Combining enhanced biomass density with reduced lignin level for improved forage quality

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Summary

To generate a forage crop with increased biomass density that retains forage quality, we have genetically transformed lines of alfalfa (*Medicago sativa* L.) expressing antisense constructs targeting two different lignin pathway biosynthetic genes with a construct for down-regulation of a WRKY family transcription factor that acts as a repressor of secondary cell wall formation in pith tissues. Plants with low-level expression of the WRKY dominant repressor construct produced lignified cell walls in pith tissues and exhibited enhanced biomass and biomass density, with an increase in total sugars in the cell wall fraction; however, lines with high expression of the WRKY dominant repressor construct exhibited a very different phenotype, with loss of interfascicular fibres associated with repression of the NST1 transcription factor. This latter phenotype was not observed in transgenic lines in which the WRKY transcription factor was down-regulated by RNA interference. Enhanced and/or ectopic deposition of secondary cell walls was also seen in corn and switchgrass expressing WRKY dominant repressor constructs, with enhanced biomass in corn but reduced biomass in switchgrass. Neutral detergent fibre digestibility was not impacted by WRKY expression in corn. Cell walls from WRKY-DR-expressing alfalfa plants with enhanced secondary cell wall formation exhibited increased sugar release efficiency, and WRKY dominant repressor expression further increased sugar release in alfalfa down-regulated in the *COMT*, but not the *HCT*, genes of lignin biosynthesis. These results suggest that significant enhancements in forage biomass and quality can be achieved through engineering WRKY transcription factors in both monocots and dicots.

Introduction

The plant cell wall is a fairly rigid but flexible layer that surrounds the protoplast. It is located outside the cell membrane and provides cells with structural support and protection, in addition to acting as a filtering mechanism. The major polysaccharides in the primary wall are cellulose, hemicellulose and pectin. The thicker and stronger plant secondary cell wall (SCW), which accounts for much of the polysaccharide in biomass, is deposited once the cell has ceased to grow. The SCWs of xylem fibres, tracheids and sclereids are further strengthened by the incorporation of lignin, a macromolecule composed of highly cross-linked phenolic units that is a major component of SCWs.

Genetic manipulation of the lignin pathway can lead to improvements in forage digestibility and/or saccharification efficiency for bioethanol production (Chen and Dixon, 2007) as illustrated in alfalfa plants with down-regulated expression of the hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT) enzyme (Shadle *et al.*, 2007) or the caffeic acid 3-*O*-methyltransferase (COMT) (Guo *et al.*, 2001a), or switchgrass down-regulated in COMT (Baxter *et al.*, 2014; Fu *et al.*, 2011) or overexpressing the lignin-repressing transcription factor MYB4

(Shen *et al.*, 2013). These plants exhibit greatly increased cell wall sugar release efficiency by enzymatic hydrolysis, potentially obviating the requirement for acid pretreatment for biofuel processing (Chen and Dixon, 2007). However, reduction in lignin levels can, in some cases, result in changes in stem vascular tissue anatomy and defects in plant growth resulting in biomass reduction (Bonawitz and Chapple, 2013; Nakashima *et al.*, 2008).

Expression of the genes involved in plant SCW biosynthesis is highly coordinated and regulated by ordered transcriptional switches. The transcription factors (TFs) that orchestrate these gene networks are only now beginning to be understood (Taylor-Teeples *et al.*, 2015; Wang and Dixon, 2012). Several closely related NAC transcription factors (TFs) act as master regulators of cellulose, lignin and hemicellulose biosynthesis genes by initiating a transcriptional signalling network to direct downstream targets, including MYB transcription factors (Mitsuda *et al.*, 2007; Zhao *et al.*, 2010).

The model legume *Medicago truncatula* possesses a negative regulator of secondary cell wall biosynthesis identified as a WRKY family TF (Wang *et al.*, 2010). Its ortholog in *Arabidopsis*, *AtWRKY12*, is expressed in stem pith and cortex, where it inhibits SCW formation by directly repressing SCW master regulators such as *NST2* (Wang *et al.*, 2010). Loss of function

of this WRKY TF results in ectopic SCW formation in the pith of both *Arabidopsis* inflorescence and *Medicago* stem, suggesting that repression, rather than activation, of SCW master regulators in specific cell types contributes to their specific spatial expression. Knockout mutation of this single *WRKY* gene leads to increased lignification, and cellulose and hemicellulose deposition, in the cell walls of the central pith tissue, leading to increases in biomass and stem density (Wang *et al.*, 2010).

Increasing stem density while at the same time reducing lignin levels presents a new approach to modify cell walls to positively impact biomass yield and forage quality. To this end, we have developed high biomass transgenic alfalfa lines by down-regulation of the *WRKY* gene in pre-existing low-lignin *COMT*- and *HCT*-down-regulated transgenic alfalfa. To test the generality of this approach in other forage and/or bioenergy crops, we have additionally generated transgenic maize and switchgrass plants with targeted silencing of their homologs of the *WRKY* gene. The effects on biomass properties resulting from silencing of this TF in different plants are discussed.

Results

Down-regulation of the WRKY TF in alfalfa

Arabidopsis AtWKRY12 belongs to group IIc of a large family of WRKY transcription factors (with more than 70 members reported), which can be divided into three groups (WRKY I, WRKY II and WRKY III) on the basis of both the number of WRKY domains and the features of their zinc finger-like motif (Eulgem *et al.*, 2000; Wang *et al.*, 2011). The WRKY IIc group presents two clades, and the one containing the *AtWRKY12* gene is itself subdivided in two clades representing monocotyledonous and dicotyledonous species (Figure S1). We cloned putative orthologs of *AtWKRY12* from alfalfa, maize and switchgrass for genetic manipulation based on the sequences available in the genome databases. To knock out/down the expression of the *WRKY* genes, we used a dominant repressor (DR) tagged strategy, driving the constructs by the constitutive CaMV 35S promoter (Figure S2) (Wang *et al.*, 2010). This strategy has been reported to successfully phenocopy the knockout mutant's secondary cell wall phenotype in *Arabidopsis* (Mitsuda *et al.*, 2007). The WRKY-DR construct was used to transform alfalfa

via *Agrobacterium tumefaciens*-mediated leaf disc transformation followed by regeneration through somatic embryogenesis (Reddy *et al.*, 2005). The transformation was performed in the wild type and previously reported low-lignin *HCT*- and *COMT*-antisense transgenic backgrounds (Guo *et al.*, 2001a; Shadle *et al.*, 2007). Lines showing the presence of the transgene in the genomic DNA were selected for further characterization, and transgenic lines transformed with empty vector were considered as wild-type control lines (WT). Additionally, the persistence of the down-regulation of *HCT* and *COMT* transcripts in their corresponding transgenic WRKY-DR lines was confirmed by qPCR (Figure S3).

