Downregulation of Maize Cinnamoyl-Coenzyme A Reductase via RNA Interference Technology Causes Brown Midrib and Improves Ammonia Fiber Expansion-Pretreated Conversion into Fermentable Sugars for Biofuels

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ABSTRACT

Conversion of lignocellulosic biomass into fermentable sugars for biofuels requires expensive pretreatment processes involving the breakdown of the cell wall structure and/or removal of lignin to increase accessibility of enzymes to the crop structural carbohydrates. Lignin is synthesized from precursors through a complex biosynthesis pathway. One of the important enzymes in this pathway is cinnamoyl-coenzyme A reductase (CCR), which catalyzes the transformation of feruloyl and *p*-coumaryl thioesters to their respective aldehydes. In an attempt to reduce lignin content and potentially accelerate deconstruction of maize (*Zea mays* L.) stover structural carbohydrates into fermentable sugars, expression of maize CCR (*ZmCCR1*; EC 1.2.1.44) was downregulated via ribonucleic acid interference (RNAi). Thirty first generation independent *ZmCCR1_RNAi* transgenic lines were produced. Among 10 out of 30 randomly tested, six lines showed significantly reduced *ZmCCR1* transcription. The second generation of these *ZmCCR1* downregulated transgenic plants exhibited brown coloration of midribs, husk, and stems and 7.0 to 8.7% reduction in Klason lignin. Also, crystalline cellulose was slightly increased in the lignin downregulated maize stover and further increased conversion of the ammonia fiber expansion (AFEX)-pretreated maize stover into fermentable sugars. The third generation of CCR downregulated plants showed further reduced CCR transcription as compared to their second generation of transgenic (T1) plants.

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Abbreviations: 4CL, 4-coumarate:coenzyme A ligase; ADL, acid detergent lignin; AFEX, ammonia fiber expansion; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-coenzyme A reductase; cDNA, complementary DNA; CoA, coenzyme A; COMT, caffeic acid *O*-methyltransferase; FPU, filter paper units; G, guaiacyl; GC, gas chromatography; HPLC, high performance liquid chromatography; NDF, neutral detergent fiber; RNA, ribonucleic acid; PCR, polymerase chain reaction; RNAi, ribonucleic acid interference; S, syringyl; T0, first generation of transgenic; T1, second generation of transgenic; T2, third generation of transgenic.

The use of lignocellulosic materials has been strongly recommended for ethanol production (Wang et al., 2011). Unlike maize grain where ethanol is derived from a storage carbohydrate (starch), for lignocellulosics, ethanol is derived from the plant cell wall structural carbohydrates, crystalline cellulose and hemicellulose (Pauly and Keegstra, 2010). In addition to the structural sugars, between 12 and 28% of the cell wall comprises lignin (Pauly and

Published in Crop Sci. 52:2687–2701 (2012).
doi: 10.2135/cropsci2012.04.0253

Keegstra, 2008), a phenylpropanoid polymer that restricts enzymatic access to the structural carbohydrates.

Lignin is formed by oxidative radical coupling of various hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl) that are synthesized via the shikimic acid and phenylpropanoid pathway (Vanholme et al., 2010). Briefly, the three monolignols are produced by deamination of phenylalanine followed by successive hydroxylation of the aromatic ring and lastly phenolic *O*-methylation and simultaneous conversion of the carboxyl to an alcohol moiety. It has long been believed that the hydroxylation and methylation reactions take place at the level of the cinnamic acid and that *p*-coumaric, ferulic, and sinapic acids are subsequently converted to the corresponding monolignols by the sequential action of 4-coumarate:coenzyme A ligase (4CL), cinnamoyl-coenzyme A reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) (Bonawitz and Chapple, 2010; Vanholme et al., 2010; Weng and Chapple, 2010; Whetten and Sederoff, 1995). Most of the enzymes and intermediates described above are differentially expressed in response to environmental cues (Lauvergeat et al., 2001), exhibiting different kinetics and substrate preferences (Harding et al., 2002).

In grasses such as maize, lignin is covalently linked to the hemicellulose glucurono-arabinoxylan via ferulate and diferulate bridges (de Buanafina, 2009; Hatfield et al., 1999; Ralph et al., 1995). In turn, the hemicelluloses are noncovalently associated with cellulose microfibrils via hydrogen bonds and van der Waals forces. These interactions result in a complex cell wall material that provides integrity and strength to both the plant cell and the plant itself. One factor that influences the mechanical strength of the walls is the type of lignin subunits and their ratios within the lignin polymer. For example, a higher syringyl (S) content as found in hardwood cell walls increases the relative stiffness (Koehler and Telewski, 2006; Reddy et al., 2005). The sheathing of the polysaccharides in the walls by lignin is considered a major impediment in the paper, pulp, and biofuels industries as it interferes with the enzyme accessibility necessary for the hydrolysis of cellulose and hemicelluloses (Vermerris et al., 2010). Hence, a major hurdle in the economical and sustainable conversion of a lignocellulosic feedstock is the ease with which it is possible to chemically or physically open up the structure of the cell wall so that the hydrolytic enzymes are able to access the biomass polysaccharides (Chundawat et al., 2010; Eggeman and Elander, 2005; Sticklen, 2008). One approach to mitigate this hurdle is by altering or reducing the lignin content or composition in the plant using plant breeding or transgenic techniques (Chen and Dixon, 2007; Hisano et al., 2009; Vanholme et al., 2008).

A large number of studies have been conducted in recent years that have analyzed the impact of altering the expression of the lignin biosynthetic enzymes on plant growth, lignin content and composition, and cell wall digestibility (ruminant or enzymatic) (Baucher et al., 2003; Hisano et al., 2009; Jung et al., 2012; Li et al., 2008; Vanholme et al., 2008). Reductions in lignin content can have a major negative impact on plant growth and development (Vermerris et al., 2010); however, it also typically has a positive impact on cell wall digestibility. A recent report showed that the ruminant digestibility of cell wall polysaccharides was improved in CAD downregulated alfalfa (*Medicago sativa* L.), suggesting that cell wall digestibility is inversely correlated with the level of lignification (Jung et al., 2011; Sticklen, 2007). Another study suggested that lignin reduction in alfalfa could bypass the need for dilute sulfuric acid pretreatment, thereby facilitating the conversion of lignocellulosic biomass into glucose with no pretreatment costs (Chen and Dixon, 2007). This research was followed by another study that reported a 30 to50% lignin reduction in alfalfa resulting on a great increase in the efficiency of saccharification (Ziebell et al., 2010). Unlike the impacts due to total lignin content, there is no clear trend with respect to the impact of lignin monomer composition on digestibility. Fu et al. (2011) found a negative correlation between S:guaiacyl (G) ratio and total sugar release; however, they determined that total lignin content was the main factor influencing sugar yields from CAD downregulated switchgrass (*Panicum virgatum* L.) (Fu et al., 2011). Another study on reduced S-lignin content alfalfa due to caffeic acid *O*-methyltransferase (COMT) downregulation showed an increase in yields following dilute ammonia pretreatment (Dien et al., 2011) while *Arabidopsis thaliana* (L.) Heynh. cell walls with S-rich lignin enhanced a pretreatment process (Li et al., 2010). Also, a study on this model plant found no impact of monomer composition on cell wall enzymatic degradation (Grabber et al., 1997); however, these model plant cell walls may have limitations in interpretations as to how accurately they represent real crop feedstock materials. Within the lignin biosynthetic pathway CCR catalyzes the conversion of the three hydroxycinnamoyl-coenzyme A (CoA) esters (*p*-coumaroyl-CoA, feruloyl-CoA, and caffeoyl-CoA) into their corresponding cinnamyl aldehydes. Two maize CCR genes have been found within the maize genome, cloned, and characterized: *ZmCCR1* (preferentially expressed in all lignifying tissues) and *ZmCCR2* (mostly expressed in roots and also induced by drought condition) (Fan et al., 2006; Pichon et al., 1998). Six other putative CCR genes have been found and annotated in maize (Guillaumie et al., 2007). In the last decade, several CCR downregulations have been successfully made in different plants. In maize, *ZmCCR1* mutants shows 31% reduction in lignin and significant changes in lignin structure, resulting in decrease in *p*-hydroxyphenyl units and a simultaneous increase in the ratio of S:G (Tamasloukht et al., 2011). Another study showed that downregulation of CCR in transgenic poplar (*Populus tremula* L. × *Populus alba* L.) revealed up to 50%

lignin reduction with an increased proportion of cellulose in transgenic plants (Leple et al., 2007). Leple et al. (2007) specifically state that the apparent increase in cellulose content is likely due to the loss of lignin, not necessarily because more cellulose is deposited. Other studies on *Arabidopsis thaliana* (Goujon et al., 2003), tomato (*Solanum lycopersicum* L.) (van der Rest et al., 2006), and pulpwood tree [*Leucaena leucocephala* (Lam.) de Wit] (Prashant et al., 2011) have proven that lignification of plant cell wall is positively correlated with the level of CCR gene expression.

