Title:
CRISPR-Cas9 Editing of CAFFEOYL SHIKIMATE ESTERASE 1 and 2 Shows Their Importance and Partial Redundancy in Lignification in *Populus tremula* x *P. alba*

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Running title:
The role of CSE1 and CSE2 in poplar

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SUMMARY

Lignins are cell-wall-located aromatic polymers that provide strength and hydrophobicity to woody tissues. Lignin monomers are synthesized via the phenylpropanoid pathway, wherein CAFFEOYL SHIKIMATE ESTERASE (CSE) converts caffeoyl shikimate into caffeic acid. Here, we explored the role of the two CSE homologs in poplar (Populus tremula x P. alba). Reporter lines showed that the expression conferred by both CSE1 and CSE2 promoters is similar. CRISPR-Cas9-generated cse1 and cse2 single mutants had a wild-type lignin level. Nevertheless, CSE1 and CSE2 are not completely redundant, as both single mutants accumulated caffeoyl shikimate. In contrast, the cse1 cse2 double mutants had a 35% reduction in lignin and associated growth penalty. The reduced lignin content translated into a four-fold increase in cellulose-to-glucose conversion upon limited saccharification. Phenolic profiling of the double mutants revealed large metabolic shifts, including an accumulation of p-coumaroyl, 5-hydroxyferuloyl, feruloyl and sinapoyl shikimate, in addition to caffeoyl shikimate. This indicates that the CSEs have a broad substrate specificity, which was confirmed by in vitro enzyme kinetics. Taken together, our results suggest an alternative path within the phenylpropanoid pathway at the level of the hydroxycinnamoyl shikimates, and show that CSE is a promising target to improve plants for the biorefinery.

INTRODUCTION

Lignocellulosic biomass is a promising renewable feedstock for the production of bio-based chemicals and fermentable sugars (Marriott et al., 2016; Van de Wouwer et al., 2018). The cell wall polysaccharides can be hydrolyzed to monosaccharides by saccharification, after which the monosaccharides can be fermented to ethanol or other
products by specific microorganisms (Vanholme et al., 2013b). However, efficient saccharification is hindered by the presence of lignin in the secondary-thickened cell walls, as lignin physically prevents the hydrolytic enzymes from accessing the cellulose surface and it also adsorbs the saccharification enzymes (Jørgensen et al., 2007; Mansfield et al., 1999). Biomass pretreatments are used to reduce the recalcitrance and thereby improve the saccharification efficiency. Nevertheless, these pretreatments are a costly step in the production of bio-based products (Aden et al., 2002; Vanholme et al., 2013b). Alternatively, plants can be engineered to contain less lignin and/or lignin with a different composition to reduce biomass recalcitrance (Chanoca et al., 2019; Chen and Dixon, 2007; De Meester et al., 2018; de Vries et al., 2018; Eudes et al., 2014; Halpin, 2019; Mansfield et al., 2012; Van Acker et al., 2014; Van Acker et al., 2013; Wilkerson et al., 2014; Zhang et al., 2012).

In angiosperms, lignin is mostly derived from the monolignols coniferyl alcohol and sinapyl alcohol, with traces of \( p \)-coumaryl alcohol. These monolignols are synthesized via the general phenylpropanoid and monolignol-specific pathways (Boerjan et al., 2003; Bonawitz and Chapple, 2010; Freudenberg, 1965; Vanholme et al., 2019; Vanholme et al., 2010). After their biosynthesis, the monolignols are deposited in the cell wall, where they are oxidized to radicals by laccases and peroxidases, and subsequently polymerized through combinatorial radical-radical coupling to create the lignin polymer (Berthet et al., 2011; Ralph et al., 2019; Ralph et al., 2004; Zhao et al., 2013). Upon incorporation into the lignin polymer, \( p \)-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol give rise to \( p \)-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. In addition to these three main monolignols, an increasing number of other aromatic compounds has been shown to couple into the lignin polymer (Mottiar et al., 2016; Ralph et al., 2019; Vanholme et al., 2019). The biosynthesis of the two main monolignols coniferyl alcohol and sinapyl alcohol passes through caffeoyl-CoA. Caffeoyl-CoA can be produced from caffeoyl-shikimate by \( p \)-HYDROXYCINNAMOYL-CoA:QUINATE/SHIKIMATE \( p \)-HYDROXYCINNAMOYLTRANSFERASE (HCT) (Franke et al., 2002; Hoffmann et al., 2004; Hoffmann et al., 2003; Schoch et al., 2001; Shadle et al., 2007). Alternatively, it can be produced from \( p \)-coumaric acid via either 4-COUMARATE 3-HYDROXYLASE (C3H; (Barros et al., 2019) or an enzyme complex involving \( p \)-COUMAROYL CoA 3′-HYDROXYLASE/CINNAMATE 4-HYDROXYLASE (C3′H/C4H), followed by the action of
4-COUMARATE:CoA LIGASE (4CL) (Chen et al., 2011). In addition to these routes, it was shown that the HCT-catalyzed conversion of caffeoyl shikimate into caffeoyl-CoA could be bypassed by the combined activities of CAFFEOLYL SHIKIMATE ESTERASE (CSE) and 4CL in Arabidopsis (Vanholme et al., 2013c). A loss-of-function mutant of CSE in Arabidopsis showed up to 36% reduced lignin content and a 30-fold increase in the relative amount of H units in the lignin polymer (Vanholme et al., 2013c). Stacking a C3H silencing construct with the cse loss-of-function mutation in Arabidopsis resulted in a further reduction of lignin content and an increase in the H unit frequency, as compared to the cse mutant (Barros et al., 2019).

The essential role of CSE in monolignol biosynthesis has also been proven in M. truncatula and P. tremula x P. alba (Ha et al., 2016; Saleme et al., 2017). The loss-of-function mutation of CSE in M. truncatula caused more severe phenotypes than those caused by the loss-of-function mutation in Arabidopsis, such as severe dwarfism, reduction of lignin levels by 80%, and levels of up to 84% H units in the lignin, as opposed to approximately 6% H units in the wild type (WT) (Ha et al., 2016). Poplar has two CSE homologs, CSE1 (corresponding to Potri.001G17500) and CSE2 (corresponding to Potri.003G059200), that share 92% identity in amino acid sequence based on the genome sequence of P. tremula x P. alba 717-1B4 from AspenDB (Xue et al., 2015; Zhou et al., 2015). Simultaneous downregulation of CSE1 and CSE2 by a hairpin approach in P. tremula x P. alba resulted in residual expression levels as low as 35% and 15%, respectively, a 25% reduction in lignin amount, and a 2-fold increase in H-unit content (Saleme et al., 2017). Although the saccharification efficiency of the hpCSE was 30% higher than that of the WT, this increase was much smaller than the 4-fold improvement observed with the Arabidopsis cse-2 loss of function mutant (Vanholme et al., 2013c). We hypothesized that the modest increase in saccharification efficiency and the small elevation in H-unit content in comparison with the Arabidopsis cse-2 were caused by the residual CSE expression in the hairpin lines. Additionally, the hairpin strategy downregulated both CSE genes simultaneously, hindering the understanding of potentially differential roles for each of the two genes individually.

Here, we investigated the role of CSE1 and CSE2 in P. tremula x P. alba, both independently and simultaneously, via the generation of knock-out mutants through
CRISPR-Cas9. Growth, lignin analysis, and metabolic profiling of the mutants provided evidence that CSE1 and CSE2 are partially redundant in poplar. By comparative metabolic profiling of WT and double mutants and in vitro enzyme kinetics, we obtained evidence for another metabolic layer in the grid-like phenylpropanoid pathway. Furthermore, the large increases in saccharification efficiency in cse1 cse2 further supported the contention that CSE is a promising target for engineering trees for the biorefinery.