UV auto-fluorescence and Maule staining of stem cross sections were used to visualize lignin for initial phenotypic screening. Based on the stem phenotypes, two very distinct groups of plants were observed independent of the genetic background in which the WRKY-DR transgene was expressed. The first group of plants showed the expected phenotype for WRKY down-regulation, namely the presence of UV auto-fluorescence and Maule staining in pith cells as a consequence of secondary cell wall deposition in this region. We term this phenotype 'increased secondary cell walls in pith' (ISCWP). Additionally, we found an unexpected increase in the differentiation of xylem vessel layers. This additional phenotype was observed in all WRKY-DR transgenic lines, whether in the WT, COMT- or HCT-antisense backgrounds (Figure 1). This phenotype was more predominant in the mature stems of plants expressing the WRKY-DR construct, which sometimes produced up to three times more xylem cell layers compared to the wild type (Figure S4). We did not find any differences in overall plant growth between WRKY-DR-expressing plants compared to the WT or COMT-antisense parentals (Figure S5). However, in the HCT-antisense: WRKY-DR population, we found some semi-dwarf plants as observed for the HCT parental, but also plants with recovered growth (Figure S6). These plants still exhibited reduced *HCT* expression when compared to wild type, but the two lines with the greatest growth restoration (#s 9 and 13) had higher *HCT* transcripts than the parental HCT-RNAi lines (Figure S3).

The second group of plants was characterized by a striking loss of secondary cell wall deposition in the interfascicular fibres (Figure S7a–d), a phenotype we term 'interfascicular fibre
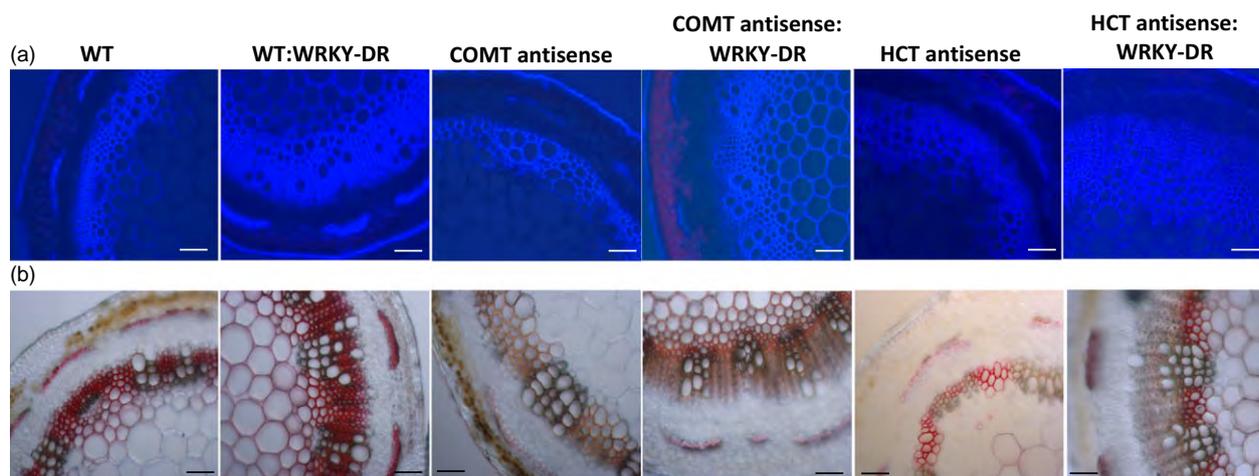

(a) WT | WT:WRKY-DR | COMT antisense | COMT antisense: WRKY-DR | HCT antisense | HCT antisense: WRKY-DR

(b)

Figure 1 Microscopic analysis of lignin deposition in stem cross sections of wild-type (WT), COMT-antisense, HCT-antisense and WRKY-DR-expressing alfalfa genotypes in the three different genetic backgrounds. The cross sections (100 μm thickness) were taken before flowering in the 4th node of the stem counting from the bottom, and size bars are 100 μm. (a) UV autofluorescence. (b) Maule staining. Plants were 2 months old at harvest time. These are representative pictures of one of three biological replicates of each genotype.

disruption' (IFD). A similar phenotype was reported when the secondary cell wall NAC family TF NST1, a direct target of the *WRKY* gene, is mutated in *Medicago truncatula* (Zhao *et al.*, 2010). Interestingly, the plants with the IFD phenotype were found only in the wild-type and COMT-antisense backgrounds, and in contrast to the NAC mutant phenotype in *M. truncatula*, which does not impact overall growth, these alfalfa plants were dwarfed and exhibited enhanced branching (Figure S7e, f).

To better understand the basis for the above phenotypes, the transcript levels of the dominant repressor transgene, and the endogenous *WRKY* and *NST1* genes, were determined in selected lines by qRT-PCR. In all transgenic plants, the transcript level of the endogenous *WRKY* gene was reduced on expression of the WRKY-DR transgene (Figure 2a, b). The reduction in transcript level of the endogenous *WRKY* gene was from 30 to 70% in the plants showing only the typical ISCWP phenotype (Figure 2b). In the case of the IFD phenotype plants, the WRKY-DR transgene level was very high, up to eight times more than the level in the plants showing only the ISCWP phenotype (Figure 2c). However, the level of silencing of the endogenous *WRKY* gene was similar in both ISCWP and IFD phenotypes (Figure 2d).

The transcript level of the *NST1* gene was increased by expression of the WRKY-DR transgene in plants with the ISCWP phenotype in the wild-type background (Figure 3a). However, expression of WRKY-DR did not appear to induce *NST1* to the same extent in the two reduced lignin backgrounds. *NST1* transcript levels were reduced in plants showing the IFD phenotype (Figure 3b). All further studies were carried out with plants exhibiting only the ISCWP phenotype.

Effect of WRKY down-regulation on biomass, lignin and sugar content in alfalfa plants

Characterization of greenhouse-grown plants showed differences in stem density and biomass production in WRKY-DR plants compared to their controls. The stem density was significantly increased in at least two independent WRKY-DR lines in the WT, COMT-antisense and HCT-antisense backgrounds (Figure 4a). Overall biomass production was also improved by down-regulation of the *WRKY* gene in the WT, COMT-antisense and HCT-antisense backgrounds (Figure 4b). This effect was most striking in the HCT-antisense background, where the semi-dwarf phenotype was reversed in several lines. The total lignin content, as determined by thioacidolysis yield, was either unaffected or increased as a result of WRKY down-regulation, whereas lignin composition was basically unaffected, with the lines in the COMT- and HCT-antisense backgrounds retaining the low S and high H monomer parental signatures, respectively (Figure 5). However, lignin levels in HCT-RNAi: WRKY-DR lines #9 and #13 were higher than in the HCT parentals, suggesting that growth restoration in these lines was most likely a consequence of partial restoration of lignin level.