There are a number of naturally occurring crop mutants with altered lignins. The most well-known examples are the brown-midrib (*bm*) maize and sorghum [*Sorghum bicolor* (L.) Moench] (*bmr*) mutants. Twelve brown midrib loci have been identified to date: five in maize (*bm1–bm5*) and four in sorghum (*bmr-2*, *bmr-6*, *bmr-12*, and *bmr-19*) (Sattler et al., 2010). In the *bm1* and *bmr6* mutants, the CAD gene is mutated (Halpin et al., 1998) while the *bm3* and *bmr12* mutants contain a mutation in the COMT gene (Zuber et al., 1977). The locations of the five remaining brown midrib loci are currently unknown (Sattler et al., 2010). However, a gene-specific maize macroarray demonstrated that in the *bm2* mutant, genes that are closely associated with several functional groups, including phenylpropanoid metabolism, transport, and trafficking as well as transcription factors and regulatory genes, are downregulated at the transcriptional level (Guillaumie et al., 2007). Since the *bm2* mutant contains lower G monolignol content, it has been hypothesized that the *bm2* mutation could affect the regulation of coniferaldehyde, the precursor for G units in lignin.

The research presented here is aimed to lower the lignin content and/or change lignin composition in maize by reducing the expression of a CCR (*ZmCCR1*; E.C.1.2.1.44; accession no. X98083). Since genetic redundancy might present an issue in this gene family, a ribonucleic acid interference (RNAi) approach was used in an attempt to reduce the prevalent forms of the *ZmCCR1*. Microscopy and cell wall composition techniques were used to determine the impact of the transgenic modifications on cell wall structure and the relative amounts of cell wall components. Three of the independent transgenic lines and one control line were subjected to ammonia fiber expansion (AFEX) pretreatment followed by enzymatic hydrolysis to determine the impact of the lignin modifications on the enzymatic hydrolysis of maize stover.

MATERIALS AND METHODS

Construction of *ZmCCR1_RNAi*

The maize *CCR1* gene (accession no. X98083) was selected for downregulation. The complementary DNA (cDNA) clone for *ZmCCR1* (X98083) was purchased from Arizona Genome Institute. The C-terminal sequence of *ZmCCR1* was chosen to design the double-stranded RNAi construct because of its specific sequence. Two pairs of primers were designed to polymerase chain reaction (PCR)-amplify two fragments for making the RNAi construct. The larger fragment (523 bp) was PCR amplified from *ZmCCR1* cDNA 748 to 1271 bp using two primers that were flanked with a restriction enzyme recognition sites for *Bgl*II and *Nco*I (underlined), ZmCCR1_748F_BglII (5′-AGATCTACATCCTCAAGTACCTGGAC-3′) and ZmCCR1_1271R_NcoI (5′-CCATGGTTTACACAGCAGGGGAAGGT-3′). The smaller fragment (285 bp) was PCR amplified from ZmCCR1 cDNA 986 to 1271 bp using ZmCCR1_986F_BglII (5′-AGATCTGGAAGCAGCCGTACAAGTTC-3′) and ZmCCR1_1271R_SacI (5′-GAGCTCTTTACACAGCAGGGGAAGGT-3′) with *Bgl*II in the forward primer and *Sac*I in the reverse primer (underlined). The amplified larger and smaller fragments were then subcloned into a pGEM-T Easy vector (Promega) following the manufacturer's instructions. The pGEM-T Easy vector containing the large fragment was digested with *Bgl*II and *Nco*I and then the excised fragment was cloned into an ImpactVector 1.1 (cytoplasm expression) (Plant Research International) containing a green-tissue specific promoter (Rubisco). Then the pGEM-T Easy vector containing the smaller fragment was digested with *Bgl*II and *Sac*I and cloned into the ImpactVector 1.1 that contained the large fragment. This formed the *ZmCCR1* RNAi construct that had a 285 bp inverted repeat sequence with a ~238 bp spacer in the middle of the inverted repeat fragments (Fig. 1).

Maize Genetic Transformation

Highly proliferating, immature-embryo-derived embryogenic Hi-II (hybrid) maize calli were co-bombarded using the Bio-Rad PSD-1000/He Particle Delivery device with a 1:1 ratio of the *ZmRNAi_RNAi* plasmid and the pDM302 plasmid, (McElroy et al., 1990) which contains the *bar* selectable marker gene encoding phosphinothricin acetyltransferase regulated by the rice (*Oryza sativa* L.) actin 1 (*Act1*) promoter and *nos* terminator. Using in vitro culture, phosphinothricin resistant callus was selected and regenerated into plantlets following standard procedures (Biswas et al., 2006). The herbicide resistant plantlets were acclimated in a growth chamber and then transferred to a greenhouse where they grew until maturity. All first generation of transgenic (T0), second generation of transgenic (T1), third generation of transgenic (T2), and wild-type control plants were grown under the same greenhouse conditions of 23°C and 16/8 h light/dark period. Fertile plants were self-pollinated and seeds were harvested when they had reached maturity, 35 to 45 d after pollination.

Transgene Integration and Transcription Analyses and Breeding of Transgenic Plants

Polymerase chain reaction analysis was performed using Eppendorf AG Thermocycler on both T0 and T1 plants to confirm the presence of the *bar* transgene. Ribonucleic acid (RNA) gel blotting was performed to confirm transcription expression levels of *ZmCCR1* gene in transgenic plants. Total RNA was isolated from putative transgenic and wild-type untransformed plants using Trizol reagent following the manufacturer's instructions (Invitrogen). The RNA gel blot analysis was performed based on the authors' previous work (Biswas et al., 2006). Transgenic

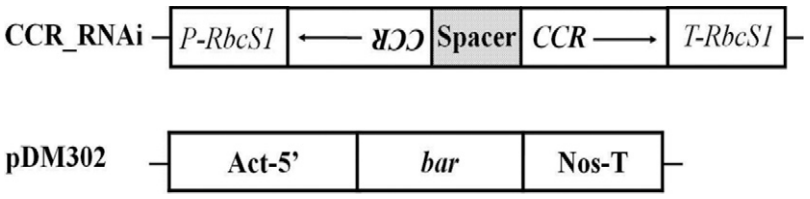


Figure 1. Ribonucleic acid interference plasmid constructs for the downregulation of the ZmCCR1 in Impactvector1.1. P-RbcS1: ribulose bisphosphate carboxylase promoter from Asteraceous chrysanthemum (*Chrysanthemum* × *morifolium* Ramat.). T-RbcS1: ribulose bisphosphate carboxylase small unit terminator from Asteraceous chrysanthemum. pDM302: a construct containing the bar gene selectable marker sequences regulated by rice actin promoter and nos terminator (McElroy et al., 1990).

plants were self-bred for development of new generation plants, and seeds were collected and tested again via northern blotting for segregation analysis of CCR downregulation. Maize breeding followed our previous research (Biswas et al., 2006).

