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***SHORT TITLE**

Lignin bioengineering in poplar through *CSE* silencing

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ARTICLE TITLE

Silencing CAFFEOYL SHIKIMATE ESTERASE affects lignification and improves saccharification

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ONE SENTENCE SUMMARY

Downregulation of *CSE* in poplar reduces lignin content, leading to a higher glucose release per plant upon saccharification.

LIST OF AUTHOR CONTRIBUTIONS

IC performed experiments, analyzed data and wrote the article with the contributions of other authors. MLSS performed most of the experiments, analyzed data and complemented the writing. LV, RVA, FCAF, AP, WV, JNJ, DP and JVD performed experiments and analyzed data. GG performed phenolic profiling and analyzed data. HK acquired and analyzed the NMR data. RV and JR analyzed data and complemented the writing. WB conceived the project, supervised and complemented the writing.

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Abstract

Caffeoyl shikimate esterase (CSE) was recently shown to play an essential role in lignin biosynthesis in *Arabidopsis thaliana* and later in *Medicago truncatula*. However, the general function of this enzyme was recently questioned by the apparent lack of CSE activity in lignifying tissues of different plant species. Here, we show that downregulation of *CSE* in hybrid poplar (*Populus tremula* × *P. alba*) resulted in up to 25% reduced lignin deposition, increased levels of *p*-hydroxyphenyl units in the lignin polymer, and a relatively higher cellulose content. The transgenic trees were morphologically indistinguishable from the WT. Ultrahigh-performance liquid chromatography-mass spectrometry (UHPLC-MS)-based phenolic profiling revealed a reduced abundance of several oligolignols containing guaiacyl and syringyl units and their corresponding hydroxycinnamaldehyde units, in agreement with the reduced flux towards coniferyl and sinapyl alcohol. These trees accumulated the CSE substrate caffeoyl shikimate along with other compounds belonging to the metabolic classes of benzenoids and hydroxycinnamates. Furthermore, the reduced lignin amount combined with the relative increase in cellulose content in the *CSE* downregulated lines resulted in up to 62% more glucose released per plant upon limited saccharification when no pretreatment was applied and by up to 86% and 91% when acid and alkaline pretreatments were used. Our results show that CSE is not only important for the lignification process in poplar but is also a

promising target for the development of improved lignocellulosic biomass crops for sugar platform biorefineries.

Keywords: *Populus* spp., lignin, caffeoyl shikimate esterase, phenolic profiling, saccharification

Introduction

Secondary cell walls account for the majority of plant lignocellulosic biomass, which constitutes an important renewable and sustainable feedstock for the production of fermentable sugars, biochemicals and biomaterials (Vanholme et al., 2013b; Marriott et al., 2016). Biorefining of plant biomass has attracted significant attention due to global climate change and the need for alternatives to fossil resources for fuels and materials. In the biorefinery, plant cell wall polysaccharides are depolymerized into simple monomeric sugars – a process called saccharification – that are subsequently fermented to ethanol or other compounds by optimized microorganisms (Zeng et al., 2014). However, the complex chemical composition and physical structure of plant cell walls hampers the efficient hydrolysis of lignocellulose, a fact known as plant biomass recalcitrance (Chen and Dixon, 2007; Cullis and Mansfield, 2010; Chundawat et al., 2011; Ding et al., 2012; Zhao et al., 2012a; Rinaldi et al., 2016). One of the major factors contributing to biomass recalcitrance is the presence of lignin, an aromatic polymer that provides strength and hydrophobicity to the cell wall. Lignin hinders the efficient enzymatic depolymerization of cellulose and hemicellulose into fermentable sugars by immobilizing the hydrolytic enzymes and physically limiting the access to their polysaccharide substrates (Chang and Holtzapple, 2000; Berlin et al., 2006; Nakagame et al., 2010; Guo et al., 2014). Although a number of pretreatments have been developed to remove lignin and consequently lower biomass recalcitrance, the pretreatment step is still a relatively expensive step in the conversion process (Zhao et al.,

2012b; Vanholme et al., 2013b). In this regard, lignin bioengineering (e.g., engineering plants that either accumulate less lignin or produce lignin polymers more amenable to chemical degradation) holds promise to tailor plants with reduced biomass recalcitrance (Chen and Dixon, 2007; Mansfield et al., 2012a; Eudes et al., 2014; Wilkerson et al., 2014; Mottiar et al., 2016). The rational engineering of lignin for the development of crops with improved bioconversion properties requires a fundamental knowledge of the phenolic metabolism, explaining the large interest in lignin research.

Lignin is a complex aromatic biopolymer mainly deposited in thickened secondary walls of specialized cell types where it provides mechanical reinforcement for the plant to stand upright and imperviousness to the cell wall, allowing long-distance water transport (Boerjan et al., 2003; Ralph et al., 2004; Bonawitz and Chapple, 2010). Lignin is derived from monomers biosynthesized in the phenylpropanoid pathway, whose primary precursor is the aromatic amino acid phenylalanine. Following the deamination of phenylalanine by the entry-point enzyme PHENYLALANINE AMMONIA LYASE (PAL), the resulting product *trans*-cinnamic acid undergoes a series of aromatic ring and propene tail modifications to yield three hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohol, differing in their degrees of methoxylation (Cesarino et al., 2012; Liu, 2012). Once incorporated into the polymer, these monolignols produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. In addition to the canonical monolignols, other less abundant units might be incorporated at varying levels, such as hydroxycinnamaldehydes, hydroxycinnamyl *p*-coumarates, and hydroxycinnamate esters (Ralph et al., 2004; Vanholme et al., 2012a). These monomers are first synthesized in the cytoplasm before being transported to the apoplast, where they are oxidized by peroxidases and/or laccases for subsequent polymerization through combinatorial radical-radical coupling (Ralph et al., 2004; Wang et al., 2013). Coupling between two electron-delocalized monolignol radicals and, more importantly,

between a monolignol radical and the growing polymer (radical) results in various linkage types, mainly β-aryl ether (β-*O*-4), phenylcoumaran (β-5) and resinol (β-β) bonding types (Freudenberg and Neish, 1968; Ralph et al., 2004; Morreel et al., 2010). Because lignin polymerization is combinatorial and not controlled by proteins, coupling reactions are thought be solely under chemical control, being affected by typical physical parameters such as pH, ionic strength and substrate supply, among others (Ralph et al., 2004; Ralph et al., 2008; Vanholme et al., 2008; Dima et al., 2015). Finally, lignification is not only developmentally regulated but also responds to specific environmental conditions, such as biotic and abiotic stresses (Moura et al., 2010; Barros et al., 2015).

The biochemical pathway leading to the production of the canonical monolignols was thought to be completely elucidated over a decade ago. When the gene encoding *P*-COUMARATE 3′-HYDROXYLASE (C3H) was finally identified in Arabidopsis and its enzymatic activity properly characterized, it was found that free *p*-coumaric acid or its CoA esters were not metabolized by the recombinant enzyme, whereas its 5-*O*-shikimate and 5-*O*-quinate esters were actively converted into their corresponding caffeate conjugates (Fig. 1) (Schoch et al., 2001). This observation suggested that the accepted version of the pathway at that time–was likely to be incorrect and would require the activity of a shikimate/quinate acyltransferase to produce the *p*-coumarate esters. Finally, in 2003, this acyltransferase was identified in tobacco and shown to catalyze the production of *p*-coumarate esters of both shikimic and quinic acids from *p*-coumaroyl-coenzyme A (CoA), and it was named *p*-HYDROXYCINNAMOYL-COA:QUINATE/SHIKIMATE *p*-HYDROXYCINNAMOYLTRANSFERASE (HCT) (Hoffmann et al., 2003). Moreover, based on *in vitro* assays using the recombinant enzyme and caffeoyl quinate (chlorogenic acid) as substrate, HCT was also suggested to catalyze a second reaction in the pathway, the conversion of caffeate esters that are products of C3H, into caffeoyl-CoA. No confirmation of

this proposed role was ever made by means of reverse genetics, but the so-called second/reverse reaction of HCT was widely accepted and thought to be conserved among plant taxa. Recently, it was demonstrated that an enzyme, named CAFFEOYL SHIKIMATE ESTERASE (CSE), is able to catalyze the conversion of caffeoyl shikimate into caffeate in *Arabidopsis thaliana* (Vanholme et al., 2013c). The combined activity of CSE with that of *p*-coumaroyl/caffeoyl-CoA ligase (4CL) producing caffeoyl-CoA would bypass the second/reverse reaction of HCT (Fig. 1). Genetic evidence for an active role of CSE in the lignification process in Arabidopsis was gained with a loss-of-function mutant showing reduced lignin content and an increased relative proportion of H units in the lignin polymer (Vanholme et al., 2013c). Because *CSE* orthologues were found in the genomes of other plant species (Vanholme et al., 2013c; Carocha et al., 2015; Ha et al., 2016), it was first suggested that this enzymatic step was conserved within the plant lineage. However, a subsequent paper indicated that many unrelated species lack a *bona fide CSE* homologue in their genomes, suggesting that CSE might not be essential for lignification in all plants (Ha et al., 2016). Apart from Arabidopsis, the unambiguous confirmation of a role for CSE in monolignol biosynthesis by means of reverse genetics was only shown in *Medicago truncatula*, for which *CSE* knockout resulted in strong negative effects on development, lower lignin levels, and preferential accumulation of H units in lignin (Ha et al., 2016).