Total sugar content was increased in some WRKY-DR-expressing plants in the WT and COMT-antisense genetic backgrounds (Figure 6a). The saccharification efficiency, measured by enzymatic hydrolysis without chemical pretreatment, was positively impacted by *WRKY* down-regulation, with a more than 30% increase in the WT background (Figure 6b). A similar effect was seen in WRKY-DR-expressing plants in the COMT-antisense background. In fact, the saccharification efficiency of the COMT-antisense: WRKY lines was enhanced 30% compared to the WT, resulting in values close to the highest cell wall saccharification efficiency reported previously for low-lignin HCT-antisense plants (Chen and Dixon, 2007). However, expression of WRKY-DR could not further increase the already very high saccharification efficiency of the parental HCT line (Figure 6b).

Silencing of MsWRKY by RNA interference

Because we obtained two different phenotypes in alfalfa using the dominant repressor strategy, we employed the RNA interference
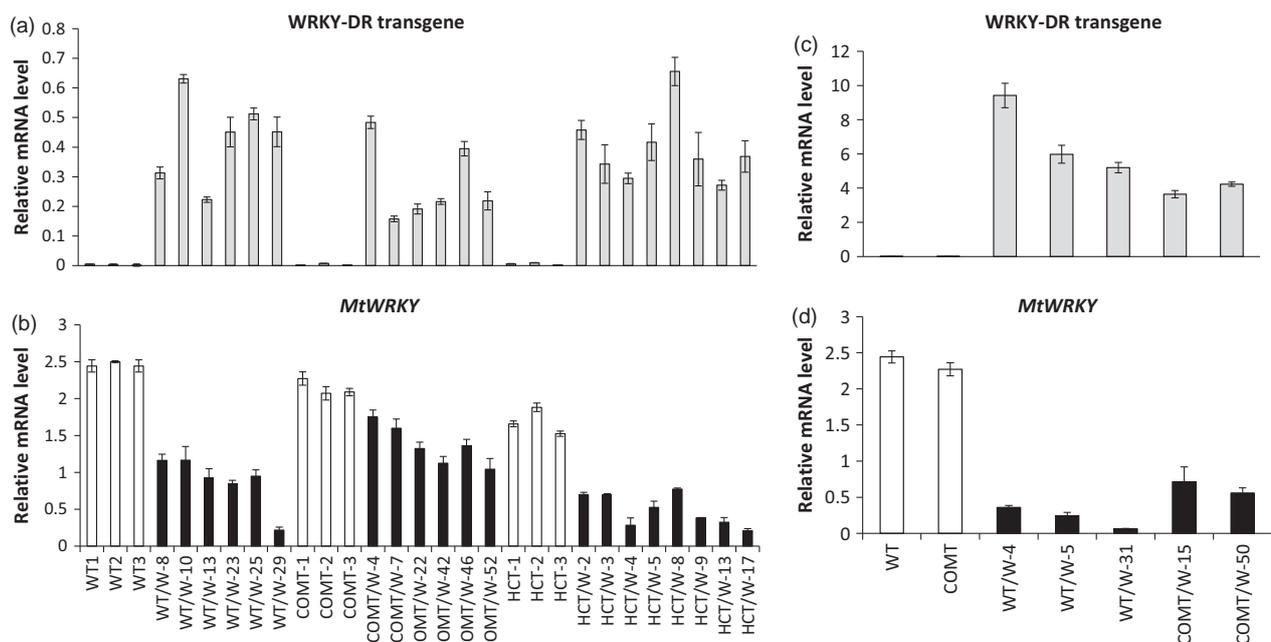

Figure 2 Analysis of transcript levels in alfalfa by qRT-PCR. (a) WRKY-DR transcripts and (b) endogenous *WRKY* transcripts in WT, COMT-antisense, HCT-antisense and WRKY-DR transgenic lines in the three different genetic backgrounds with ISCWP phenotypes. (c) WRKY-DR transcripts in lines with the IFD phenotype. (d) Endogenous *WRKY* transcripts in the IFD lines. The analysis was performed in stems (internodes 2–6). mRNA levels are expressed relative to *Actin*. Results are means ± SE of three biological replicates. White bars are genotypes without *WRKY* modification.
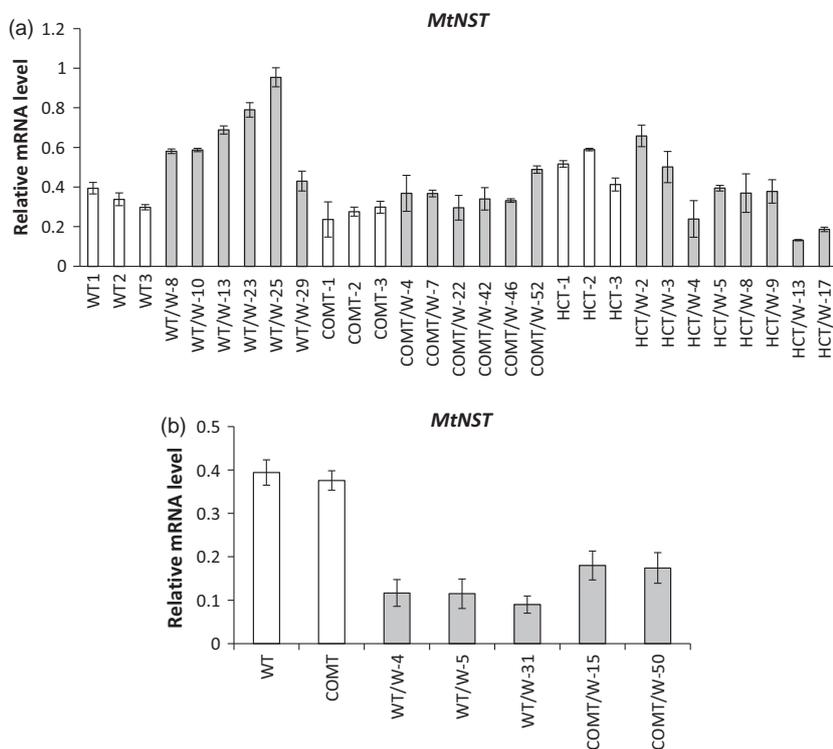
Figure 3 *NST1* transcript levels as determined by qRT-PCR. (a) Plants with the ISCWP phenotype. (b) Plants with the IFD phenotype. The analysis was performed in stems (internodes 2–6). mRNA levels are expressed relative to *Actin*. Results are means ± SE of three biological replicates. White bars are genotypes without *WRKY* modification.
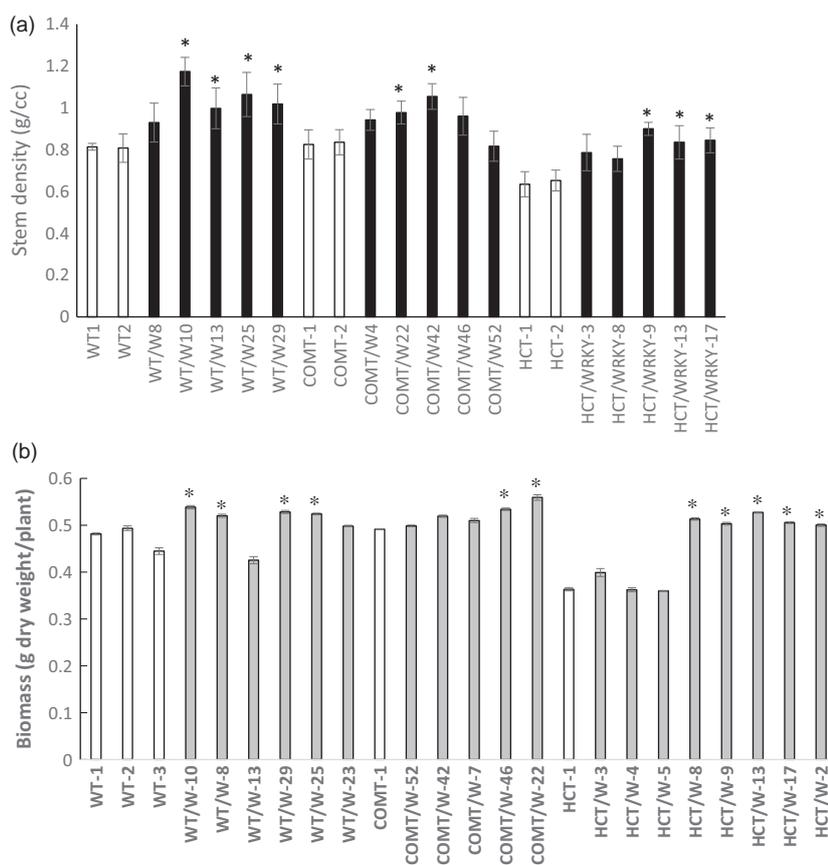

