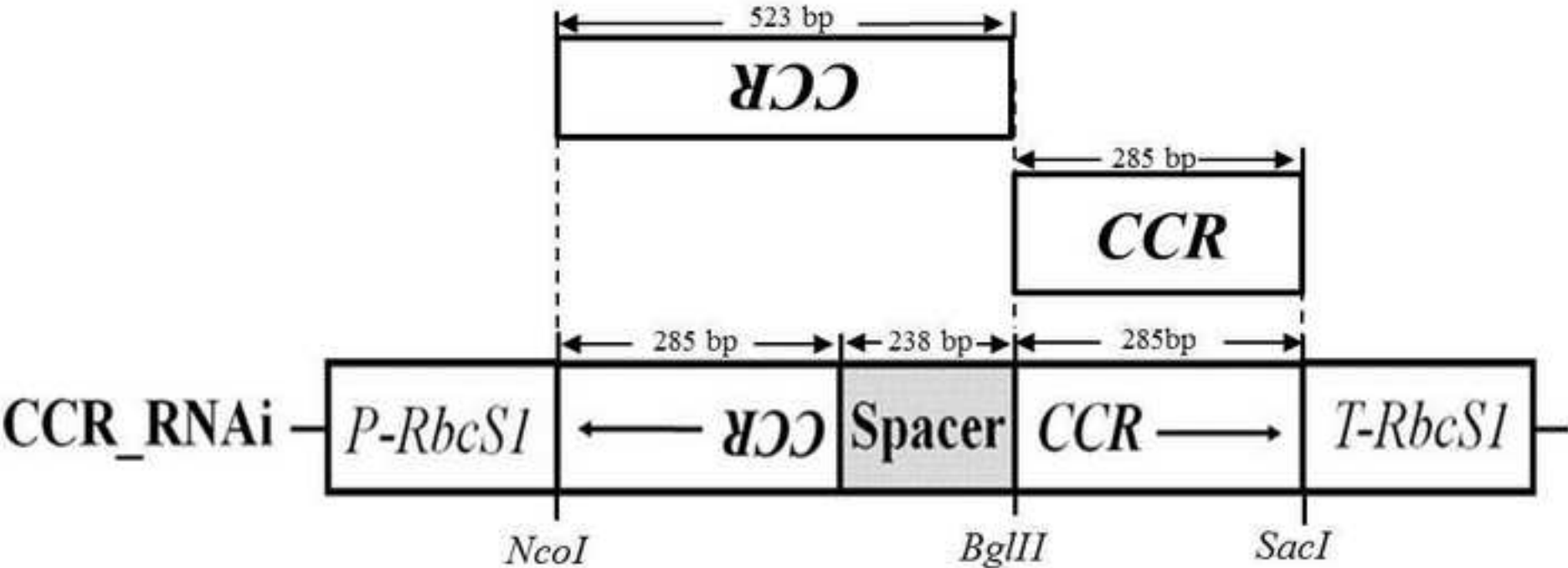
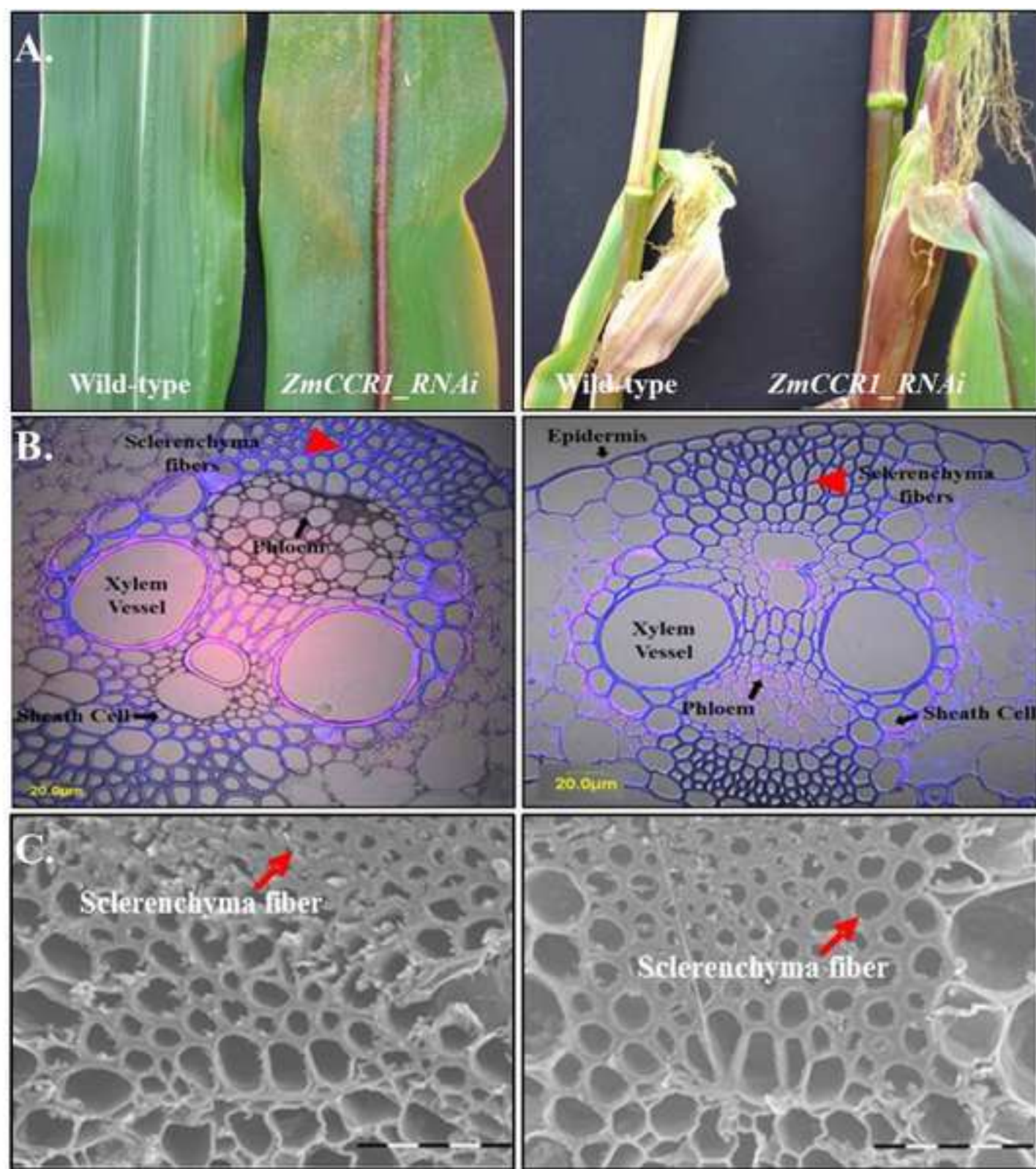