Histological Assay

Identical small sections from *ZmCCR1* transgenic and wild-type plant leaf midribs were examined using a histological assay. Briefly, the sections were fixed in 10% neutral buffered formalin, processed using an automated vacuum infiltration tissue processor, embedded in paraffin, and sectioned using a rotary microtome at 4 to 5 μm. Sections were then placed on adhesive slides and dried at 56°C. The dried sections were deparaffinized using xylene [$C_6H_4(CH_3)_2$] and hydrated using distilled water and then stained with 0.05% toluidine blue O to visualize the lignified cell wall areas.

Brightfield Microscopy

Brightfield images were collected on an Olympus IX81 inverted microscope (Olympus Corporation) configured with an MBF Bioscience CX9000 camera using the 20x UPlanFL N (0.5 numerical aperture) air and the 40x UPlanFL N (1.3 numerical aperture) oil objectives.

The images were recorded using a Zeiss PASCAL confocal laser scanning microscope with a 488 nm excitation filter, a 560 nm emission filter, and a 505 to 530 nm emission filter. Image analysis was performed using the laser scanning microscope PASCAL LSM version 3.0 SP3 software (Carl Zeiss, 2009).

Scanning Electron Microscopy

Samples were fixed at 4°C for 1 to 2 h in 4% glutaraldehyde buffered with 0.1 M Na_3PO_4 (sodium phosphate) at pH 7.4. Following a brief rinse in the buffer, samples were sequentially dehydrated in an ethanol series (25, 50, 75, and 95%) for 10 to 15 min each and then three times (10 min each) in 100% ethanol. Samples were dried in a Balzers Model 010 critical point dryer (Balzers Union Ltd.) using liquid CO_2 as the transitional fluid. Samples were mounted on Al stubs using high vacuum C tabs (SPI Supplies). After mounting, samples were coated with Au (approximately 20 nm thickness) in an Emscope Sputter Coater model SC 500 (Ashford) that was purged with Ar gas and examined in a JEOL JSM-6400V (lanthanum hexaboride electron emitter) scanning electron microscope (JEOL Ltd.). Digital images were acquired using Analysis Pro Software version 3.2 (Olympus, 2008).

Klason Lignin and Structural Sugar Analysis

The Klason lignin content and the glucan and xylan contents that were used for setting up the enzymatic hydrolysis experiments were determined using the standard National Renewable Energy Laboratory protocol (Sluiter et al., 2008). Because of small sample sizes, the material was not extracted before performing acid hydrolysis. The acid insoluble lignin analysis method was modified to use 47-mm 0.22-μm pore-size mixed-cellulose ester filter discs (Millipore Corp.) during the filtration step instead of crucibles. These discs with the filtered lignin residue were dried overnight in a desiccator before weighing. Because an extraction was not performed before acid hydrolysis and soluble sugars would interfere with the actual glucan and xylan contents, soluble glucose and xylose in the control sample were quantified as the sugars released following 72 h washing at 50°C and 200 revolutions per min^{-1} and measured by high performance liquid chromatography (HPLC) as reported in the section on enzymatic hydrolysis. These values were subtracted from the sugars released from two-stage acid hydrolysis to give the structural glucan and xylan content of the maize stover samples.

Crystalline Cellulose Assay

To determine whether crystalline cellulose production is increased when lignin content is decreased in *ZmCCR1* downregulated transgenic maize plants, the amount of crystalline cellulose present in the samples was determined using the Updegraff method (Updegraff, 1969).

Hemicellulose Composition Analysis

The monosaccharide composition of the hemicelluloses was determined as described (Foster et al., 2010). In brief, alcohol insoluble residue was prepared from freeze-dried plant material. The material was then hydrolyzed with trifuoro-acetic acid and the solubilized monosaccharides derivatized to their corresponding alditol acetates. These volatile derivatives were analyzed by a gas chromatography (GC) connected to a quadrupole mass spectrometer.

Gas chromatography analysis was performed to examine the changes in hemicellulose components in T1 *ZmCCR_RNAi* lines. Three milligrams of maize midribs were freeze-dried and hydrolyzed with trifluoroacetic acid. After acid hydrolysis, the soluble parts were reduced by adding sodium borohydrate and acetylated by adding acetic anhydride.

AFEX Pretreatment and Enzymatic Hydrolysis

Before enzymatic hydrolysis, AFEX pretreatment method of the milled maize stover was conducted at 90°C on a 0.6 g H_2O g^{-1} dry biomass for a 5 min residence time following heat-up to the set temperature. Two NH_3 (ammonia) loadings were tested for each sample: 1.0 and 1.5 g NH_3 g^{-1} dry biomass. Ammonia fiber expansion was conducted as detailed previously (Garlock et al., 2009).

Enzymatic hydrolysis was conducted in duplicate in 20 mL screw-cap vials at 1% glucan loading with a total volume of 15 mL for each of the AFEX-pretreated and untreated control samples. Samples were adjusted to pH 4.8 using a 1 M citrate buffer solution. Accelerase 1000 (61 mg mL^{-1}; Genencor Division of Danisco US, Inc.) cellulase was added at 5.5 mg protein g^{-1} glucan (2.4 FPU [filter paper units] and 49 birch (*Betula alleghaniensis* Britton) xylan FPU per gram glucan) (Fujii et al., 2009) and Multifect Xylanase (27 mg mL^{-1}; Genencor Division of Danisco US, Inc.) at 0.5 mg protein g^{-1} glucan (0.01 FPU and 467 oat (*Avena sativa* L. [syn. *Avena sativa* var. *orientalis* (Schreb.) Hook. f.])–spelt {*Triticum aestivum* L. subsp. *spelta* (L.) Thell. [syn. *Triticum aestivum* L. var. *spelta* (L.) Fiori]} xylan FPU per gram glucan) (Dien et al., 2008) were loaded. A lower enzyme loading was chosen compared to the industry standard to exhibit greater differences between the transgenic and control samples. Samples were placed in a New Brunswick Scientific (New Jersey) incubator shaker and hydrolyzed at 50°C and 200 revolutions per min^{-1} for 72 h. The samples were taken at 24 and 72 h hydrolysis, heated at 90°C for 15 min, cooled, and centrifuged at 21,952 × *g* for 5 min. The supernatant was filtered into HPLC shell vials using a 25 mm diameter 0.2-μm pore size polyethersulfone syringe filter (Whatman Inc.). Samples were then stored at −20°C until sugar analysis following a standard method (Garlock et al., 2009).

An HPLC system was used to determine the monomeric glucose and xylose concentrations of each sample following enzymatic hydrolysis. This HPLC system consisted of a Waters pump, auto-sampler, and Waters 410 refractive index detector equipped with a Bio-Rad Aminex HPX-87P carbohydrate analysis column with an attached de-ashing guard column. Degassed HPLC grade water was used as the mobile phase, at 0.6 mL min^{-1}, with the column temperature set at 85°C. Injection volume was 10 μL with a run time of 20 min per sample. Mixed sugar standards were used to quantify the amount of monomeric glucose and xylose in each hydrolysate sample.

Statistical Analysis

Statistical analyses of lignin and structural sugar contents and glucan and xylan conversions following pretreatment and enzymatic hydrolysis were conducted using Minitab15 Statistical Software (Minitab, 2006). Student's *t* tests were conducted on the structural sugar (glucan and xylan) and lignin contents and evaluated with respect to the control based on the 95% confidence interval around the mean. The percentage of lignin reduction in each transgenic line was calculated versus lignin content measured in wild-type control plants. General linear model ANOVAs were conducted on the 24 and 72 h glucose and xylose conversions from pretreated maize stover with line, pretreatment, and line × pretreatment effects. Letters were used to indicate statistically different sugar conversions based on Tukey's pairwise comparisons ($\alpha < 0.05$). Pearson correlations were conducted to determine possible correlations between sugar conversions and lignin content.

Preliminary Test on In Vitro Neutral Detergent Fiber Ruminant Digestibility

Seeds of the second generation CCR downregulated plants were grown to maturity and the leaf samples of the T2 plants were tested for transcription levels via RNA blotting. Then 92% dry leaves and husks of plants with lowest transcription levels were tested for a neutral detergent fiber (NDF) 30 h in vitro digestibility of the 92% dry CCR downregulated versus a wild-type control sample. This test was performed by the Cumberland Valley Analytical Services.