Figure 4 Impacts of WRKY down-regulation on biomass. (a) Stem density and (b) total biomass of WT, COMT-antisense, HCT-antisense and WRKY-DR transgenic lines in the three different genetic backgrounds. The analysis of stem density was performed on the second and third internodes of stems with 8 total nodes. Biomass was measured in 2-month-old plants with about 4–5 stems per plant and with at least 6 nodes before flowering. Results are means ± SE of three biological replicates. White bars are genotypes without *WRKY* modification. Values with * are significantly different ($P < 0.05$) with respect to their corresponding parental background.

(RNAi) technique to clarify whether the unexpected IFD phenotype was an artefact of the dominant repressor strategy. We cloned a 240-bp fragment of the 3'-untranslated region of the alfalfa *WRKY* gene for generation of an RNAi-silencing construct. The resulting plants exhibited a strong ISCWP phenotype, with ectopic lignification in pith cells and extended development of
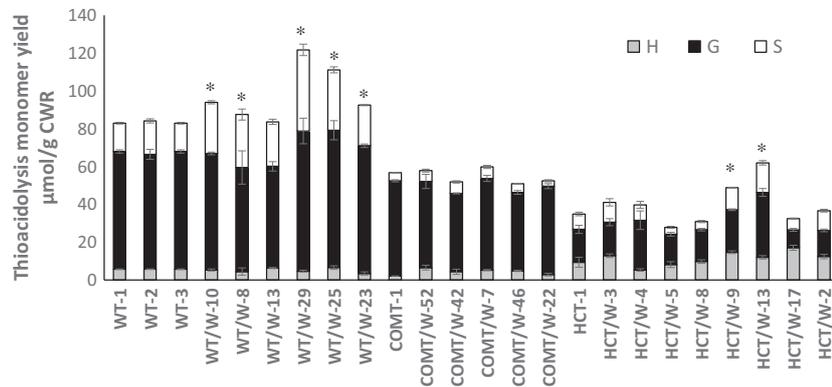
Figure 5 Lignin content and composition of WT, COMT-antisense, HCT-antisense and WRKY-DR transgenic lines in the three different genetic backgrounds. Thioacidolysis monomer composition was determined in the second nodes of stems with 6 total nodes. Results are means ± SE of three biological replicates. Values with * are significantly different (P < 0.05) with respect to their corresponding parental background.
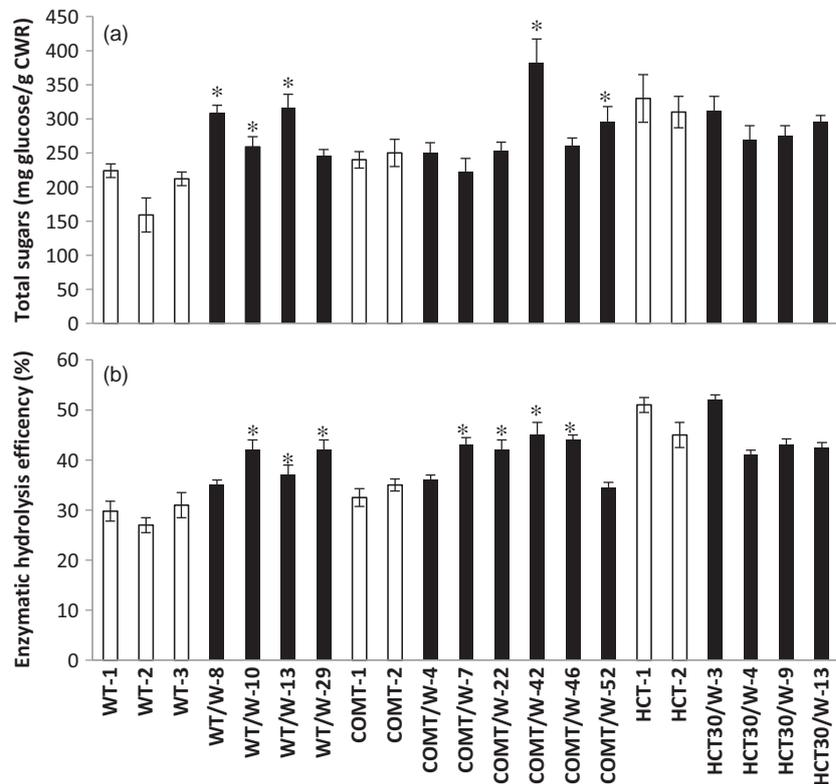

Figure 6 Sugar contents in the cell wall residues of WT, COMT-antisense, HCT-antisense and WRKY-DR transgenic lines in the three different genetic backgrounds. (a) Total sugars were measured by colorimetric analysis with phenol-sulphuric acid and are expressed as mg of glucose per g of CWR. The material for this experiment consisted of stems (internodes 2–6) from different transgenic lines before flowering. (b) Percentage of total available sugar released from the cell wall by enzymatic hydrolysis without chemical pretreatment. White bars are genotypes without WRKY modification. Results are means ± SE of three biological replicates. Values with * are significantly different (P < 0.05) with respect to their corresponding parental background.

xylem cells (Figure S8). However, we never obtained lines with the IFD phenotype or with poor plant growth. Rather, the plants appeared taller and sturdier than wild type (Figure S9). WRKY-RNAi alfalfa plants showed enhancements of about 60% and 30% for stem density and biomass yield, respectively (Table 1).