Figure
[Click here to download high resolution image](#)



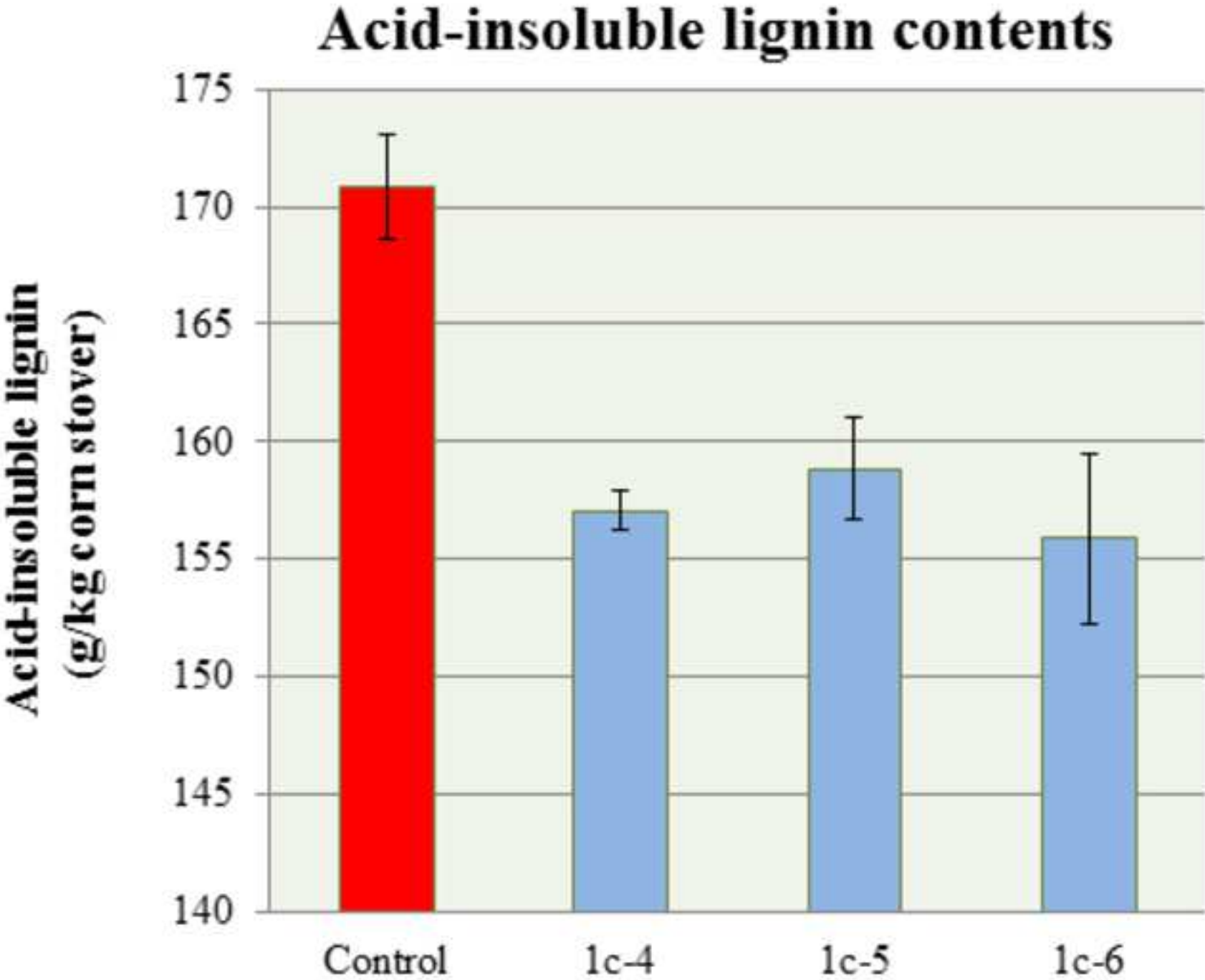
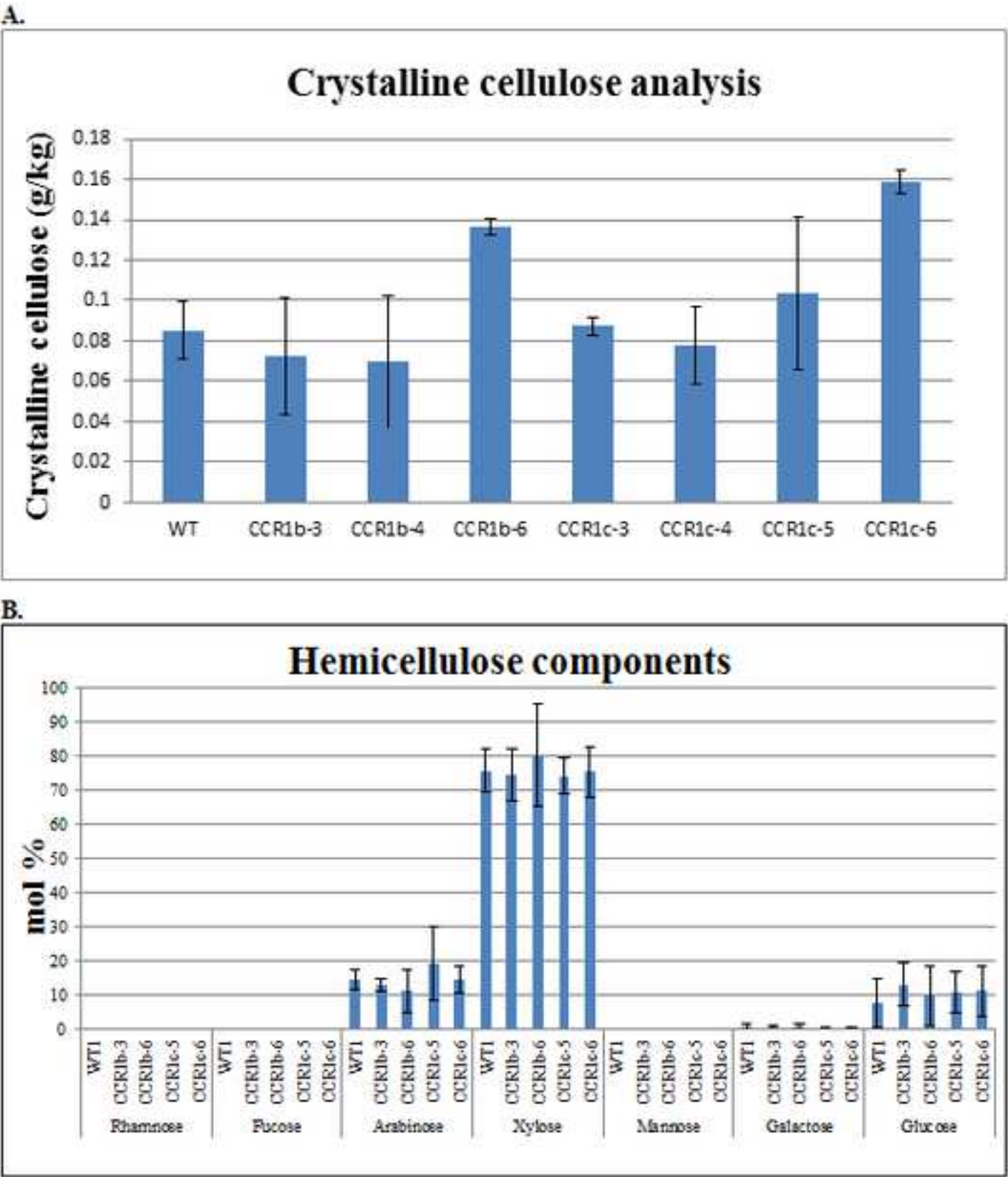


