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Expression of a bacterial 3-dehydroshikimate dehydratase reduces lignin content and improves biomass saccharification efficiency

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Summary

Lignin confers recalcitrance to plant biomass used as feedstocks in agro-processing industries or as source of renewable sugars for the production of bioproducts. The metabolic steps for the synthesis of lignin building blocks belong to the shikimate and phenylpropanoid pathways. Genetic engineering efforts to reduce lignin content typically employ gene knockout or gene silencing techniques to constitutively repress one of these metabolic pathways. Recently, new strategies have emerged offering better spatiotemporal control of lignin deposition, including the expression of enzymes that interfere with the normal process for cell wall lignification. In this study, we report that expression of a 3-dehydroshikimate dehydratase (QsuB from Corynebacterium glutamicum) reduces lignin deposition in Arabidopsis cell walls. QsuB was targeted to the plastids to convert 3-dehydroshikimate – an intermediate of the shikimate pathway – into protocatechuate. Compared to wild-type plants, lines expressing QsuB contain higher amounts of protocatechuate, p-coumarate, p-coumaraldehyde and p-coumaryl alcohol, and lower amounts of coniferaldehyde, coniferyl alcohol, sinapaldehyde and sinapyl alcohol. 2D-NMR spectroscopy and pyrolysis-gas chromatography/mass spectrometry (pyro-GC/MS) reveal an increase of p-hydroxyphenyl units and a reduction of guaiacyl units in the lignin of QsuB lines. Size-exclusion chromatography indicates a lower degree of lignin polymerization in the transgenic lines. Therefore, our data show that the expression of QsuB primarily affects the lignin biosynthetic pathway. Finally, biomass from these lines exhibits more than a twofold improvement in saccharification efficiency. We conclude that the expression of QsuB in plants, in combination with specific promoters, is a promising gain-of-function strategy for spatiotemporal reduction of lignin in plant biomass.

Introduction

Plant cells walls are the primary source of terrestrial biomass and mainly consist of cellulosic and hemicellulosic polysaccharides impregnated with lignins. Lignins are polymers of p-hydroxycinnamyl alcohols (i.e. monolignols), which are synthesized inside the cells, exported to the cell wall and ultimately undergo oxidative polymerization via laccase and peroxidase activities. The main monolignols – p-coumaryl, coniferyl and sinapyl alcohols – give rise to the p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units, respectively (Boerjan et al., 2003). Lignification generally confers mechanical strength and hydrophobicity in tissues that develop secondary cell walls, such as sclerenchyma (i.e. fibres) and xylem vessels. In addition to its essential role for upright growth, lignin also serves as a physical barrier against pathogens that degrade cell walls (Boudet, 2007).

Lignocellulosic biomass is used for pulp and paper manufacture, ruminant livestock feeding, and more recently has been considered an important source of simple sugars for fermentative production of intermediate or specialty chemicals and biofuels (Keasling, 2010). It is well-documented that lignin in plant biomass negatively affects pulp yield, forage digestibility and polysaccharide saccharification (Baucher et al., 2003; Chen and Dixon, 2007; Taboada et al., 2010). This has prompted major interest in developing a better understanding of lignin biosynthesis to reduce biomass recalcitrance by modifying lignin content and/or composition.

The shikimate pathway, which is located in plastids in plants, provides a carbon skeleton for the synthesis of phenylalanine, the precursor of the cytosolic phenylpropanoid pathway responsible for the biosynthesis of monolignols (Figure 1). All the metabolic steps and corresponding enzymes for both pathways are known and well-conserved across land plants (Fraser and Chapple, 2011; Tohge et al., 2013; Umezawa, 2010). Classic approaches to lignin reduction have relied on genetic modifications, such as transcript reduction and allelic variation of specific genes from the phenylpropanoid pathway (Li et al., 2008; Vanholme et al., 2008). However, these strategies often result in undesirable phenotypes – including dwarfism, sterility and increased susceptibility to environmental stresses – due to loss of cell wall integrity, depletion of other phenylpropanoid-related metabolites, accumulation of pathway intermediates or the constitutive activation of defence responses (Bonawitz and Chapple, 2013; Voelker et al., 2011). Such negative effects are unfortunately difficult to...
avoid because of the nontissue specificity of the strategies employed: allelic variations are transmitted to every cell of the plant during cell divisions, and small interfering RNAs generated for gene silencing generally move from cell-to-cell and over long distance in vegetative tissues (Brosnan and Voinnet, 2011).