Phenotypic effects of WRKY TF down-regulation in switchgrass and maize

We next used the dominant repressor strategy to silence corresponding WRKY TFs (Figures S1 and S2) in the monocots, switchgrass and maize. Two different phenotypes were observed among a large population of WRKY-DR-expressing switchgrass

plants according to the level of expression of the WRKY-DR transgene (Figures S10a and 7a,b).

At the E4 stage of development (stems with 4 nodes), WRKY-DR-expressing lines exhibited an increase in lignification in vascular cells based on phloroglucinol–HCL staining (Figure 7a). However, the plants fell into two groups. In the first group, for example lines #44 and #29, with a lower abundance of the WRKY-DR transcript (Figure S10a), there was no difference in plant growth (Figure S11a). The second group of plants, for example line #25, with higher expression level of the transgene but also high levels of the endogenous PvWRKY transcript (Figure S10b) showed extended ectopic lignification, with changes in the distribution of xylem vessels, tracheids and bundle

Table 1 Stem density and biomass yield of WT and WRKY-RNAi alfalfa lines. The stem density was measured in the second and third internode portion from stems with 8 total nodes. Biomass was measured by harvesting all the above-ground material. The plants were one and a half months old with 4–5 stems per plant with at least 6 nodes before flowering. Results are means ± SD of three replicates per line

Lines	Stem Density (g/cc)	Biomass (g of dry weight per plant)
WT-1	0.81 ± 0.01 a	0.51 ± 0.02 a
WT-2	0.80 ± 0.06 a	0.52 ± 0.03 a
WRKY-RNAi-1	1.22 ± 0.07 b	0.68 ± 0.04 b
WRKY-RNAi-2	1.57 ± 0.08 c	0.70 ± 0.05 b
WRKY-RNAi-3	1.58 ± 0.15 c	0.65 ± 0.03 b
WRKY-RNAi-4	1.28 ± 0.06 b	0.63 ± 0.02 b
WRKY-RNAi-5	1.35 ± 0.07 b	0.66 ± 0.03 b

Numbers with the same letters are not significantly different, $P < 0.05$.

sheath around the vascular tissue of the stem (Figure 7c). The lignification pattern of these lines was altered because lignin deposition started at the immature developmental stages of the stem. These plants had stunted growth compared to the controls (Figure S11a).

In maize, expression of the WRKY-DR transgene and corresponding down-regulation of the *ZmWRKY* gene (Figure S12) led to increased development of xylem tracheary elements (Figure 7d), particularly in older stems. This phenotype is similar to the one found previously in some of the alfalfa ISCWP lines (Figure 1) in terms of more development of xylem vascular cells. In contrast to switchgrass, however, maize plants expressing the

WRKY-DR construct had enhanced growth compared to control lines (Figure S11b).

Effect of WRKY-DR expression on biomass, lignin and sugar content in switchgrass and maize

The increase in xylem cells had an impact on the lignin content in both switchgrass and maize plants expressing the WRKY-DR construct (Figure 8). In switchgrass, enhanced lignification of about 40% was observed in both normal and dwarf lines (Figure 8a,b). This effect was more prominent in maize, where there were increases in the content of S and G lignin units of 40 and 60%, respectively (Figure 8c).

Biomass yield was clearly enhanced in maize WRKY-DR lines compared to their controls (Table 2). In contrast, biomass was not strongly affected by the down-regulation of the *WRKY* gene in switchgrass, only in the WRKY-DR dwarf plants, where the biomass was greatly compromised (Table 3).

Total sugars were increased by the introduction of the WRKY-DR transgene in both maize and switchgrass (Tables 2 and 3). However, the saccharification efficiency remained similar in maize WRKY-DR transgenic lines compared to the control (Table 2). Maize stem samples were analysed for neutral detergent fibre (NDF) digestibility by wet chemistry (Figure S13). NDF digestibility was not increased in WRKY-DR lines.

Discussion

Down-regulation of a WRKY transcription factor has been shown to increase biomass density in *Arabidopsis* through the derepression of secondary cell wall synthesis in pith tissues (Wang *et al.*, 2010). In the present study, we have assessed whether this observation can be translated for development of improved
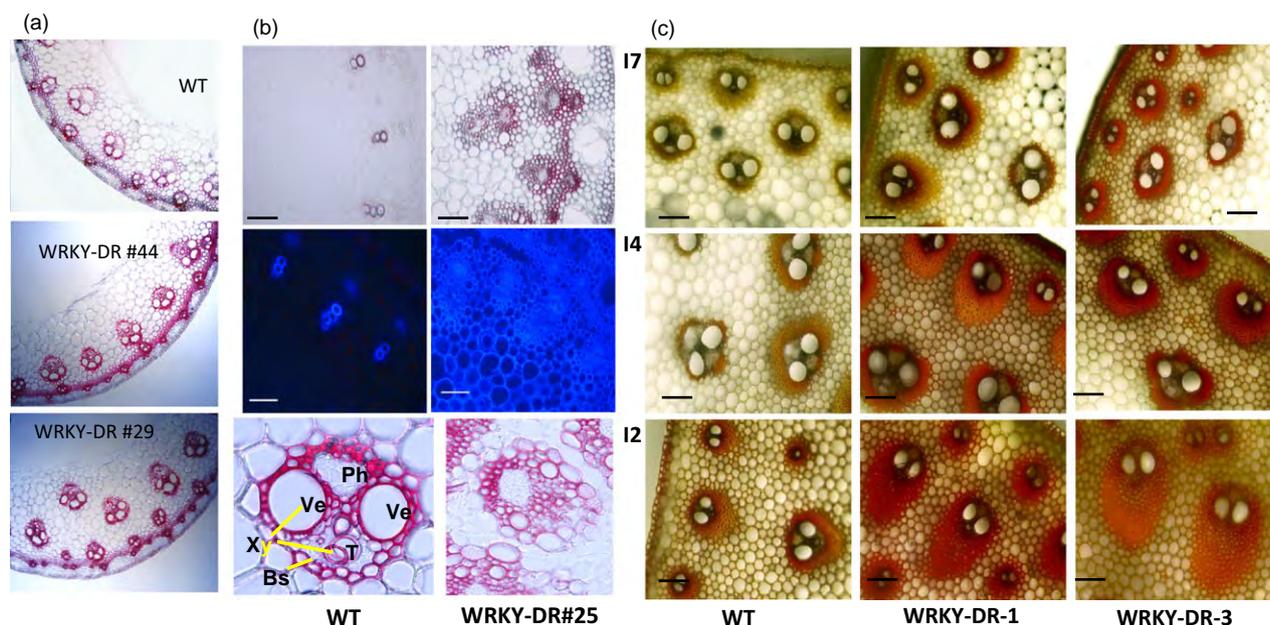