A general role for CSE in lignin biosynthesis was recently questioned by a study reporting a predictive kinetic metabolic-flux model that provides a mathematical description of the monolignol biosynthetic pathway in aspen/poplar (*Populus trichocarpa*) based on *in vitro* kinetics and absolute quantification of monolignol biosynthetic pathway proteins in the xylem (Wang et al., 2014). Although the *P. trichocarpa* genome harbors two *bona fide CSE* homologues, no CSE activity was detected in protein extracts from poplar secondary differentiating xylem, suggesting that caffeoyl shikimate is converted to caffeoyl-CoA by two

isoforms of HCT in poplar (Wang et al., 2014). Moreover, CSE activity was also not detected in protein extracts of lignifying tissues of *Eucalyptus grandis*, switchgrass, and rice, suggesting that CSE is not involved in the shikimate shunt in these species (Wang et al., 2014). Hence, to further assess a possible role for CSE in lignification in poplar (*P. tremula* × *P. alba*), we carried out a comprehensive study based on crude protein enzymatic activity assays, gene expression analysis, and multiple-level phenotyping of transgenic lines downregulated in *CSE*. Moreover, we also evaluated the biotechnological potential of *CSE* by performing saccharification assays of wood samples derived from the *CSE*-downregulated lines.

Results

Poplar xylem protein extracts show CSE activity

The generality of a role for CSE in lignification was recently questioned based on the apparent lack of CSE activity in protein extracts of poplar xylem (Wang et al., 2014). Interestingly, with the exception of poplar, all the other plant species previously analyzed (i.e., *Arabidopsis thaliana*, *Medicago truncatula*, and switchgrass) showed a positive correlation between the presence of *bona fide CSE* homologues in their genome and CSE activity in protein extracts of their lignifying tissues (Vanholme et al., 2013c; Escamilla-Treviño et al., 2014; Wang et al., 2014; Ha et al., 2016). To further address this apparent dilemma, CSE activity was assayed by feeding crude protein extracts from scraped xylem tissue of wild-type (WT) *P. tremula* × *P. alba* plants with caffeoyl shikimate and analyzing the products by UHPLC-MS. Significant levels of caffeic acid were detected after 1 h of incubation, whereas an 8-fold increase in caffeic acid amount was detected after 25 h (Fig. 2A). Extremely low levels of caffeic acid were observed when incubations were performed with boiled xylem protein extracts, suggesting that the spontaneous hydrolysis of caffeoyl

shikimate into caffeic acid is negligible. These results support the presence of CSE activity in the poplar xylem.

The poplar genome harbors 2 bona fide CSE *homologues that are highly expressed in the xylem*

A previous phylogenetic analysis of the CSE protein family in a set of different plant species has shown that these proteins group into two classes: Class I, which potentially includes *bona fide* CSE proteins, and Class II, which includes CSE homologues with low sequence identity and are therefore considered to be CSE-like proteins (Ha et al., 2016). In the genome of *P. trichocarpa*, two *bona fide CSE* homologues are found, *PtrCSE1* (Potri.001G175000) and *PtrCSE2* (Potri.003G059200), sharing 91% identity in amino acid sequence to each other and around 80% to AtCSE of *Arabidopsis thaliana*. Because not all family members for a given step of the monolignol biosynthetic pathway are necessarily involved in lignification, tissue-specific expression analysis was performed to evaluate whether the expression pattern of each *CSE* homologue in poplar correlates with developmental lignification. For such a purpose, transcript abundance of both genes was determined in four *P. tremula* × *alba* tissues differing in their degree of lignification: bark, mature leaves, axillary buds, and developing xylem. Preferential expression in the xylem, a tissue that undergoes early and strong lignification, is expected for a gene involved in developmental lignification. Accordingly, both *Ptx*a*CSE* genes were strongly and preferentially expressed in the xylem, whereas much lower transcript levels were detected in bark, leaves and especially in axillary buds (Fig. 2B). These results suggest a potential, and possibly redundant, role for the two *CSE* paralogues in poplar developmental lignification.

Downregulation of CSE *in poplar did not drastically affect plant development*

In order to gain genetic evidence for the involvement of CSE in the lignification process in poplar, constitutive downregulation of both *CSE* genes was targeted by cloning a 120 bp-fragment of *PtxaCSE2* (corresponding to Potri.003G059200) into the pK7GWIWG2II gene-silencing vector to produce *CaMV 35S*-driven *hpCSE* transgenic *P. tremula* × *P. alba* lines. The corresponding fragment in *PtxaCSE1* shared 90% identity with the cloned fragment of *PtxaCSE2*. Independent lines (137) were grown for 45 days in the greenhouse before screening. Of note, none of the transgenic lines exhibited the typical red xylem phenotype that is visible upon debarking the stems of poplars down-regulated for *CINNAMOYL-CoA REDUCTASE* (*CCR*), *CAFFEIC ACID O-METHYLTRANSFERASE* (*COMT*), or *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*) (Baucher et al., 1996; Tsai et al., 1998; Leplé et al., 2007). The *hpCSE* lines were screened for the accumulation of caffeoyl shikimate, the substrate for CSE, via UHPLC-MS after extraction of methanol-soluble phenolics from the stem. Among all analyzed lines, one particular line showed a 36-fold increase in the caffeoyl shikimate content relative to the WT, whereas the second highest amount of caffeoyl shikimate was 20-fold higher than that of the WT. In the other lines, the caffeoyl shikimate content decreased to as low as WT levels, suggesting lower silencing efficiency or complete absence of silencing. A significant increase in H-unit content and a reduction in total lignin amount are two other hallmarks of *CSE* silencing in Arabidopsis and Medicago. Relative levels of H units were therefore determined via thioacidolysis, and lignin amount was determined via acetyl bromide lignin measurements in the top 20 lines with the highest accumulation of caffeoyl shikimate. Although the relative H-unit content in lignin and the lignin amount in the transgenic lines did not differ drastically from those of the WT, these data were taken into account to select the two lines with the highest accumulation of both caffeoyl shikimate and H units and with a reduction in lignin (Supplemental Table S1). These

selected lines were named *hpCSE#1* and *hpCSE#2*, and were subjected to further multiple-level phenotyping.

Quantitative RT-PCR was used to determine the residual transcript levels of *Ptx**aCSE1* and *Ptx**aCSE2* in the developing xylem of both WT and *hpCSE* lines (Fig. 3A). This analysis showed that the two *CSE* paralogues were differentially downregulated in both *hpCSE* lines. For *Ptx**aCSE2*, 21% and 15% of residual transcript levels were observed for *hpCSE#1* and *hpCSE#2* compared to WT, whereas a much weaker down-regulation was observed for *Ptx**aCSE1,* for which 49% and 35% of residual transcript levels were found in those lines (Fig. 3A). To evaluate whether downregulation of *CSE* caused any effect on plant growth and development, the *hpCSE* lines were grown alongside the WT in the greenhouse for a period of 6 months. Plant height was followed weekly and, by the end of the growth period, the trees were harvested and biomass parameters determined. The *hpCSE* lines were morphologically indistinguishable from the WT (Fig. 3B), and no differences in either fresh or dry weight were observed after six months of growth (Fig. 3C). Stem height was similar for both lines compared to the WT during the whole growth period, but after 6 months the *hpCSE#2* plants were slightly shorter (4%) than the controls (Fig. 3D). These results suggest that these *CSE*-downregulated poplars develop normally with no severe effects on biomass accumulation.

Downregulation of* CSE *affects lignin content and structure in poplar

Silencing of a gene encoding an enzyme central to the lignin biosynthetic pathway is expected to affect lignin content and/or composition. To evaluate the impact of *CSE* downregulation on lignin deposition, crude cell wall residue (CWR) was prepared from the stem of 6-month-old *hpCSE* and WT poplars and the lignin content was determined by the Klason method. Both *hpCSE* lines showed significant reduction in (acid-insoluble) Klason lignin content, with a decrease of 19% and 25% for *hpCSE#1* and *hpCSE#2* compared to that

of the WT (Fig. 4A; Supplemental Table S2). These reductions were accompanied by a mild increase in cellulose content of 8% and 13% in *hpCSE#1* and *hpCSE#2* (Fig. 4B; Supplemental Table S2). Next, lignin structural changes were first evaluated by thioacidolysis, an analytical method that quantifies only lignin units linked by labile β-O-4 bonds. Although the absolute amount of H units remained low, the relative proportion of H-derived monomers increased by 51% in *hpCSE#1* and 113% in *hpCSE#2* (Fig. 4C; Supplemental Table S2). The relative frequencies of G- and S-derived monomers showed only mild shifts compared to control plants. The relative amount of thioacidolysis-released G units increased by 7% and 5%, whereas the relative amount of thioacidolysis-released S units decreased by 4% and 3% in *hpCSE#1* and *hpCSE#2* (Fig. 4D; Supplemental Table S2). These changes led to a mild but significant decrease in the S/G ratio from 2.06 in the WT to 1.85 and 1.90 in *hpCSE#1* and *hpCSE#2* (Fig. 4E; Supplemental Table S2). The degree of lignin condensation is inversely correlated with the thioacidolysis yield (i.e., the sum of released H, G, and S monomers). When expressed on a Klason lignin content basis, the thioacidolysis yield of the *hpCSE* lines was not significantly different from that of the WT (Fig. 4F; Supplemental Table S2).