Alternatively, there are novel and promising gain-of-function strategies that involve expression of specific proteins to reduce the production of the three main monolignols or change their ratios. Using specific promoters with restricted expression patterns, these strategies would enable the alteration of lignin at later developmental stages or, for example, only in certain tissues such as fibres — without compromising the functionality of conductive vessels for the transport of water (Voelker et al., 2011). Examples of such expressed proteins are transcription factors that act as negative regulators of lignin biosynthesis (Fornalé et al., 2010; Iwase et al., 2009; Shen et al., 2012; Yan et al., 2013); enzymes that produce alternative lignin monomers (Eudes et al., 2012; Wilkerson et al., 2014); engineered enzymes that modify monolignols into their nonoxidizable forms (Zhang et al., 2012); or proteins that mediate the post-transcriptional degradation of enzymes from the lignin biosynthetic pathway (Zhang et al., 2014). In this study, we report for the first time on the expression of a bacterial 3-dehydroshikimate dehydratase in Arabidopsis (Teramoto et al., 2009). We selected QsuB from C. glutamicum and targeted it to the plastids to convert the shikimate precursor 3-dehydroshikimate into protocatechuate (Figure 1), with the aim of reducing lignin content and modifying its composition as shikimate is required for lignin biosynthesis. Metabolomic analysis of plants expressing QsuB revealed higher amounts of p-coumaryl and of the two direct precursors of H-lignin units: p-coumaraldehyde and p-coumaryl alcohol. Conversely, the direct precursors of G and S units — coniferaldehyde, coniferyl alcohol, sinapaldehyde and sinapyl alcohol — were reduced. Lignin content was severely reduced in these transgenic lines and exhibited an enrichment of H units at the expense of G units and a lower polymerization degree. Compared to those of wild-type plants, cell walls from lines expressing QsuB released significantly higher amounts of simple sugars after cellulase treatment and required less enzyme for saccharification. Collectively, these results support the hypothesis that expression of a plastidic QsuB affects the lignin biosynthetic pathway.

Results

Targeted expression of QsuB in Arabidopsis

A sequence encoding QsuB was cloned downstream of the sequence encoding for a plastid-targeting signal peptide (SCHL) for expression in plastids. Using transient expression in tobacco, we first confirmed that QsuB was correctly targeted to the plastids by analysing its subcellular localization when fused at the C-terminus to a YFP marker (Figure S1). The schl-qsuB sequence was cloned downstream of the Arabidopsis C4H promoter for expression in lignifying tissues of Arabidopsis. Western blot analysis confirmed that QsuB was expressed in stems of several T3 plants homozygous for the pC4H::schl::qsuB (thereafter C4H::qsuB) construct (Figure 2). Based on the migration of molecular weight markers, QsuB was detected at around 70 kDa, which corresponds to the theoretical size of its native sequence after cleavage of the chloroplast transit peptide (Figure 2). Four homozygous lines with different QsuB expression levels (C4H::qsuB-1, -3, -6 and -7) were selected for biomass measurement. Although a height reduction was observed for these lines, only C4H::qsuB-1 showed a slight decrease (~18%) of biomass yield (Table 1).