Figure 7 Lignin accumulation in cross sections of young internodes of wild-type (WT) and WRKY-DR-expressing switchgrass lines with ectopic lignification. (a) Comparison of three independent WRKY-DR lines with wild type, as visualized with phloroglucinol staining. (b) Cell-type distribution of lignification in WRKY-DR lines as visualized by phloroglucinol staining (top and bottom panels—bottom panels are higher magnification) and UV autofluorescence (centre panels). Bs, bundle sheath cell; Ph, phloem; Ve, vascular element; xy, xylem. (c) Microscopic analysis of lignin deposition in stems of WT and two independent WRKY-DR maize lines. The cross sections (300 μm thickness) were cut at flowering time in three internodes of the stems, I-2, I-4 and I-7, counting from the bottom. The sections were stained with phloroglucinol. Size bars = 50 μm
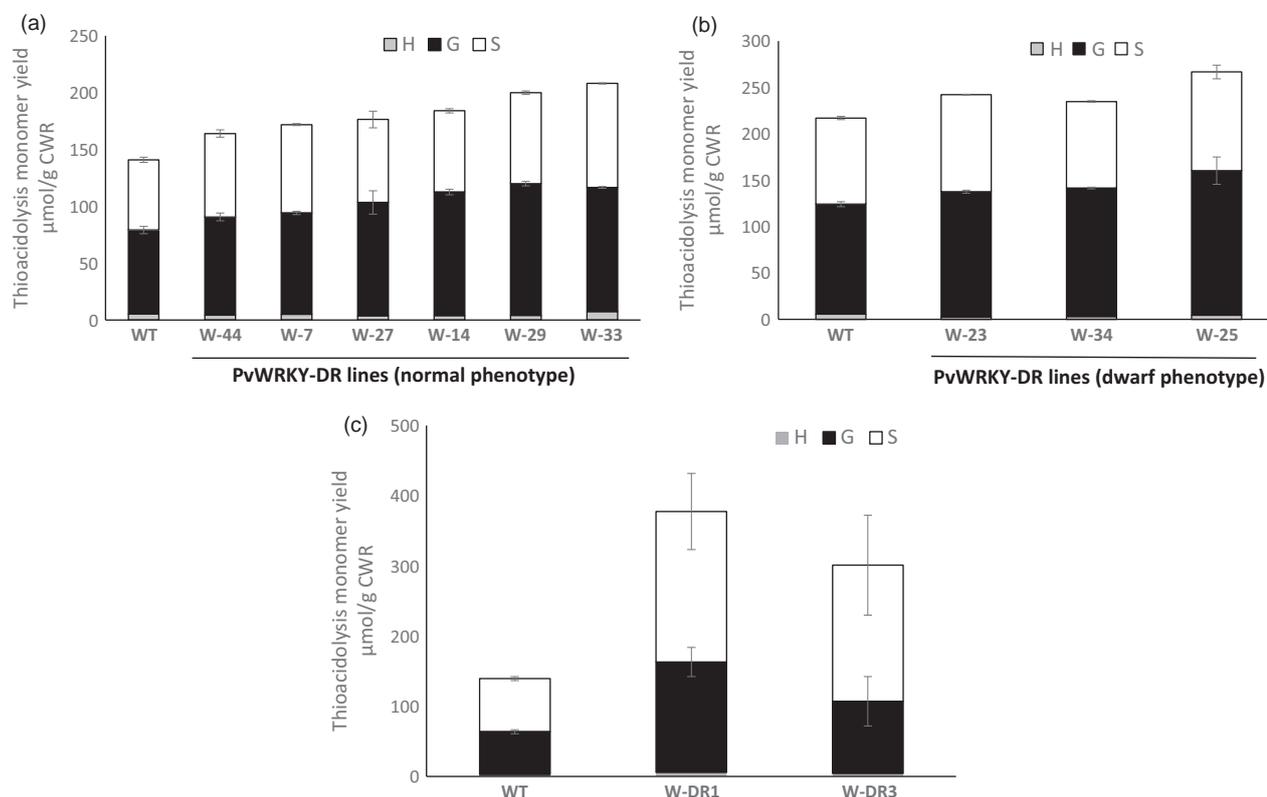
Figure 8 Lignin monomer composition of WT and WT-WRKY-DR switchgrass (a, b) and maize (c) lines. Results in switchgrass are means ± SD of two replicates per line. The analysis was performed at the E4 developmental stage and all 4 internodes were pooled for this determination. In maize, results are means ± SD of three plants per line in the case of line #1 and four in the case of line #3. The analysis was performed on node #2. Values with * are significantly different (*P* < 0.05) with respect to the parental control.

Table 2 Biomass yield and cell wall sugar release efficiency of WT and WRKY-DR maize lines. The results are means ± SD of three plants per line in the case of line #1 and four in the case of line #3. Saccharification efficiency is the percentage of total sugar in the cell wall preparation released by enzymatic hydrolysis without pretreatment. The samples used for this determination were the same as used for determination of in vitro NDF digestibility

Lines	Biomass (dry g per plant)	Total sugars (mg glucose equiv/g CWR)	Sugar release (mg glucose equiv/g CWR)	Saccharification efficiency (%)
WT	16.46 ± 3.3 a	315.63 ± 21.3 a	100.62 ± 6.2 a	32.02 ± 4.1 a
WRKY-DR-1	30.93 ± 5.8 b	397.37 ± 49.7 b	182.61 ± 33.5 b	46.85 ± 14.3 a
WRKY-DR-3	28.24 ± 7.7 b	390.68 ± 15.5 b	156.29 ± 8.5 b	40.08 ± 3.7 a

Numbers with the same letters are not significantly different, *P* < 0.05.

forages and/or lignocellulosic bioenergy crops. Theoretically, the apparently increased lignification that accompanies ectopic secondary cell wall biosynthesis in WRKY down-regulated plants might hinder the accessibility of the enhanced cellulose and hemicellulose fractions. For this reason, we generated WRKY-down-regulated transgenic lines in both wild-type and reduced lignin backgrounds. Of the two reduced lignin lines chosen, COMT-down-regulated alfalfa has an approximately 10% improvement in forage digestibility and cell wall saccharification efficiency (Chen and Dixon, 2007; Guo *et al.*, 2001b), whereas HCT-down-regulated alfalfa has up to a 20% improvement in forage digestibility (Shadle *et al.*, 2007) and 35% improvement in cell wall saccharification efficiency (Chen and Dixon, 2007). WRKY down-regulation improved the saccharification efficiency

of COMT-down-regulated alfalfa by 20%, but did not lead to further improvements in saccharification efficiency in the HCT-down-regulated lines. The reason for the improvement of saccharification efficiency as a result of WRKY expression in the COMT lines is not totally clear; possibly, the ectopically formed secondary cell walls are less recalcitrant than secondary cell walls formed under the plant's natural developmental programme in the COMT-down-regulated background. In the case of the HCT transgenics, the cell walls may have already reached a degree of recalcitrance below that of the ectopically formed secondary walls. Although *WRKY* expression restored biomass yield in a number of the HCT lines, this may have been a consequence of the partial restoration of lignin accumulation through reduced silencing of *HCT*.