In order to gain further insight into the structural changes caused by *CSE* downregulation, 2D-NMR (Nuclear Magnetic Resonance) was performed on enzyme lignins from the stems of the WT and the *hpCSE* lines. By analyzing the aromatic region of the two-dimensional ^{1}H–^{13}C correlation heteronuclear single-quantum coherence (HSQC) spectra, it is possible to visualize differences in lignin monomeric composition irrespective of the interunit linkage distribution, in contrast to thioacidolysis in which only units linked by ether bonds are determined. In general, the NMR data largely corroborated the structural changes found with thioacidolysis, with both lines showing similar spectra. Compared to the WT, the proportion of H units was increased by 100% and 110% in *hpCSE#1* and *hpCSE#2* (Fig. 5A). The

328 relative fraction of G units was increased by 6.8% and 8.4% whereas the relative frequency of
329 S units was decreased by 5.2% and 6.3% in each of these lines. Accordingly, the S/G ratio
330 decreased significantly from 1.62 in the WT to 1.43 and 1.4 in *hpCSE#1* and *hpCSE#2* (Fig.
331 5). In addition, the interunit linkage types can be deduced from the oxygenated aliphatic
332 region of the HSQC spectrum (Figure 5B). Minor shifts in the relative proportion of the major
333 unit types were observed, with a slight increase in β-aryl ether (β-O-4) units and a slight
334 decrease in resinol (β-β) units in both *hpCSE* lines (Fig. 5B), suggesting a lower proportion of
335 carbon-carbon bonds in their lignin polymers. Altogether, these results show that
336 downregulation of *CSE* in poplar not only leads to reduced lignin levels but also affects lignin
337 composition and structure, albeit mildly.
338
339 ***The phenolic profile is altered upon* CSE *downregulation in poplar***
340 A shift in the phenolic metabolism is expected upon blocking a biosynthetic step central
341 to the lignin pathway. In order to assess the consequences of *CSE* downregulation on phenolic
342 metabolism, methanol-soluble metabolites were extracted from the developing xylem of 3.5-
343 month-old *hpCSE* and WT plants and analyzed via UHPLC-mass spectrometry (MS). This
344 procedure enables the detection and relative quantification of different classes of aromatic
345 compounds, including intermediates and derivatives from the phenylpropanoid pathway, such
346 as flavonoids, benzenoids, cinnamates, and products from monolignol coupling (oligolignols).
347 A total of 2,443 peaks could be detected, aligned, and integrated in the chromatograms of
348 WT, *hpCSE#1* and *hpCSE#2* samples. Only peaks present in all replicates of at least one
349 genotype and that were on average above a 500 counts cutoff in at least one genotype, were
350 selected for statistical analysis. Some 618 peaks met these criteria. Principal component
351 analysis (PCA) showed that the phenolic profile of the *hpCSE* lines clearly separated from
352 that of the WT, indicating major metabolic differences (Supplemental Fig. S1). To further

353 select the metabolites for which the abundance was most affected by the downregulation of
354 *CSE*, filters were applied based on a significance level of 0.01 and a fold change of at least 2
355 between the *hpCSE* and the WT samples. Accordingly, the 68 peaks with a significantly
356 higher abundance in both *hpCSE* lines could be assigned to 42 compounds, of which 26 could
357 be structurally characterized (Fig. 1; Table I). Several of these compounds were only
358 observed in the transgenic lines, being below the detection limit in WT samples. The
359 identified compounds belonged to the metabolic classes of benzenoids and
360 hydroxycinnamates, of which caffeate-, ferulate- and sinapate-derived compounds were most
361 abundant. As expected, one of the top-accumulating compounds was the CSE substrate
362 caffeoyl shikimate (two free and two hexosylated forms), which is consistent with a reduced
363 *CSE* expression. In addition, *p*-coumaroyl shikimate, caffeoyl quinate, feruloyl shikimate,
364 feruloyl quinate and sinapoyl shikimate also accumulated in the *hpCSE* lines. Among these 26
365 identified compounds with higher abundance in the *hpCSE* lines, a total of 12 metabolites
366 were hexosylated, presumably to reduce their toxicity and allow for their sequestration in the
367 vacuole (Dima et al., 2015).

368 The 26 peaks with significantly lower abundance (average relative abundance < 0.5) in
369 the *hpCSE* lines compared to the WT could be assigned to 26 compounds, of which 14 could
370 be structurally characterized (Fig. 1; Table II). Interestingly, with the exception of
371 hexosylated protocatechuic acid, all the metabolites with reduced abundance were
372 oligolignols containing G and S units or their corresponding hydroxycinnamaldehyde analogs,
373 in agreement with the reduced lignin deposition upon *CSE* downregulation. Altogether, the
374 metabolic alterations observed in the xylem of *CSE*-downregulated poplars (e.g.,
375 accumulation of caffeoyl shikimate and reduced amounts of oligolignols) suggest that the
376 function of CSE in the lignin pathway is conserved in poplar.
377

CSE *downregulation improves saccharification efficiency of poplar stems*

Previously, we have shown that *CSE* loss-of-function in *Arabidopsis thaliana* resulted in a fourfold increase in cellulose-to-glucose conversion of senesced stems when compared to the WT (Vanholme et al., 2013c), one of the highest improvements in saccharification efficiency without biomass pretreatment ever reported. In order to evaluate whether the cell wall changes caused by *CSE* downregulation also lead to improved saccharification efficiency in poplar, cellulose-to-glucose conversion of the wood from the *hpCSE* lines was compared in limited-saccharification assays to that of the WT. Saccharification assays were performed with debarked stems without and with acid (1 M HCl, 80 °C, 2 h) or alkaline (62.5 mM NaOH, 90 °C, 3 h) pretreatment, with glucose release measured after 3, 6, 24, 30 and 48 h. The cellulose-to-glucose conversion was calculated based on the amount of glucose released upon saccharification and the cellulose content measured for each sample (Fig. 6; Supplemental Table S3). When no pretreatment was applied, the cellulose-to-glucose conversion of line *hpCSE#1* was significantly higher compared to that of the WT already after 6 h of saccharification, whereas no significant differences were observed for *hpCSE#2* (Fig. 6A). After 48 h, the cellulose-to-glucose conversion from the stems of line *hpCSE#1* was 31% higher than that of the WT (Fig. 6A; Supplemental Table S3). Both lines performed better than the WT in saccharification assays when the pretreatments were performed, and the differences were significant at all analyzed time-points. The cellulose-to-glucose conversion after 48 h was increased by 48% and 46% in *hpCSE#1* and *hpCSE#2* lines when an acid pretreatment was applied (Fig. 6C; Supplemental Table S3), whereas alkaline pretreatment resulted in an increase of 51% and 40% compared to the WT (Fig. 6E; Supplemental Table S3).

In Arabidopsis, the inflorescence stems of the *cse2* null mutant are 37% smaller and 42% lighter at senescence than those of the WT, revealing a substantial yield penalty upon *CSE*

loss-of-function. Because downregulation of *CSE* did not affect biomass accumulation in poplar, and because the *hpCSE* lines have slightly more cellulose, we anticipated that the saccharification yield on a plant basis would be substantially increased in the *hpCSE* lines. Accordingly, we compared the total glucose release per plant among all genotypes with and without pretreatment. Similarly to the cellulose-to-glucose conversion results, only the *hpCSE#1* line showed increased glucose release on a plant basis without pretreatment, releasing 62% more than the WT (Fig. 6B). Additionally, *hpCSE#1* and *hpCSE#2* lines released 86% and 57% more glucose than the WT on a plant basis when acid pretreatment was applied (Fig. 6D), whereas the improvements were of 91% and 50% with alkaline pretreatment (Fig. 6F). These results show that recalcitrance is reduced and glucose release is improved in poplar stems downregulated for *CSE*.

Discussion

CSE is important for lignification in poplar

The identification of CSE as an enzyme central to the lignin biosynthetic pathway in Arabidopsis led to a major question on whether this catalytic step is conserved across plant lineages. The fact that many unrelated plant species lack a *bona fide CSE* homologue in their genomes suggests that CSE might not be essential for lignification in all plants. Interestingly, from the plant species analyzed to date, the presence of *bona fide CSE* homologues in the genome was positively correlated with the detection of CSE activity in protein extracts prepared from lignifying tissues. Weak esterase activity was detected in protein preparations from stems of *Brachypodium distachyon* and maize, whose genomes do not possess a *bona fide CSE* gene, whereas high CSE activity was found in Arabidopsis and switchgrass that harbor *CSE* homologues (Vanholme et al., 2013c; Ha et al., 2016). The exception was *P. trichocarpa,* for which no CSE enzymatic activity could be detected in extracts prepared from

developing secondary xylem (Wang et al., 2014), even though its genome possesses two *bona fide CSE* homologues. These results led those authors to conclude that there was no direct evidence for a role for CSE in lignification in poplar and that caffeoyl shikimate is likely converted to caffeoyl-CoA by two xylem-specific HCT isoforms (Wang et al., 2014). However, the apparent lack of CSE activity in protein extracts from *P. trichocarpa* xylem is difficult to reconcile with the recent observation that both recombinant CSEs from *Populus deltoides*, which share 98% amino acid identity with their orthologues from *P. trichocarpa*, show high esterase activity *in vitro* (Ha et al., 2016). In addition, CSE activity in lignifying tissues was not detected in switchgrass, whereas a previous study had already shown that protein extracts prepared from switchgrass stems produce caffeic acid, instead of caffeoyl-CoA, when incubated with caffeoyl shikimate and CoA (Escamilla-Treviño et al., 2014). Subsequent work from the same group confirmed high CSE activity in crude extracts from switchgrass stems (Ha et al., 2016). Here, we showed not only that both poplar *CSE* homologues are preferentially expressed in developing xylem but also that protein preparations from this tissue show CSE activity, providing supporting evidence for a role for CSE in lignification in poplar.