RESULTS

Generation of cse1 and cse2 Single Mutants and cse1 cse2 Double Mutants via CRISPR-Cas9 and expression analyses

Based on the genome sequence of the P. tremula x P. alba 717-1B4 from AspenDB (Figure S1) (Xue et al., 2015; Zhou et al., 2015), the two CSE paralogs share 92% identity in amino acid sequence. In order to investigate the specific roles of CSE1 and CSE2 in poplar, we generated cse1 and cse2 single mutants, as well as cse1 cse2 double mutants via CRISPR-Cas9. Four gRNAs were designed to target the DNA sequence upstream of the codon for the last amino acid of the catalytic triad so that a frameshift mutation would result in a complete loss of enzyme activity (Figure S2, Supplemental Experimental Procedures). After transformation and regeneration, we obtained independent poplar transformants with mutations caused by gRNA1 solely targeting CSE1 (Table S1), gRNA2 solely targeting CSE2 (Table S2), and gRNA4 targeting both CSE1 and CSE2 (Table S4). No bi-allelic double mutants targeting both CSE1 and CSE2 were observed with gRNA3 (Table S3). Only the lines that had bi-allelic frame shift mutations were analyzed further.

Based on GUS expression analyses, CSE1 and CSE2 promoters (2kb upstream sequence from start codon) conferred overlapping expression patterns (Supplemental Results 1.1, Figure S3).

Simultaneous Mutation of CSE1 and CSE2 Affects Plant Development
Perturbations of lignin biosynthesis often lead to a yield penalty in plants (Bonawitz and Chapple, 2013; Li et al., 2010; Muro-Villanueva et al., 2019). No differences were observed for the biomass of cse1 and cse2 as compared to WT plants, with respect to height, stem diameter, fresh and dry weight after 4 months of growth in the greenhouse (Table S5, Figure S4). In an independent growth experiment, cse1 cse2 were grown alongside WT. In contrast to the single mutants, the height of the cse1 cse2 was reduced by 35%, the stem diameter by 14%, stem fresh weight (not debarked) by 52%, and stem dry weight (debarked) by 69% as compared to WT (Table S5, Figure S4). These data suggest redundancy of CSE1 and CSE2 in poplar.

**Altered Phenolic Profile in cse1 and cse2 Single, and cse1 cse2 Double Mutant Poplars**

Next, we analyzed the changes in the phenolic metabolism as a consequence of blocking CSE1 and/or CSE2 to gain insights into the individual roles of these genes. Methanol extracts of developing xylem and bark from 4-month-old WT and mutant lines were analyzed via reverse-phase ultra-high-performance liquid chromatography – mass spectrometry (UHPLC-MS).

In a first experiment, phenolic profiles of xylem from cse1 (n=24) and cse2 (n=15) were compared with those from WT (n=10), yielding a total of 6681 m/z peaks (1850 compounds estimated, Supplemental Experimental Procedures). Principal component analysis (PCA) on the 6681 peaks showed that the first two principal components (PCs) were not able to differentiate the single mutants from WT (Figure S5a). Nevertheless, ANOVA revealed 20 differential peaks (11 compounds, estimated) with a minimum 2-fold change between the WT, and either cse1 or cse2. For cse1 and cse2, a total of 7 and 10 compounds, respectively, were higher in abundance compared to WT. All 7 compounds accumulating in cse1 also accumulated in cse2, but none could be structurally characterized. For cse2, 2 compounds were characterized based on their MS/MS fragmentation spectra (Table 1, Dataset S1, Figure S6). Caffeoyl shikimate (16), the known substrate of CSE, increased 2.65-fold in cse2 and 1.44-fold in cse1 (below 2-fold threshold), as compared to WT. In addition, sinapoyl hexose (10) increased 5.30-fold in
cse2 in comparison to WT. One unknown compound was significantly decreased in cse1, compared to WT. No significantly decreased compounds were detected for cse2.

A comparative profiling of xylem extracts of cse1 cse2 mutants (n=27) versus WT (n=5) yielded a total of 4270 m/z peaks (2356 compounds, estimated). PCA on the 4270 peaks showed that PC1 (counting for 11.5% of the variation) allowed separation between the cse1 cse2 lines and the WTs (Figure S5b). Following ANOVA, 749 differential peaks (560 compounds, estimated) with a minimal 10-fold change were selected (see Supplemental Experimental Procedures for selection criteria). Of these, 316 compounds accumulated in the cse1 cse2 mutant lines as compared to WT. Of the accumulating compounds, 39 (1-25, including 14 isomers) could be structurally characterized based on their MS/MS fragmentation spectra (Table 2, Figure 1, Dataset S2, Figure S6). Among the accumulating compounds were metabolites with a p-coumarate, caffeate, ferulate, or sinapate moiety (4-25), or derivates thereof (1-3). These included caffeoyl shikimate (16), the known substrate of CSE, but also p-coumaroyl shikimate (15), feruloyl shikimate (18), and sinapoyl shikimate (22). In total, 21 characterized metabolites (including isomers) were hexosylated (1-14, 17, 20, 21, 23), most likely to reduce their toxicity and for storage in the vacuole (Desmet et al., 2021; Dima et al., 2015; Le Roy et al., 2016). There were 244 metabolites that were significantly decreased (with a minimal 10-fold change) in the mutants versus WTs, of which 36 could be characterized (26-51, including 13 isomers; Table 2, Figure 1, Dataset S2, Figure S6). In agreement with the reduction in lignin amount in the cse1 cse2 lines (see below), all of these compounds were oligolignols. These were mainly composed of units derived from coniferyl alcohol, sinapyl alcohol, coniferaldehyde, and sinapaldehyde, connected by 8-O-4, 8-5 and 8-8 linkages (26-42) (Morreel et al., 2010a; Morreel et al., 2010b). Aliphatic end-groups were sometimes oxidized (43-45), and units with 8-5 linkages were reduced (46). Also units derived from sinapyl p-hydroxybenzoate (pHBA) (48-50), typical for poplar lignin (Morreel et al., 2010a; Morreel et al., 2004), were reduced in abundance. Furthermore, the abundance of syringyl glycerol p-hydroxybenzoate ester (48) in cse1 cse2 lines was less than 4% relative to levels in WT. This compound has not been reported before. In addition, one compound that was reduced in abundance was characterized as G(8-O-4)G(8-O-4)p-hydroxybenzyl alcohol (51), in which the p-hydroxybenzyl alcohol apparently acted as yet another alternative lignin monomer (Ralph et al., in preparation).
In a similar set-up, both single and double mutant bark tissue was subjected to phenolic profiling. Analogously to the xylem samples, hydroxycinnamoyl shikimates [caffeoyl shikimate (16), feruloyl shikimate (18), and 5-hydroxyferuloyl shikimate (66)] and several derivatives thereof accumulated in the cse1 cse2 bark samples (17, 19, 21, 63-67) (Figure 1, Supplemental Results 1.2).

In short, large metabolic shifts (~20% of the estimated number of compounds) were observed in the profiles of the cse1 cse2 mutants, including the accumulation of various hydroxycinnamoyl shikimic acid derivatives. This indicates that CSE is able to convert hydroxycinnamoyl shikimates other than caffeoyl shikimate to their respective acid forms. The fact that caffeoyl shikimate (the known substrate of CSE) accumulates in each of the cse1 and cse2 mutants indicates that the CSE1 and CSE2 are not fully redundant at the metabolic level.