RESULTS AND DISCUSSION

Regeneration of *ZmCCR1_RNAi* Transgenic Plants

A total of 30 mature independent *ZmCCR1_RNAi* transgenic maize lines (T0) were produced showing the *bar* gene integration. Among them, 10 independent transgenic lines were examined for their *ZmCCR1* transcription levels. The results showed that 6 out of 10 lines (*ZmCCR1_RNAi*-1a, *ZmCCR1_RNAi*-1b, *ZmCCR1_RNAi*-1c, *ZmCCR1_RNAi*-4c, *ZmCCR1_RNAi*-6a, and *ZmCCR1_RNA*-6b) had significantly reduced *ZmCCR1* gene expression levels (Fig. 2A).

Over 100 generations (T1) of *ZmCCR1_RNAi* plants were obtained as a result of self-pollination of the T0 plants of *ZmCCR1_RNA*-1b and *ZmCCR1_RNA*-1c lines. When RNA blot (Northern blot) hybridization was performed to examine whether the *ZmCCR1* downregulation was vertically transmitted to the next generation, several different T1 lines, *ZmCCR1_RNAi*-1b (27, 31, 34, 39, 44, 46, 47, and 48) and *ZmCCR1_RNAi*-1c (2, 3, 4, 5, 6, 10, 16, 19, and 24), showed a significant transcriptional reduction of *ZmCCR1* gene. Figure 2B shows both the range of downregulation of the *ZmCCR1* gene in T1 generations and some of the progenies with significantly reduced levels of *ZmCCR1* transcription. Also, the presence of the *bar* gene was confirmed in all *ZmCCR1_RNAi* maize lines tested (data not shown).

Phenotypic Analysis

The seed germination, growth, and development of downregulated *ZmCCR1* T0, T1, and T2 lines were visually monitored and no detrimental effects were observed under either the growth chamber or greenhouse conditions. Table 1 shows plant height and stem diameters at 13 cm from soil surface of T2 lines versus those of wild-type control plants. There was no significant phenotypic difference other than colorations between wild-type and downregulated *ZmCCR1* plants, implying that low level downregulation of *ZmCCR1* and lignin reduction presented in this report might not have a significant effect on maize stover biomass.

When compared to wild-type nontransgenic plants, the T1 and T2 *ZmCCR1_RNAi* transgenic lines appeared to be normal in their seed germination, growth, and development. However, about 5% of T1 plants, which may had carried *ZmCCR_RNAi* homozygous recessive alleles, were stunted with curly leaves and aborted early flowering. These abnormalities of the T1 plants could be

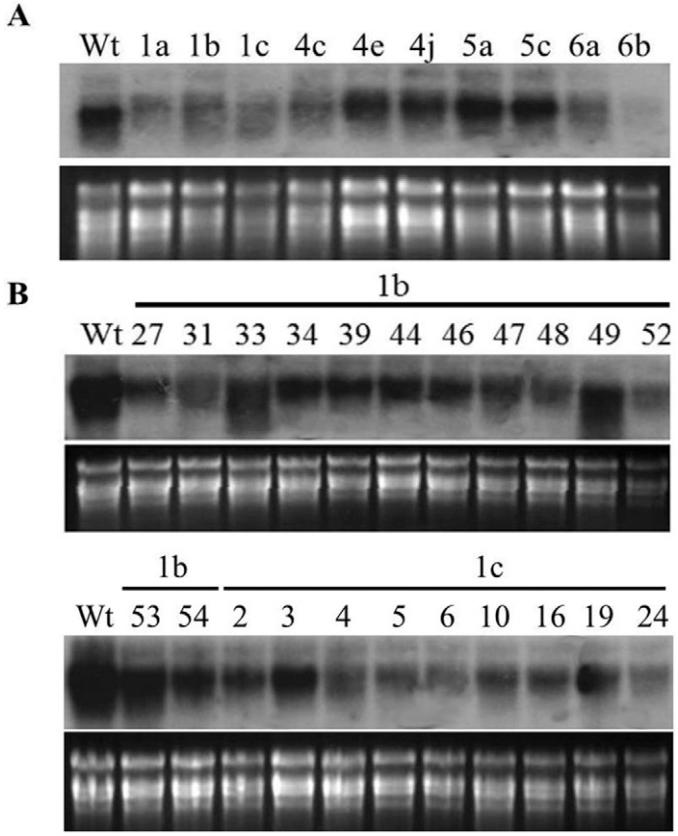


Figure 2. Transcriptional analysis of wild-type (Wt) corn and *ZmCCR1_RNAi* transgenic corn lines. A. Downregulation of *ZmCCR1* transcripts on first generation of transgenic (T0) corn. B. Downregulation of *ZmCCR1* transcripts on second generation of transgenic (T1) corn. Ribonucleic acid (RNA) loading control is located underneath of each RNA gel (15 µg of total RNA).

Table 1. Comparison of plant height and diameter between wild-type maize and *ZmCCR1_RNAi*-1c third generation of transgenic (T2) lines. Plant height was measured from the soil base line to the top of the tassel and stem diameter was measured 13 cm distal from the soil base line. Mean ± standard deviation ($P > 0.05$).

	Wild-type (Hi-II)	*ZmCCR1_RNAi*-1c
Height, cm	153.92 ± 6.37 (n = 5)	159.2 ± 14.41 (n = 8)
Diameter, cm	2.78 ± 0.82 (n = 5)	2.26 ± 0.36 (n = 8)

due to either higher level of lignin reduction (Vanholme et al., 2010) or somaclonal variations, which are common in maize tissue culture.

Six out of 20 of T1 lines (30%) showed phenotypic variation with brown coloration in their leaf midribs, stems, and husks without any other apparent abnormalities while none of the T0 plants showed brown colorations (Fig. 3). The segregation data for midribs brown pigmentation in T1 *ZmCCR1_RNAi* transgenic lines supports a previous report indicating that the *CCR1* allele is a recessive trait (Cherney et al., 1991). Unlike the naturally occurring *bm* mutants of maize, where brown coloration is typically localized to the leaf midribs, the brown coloration seen in the maize CCR1 transgenic lines also occurred in the stems and husks (Fig. 3).

Other studies on poplar (*Populus tremuloides* Michx.) (Leple et al., 2007) and tobacco (*Nicotiana tabacum* L.) (Chabannes et al., 2001) also showed brown coloration in xylems of lignin downregulated transgenic lines. It is well known that the intermediates (e.g., phenylalmine) involved in lignin biosynthesis pathways are closely associated with anthocyanin synthesis (Whetten and Sederoff, 1995) and brown midribs coloration is a phenomenon that results when certain lignin biosynthesis-related genes are defective or less functional (Ambavaram et al., 2010; Voelker et al., 2010, 2011). The discoloration is a result of the oxidization of accumulated soluble phenolic compounds that form brown polymers (Ke and Saltveit, 1988). Therefore, we believe that the brown coloration in lignin downregulated *ZmCCR1_RNAi* transgenic plants is due to a buildup of phenolic precursors.

Histological Assay

Histological assays were performed on lignified tissues of wild-type and the T1 plant leaf midribs of 1c line that showed low CCR transcription. The main vascular system (i.e., xylem vessel, phloem, and sheath cells) showed no significant differences between two samples except the sclerenchyma fiber. The cross section of a wild-type leaf midrib shows much thicker sclerenchyma fiber cell walls than that of the downregulated *CCR1* transgenics (Fig. 3). This assay demonstrates that although the *ZmCCR1* downregulated plants contain fewer fibers in their sclerenchyma cell walls, xylem vessel and phloem cells are not defected in structure. It suggests that vascular system functions normally without any defects in water and nutrient transmission. However, there might be possible reduction of physical strength of the stems. In the greenhouse condition, no structural damage was observed but field testing might be required to measure *CCR1* mutant stem rigidity and resistance to lodging as the result of wind.

