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RESEARCH PAPER

# Characterization of a cinnamoyl-CoA reductase 1 (CCR1) mutant in maize: effects on lignification, fibre development, and global gene expression

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## Abstract

**Cinnamoyl-CoA reductase (CCR), which catalyses the first committed step of the lignin-specific branch of monolignol biosynthesis, has been extensively characterized in dicot species, but few data are available in monocots. By screening a *Mu* insertional mutant collection in maize, a mutant in the *CCR1* gene was isolated named *Zmccr1*⁻. In this mutant, *CCR1* gene expression is reduced to 31% of the residual wild-type level. *Zmccr1*⁻ exhibited enhanced digestibility without compromising plant growth and development. Lignin analysis revealed a slight decrease in lignin content and significant changes in lignin structure. *p*-Hydroxyphenyl units were strongly decreased and the syringyl/guaiacyl ratio was slightly increased. At the cellular level, alterations in lignin deposition were mainly observed in the walls of the sclerenchymatic fibre cells surrounding the vascular bundles. These cell walls showed little to no staining with phloroglucinol. These histochemical changes were accompanied by an increase in sclerenchyma surface area and an alteration in cell shape. In keeping with this cell type-specific phenotype, transcriptomics performed at an early stage of plant development revealed the down-regulation of genes specifically associated with fibre wall formation. To the present authors' knowledge, this is the first functional characterization of CCR1 in a grass species.**

**Key words:** Affymetrix array 18K, CCR, cell wall, digestibility, lignin, sclerenchyma, *Zea mays*.

## Introduction

Lignin biosynthesis has received growing attention in the cell wall field because lignin is a limiting factor in a number of agro-industrial processes such as pulping, forage digestibility, and lignocellulosic-to-bioethanol conversion processes. Cinnamoyl-CoA reductase (CCR) is the entry point for the lignin-specific branch of the phenylpropanoid pathway and is considered to be a key enzyme controlling the quantity and quality of lignins (Piquemal *et al.*, 1998; Jones *et al.*, 2001; Goujon *et al.*, 2003; Kawasaki *et al.*, 2006; Leple *et al.*, 2007; Wadenback *et al.*,

Abbreviations: CCR, cinnamoyl-CoA reductase; C3H, *p*-coumaroyl ester 3-hydroxylase; DFR, dihydroflavonol reductase; G, guaiacyl; H, *p*-hydroxyphenyl; QTL, quantitative trait locus; S, syringyl.

2008; Zhou *et al.*, 2010). However, most of the functional analysis of CCR has been performed on non-grass species.

The first transgenic plants with down-regulated CCR activity were obtained in tobacco (Piquemal *et al.*, 1998) using an antisense strategy. Lignin content was decreased to 50% of the wild type in transgenic lines exhibiting severe reduction of CCR activity. This dramatic lignin decrease provoked deleterious effects on plant development including stunting and collapsed xylem vessels. Molecular phenotyping demonstrated that decreasing CCR expression in tobacco affected not only cell wall synthesis, but also other metabolic processes such as photorespiration and photo-oxidative stress (Dauwe *et al.*, 2007). In CCR down-regulated poplar, transcript and metabolite profiling suggested that, in addition to altered lignification, CCR deficiency resulted in a decrease in hemicellulose and pectin biosynthesis (Leple *et al.*, 2007).

Many data concerning the role of CCR1 have been obtained in *Arabidopsis*. The first CCR mutant in *Arabidopsis* was identified based on its collapsed xylem phenotype (Jones *et al.*, 2001) and named *irx4* (for *irregular xylem 4*). *Irx4* exhibits a strong decrease in lignin content (50%) associated with a severe defect in secondary cell wall formation. Its interfascicular fibres are characterized by an expanded cell wall in the interior of the cell. More recently, further studies carried out on *irx4* suggested that lignification was delayed during plant development (Patten *et al.*, 2005; Laskar *et al.*, 2006). In addition to *irx4*, antisense CCR lines and allelic *ccr1* mutants have been obtained (Goujon *et al.*, 2003; Mir Derikvand *et al.*, 2008). Analyses of feruloyl derivatives in these lines suggested that feruloyl-CoA, a substrate of CCR, was redirected to cell wall-bound ferulate esters and soluble feruloyl malate. In addition, the increased recovery of 1,2,2-trithioethyl ethylguaiacol from thioacidolysis of CCR-deficient angiosperms (*Arabidopsis*, poplar, tobacco) revealed increased incorporation of free ferulic acid in lignins by *bis*-8-*O*-4 (cross) coupling (Ralph *et al.*, 2008).

In grasses, the role of CCR in controlling the flux of phenylpropanoid metabolites to lignins has never been demonstrated. cDNAs encoding *CCR* genes have been identified in many monocot species including maize (Pichon *et al.*, 1998), sugarcane (Selman-Housein *et al.*, 1999), perennial ryegrass (McInnes *et al.*, 2002; Tu *et al.*, 2010), rice (Bai *et al.*, 2003; Kawasaki *et al.*, 2006), barley (Larsen, 2004), wheat (Ma and Tian, 2005; Ma, 2007), and more recently, switchgrass (Escamilla-Trevino *et al.*, 2010). As is the case in dicots, all grass CCR proteins possess two conserved functional domains: an NADPH binding site and a CCR amino acid signature, NWYCY (Lacombe *et al.*, 1997). This NWYCY motif has recently been shown to be essential for CCR activity in two switchgrass CCRs, *Pv*CCR1 and *Pv*CCR2 (Escamilla-Trevino *et al.*, 2010). *Pv*CCR1, which is involved in constitutive lignification, prefers feruloyl-CoA as substrate, whereas *Pv*CCR2 is more related to defence and its preferred substrates are caffeoyl and 4-coumaroyl-CoA. In wheat, two cDNAs, *Ta-CCR2* and *Ta-CCR1*, have been characterized (Bai *et al.*, 2003; Ma and Tian, 2005; Kawasaki *et al.*, 2006; Ma, 2007). Both corresponding recombinant enzymes could use feruloyl, 5-OH-feruloyl, sinapoyl, and caffeoyl-CoAs as substrates; however, as in switchgrass, *Ta*-CCR1 used feruloyl-CoA with the greatest efficiency (Ma and Tian, 2005).