Figure
[Click here to download high resolution image](#)



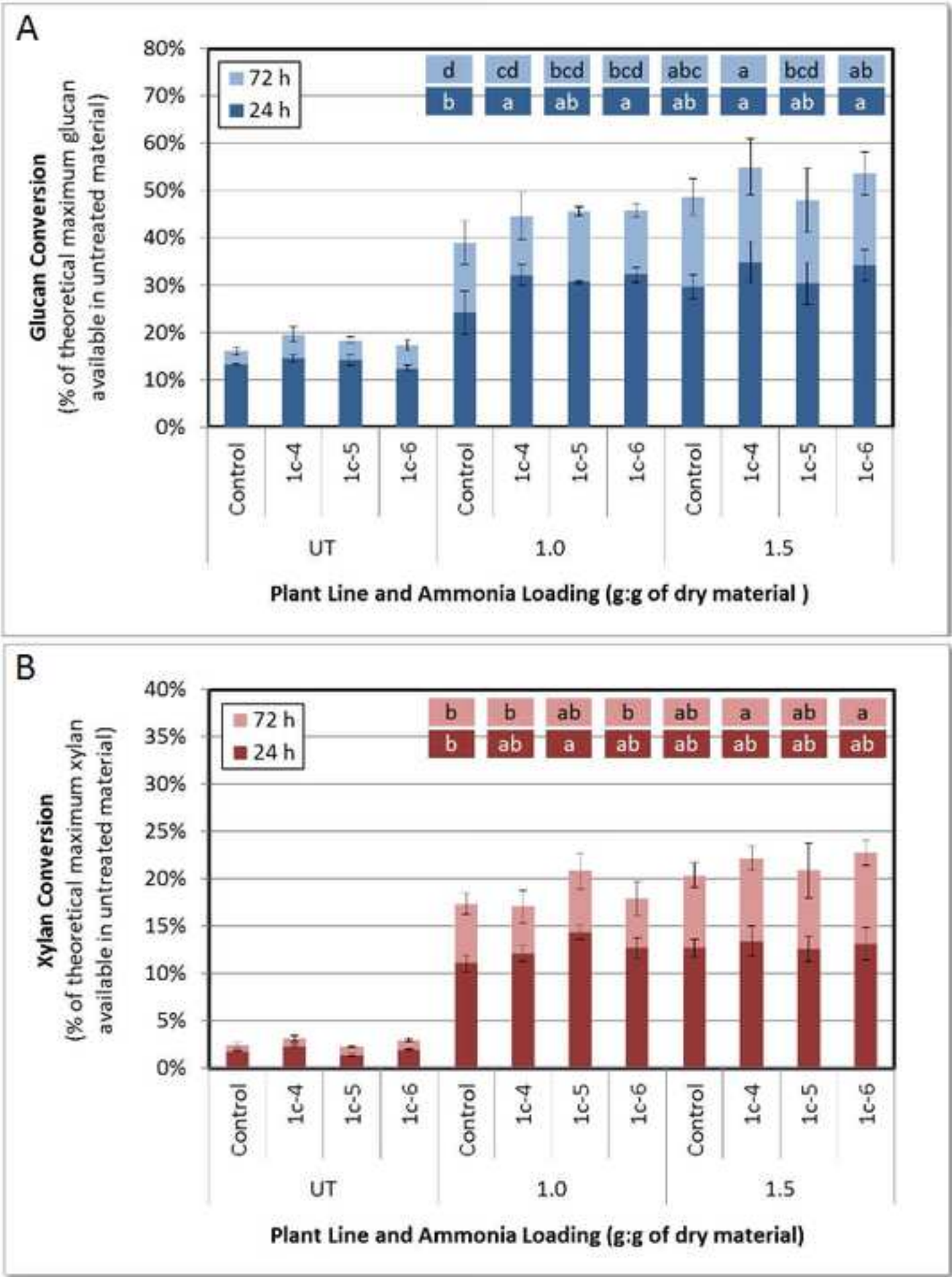


Table of Specific Materials
Media
N6OSM (Osmotic medium)
N6E (Callus induction)
N6S media (Selection media)
Regeneration medium
Rooting medium

Specific materials

Screw-top high pressure tubes

10% Neutral buffered formalin
(1 liter)

Chemical compositions

4 g/l N6 salts
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
2.5g/l gelrite, pH 5.8
Add filter sterilized silver nitrate (25uM) after autoclaving

4 g/l N6 salts
1 ml/l (1000X) 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
2.5g/l gelrite, pH 5.8.
Add filter sterilized silver nitrate (25uM) after autoclaving

4 g/l N6 salts
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
2.5g/l gelrite, pH 5.8
Add filter sterilized silver nitrate (25uM) after autoclaving

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 (100x25 mm petri-plates)
Add filter sterilized bialaphos (3 mg/L) added after autoclaving.

4.3 g/L MS salts
1 ml/L MS vitamin stock
100 mg/L myo-inositol
30 g/L sucrose

3g/L gelrite, pH 5.8 (100x25 mm petri-plates).

Pressure tube (#8648-27); Ace Glass, Vineland, NJ
Plug (#5845-47); Ace Glass, Vineland, NJ

100ml of formalin
900ml of ddH₂O
4.0 g of Sodium dihydrogen phosphate, monohydrate
(NaH₂PO₄.H₂O)

Equipments
Bio-Rad PSD-1000/He Particle Delivery device (Hercules, CA, United States)
Zeiss PASCAL confocal laser scanning microscope (Carl Zeiss, Jena, Germany)
Excelsior ES Tissue Processor (Thermo Scientific, Pittsburgh, PA, United States).
HistoCentre III Embedding Station (Thermo Scientific, Pittsburgh, PA, United States)
Microtome Model Reichert 2030 (Reichert, Depew, NY, United States)
Emscope Sputter Coater model SC 500 (Ashford, Kent, England)
JEOL JSM-6400V Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan)
Fitzpatrick JT-6 Homoloid mill; Continental Process Systems, Inc., Westmont, IL
MA35 Moisture Analyzer; Sartorius
Critical point dryer, Balzers CPD (Leica Microsystems Inc, Buffalo Grove, IL, United States)

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

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

Author(s):

Sang-Hyuck Park, Rebecca Garlock Ong, Chuansheng Mei, and Mariam Sticklen

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict

shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or

damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name:

Sang-Hyuck Park

Department:

The School of Plant Sciences

Institution:

University of Arizona

Article Title:

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

Signature:



Date:

01/27/2014

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy as a PDF to the JoVE submission site upon manuscript submission (preferred);
- 2) Fax the document to +1.866.381.2236; or
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

Dear Dr. Park,

Your manuscript JoVE51340 'Lignin Down-regulation of Zea mays and Cell Wall Compositional Analyses' has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

Editorial comments:

- 1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.
- 2) Prior to peer review, the protocol length is over our 3 page limit. Please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) to identify which portions of the procedure are most important to include in the video, i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. See JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
- 3) Section 1 would require extensive conceptualization in order to be filmed. If you want to include it in the video, then you will need to provide graphics to accompany that material.
- 4) If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the summary briefly mention the limitation of this gene disruption technique.

The limitations are mentioned in the introduction (line 101-102).

