Title: Cell wall modifications triggered by the down-regulation of Coumarate 3-hydroxylase-1 in maize.

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Abstract

Coumarate 3-hydroxylase (C3H) catalyzes a key step of the synthesis of the two main lignin subunits, guaiacyl (G) and syringyl (S) in dicotyledonous species. As no functional data are available in regards to this enzyme in monocotyledonous species, we generated C3H1 knock-down maize plants. The results obtained indicate that C3H1 participates in lignin biosynthesis as its down-regulation redirects the phenylpropanoid flux: as a result, increased amounts of *p*-hydroxyphenyl (H) units, lignin-associated ferulates and the flavone triclin were detected in transgenic stems cell walls. Altogether, these changes make stem cell walls more degradable in the most *C3H1*-repressed plants, despite their unaltered polysaccharide content. The increase in H monomers is moderate compared to C3H deficient Arabidopsis and alfalfa plants. This could be due to the existence of a second maize C3H protein (C3H2) that can compensate the reduced levels of C3H1 in these *C3H1*-RNAi maize plants. The reduced expression of *C3H1* alters the macroscopic phenotype of the plants, whose growth is inhibited proportionally to the extent of *C3H1* repression. Finally, the down-regulation of C3H1 also increases the synthesis of flavonoids, leading to the accumulation of anthocyanins in transgenic leaves.

Keywords: Cell wall polysaccharides, degradability, flavonoids, lignin, maize.

1. Introduction

In plants, 20 to 30% of photosynthetically fixed carbon is directed toward lignin and other phenylpropanoid compounds, being lignin the second most abundant organic polymer after cellulose [1].

Lignin is a heterogeneous phenolic biopolymer formed by the oxidative coupling of three main hydroxycinnamyl alcohols (monolignols): *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, forming the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin subunits respectively [1, 2, 3]. Recently, it has been described that monolignols can be acylated through the action of different acyl-transferases prior to their polymerization [4, 5, 6].

Lignin is produced by the phenylpropanoid pathway (Fig. 1), together with a broad range of specialized metabolites such as flavonoids, hydroxycinnamic acids and esters, tannins, suberin, cutin and stilbenes [7] and many of them, including flavonoids and stilbenes, possess extraordinary antioxidant activity and therefore a putative health-protecting function [8, 9].

The synthesis of monolignols starts from phenylalanine and consists of coordinated reactions of deamination, hydroxylation, methylation, acylation and reduction and the genes involved in this process are well established [2, 7, 10, 11] (Fig. 1).

Once produced, lignin is irreversibly deposited into the cell wall where it interacts covalently with hemicelluloses, generating a strong network that limits the access to cell wall sugars. These interactions reduce the digestibility of the lignocellulosic biomass for its use as forage, reduce the efficiency of the industrial processing for the pulp and paper industry and increase its recalcitrance for bioethanol production [12, 13]. Many studies showed that the chemical and physical properties of the lignin polymer are affected by the relative amount of the three main

subunits [1, 12, 14] and an increasing amount of transgenic plants in which the expression of monolignol biosynthetic genes have been altered have been characterized [7, 15, 16].

Within the lignin pathway, three cytochrome P450 enzymes, the general phenylpropanoid cinnamate 4-hydroxylase (C4H) and the lignin-specific *p*-coumarate 3-hydroxylase (C3H) and ferulate 5-hydroxylase (F5H) catalyze the corresponding hydroxylations of the aromatic ring (Fig. 1). Recent studies showed that at least C4H and C3H co-localize in the endoplasmic reticulum, forming protein complexes that interact with the soluble HCT and 4CL. These findings suggest the existence of a cluster of membrane proteins acting as a scaffold for further looser associations of soluble partners, leading to the creation of dynamic metabolons that drive the synthesis of a specific monolignol [17, 18].

The role of C3H in lignification has been clearly demonstrated by the characterization of two *Arabidopsis thaliana* C3H mutants [19, 20, 21]. A point mutation in the C3H coding sequence of the *ref8* mutant is sufficient to produce a strong impact on plant growth and inhibited the formation of G and S lignin monomers [20], but the T-DNA insertion mutant *cyp98A3* was found to have an even greater impact [21]. In the latter, the complete lack of C3H results in a strong reduction of lignin that is almost exclusively composed of H monomers (95%), a reduced cell expansion, altered cell wall sugar composition and decreased levels of crystalline cellulose. In addition, the authors reported a higher accumulation of flavonoids, indicating the occurrence of a cross-talk between hydroxycinnamic acid/lignin and flavonoid pathways [21]. Two additional CYP98A genes (*AtC3H2* and *AtC3H3*) have been identified in the *A. thaliana* genome [10] but are more divergent and constitute a separate class and do not appear to hydroxylate shikimate and quinate esters of *p*-coumaric acid [22].

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4 Heavily *C3H*-downregulated alfalfa plants (*Medicago sativa*), a forage legume, have been
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6 generated and characterized [23, 24]. Despite the only 5% residual C3H activity, these plants did
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8 not show severe growth and phenotypic impairments. Nevertheless, the huge increase in H lignin
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10 units and the reduction of the total lignin content highly increase the *in vitro* digestibility of their
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12 cell walls [23]. Similarly, one of the characterized *C3H*-repressed poplar plants [25, 26] showed
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14 that *C3H* down-regulation leads to 45% reduction of lignin but with a 20% accumulation of H
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16 monomers that occurs at expenses of the G subunits [26].
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21 Thus, the existing studies on *C3H* down-regulation performed in dicotyledonous species
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23 (Arabidopsis, poplar and alfalfa) revealed striking differences in the extent of the perturbations
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25 produced on lignin biosynthesis. As the only data available on the effects of C3H repression
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27 came from dicotyledonous plants, we addressed this issue in the monocotyledonous maize plant.
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29 Like other grasses, the structural organization of maize cell wall is significantly different from
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31 those of dicotyledonous plants in terms of lignin (with higher amounts of H subunits), higher
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33 hydroxycinnamates content [27, 28] and hemicellulose composition [27, 29]. In addition, it has
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35 been recently shown that the flavone tricetin is also a structural component of the lignin polymer in
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37 grasses [30, 31].
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43 Therefore, any alteration of one of these biopolymers can produce effects that differ from the
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45 ones already described in dicotyledonous species.
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48 Although the maize genome contains two *C3H* genes, *ZmC3H1* (GRMZM2G138074) and
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50 *ZmC3H2* (GRMZM2G140817), it was initially thought that ZmC3H was encoded by a single
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52 gene whose expression pattern is compatible with a possible involvement in lignification [32] and
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54 association studies of the maize genetic variation in the phenylpropanoid pathway and forage
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quality identified a non-synonymous SNP in the terminal exon of *C3H1* associated with the *in vitro* digestibility of organic matter (IVDOM) [33].

Thus, in view of the importance of the composition and cross-linkage of lignin and hemicelluloses in the chemical properties of cell walls, we undertook the study of how *C3H1* repression affects the synthesis of lignin and of the main phenylpropanoids in maize. We generated and characterized transgenic *C3H1*-RNAi plants, paying special attention to the changes produced in the composition of the final lignin polymer and of the major components of the cell wall and analyzed whether these alterations affect cell wall degradability.

2. Methods

2.1. Generation of the double transgenic *A. thaliana* *atc3h/ZmC3H1* plants

The full coding sequence of *ZmC3H1* (1656 bp) was cloned (GRMZM2G138074_T01), placed under the control of the double CaMV 35S gene promoter and the pA35S transcription terminator. The construct was cloned into the pCAMBIA1300 vector and transferred into the *A. tumefaciens* (C58C1 strain) to transform *A. thaliana* plants (Col-0) by floral dip [34]. Transgenic plants expressing the maize *C3H1* were selected by growing them on MS medium supplied with hygromycin (32 mg/L) and then transferred to the greenhouse (25°C day/22°C night, 50% humidity). Homozygous seed stocks were obtained for three lines.

The *A. thaliana* *c3h* mutant (SALK_048706) [35] was grown on MS supplied with kanamycin (40 mg/L). As homozygous *c3h* are sterile, the heterozygous (flowering) *c3h* plants were cross-pollinated with the homozygous 35S::*ZmC3H1* ones. The resulting seeds were germinated on MS medium supplied with both hygromycin and kanamycin to select the double heterozygous *atc3h/ZmC3H1*. The offspring of these double heterozygous plants was then re-selected in vitro using the same two antibiotics, transferred to the greenhouse and rosette leaves were used to extract genomic DNA [36] to identify the double homozygous *atc3h/ZmC3H1* plants by genotyping. All the gene primers used are shown in Supplementary Fig. S4.

2.2. Generation of transgenic maize *C3H1-RNAi* plants

A genomic fragment spanning the first exon, the first intron and part of the second exon of the *C3H1* gene was cloned and used to generate the RNAi construct following the scheme of Supplementary Fig. S5. The insert was then cloned downstream of the maize ubiquitin gene

promoter and upstream of the nopaline synthase gene terminator of the pAHC25 vector [37] for maize transformation.