Table 3 Biomass yield and total sugar content of WT and WRKY-DR switchgrass lines. Biomass was calculated for the first cut of the total tillers (leaves and stem) at R1 stage of T0 plants. Total sugars were determined in the tillers of plants with 4 nodes before flowering. The results are means ± SE of at least two technical replicates

Lines	Biomass (dry g per plant)	Total sugars (mg of equiv glucose/g CWR)
WT	26.06	565.59 ± 21.2 a
WRKY-DR-33	34.65	634.00 ± 59.8 a,b
WRKY-DR-7	21.74	621.98 ± 88.2 a,b
WRKY-DR-29	26.94	N/A
WRKY-DR-44	26.45	661.75 ± 80.7 b
WRKY-DR-34	22.36	N/A
WRKY-DR-23	8.68	N/A
WRKY-DR-25	11.77	N/A
WRKY-DR-5	N/A	726.71 ± 99 b
WRKY-DR-3	N/A	660 ± 71 b

N/A, data not available.

Numbers with the same letters are not significantly different, $P < 0.05$.

The *WRKY* genes selected as targets in the present work were chosen based on the available genome information at the time. A more recent phylogenetic analysis performed in June 2015, and shown in Figure S1, revealed the presence of additional close orthologs of the Medicago, corn and switchgrass *WRKY12* genes (one additional gene in each species). WRKY transcription factors are known to exhibit redundant functions (Eulgem *et al.*, 2000), and it is also possible that the different phenotypes observed between the three species may result from down-regulation of genes that are not functionally orthologous. However, it should be noted that the ISCWP phenotype observed in alfalfa appears almost identical to the phenotype resulting from complete loss of function in the Medicago *wrky* mutant (Wang *et al.*, 2010) and that the phenotypes observed in both corn and switchgrass also involve ectopic secondary wall formation.

By targeting the endogenous *WRKY* genes in three species, alfalfa, corn and switchgrass, we have shown that this approach can result in ectopic SCW formation (on the basis of appearance of lignified walls) not only in dicots, but also in monocots. Furthermore, two new observations have been made that were not reported in earlier work with *M. truncatula* and *Arabidopsis*; first, in addition to ectopic lignification of pith cells, plants with reduced *WRKY* expression exhibit extended development of lignified vascular cells towards the centre of the stem. Second, high-level expression of the WRKY-DR construct can lead to a different phenotype in which the *NST1* transcription factor, which controls lignification in interfascicular fibres (Zhao *et al.*, 2010), is repressed. We have confirmed that these plants do indeed show repression of *NST1*. Because *WRKY12* is a repressor of *NST1* in *Arabidopsis* and *M. truncatula* (Wang *et al.*, 2010), this suggests that, at high expression level, the WRKY-DR construct is behaving as a simple overexpression construct rather than as a dominant repressor. This confirms the conclusion that cell wall TFs may function differentially in a dose-dependent manner (Taylor-Teeples *et al.*, 2015). In contrast, the RNAi-silencing strategy appears to be more straightforward and not lead to unexpected phenotypes. The dominant repressor strategy has previously been used primarily as a knock-down technique only in TFs that positively regulate the transcription of their target genes (Liu

et al., 2013). Further work is in progress to understand the mechanism of WRKY-DR action in alfalfa, but future biotechnological development in either monocots or dicots utilizing WRKY down-regulation for biomass densification will likely be more straightforward if performed using direct RNA silencing or genome editing rather than the dominant repressor strategy.

We were able to generate transgenic alfalfa lines with combined silencing of *WRKY*, *COMT* and *HCT* genes. Silencing of WRKY alone in alfalfa resulted in increased lignin content, biomass and biomass density. The lignin thioacidolysis signatures of COMT- and HCT-down-regulated plants, namely reduction of S units and increase of H units, respectively, were maintained in all the reduced lignin lines. Importantly, the semi-dwarf phenotype of HCT-silenced plants was not observed in a subset of the WRKY-/HCT-silenced plants, in which *HCT* transcripts and lignin levels remained low. This growth recovery might be a result of the increase in xylem vessels which might enhance water transport and attenuate potential water stress conditions in these plants.

Other than the growth enhancement of HCT lines, combining reduced lignin with WRKY silencing in alfalfa did not, overall, result in additive improvements. This appears to be because WRKY silencing, in spite of increased lignification, itself resulted in enhanced sugar release efficiency from cell walls.

WRKY silencing resulted in increased lignification in both maize and switchgrass. However, the final outcome was different in the two species. Maize deposited more lignin in the older region of the stem, close to the bottom, and biomass production was improved. In contrast, the ectopic lignification in switchgrass began in young tissues, resulting in inhibition of cell expansion and therefore both shorter and thinner stems. Based on the present data, WRKY down-regulation does not seem to be a useful strategy for improvement of switchgrass as a bioenergy crop.

Reduced lignin transgenic alfalfa has recently been deregulated by USDA-APHIS (Federal Register Vol. 79, No. 217, Monday, November 10, 2014). Natural breeding of this out-crossing species to incorporate WRKY down-regulation into a reduced lignin genetic background could generate significantly more phenotypic variation in biomass density and sugar release efficiency than obtained in the present limited transgenic experiments, leading to selection of further improved forage phenotypes.

Experimental procedures

Plant transformation

To make the dominant repressor construct for *Medicago* (*MtWRKY-DR*), we amplified the *MtWRKY* coding sequence using forward primer MtWRKYFw: CACCATGGATGGAGAAA GAGATGTTC and reverse primer with incorporated DR sequence MtWRKYRe: CGTCTTTTTGATTAAGCGAAACCCAAACGGAGTTC TAGATCCAGATCCAG. The PCR fragment was then ligated to the TOPO/D vector (Invitrogen, Carlsbad, CA), and confirmed with sequencing. The p-Entry vector with *MtWRKY-DR* was then recombined to a pK2GW7 binary vector. The WRKY-RNAi construct was made by cloning a fragment of 240 bp from *MsWRKY,* using the primers: 3′-F-RNAi2: CACCTCTCCTTGTGAC-GACTCTAATTCTT and 3′-R-RNAi2: CATACGTAAATG-GATCTTTTTTATAAACAAC. The PCR fragment was then ligated to the TOPO/D vector (Invitrogen). The p-Entry vector with the WRKY-RNAi fragment was then recombined to a pB7GWIWG2(I) vector. These constructs were used to transform *Agrobacterium tumefaciens* strain AGL1 as previously described (Shadle *et al.*,

2007). Alfalfa transformation was performed according to Reddy *et al.* (2005).