Major Concerns:

The lignin content in biomass is presented as g/kg of corn stover. However, when reporting the cellulose and hemicellulose content in the genetically modified maize plants (figure 2A and 2B), it is given as ng/mg of ?. It will be appropriate if the cellulose and hemicellulose content is presented as g/kg of corn stover.

The ng/mg has been converted to g/kg in Figure 4A.

Provide brief protocol about carbohydrate analysis instead of just referring to a manuscript.

Brief protocol summary has been described in the line 399-402.

Editorial comment:

[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments(use short steps, imperative tense, proper spacing, etc).]

The authors claim that down regulating lignin will increase the digestibility of corn stover. However, no experimental evidence is provided. Additional data (enzyme hydrolysis and sugar conversion data) to prove that the down regulation of lignin increase the biomass digestibility is necessary.

The digestibility test has been added as in Figure 5.

Picture of the maize plants (wild type versus down regulated plants should be provided). This will help people who read this article to visualize any physical changes in the plants and their respective growth.

The picture of the maize plants has been added to Figure 2.

Reviewer #2:

Manuscript Summary:

The manuscript entitled "Lignin Down-regulation of Zea mays and Cell Wall Compositional Analyses" describes the down-regulation of a lignin biosynthetic gene (CCR1) in maize using a dsRNAi approach. The authors describe a method for (i) construction of a dsRNAi construct, (ii) creation of transgenic maize plants by particle bombardment and (iii) analysis of cell wall structures in transgenic maize plants. Overall this article gives a detailed technical overview of the above mentioned methods and it will be of interest to many plant scientists working in the field of maize genetics and cell wall structure.

Minor Concerns:

To better reflect the focus of the manuscript, the authors might consider rephrasing the title and include keywords such as "dsRNAi", "particle bombardment" or similar in the title. This would help readers to immediately see which methods are described in the video.

The title has been changed to "Lignin Down-regulation of *Zea mays* via dsRNAi technique and Lignin Analysis"

The introduction gives background information for all methods described in the manuscript. However, it misses giving information about the SEM analysis mentioned in the manuscript. The Authors should add a brief paragraph about the SEM technique.

Brief SEM information has been added in lines 112 to 114.

1.1: The authors should briefly explain how the silencing sequence for the dsRNAi approach was selected and which rules need to be followed to obtain successful silencing. A reference might be useful for viewers, who are interested in this technique.

The reason chosen for the sequence is briefly added to the introduction. Please see the lines 120-123.

2.7, 2.9, 2.11: replace "ul" with "µl"

The units have been replaced.

3.1: Please list the composition of "10% neutral buffered formalin" in the "Table of specific material"

The compositions have been added to the "Table of specific material"

3.14: What kind of critical point dryer has been used, add to "Equipments" section

The information (Critical point dryer, Balzers CPD (Leica Microsystems Inc, Buffalo Grove, IL, United States) has been added to "Equipments" section.

4.1: What kind of mill has been used, add to "Equipments" section

The information (Fitzpatrick JT-6 Homoloid mill; Continental Process Systems, Inc., Westmont, IL) has been added to Equipments section.

4.2: What kind of moisture analyzer has been used, add to "Equipments" section

The information (MA35 Moisture Analyzer; Sartorius) has been added to Equipments section.

4.4: Please give information about the screw-top high pressure tubes in the "Table of specific material"

The information (#8648-27 Pressure tube with #5845-47 Plug; Ace Glass, Vineland, NJ) has been added to the 'Table of specific material'

Legend of figure 4: "gas Chromatography" should be "gas chromatography"

The "C" has been changed to "c"

Discussion, 4th paragraph, line 7: "chagnes" should be "changes"

The error has been fixed.

Figure 3 title: "Acid-Insuluble Lignin Contents" should be "Acid-Insoluble Lignin Contents"

The error has been fixed.

Reviewer #3:

1) As the paper describes a protocol for maize transformation, the reader could be interested in the transformation efficiency and or the ratio between stable and transient transformants using this protocol? It showed ~30% transformation efficiency. The ratio between stable and transient transformants was not measured in the original research (Lines 410-411).

2) Another interesting question is on the silencing stability in the plant and whether CCR is down over several generations? The silencing effects were consistently observed in several generations (T0-T2). Please see the lines 411-412.

Related to this question: Was transcription level of CCR checked in the different lines (based on the previous paper I know it was, but it is not mentioned here).