Maize HII (A188 x B73) x B73 calli were transformed and transgenic plants were regenerated following the protocols of the Plant Transformation Facility, Iowa State University (<http://www.agron.iastate.edu/ptf/#>) and the scheme shown in Supplementary Fig. S5.

PCR analyses showed that 8 out of 12 transformation events contained at least one full copy of the *C3H1*-RNAi sequence integrated into the genome, and all of them were able to regenerate viable plants (R0 generation).

RT-PCR analyses showed that only three transgenic lines displayed a reduced *C3H1* expression and were thus selected for further studies (line *C3H1*-RNAi-2, *C3H1*-RNAi-4 and *C3H1*-RNAi-14, Supplementary Figure 5). Of these, two lines (*C3H1*-RNAi-2 and *C3H1*-RNAi-4) produced fertile pollen and were selfed. The R1 offspring of *C3H1*-RNAi-2 and *C3H1*-RNAi-4 plants was treated with the herbicide bialaphos to discard wild type plants but no selection was made between homozygous and heterozygous stage. The *C3H1*-RNAi-14 line was male-sterile and was thus partially characterized at the R0 stage.

Maize transgenic and wild-type plants were grown under standard greenhouse conditions (25°C day and 22°C night with 60% humidity) and a 16/8 h photoperiod.

2.3. Gene expression analyses: RT-PCR and qRT-PCR and phylogenetic analyses

Total RNA was extracted with Trizol Reagent (Invitrogen) from young leaves (2nd from the apex) taken from 1-month old plants of the R1 generation and 1.5 µg of total RNA were reverse-transcribed using an oligo(dT)15 primer and M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. RT-PCR assays were run to select the *C3H1*-repressed

transgenic plants and qPCR assays were run to quantify the expression of the *C3H1* gene in each individual plant of the two *C3H1*-RNAi lines selected for this study. Three technical replicates were made and qPCR reactions were run using the Light Cycler 480 (Roche) and SYBR Green dye (Roche) and maize *Leuning* (*LUG*) gene was used for data normalization (38). qPCR assays were also run to analyze the expression of the main genes involved in lignin synthesis using two plants for each line. Gene-specific primers were designed using primer-blast program from the NCBI webpage. In all cases, PCR conditions included an initial denaturation step at 95°C for 10 min, followed by 45 cycles of a denaturation step at 95°C for 10s, an annealing step at 60°C for 30s and an extension step at 72°C for 30s. The gene-specific primers used to analyse the transcript levels of the phenylpropanoid genes are shown in Supplementary Fig. S4.

Phylogenetic analyses of the maize C3H1 and C3H2 with other C3H already characterized from other plant species was done using the online ClustalW program (<http://www.genome.jp/tools/clustalw/>) and the neighboring-joining method using Treecon program (39).

2.4. Lignin analysis

To observe the amount and distribution of the lignified cells in midrib and stems, transgenic and wild-type plants were grown to silage state. The third internode (ground level) and the third leaf (from the top) of four plants from each line were excised. Hand-cross sections were prepared from all these samples, stained with phloroglucinol and observed under light microscopy. Sections from leaves and stems of two viable plants from the *C3H1*-RNAi-14 line were also analysed.

Lignin content and composition were determined in mature midribs and whole stems of *C3H1*-RNAi and wild-type plants. Thioacidolysis and GC-MS were used to determine lignin composition according to the method published by [40] and total lignin content was gravimetrically determined by the Klason procedure [41].

For NMR analyses, 50-60 mg of finely divided (ball-milled) whole stem cell wall material taken from two wild type, *C3H1*-RNAi-2 and *C3H1*-RNAi-4 plants, was swollen in 0.75 mL of DMSO-*d*₆ according to the method previously described [42, 43]. NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 MHz instrument equipped with a cryogenically-cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). HSQC (heteronuclear single quantum coherence) experiments used Bruker's 'hsqcetgpsisp.2' pulse program (adiabatic-pulsed version) with spectral widths of 5000 Hz (from 10 to 0 ppm) and 20 843 Hz (from 165 to 0 ppm) for the ¹H- and ¹³C-dimensions. The number of collected complex points was 2048 for the ¹H-dimension with a recycle delay of 1s. The number of transients was 64, and 256 time increments were always recorded in the ¹³C-dimension. The ¹J_{CH} used was 145 Hz. Processing used typical matched Gaussian apodization in the ¹H dimension and squared cosine-bell apodization in the ¹³C dimension. Prior to Fourier transformation, the data matrixes were zero-filled up to 1024 points in the ¹³C-dimension. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.49). HSQC correlation peaks were assigned by comparing with the literature [30, 42, 44, 45, 46, 47, 48, 49, 50]. A semiquantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 processing software. In the aromatic/unsaturated region, C₂-H₂ correlations from H, G and S lignin units and from *p*-coumarates and ferulates, and C₆-H₆ correlations from tricetin, were used to estimate their relative abundances.

2.5. Soluble phenolics determination.

Total phenolics were determined using a modified Folin-Ciocalteu colorimetric method [51]. One mature leaf (2nd from the bottom) was taken from four 1-month old plants of the R1 generation (R0 in the case of *C3H1*-RNAi-14 line) and fresh leaf tissue (100 mg) was extracted in 1 ml ethanol (80%), incubated 2h at 4°C in the dark and then centrifuged to remove cell debris. Distilled water was added to aliquots of supernatant to reach a final volume of 3 ml. Then 0.5 ml Folin Ciocalteu reagent (1:1 with water) and 2 ml of Na₂CO₃ (20%) were added. The solution was warmed 15 min at 45°C, cooled to room temperature and the absorbance was measured at 650 nm. Total flavonols were determined according to [52]. Leaf tissues were extracted in 80% ethanol at 4°C for two hours. After centrifugation, methanol was added to aliquots of supernatant to reach a final volume of 2 ml and sequentially mixed with 0.1 ml aluminium chloride (10% water solution), 0.1ml K-acetate 1M and 2.8 ml distilled water. After 30 min incubation at room temperature, absorbance at 415 nm was recorded. Total anthocyanins were determined according to [53]. Leaf tissues (100 mg) were extracted with 1 ml of extraction solvent (methanol, water, hydrochloric acid, 7:2:1) at 4°C for 20h and centrifuged (20 min, 10000 rpm, 4°C) and the absorbance of the supernatants was measured at 530 nm.

2.6. Cell wall analysis

Dried stems from wild-type and transgenic plants were ground to a fine powder, extracted with 20 volumes of methanol, and filtered through GC. This alcohol insoluble residue (AIR) was dried at 60°C for 2 days. AIR was then de-starched, treated with acidified phenol and washed with organic solvents to obtain the cell wall residue as previously described [54, 55].

Neutral sugar analysis was performed according to [56]. Dried cell walls were hydrolyzed with 2M TFA (trifluoroacetic acid) for 1 h at 121°C and the resulting sugars were derivatized to alditol acetates and analyzed by gas chromatography (GC). Uronic acid contents were determined by the m-hydroxybiphenyl method [57], with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method [58] with the hydrolytic conditions described by [59] and quantification of the glucose released by the anthrone method [60] with glucose as a standard.

For the cell wall degradability assays, cell walls were hydrolyzed (20 mg/1.5 ml) in a mixture of Cellulase R10 (1%); Macerozyme R-10 (0.5%) and purified Driselase (0.1%) dissolved in sodium acetate 20mM (pH 4.8). Aliquots were taken at 6, 48 and 72 h, clarified by centrifugation and assayed for total sugars [61].

For cell wall fractionation, dry cell walls were extracted (2.5 ml/ 1mg cell wall) at room temperature with 0.1 M KOH + 20 mM NaBH₄ for 24 h. The soluble fraction after 0.1M KOH treatment was collected by filtration and the resulting residue was washed with distilled water. Soluble fraction and washing were mixed. Further, 4 M KOH + 20 mM NaBH₄ was added to the residue, extracted at room temperature for 24 h, and washed with distilled water as described above. The extracts were acidified to pH 5.0 with acetic acid and dialysed representing KOH 0.1N (KI) and KOH 4N (KII) fractions, respectively.

Ester-linked ferulate and p-coumarate monomers and ferulate dimers (diferulates) were extracted from alcohol insoluble residues with 2 M NaOH at room temperature and quantified by HPLC based on a procedure previously described [62]. Total diferulates were calculated as the sum of four identified and quantified dimers: 8-5-open (non-cyclic), 8-5-benzofuran (cyclic), 8-*O*-4, and 5-5.

3. Results

3.1. *ZmC3H1* partially rescues the severe phenotype of the *A. thaliana atc3h* mutant.