The reactions of CSE need to be augmented by an additional 4CL step to regenerate the activated caffeoyl-CoA from the CSE product, caffeic acid. As an additional molecule of ATP is required, in contrast to the transesterification reaction catalyzed by HCT, the involvement of CSE in monolignol biosynthesis might seem counterintuitive. Nevertheless, this second "reverse" reaction of HCT has not yet been supported by genetic evidence, because *HCT* loss-of-function also affects the conversion of *p*-coumaroyl-CoA to *p*-coumaroyl-shikimate by HCT upstream of the pathway. In addition, kinetic analyses of recombinant enzymes have shown that, although poplar xylem-specific HCT isoforms can convert caffeoyl shikimate into caffeoyl-CoA, the catalytic efficiency of this reaction is 20-fold lower than that of the

453 "forward" acyltransferase HCT reaction, i.e., the formation of caffeoyl shikimate from
454 caffeoyl-CoA and shikimate (Wang et al., 2014). Similar results were found with recombinant
455 HCT enzymes from switchgrass, which show preferences of around 15-fold for the forward
456 compared to the reverse reaction (Escamilla-Treviño et al., 2014). When Hoffmann and
457 collaborators (Hoffmann et al., 2003) first described and characterized HCT in tobacco, the
458 conversion of caffeoyl-CoA to caffeoyl shikimate was also shown to be the most efficient
459 reaction performed by the recombinant enzyme. Therefore, it seems counterintuitive that the
460 reverse transesterification reaction of HCT has an important role *in vivo*, as the production of
461 caffeoyl shikimate from caffeoyl-CoA would be highly favored. These observations suggest
462 that caffeoyl-CoA is produced from caffeoyl shikimate by a two-step reaction catalyzed by
463 CSE and 4CL instead of the single HCT-dependent transesterification reaction, at least in
464 plant species harboring a *bona fide* CSE homologue.

465 Our data clearly demonstrate that *CSE* plays an important role in lignification in poplar.
466 The *hpCSE* lines deposited up to 25% less lignin than control plants and the relative level of
467 H units was slightly increased, which is in line with a function for CSE after the branch where
468 the biosynthesis of H units diverges from that of G and S units. These lignin changes were not
469 associated with any drastic effect on plant growth and development. This mild phenotype is
470 likely due to residual expression of both *PtxaCSE* paralogues observed in the *hpCSE* lines.
471 Accordingly, although the Arabidopsis loss-of-function *cse-2* mutant exhibited a substantial
472 developmental arrest, a 36% reduction in lignin amount and a 30-fold increase in H units
473 content, the knockdown *cse-1* mutant, with a T-DNA insertion in the promoter and 6.3% of
474 residual *CSE* expression, did not show any developmental abnormality, with total lignin
475 reduced by 17% and only a 4-fold increase in H-unit content (Vanholme et al., 2013c).
476 Interestingly, the loss-of-function phenotype of *Medicago truncatula cse* mutants is even
477 more severe than that of Arabidopsis, with lignin levels reduced by over 80% and H-unit

content increased by around 50-fold (Ha et al., 2016). Given that both are knock-out mutants, the contrasting phenotypes are probably due to differential catalytic properties of HCT in converting caffeoyl shikimate into caffeoyl-CoA in these two species. Although the kinetics of this reaction are unknown for Medicago HCT, Arabidopsis HCT has been shown to catalyze this conversion *in vitro*, albeit with a much lower affinity for caffeoyl shikimate than the Arabidopsis CSE (Vanholme et al., 2013c). Therefore, a lower catalytic efficiency of Medicago HCT might result in a lower flux towards G and S units from the accumulated caffeoyl shikimate pool caused by CSE loss-of-function, resulting in a more drastic phenotype when compared to Arabidopsis. In poplar, the efficiency of recombinant HCT1 and HCT6 in converting caffeoyl shikimate into caffeoyl-CoA was shown to be very low (Wang et al., 2014), which explains why even intermediate levels of *CSE* down-regulation in this species still result in the accumulation of caffeoyl shikimate and a significantly lower lignin content (this work). Altogether, these data suggest that only strong reductions in *CSE* mRNA levels cause developmental abnormalities, whereas intermediate suppression does not necessarily affect plant yield but could still lead to alternations in lignin deposition that might be beneficial for biomass processing. These results highlight the biotechnological potential of *CSE* manipulation for the development of bioenergy feedstock with improved extractability.

CSE *downregulation in poplar results in a shift in phenolic metabolism*

Lignin loss-of-function mutants and down-regulated plants often accumulate the substrate of the corresponding deficient enzyme along with other pathway intermediates or derivatives, which results in conspicuous shifts in their phenolic profiles (Morreel et al., 2004; Rohde et al., 2004; Coleman et al., 2008; Vanholme et al., 2012b; Vanholme et al., 2013c). Therefore, phenolic profiling is a powerful tool to gain insights into the potential *in vivo* substrate of a given enzyme, as well as to evaluate how the carbon flux through the phenylpropanoid

503 pathway is redirected in response to a perturbation (i.e., upon blocking a particular step in the
504 pathway). The majority of the compounds with significantly reduced abundance in *hpCSE*
505 poplars were oligolignols mainly composed of G and/or S units and their corresponding
506 cinnamaldehydes, which is consistent with the reduced flux towards the main monolignols if
507 CSE is a pathway enzyme utilized in poplar lignification. Interestingly, upon *CSE*
508 downregulation, the carbon flux was not massively redirected to the biosynthesis of H units,
509 and no differences in the relative amount of H-containing oligolignols were observed in the
510 metabolome of *hpCSE* plants: the relative amount of H units in the lignin polymer doubled
511 but remained very low overall. This is in contrast with the results found in Arabidopsis, in
512 which three H-containing oligolignols accumulated to significant levels in both *cse-1* and *cse-*
513 *2* mutants (Vanholme et al., 2013c). This discrepancy might again be explained by the higher
514 residual *CSE* expression in comparison with the corresponding Arabidopsis mutants, but other
515 explanations are also possible. For example, the metabolite profiling revealed the
516 accumulation of *p*-coumaroyl shikimate and caffeoyl shikimate in the *hpCSE* lines, and both
517 compounds have been described to inhibit poplar 4CL activities (Lin et al., 2015). Such
518 inhibition could cause the observed accumulation of ferulic acid and derivatives of caffeic
519 acid (e.g., caffeic acid glucosides) in the *hpCSE* lines, but could also be the reason for a lower
520 flux towards H units in the *hpCSE* lines. Furthermore, the differences between the redirection
521 of the carbon flux in Arabidopsis *cse-2* and poplar *hpCSE* might be due to intrinsic
522 differences in the phenolic metabolism or different regulatory mechanisms to compensate for
523 the lower amount of lignin. Accordingly, it has been shown that C4H physically interacts with
524 C3H in poplar xylem and that this protein complex is able to catalyze the 3-hydroxylation of
525 *p*-coumaric acid into caffeic acid, providing an alternate pathway to the production of G and S
526 units that bypasses the shikimate shunt in poplar (Chen et al., 2011). Such a pathway might

527 also explain the lower redirection of carbon skeletons towards H units in the *hpCSE* lines as
528 compared to Arabidopsis.

529 The observed accumulation of *p*-coumaroyl shikimate in the *hpCSE* lines might logically
530 be explained by product inhibition of C3H activity. In addition to *p*-coumaroyl shikimate,
531 feruloyl shikimate and sinapoyl shikimate also accumulated in the *hpCSE* lines. It is unlikely
532 that feruloyl shikimate is made via 4CL and HCT activities from their corresponding acids,
533 because the intermediate feruloyl-CoA is rather expected to be used by CCR and thereby
534 shunted into the monolignol-specific pathway. More likely, feruloyl shikimate and sinapoyl
535 shikimate are synthesized via hydroxylation and methylation of the accumulating caffeoyl
536 shikimate. If confirmed, these reactions add another layer in the complex grid of
537 phenylpropanoid derivatizations. Besides hydroxylation and methylation at the free-acid
538 level, the CoA-thioester level, and the aldehyde level, these reactions now also appear to
539 occur at the shikimate ester level. However, the relevance of this route in WT poplars remains
540 to be further elucidated. Notably, the accumulation of feruloyl shikimate and sinapoyl
541 shikimate in the *hpCSE* lines suggests that these compounds are also *in vivo* CSE substrates.

542 Eight phenylpropanoid derivatives were detected in their hexosylated forms. The
543 accumulation of these compounds hints to a detoxification route for potentially harmful
544 pathway intermediates (phenolic acids) that accumulate upon *CSE* perturbation (Vanholme et
545 al., 2010; Sundin et al., 2014; Dima et al., 2015).

546

547 ***Total glucose release per plant is remarkably increased upon* CSE *downregulation in***
548 ***poplar***

549 Despite its essential role in plant growth and development, lignin is widely recognized as
550 a major limiting factor that hinders the efficient processing of plant biomass into fermentable
551 sugars (Zhao et al., 2012a; Cesarino et al., 2016). The recalcitrant nature of this phenolic

552 polymer has stimulated biotechnological approaches to improve polysaccharide extraction
553 and processing in bioenergy crops via genetic modification of lignin biosynthesis, either by
554 reducing lignin amounts or manipulating lignin composition (Chen and Dixon, 2007;
555 Vanholme et al., 2012a; Vanholme et al., 2013b; Van Acker et al., 2014; Mottiar et al., 2016;
556 Sibout et al., 2016). Accordingly, a fourfold increase in saccharification yield was observed
557 for the Arabidopsis *cse-2* loss-of-function mutant, which shows a 36% reduction in lignin
558 content (Vanholme et al., 2013c). However, the improvement in glucose release was
559 accompanied by a substantial biomass yield penalty, with a reduction of 37% and 42% in the
560 inflorescence height and weight at senescence, respectively. Indeed, drastic reductions in the
561 biosynthesis of lignin are typically followed by deleterious effects on plant development,
562 which might significantly outweigh the gains in fermentable sugar yield (Gallego-Giraldo et
563 al., 2011; Bonawitz and Chapple, 2013; Yang et al., 2013; Van Acker et al., 2014). Although
564 the underlying molecular mechanisms that result in developmental arrest in lignin deficient
565 plants are largely unknown, it is likely that they depend on the particular step of the pathway
566 that is blocked (Bonawitz and Chapple, 2013). Recently, we found evidence that vasculature
567 collapse underlies the yield penalty found in the Arabidopsis *cse-2* mutant, as restoring *CSE*
568 expression specifically in vessels also restored vasculature morphology and final stem weight,
569 leading to an even higher total glucose release per plant (Vargas et al., 2016). In poplar, *CSE*
570 downregulation resulted in higher amounts of glucose released upon saccharification with and
571 without pretreatment, which is likely associated with reduced lignin amounts combined with
572 increased relative cellulose contents. Similar results (i.e., an inverse relation between lignin
573 and cellulose) have also been found when other biosynthetic genes, such as *CINNAMATE 4-*
574 *HYDROXYLASE* (*C4H*), *4CL*, *C3H*, *CCR* and *COMT* (Jouanin et al., 2000; Leplé et al., 2007;
575 Coleman et al., 2008; Bjurhager et al., 2010; Voelker et al., 2010), were downregulated in
576 poplar. However, increases in cellulose content were not found in a set of Arabidopsis