**A broader role for CSE in the phenylpropanoid pathway**

The phenolic profiling showed an accumulation in the cse1 cse2 mutants of p-coumaroyl shikimate (15), caffeoyl shikimate (17), feruloyl shikimate (18), 5-hydroxyferuloyl shikimate (66), and sinapoyl shikimate (22), suggesting that CSE has a broad substrate specificity. Furthermore, *in silico* protein structure prediction through Phyre2 revealed a L189F substitution for CSE2 in the substrate binding site (Supplemental Results 1.3, Figure S7). To investigate whether CSE1 and CSE2 have different catalytic properties, enzyme kinetics were performed on CSE1 and CSE2 derived from *Populus trichocarpa* [PtrCSE1 has a 98% amino acid sequence identity compared with CSE1 encoded by both the *P. alba* and *P. tremula* alleles; PtrCSE2 has a 97% and 98% amino acid sequence identity compared with CSE2 encoded by the *P. alba* and *P. tremula* alleles, respectively]. The Michaelis-Menten kinetic parameter (K\textsubscript{m}), the turnover number (k\textsubscript{cat}), and the catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}) of PtrCSE1 and PtrCSE2 were determined by *in vitro* assays for four hydroxycinnamoyl shikimates (15, 16, 18 and 22) at each of the enzyme’s respective optimal pH (Figure S8a, b), optimal temperature (Figure S8c, d) and with the protein concentrations mentioned (Figure S8e-f, Table S8). The four tested hydroxycinnamoyl shikimates were all substrates of PtrCSE1 and PtrCSE2 (Figure S9). PtrCSE1 had the highest k\textsubscript{cat}/K\textsubscript{m} for caffeoyl shikimate, followed by feruloyl shikimate, p-
coumaroyl shikimate, and sinapoyl shikimate (Table 3). PtrCSE2 had the highest catalytic efficiency for feruloyl shikimate followed by caffeoyl shikimate, p-coumaroyl shikimate, and sinapoyl shikimate (Table 3). Together with the phenolic profiling data, these results suggest an extra metabolic layer and a role for the different hydroxycinnamoyl shikimates in the phenylpropanoid pathway (Figure 1).

**Reduced Lignin Amount and Altered Lignin Composition in cse1 cse2 Mutants**

Next, we investigated the effect of mutating *CSE1* and/or *CSE2* on lignin amount and composition. First, the cell wall residue (CWR) of wood from the mutants was determined by removing soluble compounds in a sequential solvent extraction. No differences in % CWR were observed between *cse1*, *cse2*, *cse1 cse2* and WT (Figure 2a and 2b, Figure S11 for individual values). Whereas no differences in Klason-insoluble lignin were observed between *cse1* and *cse2* and WT poplars (Figure 2c), *cse1 cse2* poplars showed a significant reduction of 35% in Klason lignin compared to WT (Figure 2d).

Because the *cse1* and *cse2* mutants did not show a difference in lignin amount, these samples were not further investigated. The lignin composition of *cse1 cse2* mutants was determined via thioacidolysis (releasing only 8-O-4 linked monomers) and two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy. The relative amount of thioacidolysis-released G units did not differ significantly and was about 30% in both the *cse1 cse2* mutant and WT samples (Figure 2e, Figure S11 for individual values). However, there was a decrease in thioacidolysis-released S units from 70% in WT to 66% in the *cse1 cse2* mutant. The thioacidolysis-released H units increased about 9-fold, from 0.45% in the WT to 4.05% in the *cse1 cse2* mutant (Figure 2e, Figure S11 for individual values).

Next, NMR was performed on 3 pools of *cse1 cse2* mutants and 3 pools of corresponding WT samples (2 plants per pool). Minor differences were evident between the NMR spectra of enzyme lignin from *cse1 cse2* mutants and that of WT (Figure 2f and 2g, Figure S10). The relative intensities of signals from S units were lower in the *cse1 cse2* poplars compared to WT, whereas the relative intensities of signals from G units were higher; the S/G ratio was therefore lower in the double mutants. The intensities of signals assigned to H units were detected at a trace level (0.6%) in WT, as usual, but their relative contribution was considerably elevated to 4.3% (7-fold) in the *cse1 cse2* poplars.
poplars (Figure 2f). The relative intensity of the signal assigned to pHBA was similar in spectra from cse1 cse2 mutant samples and those of the WT (Figure 2f, Figure S10). Due to the changed lignin composition with the change in H levels, the aliphatic regions also differed (Figure 2g, Figure S10c, d). There is a relative decrease in the 8-5 lignin interunit level, a relative increase in the end-groups, and a slight relative increase in the 8-O-4 (β-O-4) interunit levels in the lignin of cse1 cse2 mutant as compared to those of the WT.

Compromised Vessels in cse1 cse2 Mutants

Microscopy was performed to investigate the cell anatomy and lignin distribution in the xylem. The bottom part of 4-month-old stems of cse1 cse2 and WT was debarked, sectioned, and investigated via phloroglucinol and Mäule staining and via fluorescence microscopy. The cse1 cse2 mutant stems were considerably softer and more pliable than those of WT; upon sectioning, the tissue often got crushed. Phloroglucinol staining (staining hydroxycinnamaldehyde end-groups is considered to reflect the total amount of lignin) was less intense in the cse1 cse2 mutant (Figure 3a). Both in cse1 cse2 and WT, the vessel cell walls showed a more intense staining than the fibers. Vessel morphology appeared compromised in the double mutants. WT showed the expected Mäule staining pattern, with fibers (rich in S-units) stained in red and vessels (rich in G-units) in tan. The cse1 cse2 mutant, however, showed a strong reduction in staining, with fibers and vessels staining in a light orange tone. Moreover, cse1 cse2 mutant xylem showed uneven staining and stronger signal in ray parenchyma cells compared to other cells. Finally, imaging of lignin autofluorescence by confocal microscopy demonstrated that, in addition to irregular vessel walls, the structure and organization of the xylem fibers were also compromised in the cse1 cse2 mutant. Accordingly, vessel circularity, determined on microscopy sections visualized via autofluorescence, was reduced (Figure 3b).

Increased Cellulose-to-Glucose Conversion in cse1 cse2 Mutants

To evaluate whether the reduced lignin amount in the cse1 cse2 poplars leads to an increase in saccharification efficiency, as in Arabidopsis cse mutants and CSE-downregulated poplars (Saleme et al., 2017; Vanholme et al., 2013c), the cellulose-to-
glucose conversion of dried debarked wood of cse1 cse2 mutants and WT was determined using limited-saccharification assays. Cellulose content was measured for each sample via the Updegraff method (Figure 2h, Figure S11 for individual values). No significant difference in cellulose content between cse1 cse2 and WT was observed. Saccharification assays were performed without pretreatment, and with acid and alkaline pretreatment (Figure 4, Figure S12 for individual values). To determine the cellulose-to-glucose conversion, glucose release was measured 3, 6, 24, 30, 48 and 72 h after addition of the saccharification enzymes. Whereas in WT samples the cellulose-to-glucose conversion was 15%, 20% and 20% without pretreatment, with an acid and alkaline pretreatment, respectively, the conversion of cse1 cse2 poplars was significantly higher at all timepoints and for all conditions. The cellulose-to-glucose conversion after 72 h was 63%, 88% and 84% without, with acid, and with alkaline pretreatments, respectively, for the cse1 cse2 mutants. This means that, independently of the pretreatment, the cellulose-to-glucose conversion increased approximately 4-fold in the cse1 cse2 mutants. However, because of the general biomass yield penalty, no increase was observed when the glucose yield was calculated on a per plant basis (Figure S13).