Only one sequence corresponding to a putative *C3H* (here defined as *ZmC3H1*) was initially found in maize [32, 33]. The *ZmC3H1* gene exhibited relatively low levels of expression in all organs studied (roots, young stem, leaves, basal and ear internodes), with slightly higher levels in the ear internode, where a striking switch of gene expression toward phenylpropanoid metabolism occurs, suggesting a high metabolic demand for lignin precursors [32]. In addition, gene expression analyses from microarray databases [63] show that *ZmC3H1* is mainly expressed in the aerial parts of the maize plants and is undetectable in non-lignifying tissues (Supplementary Fig. S1). More recently a second sequence, encoding a putative *C3H* has been identified in the maize genome (here defined as *ZmC3H2*) being its protein sequence 79% identical to *ZmC3H1*. The phylogenetic analysis shows that *ZmC3H1* and *ZmC3H2* are more closely related to the lignin-related *C3H* proteins described in *A. thaliana*, poplar and alfalfa than the two *AtC3H* proteins not involved in lignification (Supplementary Fig. S1). Gene expression analyses from microarray databases [63] show that *ZmC3H2* is ubiquitously expressed, especially in roots and in several non-lignifying tissues (Supplementary Fig. S1).

To functionally assess whether *ZmC3H1* is involved in the synthesis of lignin, we expressed this maize enzyme in the *A. thaliana c3h* mutant. Therefore we produced *A. thaliana* plants constitutively expressing *ZmC3H1* that were cross-pollinated with the hemizygous *atc3h* mutant to further isolate plants expressing *ZmC3H1* in the homozygous *atc3h* background. While the 35S::*ZmC3H1* plants did not display phenotypic alterations, the homozygous *atc3h* mutant showed the already reported extreme phenotype (Supplementary Fig. S2). Nevertheless, the

expression of *ZmC3H1* was able to rescue the dwarf phenotype of the homozygous *atc3h*. These plants, even if smaller than the wild-type, are able to complete their growth cycle, and flowers normally (Supplementary Fig. S2).

3.2. *C3H1* repression affects plant growth and fertility in maize

To study the role of *C3H1* in lignin synthesis, we produced transgenic RNAi maize plants to down-regulate this gene. Among all, three R0 transgenic lines showed a reduction of *C3H1* gene expression and were named *C3H1*-RNAi-2, *C3H1*-RNAi-4 and *C3H1*-RNAi-14 (Supplementary Fig. S5). These plants were grown in the greenhouse for their characterization. No changes in plant size were observed during the early growth stages (Fig 2A, E, I), yet a clear reduction of stem growth was observed in line 4 and 14 starting from one month of growth (Fig. 2F, J). The macroscopic alterations displayed by the three *C3H1*-repressed lines suggested an increasing down-regulation of *C3H1* gene expression. Therefore, young leaves from four one month-old *C3H1*-RNAi-2 and *C3H1*-RNAi-4 R1 plants were analysed for their residual *C3H1* gene expression by qRT-PCR. The results indicated that both *C3H1*-RNAi-2 and *C3H1*-RNAi-4 leaves retained approximately 40% residual *C3H1* expression at this leaf growing stage (Table 1). We also analyzed the expression of *C3H2* and determined that its gene expression does not statistically change in these transgenic lines (Table 1). Finally, using the same leaf material, we analyzed the expression of the main lignin biosynthetic genes to minimize the impact of differences in plant development and to catch a picture of the *C3H1* repression at its onset. RNA from two wild-type and two *C3H1*-RNAi-2 and *C3H1*-RNAi-4 lines was used for qPCR assays and the results obtained indicated that a common feature of these *C3H1*-repressed plants is the

up-regulation of the early-phenylpropanoid genes *PAL1*, while *4CL1* gene expression is induced only in leaves of *C3H1*-RNAi-2 line. (Sup Fig. S3).

At anthesis, while the average height of *C3H1*-RNAi-2 plants was similar to wild type, *C3H1*-RNAi-4 plants were 40% shorter than wild-type plants (Fig. 2B, F, J and Table 1) and produced in almost all cases an abnormal and sterile male inflorescence (Fig. 2H). In the case of *C3H1*-RNAi-14 plants, these plants were sterile and displayed a 70% growth reduction, accompanied by both a reduced number and length of internodes.

A feature common to all the *C3H1*-repressed plants was the appearance of a red coloration possibly due to the accumulation of anthocyanins in different parts of the plant. Thus, *C3H1*-RNAi-2 plants accumulated higher levels of pigments in the anthers, while *C3H1*-RNAi-4 and *C3H1*-RNAi-14 plants showed an unusual red coloration in large parts of mature leaf tissues (Fig. 2D, G, J). Senescent areas longitudinally crossed the leaf surface of *C3H1*-RNAi-2 mature leaves were also observed (Fig. 2C).

3.3. *C3H1* repression increases the accumulation of anthocyanins in a dose-dependent fashion

As expected from the phenotypic observation, the quantification of anthocyanins in leaf extracts indicates that *C3H1*-RNAi-4 and *C3H1*-RNAi-14 plants accumulate high amounts of these compounds, the endogenous levels being 3 and 9-fold higher compared to wild-type leaves respectively. No changes were observed in the case of *C3H1*-RNAi-2 leaves (Fig. 3A).

These alterations prompted us to investigate the effects on the accumulation of the other main products of the flavonoid pathway. Thus, in addition to anthocyanin, we quantified the endogenous levels of flavonols and soluble phenolics in mature leaves of transgenic and wild-

type plants. The quantification of the relative amount of flavonols indicates that no changes occur in transgenic leaves (Fig. 3B).

Finally the level of soluble phenolics increased proportionally to the extent of the phenotypic alterations. Thus, in *C3H1*-RNAi-2 leaves a 20% higher content was found, while in the *C3H1*-RNAi-4 and *C3H1*-RNAi-14 ones this was respectively 60% and 80% higher compared to wild-type leaves (Fig. 3C). Altogether, the results obtained suggest that the down-regulation of *C3H1* produces an enhancement of the metabolic flow towards the flavonoid branch of the phenylpropanoid pathway.

3.4. *C3H1*-RNAi stems display alterations in lignin content and composition

The effects of *C3H1* repression on lignin synthesis was assessed in midrib and whole stems of *C3H1*-RNAi-2, *C3H1*-RNAi-4, *C3H1*-RNAi-14 and wild-type plants collected at the silage state. Hand-cross sections from internodes and midribs of mature leaves were stained with phloroglucinol to analyse the distribution of lignified tissues. The results showed no significant differences in the distribution of lignified tissues in transgenic midribs, except for a lighter staining of *C3H1*-RNAi-4 samples, indicative of possible reduced lignin content (Fig. 4). On the other hand, *C3H1*-RNAi-4 and *C3H1*-RNAi-14 transgenic stems presented a reduced size of vascular bundles (Fig. 4).

The quantitative analysis of lignin indicated that *C3H1* repression did not significantly alter the lignin content of transgenic midribs, while *C3H1*-RNAi stems tend to accumulate less lignin compared to wild-type even if this tendency is statistically significant only at $p < 0.1$ (Table 2). *C3H1*-RNAi-14 line did not produce sufficient material to determine its Klason lignin.

The sum of the main subunits (H, G, and S) obtained by thioacidolysis indicated a significant reduction of their yield in *C3H1*-RNAi-4 plants. This yield is even more reduced in the *C3H1*-RNAi-14 line, despite only one plant could be analyzed (Table 2).

The determination of the monomeric composition of both midribs and stems indicated that no changes were detected in transgenic *C3H1*-RNAi midribs (Table 2). On the other hand, transgenic stems present an increase of H monomers that is proportional to the extent of the phenotypical alterations observed (Table 2). Thus, a 1.5 fold and 2.5 fold increase in H monomer was detected in *C3H1*-RNAi-2 and *C3H1*-RNAi-4 stems, respectively. In addition, this trend was reinforced by a 4 fold increase in H monomers in the stems of the only one *C3H1*-RNAi-14 plant that could be analyzed. In addition, our results also show a slight decrease of S monomers in *C3H1*-RNAi plants. In the case of *C3H1*-RNAi-2 stems, we also detected a slight increase of G subunits. However, this perturbation was not observed in the other *C3H1*-RNAi lines with a more severe phenotype (Table 2).

To deepen in the study of the modifications produced by *C3H1* repression on lignin polymer, we also performed 2D-NMR assays on the whole stem cell walls. The aromatic/unsaturated (δ_C/δ_H 90-150/5.5-8.5) regions of the HSQC NMR spectra of the whole cell-walls from the wild-type and *C3H1*-RNAi-2 and *C3H1*-RNAi-4 transgenic stems are shown in Figure 5 and the main correlation signals in this region are listed in Table 3.