mutants defective in lignin biosynthesis, in which lower deposition of lignin was compensated for by a relative increase in hemicellulose content (Van Acker et al., 2013). Also in the case of the Arabidopsis *cse-2* mutant, the lower lignin content was associated with a decrease in cellulose content (Vanholme et al., 2013c), i.e., the opposite to what we found for transgenic poplars downregulated in *CSE*. Of particular note, the improved polysaccharide extractability of *hpCSE* plants was not accompanied by severe negative effects on plant yield when grown for 6 months under greenhouse conditions. Consequently, in limited-saccharification experiments, the total glucose released per plant was increased by up to 62% when no pretreatment was applied and by up to 86% and 91% when acid and alkaline pretreatments were used, compared to the controls. Overall, the observation that *CSE* silencing results in both significantly improved sugar yield without drastic effects in biomass production, at least under greenhouse conditions, opens the possibility for the exploitation of this gene as a target for genetic engineering of lignin biosynthesis in bioenergy crops.

The improvements in saccharification yield observed in the *hpCSE* lines were not as remarkable as those observed for the Arabidopsis *cse-2* mutant, which were among the highest saccharification efficiencies ever reported (Van Acker et al., 2013; Vanholme et al., 2013c). However, the *cse-2* plants exhibited not only more drastic reductions in lignin levels but the lignin polymer was also massively enriched in H units, which might help explain their higher performance in saccharification assays. Because *p*-coumaryl alcohol is devoid of methoxy groups that, as in *p*-coumarate, help stabilize the free radical, it favors radical transfer reactions (Hatfield et al., 2008; Ralph, 2010). Consequently, *p*-coumaryl alcohol frequently occurs as free-phenolic endgroups when incorporated into lignin (Lapierre and Monties, 1988; Russell et al., 2006; Mottiar et al., 2016). Therefore, H-rich lignins have a lower degree of polymerization (Lapierre and Rolando, 2009; Ziebell et al., 2010). Shorter lignin chains would be presumably less cross-linked, have potentially fewer connections to

602 the cell wall ultra-structure and have a greater solubility (Ziebell et al., 2010). Therefore,
603 increasing the incorporation of H units may increase lignin extractability and improve
604 biomass digestibility. Because the relative amount of H units in the *hpCSE* lines was only
605 slightly increased, it is unlikely that this parameter has contributed to the improved
606 saccharification efficiency found in these trees. Nevertheless, boosting H-unit incorporation in
607 order to produce shorter lignin polymers remains a promising strategy to increase cellulose-
608 to-glucose conversion in dedicated crops (Vanholme et al., 2012a; Bonawitz et al., 2014;
609 Mottiar et al., 2016). In this regard, the generation of *CSE* knockouts in poplar via
610 CRISPR/Cas9 would likely produce plants with lower lignin amounts and much higher
611 incorporation of H units into lignin.
612
613 ***CSE activity might not be essential for lignification in all plant species***
614 The recent characterization of CSE in *Medicago truncatula* (Ha et al., 2016) and poplar
615 (this work) is shedding some light onto the generality of CSE function in monolignol
616 biosynthesis. Reverse genetics has unambiguously confirmed to date an essential role for CSE
617 in lignification only in plants harboring *bona fide* class I homologues in their genomes. How
618 caffeate esters are channeled into the production of G and S lignin units in plants that do not
619 possess genes encoding class I CSE enzymes, which is the case for most grasses, therefore
620 remains an open question. One possible explanation is that, in those plants, HCT has a higher
621 efficiency in converting caffeoyl shikimate into caffeoyl-CoA than its counterparts in CSE-
622 harboring species. Unfortunately, limited data on kinetic parameters for HCT are available,
623 especially for those species shown to lack a class I CSE. Hitherto, the only study
624 characterizing the reaction mechanism of HCT in a plant that lacks a *bona fide* CSE focused
625 exclusively on the forward reaction, with no information on the reverse transesterification
626 reaction (Walker et al., 2013). Alternatively, the phylogenetically distant class II CSE

enzymes might be catalytically able to fulfill this enzymatic step. Interestingly, switchgrass seems to employ an HCT-like enzyme for the biosynthesis of chlorogenic acids (CGAs) (Escamilla-Treviño et al., 2014). In plants that accumulate CGAs, such as tobacco and coffee, the enzyme HYDROXYCINNAMOYL-COA:QUINATE HYDROXYCINNAMOYLTRANSFERASE (HQT) is normally responsible for the formation of CGA from caffeoyl-CoA and quinic acid, but close HQT orthologues were not found in switchgrass. In contrast, a HCT-like enzyme phylogenetically distant from HCTs or HQTs was discovered from transcriptome analysis of lignifying switchgrass cell suspensions and elongating internodes (Shen et al., 2013) and shown to exhibit HQT activity, preferring quinate instead of shikimate as its acyl acceptor (Escamilla-Treviño et al., 2014). A similar scenario may occur for class II CSE in lignin biosynthesis. In support of this hypothesis, it has recently been shown that the class II CSEs from maize and sorghum are targeted by the lignin repressors MYB31 and MYB42 in chromatin immunoprecipitation (ChIP) experiments (Agarwal et al., 2016), suggesting a role for these genes in lignification. Conversely, the only Arabidopsis class II *CSE* gene was recently characterized and shown to encode a monoacylglycerol lipase that catalyzes the last step of triacylglycerol breakdown, the hydrolysis of monoacylglycerol to fatty acid and glycerol (Kim et al., 2016). It is therefore likely that the lack of class I CSEs contributed to the process of sub- or neofunctionalization of class II CSEs, allowing these enzymes to be part of the lignin toolbox in certain plants only. Further genetic analysis is necessary to determine how the shikimate shunt works in plants lacking class I *CSE* genes.

Material and Methods

Plant material and generation of transgenic hpCSE lines

651 The first 120 bp of the *PtxaCSE2* coding sequence from *P. tremula* × *P. alba* (INRA 717-
652 1B4), corresponding to *P. trichocarpa* Potri.003G059200, was PCR-amplified from cDNA
653 obtained from *P. tremula* × *P. alba* stems using iProof™ High Fidelity DNA Polymerase and
654 cloned into the pDONR221 vector using BP Clonase (Invitrogen). Sequence identity was
655 confirmed by sequencing. The fragment was subsequently introduced via LR Clonase
656 (Invitrogen) into the pK7GWIWG2II vector suited for *CaMV 35S*-driven intron-spliced
657 hairpin RNA-mediated gene silencing. Restriction analysis with XbaI (New England Biolabs)
658 was performed to confirm that intron flip, which may reduce silencing efficiency, did not
659 occur. The recombinant vector was transferred into *Agrobacterium tumefaciens* strain C58C1
660 PMP90 by electroporation. *Agrobacterium*-mediated transformation of *P. tremula* × *P. alba*
661 was performed according to Leplé et al. (1992).
662 In total, 137 transgenic lines and their corresponding WT were individually transferred to
663 soil in pots of 5.5 cm diameter, placed in a tray filled with water and covered with a cage liner
664 (Tecniplast APET disposable cage liner for cage body 1291H) for acclimatization. After 2
665 weeks, one side of the cage liner was lifted and kept accordingly for 1 day, after which the
666 other side was also lifted. The next day, the acclimatized plants were transferred to bigger
667 pots (24 cm diameter) and grown for 45 days in the greenhouse. A 5-cm debarked stem
668 fragment was collected at the base of each plant for the first screening (relative increase of
669 caffeoyl shikimate). The debarked stem fragment was scraped and the scrapings subsequently
670 extracted with 1 mL methanol at 70 °C for 15 min under 1000 rpm shaking. After
671 centrifugation, 800 µL of the liquid phase was dried in a SpeedVac and the resulting pellet
672 was resuspended in a mixture of water:cyclohexane (100 µL: 100 µL). The tubes were
673 vortexed and centrifuged at 20,000 x g for 10 min, after which a 15 µL aliquot of the lower
674 water phase was analyzed via UHPLC-MS. Mass spectrometric analysis and caffeoyl
675 shikimate relative quantification were performed as previously described (Vanholme et al.,

2013c). The top 20 lines with highest accumulation of caffeoyl shikimate were chosen for the second screening, which was performed with a debarked 5-cm stem fragment harvested immediately above the fragment used for the first screening. The stem fragment was dried at room temperature for 2 weeks, ground in 2-mL Eppendorf tubes using a Retsch MM300 mill (20 Hz, 4-mm bead) and subjected to sequential extraction to obtain the cell wall residue (CWR) (Van Acker et al., 2013). Lignin content was determined using the acetyl bromide method adapted to small samples (Johnson et al., 1961; Van Acker et al., 2013), whereas thioacidolysis (Robinson and Mansfield, 2009) was performed to determine the relative level of H units in each sample. The best two lines with highest accumulation of caffeoyl shikimate, and the lowest lignin content with the highest level of H units were chosen for further multiple-level phenotyping.