DISCUSSION

Biosynthesis of Phenylpropanoids and Lignin in cse1 cse2 Mutant Poplar

We have previously shown that downregulation of CSE in poplar using a hairpin approach resulted in up to 25% reduced lignin content, a 100-110% increase in H units and an 62%-91% increase in saccharification efficiency on a plant basis, depending on the pretreatment (Saleme et al., 2017). However, the relatively modest increases in H units and saccharification efficiency compared with the Arabidopsis cse-2 mutant suggested that a full knock-out of CSE generated by CRISPR-Cas9 could result in stronger reductions in lignin content and associated improvements in saccharification efficiency (Vanholme et al., 2013c). In addition, CRISPR-Cas9 generated cse knock-out mutants could reveal the role of the individual CSE genes. Here we show that lignin content is not affected in poplar cse1 and cse2 mutants, and is reduced in cse1 cse2 mutants by about 35% in comparison to WT. The remaining 65% lignin in cse1 cse2
mutants imply other routes towards the biosynthesis of lignin in the absence of functional CSE in poplar. One possible route towards coniferyl and sinapyl alcohol could be through HCT. The activity of recombinant poplar HCTs involved in lignification (HCT1 and HCT6) is very low towards caffeoyl shikimate (Wang et al., 2014), but because caffeoyl shikimate increased to very high levels in the cse1 cse2 mutants, HCT enzymes might work near their Vmax in these mutants. Despite that, accumulation of caffeoyl shikimate (16) and caffeoyl shikimate hexoside (17) in cse1 cse2 mutants, showed that HCT was unable to convert all caffeoyl shikimate to caffeoyl-CoA, and that part of it was detoxified via glycosylation or converted to other hydroxycinnamoyl shikimate derivatives. A second possible route towards coniferyl and sinapyl alcohol is the bypass via either the recently characterized ascorbate peroxidase C3H enzyme or the C3’H/C4H heteromeric complex, whereby p-coumaric acid is converted to caffeic acid (Barros et al., 2019; Chen et al., 2011). A third possibility is that a CSE-like protein has partially taken over the role of CSE1 and CSE2, as discussed previously (Ha et al., 2016; Saleme et al., 2017).

Although the frequency of H units had increased by 7-fold in cse1 cse2 mutants (based on NMR data), the total incorporation of H units remained relatively low (approximately 4% of the total H+G+S units), whereas in M. truncatula, a knock-out of CSE dramatically increased the frequency of H units in lignin from 6% up to 84% and in Arabidopsis from almost 0% up to 44% (Ha et al., 2016; Vanholme et al., 2013c). A possible reason for the relatively low frequency of H units in the lignin in cse1 cse2 mutant poplars in comparison with the M. truncatula mutant could be that CCR2 in poplar is not efficient in catalyzing the reaction from p-coumaroyl-CoA to p-coumaraldehyde \( \frac{k_{cat}}{K_m} \) of 2.51 µM\(^{-1}\) min\(^{-1}\) for feruloyl-CoA vs 0.15 for p-coumaroyl-CoA (Wang et al., 2014)], whereas for M. truncatula CCR2, p-coumaroyl-CoA is actually the preferred substrate with the highest \( \frac{k_{cat}}{K_m} \) [0.90 versus 0.40, 0.37 and 0.49 µM\(^{-1}\) min\(^{-1}\) for feruloyl-CoA, sinapoyl-CoA, and caffeoyl-CoA respectively (Zhou et al., 2010)]. Nevertheless, for Arabidopsis the preferred substrate is feruloyl-CoA \( \frac{k_{cat}}{K_m} \) of 4.52 µM\(^{-1}\) min\(^{-1}\) vs 3.03 and 0.72 for sinapoyl-CoA and p-coumaroyl-CoA, respectively (Baltas et al., 2005)]. However, the \( \frac{k_{cat}}{K_m} \) is (relatively) still much higher for Arabidopsis CCR1 compared to poplar CCR2. These observations could suggest that CCR (and/or CAD) cannot efficiently catalyze the biosynthesis of p-coumaryl alcohol in poplar, resulting in a relatively low accumulation of H units in the cse1 cse2 knockouts.
The cse1 cse2 lines accumulate several hydroxycinnamoyl shikimates in xylem and bark tissues, such as p-coumaroyl shikimate (15), caffeoyl shikimate (16), feruloyl shikimate (18), 5-hydroxyferuloyl shikimate (66), sinapoyl shikimate (22), and several derivates thereof (17, 20, 21, 23, 63-65, 67). The accumulation of these hydroxycinnamoyl shikimates suggests that they are also substrates of CSE. Indeed, our enzyme kinetics studies of PtrCSE1 and PtrCSE2 showed that both CSE1 and CSE2 are able to convert shikimates other than caffeoyl-shikimate, such as p-coumaroyl, feruloyl, and to a lesser extent also sinapoyl shikimate in vitro (Table 3, Figure S9). Together with the observed accumulation of the hydroxycinnamoyl shikimates and their derivatives in cse1 cse2 mutant lines, the enzyme kinetics hint at the existence of another metabolic layer in the phenylpropanoid pathway, in which hydroxylation and methylation occur at the shikimate-ester level, besides the free-acid level, the CoA-thioester level, and the aldehyde level, as recently suggested (Saleme et al. 2017). Moreover, cell lysates of yeast lines co-expressing Populus nigra HCT1 and Arabidopsis thaliana 4CL4 were able to convert caffeic and ferulic acid to the corresponding shikimate esters, illustrating that Populus nigra HCT1 is able to use caffeoyl-CoA and feruloyl-CoA (formed by 4CL4 from the acids) as substrates, albeit to a lesser extent than p-coumaroyl-CoA (Vanholme et al., 2013a). These results suggest a route in the phenylpropanoid pathway from the acids, through the CoA-thioester intermediates, towards the shikimate-esters that, in their turn, could be reconverted back to their acid forms by the CSE enzymes and in this way potentially form a substrate cycle. Such substrate cycles play an important role in cellular homeostasis, allowing for fluctuations in the cycle without directly affecting other fluxes in the metabolic network (Sridharan et al., 2015), and in this particular case could be important in maintaining the levels of free CoA by regulating the accumulation of CoA-ester intermediates such as caffeoyl-CoA.

CSE1 and CSE2 Are Partially Redundant in Poplar

Both CSE promoters confer similar expression patterns (Figure S3a-I), and both CSE proteins were shown to have similar relative activities towards caffeoyl shikimate (Ha et al., 2016; Figure S9), suggesting that CSE1 and CSE2 are redundant in poplar. Despite the normal development and lignin accumulation of the cse1 and cse2 mutants, they show metabolic shifts in xylem and bark, including the accumulation of the CSE
substrate, caffeoyl shikimate. Furthermore, cse2 mutants showed more differential compounds in both bark and xylem tissues in comparison to cse1 mutants, demonstrating that the redundancy of CSE1 and CSE2 is incomplete at the metabolic level. These results, in combination with the difference in catalytic efficiency of PtrCSE1 and PtrCSE2 (Table 3), suggest that CSE1 and CSE2 might be undergoing subfunctionalization. Moreover, our data indicate that the conversion of caffeoyl shikimate to caffeic acid (and potentially the conversion of p-coumaroyl shikimate, feruloyl shikimate, and sinapoyl shikimate to p-coumaric acid, ferulic acid, and sinapic acid, respectively), is not the rate-limiting step in the pathway flux to lignin in WT plants, as its decrease in efficiency in the cse1 and cse2 mutants, substantiated by the product accumulation, does not result in notable changes in lignin amount.

**CSE as a Target for Engineering Low-Lignin Trees for the Biorefinery**

A ~4-fold improvement in cellulose-to-glucose conversion was observed in cse1 cse2 poplars, independent of pretreatment. However, due to the significant biomass penalty this improvement does not persist when the glucose yield is expressed on a plant basis. Nevertheless, if the yield penalty of the plants could be overcome, CSE-edited trees would represent a significantly improved feedstock for the biorefinery. The vessel cell morphology is compromised in cse1 cse2 lines, as observed in several other plants with perturbations in the lignin biosynthetic pathway (Coleman et al., 2008; Jones et al., 2001; Leplé et al., 2007; Schilmiller et al., 2009; Vanholme et al., 2013c; Voelker et al., 2010). This is a possible reason for the observed yield penalty, either because the function of the vessel cell wall is impaired, or because its physico-chemical defects signal a stress response (Gallego-Giraldo et al., 2020; Gallego-Giraldo et al., 2018; Ha et al., 2021; Muro-Villanueva et al., 2019); restoring lignification in the vessel cells of Arabidopsis lignin mutants has been successfully used to recover the vessel cell morphology and overall biomass, while still maintaining the enhanced saccharification efficiency of the mutant biomass (De Meester et al., 2018; Vargas et al., 2016; Yang et al., 2013). However, implementing this strategy in trees might not be straightforward to rescue the biomass yield penalty. It has been shown that in poplar, the fibers adjacent to vessels have a similar lignin composition as the vessels, suggesting that monolignols diffuse from vessels to fibers (Gorzsás et al., 2011). Although it is not clear to what extent this
diffusion could re-lignify poorly lignified fibers, such as in the Arabidopsis cse mutants, it should be plausible to screen for transgenic lines that have normal-shaped and lignified vessels but hypolignified fibers. Such biomass would still present advantages over wild-type biomass.