The main cross-signals in the aromatic region of the HSQC spectra corresponded to the aromatic rings and unsaturated side-chains of the different H, G and S lignin units, and to *p*-coumarates (PCA) and ferulates (FA) that are associated to lignin. The S-lignin units showed a prominent signal for the C_{2,6}-H_{2,6} correlation at δ_C/δ_H 103.8/6.69, whereas the G-lignin units showed different correlations for C₂-H₂ (δ_C/δ_H 110.9/6.99) and C₅-H₅/C₆-H₆ (δ_C/δ_H 114.9/6.72 and δ_C/δ_H

118.9/6.76). Signals corresponding to C_{3,5}–H_{3,5} correlations in H-lignin units were observed at δ_C/δ_H 114.9/6.74 overlapping with other lignin signals, whereas the C_{2,6}–H_{2,6} correlations were observed at δ_C/δ_H 128.0/7.23. Signals corresponding to *p*-coumarate structures (PCA) were observed in the spectra of the whole cell-walls. Cross-signals corresponding to the C_{2,6}–H_{2,6} at δ_C/δ_H 129.9/7.44 and C_{3,5}–H_{3,5} at δ_C/δ_H 115.5/6.76 correlations of the aromatic ring and signals for the correlations of the unsaturated C _{α} –H _{α} at δ_C/δ_H 144.6/7.56 and C _{β} –H _{β} at 113.6/6.25 of the *p*-coumarate units were observed in this region of the HSQC spectra. Signals corresponding to the C₂–H₂ and C₆–H₆ correlations of ferulate moieties (FA) were also observed at δ_C/δ_H 110.9/7.30 and 123.1/7.12 in the spectra. The signals corresponding to the unsaturated C _{α} –H _{α} and C _{β} –H _{β} correlations overlapped with those of the *p*-coumarates. Moreover, the signals for the C _{β} –H _{β} correlations of *p*-coumarates and ferulates indicated that the carboxylic groups were not in free but in ester form. Interestingly, in this region of the HSQC spectra, it was also possible to detect the two distinctive signals at δ_C/δ_H 94.0/6.56 and 98.7/6.22 corresponding to the C₈–H₈ and C₆–H₆ correlations of the flavone triclin (T) [30]. The HSQC spectra also show the C₃–H₃ correlation at δ_C/δ_H 104.5/7.03 while the correlations for C₂–H₂ and C₆–H₆ are observed at δ_C/δ_H 103.9/7.30.

The molar abundances of the different lignin units (H, G and S), *p*-hydroxycinnamates (*p*-coumarates and ferulates) and the flavone triclin, in the wild-type and *C3H1*-RNAi-2 and *C3H1*-RNAi-4 transgenic plants, all estimated from volume integration of contours in the HSQC spectra, are shown in Table 4. In general terms, the results are in close agreement with those observed by thioacidolysis, and the most important feature is the significant and gradual enrichment in H-lignin units in the *C3H1*-RNAi stems, with respect to the wild-type, whereas the S/G ratio remains constant. Regarding the content of *p*-hydroxycinnamates, a higher abundance

of *p*-coumarates than ferulates was observed in the wild-type stem, with *p*-coumarates accounting for 58% and ferulates accounting for 24% of the total lignin units. *p*-Coumarates are mostly acylating the γ -OH of the lignin side-chain, whereas ferulates and diferulates acylate cell wall polysaccharides and participate in both polysaccharide-polysaccharide and lignin-polysaccharide cross-coupling reactions, in the latter case becoming integrally bound into the lignin polymer [64]. While the abundance of *p*-coumarates remains constant in the wild-type and the *C3H1*-RNAi stems, the abundance of ferulates gradually increased in the *C3H1*-RNAi stems with respect to the wild-type, which is reflected in the steadily decrease in the *p*-coumarate/ferulate ratio from 2.4 in the wild-type stem to 2.2 in the *C3H1*-RNAi-2 stems and 1.9 in the *C3H1*-RNAi-4 stems. Finally, tricetin units account for 7% of total lignin units in the wild-type stem, and its abundance increases up to 10% in the stems of the *C3H1*-RNAi plants.

3.5. Cell wall polysaccharides, cross-linkage and *in vitro* degradability

Wild-type and transgenic stems were analyzed for their cell wall polysaccharide composition and no major changes were detected in the cell walls of *C3H1*-RNAi stems (Table 5). In order to insight into cell wall features, the levels of cross-linkage of the cell wall polymers were also analyzed. Thus, the levels of esterified ferulates cross-linked to hemicelluloses did not show significant changes in transgenic cell walls compared to wild type plants (Table 5). Finally, we performed *in vitro* assays to analyze the response of these transgenic cell walls to enzymatic degradation and while no significant changes were observed in *C3H1*-RNAi-2 stems, a 30% increase was observed in the most repressed *C3H1*-RNAi-4 cell walls. This result is in line with the cell wall fractionation with alkali assays that revealed an increase of mild-alkali extractable hemicelluloses in the most repressed *C3H1*-RNAi-4 plants (Table 5).

4. Discussion

The existing functional redundancy of many lignin biosynthetic enzymes and their complex regulatory mechanisms lead to unexpected effects when the corresponding genes are down-regulated. However, the repression of the early stages of lignin synthesis in tobacco and *A. thaliana* always resulted in lignin reduction [21, 65, 66, 67].

Within this early-lignin biosynthetic pathway, C3H has been shown to be essential in the biosynthesis of G and S lignin monomers in several dicotyledonous plants [19, 20, 21, 23, 24, 25]. Moreover, the extremely severe phenotype of the only knock-out existing mutants [19, 21], suggests that the function of C3H in lignin synthesis cannot be compensated by other meta-hydroxylases.

The maize *C3H* has been initially identified as single copy gene (*ZmC3H1*) whose gene expression pattern is compatible with a possible involvement in lignification [32]. Recently, a second gene putatively encoding a C3H enzyme (*ZmC3H2*) has been identified in the maize genome. This maize *C3H2* displays a broad gene expression pattern that includes non-lignifying tissues and is highly expressed in roots, where *C3H1* is poorly detectable (Supplementary Fig. S1). In this work we undertook the functional characterization of the maize C3H1 and we showed that it is able to partially rescue the severe phenotypic alterations of the *A. thaliana c3h* mutant. To investigate the role of ZmC3H1, we generated maize transgenic plants down-regulated for this gene. In accordance with the role of C3H in the synthesis of G and S subunits in dicotyledonous, all the *C3H*-repressed plants described so far (alfalfa and poplar) presented reduced content of lignin that is enriched in H monomers [23, 25]. This trait is extreme in the knock-out *A. thaliana* mutant *ref8* in which the lignin formed is exclusively made of H units [19]. The maize *C3H1*-

RNAi plants characterized in this work only have a slight decrease in S units and an increase in H units in stems. This mild effect on the synthesis of these lignin subunits could be explained by the presence of C3H2 and/or the remaining C3H1 enzymatic activity.

On the other hand, no alterations were detected in transgenic midribs. A different effect on stems and midribs has already been described in *CAD*-RNAi maize plants [68] and together with previous studies indicates the occurrence of cell-type specific effects in lignin-repressed plants [15, 69, 70]. This can be attributed to the tight regulation of this metabolic pathway and, especially in the case of early genes, to the interplay of the lignin and flavonoid branches that can reallocate the flow of precursors according to the plant tissue/organ.

It has been reported that severely down-regulated *C3H1*-RNAi poplar lines did not survive the *in vitro* tissue culture process [25] and similarly, the maize *C3H1*-RNAi-14 plants failed to reach maturity. On the other hand, it has been shown that alfalfa can survive to practically minimum levels of C3H activity [23]. This can be due to the promoter used to make the gene construct (the vascular tissue-specific bean *PAL2* gene promoter) and/or to the herbaceous bearing of alfalfa in which the contribution of lignin to plant stiffness is limited compared to maize. It has been shown that a slight perturbation in lignin biosynthesis can trigger severe growth impairments, such as the case of the double brown-midrib *bm2-bm4* maize mutants [71].

C3H1-RNAi maize plants did not display alterations at the early stages of their development, while these effects became visible when the plant increased its height. These plants presented growth reduction, over-accumulation of anthocyanins and sterility. These phenotypic traits are similar to the ones described in other heavily *C3H*-repressed [19, 21, 23] or lignin-down regulated plants [72, 73].

The higher levels of leaf soluble phenolics indicate that the repression of *C3H1* expression produced both the accumulation of precursors and the redirection of *p*-coumaroyl-CoA into the flavonoid pathway. In this sense, our data indicate that the abundance of tricetin increases in the stems of the *C3H1*-RNAi plants. Tricetin is a flavone that is known to be incorporated into the lignin polymer in grasses and participates in cross-coupling reactions with monolignols [30]. Its increased levels are consistent with the redirection of the carbon flux through the synthesis of flavonoids observed in *C3H1*-RNAi transgenic maize plants. As tricetin can undergo cross-coupling with monolignols and must be present at the start of lignification acting as the initiation site [30], the relative increase of tricetin in *C3H1*-RNAi lignin polymer could stimulate and promote the formation of shorter lignin chains.

The polysaccharides composition of the transgenic cell walls remained substantially unchanged in *C3H1*-RNAi stems. However, the percentage of mild-alkali extractable hemicelluloses in the cell walls of the most repressed *C3H1*-RNAi-4 plants is increased. Accordingly, the cell walls of these transgenic plants are 30% more degradable than the ones of wild type plants.