In maize, two cDNAs, *ZmCCR1* and *ZmCCR2*, have been reported (Pichon *et al.*, 1998). Whereas *ZmCCR1* was preferentially expressed in all lignifying tissues, *ZmCCR2* was detected mainly in roots and it was shown to be induced by drought conditions (Fan *et al.*, 2006). A more recent study identified six other maize contigs that were annotated as putative *CCR*s (Guillaumie *et al.*, 2007). Expression data indicated that although all of them were expressed in lignifying ear internodes, *ZmCCR1* was the most highly expressed. Therefore, in order to study the role of *CCR1* in the formation and digestibility of lignified cell walls in maize, a maize *CCR1* mutant, *Zmccr1*$^{-}$ has been isolated and characterized. The characterization of *Zmccr1*$^{-}$ revealed that despite only a slight decrease in lignin content, there were significant changes in lignin structure. Histochemical and immunocytochemical studies suggested that these lignin alterations were tissue specific. Finally, these modifications in lignin structure had a positive impact on the digestibility of maize stems without provoking any detrimental repercussions on plant growth and development.

## Materials and methods

### *Heterologous expression of* ZmCCR1 *in* Escherichia coli *and CCR activity assay*

The full-length *ZmCCR1* cDNA was cloned in pT7-7 vector for expression in BL21 *E. coli*. To facilitate cloning, an *Nde*I site overlapping the ATG start codon (5'-CGTCGCCCATATGACCGTCG-3') and *Bam*HI site (5'-GGGCGAATTGGATCCCGGGC-3') were introduced by PCR. Recombinant protein production was performed according to the protocol described by Lacombe *et al.* (1997). CCR activity measurements and $K_m$ determinations were performed as previously described by Goffner *et al.* (1994).

### *Field experiments*

Field experiments were carried out over 2 years in block designs with two replicates. Row spacing was 0.75 m, and the density was 90 000 plants ha$^{-1}$. Whole-plant biomass, excluding the ears, was collected at the silage stage and subjected to chemical and digestibility assay after drying the samples in a ventilated oven at 65 °C.

### *Isolation of* Zmccr1$^{-}$

One insertion event was isolated using a resource of 27 500 maize lines following the procedure described by Kärkönen *et al.* (2005). Mutant screens were accomplished through a PCR-based approach using a *Mu*-specific primer called OMuA: 5′-CTTCGTCCATAATGGCAATTATCTC-3' in combination with CCR primers (forward1: 5′-GTCGCCAGGATGACC-3′, forward2: 5′-GTACATCGCCTCGTGGTTAG-3′ reverse: 5′-GAGTTCTGCAAGAGAACGAG-3′) that are specific to *ZmCCR1*. Because of the process used to make the maize mutant collection, each plant

from the F2 generation for each family has a heterogeneous genetic background. Therefore, to minimize phenotypic variation between plants belonging to the same family, each mutant was crossed with a standard elite line adapted to the European climate. Thus, at each generation, there is a genetic segregation for the mutant allele; therefore, the presence of a *Mu* insertion was verified for each plant at each generation. After three rounds of crosses, heterozygous mutants were self-pollinated and analysed.

### *Lignin analysis*

Ear internode and whole plants at silage stage were lyophilized at harvest and ground to a fine powder. Lignin analyses were performed on extract-free cell wall residue. Lignin content was estimated by the Klason procedure (Whiting *et al.*, 1981). Lignin monomeric composition was determined by thioacidolysis followed by GC-MS of lignin-derived monomer trimethylsilyl derivatives (Lapierre *et al.*, 1986). Determination of *p*-hydroxycinnamic esters linked to lignin was performed by mild alkaline hydrolysis according to Jacquet *et al.* (1995).

### *Digestibility measurements*

Digestibility measurements were performed as previously described in Pichon *et al.* (2006) on plants grown in field conditions at silage stage. All parts of the plant except the ears were collected for analysis.

### *Lignin histochemical staining*

Transverse sections (100 μm) were made using a vibratome from internodes of plants grown under field conditions at flowering stage. Phloroglucinol staining was performed according to standard protocols (Nakano, 1992). Sections were observed using an inverted microscope (Leitz DMRIBE; Leica Microsystems, Wetzlar, Germany). Images were registered using a CCD camera (Color Coolview; Photonic Science, Milham, UK).

### *Quantitative analysis of vascular bundles*

Ear internodes from plants collected at the flowering stage were sectioned (100 μm) using a vibratome, stained with phloroglucinol, and scanned (2400 dpi). The image was then calibrated: 20×20 mm corresponds to 1889×1889 pixels. The sizes of 122 and 110 vascular bundles located under the epidermis in the lignified parenchyma zone were measured for wild type and *Zmccr1*$^{-}$ mutant, respectively. The number of vascular bundle and their surface area were determined with Image PRO-Plus software (Media Cybernetics, Silver Spring, MD, USA).

### *Immunohistochemistry*

Immunohistochemistry experiments were performed as described in Chavez Montes *et al.* (2008) with some modifications. Samples of maize tissues were fixed in 80% (v/v) ethanol. They were dehydrated in two successive ethanol 100% and embedded in LR White resin (Electron Microscopy Sciences; 33%, 50%, 66%, and 100% in ethanol). Primary antibodies against *p*-hydroxyphenylpropane (H) epitopes were diluted 1:50 (v/v) (Joseleau and Ruel, 1997). The secondary antibody was a goat anti-rabbit IgG coupled to the fluorescent dye Alexa Fluor 633 (Molecular Probes) and was used at a 1:10 (v/v) dilution. For each experiment, two plants per line (wild type and mutant) and two sections per plant were observed.

### *RT-PCR analysis*

RNA was extracted from piled-up internodes of 20-d-old plants with RNeasy midi kit (Qiagen) and reverse transcribed using MMLV (Promega enzyme). CCR1 gene expression was monitored by RT-PCR using specific primers (forward 5′-TCCTCGCCAAGCTCTTCCCCGA-3′ and reverse 5′-AAGAACGAACATGACGTTACAAGTCTTAGG-3′) designed in the 3′UTR region. The amplification of GAPDH was performed as a control (forward 5′-CCATGGAGAAGGCTGGGG-3′ and reverse 5′-CAAAGTCATGGATGACC-3′).