Figure 4 shows scanning electron microscope images of *ZmCCR1* downregulated maize midribs compared with that of wild-type. No differences were found in major xylem vessel, phloem, and sheath cells in both maize lines. However, the sclerenchyma fibers in *ZmCCR1* downregulated leaves showed relatively thinner cell walls (red arrow in right bottom) compared to that of wild-type control plant sample (red arrow in left bottom). This also indicates that the downregulation of *ZmCCR1* via RNAi reduced lignin contents might have only occurred in sclerenchyma fiber cells. Although lignin reduction might cause structural and/or defense damages to plants, one might wish to reduce lignin further by downregulating more than one lignin biosynthesis pathway enzymes. For example, a research on double mutations of CCR and CAD in tobacco showed synergistic reduction in lignin contents and had no alterations

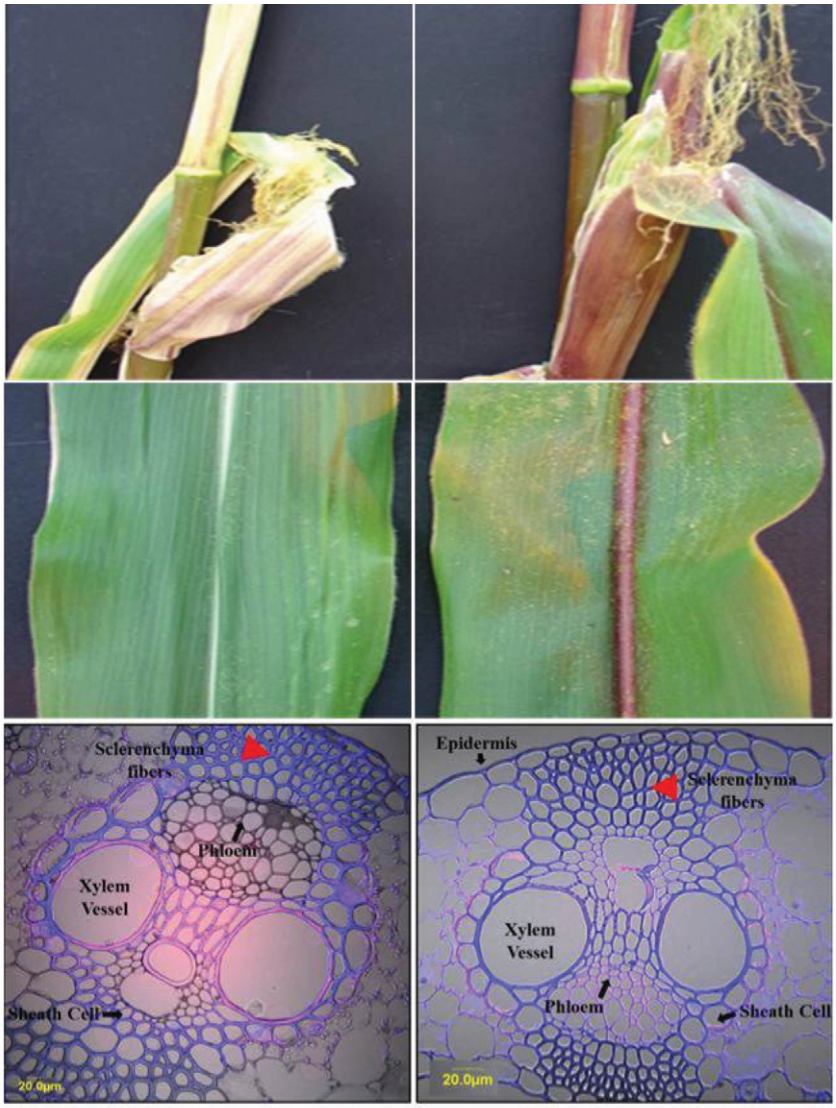


Figure 3. Phenotypic analysis of wild-type and the second generation of transgenic (T1) *ZmCCR1_RNAi* maize line. Brown coloration was seen in *ZmCCR1* downregulated leaf midribs, stems, and husks. Histological assay (brightfield images) of wild-type (HI-II) and a T1 *ZmCCR1_RNAi*-1c-5 leaf midrib; each leaf midrib was cross-sectioned via rotary microtome. The cross-sectioned maize leaf midribs 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.

of plant normal development under controlled conditions (Chabannes et al., 2001). The limited lignin reduction in sclerenchyma fibers enables maize plants to grow normally without the interruption of the integrity of nutrient and water transporting system. Although no pathogenic infection was observed during the experimental period at the controlled environment greenhouse conditions, field studies are needed to sure that the defense system of *ZmCCR1_RNAi* lines still properly function.

Lignin and Sugar Compositional Analysis

For forage research, lignin concentrations are mostly determined by one of two lignin methods, the Klason method or the acid detergent lignin (ADL) method, although it is also possible to measure lignin using acetyl bromide. The Klason lignin method is generally higher than the acid detergent lignin concentration due to the loss of acid soluble lignin fraction during ADL method (Jung et al., 1997). However, the amount of lignin measured in CCR downregulated maize stover via the Klason and acid detergent methods are positively correlated. It is known that Klason lignin provides a more accurate estimation of plant cell wall lignin content than the ADL method (Hatfield et al., 1994). Therefore, a Klason lignin method was used for the lignin measurement in this study. Future studies might be needed to show how lignin structure of composition may have changed due to CCR downregulation.

Figure 5 shows the plant biomass dry weight, corresponding compositional sugars (glucan and xylan), and

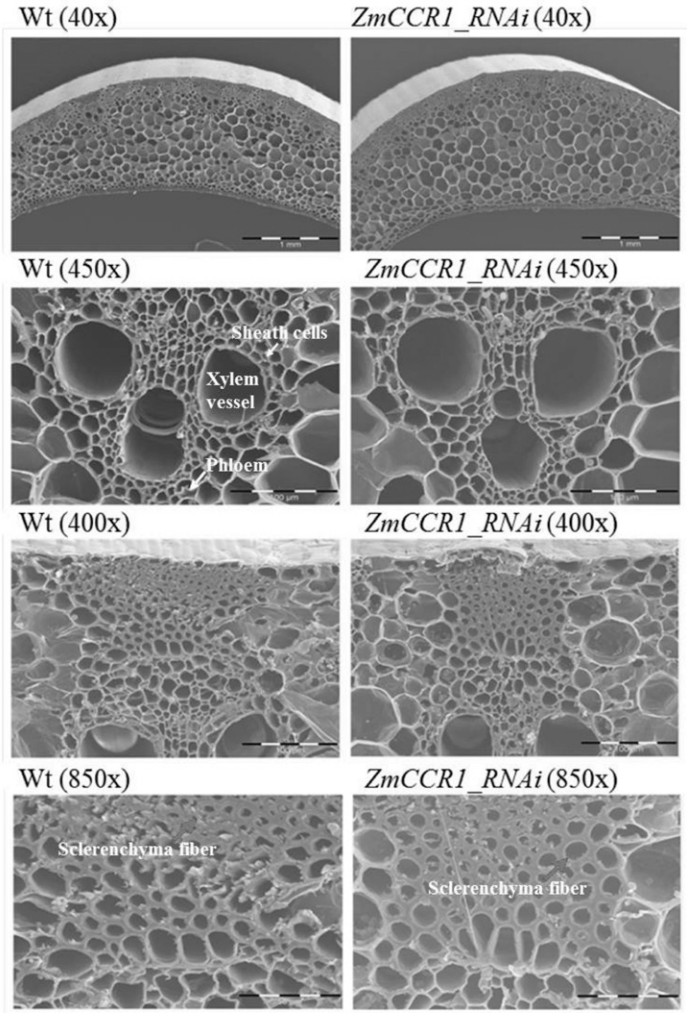


Figure 4. Scanning electron microscopy of *ZmCCR1* downregulated transgenic maize leaf midrib as compared to that of wild-type (Wt) nontransgenic control plant. The red arrow points to the sclerenchyma fibers.

lignin per plant for a number of different T1 lines. Based on *t* test 95% confidence intervals, $P < 0.05$, there was no statistically significant effect on structural sugar content (percent of dry matter) compared to the controls. The majority of the transgenic lines had similar dry matter per plant or higher compared to the wild-type control line. Only three of these lines (*ZmCCR1_RNAi*-1c-4, *ZmCCR1_RNAi*-1c-5, and *ZmCCR1_RNAi*-1c-6; indicated as a red asterisk) had statistically significant lower lignin content (8.5, 7.5, and 9.2%) and dry matter compared with the wild-type control plants. Such low lignin reduction in the *ZmCCR1_RNAi*-1c lines did not affect seed germination, growth, and development of transgenic plants.