The results obtained in this work show that *C3H1* can participate in lignin synthesis as its down-regulation affects lignin biosynthesis in stems. In addition, *C3H1* repression affects plant development and growth and increases their flavonoid content, possibly as the result of a redirection of the metabolic flow into the flavonoid biosynthetic pathway. As *C3H1* gene repression has been achieved using a constitutive gene promoter, it could not be excluded that some of the phenotypical alterations observed in the maize *C3H1*-RNAi plants are related to the involvement of *C3H1* in some metabolic processes other than lignin biosynthesis.

In sum, our results indicate that the changes produced in the cell walls of the *C3H1*-RNAi plants are essentially committed to perturbations of the lignin polymer composition; reduced

thioacidolysis yield, increased levels of H units, triclin and lignin-associated ferulates. These changes may generate weaker interactions between lignin and cell wall polysaccharides that lead to an enhanced *in vitro* degradability of the transgenic *C3H1*-RNAi cell walls.

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Disclosures

The authors declare that there are no conflicts of interest.

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References

- [1] W. Boerjan, J. Ralph, M. Baucher, Lignin biosynthesis, *Annu. Rev. Plant Biol.* 54 (2003) 519-546.
- [2] R. Vanholme, B. Demedts, K. Morreel, J. Ralph, W. Boerjan, Lignin biosynthesis and structure, *Plant Physiol.* 153 (2010) 895-905.
- [3] Y. Wang, M. Chantreau, R. Sibout, S. Hawkins, Plant cell wall lignification and monolignol metabolism, *Front. Plant Sci.* 4 (2013) 220. doi: 10.3389/fpls.2013.00220.
- [4] A.T. Martínez, J. Rencoret, G. Marques, A. Gutiérrez, D. Ibarra, J. Jiménez-Barbero, J.C. del Río, Monolignol acylation and lignin structure in some nonwoody plants: A 2D NMR study, *Phytochem.* 69 (2008) 2831-2843.
- [5] L.E. Bartley, M.L. Peck, S.-R. Kim, B. Ebert, C. Manisseri, D.M. Chiniquy, R. Sykes, L. Gao, C. Rautengarten, M.E. Vega-Sánchez, P.I. Benke, P.E. Canlas, P. Cao, S. Brewer, F. Lin, W.L. Smith, X. Zhang, J.D. Keasling, R.E. Jentoff, S.B. Foster, J. Zhou, A. Ziebell, G. An, H.V. Scheller, P.C. Ronald, Overexpression of a BAHD Acyltransferase, OsAt10, alters rice cell wall hydroxycinnamic acid content and saccharification, *Plant Physiol.* 161 (2013) 1615-1633.
- [6] F. Lu, J. Ralph, Novel tetrahydrofuran structures derived from β - β -coupling reactions involving sinapyl acetate in Kenaf lignins, *Org. Biomol. Chem.* 6 (2008) 3681-3694.

- [7] N.D. Bonawitz, C. Chapple, The genetics of lignin biosynthesis: connecting genotype to phenotype, *Annu. Rev. Genet.* 44 (2010) 337-363.
- [8] G. Forkmann, S. Martens, Metabolic engineering and applications of flavonoids, *Curr. Opin. Biotechnol.* 12 (2001) 155-160.
- [9] M.L. Falcone Ferreyra, S.P. Rius, P. Casati, Flavonoids: biosynthesis, biological functions, and biotechnological applications, *Front. Plant Sci.* 3 (2012) 222. doi:10.3389/fpls.2012.00222.
- [10] J. Raes, A. Rohde, J.H. Christensen, Y. Van de Peer, W. Boerjan, Genome-wide characterization of the lignification toolbox in *Arabidopsis*, *Plant Physiol.* 133 (2003) 1051-1071.
- [11] R. Vanholme, I. Cesarino, K. Rataj, Y. Xiao, L. Sundin, G. Goeminne, H. Kim, J. Cross, K. Morreel, P. Araujo, L. Welsh, J. Hastraete, C. McClellan, B. Vanholme, J. Ralph, G.G. Simpson, C. Halpin, W. Boerjan, Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in *Arabidopsis*, *Science* 341(2013) 1103-1106.
- [12] F. Chen, R.A. Dixon, Lignin modification improves fermentable sugar yields for biofuel production, *Nature Biotech.* 25 (2007) 759-761.

- [13] B.A. Simmons, D. Logue, J. Ralph, Advances in modifying lignin for enhanced biofuel production. *Curr. Opin. Plant Biol.* 13 (2010) 313-320.
- [14] X. Li, J.K. Weng, C. Chapple, Improvement of biomass through lignin modification, *Plant J.* 54 (2008) 569-581.
- [15] J. Nakashima, F. Chen, L. Jakson, G. Shadle, R.A. Dixon, Multi-site genetic modification of monolignol biosynthesis in alfalfa (*Medicago sativa*): effects on lignin composition in specific cell types, *New Phytol.* 179 (2008) 738-750.
- [16] R. Vanholme, K. Morreel, J. Ralph, W. Boerjan, Lignin engineering, *Curr. Opin. Plant Biol.* 11 (2008) 1-8.
- [17] H.C. Chen, Q. Li, C.M. Shuford, J. Liu, D.C. Muddiman, R.R. Sederoff, V.L. Chiang, Membrane protein complexes catalyze both 4- and 3-hydroxylation of cinnamic acid derivatives in monolignol biosynthesis, *Proc. Natl. Acad. Sci. U S A.* 108 (2011) 21253-21258.
- [18] J.E. Bassard, L. Richert, J. Geerinck, H. Renault, F. Duval, P. Ullmann, M. Schmitt, E. Meyer, J. Mutterer, W. Boerjan, G. De Jaeger, Y. Mely, A. Goossens, D. Werck-Reichhart, Protein-Protein and Protein-Membrane Associations in the Lignin Pathway, *Plant Cell* 24 (2012) 4465-4482.

- [19] R. Franke, M.R. Hemm, J.W. Denault, M.O. Ruegger, J.M. Humphreys, C. Chapple, Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of *Arabidopsis*, *Plant J.* 30 (2002a) 47-59.
- [20] R. Franke, J.M. Humphreys, M.R. Hemm, J.W. Denault, M.O. Ruegger, J.C. Cusumano, C. Chapple, The *Arabidopsis* REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism, *Plant J.* 30 (2002b) 33-45.
- [21] N. Abdulrazzak, B. Pollet, J. Ehlting, K. Larsen, C. Asnaghi, S. Ronseau, C. Proux, M. Erhardt, V. Seltzer, J.P. Renou, P. Ullmann, M. Pauly, C. Lapierre and D. Werck-Reichhart, A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of nonredundant meta-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth, *Plant Physiol.* 140 (2006) 30-48.
- [22] G. Schoch, S. Goepfert, M. Morant, A. Hehn, D. Meyer, P. Ullmann, D. Werck-Reichhart, CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway, *J Biol Chem.* 276 (2001) 36566-36574.
- [23] M.S.S. Reddy, F. Chen, G. Shadle, L. Jackson, H. Aljoe, R.A. Dixon, Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.), *Proc. Natl. Acad. Sci. USA* 102 (2005) 16573-16578.

- [24] J. Ralph, T. Akiyama, H. Kim, F. Lu, P.F. Schatz, J.M. Marita, S.A. Ralph, M.S.M. Reddy, F. Chen, R.A. Dixon, Effects of coumarate 3-hydroxylase down-regulation on lignin structure, *J. Biol. Chem.* 281(2006) 8843-8853.
- [25] H.D. Coleman, J.Y. Park, R. Nair, C. Chapple, S.D. Mansfield, RNAi-mediated suppression of *p*-coumaroyl-CoA 3'-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism, *Proc. Natl. Acad. Sci. USA* 105 (2008) 4501-4506.
- [26] J. Ralph, T. Akiyama, H.D. Coleman, S.D. Mansfield, Effects on lignin structure of Coumarate 3-Hydroxylase downregulation in poplar, *Bioenerg. Res.* 5 (2012) 1009-1019.
- [27] Y. Barrière, C. Riboulet, V. Méchin, S. Maltese, M. Pichon, A. Cardinal., C. Lapierre, T. Lübberstedt, J.P. Martinant, Genetics and genomics of lignification in grass cell walls based on maize as model species, *Genes, Genomes and Genomics*. 1 (2007) 133-156.
- [28] J.M. Marita, R.D. Hatfield, D.M. Rancour, K.E. Frost, Identification and suppression of the *p*-coumaroyl CoA:hydroxycinnamyl alcohol transferase in *Zea mays L.*, *Plant J.* 78 (2014) 850-864.
- [29] N.C. Carpita, Structure and biogenesis of the cell walls of grasses, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 47 (1996) 445-476.