### *Affymetrix array hybridization*

Hybridization experiments were performed as described in Cossegal *et al.* (2008) with RNA extracted from piled-up internodes of 20-d-old plants using RNeasy midi kit. All raw and normalized data are available through the CATdb database (AFFY_CCR_Maize) (Gagnot *et al.*, 2008) and from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) (Barrett *et al.*, 2007): accession number GSE 11531.

## Results

### *Characterization of* CCR1 *gene structure and mutant isolation in maize*

This study focuses on *ZmCCR1* since it was originally shown that its expression was correlated with lignifying tissues (Pichon *et al.*, 1998). In this study, recombinant CCR1 protein was produced in *E. coli* and was active against all CCR substrates tested, with a slightly higher affinity for *p*-coumaroyl-CoA ($K_m$ for *p*-coumaroyl-CoA: 2.8 μM, sinapoyl-CoA: 6.58 μM, feruloyl-CoA: 9.26 μM). The availability of the maize genome sequence allowed us to identify the complete genomic sequence for *ZmCCR1*. The *ZmCCR1* gene contains five exons and four introns (Fig. 1A). In comparison with other grasses (rice, sorghum, ryegrass, *Brachypodium*) and dicots (*Arabidopsis*, poplar), the intron/exon positions of the *ZmCCR1* gene are well conserved (Supplementary Fig. S1 available at *JXB* online).

To identify a CCR1 mutant, a *Mu* collection of 27 500 maize lines was screened using a PCR-based approach with a *Mu*-specific primer that binds the terminal-inverted repeat sequence of the *Mu* element together with specific *CCR* primers. An insertional *CCR1* mutant, *Zmccr1*$^{-}$, was identified. Sequence analysis of the flanking region surrounding the *Mu* element indicated that the mutation occurred in the first intron (Fig. 1A). To correlate *Zmccr1*$^{-}$ phenotype with the *Mu* insertion, *Zmccr1*$^{-}$ was backcrossed to an elite line devoid of active *Mu* element. The mutation was tracked by PCR-based markers and, after five backcrosses, selfing was performed in order to obtain homozygous plants for a wild-type allele or the *ccr1* mutation.

RT-PCR was performed to determine the effect of the *Mu* insertion on *CCR* gene expression. *CCR1* transcripts were detectable in the *Zmccr1*$^{-}$ mutant but in lower amounts compared with the wild type (Fig. 1B). Further transcriptomic data allow us to estimate that *CCR1* gene expression in *Zmccr1*$^{-}$ was reduced to 31% of the residual wild-type level.

### *The* Zmccr1⁻ *mutant displays normal growth and development but modified lignin structure and cell wall digestibility*

At all developmental stages, field-grown *Zmccr1*⁻ was phenotypically indistinguishable from wild-type plants (data not shown). The effect of CCR1 down-regulation on lignin content and composition and cell wall digestibility was determined in field-grown plants at the silage stage (Table 1). The *Zmccr1*⁻ mutation had very little effect on lignin content. Whereas lignin content of the mutant and wild-type ear-bearing internodes was similar, the whole-plant biomass of *Zmccr1*⁻ displayed a slight reduction in lignin content (reduction by ~10%, Table 1). This slightly reduced lignin level was associated with a significant increase in cell wall digestibility. Lignin structure was then investigated by thioacidolysis, in ear-bearing internodes and whole-plant biomass (Table 1). The yield of thioacidolysis, *p*-hydroxyphenyl (H), guaiacyl (G), syringyl (S) monomers, reflects the frequency of lignin units involved in labile β-*O*-4 bonds. These monomers were released in similar amounts in *Zmccr1*⁻ and wild-type plants, suggesting that the overall frequency of lignin units involved in labile β-*O*-4 bonds is not affected by the mutation or, conversely, that the frequency of resistant interunit bonds, referred to as the lignin condensation degree, is similar in the mutant and control samples. Whereas the lignin content is very little affected in *Zmccr1*⁻, significant changes were observed in the relative frequency of S, G, and H monomers. Down-regulation of maize CCR1 resulted in an increase in the S/G ratio of both the ear-bearing internodes and whole-plant biomass (Table 1). Another striking difference concerned the H lignin units. H units were released in lower amounts from *Zmccr1*⁻compared with the wild-type plants. According to a recent study, the lignins of CCR-deficient poplar, tobacco, and *Arabidopsis* contain higher amounts of G-CHSEt-$CH_2(SEt)_2$, a compound that originates from the increased incorporation of ferulic acid by *bis*-β-*O*-4 ethers (Ralph *et al.*, 2008). Concomitantly with the accumulation of this ferulic acid-derived marker compound, cell walls of CCR-deficient plants release higher amounts of ferulic acid when subjected to thioacidolysis and/or to mild alkaline hydrolysis (Chabannes *et al.*, 2001; Leple *et al.*, 2007; Mir Derikvand *et al.*, 2008). In contrast to these CCR-deficient dicots, the *Zmccr1*⁻ samples analysed in the present study did not release higher amounts of ferulic acid or of the G-CHSEt-$CH_2(SEt)_2$ marker compound when subjected to thioacidolysis and as compared with the wild-type samples. When subjected to alkaline hydrolysis, *Zmccr1*⁻ cell walls

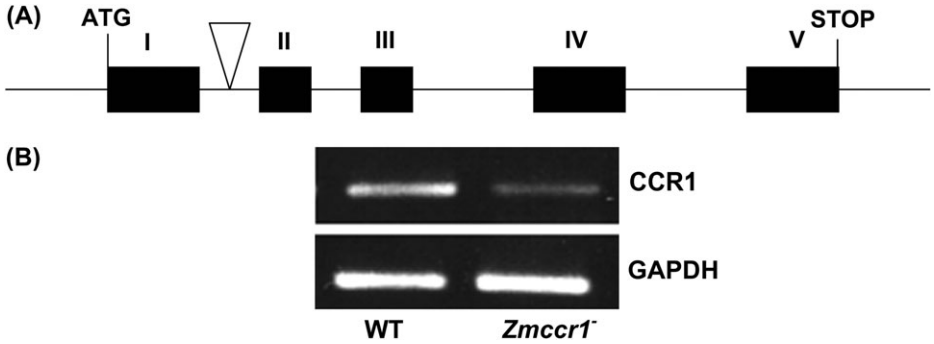


**Fig. 1.** *ZmCCR1* gene structure and impact of the *Mu* mutation on CCR expression. (A) Exon and intron organization of the *ZmCCR1* gene. Black boxes indicate exons and lines between boxes indicate introns. Insertion of the *Mu* element is indicated by an open arrowhead. The references for *ZmCCR1* are as follows: gene ID 542463 in NCBI, 199139 in Maize GDB, and GRMZM2G131205 in maizesequence.org. (B) RT-PCR of *CCR1* expression in piled-up internodes of 20-d-old wild-type and *Zmccr1*⁻ plants.