Two *CSE*-downregulated lines (*hpCSE#1* and *hpCSE#2*) were selected and, together with the corresponding WT, simultaneously propagated and grown randomly in the greenhouse to obtain enough biological replicates of each line. For the analysis of biomass parameters, WT (n = 25), *hpCSE#1* (n = 23) and *hpCSE#2* (n = 23) plants were grown under 16 h-light / 8 h-dark photoperiod, at ± 21 °C in 10-L pots filled with a Saniflor® commercial soil (Van Israel). The height of the trees was measured weekly for a period of six months. At the end of the growth period, the stems were harvested to determine their final fresh and dry weights.

For phenolic profiling, stems from 7 replicates of each genotype grown for approximately 6 months under the same greenhouse conditions were cut 10 cm above the soil. A basal 10-cm stem fragment was debarked, frozen in liquid nitrogen and stored at -70 °C until use. For cell wall analyses (n = 5, per line), 2D-NMR (n = 3, per line) and saccharification assays (n = 10, per line), the stems of another set of 6-months old plants grown under the same conditions were cut 10 cm above the soil. The stems were debarked, air-dried, ground in a ball mill, and

700 sieved to pass a mesh of 0.5 mm, after which the CWR was prepared for each sample as
701 previously described (Van Acker et al., 2013).
702
703 ***CSE activity assays of poplar xylem***
704 Preparation of crude protein extracts of xylem from 3.5-month old *P. tremula* × *P. alba*
705 WT plants was performed as previously described for Arabidopsis (Vanholme et al., 2013a).
706 The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.0, 1 mM DTT, 100 μM
707 caffeoyl shikimate and 10 μg soluble xylem proteins in a total volume of 40 μL. Samples
708 were incubated at 25 °C for 1 h or 25 h and the reaction was terminated by boiling for 5 min.
709 Boiled xylem protein extracts were used as negative control. Quantification of caffeic acid
710 was performed as previously described (Vanholme et al., 2013c).
711
712 ***Gene expression analysis via RT-qPCR***
713 Tissue-specific expression analysis was performed in bark, mature leaves, axillary buds
714 and developing xylem of 6-month-old *P. tremula* × *P. alba* WT plants. A 10-cm stem
715 fragment was harvested in a region 40 cm below the apex of the plants, debarked and
716 immediately frozen in liquid nitrogen. The bark, leaves and axillary buds were harvested and
717 also frozen in liquid nitrogen. All plant materials were ground in 2-mL Eppendorf tubes using
718 a Retsch MM300 mill (20 Hz, 4-mm bead). Total RNA was extracted using RNeasy® Plant
719 Mini Kit (Qiagen) and treated with Ambion® DNA-free™ (Life Technologies) to remove
720 contaminating genomic DNA. Total RNA (1 μg) was used as template for the synthesis of
721 cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). cDNAs from two plants were pooled
722 to constitute a biological replicate and a total of four biological replicates for each line were
723 used in the analysis. The transcript levels of *PtxaCSE1* (corresponding to Potri.001G175000)
724 and *PtxaCSE2* (corresponding to Potri.003G059200) were determined via quantitative RT-

PCR as previously described (Vargas et al., 2016). Poplar *18S RIBOSOMAL RNA* gene (*Pt18S*; AF206999), *POLYUBIQUITIN* (*PtUBQ*; BU879229) and *LEAFY/FLORICAULA* (*PtLF*; Potri.015G106900) were used as reference genes (Brunner et al., 2004; Li et al., 2009). In addition, transcript levels of *Ptx**aCSE1* and *Ptx**aCSE2* were determined in the *hpCSE* lines in order to evaluate the level of *CSE* downregulation. This analysis was performed essentially as described above, but total RNA was extracted from scraped xylem of a stem fragment harvested 60-cm below the top of 6-month old plants. All primers used in this study are listed in Supplemental Table S4.

Cell wall analysis and saccharification assays

Lignin content was measured via the acetyl bromide lignin method to screen the transformed lines and via the Klason method to characterize *hpCSE#1* and *hpCSE#2*. Acetyl bromide lignin quantification was performed as described in Van Acker et al. (2014). The Klason method was performed via a protocol modified from Van den Bosch et al. (Van den Bosch et al., 2015). First, 1.5 mL of 72% H_2SO_4 was added to 100 mg of purified CWR in 15 mL glass vials and incubated at room temperature for 2 h under magnetic stirring. Next, the content was transferred to 100 mL bottles and diluted to 3% H_2SO_4 using 36 mL of water. The diluted solution was then autoclaved for 1 h (1 bar, 121 °C), filtered and washed with hot water through a pre-weighed filter paper (Sartorius AG) using a Büchner filter system (Merck Millipore). The filter paper containing the extracted lignin was placed in a Petri dish and oven-dried at 80 °C overnight. After cooling for 1 h at room temperature, Klason lignin was determined gravimetrically. Cellulose was measured according to a protocol modified from Brendel et al. (Brendel et al., 2000). In 5 mL autoclavable borosilicate vials with PTFE-coated screw caps (Supelco/Sigma-Aldrich), 2 mL of 80% acetic acid and 0.2 mL of 69% nitric acid (HNO_3) were added to 100 mg of milled stem and the mixture was autoclaved for 20 min (1

bar, 121 °C). After cooling at room temperature, 2.5 mL of 99% ethanol was added and the samples were filtered through a pre-weighed filter paper (Sartorius AG) using a Büchner filter system (Merck Millipore). The pellet was sequentially washed as follows: (1) twice with 5 mL 99% ethanol; (2) twice with 5 mL water; (3) once with 5 mL 99% ethanol; (4) once with 4 mL acetone. The filter paper with extracted cellulose was placed in a Petri dish and oven-dried at 50 °C overnight. After cooling for 1 h at room temperature, cellulose was determined gravimetrically. Saccharification assays were performed as previously described (Van Acker et al., 2016). Total amount of glucose release on a plant basis was calculated with the formula: (glucose release/dry weight)*average dry weight of the stem.

2D-Nuclear Magnetic Resonance

Preparation of whole plant CW gel-state NMR samples in DMSO-d_6/pyridine-d_5 (4:1) and NMR experiments were performed as previously described (Kim et al., 2008). NMR spectra were recorded on a Bruker Biospin AVANCE 700-MHz spectrometer fitted with a cryogenically-cooled 5-mm quadruple-resonance $^{1}H/^{31}P/^{13}C/^{15}N$ QCI gradient probe with inverse geometry (proton coils closest to the sample). Bruker's TopSpin 3.2 (Mac version) software was used to process the spectra and volume integration of contours in HSQC plots was performed on data processed without linear prediction. The peaks characteristic of the lignin monomers (aromatic distributions) and linkages (side-chain distributions) were selected and estimated after the contour level adjustment to achieve optimal peak separation. As end-groups, in particular, are substantially overestimated (Mansfield et al., 2012b), the integrals were used in relative comparisons and do not represent true absolute quantification. This relative quantification was performed for all three genotypes using 3 biological replicates.

Phenolic profiling

775 The debarked 10-cm basal stem fragments from 3.5-month old *P. tremula* × *P. alba* WT
776 and *hpCSE* plants were scraped and the resulting developing xylem was ground using mortar
777 and pestle. Soluble phenolic compounds were extracted from approximately 100 mg of
778 scraped and ground xylem with 1 mL methanol at 70 °C for 15 min under 1000 rpm shaking.
779 After centrifugation at room temperature and maximum speed, 800 µL of the supernatants
780 was dried under vacuum and the pellet redissolved in 200 µL milliQ water/cyclohexane (1:1,
781 v/v). The tubes were vortexed and centrifuged at 14,000 rpm (20,000 x g) for 10 min, after
782 which a 15 µL aliquot of the aqueous phase was injected on a UHPLC system (Waters
783 Acquity UPLC®) equipped with a BEH C18 column (2.1 x 150 mm, 1.7 µM, Waters) and
784 hyphenated to a time-of-flight mass spectrometer (TOF MS, Synapt Q-Tof, Waters
785 Corporation, Milford, Massachusetts, USA), using gradient elution. Gradient elution
786 information, negative mode mass spectrometry setting, chromatogram integration and
787 alignment via Progenesis QI software (Waters) were performed as previously described (Eloy
788 et al., 2016). Peak abundances were normalized to the dry weight (mg) of the pellet remaining
789 after methanol extraction and dried in the SpeedVac. The criteria to select peak/metabolites
790 with significantly higher or lower relative abundance in *hpCSE* lines compared to the WT
791 were the following: (1) present in all samples of at least one of the three genotypes; (2)
792 average normalized abundance higher than 500 counts in at least one of the three genotypes;
793 (3) at least 2-fold increased or 50% decreased peak area in *hpCSE* versus WT; and (4)
794 Student's t test $P < 0.01$. PCA was performed in R software via the 'prcomp' command.
795 Annotation of compounds matching these criteria was based on accurate *m/z*, isotope
796 distribution, and MS/MS similarities. The MS/MS spectra were structurally elucidated based
797 on their similarity with commercially available standards and previously identified
798 metabolites that have already been described in the literature. For targeted analysis of the
799 oligolignols, compounds were identified via *m/z*, retention time, and MS/MS similarity.

Supplemental Material

The following supplemental materials are provided.

Supplemental Table S1. Caffeoyl shikimate abundance, thioacidolysis monomeric composition, and lignin quantification used to screen the transgenic *hpCSE* lines.

Supplemental Table S2. Cell wall parameters measured in both *hpCSE* lines and the WT.

Supplemental Table S3. Saccharification results from limited-saccharification tests.

Supplemental Table S4. List of primers used for cloning and quantitative RT-PCR.

Supplemental Figure S1. Principal Component Analysis (PCA) plot.

Supplemental Figure S2. MS/MS spectra of the characterized metabolites.

Tables

Table I. List of compounds for which the relative abundance was significantly higher in both *hpCSE* lines compared to the WT.