Metabolite analysis of C4H::qsuB lines

Methanol-soluble metabolites from stems of the four homozygous C4H::qsuB lines were extracted for analysis (Table 2, Figure S2). Compared to wild-type plants, protocatechuate content was increased 67- to 113-fold in the transgenic lines. However, no significant reduction was observed for the content of several metabolites derived from the shikimate pathway such as salicylate and aromatic amino acids (i.e. phenylalanine, tyrosine and tryptophan). Interestingly, several metabolites from the phenylpropanoid pathway were increased in the transgenic lines; p-coumaraldehyde and p-coumaryl alcohol, the two direct precursors of H-lignin units, were increased 5.7- to 16.4-fold and 12.2- to 13.7-fold, respectively. Similarly, p-coumarate content was increased 6.4- to 9.5-fold compared to wild type. In contrast, the direct precursors of G- and S-lignin units were negatively altered in transgenic lines. Coniferaldehyde and coniferyl alcohol were reduced by 33–50% and 36–68%, respectively. Sinapaldehyde and sinapyl alcohol were decreased by 45–77% and 73–87%, with the exception of line C4H::qsuB-1 which showed no significant difference for sinapaldehyde compared to wild type (Table 2).

Cell wall-bound p-coumarate and ferulate released from cell wall residues by mild alkaline hydrolysis were also analysed (Table 3). The content of p-coumarate was significantly increased in the C4H::qsuB lines (1.75–3-fold), whereas ferulate was reduced (1.8–2.9-fold). In addition, bound p-coumaraldehyde could be detected in cell wall samples from the transgenic lines but not in those from wild type (Table 3).

Lignin content and monomeric composition in C4H::qsuB lines

The Klasson method was used to measure the lignin content: a reduction ranging from 45% (C4H::qsuB-7) to 52% (C4H::qsuB-1) of biomass yield (Table 1).
Asterisks indicate significant differences from the wild type using the unpaired Student’s t-test (\(P < 0.005; **P < 0.001\)).

**Table 1** Height and dry weight of the main inflorescence stem of senesced mature wild-type (WT) and pC4H::schl::qsuB (C4H::qsuB) plants. Number, \(n\), of plants analysed

| Plant line | Height (cm) Mean ± SE | Dry weight (mg) Mean ± SE | \(n\) |
|------------|-----------------------|---------------------------|------|
| WT         | 47.3 ± 0.8            | 271.0 ± 11.1              | 24   |
| C4H::qsuB-1| 36.6 ± 1.0**          | 221.3 ± 11.0*             | 20   |
| C4H::qsuB-3| 38.8 ± 0.7**          | 244.4 ± 13.4              | 20   |
| C4H::qsuB-6| 35.9 ± 0.9**          | 254.1 ± 12.7              | 20   |
| C4H::qsuB-7| 41.0 ± 0.9**          | 251.3 ± 17.4              | 20   |

**Table 2** Quantitative analysis of methanol-soluble metabolites in stems from 5-week-old wild-type (WT) and pC4H::schl::qsuB (C4H::qsuB) plants. Values in brackets are the SE from four biological replicates (\(n = 4\))