Crystalline Cellulose and Hemicelluloses Composition Analyses

The content of crystalline cellulose and the hemicelluloses were determined to examine whether the C flux was shifted from lignin to the wall carbohydrates. The crystalline cellulose was increased 1.5-fold ($P > 0.05$) and 1.8-fold ($P < 0.05$) higher in two of the transgenic lines, *ZmCCR1*-1b-6 and *ZmCCR1*-1c-6 (Fig. 6A). However, cellulose content of the other lines, including the lines with reduced lignin content (*ZmCCR1*-1c-4 and *ZmCCR1*-1c-5), was not affected.

Compensation effects of lignin reduction on an increase in cellulose have been observed in cellulose-deficient *Arabidopsis thaliana* mutants (CESA3), which exhibited ectopic lignification via the jasmonate and ethylene signaling pathways (Cano-Delgado et al., 2003). Also, a report (Boudet et al., 2003) indicates that lignin reduction often results in the concomitant increase in cellulose to perhaps compensate for the loss of cell wall strength by shifting more C into cellulose. For example, an older report indicates that downregulation of the *4CL* gene of poplar resulted in a 45% decrease in lignin content with a 15% increase in cellulose without apparent damage to plant growth and development (Hu et al., 1998). However, more studies are needed to adequately show that the cellulose content increased on a mass basis per plant for lignin-modified transgenics. This is because, by measuring the total plant weight, one cannot judge whether the cellulose content has actually increased or the loss of lignin has lead to a decrease in total plant weight and (as would be expected) an increase in the relative percentage of cellulose.

Five major hemicelluloses were analyzed in lignin downregulated versus wild-type control maize stover. The hemicelluloses present in the samples were assessed by hydrolysis of their monosaccharides (Fig. 6B). The main monosaccharides found were xylose (75%) and arabinose (10%), representing arabinoxylan, that is, the dominant hemicellulose in grasses (Subramaniyan and Prema, 2002), as well as some glucose (less than 10%) probably representing amorphous cellulose. Fructose and mannose were not detected in GC analysis.

Figure 6B shows that arabionose, xylose, and a small amount of rhamnose and galactose were detected by GC analysis. Grass hemicelluloses are largely glucuronoarabinoxylans with some mixed-linkage glucans (Scheller and Ulvskov, 2010), so the fact that these four hemicellulose sugars are present and that xylose is the most prominent is not surprising. None of these monosaccharaides were significantly altered in the *ZmCCR1* downregulated lines (Tukey's pairwise comparisons, $P > 0.05$, $n = 3$) compared to wild-type control plant. The hemicellulose composition analysis was repeated (data not shown) and the results were consistent in both cases. Slight reduction in lignin content may not have been sufficient to trigger significant increase in biosynthesis of hemicelluloses.

Enzymatic Hydrolysis of AFEX-Pretreated Maize Stover

Figure 7 shows the respective maize stover glucan and xylan conversions after AFEX pretreatment and enzymatic hydrolysis for three of the transgenic lines and the wild-type control line. Under the same pretreatment condition, only the 24

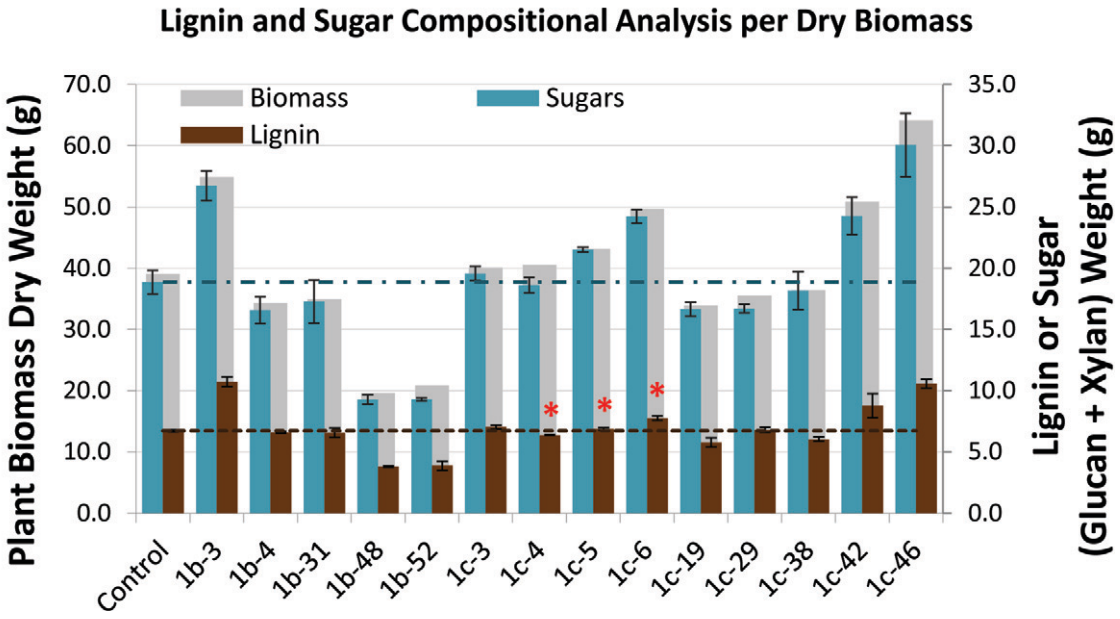


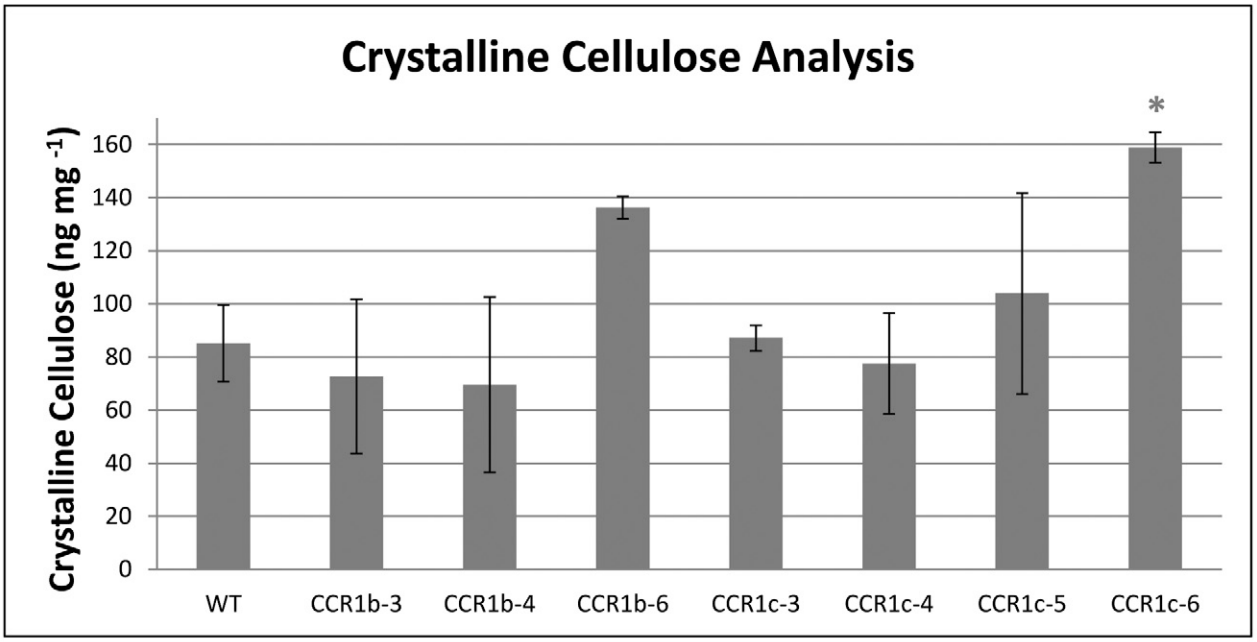
Figure 5. Lignin and sugar (glucan and xylan) compositional analysis of wild-type control and *ZmCCR1_RNAi* second generation of transgenic (T1) maize lines. Acid-insoluble lignin was measured via the Klason method. The red asterisk on the lines of *ZmCCR1_RNAi*-1c, *ZmCCR1_RNAi*-1c-5, and *ZmCCR1_RNAi*-1c-6 indicates that the lines 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 ± standard deviation ($P < 0.05$, $n = 3$).