Downregulation of CSE1 and CSE2 expression in poplar through RNA interference (hpCSE) did not result in a yield penalty, even though the lignin amount was reduced by 25% (Saleme et al., 2017). This suggests that there are no phenotypic effects as long as the lignin amount stays above a certain threshold in CSE downregulated lines. The saccharification efficiency of the hpCSE-downregulated poplars was increased by 31% when no pretreatment was used (Saleme et al., 2017), whereas that of the cse1 cse2 mutated poplars had an increase of 320% when no pretreatment was applied. To achieve a high saccharification efficiency while at the same time avoiding a yield penalty, instead of knocking-out CSE1 and CSE2, weak alleles may be generated via CRISPR-Cas9, as recently illustrated for the CINNAMOYL-CoA REDUCTASE 2 gene by De Meester et al. (2021).

EXPERIMENTAL PROCEDURES

Generation of CSE Mutations via CRISPR-Cas9, Poplar Transformation and Plant Material

CSE1 and CSE2 sequences of P. tremula x P. alba and a list of predesigned gRNAs (used as basis for gRNA design) was obtained from AspenDB (Xue et al., 2015; Zhou et al., 2015). The gRNAs were further selected based on criteria outlined in Supplemental Experimental Procedures. For targeting CSE1 and CSE2 individually, one gRNA per gene fulfilled these criteria. To design gRNAs that target CSE1 and CSE2 simultaneously, an alignment of CSE1 and CSE2 was made in CLC Main Workbench. A search on the PAM-sequence (5’-NGG-3’) was performed, resulting in a list of thirteen possible gRNAs that target both CSE1 and CSE2 simultaneously. Eight gRNAs fulfilled the selection criteria (Supplemental Experimental Procedures), of which two were chosen. The gRNAs were then cloned via Gibson Assembly into the p201N-Cas9 vector, as previously reported (Jacobs and Martin, 2016).
The different constructs were transformed via electroporation in the Agrobacterium tumefaciens strain C58C1. Transformation of P. tremula x P. alba cv 717-1B4 was performed as previously reported (Leplé et al., 1992). The transformed poplars and their corresponding WT were transferred to soil in pots of 5.5 cm diameter and covered with a cage liner for acclimatization. DNA was extracted from a leaf via the Edwards procedure (Edwards et al., 1991), and amplified and Sanger sequenced around the different target sites (Table S9 for primer sequences). The Sanger sequences of the different transformed poplar lines were analyzed for possible mutations Via TIDE (Brinkman et al., 2014).

The transformed poplars that carried mutations (caused by gRNA4) were first propagated to obtain enough biological replicates. After propagation, the poplars were transferred to bigger pots (24 cm diameter) and their heights were measured weekly over 12 weeks (n=30 for cse1 cse2 and n=6 for WT, n=31 for cse1, n=33 for cse2 and n=17 for WT). After 4 months of growth, the poplars were harvested and their diameters (5 cm above the ground) and weights were measured.

**Phenolic Profiling**

Bark and wood from 10-cm basal stem fragments from 4-month-old P. tremula x P. alba cse1 cse2, cse1, cse2 and WT were subjected to metabolic profiling according to Saleme et al. (2017) with the following adaptations: 250 mg of ground tissue was used instead of 100 mg, and 600 µL of methanol supernatants was dried instead of 800 µL (Supplemental Experimental Procedures for adaptations and metabolite selection-criteria).

Structural characterization was performed using MS/MS spectral matching against an in-house spectral database, spectral elucidation using published MS fragmentation pathways (Morreel et al., 2014), and de novo spectral elucidation software [CSI:FingerID (Dührkop et al., 2015); CFM-ID (Allen et al., 2015)].

**Lignin Amount and Thioacidolysis**

Poplars stems were harvested and dried in a Memmert UF 750 oven at 30 °C for five days and subsequently ground into powder (Fritsch Funnel P-19 for long and bulk solids).
This powder was used for cell wall analysis and saccharification. To obtain purified CWR, the samples were subjected to sequential extraction as described in (Saleme et al., 2017). Lignin quantification was performed via a modified Klason protocol following Saleme et al. (2017). Thioacidolysis was performed as previously described (Robinson and Mansfield, 2009). For quantification of the conventional lignin units H, G and S, response factors were used as reported earlier (Yue et al., 2012). To determine the lignin composition regardless of the interunit linkages, 2D-NMR was used as previously described (Kim et al., 2008). For 2D-NMR, three biological replicates were analyzed from cse1 cse2 and WT; each of the biological replicates comprised wood derived from two different lines.

Isolation of Enzyme Lignin (EL) and NMR
Ball-milled cell wall was prepared and enzyme lignin (EL) was isolated as described previously (Kim et al., 2017). NMR spectra were acquired according to (Saleme et al., 2017) with slight adaptations (Supplemental Experimental Procedures).

Lignin Staining and Vessel Circularity Analyses
Lignin staining with Wiesner and Mäule reagents was performed as previously described (Pradhan Mitra and Loqué, 2014). Sections of 100 µm were obtained in a Leica Vibratome, mounted in 20 mM Tris pH 7, 50% glycerol and visualized using a Zeiss Axioscope. Vessel circularity was determined using the measure command in ImageJ, using 20X thresholded images. Circularity is calculated as circularity = 4π(area/perimeter²). Thirty vessels were measured for each genotype.

Cellulose Quantification
Cellulose content was estimated via the colorimetric Updegraff method on CWR as previously described (Foster et al., 2010). The absorbance was measured at room temperature at 625 nm.

Saccharification Assays
Saccharification assays were performed as previously described (Van Acker et al., 2016; Van Acker et al., 2013), without pretreatment, acid pretreatment 1 M HCl at 80 °C for 2 h and alkaline pretreatment (62.5 mM NaOH at 90 °C for 3 h). Glucose measurements were performed at 3, 6, 24, 48 and 72 h after the saccharification enzymes were added. The enzyme activity added to each aliquot was 0.015 FPU. To calculate the glucose yield per plant, the cellulose-to-glucose conversion at timepoint 72 h was multiplied by the average dry weight of debarked stems and the average cellulose content.

Recombinant Protein Expression and Purification

Full-length coding regions of *PtrCSE1* (Potri.003G059200) and *PtrCSE2* (Potri.001G175000) were amplified from cDNA of *P. trichocarpa* (Nisqually-1) stem differentiating xylem and verified by Sanger sequencing. *PtrCSE1* and *PtrCSE2* coding regions were then cloned into the pET-101/D-TOPO vector (Invitrogen, Carlsbad, CA) to express recombinant proteins fused with C-terminal 6×histidine tag. The assembled constructs (*PtrCSE1-pET101* and *PtrCSE2-pET101*) were transformed into *E. coli* Rosetta 2 (DE3) (EMD Millipore Corp., Billerica, MA), and induced for protein expression using 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22 °C for 16 h. Recombinant proteins were purified using the Probond Purification Kit (Invitrogen, Carlsbad, CA) as described previously (Shuford et al., 2012) and stored at -80 °C before enzyme kinetic analysis.