For each metabolite, a unique number (No.), mass-to-charge ratio (*m/z*), retention time (RT), peak area (as mean ± SD), and ratio of the peak area in the *hpCSE* lines and the WT are given. For the structural elucidation of the compounds, see Supplemental Figure S2.

Table II. List of compounds for which the relative abundance was significantly lower in both *hpCSE* lines compared to the WT.

For each metabolite, a unique number (No.), mass-to-charge ratio (*m/z*), retention time (RT), peak area (as mean ± SD), and ratio of the peak area in the *hpCSE* lines and the WT are given. For the structural elucidation of the compounds, see Supplemental Figure S2.

823

824 Table I

825

				WT				*hpCSE#1*			ratio		*hpCSE#2*			ratio
No.	***m/z***	**RT (min)**	**Name**	**average**	**±**	**SD**		**average**	**±**	**SD**	***hpCSE#1*/WT**		**average**	**±**	**SD**	***hpCSE#2*/WT**
			***p*-coumaric acid derivatives**													
1	319.0833	7.43	*p*-coumaroyl shikimate	0	±	0		482	±	545	>100		805	±	741	>100
2	321.0679	3.28	dihydro-*p*-coumaroyl shikimate	2049	±	513		6785	±	1213	3.3		6514	±	817	3.2
3	495.1314	18.32	*p*-coumaroyl feruloyl shikimate	0	±	0		1745	±	1207	>100		3623	±	3388	>100
			caffeic acid derivatives													
4	341.0895	4.10	caffeic acid 3/4-*O*-hexoside 1	2628	±	334		6561	±	1431	2.5		6785	±	1193	2.6
5	341.0925	5.57	caffeic acid 3/4-*O*-hexoside 2	248	±	104		764	±	221	3.1		778	±	247	3.1
6	335.0772	6.23	caffeoyl shikimate 1	300	±	206		24163	±	8550	80		26146	±	17480	87
7	335.0780	7.72	caffeoyl shikimate 2	0	±	0		875	±	487	>100		1152	±	1116	>100
8	497.1312	3.75	caffeoyl shikimate 3/4-*O*-hexoside 1	0	±	0		831	±	687	>100		1330	±	754	>100
9	497.1313	5.17	caffeoyl shikimate 3/4-*O*-hexoside 2	0	±	0		2309	±	890	>100		2687	±	821	>100
10	353.0887	4.28	caffeoyl quinate [chlorogenic acid]	83	±	115		1475	±	815	18		1131	±	779	14
			ferulic acid derivatives													
11	329.0877	2.29	vanillic acid 4-*O*-hexoside	7748	±	796		25854	±	3977	3.3		25260	±	2298	3.3
12	193.0506	8.40	ferulic acid	0	±	0		575	±	729	>100		669	±	669	>100
13	349.0941	8.03	feruloyl shikimate 1	0	±	0		737	±	838	>100		964	±	905	>100
14	349.0938	8.21	feruloyl shikimate 2	6	±	15		5015	±	4305	>100		5646	±	3729	>100
15	349.0928	9.17	feruloyl shikimate 3	759	±	691		30521	±	19272	40		31096	±	20848	41
16	349.0937	10.27	feruloyl shikimate 4	118	±	167		8590	±	5677	73		10284	±	8429	87
17	511.1467	4.64	feruloyl shikimate + hexose 1	0	±	0		2243	±	1507	>100		4763	±	1064	>100
18	511.1474	7.04	feruloyl shikimate + hexose 2	0	±	0		265	±	478	>100		1148	±	336	>100
19	367.1048	6.80	feruloyl quinate	0	±	0		1110	±	1118	>100		922	±	1077	>100
20	487.1473	5.68	ferulic acid + 294 Da	65	±	111		696	±	459	11		845	±	614	13.1
			sinapic acid derivatives													
21	385.1137	4.48	sinapic acid 4-*O*-hexoside 1	1064	±	571		30844	±	4722	29		35447	±	2908	33
22	385.1145	6.18	sinapic acid 4-*O*-hexoside 2	190	±	140		10965	±	2100	58		15412	±	1665	81
23	593.1739	3.10	sinapoyl hexose 4-*O*-hexoside (formic acid adduct)	0	±	0		1354	±	666	>100		2196	±	1648	>100
24	815.2274	7.42	sinapic acid + vanillic acid + pentose + hexose + hexose	0	±	0		2153	±	760	>100		2834	±	966	>100
25	379.1044	9.11	sinapoyl shikimate	6	±	15		4335	±	4372	>100		5620	±	3167	>100
26	387.1303	5.36	dihydrosinapoyl hexose	0	±	0		625	±	335	>100		978	±	220	>100

826 Table II
827

				WT				*hpCSE#1*			ratio		*hpCSE#2*			ratio
No.	***m/z***	**RT (min)**	**Name**	**average**	**±**	**SD**		**average**	**±**	**SD**	***hpCSE#1*/WT**		**average**	**±**	**SD**	***hpCSE#2*/WT**
27	405.1201	12.63	protocatechuic acid + hexose + 90 Da	732	±	353		3	±	10	0.005		2	±	6	0.003
28	555.1898	15.41	G(β-O-4)G(redβ-5)G	757	±	157		103	±	108	0.136		28	±	38	0.037
29	599.2138	14.04	G(β-O-4)G(β-O-4)S' 1	5568	±	339		2757	±	760	0.495		1389	±	723	0.249
30	599.2142	14.86	G(β-O-4)G(β-O-4)S' 2	1306	±	395		608	±	258	0.465		241	±	211	0.185
31	515.1939	7.01	G(β-O-4)G(β-O-4)hydroxybenzyl alcohol 1	1778	±	381		147	±	147	0.083		28	±	45	0.016
32	515.1940	7.47	G(β-O-4)G(β-O-4)hydroxybenzyl alcohol 2	2051	±	319		340	±	282	0.166		106	±	138	0.052
33	611.2146	16.67	S(β-O-4)S(β-5)G'	5672	±	702		2550	±	328	0.450		2385	±	974	0.421
34	779.2940	15.19	G(β-O-4)G(β-O-4)S(β-5)G 1	2090	±	881		273	±	259	0.130		26	±	48	0.012
35	779.2939	15.30	G(β-O-4)G(β-O-4)S(β-5)G 2	601	±	203		27	±	66	0.045		8	±	23	0.013
36	779.2933	17.26	G(β-O-4)G(β-β)S(4-O-β)G	2677	±	770		976	±	359	0.364		1032	±	896	0.385
37	809.3044	16.74	G(β-O-4)S(β-O-4)S(β-5)G	2365	±	624		734	±	630	0.310		10	±	29	0.004
38	839.3155	18.64	G(β-O-4)S(β-β)S(4-O-β)G	3899	±	353		1561	±	414	0.400		1533	±	614	0.393
39	869.3261	17.80	S(β-O-4)S(β-O-4)S(β-β)S	662	±	265		29	±	54	0.044		64	±	121	0.097
40	869.3256	17.90	S(β-O-4)S(β-β)S(4-O-β)S	1207	±	326		81	±	135	0.067		133	±	227	0.111

828 **Figures Captions**

829 **Figure 1**. Metabolic map of the general phenylpropanoid and monolignol-specific pathways
830 showing the changes in phenolic metabolism upon *CSE* down-regulation in poplar.
831 Metabolites found to have higher abundance in the *hpCSE* lines are highlighted in red, while
832 those with decreased abundance are shown in blue. Metabolites belonging to the same class
833 are framed with a box. Solid and dashed arrows represent enzymatic conversions validated by
834 experimental evidence and suggested conversions, respectively. Two successive arrows
835 represent two or more metabolic steps. PAL, PHENYLALANINE AMMONIA LYASE;
836 C4H, CINNAMATE 4-HYDROXYLASE; 4CL, 4-COUMARATE:COENZYME A
837 LIGASE; HCT, *p*-HYDROXYCINNAMOYL-COENZYME A:QUINATE/SHIKIMATE *p*-
838 HYDROXYCINNAMOYLTRANSFERASE; C3H, *p*-COUMARATE 3-HYDROXYLASE;
839 CSE, CAFFEOYL SHIKIMATE ESTERASE; CCoAOMT, CAFFEOYL-COENZYME A *O*-
840 METHYLTRANSFERASE; CCR, CINNAMOYL-COENZYME A REDUCTASE; F5H,
841 FERULATE 5-HYDROXYLASE; COMT, CAFFEIC ACID *O*-METHYLTRANSFERASE;
842 CAD, CINNAMYL ALCOHOL DEHYDROGENASE; HCALDH,
843 HYDROXYCINNAMALDEHYDE DEHYDROGENASE.

844

845 **Figure 2.** Poplar xylem CSE activity and *CSE* gene expression. A) CSE activity in crude
846 poplar xylem protein extracts. Total poplar xylem protein was added to reaction buffer
847 containing 100 µM caffeoyl shikimate, and caffeate was measured via UHPLC-MS after 1 h
848 and 25 h. Boiled poplar xylem protein was used as negative control. Error bars indicate the
849 standard error. Differences in caffeic acid abundance were assessed with Student's *t* test (n =
850 4; **$0.01 > P > 0.001$). B) Expression analysis of *CSE* homologues in different tissues of
851 poplar as determined via RT-qPCR. Error bars indicate the standard error (n = 4).