**Table 1.** Impact of CCR1 down-regulation on lignin content and composition and cell wall digestibility at the silage stage

All values are the means from three wild-type and three mutant (*Zmccr1*⁻) plants. The lignin content is measured as Klason Lignin (KL) and expressed as a weight percentage of the extract-free sample. Lignin structure is evaluated by determining the H, G, and S thioacidolysis monomers. *Significant differences between wild-type and mutant parameters are indicated by Student *t*-test $P<0.05$. ND: not determined

| Line | KL (%) | dNDF (%) | Thioacidolysis yield (μmol $g^{-1}$ KL) | Relative frequency of thioacidolysis monomers (% molar) | | | S/G |
|---|---|---|---|---|---|---|---|
| | | | | H | G | S | |
| Ear internode | | | | | | | |
| Wild type | 12.89 | ND | 1117 | 1.24* | 39.9* | 58.9* | 1.48* |
| *Zmccr1*⁻ | 12.31 | ND | 1015 | 0.92* | 37.6* | 61.5* | 1.64* |
| Whole-plant without ear | | | | | | | |
| Wild type | 13.04* | 23.96* | 660 | 2.27* | 43.9* | 53.4* | 1.22* |
| *Zmccr1*⁻ | 11.46* | 28.25* | 620 | 1.23* | 40.6* | 57.8* | 1.43* |

released similar amounts of ferulic and diferulic acids to the wild-type samples (Supplementary Table S1 at *JXB* online).

### *Down-regulation of* ZmCCR1 *alters schlerenchymatic fibre morphology and cell wall structure*

Transverse sections were prepared from field-grown, ear-bearing internodes at the flowering stage. Microscopic observations were made from the upper and lower portions of the internode, corresponding to the older and younger zones of the internode, respectively (Fig. 2). In the upper and lower portion of wild-type internodes stained with phloroglucinol, xylem vessels, sclerenchyma cells surrounding vascular bundles and situated directly under the epidermis stained red, confirming that all these cells are lignified at both stages of development (Fig. 2A, C). In the basal portion of *Zmccr1*$^{-}$ internodes, only the xylem stained red (see arrow on Fig. 2D). The sclerenchyma cells surrounding the vascular bundles were not only unstained (Fig. 2D), but their shape appeared more oblong than the equivalent wild-type cells (Fig. 2E, F). In the upper portion of *Zmccr1*$^{-}$ internodes, all lignified cell types exhibited a reddish-pink coloration, albeit less intense than in the wild-type cells at the same stage (Fig. 2A, B).

Since biochemical data indicated a lower frequency of H lignin units in *Zmccr1*$^{-}$, their spatial distribution in immunolocalization experiments was examined with a specific H-unit antibody (Joseleau and Ruel, 1997). In the upper portion of wild-type internodes, H units were detected in all cell types (Fig. 3A). A close-up of sclerenchyma cells surrounding the vascular bundles indicated that H units were preferentially localized in the middle lamella and, more precisely in the tricellular junctions (boxed-in area of Fig. 3A) in agreement with a previous study (Joseleau and Ruel, 1997). In the basal portion of wild-type internodes, the walls of all cell types were also labelled, but H units appeared to be more evenly distributed throughout the walls (Fig. 3C). In *Zmccr1*$^{-}$, very little labelling was observed in