Enzyme Reaction Conditions and HPLC Analysis

Enzyme kinetic analysis using p-coumaroyl, caffeoyl, feruloyl, and sinapoyl shikimates, synthesized following (Timokhin et al., in preparation), as substrates for *PtrCSE1* and *PtrCSE2*, were performed using purified recombinant proteins and the optimal reaction temperature and pH for each enzyme (Supplemental Experimental Procedures). The enzyme assays were performed as described for optimal temperature and pH determination, using different concentrations of each substrate ranging from 10 to 1000 µM, and reaction time was adjusted between 10 and 240 min to ensure initial reaction velocity was measured. Three to five technical replicates were performed for each enzyme-substrate combination. Kinetic parameters (K_m and K_cat) were obtained using
Prism 8 (GraphPad, San Diego, CA) by applying the nonlinear Michaelis-Menten curve-fitting function.

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CONFLICT OF INTEREST

R.V. and W.B. are inventors of patent number 9834776.

AUTHOR CONTRIBUTION

L.d.V., M.B., A.C., R.V. and W.B. designed the research; L.d.V, M.B., A.C., H.K., M.R.R., V.I.T., Y.S., B.D.M., J.V.D., and G.G. performed the experiments; L.d.V., M.B., A.C., H.K., V.L.C., J.P.W., J.R., K.M., and R.V., performed data analysis and L.d.V., M.B., A.C., R.V., and W.B. wrote the article.

SUPPORTING INFORMATION

1. Supporting results:
   1.1 CSE1 and CSE2 Have Overlapping Promoter Activity Patterns
   1.2 Bark metabolic profiling
   1.3 Protein structure prediction with Phyre2

2. Supporting discussion:
   2.1 Comparison of hpCSE1 and hpCSE1 with cse1 cse2 double mutants

3. Supporting Figures:
Figure S1. *P. tremula* x *P. alba* sequences of CSE1 and CSE2

Figure S2. Schematic Representation of *P. tremula* x *P. alba* CSE1 and CSE2 with the Location of the possible gRNAs.

Figure S3. GUS Staining of pCSE1:GUS and pCSE2:GUS Plants

Figure S4. Phenotype of representative poplars grown in the greenhouse for four months and individual values of height measurements

Figure S5. Principal Component Analysis (PCA) plots of phenolic profiling experiments

Figure S6. Molecular structures of characterized compounds that are higher or lower in abundance in the cse1 cse2 double mutant, or in the cse1 and cse2 single mutant xylem and/or bark extracts compared to WT

Figure S7. Protein modeling of the *P. tremula* and *P. alba* CSE1 and CSE2 proteins

Figure S8. Optimization of pH and temperature for enzyme kinetics

Figure S9. Enzyme kinetics of PtrCSE1 and PtrCSE2

Figure S10. Lignin Composition Analyzed by 2D-NMR

Figure S11. Individual values of cell wall characteristics for WT and cse1 cse2

Figure S12. Individual values for saccharification efficiency at 72 h timepoint

Figure S13. Released glucose per plant of WT (n=5) and cse1 cse2 (n=16)

4. Supporting Tables:

Table S1. Sequencing results of *P. tremula* x *P. alba* poplars around target site of gRNA1 targeting CSE1
Table S2. Sequencing results of *P. tremula* x *P. alba* poplars around target site of gRNA2 targeting CSE2

Table S3. Sequencing results of *P. tremula* x *P. alba* poplars around target site of gRNA3 targeting both CSE1 and CSE2

Table S4. Sequencing results of *P. tremula* x *P. alba* poplars around target site of gRNA4 targeting both CSE1 and CSE2

Table S5. Biomass Yield of *cse1* and *cse2* Single Mutants and *cse1 cse2* Double Mutants

Table S6. List of characterized compounds in bark extracts of *cse1* and *cse2* single mutants

Table S7. List of characterized compounds in bark extracts of *cse1 cse2* double mutants

Table S8. Protein concentrations of CSE1 and CSE2 used for enzyme kinetic assays

Table S9. Primers used for sequencing CSE1 and CSE2 to confirm mutations caused by CRISPR-Cas9 and primers used for confirming destination vector pCSE1:GUS and pCSE2:GUS

Table S10. gRNA1 alignments with the 25 closest potential off-targets

Table S11. gRNA2 alignments with the 25 closest potential off-targets

Table S12. gRNA4 alignments with the 25 closest potential off-targets

5. Supporting Experimental procedures

Supporting datasets:

- **Dataset S1.** List of structurally characterized metabolites with a different abundance in the *cse1* and *cse2* single mutant xylem extracts as compared to WT
• **Dataset S2.** List of structurally characterized metabolites with a different abundance in the *cse1 cse2* double mutant xylem extracts as compared to WT

• **Dataset S3.** List of structurally characterized metabolites with a different abundance in the *cse1* and *cse2* single mutant bark extracts as compared to WT

• **Dataset S4.** List of structurally characterized metabolites with a different abundance in the *cse1 cse2* double mutant bark extracts as compared to WT

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**FIGURE LEGENDS**

**Figure 1.** The General Phenylpropanoid and Monolignol-Specific Pathways, with the Changes in Phenolic Metabolism Indicated for *cse1 cse2* xylem and/or Bark Extracts Compared to WT.

Red and blue names indicate metabolites that are up or down, respectively, in *cse1 cse2* xylem and/or bark extracts as compared to WT. Characterised metabolites uniquely detected in bark or xylem profiles are
indicated with a (b) or (x) respectively. Co-occurring metabolites are indicated by (x, b). Metabolites that are framed in a box belong to the same class. The flow through the pathway towards the conventional monolignols is in black in WT, whereas (putative) alternative flows are in grey. Successive arrows show two or more metabolic steps. Solid arrows show enzymatic conversions that are validated by experimental evidence, whereas dashed arrows show suggested conversions (Saleme et al., 2017; Van Acker et al., 2017). * The catalytic activity of CSE1 and CSE2 is suggested in planta and proven in vitro in this work. Each metabolite is indicated with a unique number, corresponding to the metabolites mentioned in the manuscript and Tables 1 and 2 and Supplemental Tables S6 and S7. Their structures are given in Figure S6. CAD, CINNAMYL ALCOHOL DEHYDROGENASE; CCoAOMT, CAFFEOYL-CoA O-METHYLTRANSFERASE; CCR, CINNAMOYL-CoA REDUCTASE; CHS, CHALCONE SYNTHASE; COMT, CAFFEIC ACID O-METHYLTRANSFERASE; F5H, FERULATE 5-HYDOXYLASE; PAL, PHENYLALANINE AMMONIA-LYASE.

Figure 2. Cell wall characteristics
(a) CWR of WT, cse1 and cse2 and (b) of WT and cse1 cse2 was determined gravimetrically after extraction (c) Acid-insoluble Klason lignin of WT, cse1 and cse2 and (d) WT and cse1 cse2. (e, f) Lignin composition analyzed by thioacidolysis (e) and 2D-NMR (NMR) (f) of cse1 cse2, values are expressed in percentage of the respective released lignin unit relative to the sum of H+G+S. S* units are only observed via NMR. These are S units with alpha-keto functionalities that are thought to be derived from S units during the ball-milling process (see Figure 3). (g) Lignin aliphatic region analyzed by NMR, values are expressed in percentage of the respective lignin interunit level relative to the sum of all the lignin interunits. 8-O-4: 8-aryl ether; 8-5: phenylcoumaran; 8-8: resinol; X: cinnamyl alcohol (endgroup). (h) Cellulose content of WT and cse1 cse2, determined via the colorimetric Updegraff method. For cse1 (n=15), cse2 (n=15) and their corresponding WT (n=10) statistical differences were assessed with ANOVA. For acid-insoluble lignin of cse1 cse2 n=16 [6 independent lines (line 3, 5.2 5.3, 7.1, 26 and 28] and its corresponding WT n=5, for thioacidolysis and cellulose content: cse1 cse2 n=16 [6 independent lines (line 3, 5.2 5.3, 7.1, 26 and 28] and WT n= 6 and for NMR cse1 cse2 n=3 (each sample is a pool of wood derived from 3 poplars) and WT n=3 (each sample is a pool of wood derived from 3 poplars), differences were assessed via the Student’s t-test. Error bars represent standard deviation. *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; and ***, P < 0.001.