| Metabolites | WT          | C4H::qsuB-1 | C4H::qsuB-3 | C4H::qsuB-6 | C4H::qsuB-7 |
|-------------|-------------|-------------|-------------|-------------|-------------|
| Protocatechuate\(a\) | 1.2 (0.6)   | 110.4 (15.4)*** | 133.4 (14.0)*** | 79.7 (15.9)*** | 118.7 (16.2)*** |
| Tryptophan\(a\) | 3.5 (0.6)   | 2.9 (0.1)   | 3.4 (0.5)   | 3.1 (0.7)   | 3.0 (0.3)   |
| Phenylalanine\(a\) | 4.9 (0.5)   | 4.9 (0.9)   | 4.1 (0.5)   | 4.1 (0.4)   | 4.5 (0.3)   |
| Tyrosine\(a\) | 7.3 (1.0)   | 6.7 (0.6)   | 8.2 (0.5)   | 6.7 (1.3)   | 6.4 (0.6)   |
| Salicylate\(b\) | 755.4 (33.1) | 762.9 (59.8) | 732.7 (54.4) | 695.6 (25.5) | 665.9 (26.9) |
| p-coumaraldehyde\(b\) | 0.8 (0.2)   | 4.8 (1.6)*   | 11.7 (2.2)**  | 11.7 (2.2)**  | 8.5 (0.7)**   |
| p-coumaryl alcohol\(b\) | 13.2 (1.4)  | 181.1 (20.9)*** | 180.3 (52.4)*  | 160.4 (46.1)*  | 175.9 (33.0)** |
| p-coumarate\(b\) | 5.9 (0.4)   | 55.9 (8.7)** | 47.8 (13.4)*  | 41.7 (13.5)*  | 37.6 (6.5)** |
| Coniferaldehyde\(b\) | 18.0 (1.0)  | 12.0 (1.5)*   | 9.6 (2.4)*   | 9.1 (1.1)**   | 11.3 (1.5)*   |
| Coniferol alcohol\(b\) | 792.6 (87.0) | 504.5 (70.1)* | 363.3 (101.9)* | 255.0 (26.3)** | 325.4 (7.3)** |
| Sinapaldehyde\(b\) | 14.7 (1.6)  | 12.8 (1.5)   | 8.1 (2.7)*   | 3.4 (1.3)**   | 5.7 (1.2)**   |
| Sinapyl alcohol\(b\) | 2752.8 (334.9) | 731.5 (101.1)** | 357.4 (123.8)*** | 350.6 (171.7)*** | 540.1 (57.8)*** |

Asterisks indicate significant differences from the wild type using the unpaired Student’s t-test (*\(P < 0.05; **P < 0.005; ***P < 0.001\)).
Table 3 Quantitative analysis of cell wall-bound aromatics in stems from extractive-free senesced mature wild-type (WT) and pC4H::schl::qsuB (C4H::qsuB) plants. Values are means of three biological replicates (n = 3)

| Plant line        | p-coumaratea | Ferulata | p-coumaraldehydeb |
|-------------------|--------------|----------|-------------------|
| WT                | 5.4 ± 0.6    | 41.8 ± 4.3 | ND                |
| C4H::qsuB-1      | 9.4 ± 1.2*   | 14.5 ± 0.8** | 47.6 ± 13.0**    |
| C4H::qsuB-3      | 15.4 ± 1.9** | 19.3 ± 1.3** | 64.8 ± 6.6**     |
| C4H::qsuB-6      | 16.5 ± 2.6*  | 20.8 ± 2.4*  | 96.5 ± 19.0**    |
| C4H::qsuB-7      | 14.5 ± 0.9** | 22.9 ± 1.8*  | 62.1 ± 0.4**     |

ND, not detected.

Asterisks indicate significant differences from the wild type using the unpaired Student’s t-test (*P < 0.05; **P < 0.01).

Table 4 Lignin content and composition in senesced mature stems from wild-type (WT) and pC4H::schl::qsuB (C4H::qsuB) plants. Values in brackets are the SE from three biological replicates (n = 3)

| Plant line        | Klason lignin (mg/g cell wall) | %H     | %G     | %S     |
|-------------------|-------------------------------|--------|--------|--------|
| WT                | 177.8 (18.2)                  | 3.3 (0.2) | 64.1 (1.9) | 32.6 (2.0) |
| C4H::qsuB-1      | 85.0 (4.6)**                  | 15.5 (0.2)** | 38.9 (0.6)** | 45.6 (0.5)* |
| C4H::qsuB-3      | 95.4 (1.5)**                  | 10.8 (0.4)** | 39.4 (1.2)** | 49.8 (0.9)* |
| C4H::qsuB-6      | 91.4 (6.4)**                  | 20.0 (1.0)** | 36.9 (2.8)*  | 43.1 (3.5)* |
| C4H::qsuB-7      | 97.8 (1.2)**                  | 12.8 (1.8)*  | 43.8 (1.3)** | 43.4 (1.9)* |