- [30] J.C. del Río, J. Rencoret, P. Prinsen, Á.T. Martínez, J. Ralph, A. Gutiérrez, Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods, *J. Agric. Food Chem.* 60 (2012a) 5922-5935.
- [31] W. Lan, F. Lu, M. Regner, Y. Zhu, J. Rencoret, S.A. Ralph, U.I. Uzma, K. Morreel, W. Boerjan, J. Ralph, Tricin, a flavonoid monomer in monocot lignification, *Plant Physiol.* 167 (2015) 1284-1295.
- [32] S. Guillaumie, H. San-Clemente, C. Deswarte, Y. Martinez, C. Lapierre, A. Murigneux, Y. Barrière, M. Pichon, D. Goffner, MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize, *Plant Physiol.* 143 (2007) 339-363.
- [33] J.R. Andersen, I. Zein, G. Wenzel, B. Darnhofer, J. Eder, M. Ouzunova, T. Lübberstedt, Characterization of phenylpropanoid pathway genes within European maize (*Zea mays* L.) inbreds, *BMC Plant Biol.* 8 (2008) 2. doi:10.1186/1471-2229-8-2.
- [34] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, *Plant J.* 16 (1998) 735-743.
- [35] J.M. Alonso, A.N. Stepanova, T.J. Leisse, C.J. Kim, H. Chen, P. Shinn, D.K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C.C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M.

Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D.E. Carter, T. Marchand, E. Risseuw, D. Brogden, A. Zeko, W.L. Crosby, C.C. Berry, J.R. Ecker, Genome-wide insertional mutagenesis of *Arabidopsis thaliana*, Science 301 (2003) 653-657.

[36] K. Edwards, C. Jonhstone, C. Thompson, A simple and rapid method for the preparation of plant genomic DNA for PCR analyses, Nucl. Ac. Res. 19 (1991) 1349.

[37] A.H. Christensen, P.H. Quail, Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants, Transgenic Res. 5 (1996) 213-218.

[38] A. Manoli, A. Sturaro, S. Trevisan, S. Quaggiotti, A. Nonis, Evaluation of candidate reference genes for qPCR in maize. J. Plant Phys. 169 (2012) 807-815.

[39] Y. Van de Peer, R. De Wachter, TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10 (1994) 569-570.

[40] C. Lapierre, C. Rolando, B. Monties, Thioacidolysis of poplar lignins: identification of monomeric syringyl products and characterization of guaiacyl syringyl fractions, Holzforschung 40 (1986): 113-118.

- [41] C. Dence, Lignin determination, in: C. Dence, C. Lin (Eds.), *Methods in Lignin Biochemistry*, Springer Verlag, Berlin, 1992, pp. 33-61.
- [42] H. Kim, J. Ralph, T. Akiyama, Solution-state 2D NMR of ball-milled plant cell-wall gels in DMSO-*d*₆, *Bioenergy Res.* 1 (2008) 56-66.
- [43] J. Rencoret, G. Marques, A. Gutiérrez, L. Nieto, J.I. Santos, J. Jiménez-Barbero, A.T. Martínez, J.C. del Río HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state, *Holzforschung* 63 (2009) 691-698.
- [44] J. Ralph, J.M. Marita, S.A. Ralph, R.D. Hatfield, F. Lu, R.M. Ede, J. Peng, S. Quideau, R.F. Helm, J.H. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H.-J.G. Jung, L.L. Landucci,, J.J. MacKay, R.R. Sederoff, C. Chapple, A.M. Boudet, Solution-state NMR of lignin, in: D. S. Argyropoulos (Ed.) *Advances in Lignocellulosics Characterization*, Tappi Press, Atlanta, 1999, pp. 55-108.
- [45] J. Ralph, L.L. Landucci, NMR of Lignins, in: C. Heitner, D. R. Dimmel, J. A. Schmidt, (Eds.), *Lignin and lignans, advances in chemistry*, CRC Press (Taylor & Francis Group), Boca Raton, FL, 2010, pp. 137-234.

- [46] S.A. Ralph, J. Ralph, L. Landucci, NMR database of lignin and cell wall model compounds, US Forest Prod. Lab., One Gifford Pinchot Dr., Madison, WI 53705, (<http://ars.usda.gov/Services/docs.htm?docid=10491>) (2004) (accessed: January 2009).
- [47] J.C. del Río, J. Rencoret, G. Marques, A. Gutiérrez, D. Ibarra, J.I. Santos, J. Jiménez-Barbero, L. Zhang, A.T. Martínez, Highly acylated (acetylated and/or *p*-coumaroylated) native lignins from diverse herbaceous plants, *J. Agric. Food Chem.* 56 (2008) 9525-9534.
- [48] J.C. del Río, P. Prinsen, J. Rencoret, L. Nieto, J. Jiménez-Barbero, J. Ralph, A.T. Martínez, A. Gutiérrez, Structural characterization of the lignin in the cortex and pith of elephant grass (*Pennisetum purpureum*) stems, *J. Agric. Food Chem.* 60 (2012b) 3619-3634.
- [49] J. Rencoret, A. Gutiérrez, L. Nieto, J. Jiménez-Barbero, C.B. Faulds, H. Kim, J. Ralph, A.T. Martínez, J.C. del Río, Lignin composition and structure in young versus adult *Eucalyptus globulus* plants, *Plant Physiol.* 155 (2011) 667-682.
- [50] J. Rencoret, J. Ralph, G. Marques, A. Gutiérrez, A.T. Martínez, J.C. del Río, Structural characterization of lignin isolated from coconut (*Cocos nucifera*) coir fibers, *J. Agric. Food Chem.* 61 (2013) 2434-2445.
- [51] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic.* 16 (1965) 144-158.

- [52] C.C. Chang, M.H. Yang, H.M. Wen, J.C. Chern, Estimation of total flavonoid content in propolis by two complementary colorimetric methods, *J. Food Drug Anal.* 10 (2002) 178-182.
- [53] R.A.E. Laitinen, M. Ainasoja, S.K. Broholm, T.H. Teeri, P. Elomaa, Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*, *J. Exp. Bot.* 59 (2008) 3691-3703.
- [54] A. Encina, J.M. Sevillano, J.L. Acebes, J. Alvarez, Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil, *Physiol. Plant.* 114 (2002) 182-191.
- [55] H. Mérida, A. Encina, J. Álvarez, J.L. Acebes, D. Caparrós-Ruiz, Unraveling the biochemical and molecular networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil, *Mol. Plant* 3 (2010) 842-853.
- [56] P. Albersheim, D.S. Nevins, P.D. English, A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography, *Carbohydr. Res.* 5 (1967) 340-345.
- [57] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, *Anal. Biochem.* 5 (1973) 484-489.
- [58] D.M. Updegraff, Semi-micro determination of cellulose in biological materials, *Anal. Biochem.* 32 (1969) 420-424.

- [59] J.F. Saeman, W.E. Moore, M.A. Millett, in: R.L. Whistler (Ed.), Methods in carbohydrate chemistry Vol. 3, Academic Press, New York, 1963, pp.54-69.
- [60] Z. Dische, Color reactions of carbohydrates, in: R.L. Whistler, M.L. Wolfrom (Eds.), Methods in Carbohydrate Chemistry Vol. 1, Academic Press, New York, 1962, pp. 475-514.
- [61] M. Dubois, K.A. Gilles, J.K. Hamilton, D.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350-356.
- [62] R. Santiago, A. Butrón, J.T. Arnason, L.M. Reid, X.C. Souto, R.A. Malvar, Putative role of pith cell wall phenylpropanoids in *Sesamia nonagrioides* (Lepidoptera : Noctuidae) resistance, *J. Agric. Food Chem.* 54 (2006) 2274-2279.
- [63] R.S. Sekhon, H. Lin, K.L. Childs, C.N. Hansey, C.R. Buell, N. de Leon, S.M. Kaeppler, Genome-wide atlas of transcription during maize development, *Plant J.* 66 (2011) 553-563.
- [64] J. Ralph, Hydroxycinnamates in lignification, *Phytochem. Rev.* 9 (2010) 65-83.
- [65] S. Kajita, Y. Katayama, S. Omori, Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate: coenzyme A ligase, *Plant Cell Phys.* 37 (1996) 957-65.