Figure 3. Cellular histology of cse1 cse2 Double Mutants and WT
(a) Phloroglucinol, Mäule, and autofluorescence pictures of a representative sample of WT and cse1 cse2 show compromised vessels in the cse1 cse2 double mutant. Sections stained with phloroglucinol and Mäule were analyzed by a light microscope, autofluorescence was analyzed by a confocal microscope. V: vessels; F: fibers; CR: contact ray parenchyma; IR: isolation ray parenchyma. (b) Vessel circularity was calculated as circularity = 4π(area/perimeter2). Thirty vessels were measured for each genotype, and
differences were assessed via the Student's t-test. Error bars represent standard deviation. ***, P < 0.001.
Bar = 50 μm.

Figure 4. Saccharification Efficiency of cse1 cse2 wood
Wood from cse1 cse2 (n=16, 6 independent lines (line 3, 5.2 5.3, 7.1 26 and 28]) and WT (n=5) was tested for saccharification efficiency at 3, 6, 24, 30, 48 and 72 h, either without pretreatment (a), with acid pretreatment (b) or with alkaline pretreatment (c). Error bars represent standard deviations. Significant differences were assessed with Student's t test (***, P < 0.001).
**TABLES**

Table 1. List of characterized compounds in xylem extracts of *cse2* single mutants. Column “No.”: the number the compound was assigned in Figure 1 and the text. tR: retention time in minutes. Fold changes were calculated as the ratio of average normalized abundance in mutants/average normalized abundance in WT. The structures are given in Figure S6.

| No. | tR   | m/z     | name                  | average normalized abundance in WT | average normalized abundance in mutants | fold change |
|-----|------|---------|-----------------------|-------------------------------------|-----------------------------------------|-------------|
| UP  |      |         |                       |                                     |                                         |             |
| 10  | 4.39 | 385.113 | sinapoyl hexose       | 826                                 | 4381                                    | 5.3         |
| 16  | 6.12 | 335.075 | caffeoyl shikimate    | 5331                                | 14134                                   | 2.7         |
Table 2. List of characterized compounds in xylem extracts of cse1 cse2 double mutants. Column “No.”: the number the compound was assigned in Figure 1 and the text. tR: retention time in minutes. Fold changes were calculated as the ratio of average normalized abundance in mutants/average normalized abundance in WT. The structures are given in Figure S6. Superscripts: 1 = feature detected as ion-neutral complex, 2 = feature detected as formate adduct, 3 = in source fragment of unknown origin. b.d.l. = below detection limit, * = exact position of OH-groups could not be inferred from the fragmentation spectra, biochemical knowledge was used to support the characterization.

| No. | tR  | m/z   | name                              | average normalized abundance in WT | average normalized abundance in mutants | fold change |
|-----|-----|-------|-----------------------------------|-------------------------------------|----------------------------------------|-------------|
| **UP** | | | | | | |
| 1 | 1.8 | 315.072 | protocatechoyl hexose* | 299 | 5185 | 17 |
| 2 | 2.3 | 659.177 | vanillic acid hexoside\(^1\) | 2099 | 99993 | 48 |
| 3 | 3.6 | 461.128 | vanillic acid pentosyl hexoside | 81 | 2488 | 31 |
| 4 | 14. | 475.124 | hydroxybenzoyl (feruloyl) hexoside | b.d.l. | 6797 | > 100 |
|   |   |   |                          |        |   |   |
|---|---|---|--------------------------|--------|---|---|
| 5 | 97| 477.139 | dihydroxybenzyl alcohol (feruloyl) hexoside | b.d.l. | 11459 | > 1000 |
| 6 | 5 | 355.103 | feruloyl hexose | 9234 | 154450 | 17 |
| 6 | 6 | 355.103 | feruloyl hexose | 611 | 28801 | 47 |
| 6 | 3 | 355.103 | feruloyl hexose | 592 | 17342 | 29 |
| 7 | 75 | 531.149 | differeduloyl hexose | b.d.l. | 28838 | > 1000 |
| 8 | 9 | 491.119 | protocatechuic acid ferulic acid hexoside* | 256 | 16260 | 63 |
| 9 | 6 | 343.104 | dihydrocaffeoyl hexoside | 129 | 3711 | 29 |
| 10 | 6 | 385.113 | sinapoyl hexose | 1746 | 78843 | 45 |
| 10 | 4 | 385.114 | sinapoyl hexose | 378 | 5137 | 14 |
| 11 | 8 | 535.144 | sinapoyl (vanilloyl) hexose | b.d.l. | 6502 | > 100 |