Asterisks indicate significant differences from the wild type using the unpaired Student’s t-test (*P < 0.05; **P < 0.01).
and p-coumaryl alcohol, the precursors of H-lignin units, was observed in the transgenic lines (Table 2 and Figure S2). Analysis of the lignin monomeric composition using 2D NMR spectroscopy and pyro-GC/MS unequivocally demonstrated an increase in H units in plants expressing QsuB (Figure 3; Tables 4 and S1). These data could explain the reduced degree of polymerization of these lignins, which has been previously observed in various lignin mutants that exhibit high content of H units, incorporation of which typically slows or stops lignin-chain elongation (Sangha et al., 2014; Ziebell et al., 2010; Figure 4). Therefore, reduced lignin–polysaccharide cross-linking within the biomass of the transgenic lines is expected, and this could contribute to its superior enzymatic digestibility (Ralph et al., 2004).

A low lignin content rich in H units and higher S/G corresponds to a phenotype previously characterized in plants down-regulated for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), p-coumarate 3-hydroxylase (C3H) or caffeoyl shikimate esterase (CSE) (Ralph et al., 2006; Vanholme et al., 2013; Ziebell et al., 2010). Moreover, reduction of HCT activity results in the accumulation of free and bound p-coumaraldehyde in cucumber and of p-coumarate in alfalfa, presumably due to the build-up of coumaroyl-CoA (Gallego-Giraldo et al., 2011; Varbanova et al., 2011). This suggests that an alteration of these biosynthetic steps has occurred in the C4H::qsuB lines. However, no particular reduction of transcript abundance for HCT, C3H and CSE was observed in the transgenic lines compared to wild type (Figure S6). A possible explanation is that QsuB activity, which consumes 3-dehydroshikimate in lignifying tissues, affects indirectly the amount of shikimate available for HCT in the cytosol. Although some enzymes of the shikimate pathway exist in the cytosol, there is so far no evidence for a complete alternative extra-plastidial shikimate biosynthetic pathway. Instead, a yet-unidentified transporter probably mediates the export of shikimate from the plastid to the cytosol (Maeda and Dudareva, 2012). If such transport system is only active at a narrow range of concentrations, a reduction of shikimate content in plastids (as anticipated in plants expressing QsuB) would compromise its export to the cytosol. Moreover, it is possible that the large amount of protocatechuate generated by QsuB activity in plastid competes with shikimate export. The distribution of shikimate between plastids and the cytosol is still poorly understood, and shikimate levels were below the detection limit in our stem extracts from wild type and transgenic plants. Alternatively, because previous studies reported a substrate flexibility of HCTs

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**Figure 3** Partial short-range $^{13}$C–$^1$H (HSQC) spectra (aromatic region) of cell wall material from mature senesced stems of wild-type (WT) and pC4H::schl::qsuB-1 plants. Lignin monomer ratios are provided on the figures.

**Figure 4** Polydispersity of cellulolytic enzyme lignins from wild-type and pC4H::schl::qsuB-1 plants. Cellulolytic enzyme lignins were purified from mature senesced stems of wild-type (black line) and pC4H::schl::qsuB-1 (red line) plants and analysed for polydispersity by size-exclusion chromatography (SEC). SEC chromatograms were obtained using UV-F fluorescence (Ex$_{250}$/Em$_{450}$). m, molecular mass.
made on Murashige and Skoog vitamin medium (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), supplemented with 1% sucrose, 1.5% agar and 50 µg/mL kanamycin.

**Generation of binary vectors**

The promoter p255, with a single enhancer, was amplified by PCR from pRT100 with phosphonlated primers F-p255 (5′-GTCAACATGTGGAGCACGACAC-3′) and R-p255 (5′-CGAGACATCCTAGATTGTCCTCTCCAAATGAAATGAACCTC-3′), and cloned into a Smal-digested dephosphonylated pTKan vector (Yuan et al., 2009) to generate a pTKan-p255 vector. Subsequently, a GW-YFP cassette was extracted from the pX-YFP vector (Kim et al., 2009) by XhoI/Spel digestion and ligated into a XhoI/Spel-digested pTKan-p255 vector to generate the pTKan-p255-GWR1R2-YFP vector.