How many CCR genes do you have in maize, and do you target them all with the construct used? Two CCR genes (ZmCCR1 and ZmCCR2) were characterized in maize. ZmCCR1 was targeted for gene silencing in this paper. Please see the lines 116-117.

3) It could be wise to change the title. Especially since I'm not convinced the cell wall composition analysis was performed in an accurate way (for sure, "cell wall analysis" was not performed on all samples). Why not sticking to the transformation and lignin analysis? of course, the conclusions on the carbon flux could not be made in that case, but even with the presented data I'm not convinced it is appropriate to make this statement (see later)

The title has been changed to "Lignin Down-regulation of Zea mays via dsRNAi and Lignin Analysis"

4) Related to previous remark: At the end of the introduction, the authors mentioned "Here we describe a procedure for ? . ? and for analysis of the plant cell wall components of these materials." I have some problems with the last statement, as only the analysis of lignin is explained. For hemicellulose and cellulose only a reference is given. In line with previous remark I would suggest to remove point 5 (Carbohydrate analysis), as this is only a reference. Similar reference is given in the introduction. Also hemicellulose analysis is only briefly mentioned in the introduction, but not explained in the protocol-section.

Yes, the authors agree with the reviewer's comment, and the cell wall carbohydrate analysis part was removed from the last paragraph. Please see the lines 151-153.

5) Still on the cell wall analysis. Lignin was determined in following samples 1C4, 1C5, 1C6; cellulose measurements were done on the same and some additional samples; and hemicellulose still on a different subset. As a result for line 1B3 and 1B6 we only have cellulose data, and for 1B4 we have no lignin data. This makes it dangerous to make strong conclusions. For example, the conclusion that there is a carbon flow from lignin too cellulose is based on one of the seven ccr samples. And although we do have lignin and cellulose data for 1C4 and 1C5, we don't see this "carbon flow" from lignin towards cellulose. Maybe the authors put their statement a little too strong.

Yes, the authors agree with the comments. There was no direct evidence of shifting of carbon flow from lignin to cellulose. Thus the conclusion sentence in the long abstract has been changed. Please see lines 58-59.

6) For the protocol: specify the characteristics of the rupture disk (which pressure), and what distance was used?

The pressure and distance were added in the transformation protocol. Please see lines 242and 249.

7) The protocol jumps from 4.13 -> 4.18

The numbering has been fixed.

8) In the listed formula, it seems like the authors multiply by 1, which is most likely not the case. Brackets should be added to clarify this.

Brackets have been added to the formula.

9) The number of biological/ technical replicates should be mentioned.

The technical replicates have been added in Figure 3.

10) I wonder whether the units in fig4A are correct. Do you really find only 0.01% crystalline cellulose in the cell wall (or biomass.... It is not clear what the "mg" is referring to) or should ng be μg ?

The units have been changed to g/kg to be consistent with figure 3A. Based on our GC result, around 0.01% crystalline cellulose was measured in biomass.

11) As for all pictures and graphs, Fig4 (at least panel A) was published before, but that's not the point here. I have a problem with panel B. Not only is the variation extremely high, making it impossible to make the strong conclusion/statement made by the authors, that there is no effect on hemicellulose. Another issue of particular concern is the fact that these data are different from previously published values. In the original paper variation are much smaller.

The authors have replaced the Figure 4B to the previous one. Thank you for the correction.

12) It is not clear whether the different samples tested are different plants, different lines, different individuals, transformed with different construct... and what is the difference between "b" and "c" in the nomenclature.

The authors generated >30 1st generation (T0) transgenic maize. Among them, 3 transgenic lines showed significant ZmCCR1 transcript down-regulation and the lines have been annotated as 1a, 1b, and 1c. The progenies (T1) produced from T0 plants have been annotated as 1a-#, 1b-#, and 1c-#.

13) Rather than recycling figures, a new figure describing the cloning strategy could be added.

More cloning details have been added to Figure 1.

14) The first step of the bombardment needs some more explanation for a technical paper. "subculture and grow highly proliferating immature-embryo-derived Hi-II embryonic maize calli..." is rather abstract if you are not familiar with tissue cultures. How do you do this? medium? Conditions? Handling?

The authors appreciate the comments and we decided to remove the subculture part to put more focus on the bombardment procedures. Thank you.

Reprint permission

[Click here to download Supplemental File \(as requested by JoVE\): Reprint permission.docx](#)