To make the dominant repressor construct for the orthologous maize gene (*ZmWRKY-DR*), we amplified the *ZmWRKY* coding sequence using the forward primer ZmWRKYFw: CACCGAGCTC ATGCTTCTGCAGATGCCGTTTGTC and reverse primer with incorporated DR sequence, ZmWRKYRe: TACTAGTTTAAGCGAA ACCCAAACGGAGTTCTAGATCCAGATCCAGGAGCGACGTGAA AGCGCAG. The PCR fragment was then ligated to the TOPO/D vector (Invitrogen) and confirmed with sequencing. The *ZmWRKY-DR* fragment was digested with *Sac*I and *Spe*I, and inserted into the PMCG1005 vector. The resulting construct was used for *Agrobacterium*-mediated transformation into the maize genotype Hi II at the Iowa State University Plant Transformation Facility. A similar strategy was used to make the switchgrass *PvWRKY*-DR dominant repressor construct. The primers used for *PvWRKY* cloning were as follows: forward primer PvWRKYFw, CACCATGGAGGGGAGCAGCCAGTTTG, and reverse primer PvWRKYRe, TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGA TCCAGGAGCGAGGTGAAGGCGCAGC. After sequence confirmation, the PvWRKY-DR sequence was inserted into the destination vector pANIC10A (https://www.arabidopsis.org/servlet/TairObject?id=1001200382&type=vector) using LR reaction (Invitrogen). Agrobacterium-mediated transformation of switchgrass was performed at the Transformation Facility at the Samuel Roberts Noble Foundation according to a previously described protocol (Xi *et al.*, 2009).

Plant growth conditions in the greenhouse for alfalfa (cv Regen SY4D) were 22 °C under long-day photoperiod (16 h light, 8 h dark) with an average day light intensity of 240 μmol/m^2/s. Plant growth conditions for switchgrass and maize were as described previously (Shen *et al.*, 2009).

Microscopic analysis

Alfalfa stem cross sections were made using a vibratome (Thermo Scientific Microm large field magnifier; Thermo Fisher Scientific, Walldorf, Germany) and subjected to Maule staining as described previously (Guo *et al.*, 2001a). Photographs were taken using an EVOS Xl Core Digital Inverted Microscope (AMG Advanced Microscopy Group, Fisher Scientific). In the case of monocot species, the sections were subjected to phloroglucinol–HCL staining as described previously (Shen *et al.*, 2009).

Gene expression analysis

The transcript levels of different genes were determined by qRT-PCR as described previously (Gallego-Giraldo *et al.*, 2011). Gene-specific primers are listed in Table S1.

Cloning of the 5′ and 3′ end sequences of MsWRKY

To determine the expression level of the endogenous *MsWRKY* gene in the silenced transgenic alfalfa lines, we cloned the nontranslated 5′ and 3′ end sequence using the RNA 5′/3′-Full RACE Core Kit (Clontech, Mountain View, CA) according to the manufacturer's specifications. The primer used to clone the 5′ end was GSP-At5′-MsWRKY: TTCCTCCTCACTTTCGCCTTGCTCTTCT and for the 3′ end was GSP-S-3′MsWRKY: GCTGTGAGTGATGAGAATTGCACTGGAA.

Lignin analysis

Cell wall residue (CWR) was obtained from about 1 g of ground frozen plant material. Sequential extractions with methanol (100%), chloroform: methanol (2:1) and methanol (100%) were used to extract the cell walls. Around 15 mg of CWR was used for determination of lignin content and composition by thioacidolysis in alfalfa and maize as described previously (Shadle *et al.*, 2007). Twenty-five mg of CWR was subjected to the thioacidolysis protocol in switchgrass.

Determination of total sugars

One hundred mg of CWR and 15 mg of CWR were used for determination of total sugar release and enzymatic saccharification without acid pretreatment, in switchgrass and alfalfa, respectively, as previously described (Shen *et al.*, 2009;). In the case of maize and switchgrass, 100 mg of CWR was used for this determination.

Determination of stem density

Stem density in alfalfa was determined using the cylinder formula for volume calculation. The density was calculated by dividing the fresh weight value of the stem portion by the volume.

Determination of forage digestibility

Determination of NDF digestibility was performed by the Forage Lab (Hagerstown Maryland, http://www.foragelab.com/Lab-Services/Forage-and-Feed/Invitro-Digestibility/).

Statistical analysis

Statistical treatment of data was performed by analysis of variance using Fisher's LSD procedure for multiple comparison tests (STATISTICA, Statsoft, DELL Software).

Acknowledgements

Author contributions

R.A.D. and L.G-G. conceived the project. L.G-G., G.S., H.S., J.B-R., S.F.C and H.W. performed the experiments and analysed data. R.A.D supervised the project and R.A.D and L.G-G. prepared the study.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Phylogenetic tree of plant *WRKY* genes.

Figure S2 WRKY dominant repressor constructs.

Figure S3 Transcript levels of *HCT* and *COMT* in HCT antisense: WRKY-DR and COMT antisense: WRKY-DR transgenic alfalfa as determined by qRT-PCR.

Figure S4 Details of xylem vessel development in WRKY-DR transgenic alfalfa lines.

Figure S5 Overall growth phenotypes of 3 month old wild-type and COMT-antisense alfalfa plants with and without expression of WRKY-DR.

Figure S6 Overall growth phenotypes of 3 month old wild-type and HCT-antisense alfalfa plants with and without expression of WRKY-DR.

Figure S7 The interfascicular fiber disruption (IFD) phenotype.

Figure S8 Microscopic analysis of lignin deposition in stem cross sections of wild type (WT) and WRKY-RNAi alfalfa lines.

Figure S9 Comparison of overall growth of wild type and WRKY-RNAi alfalfa lines.

Figure S10 Transcript levels of the WRKY-DR transgene and the endogenous *PvWRKY* gene in switchgrass WRKY-DR plants as determined by qPCR.

Figure S11 Overall growth of switchgrass and maize WRKY-DR plants.

Figure S12 Transcript levels of the WRKY-DR transgene and the endogenous *ZmWRKY* gene in maize WRKY-DR plants as determined by qPCR.

Figure S13 In vitro neutral detergent fiber digestibility (NDFD) of wild-type maize and maize WRKY-DR lines at different times of incubation in rumen fluid.

Table S1 List of primers used in the present work.