A chimeric DNA construct was synthesized (GenScript, Piscataway, NJ, USA); it was flanked by the gateway sequences attB4r (5′-end) and attB3r (3′-end), and contained, in the following order, the tG7 terminator; the restriction sites Smal, KpnI, HindIII and Xhol; a 2.9-Kb sequence corresponding to the Arabidopsis C4H promoter (pC4H); and a sequence encoding a plastid-targeting signal (SCHL; Lebrun et al., 1992). This attB4r-tG7-pC4H-schl-attB3r construct was then subcloned into the Gateway pDONR221-P4P3r entry vector by BP recombination (Life technologies, Foster City, CA, USA) to generate pENTR-L4-tG7-pC4H-schl-L3. An LR recombination reaction was performed with pTKan-pLRXS-GW (Eudes et al., 2012), pENTR-L1-plac-lacZalpha-L3 (Life technologies), pENTR-L3-plac-Tet-L2 (Life technologies) and pENTR-L4-tG7-pC4H-schl-L3. The obtained construct was subsequently digested by Smal to remove the plac-lacZalpha and tG7 fragments. The plac-Tet fragment was replaced by the gateway cassette using BP recombination to generate the pTKan-pC4H::schl-GWR3R2 vector.

**Generation of a pTKan-pC4H::schl::qsuB plasmid and plant transformation**

A gene sequence encoding QsuB from C. glutamicum (GenBank Accession Number YP_001137362.1) without stop codon and flanked with the Gateway attB3 (5′-end) and attB2 (3′-end) recombination sites was synthesized for expression in Arabidopsis (GenScript) and cloned into the Gateway pDONR221-P3P2 entry vector by BP recombination (Life technologies). A sequence-verified entry clone was LR recombined with the pTKan-pLRXS-GW (Eudes et al., 2012), pENTR-L1-plac-lacZalpha-L3 (Life technologies), pENTR-L3-plac-Tet-L2 (Life technologies) and pENTR-L4-tG7-pC4H::schl-L3. The obtained construct was subsequently digested by Smal to remove the plac-lacZalpha and tG7 fragments. The plac-Tet fragment was replaced by the gateway cassette using BP recombination to generate the pTKan-pC4H::schl-GWR3R2 vector.

**Western blot analysis**

Proteins from Arabidopsis stems were extracted using a buffer containing 250 mM Tris-HCl pH 8.5, 25 mM EDTA, 2 mM DTT, 5 mM β-mercaptoethanol and 10% sucrose, and were quantified using the Bradford method (Bradford, 1976). Proteins (15 µg) were separated by SDS-PAGE, blotted and immunodetected using a universal antibody, as previously described (Eudes et al., 2011).

**Methanol-soluble metabolites extraction**

Arabidopsis stems of 5-week-old wild-type and T3 homozygous C4H::qsuB lines were collected in liquid nitrogen and stored at −80 °C until further utilization. Prior to metabolite extraction, collected stems were pulverized in liquid nitrogen. For extraction of methanol-soluble metabolites, 700–1000 mg of frozen stem
powder was mixed with 2 mL of 80% (v/v) methanol–water and mixed (1400 rpm) for 15 min at 70 °C. This step was repeated four times. Pooled extracts were cleared by centrifugation (5 min, 20 000 g, at room temperature), mixed with 4 mL of analytical grade water and filtered using Amicon Ultra centrifugal filters (10 000 Da MW cut-off regenerated cellulose membrane; EMD Millipore, Billerica, MA, USA). Filtered extracts were lyophilized and the resulting pellets dissolved in 200 μL 50% (v/v) methanol–water prior to LC-MS analysis. An acid hydrolysis of the samples was performed for the quantification of protocatechuic and salicylate; an aliquot of the filtered extracts was dried under vacuum, resuspended with 1 N HCl and incubated at 95 °C for 3 h. The mixture was subjected to three ethyl acetate partitioning steps. Ethyl acetate fractions were pooled, dried in vacuo and resuspended in 50% (v/v) methanol–water prior to LC-MS analysis.