- [66] K. Blee, J.W. Choi, A. O'Connell, S.C. Jupe, W. Schuch, N.G. Lewis, G.P. Bolwell, Antisense and sense expression of cDNA coding for CYP73A15, a class II cinnamate 4-hydroxylase, leads to a delayed and reduced production of lignin in tobacco, *Phytochem.* 57 (2001) 1159-1166.
- [67] V.J.H. Sewalt, W. Ni, J.W. Blount, H.G. Jung, S.A. Masoud, P.A. Howles, C. Lamb, R.A. Dixon, Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase, *Plant Physiol.* 115 (1997) 41-50.
- [68] S. Fornalé, M. Capellades, A. Encina, K. Wang, S. Irar, C. Lapierre, K. Ruel, J.P. Joseleau, J. Berenguer, P. Puigdomènech, J. Rigau, D. Caparrós-Ruiz, Altered lignin biosynthesis improves cellulosic bioethanol production in transgenic maize plants down-regulated for cinnamyl alcohol dehydrogenase, *Mol. Plant* 5 (2012) 817-830.
- [69] C. Chen, H. Meyermans, B. Burggraeve, R.M. De Rycke, K. Inoue, V. De Vleeschauwer, M. Steenackers, M.C. Van Montagu, G.J. Engler, W. Boerjan, Cell-specific and conditional expression of caffeoyl-coenzyme A-3-O-methyltransferase in poplar, *Plant Physiol.* 123 (2000) 853-868.
- [70] J.C. Leplé, R. Dauwe, K. Morreel, V. Storme, C. Lapierre, B. Pollet, A. Naumann, K.Y. Kang, H. Kim, K. Ruel, A. Lefèbvre, J.P. Joseleau, J. Grima-Pettenati, R. De Rycke, S. Andersson-Gunnerås, A. Erban, I. Fehrle, M. Petit-Conil, J. Kopka, A. Polle, E. Messens, B.

Sundberg, S.D. Mansfield, J. Ralph, G. Pilate, W. Boerjan, Downregulation of cinnamoyl-coenzyme A reductase in poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure, *Plant Cell* 19 (2007) 3669-3691.

[71] W. Vermerris, D.M. Sherman, L.M. McIntyre, Phenotypic plasticity in cell walls of maize brown midrib mutants is limited by lignin composition, *J Exp Bot.* 61 (2010) 2479-2490.

[72] J. Gui, J. Shen, L. Li, Functional characterization of evolutionarily divergent 4-Coumarate:Coenzyme A Ligases in rice, *Plant Physiol.* 157 (2011) 574-586.

[73] J. Thevenin, B. Pollet, B. Letarnec, L. Saulnier, L. Gissot, A. Maia-Grondard, C. Lapierre, L. Jouanin, The simultaneous repression of CCR and CAD, two enzymes of the lignin biosynthetic pathway, results in sterility and dwarfism in *Arabidopsis thaliana*, *Mol. Plant* 4 (2011) 70-82.

Figure Legends

Figure 1: The phenylpropanoid pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; C3H, 4-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F5H, ferulate-5-hydroxylase, HCALDH, hydroxycinnamaldehyde dehydrogenase; AMT, acetyl-CoA:monolignol transferase; PMT, p-coumarate:monolignol transferase; pCAT, p-coumaroyl CoA:hydroxycinnamyl alcohol transferase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FNSII, flavone synthase II; F3'5'H, flavonoid 3',5'-hydroxylases; pCA-Lignin, p-coumarate linked to the lignin polymer. The metabolic steps required to produced flavonoids from dihydrokaempferol are represented by single broken arrows. Grey dotted arrows represent putative pathways through which ferulic acid can be synthesized. The enzymatic steps involved in the production of three main lignin-related metabolites (p-coumaryl alcohol, ferulic acid and triclin) and flavonoids that are over-accumulated in *C3H1*-RNAi plants are shown under a grey background (only the possible pathways that could produce ferulic acid without the involvement of C3H are marked). Enzymes with asterisks refer to enzymatic steps that have been described in *Arabidopsis thaliana* but still not described in maize or in any grass plant species.

Figure 2: Macroscopic phenotype of mature wild-type and *C3H1*-RNAi 2, 4 and 14 lines along their development. Phenotype of *C3H1*-RNAi-2 (A), *C3H1*-RNAi-4 (E) and *C3H1*-RNAi-14 (I)

plantlets. Phenotype of 45 days-old *C3H1*-RNAi-2 (B) and *C3H1*-RNAi-4 (F). Detail of a mature leaf of *C3H1*-RNAi-2 (C) and *C3H1*-RNAi-4 (G). Anthers of *C3H1*-RNAi-2 (D). Sterile male inflorescence of *C3H1*-RNAi-4 (H). Phenotype of *C3H1*-RNAi-14 at final stage of development (J). Bottom: size of whole plants and internodes at anthesis. Data represent the mean $\pm$ SD of 4 different plants (2 in the case of *C3H1*-RNAi-14 line). Asterisks refer to a significant difference compared to wild type by Student-t test. * $p < 0.05$ and ** $p < 0.01$.

Figure 3: Macroscopic leaf phenotype and phenolics contents of wild-type and *C3H1*-RNAi mature leaves. Relative quantification of anthocyanins (A), flavonols (B) and soluble phenolics (C). Samples were taken from 4 plants of each line (2 in the case of *C3H1*-RNAi-14 line) and data correspond to the mean of the values obtained $\pm$ SD. Asterisks refer to a significant difference compared to wild type by Student-t test. * $p < 0.05$.

Figure 4: Lignin staining of hand-cross sections of wild-type and *C3H1*-RNAi internodes and midribs. Wiesner staining was used to visualize the lignified tissues. Pictures of the abaxial and adaxial sides of midribs and of internodes were taken. Scale bar corresponds to 500 μ m. Measurement of the major diameter of vascular bundles was done using the ImageJ 1.48v program (<http://rsb.info.nih.gov/ij/>). Sections from 4 plants of each line were used (only one plant available for *C3H1*-RNAi-14. n refers to the number of vascular bundles measured. Data correspond to the mean of the values obtained $\pm$ SD. Asterisks refer to a significant difference compared to wild type by Student-t test. * $p < 0.001$.

Figure 5: Aromatic/unsaturated region (δ_C/δ_H 90-150/5.5-8.5) of the HSQC NMR spectra of the whole cell walls of wild-type (A), *C3H1*-RNAi-2 (B) and *C3H1*-RNAi-4 stems (C). Main lignin units present in the HSQC NMR spectra of the wild-type and *C3H1*-RNAi stems: (PCA) *p*-coumarates; (FA) ferulates; (H) *p*-hydroxyphenyl units; (G) guaiacyl units; (S) syringyl units; (T) triclin. (See Table 3 for lignin signal assignment).

Supplementary Data

Supplementary Fig. S1: (A) Phylogenetic relationship of the maize C3H1 and C3H2 with the already characterized lignin-related C3H of several species. Numbers on branches refer to the bootstrap proportion. The scale bar represents 0.1 substitutions per position (B) Gene expression analysis of the maize *C3H1* and *C3H2* obtained from microarrays databases [61].

Supplementary Fig. S2: The maize C3H1 partially rescues the dwarf phenotype of the *A. thaliana c3h1* mutant.

Supplementary Fig. S3: Gene expression analysis of the main phenylpropanoid genes in wild-type and *C3H1*-RNAi leaves: qPCR assays were run using *LUG* as internal standard. Two plants for each line were analyzed separately and data are the mean $\pm$ SD of three replicates. (White bars: WT; dark-grey bars: *C3H1*-RNAi-2; light-grey bars: *C3H1*-RNAi-4).

Supplementary Fig. S4: List of the primers used in this work.

Supplementary Fig. S5: Flowchart of the production of the *C3H1*-RNAi maize plants. The repression of *C3H1* gene in three R0 transgenic plants is shown by RT-PCR. Ubiquitin was used as a control gene.

Table 1: Gene expression analysis of *C3H1* and *C3H2* in wild-type and transgenic plants. qRT-PCR assays were run using young leaves taken from 4 plants of each line (2nd from the apex, taken from 1-month old plants). *LUG* was used as house-keeping gene. Plants were analyzed separately (three technical replicates) and data represent the mean of the values obtained $\pm$ SD. Asterisks refer to a significant difference compared to wild type by Student-t test. * $p < 0.05$ and ** $p < 0.01$.

	<i>C3H1</i> Relative Expression	<i>C3H2</i> Relative Expression
WT	100% $\pm$ 23	100% $\pm$ 10
<i>C3H1</i>RNAi-2	43% $\pm$ 39*	82% $\pm$ 11
<i>C3H1</i>RNAi-4	42% $\pm$ 17**	137% $\pm$ 20
<i>C3H1</i>RNAi-14	n.d.	n.d.

Table 2: Analysis of lignin content and its monomeric composition in wild-type and *C3H1*-RNAi stems (top) and midrib (bottom). Lignin content was quantified using the Klason assay and the results expressed as mg per gram of Alcohol Insoluble Residue (AIR). H+G+S refers to the thioacidolysis yield expressed as the total nmols of the three subunits per gram of AIR. Data represent the mean $\pm$ SD of the values obtained by 4 plants of each line. Lignin composition was determined by thioacidolysis and GC-MS analysis of the AIR obtained from midribs and whole stems of four plants of each line. The relative molar frequencies of each lignin monomer are expressed as percentage of the total. KL refers to Klason lignin. Asterisks refer to a significant difference compared to wild type by Student-t test. * $p < 0.05$ and ** $p < 0.01$. n.d. refers to not determined. (1) Only one plant from this line could be analyzed.