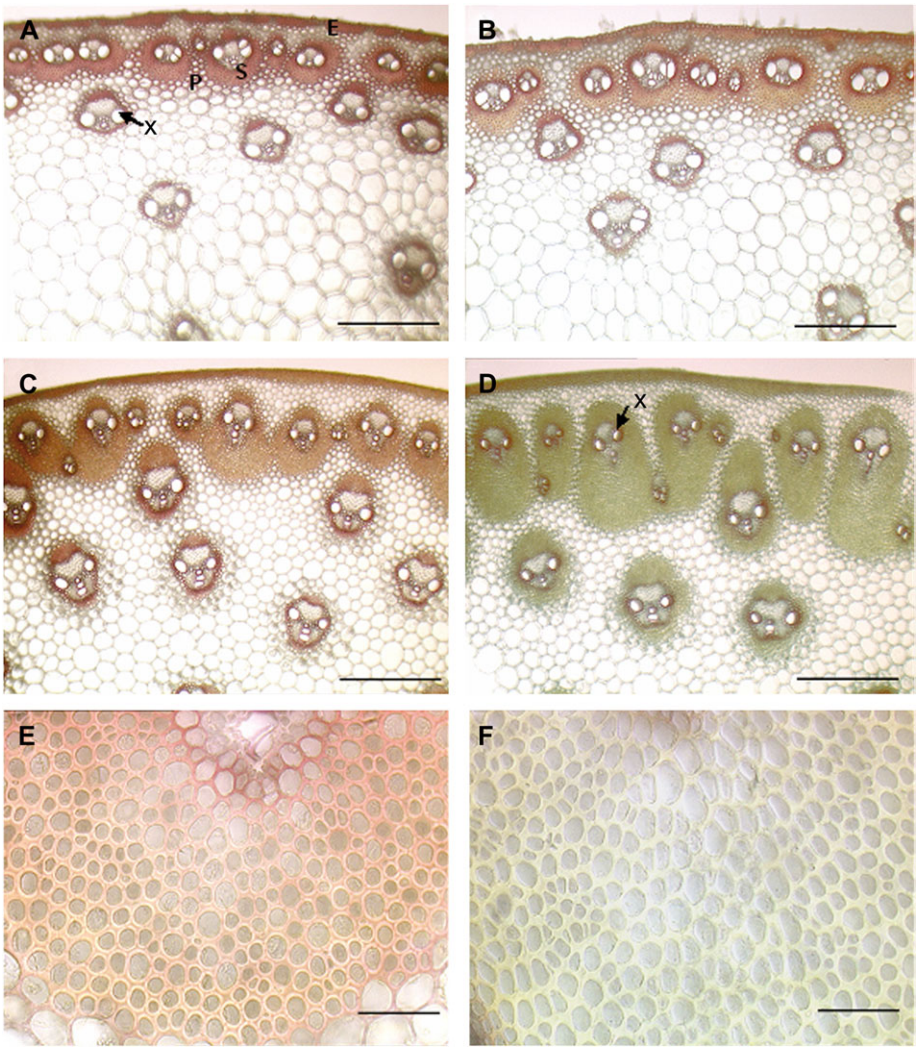


**Fig. 2.** Histochemical staining of lignin in wild type (A, C, E) and *Zmccr1*$^{-}$ (B, D, F). Light micrographs of transverse sections stained with phloroglucinol from the top (A, B) and bottom (C, D) parts of the ear-bearing internode. (E and F) Enlargement of sclerenchyma located around vascular bundles. Black arrow in (D) indicates xylem vessels stained red with phloroglucinol. Bars: 500 μm (A–D), 50 μm (E, F). P, parenchyma; S, sclerenchyma; E, epidermis; X, xylem vessels.

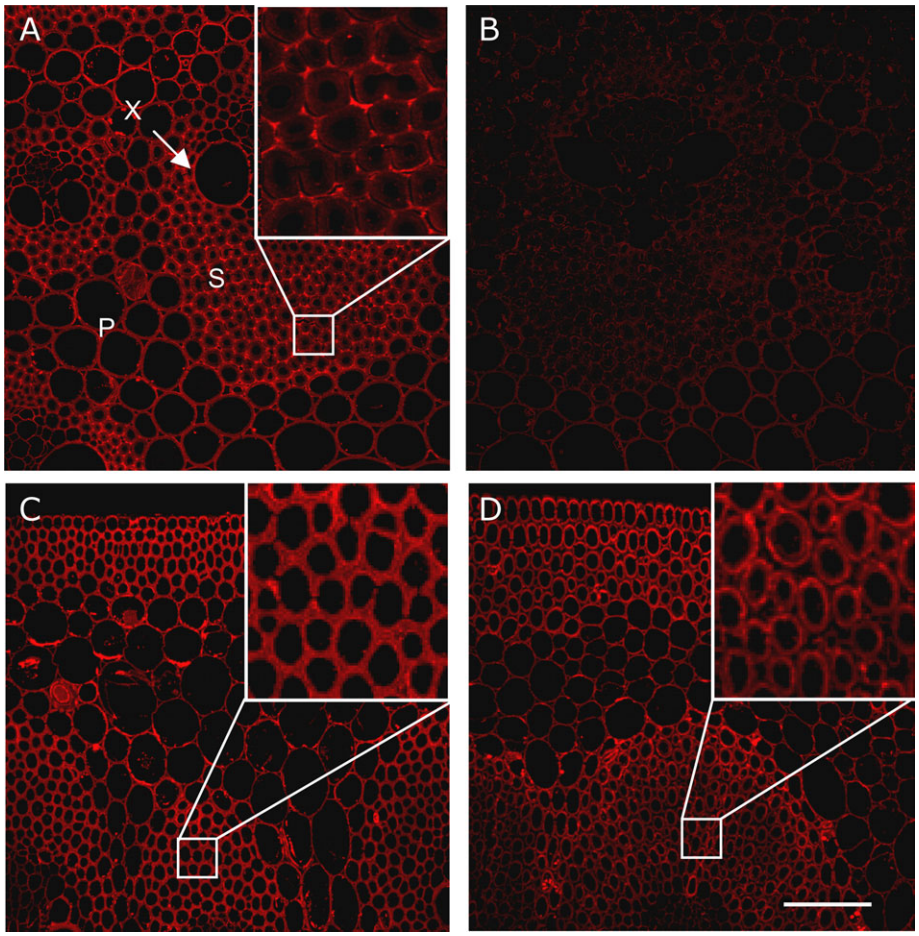


**Fig. 3.** Immunolocalization of H unit lignins in wild type (A, C) and *Zmccr1*⁻ (B, D). Indirect immunofluorescence micrographs of resin-embedded ear-bearing internode sections. Sections were performed in the top (A, B) and bottom (C, D) portions of internodes and labelled with anti-H antibodies. White boxes in A, C, D: enlargement of sclerenchyma cells. Anti-H label is indicated in red. P, parenchyma; S, sclerenchyma; X, xylem vessels. Bar: 50 μm (A-D).

the upper portion of the internode (Fig. 3B) and in the basal portion, H units were located in all cell types (Fig. 3D). However, in the basal portion, the label in sclerenchyma cells was less intense than the wild type and unevenly distributed within the wall (boxed-in area of Fig. 3D).

Another striking feature of *Zmccr1*⁻ was the difference in vascular bundle size as compared with the wild type (Figs 2, 4). Although the overall spatial organization of the different cell types within the bundle was conserved *Zmccr1*⁻ bundles were larger. In the wild-type sample, the mean size of the vascular bundles ranged between 20 000 and 60 000 μm$^2$, whereas in *Zmccr1*⁻ the distribution of vascular bundle size was larger, ranging from 40 000 to 160 000 μm$^2$ (Fig. 4). This difference was mainly due to an increase in surface area of sclerenchyma cells surrounding the bundle and not the xylem or phloem themselves.

### *Transcriptomics analysis*

To determine the influence of the CCR mutation on global gene expression, transcriptome profiling was performed on young piled-up internodes of 20-d-old *Zmccr1*⁻ and wild-type plants. A total of 167 genes were differentially expressed between the two genotypes: 107 genes were up-regulated and 60 were down-regulated (Supplementary Table S2A, B at *JXB* online). A subset of the data relevant to cell wall, phenylpropanoid metabolism, and transcription factors are shown in Table 2.