LC-MS analysis
Phenolic acids, phenolic aldehydes, and aromatic amino acids were analyzed using high-performance liquid chromatography (HPLC), electrospray ionization (ESI), and time-of-flight (TOF) mass spectrometry (MS) as previously described in Eudes et al. (2013) and Bokinsky et al. (2013), respectively. Aromatic alcohols were analysed by HPLC – atmospheric pressure chemical ionization (APCI) – TOF MS. Their separation was conducted on an Agilent 1200 Series Rapid Resolution HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) using a Phenomenex Kinetex XB-C18 (100 mm length, 2.1 mm internal diameter and 2.6 μm particle size; Phenomenex, Torrance, CA, USA). The mobile phase was composed of 0.1% formic acid in water (solvent A) and methanol (solvent B). The elution gradient was as follows: from 5% B to 25% B for 6 min, 25% B to 5% B for 1 min and held at 5% B for a further 3 min. A flow rate of 0.5 mL/min was used throughout. The column compartment and sample tray were set to 50 and 25 °C, respectively, and a capillary voltage of 330 V was also used. Fragmentor and OCT 1 RF voltages were set to 350 °C was maintained at 300 °C. The compounds were identified by comparing their mass spectra with those of the NIST library and those previously reported (Del Rio and Gutiérrez, 2006; Ralph and Hatfield, 1991). Peak molar areas were calculated for the lignin degradation products, and the summed areas were normalized.

Cell wall-bound aromatics extraction
The biomass from senesced wild-type plants and T3 homozygous C4H::qsuB lines was used to measure cell wall-bound aromatics. Extracted biomass (10 mg) was mixed with 500 μL of 2 N NaOH and shaken at 1400 rpm for 24 h at 30 °C. The mixture was acidified with 100 μL of concentrated HCl and subjected to three ethyl acetate partitioning steps. Ethyl acetate fractions were pooled, dried in vacuo and suspended in 50% (v/v) methanol–water prior to LC-MS analysis.

2D 13C−1H heteronuclear single-quantum coherence (HSQC) NMR spectroscopy
Stem material from wild-type and pC4H::scl::qsuB-1 plants was extracted and ball-milled as previously described (Kim and Ralph, 2010; Mansfield et al., 2012). The gels were formed using DMSO-d_{6}/pyridine-d_{5} (4:1) and sonicated until homogenous in a Branson 2510 table-top cleaner (Branson Ultrasonic Corporation, Danbury, CT, USA). The temperature of the bath was closely monitored and maintained below 55 °C. The homogeneous solutions were transferred to NMR tubes. HSQC spectra were acquired at 25 °C using a Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient H^{13}C cryoprobe using a hsqcetgpsio2.2 pulse programme (ns = 400, ds = 16, number of increments = 256, d1 = 1.0 s) (Heikkinen et al., 2003). Chemical shifts were referenced to the central DMSO peak (δ_{H} = 39.5 ppm). Assignment of the HSQC spectra was described elsewhere (Kim and Ralph, 2010; Yelle et al., 2008). A semi-quantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker’s Topspin 3.1 (Windows) processing software. A Gaussian apodization in F_{2} (LB = −0.50, GB = 0.001) and squared cosine-bell in F_{1} (LB = −0.10, GB = 0.001) were applied prior to 2D Fourier transformation.

Isolation of cellulolytic enzyme lignin
Stem material from wild-type and pC4H::scl::qsuB-1 plants was extracted and ball-milled for 3 h per 500 mg of sample (in 10 min on/10 min off cycles) using a PM100 ball mill (Retsch, Newtown, PA, USA) vibrating at 600 rpm in zirconium dioxide vessels (50 mL) containing ZrO_{2} ball bearings (10 x 10 mm). Ball-milled walls were digested four times over 3 days at 50 °C with the polysaccharidases Cellic CTec2 and HTeC2 (Novozymes, Davis, CA, USA) and pectinase from Aspergillus niger (Sigma-Aldrich, St. Louis, MO, USA) in sodium citrate buffer (pH 5.0). The
obtained cellulosic lignin was washed with deionized water and lyophilized overnight.