STEM				
	WT	<i>C3H1</i> -RNAi-2	<i>C3H1</i> -RNAi-4	<i>C3H1</i> -RNAi-14
KL (mg/gAIR)	319.96 $\pm$ 57.1	245.0 $\pm$ 24.8	257.6 $\pm$ 23.2	n.d.
H+G+S (mmol/gAIR)	1.8 $\pm$ 0.4	1.3 $\pm$ 0.4	0.9 $\pm$ 0.4**	0.7 $\pm$ 0.1 ⁽¹⁾
%H	2.0 $\pm$ 0.4	3.3 $\pm$ 0.9*	5.1 $\pm$ 2.1**	7.8 $\pm$ 0.1 ⁽¹⁾
%G	44.4 $\pm$ 1.3	52.5 $\pm$ 3.3**	44.0 $\pm$ 2.6	37.5 $\pm$ 0.2 ⁽¹⁾
%S	53.6 $\pm$ 1.6	44.3 $\pm$ 4.1**	51.0 $\pm$ 1.9*	54.7 $\pm$ 0.3 ⁽¹⁾
MIDRIB				
	WT	<i>C3H1</i> -RNAi-2	<i>C3H1</i> -RNAi-4	<i>C3H1</i> -RNAi-14
KL (mg/gAIR)	280.7 $\pm$ 74.1	218.7 $\pm$ 46.8	264.7 $\pm$ 35.6	n.d.
H+G+S (mmol/gAIR)	1.1 $\pm$ 0.2	1.5 $\pm$ 0.2	1.2 $\pm$ 0.2	1.7 $\pm$ 0.4 ⁽¹⁾
%H	5.2 $\pm$ 0.9	4.5 $\pm$ 0.4	6.1 $\pm$ 0.8	5.0 $\pm$ 1.0 ⁽¹⁾
%G	70.2 $\pm$ 6.3	72.6 $\pm$ 2.8	68.4 $\pm$ 2.1	67.0 $\pm$ 3.0 ⁽¹⁾
%S	24.7 $\pm$ 7.0	22.9 $\pm$ 2.9	25.6 $\pm$ 1.5	28.0 $\pm$ 2.0 ⁽¹⁾

Table 3: Assignments of the lignin ^{13}C - ^1H correlation signals in the HSQC spectra of wild-type and *C3H1*-RNAi stems.

Labels	$\delta_{\text{C}}/\delta_{\text{H}}$ (ppm)	Assignment
<i>Lignin correlations</i>		
T ₈	94.0/6.56	C ₈ -H ₈ in tricin (T)
T ₆	98.7/6.22	C ₆ -H ₆ in tricin (T)
S _{2,6}	103.8/6.69	C _{2,6} -H _{2,6} in etherified syringyl units (S)
T _{2',6'}	103.9/7.30	C _{2',6'} -H _{2',6'} in tricin (T)
T ₃	104.5/7.03	C ₃ -H ₃ in tricin (T)
G ₂	110.9/6.99	C ₂ -H ₂ in guaiacyl units (G)
FA ₂	110.9/7.30	C ₂ -H ₂ in ferulate (FA)
PCA _β /FA _β	113.6/6.25	C _β -H _β in <i>p</i> -coumarates (PCA) and ferulates (FA)
H _{3,5}	114.9/6.74	C _{3,5} -H _{3,5} in <i>p</i> -hydroxyphenyl units (H)
G _{5/6}	114.9/6.72	C ₅ -H ₅ and C ₆ -H ₆ in guaiacyl units (G)
FA ₅	115.4/6.86	C ₅ -H ₅ in ferulate (FA)
PCA _{3,5}	115.5/6.76	C _{3,5} -H _{3,5} in <i>p</i> -coumarates (PCA)
G ₆	118.9/6.76	C ₆ -H ₆ in guaiacyl units (G)
FA ₆	123.1/7.12	C ₆ -H ₆ in ferulates (FA)
H _{2,6}	128.0/7.23	C _{2,6} -H _{2,6} in <i>p</i> -hydroxyphenyl units (H)
PCA _{2,6}	129.9/7.44	C _{2,6} -H _{2,6} in <i>p</i> -coumarates (PCA)
PCA _α /FA _α	144.6/7.56	C _α -H _α in <i>p</i> -coumarates (PCA) and ferulates (FA)

Table 4: Analysis of lignin composition (relative molar composition of the H, G, and S-lignin aromatic units, S/G ratio, *p*-hydroxycinnamates, *p*-coumarate/ferulate ratio, and tricetin) in wild-type and *C3H1*-RNAi stems. Lignin composition was determined from integration of ^{13}C - ^1H correlation signals in the by 2D-NMR (HSQC) of the AIR samples obtained from whole stems. ^aMolar percentages (H+G+S=100). ^b*p*-Hydroxycinnamate and tricetin levels expressed as a fraction of lignin content (H+G+S).

Lignin aromatic units ^a	WT	<i>C3H1</i> -RNAi-2	<i>C3H1</i> -RNAi-4
H (%)	6	15	21
G (%)	41	40	35
S (%)	53	45	44
S/G ratio	1.3	1.1	1.3
Hydroxycinnamates ^b			
<i>p</i> -coumarates (%)	58	58	59
ferulates (%)	24	26	31
coumarates/ferulates ratio	2.4	2.2	1.9
Tricetin ^b	7	10	10

Table 5: Cell wall sugar and ferulates determination and degradability assays of *C3H1*-RNAi and wild type stems. Whole stems from 4 wild-type plants and 4 plants of each transgenic line were analyzed. Data are expressed as mean of the value obtained $\pm$ SD. (H/C, Hemicellulose/Cellulose). Cell wall degradability assay was performed using whole stems taken from 4 wild-type plants and 4 plants of each *C3H1*-RNAi line. Data correspond to the mean of the individual values obtained $\pm$ SD. (CW, cell wall). Diferulates were calculated as the sum of 5-5, 8-*O*-4, 8-5-open and 8-5 benzofurans forms. Data correspond to the mean $\pm$ SD of n=2-5. Total sugar content in hemicellulose fractions from cell walls of *C3H1*-RNAi and wild-type stems. Data correspond to the mean $\pm$ SD of 4 plants each line. K-I refers to hemicelluloses extracted with KOH-0.1N, K-II refers to hemicelluloses extracted with KOH-4N. Asterisks refer to a significant difference compared to wild type by Student-t test. *p<0.001 and **p<0.05.

(μ g/mgCW)	WT	<i>C3H1</i> -RNAi-2	<i>C3H1</i> -RNAi-4
Rhamnose	0.94 $\pm$ 0.98	0.85 $\pm$ 0.43	0.48 $\pm$ 0.37
Fucose	0.57 $\pm$ 0.33	0.67 $\pm$ 0.07	0.52 $\pm$ 0.45
Arabinose	27.40 $\pm$ 15.21	41.39 $\pm$ 13.20	14.82 $\pm$ 6.6
Xylose	299.53 $\pm$ 23.9	317.94 $\pm$ 14.8	296.64 $\pm$ 79.1
Mannose	1.64 $\pm$ 0.76	1.96 $\pm$ 0.55	1.17 $\pm$ 1.00
Galactose	5.90 $\pm$ 3.6	10.91 $\pm$ 4.2	4.56 $\pm$ 2.7
Glucose	10.58 $\pm$ 5.0	20.91 $\pm$ 6.0	14.52 $\pm$ 8.8
Uronic acids	20.81 $\pm$ 7.6	46.27 $\pm$ 5.7	26.93 $\pm$ 15.3
Cellulose	492.05 $\pm$ 80.3	405.78 $\pm$ 38.3	477.00 $\pm$ 32.6
Ferulates	3.80 $\pm$ 1.3	3.74 $\pm$ 0.1	3.06 $\pm$ 1.1
Diferulates	0.38 $\pm$ 0.2	0.58 $\pm$ 0.0	0.35 $\pm$ 0.2
degradability (mg sugars/ gCW)	547.96 $\pm$ 56.6	512.93 $\pm$ 35.2	722.28 $\pm$ 134*
K-I fraction	83.5 $\pm$ 17.0	87.2 $\pm$ 16.5	127.8 $\pm$ 29.4**
K-II fraction	269.8 $\pm$ 19.6	293.9 $\pm$ 26.2	288.0 $\pm$ 54.5

Figure 1

Figure 2

	Height at anthesis (cm)	Number of developed internodes	Internode length (cm)
WT	185 ±17	14 ±1	13 ±1
<i>C3H1</i> -RNAi-2	215 ±21	13 ±1	16 ±2
<i>C3H1</i> -RNAi-4	110 ±47*	13 ±1	9 ±4
<i>C3H1</i> -RNAi-14	52 ±8**	6 ±1**	9 ±1**

Figure 3

Figure 4

Stem vascular bundles diameter (μm)

Wild type (n=21)	177 ± 53
C3H1-RNAi-2 (n=29)	183 ± 35
C3H1-RNAi-4 (n=49)	123*± 28
C3H1-RNAi-14 (n=18)	106*± 10

Figure 5