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|   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|
| 12 | 97 | 537.160 | hydroxyvanillyl alcohol (sinapoyl) hexoside | b.d.l. | 7646 | > 100 |
|   | 1.6 |   |   |   |   |   |   |
| 13 | 6 | 506.131 | 1,2,3,4,5-pentahydroxy-(cysteinyl-1-S)-hexanyl sinapic acid | b.d.l. | 10669 | > 1000 |
|   | 2.9 |   |   |   |   |   |   |
| 14 | 1 | 593.166 | 4-O-hexosyl-sinapoyl hexose² | b.d.l. | 2032 | > 100 |
|   | 3.1 |   |   |   |   |   |   |
| 14 | 7 | 593.168 | 4-O-hexosyl-sinapoyl hexose² | 82 | 11325 | 138 |
|   | 7.3 |   |   |   |   |   |   |
| 15 | 8 | 319.082 | p-coumaroyl shikimate | 372 | 36405 | 98 |
| 16 | 2 | 335.079 | caffeoyl shikimate | 11 | 1330 | 120 |
|   | 5.7 |   |   |   |   |   |   |
| 16 | 8 | 335.077 | caffeoyl shikimate | 1871 | 48164 | 26 |
| 16 | 6.2 | 335.077 | caffeoyl shikimate | 20360 | 308659 | 15 |
|   | 7.7 |   |   |   |   |   |   |
| 16 | 1 | 335.078 | caffeoyl shikimate | 703 | 11941 | 17 |
|   | 3.7 |   |   |   |   |   |   |
| 17 | 8 | 995.257 | caffeoyl shikimate hexoside¹ | b.d.l. | 6625 | > 100 |
|   | 4.9 |   |   |   |   |   |   |
| 17 | 6 | 497.128 | caffeoyl shikimate hexoside | b.d.l. | 58902 | > 1000 |
|    |    |    |                  |    |    |    |
|----|----|----|------------------|----|----|----|
| 17 | 7.2|    | caffeoyl shikimate hexoside | 28 | 4675 | 166 |
| 18 |    | 4.6| feruloyl shikimate      | 222| 9958 |  45 |
| 18 |    | 5.6| feruloyl shikimate      | 12 | 1676 | 135 |
| 18 |    | 8.1| feruloyl shikimate      | 2265| 40966|  18 |
| 18 |    | 8.3| feruloyl shikimate      | 31 |  4885| 158 |
| 18 |    | 9.1| feruloyl shikimate      | 10196|135217|  13 |
| 19 |    |    | sulfoferuloyl shikimate | 32 |  2147|  67 |
| 20 |    | 5.1| 5-hydroxyferuloyl shikimate hexoside | b.d.l.|6816 | 682 |
| 21 |    | 6.3| feruloyl shikimate hexoside | b.d.l.|10136|1014 |
| 22 |    | 9.0| sinapoyl shikimate      | 1736| 36183|  21 |
| 23 |    | 5.1| sinapoyl (shikimoyl) hexose | b.d.l.|14722|1473 |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 24 | 6.8 | 367.103 | feruloyl quinate | 19 | 1684 | 88 |
|   | 2.4 |   |   |   |   |   |
| 25 | 5   | 470.110 | cysteinyl feruloyl quinate | b.d.l. | 17458 | 1746 |
| 25 | 2.8 | 470.110 | cysteinyl feruloyl quinate | b.d.l. | 17685 | 1769 |
| **DOWN** |   |   |   |   |   |   |
| 26 | 15. |   |   |   |   |   |
| 26 | 15 | 779.288 | G(8-O-4)G(8-O-4)S(8-5)G | 4774 | 13 | 0.0028 |
| 26 | 28 | 779.288 | G(8-O-4)G(8-O-4)S(8-5)G | 839 | b.d.l. | < 0.1 |
| 27 | 54 | 809.301 | G(8-O-4)G(8-O-4)S(8-8)S | 5981 | 101 | 0.0170 |
| 27 | 96 | 1005.373 | G(8-O-4)G(8-O-4)S(8-8)S(4-O-8)G | 13342 | 491 | 0.0368 |
| 28 | 16 | 809.300 | G(8-O-4)S(8-8)S(4-O-8)G | 152444 | 13896 | 0.0912 |
| 29 | 16 | 809.300 | G(8-O-4)S(8-8)S(4-O-8)G | 71685 | 5293 | 0.0738 |
| 29 | 86 | 809.300 | G(8-O-4)S(8-8)S(4-O-8)G | 1588 | b.d.l. | < 0.01 |
| 30 | 04 | 809.297 | G(8-O-4)S(8-O-4)S(8-5)G | 6879 | 15 | 0.0021 |
| 30 | 16 | 809.299 | G(8-O-4)S(8-O-4)S(8-5)G |   |   |   |
|  |  |  |  |  |
|---|---|---|---|---|
| 31 | 15. | 71 | 583.215 | G(e8-O-4)S(8-5)G | 23863 | 386 | 0.0162 |
| 32 | 15. | 28 | 553.206 | G(t8-O-4)G(8-8)G³ | 48422 | 2654 | 0.0548 |
| 33 | 17. | 89 | 839.310 | S(8-O-4)S(8-8)S(4-O-8)G | 25988 | 1918 | 0.0738 |
| 34 | 17. | 59 | 839.311 | S(8-O-4)S(8-8)S(4-O-8)G³ | 13430 | 355 | 0.0264 |
| 35 | 18. | 88 | 869.320 | S(8-O-4)S(8-O-4)S(8-8)S | 5703 | 222 | 0.0390 |
| 36 | 18. | 32 | 869.320 | S(8-O-4)S(8-O-4)S(8-8)S | 7257 | 92 | 0.0126 |
| 37 | 14. | 67 | 613.224 | S(t8-O-4)S(8-5)G | 8700 | 124 | 0.0142 |
| 38 | 16. | 95 | 777.273 | G(8-O-4)G(8-O-4)S(8-5)G’ | 10751 | 179 | 0.0166 |
| 39 | 15. | 85 | 825.293 | G(8-O-4)G(8-O-4)S(8-O-4)S’ | 38278 | 441 | 0.0115 |
| 40 | 16. | 825.294 | G(8-O-4)G(8-O-4)S(8-O-4)S’ | 15730 | 360 | 0.0229 |
|    |   |     |             |       |   |     |
|----|---|-----|-------------|-------|---|-----|
| 38 | 14.| 83  | 599.210     | G(8-O-4)G(8-O-5)S' | 6577 | 23  | 0.0036 |
| 39 | 15.| 58  | 629.220     | G(e8-O-4)S(8-O-4)S' | 5858 | 184 | 0.0314 |
| 40 | 16.| 93  | 581.201     | G(t8-O-4)S(8-5)G'  | 164401 | 8753 | 0.0532 |
| 41 | 14.| 84  | 629.221     | G(t8-O-4)S(8-O-4)S' | 38762 | 1220 | 0.0315 |
| 42 | 14.| 7   | 659.231     | S(8-O-4)S(8-O-4)S' | 14655 | 246 | 0.0168 |
| 43 | 10.| 66  | 813.290     | G(8-O-4)G(8-O-4)S(8-5)G-glycerol | 6216 | 77 | 0.0124 |
| 44 | 5.6| 9   | 439.159     | G(8-O-4)S-glycerol  | 6586 | 76  | 0.0116 |
| 45 | 12.| 45  | 599.210     | G'(8-O-4)G(8-O-4)S-OH | 10721 | 59 | 0.0055 |
| 46 | 12.| 96  | 555.221     | G(8-O-4)G(red8-5)G  | 61815 | 6121 | 0.0990 |
| 46 | 13.| 555.221 | G(8-O-4)G(red8-5)G | 40959 | 1281 | 0.0313 |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 46 | 7 | 555.221 | G(8-O-4)G(red8-5)G<sup>3</sup> | 5570 | 26 | 0.0047 |
| 47 | 46 | 737.241 | G(8-O-4)G(8-O-4)alpha-oxoS-pHBA | 15724 | 418 | 0.0266 |
| 48 | 1 | 363.109 | S-glycerol pHBA | 11431 | 452 | 0.0396 |
| 49 | 31 | 559.180 | G(8-O-4)S-glycerol-pHBA | 18713 | 11 | 0.0006 |
| 49 | 96 | 559.180 | G(8-O-4)S-glycerol-pHBA | 12764 | 19 | 0.0015 |
| 49 | 08 | 559.180 | G(8-O-4)S-glycerol-pHBA | 16551 | 78 | 0.0047 |
| 50 | 95 | 589.189 | S(8-O-4)S-glycerol-pHBA | 8562 | 11 | 0.0013 |
| 51 | 1 | 515.190 | G(8-O-4)G(8-O-4)hydroxybenzyl alcohol | 21700 | 147 | 0.0068 |
Table 3. Enzyme kinetics of PtrCSE1 and PtrCSE2. Corresponding $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values with standard deviations for 3 to 5 technical replicates are shown for each tested substrate. CSE1 shows the highest $k_{cat}/K_m$ for caffeoyl shikimate, whereas CSE2 shows highest $k_{cat}/K_m$ for feruloyl shikimate.

| Substrates               | $K_m$ (µM) | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ (µM) | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------------------|------------|-----------|---------------|------------|-----------|---------------|
| $p$-coumaroyl shikimate  | 234 ± 35   | 0.43 ± 0.05 | 1.78 x $10^{-3}$ ± 0.41 x $10^{-3}$ | 49.40 ± 7.90 | 1.27 ± 0.06 | 2.56 x $10^{-2}$ ± 0.20 x $10^{-2}$ |
| Caffeoyl shikimate       | 111 ± 22   | 2.05 ± 0.19 | 1.87 x $10^{-2}$ ± 0.03 x $10^{-2}$ | 30.00 ± 3.90 | 1.70 ± 0.03 | 5.65 x $10^{-2}$ ± 0.65 x $10^{-2}$ |
| Feruloyl shikimate       | 13.0 ± 0.9 | 6.54 x $10^{-2}$ ± 0.02 x $10^{-2}$ | 5.06 x $10^{-3}$ ± 0.41 x $10^{-3}$ | 0.85 ± 0.11 | 7.24 x $10^{-1}$ ± 0.80 x $10^{-1}$ | 0.86 ± 0.10 |
| Sinapoyl shikimate       | 618 ± 67   | 3.84 x $10^{-2}$ ± 0.14 x $10^{-2}$ | 6.21 x $10^{-5}$ ± 0.07 x $10^{-5}$ | 95.30 ± 8.90 | 1.16 x $10^{-1}$ ± 0.05 x $10^{-1}$ | 1.21 x $10^{-3}$ ± 0.15 x $10^{-3}$ |