Size-exclusion chromatography

Lignin solutions, 1% (w/v), were prepared in analytical grade 1-methyl-2-pyrrolidinone (NMP). The polydispersity of dissolved lignin was determined using analytical techniques involving SEC UV-F$_{250-400}$ as previously described (George et al., 2011). An Agilent 1200 series binary LC system (G1312B) equipped with diode-array (G1315D) and fluorescence (G1321A) detectors was used. Separation was achieved with a Mixed-D column (5 μm particle size, 300 mm × 7.5 mm i.d., linear molecular mass range of 200 to 400 000 u, Agilent Technologies Inc.) at 80 °C using a mobile phase of NMP at a flow rate of 0.5 mL/min. Absorbance of materials eluting from the column was detected using UV-ﬂuorescence (Ex$_{355}$/Em$_{450}$). Spectral intensities were area-normalized, and molecular mass estimates were determined after calibration of the system with polystyrene standards.

Cell wall pretreatments and saccharification

Ball-milled senesced stems (10 mg) were mixed with 340 μL of H$_2$SO$_4$ (1.2%, w/v) or 340 μL of NaOH (0.25%, w/v) for hot water, dilute acid or dilute alkali pretreatments, respectively, shaken at 1400 rpm (30 °C, 30 min) and autoclaved at 120 °C for 1 h. Samples pretreated with dilute acid were neutralized with 5 N NaOH (25 μL). Saccharification was initiated by adding 650 μL of 100 μm sodium citrate buffer pH 5 (for hot water- and dilute alkali-pretreated samples) or 625 μL of 80 μm sodium citrate buffer pH 6.2 (for dilute acid-pretreated samples) containing 80 μg/mL tetracycline and 1% w/v or 0.2% w/w Celic C Tec2 cellulase (Novozymes). After 72 h of incubation at 50 °C with shaking (800 rpm), samples were centrifuged (20 000 g, 3 min) and 10 μL of the supernatant was collected for measurement of reducing sugars using the 3,5-dinitrosalicylic acid assay and glucose solutions as standards (Miller, 1959).

Conflict of interests

JDK has financial conflict of interests in Amyris, LS9 and Lygos. DL has financial conflict of interests in Afingen.

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Expression of 3-dehydroshikimate dehydratase to repress lignin biosynthesis

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Supporting information

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

Figure S1 Subcellular localization of SCHEL::QsuB.

Figure S2 Summary of the fold changes observed for the 3-dehydroshikimate dehydratase enzyme.

Figure S3 Partial short-range 13C–1H (HSQC) spectra (aliphatic region) of cell wall material from mature senesced stems of wild-type and pC4H::schl::qsuB-1 plants.

Figure S4 Lignin staining by phloroglucinol-HCl of stem sections from 5-week-old wild-type and pC4H::schl::qsuB-1 plants.

Figure S5 Picture of 12-week-old wild-type (WT) and pC4H::schl::qsuB-1 plants.

Figure S6 Detection by RT-PCR of HCT, C3H and CSE transcripts using stem mRNA from 5-week-old wild-type (WT) and pC4H::schl::qsuB-1 plants.
Tub8-specific primers were used to assess cDNA quality for each sample. **Figure S7** Representative LC-MS chromatograms obtained from solutions of standard compounds and from metabolite (methanol-soluble or cell wall-bound) extracts from wild-type (WT) and/or pC4H::chl::qsuB (C4H::qsuB) plants. **Table S1** Characteristics and relative molar abundances (%) of the compounds released after Pyro-GC/MS of extractive-free senesced mature stems from wild-type (WT) and pC4H::chl::qsuB (C4H::qsuB) plants. Values in brackets are the SE from duplicate analyses. **Data S1** Supporting experimental procedures for supplemental data.