Besides CCR1 itself, no other lignification genes were down-regulated in *Zmccr1*⁻. As for cell wall modifying enzymes, genes encoding a cellulase, a glucan endo-1,3-β-glucosidase, and a glycosyltransferase 6 were also found to be down-regulated in the mutant. In relation to sclerenchyma formation, a katanin p80 gene was the second most significantly reduced gene in *Zmccr1*⁻ (Table 2). In *Arabidopsis*, *At*KN1 is essential for normal cortical microtubule patterning (Burk *et al.*, 2001). The corresponding mutant, fragile fibre 2 (*fra2*), exhibits altered cellulose microfibril deposition and cell wall biosynthesis in fibres (Burk and Ye, 2002). Interestingly, another gene in relation to cellulose microfibril deposition, a kinesin (corresponding to the *fra1 Arabidopsis* mutant) (Zhong *et al.*, 2002), was also down-regulated in *Zmccr1*⁻. Elsewhere, it was noted

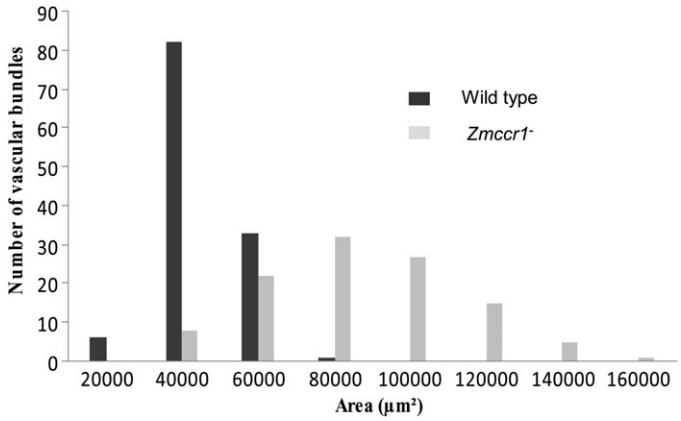


**Fig. 4.** Distribution of vascular bundle surface area in internodes of wild type and *Zmccr1*⁻. Vascular bundle size was measured from 100 μm sections from the basal portion of the ear-bearing internode. Sections were stained with phloroglucinol reagent and scanned. Using Image Pro software each vascular bundle located in the external zone of the internode was numerized for counting. A total of 122 and 110 vascular bundles for wild type and *Zmccr1*⁻ mutant respectively were counted. Black and grey bars on the histogram represent, respectively, wild-type and *Zmccr1*⁻ vascular bundle sizes.

that the most down-regulated gene in the complete data set codes for an extracellular matrix structural constituent (Supplementary Table S2A at *JXB* online).

Among the up-regulated genes in *Zmccr1*⁻, six structural proteins including five proline-rich proteins and a hydroxyproline-rich protein were identified (Table 2). In addition, genes involved in flavonoid metabolism, such as flavonoid 3'-hydroxylase, two dihydroflavonol-4-reductases, and an anthocyanidin 3-*O*-glucosyltransferase, were also up-regulated. Another striking feature of *Zmccr1*⁻ gene expression is the up-regulation of seven members of the MADS-box transcription factor family. These transcription factors have been studied mainly in the context of floral development (Hernandez-Hernandez *et al.*, 2007). Although a link between the MADS-box transcription factors and CCR down-regulation is difficult to establish, Liljegren *et al.* (2000) reported a role for a MADS-box transcription factor in the lignification of the *Arabidopsis* silique that would enable dehiscence.

## Discussion

### *ZmCCR1 plays a role in dictating lignin structure in maize*

A major conclusion of previous studies on dicot species was that strong down-regulation of the *CCR* gene dramatically affects lignin content. For example, in *irx4* (Jones *et al.*, 2001) and the knock-out CCR1 *Arabidopsis* mutant (Mir Derikvand *et al.*, 2008), the lignin content of mature floral stems was reduced to ~50% of the wild-type level. Concomitantly, feruloyl-CoA, which is a substrate of CCR in dicots, was redirected either to ferulic acid ester-linked to the cell walls or incorporated into lignins, or, in the case of *Arabidopsis*, to soluble feruloyl malate (Mir Derikvand *et al.*, 2008; Ralph *et al.*, 2008). In maize, the *Mu* insertion in the first intron of the *CCR* gene led to a slight decrease in CCR expression. As a consequence, the lignin content was slightly affected and the pool of ferulic acid was not altered. A small decrease in lignin content has also been reported in transgenic Norway spruce expressing the *CCR* gene in antisense orientation. In that case, the transcript abundance of CCR was reduced by 35% (Wadenback *et al.*, 2008). The most important effect observed in the internodes of the *Zmccr1*⁻ mutant was an increase in the S/G ratio as well as a decrease in H lignin units. Moreover, the use of an H unit antibody revealed changes in both the amount and the distribution of H units in the ear internode. Radiotracer experiments performed in grass and non-grass species have revealed that lignins deposited in the middle lamella and at the early stage of lignification are enriched in H units (Terashima, 1993). In addition, these H units are more abundant in the lignins of compression wood (Bailleres *et al.*, 1997) and in the stress lignins formed in response to a fungal elicitor (Lange *et al.*, 1995) or to ozone exposure (Cabane *et al.*, 2004). In transgenic Norway spruce displaying moderate CCR down-regulation, a significant decrease in the minor H units has been reported (Wadenback *et al.*, 2008). A similar reduction in H lignin has been reported in *Arabidopsis* (Goujon *et al.*, 2003) and alfalfa (Nakashima *et al.*, 2008*a*) antisense CCR plants. Interestingly, in all plant species (gymnosperms, angiosperms, dicots, and monocots) and for all degrees of down-regulation (moderate or severe), CCR down-regulation systematically reduces the frequency of H lignin units, which are more specific to the early lignification stage or stress lignins. By contrast, the down-regulation of *p*-coumaroyl ester 3-hydroxylase (C3H) in *Arabidopsis* led to lignin essentially comprised of H units (Abdulrazzak *et al.*, 2006). In a similar manner, down-regulation of C3H in alfalfa (Ralph *et al.*, 2006) and in poplar (Coleman *et al.*, 2008) led to an increased proportion of H units. Thus, it seems that CCR1 and C3H have opposing roles in establishing H unit content in plant lignins. In maize, it was demonstrated that although CCR1 recombinant protein uses all the CCR substrates tested, its preferred substrate is *p*-coumaroyl-CoA. This result is quite different from those obtained in switchgrass (Escamilla-Trevino *et al.*, 2010) and rice (Ma and Tian, 2005; Ma, 2007) in which CCR1 exhibited a higher affinity for feruloyl-CoA. This difference could be related to the fact that these grass species belong to divergent clades (Supplementary Fig. S2 at *JXB* online). Taken together these results suggest that CCR1 plays a role in regulating lignin monomeric composition in maize and, more particularly, the formation of the minor H lignin units. Albeit occurring in relatively minor amounts (<5% of the lignin units, except in compression wood), these H units may have a locally higher concentration and a pivotal role in modulating plant cell wall properties, particularly in the middle lamella region. In vascular and supporting tissues, this cell wall region has high lignin content and contains