h glucan (Fig. 7A; red asterisk) and xylan (Fig. 7B; red asterisk) conversions showed any statistical difference between the transgenic lines and the wild-type line. Compared to the wild-type, *ZmCCR1_RNAi*-1c-4 and *ZmCCR1_RNAi*-1c-6 had the lowest lignin content and the highest 24 h glucan conversion. The *ZmCCR1_RNAi*-1c-5 line showed the highest xylan conversion. Of all the sugar conversions, 24 h glucan is the most strongly correlated to lignin content ($r = -0.601$, $P = 0.000$) based on the Pearson correlation across both pretreatment conditions. Interestingly, the correlation decreases for the 72 h sample ($r = -0.370$, $P = 0.037$). This may indicate that sugar yields even out over the longer hydrolysis time and the strongest effect by lignin is early in hydrolysis. The 1c-5 line had the smallest decrease in lignin content compared to the control of the three lines tested, and it also had no increase in glucan yields at the higher NH_3 loading compared to the lower. Ammoniation of active methoxyl sites in lignin (Sewalt et al., 1996) is one of the possible modes by which AFEX decreases the recalcitrance of lignocellulosic biomass by decreasing the hydrophobicity and binding capacity of AFEX-treated lignin toward enzymes (Gao et al., 2011). Therefore, materials with lower lignin content could be less affected by a higher NH_3 loading than those with a higher lignin content.

Many scientists have found an inverse relationship between pretreated biomass lignin content and enzymatic hydrolysis conversions (Chen and Dixon, 2007; Fu et al., 2011; Jackson et al., 2008; Shen et al., 2009; Zhu et al., 2008). Unlike what has been found by other methods of lignin downregulation (Chen and Dixon, 2007), in our study there was not a large impact on the enzymatic hydrolysis of the untreated transgenics compared to the control. Relatively small increases in yields from untreated transgenics compared to their control samples has also been observed for downregulation of other enzymes that occur later in the lignin biosynthesis pathway: caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), CCR, and CAD (Chen and Dixon, 2007; Fu et al., 2011; Jackson et al., 2008).

To date, only one article has been published that examined the effect of CCR downregulation on enzymatic conversion of alfalfa lignocellulosic biomass (Jackson et al., 2008). In this article, improvements in dry matter in vitro digestibility and saccharification efficiency following dilute acid pretreatment of CCR downregulated alfalfa were related to the reduction in total lignin contents. The decrease in lignin content and increase in saccharification yields due to transgenic modification (~55 mg lignin g^{-1} cell wall residue; 20–25% increase in total sugar conversion) was much larger compared to what was observed in our studies (~15 mg lignin g^{-1} dry biomass; 7–8% increase in total sugar conversion). This may indicate that if we were able to more strongly suppress CCR activity, it may have been possible to more strongly decrease lignin content and correspondingly increase sugar conversions. Of course, the danger of decreasing lignin content further would be the possibility of reducing plant structural strength and viability. It has been reported that a minor reduction of lignin content (<10%) has little effect on the health of transgenics while a reduction in lignin content of more than 20% generally causes not only a loss of biomass but also xylem conductivity and mechanical support of the stem in hybrid poplar (Voelker et al., 2010, 2011). As shown on Fig. 5, a number of CCR downregulated plants contained lower lignin as compared to their wild-type control plants.

A.



B.

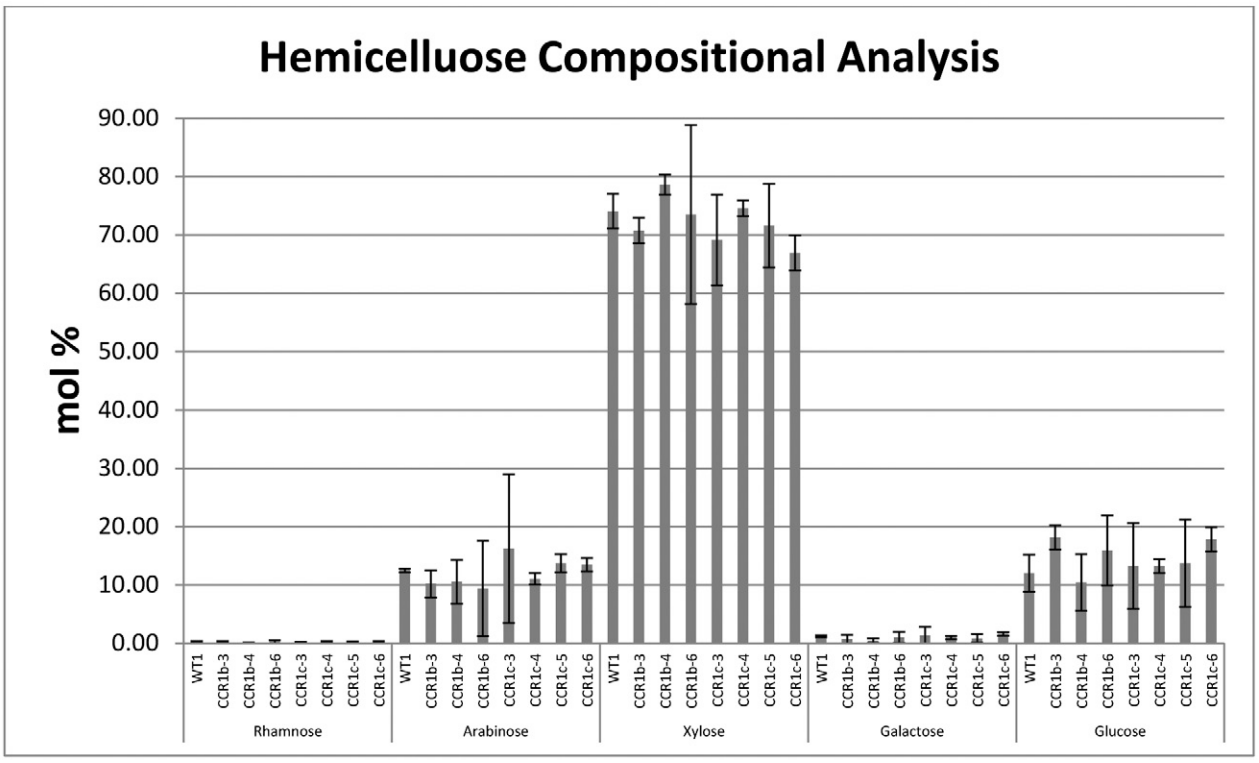


Figure 6. Crystalline cellulose (Tukey's pairwise comparisons, $P < 0.05$, $n = 3$) (A) and hemicellulose compositional analysis (Tukey's pairwise comparisons, $P > 0.05$; $n = 3$) (B) from the leaf midribs of wild-type (WT) maize versus the *ZmCCR_RNAi* lines.

Results presented in this research show that *ZmCCR1_RNAi* transgenic lines undergo normal development under controlled greenhouse conditions with brown pigmentation in midribs, stems, and husks that may have resulted from lignin reduction in sclerenchyma fiber. Such lignin downregulation increased glucose conversion during enzymatic hydrolysis of AFEX-pretreated maize stover. Should the lignin downregulated plants presented here show similar effects at the field level, reduction in lignin level might allow for a reduction in hydrolysis enzyme loading and associated costs.