**Table 2.** Differential expression of genes involved in cell wall and phenylpropanoid metabolism and transcription factors between *Zmccr1*⁻ and wild-type plants

Experiments were performed with RNA extracted from 20-d-old plants.

| Gene | Accession No. | Log2 ratio | Bonferroni |
|---|---|---|---|
| **Cell wall and phenylpropanoid metabolism** | | | |
| **Down-regulated genes in *Zmccr1*⁻** | | | |
| Extracellular matrix structural constituent. | gb\|CF647864 | –8.18 | 0.00E+0 |
| Katanin p80 (*fra2*) | gb\|BG873873 | –4.53 | 0.00E+0 |
| Cinnamoyl-CoA reductase 1 | gb\|CF632382 | –1.69 | 0.00E+0 |
| Actin-related protein 7 | gb\|AY107222.1 | –1.23 | 2.54E–7 |
| Cellulase 1 | gb\|AY108307.1 | –1.14 | 7.33E–6 |
| Glucan endo-1,3-β-glucosidase-related | gb\|CO522465 | –1.00 | 7.83E–4 |
| Glycosyltransferase 6 | gb\|AI665306 | –1.00 | 7.29E–4 |
| Kinesin (*fra1*) | gb\|BM380170 | –0.88 | 2.04E–2 |
| **Up-regulated genes in *Zmccr1*⁻** | | | |
| Pectinesterase | gb\|AY112091.1 | 7.55 | 0.00E+00 |
| Proline-rich protein APG precursor | gb\|AW573375 | 6.5 | 0.00E+00 |
| Flavonoid 3'-hydroxylase | gb\|BG873885 | 2.13 | 0.00E+00 |
| Polygalacturonase inhibitor 1 | gb\|CK371299 | 1.82 | 0.00E+00 |
| Dihydroflavonol-4-reductase | gb\|CK827965 | 1.81 | 0.00E+00 |
| Dihydroflavonol-4-reductase | gb\|CO523092 | 1.76 | 0.00E+00 |
| Hydroxyproline-rich glycoprotein | gb\|BM350630 | 1.72 | 0.00E+00 |
| UDP-glucose 4-epimerase | gb\|AY303682.1 | 1.31 | 1.08E–08 |
| Proline-rich protein | gb\|BM380341 | 1.3 | 2.16E–08 |
| Chorismate mutase | gb\|AI673851 | 1.23 | 2.29E–07 |
| Anthocyanidin 3-*O*-glucosyltransferase | gb\|CO525742 | 1.21 | 5.80E–07 |
| Proline-rich protein APG precursor | gb\|CO532055 | 1.17 | 2.41E–06 |
| Peroxidase 27 | gb\|CK144844 | 1.08 | 5.35E–05 |
| Pectinesterase | gb\|CF629045 | 1.05 | 1.26E–04 |
| Proline-rich protein APG | gb\|BM382516 | 0.95 | 3.30E–03 |
| Cell wall protein | gb\|M36914.1 | 0.85 | 4.97E–02 |
| **Transcription factors** | | | |
| **Down-regulated genes in *Zmccr1*⁻** | | | |
| AtMYB59 | gb\|BU571552 | –0.98 | 1.37E–3 |
| bZIP transcription factor HBP-1b | gb\|X69152.1 | –0.89 | 1.99E–2 |
| Transcription factor HBP-1b | gb\|X69152.1 | –0.86 | 4.14E–2 |
| **Up-regulated genes in *Zmccr1*⁻** | | | |
| CHY zinc finger family protein | gb\|BQ538104 | 4.86 | 0.00E+00 |
| MADS-box transcription factor 4 | gb\|AW055920 | 3.43 | 0.00E+00 |
| MADS-box transcription factor 16 | gb\|AF181479.1 | 3.06 | 0.00E+00 |
| MADS-box transcription factor 2 | gb\|BM078498 | 2.99 | 0.00E+00 |
| MADS-domain transcription factor | gb\|CF649598 | 2.8 | 0.00E+00 |
| MADS-box transcription factor 8 | gb\|BQ703314 | 1.99 | 0.00E+00 |
| Zinc finger (HIT type) family | gb\|BU571579 | 1.49 | 6.98E–12 |
| MADS-box transcription factor 34 | gb\|AI691625 | 0.94 | 4.30E–03 |
| MADS-box transcription factor 15 | gb\|AF112150.1 | 0.86 | 4.26E–02 |

lignins that are rich both in H units and in condensed bonds, which probably favours their cementing function. The fact that CCR1 down-regulation specifically affects the formation of the minor H lignin units in all plant species might suggest that this down-regulation is more effective during the early stages of lignification. Another hypothesis would be that the formation of H lignins would proceed by different mechanisms than the formation of G or S constitutive lignins. In agreement with the fact that stress lignins are often enriched in H units (Lange *et al.*, 1995), one can imagine that, due to its higher redox potential and as compared with coniferyl or sinapyl alcohol, the effective incorporation of *p*-coumaryl alcohol into the lignin polymers requires harsher oxidative conditions, such as those occurring during plant defence and in the presence of high concentrations of reactive oxygen species. In non-stress conditions, the baseline level of these reactive oxygen species would allow the incorporation of some H units whereas stress conditions would increase this incorporation. To further support the relationship of grass CCR1 to stress lignins, recent work has revealed that rice CCR1 is an effector of the small GTPase Rac acting in plant defence (Kawasaki *et al.*, 2006; Nakashima *et al.*, 2008*b*).

### *Down-regulation of* CCR1 *altered lignin structure in a cell type-specific manner*

In the younger portion of *Zmccr1*⁻ internodes, the cell walls of sclerenchyma cells surrounding the vascular bundles were poorly stained with phloroglucinol and H units were not uniformly distributed within the wall. Since total lignin content was only affected slightly in *Zmccr1*⁻, the absence of phloroglucinol staining was somewhat surprising. Interestingly, in the maize *brittle stalk-2* mutant in which cellulose deposition was modified, sclerenchyma cells did not react with phloroglucinol stain despite higher amounts of lignin and hydroxycinnamic acids in internodes (Sindhu *et al.*, 2007). In the case of *Zmccr1*⁻, one explanation could be that lignification is delayed in the sclerenchyma cells. This hypothesis is supported by the fact that the upper (older) portion of the internode reacted positively to phloroglucinol staining. A delay in lignification and development has been reported for the CCR *Arabidopsis* mutant, *irx4* (Laskar *et al.*, 2006). Moreover, the sclerenchyma cell shape in the basal portion of the ear internode appeared more oblong in *Zmccr1*⁻ as compared with the wild type. Modifications in cell shape have been previously reported in CCR-down-regulated alfalfa (Nakashima *et al.*, 2008*a*). In contrast, xylem vessels of *Zmccr1*⁻ were not affected by the CCR mutation. The observation that xylem vessels are stained red with phloroglucinol and are correctly formed in *Zmccr1*⁻ is quite different from that described in other CCR down-regulated angiosperm species displaying a collapsed xylem phenotype (Jones *et al.*, 2001; Mir Derikvand *et al.*, 2008). To date the only xylem-deficient mutant in maize that has been described is the wilted mutant (Postlethwait and Nelson, 1957). In this mutant, some of the vascular bundles are characterized by immature, non-functional metaxylem elements.

### *Transcriptomics in* Zmccr1⁻ *revealed cross-talk in phenypropanoid metabolism and cellulose deposition in cell wall*

Transcriptomic analysis of 20-d-old plants revealed that, except for the down-regulation of *CCR1*, no other genes involved in the lignification pathway were deregulated. On the contrary, up-regulation of several flavonoid biosynthetic genes including a chorismate mutase, two dihydroflavonol reductases (*DFR*), and a flavonoid 3'-hydroxylase (*F3H*) was observed. Chorismate mutase and *DFR* were also differentially expressed in CCR-down-regulated tobacco (Dauwe *et al.*, 2007). Interestingly, *DFR* and *F3H* were also up-regulated in C3H-down-regulated *Arabidopsis*. As *CCR1*, the *HCT*/*C3H* couple uses coumaroyl-CoA as substrate. When one of these enzymes is disrupted, the pool of coumaroyl-CoA, which serves usually for the synthesis of H unit at the early stage of plant development, is probably redirected towards the flavonoid pathway as indicated by the up-regulation of both *DFR* and *F3H*. *Zmccr1*⁻ transcriptomic data also indicated deregulation of two genes involved in cellulose microfibril deposition, kinesin and katanin. The corresponding *Arabidopsis* mutants, *fra1* and *fra2* respectively, exhibited defects in cell wall formation specifically in sclerenchyma cells (Burk and Ye, 2002; Zhong *et al.*, 2002). More recently, Zhang *et al.* (2010) reported that alteration in a *kinesin-4* gene in rice led to modification of sclerenchyma cell wall structure and properties including randomly oriented cellulose microfibrils and an increase in lignin and arabinoxylan content. Thus, the deregulation of genes involved in cellulose microfibril deposition, together with the observed modification of H unit distribution in sclerenchyma cell walls suggests that the cellulose–lignin network may be altered in *Zmccr1*⁻.

### *Moderate down-regulation of* CCR1 *significantly improved cell wall digestibility in maize*

To the present authors' knowledge, this is the first report that the down-regulation of a gene in the lignin biosynthetic pathway led to a significant increase in cell wall digestibility without severely modifying lignin content. In keeping with the improved digestibility in *Zmccr1*⁻, the *CCR1* gene in maize is located on chromosome 1 and co-localizes with a quantitative trait locus (QTL) of cell wall digestibility explaining 12.1% of this trait in F838XF286 RIL progeny (Barriere *et al.*, 2008). Co-localization between herbage quality and *CCR1* has also been reported in perennial ryegrass (Cogan *et al.*, 2005). Thumma *et al.* (2005) also reported that *CCR* polymorphism was associated with variation in microfibril angle in eucalyptus. Recently, a single non-coding two-state marker in CCR1 has been found in poplar (Wegrzyn *et al.*, 2010).

In *Zmccr1*⁻, biochemical data indicated that lignin content (10% lower than wild-type plants) and structure (lower H unit content) were significantly modified. These modifications are associated with significantly improved polysaccharide cell wall degradability [digestibility of neutral detergent fibre (dNDF) increase from 24% to 28%]. In agreement with biochemical data, cytological observations indicated specific changes in sclerenchyma cell walls including greatly reduced phloroglucinol staining and modification of H distribution within the wall. Even if H units are in low proportion in cell wall, they are the first to be incorporated during plant development and may subsequently modify the establishment of the lignin–polysaccharide network. The next step is to determine the precise nature of the changes in sclerenchyma wall chemistry and to establish how these changes have a beneficial impact on plant biomass properties.

Finally, *Zmccr1*⁻ mutant is of particular interest for breeders because the increase in digestibility is not associated with undesirable agronomics traits when plants are grown in field conditions.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** *CCR1* gene structure in different species.

**Supplementary Fig. S2.** *CCR1* gene family phylogeny in grasses.

Supplementary Table S1. Recovery of ferulic and *p*-coumaric acids by alkaline hydrolysis of extract-free whole plant biomass without ear collected at the silage stage.

Supplementary Table S2. Complete list of down-regulated and up-regulated genes in *Zmccr1*⁻.

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