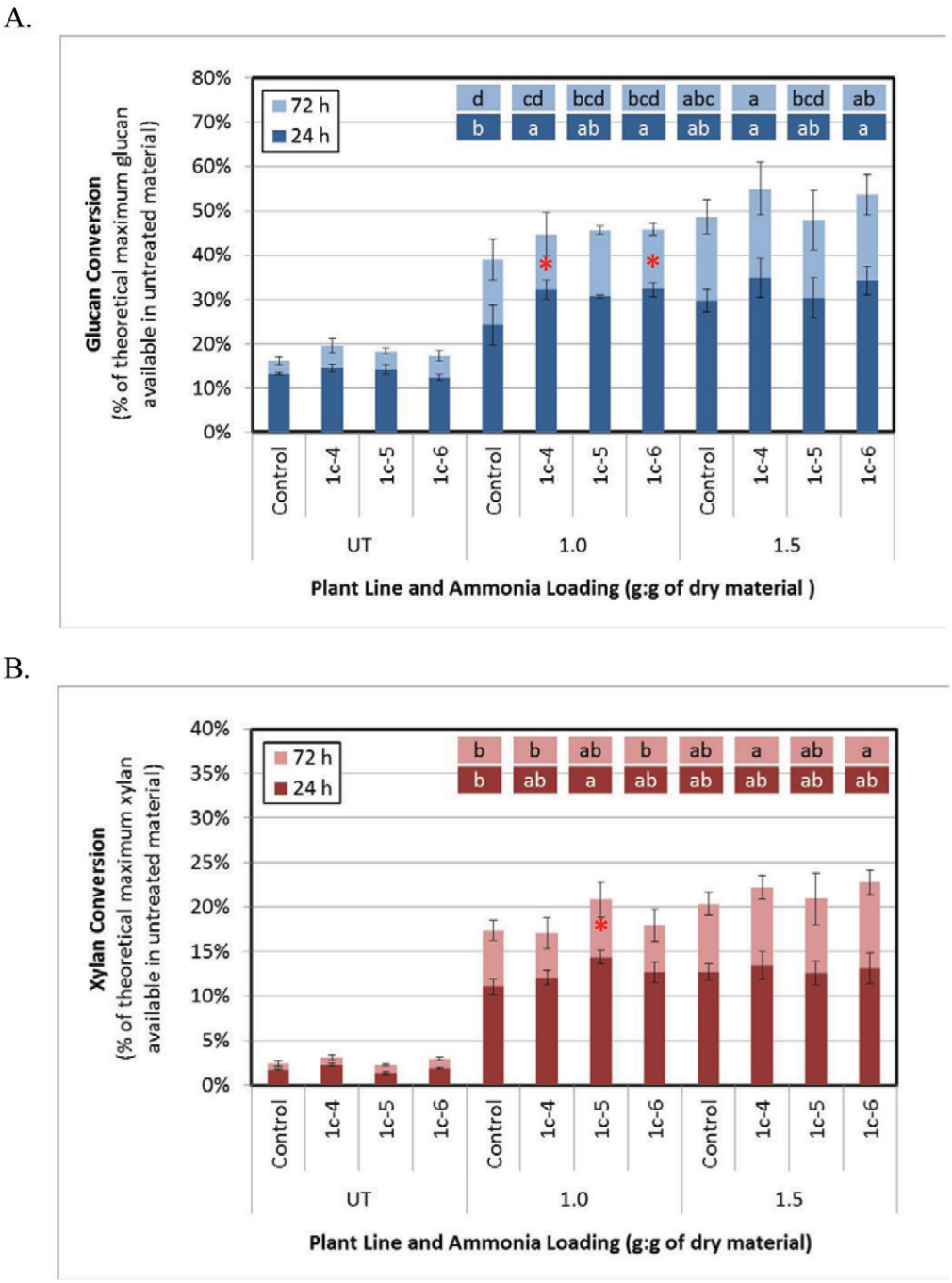
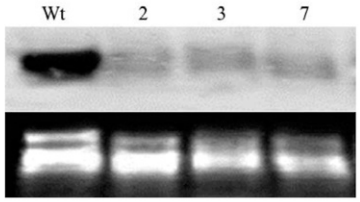


Figure 7. Percent sugar (glucan and xylan) conversions for untreated (UT) and ammonia fiber expansion (AFEX)-pretreated (90°C for 5 min) corn stover at different concentrations of ammonia (1.0:1.0 g NH_3:g dry biomass and 1.5:1.5 g NH_3:g dry biomass). The AFEX-pretreated maize stover was hydrolyzed using Accelerase 1000 (5.5 mg protein per gram glucan) and Multifect Xylanase (0.5 mg protein per gram glucan). 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 or 72 h) labeled with different letters are statistically different based on Tukey's pairwise comparisons ($P < 0.05$).

Preliminary Test on Dry Matter In Vitro Digestibility

The preliminary observational result of the in vitro NDF digestibility of the 92% dry T2 CCR downregulated maize leaves and husks versus their wild-type control dry matter sample showed an increase in level of digestibility in CCR downregulated samples (Fig. 8). A repeated comprehensive test was not performed due to the limited number of T2 seeds produced. Therefore, it is important to note that this part of work was performed for the purpose of observation. Many seeds have been collected from the T2 CCR downregulated plants for production of fourth generation of transgenic (T3) plants for a comprehensive NDF in vitro digestibility and other analytical tests.

A.



	T2 plants	Cross pollination for T3 seeds
2	♀ *ZmCCR1_RNAi*-1a x ♂ Self	*ZmCCR1_RNAi* -1c x ♂ Self
3	♀ *ZmCCR1_RNA* -1b x ♂ *ZmCCR1_RNAi* -1b	*ZmCCR1_RNAi* -4c x ♂ Wild-type
7	♀ *ZmCCR1_RNA* -1b x ♂ *ZmCCR1_RNAi* -1b	*ZmCCR1_RNAi* -1b x ♂ *ZmCCR1_RNAi* -1b

B.

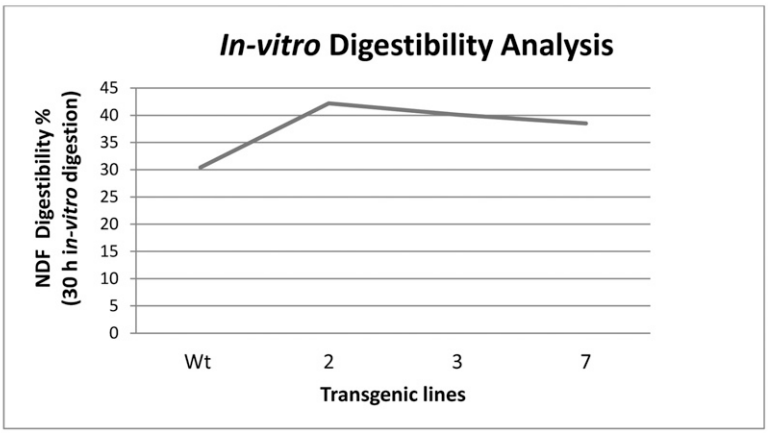


Figure 8. A. Northern blot analysis of third generation of transgenic (T2) *ZmCCR1_RNAi* plants, which were either self or crossed with other CCR downregulated plants (table in the middle), B. Preliminary result on in vitro neutral detergent fiber (NDF) digestibility of 92% dry plants that had shown least transcription in the Northern blot analysis as compared to a wild-type control plant (A). The digestibility of NDF was measured after 30 h in vitro digestion. T3, fourth generation of transgenic.

Over all we conclude that the research presented here might be one step forward toward improvement of cellulosic biofuels and on making lignocellulosic ethanol production more commercially competitive with petroleum fuel.

Acknowledgments

The microscopic imaging was conducted via the services of the Michigan State University Center for Advanced Microscopy. Maize callus was obtained 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). AFEX is a registered trademark of MBI International, Lansing, MI

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