852

853 **Figure 3**. Residual expression of both *CSE* genes and growth analyses of poplar trees
854 downregulated in *CSE*. A) Residual expression levels of *Ptx**aCSE1* and *Ptx**aCSE2* in the
855 xylem of WT poplar plants and the *hpCSE* lines were determined by RT-qPCR. The
856 expression of each *Ptx**aCSE* gene was normalized to that of the wild type. Error bars indicate
857 the standard error. Differences in gene expression were assessed with Student's *t* test (*0.05 >
858 $P > 0.01$; **0.01 > $P > 0.001$; *** $P < 0.001$; n = 4). B) Photograph of representative plants
859 grown for 6 months in the greenhouse. C) Fresh and dry weights of stems harvested from WT
860 and *hpCSE* plants at the end of the 6-month growth period. No statistical difference was
861 found among the genotypes. Error bars indicate the standard deviation. D) Growth curves of
862 the WT and the *hpCSE* lines. Height was monitored every week for a period of 6 months. The
863 average growth in each month was used to plot the graphic. Error bars indicate the standard
864 deviation. Differences in growth parameters between the WT and the transgenic lines were
865 assessed with Student's *t* test (*0.05 > $P > 0.01$; **0.01 > $P > 0.001$; WT, n = 25, *hpCSE#1*, n
866 = 23 and *hpCSE#2*, n = 23). Asterisks above the data point represent statistical difference for
867 line *hpCSE#1* whereas asterisks below represent statistical difference for line *hpCSE#2*.
868
869 **Figure 4**. Lignin content and composition and cellulose content of stems from *hpCSE* lines
870 and control plants. A) Total acid-insoluble lignin content determined by the Klason method.
871 Data are expressed as percentage of CWR. B) Cellulose content was determined
872 gravimetrically and is expressed as a percentage of CWR. C) Relative levels of releasable H
873 monomers determined by thioacidolysis. D) Relative levels of releasable G and S monomers
874 determined by thioacidolysis. E) S/G ratio determined based on thioacidolysis data. F) Total
875 thioacidolysis yield as determined from the sum of released H, G and S monomers, expressed
876 in µmoles per gram of Klason lignin. For all the analyses, data are means ± SD (n = 5), except
877 for thioacidolysis yield for which standard errors are shown. Differences in cell wall

878 parameters were assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; *** *P* <
879 0.001).
880
881 **Figure 5**. Structural characterization of lignin by NMR. HSQC spectra of the aromatic
882 regions (A) and the oxygenated aliphatic regions (B) of whole cell walls from stems of WT
883 and the two *hpCSE* lines, along with a synthetic lignin synthesized biomimetically from *p*-
884 coumaryl alcohol to help validate the H-unit assignments (H-DHP). Integrated values for each
885 monomeric unit **H**, **G** and **S** and the α-C/H correlation peaks from the major lignin interunit
886 structures **A-C** are provided. The colors of the substructures shown match those of the
887 corresponding signals in the HSQC spectra (where they are resolved).
888
889 **Figure 6**. Saccharification efficiency of stem biomass from WT and *hpCSE* plants. Cellulose-
890 to-glucose conversion during limited saccharification of stems from the WT (solid lines) and
891 the *hpCSE* lines (dashed lines) without (A) and with acidic (C) and alkaline (E) pretreatments.
892 Cellulose-to-glucose conversion was calculated based on the amounts of released glucose and
893 the quantified amount of cellulose. Relative glucose release per plant after 48 h of
894 saccharification without (B) and with acid (D) and alkaline (F) pretreatments, normalized to
895 the values of the WT. Error bars indicate the standard error. Significant differences were
896 assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; *** *P* < 0.001; n = 20).
897
898 **Supplemental Table S1.** Caffeoyl shikimate abundance, thioacidolysis monomeric
899 composition and lignin quantification to screen the transgenic *hpCSE* lines. H units
900 (determined via thioacidolysis) and acetyl bromide lignin were measured in the 20 lines with
901 the highest signals for caffeoyl shikimate. Based on the combination of these three parameters
902 (high abundance of caffeoyl shikimate, high relative H-unit content, and low lignin level), two

903 lines (6_80_1 and 6_81_1) were selected and were named *hpCSE#1* and *hpCSE#2*,
904 respectively, for convenience.
905
906 **Supplemental Table S2.** Cell wall parameters measured in both *hpCSE* lines and the WT.
907 CWR: cell wall residue; DW: dry weight; SD: standard deviation. The cell wall residue
908 (CWR) was determined gravimetrically after a sequential extraction and is expressed as
909 %DW. Cellulose was determined gravimetrically and lignin content was measured with the
910 Klason method. Both parameters are expressed as %CWR. Lignin composition was
911 determined with thioacidolysis. Increases and decreases compared to the WT are in bold and
912 italics, respectively (Student's *t* test, $P < 0.05$).
913
914 **Supplemental Table S3.** Saccharification results. The average (± SD) glucose released after
915 48 h for the two *hpCSE* poplar lines and WT using three different pretreatment conditions (n
916 = 10). Cellulose-to-glucose conversions were calculated based on the amount of cellulose
917 (Supplemental Table S2) and glucose released during saccharification. Increases (%) are only
918 shown for the conditions that resulted in significant improvements and are compared to the
919 values of WT trees within the same treatment condition. Bold, significantly increased
920 compared to WT (Dunnett adjusted T-test, $P < 0.05$).
921
922 **Supplemental Table S4.** List of primers used for cloning and quantitative RT-PCR.
923
924 **Supplemental Figure S1.** Principal Component Analysis (PCA) plot. PCA was performed on
925 the 618 integrated and aligned peaks which were present in all replicates of at least one
926 genotype and that were above a 500-count cutoff in at least one genotype. PCA revealed that
927 the first principal component (PC1, 33.9% of the variation) explained mainly the difference

between the *hpCSE* lines and WT, while PC2 (16.6%) represents other (e.g., biological) variation. No separation between *hpCSE#1* and *hpCSE#2* could be observed.

Supplemental Figure S2. MS/MS spectra of the characterized metabolites. Most metabolites have been structurally characterized based on their *m/z*, retention time and MS/MS. Caffeoyl shikimate, *p*-coumaroyl shikimate and ferulic acid have been characterized based on *m/z* and retention time match with their authentic chemical standards.

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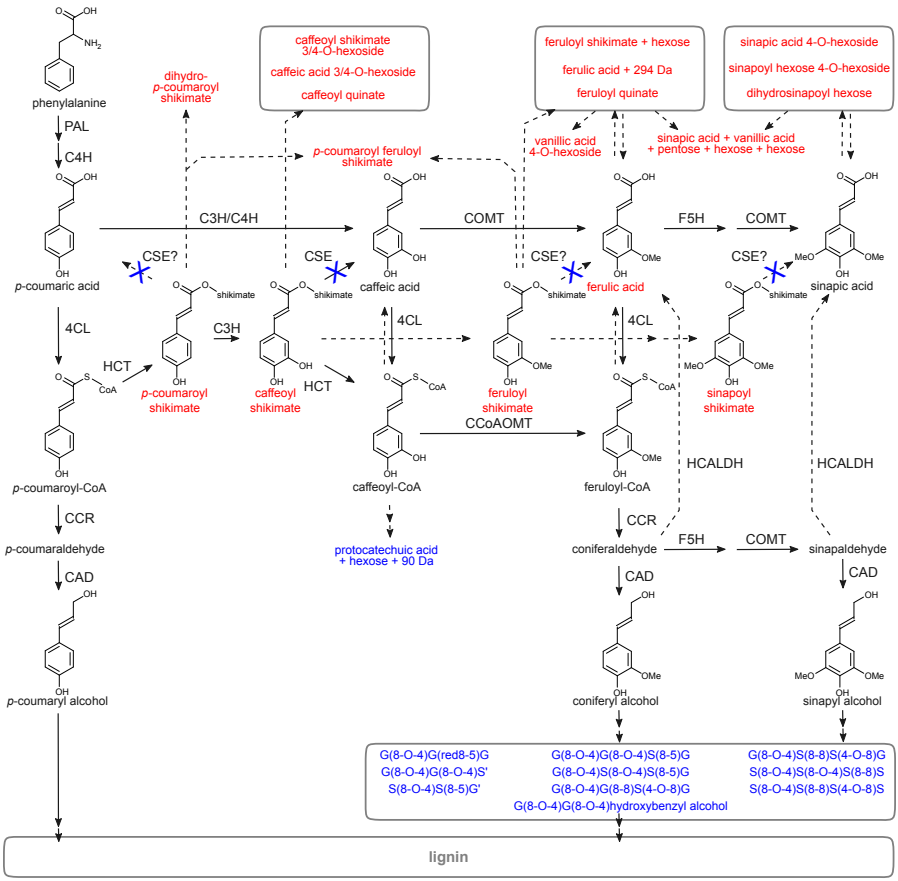


Figure 1. Metabolic map of the general phenylpropanoid and monolignol-specific pathways showing the changes in phenolic metabolism upon *CSE* down-regulation in poplar. Metabolites found to have higher abundance in the *hpCSE* lines are highlighted in red, while those with decreased abundance are shown in blue. Metabolites belonging to the same class are framed with a box. Solid and dashed arrows represent enzymatic conversions validated by experimental evidence and suggested conversions, respectively. Two successive arrows represent two or more metabolic steps. PAL, PHENYLALANINE AMMONIA LYASE; C4H, CINNAMATE 4-HYDROXYLASE; 4CL, 4-COUMARATE:COENZYME A LIGASE; HCT, *p*-HYDROXYCINNAMOYL-COENZYME A:QUINATE/SHIKIMATE *p*-HYDROXYCINNAMOYLTRANSFERASE; C3H, *p*-COUMARATE 3-HYDROXYLASE; CSE, CAFFEOYL SHIKIMATE ESTERASE; CCoAOMT, CAFFEOYL-COENZYME A *O*-METHYLTRANSFERASE; CCR, CINNAMOYL-COENZYME A REDUCTASE; F5H, FERULATE 5-HYDROXYLASE; COMT, CAFFEIC ACID *O*-METHYLTRANSFERASE; CAD, CINNAMYL ALCOHOL DEHYDROGENASE; HCALDH, HYDROXYCINNAMALDEHYDE DEHYDROGENASE.

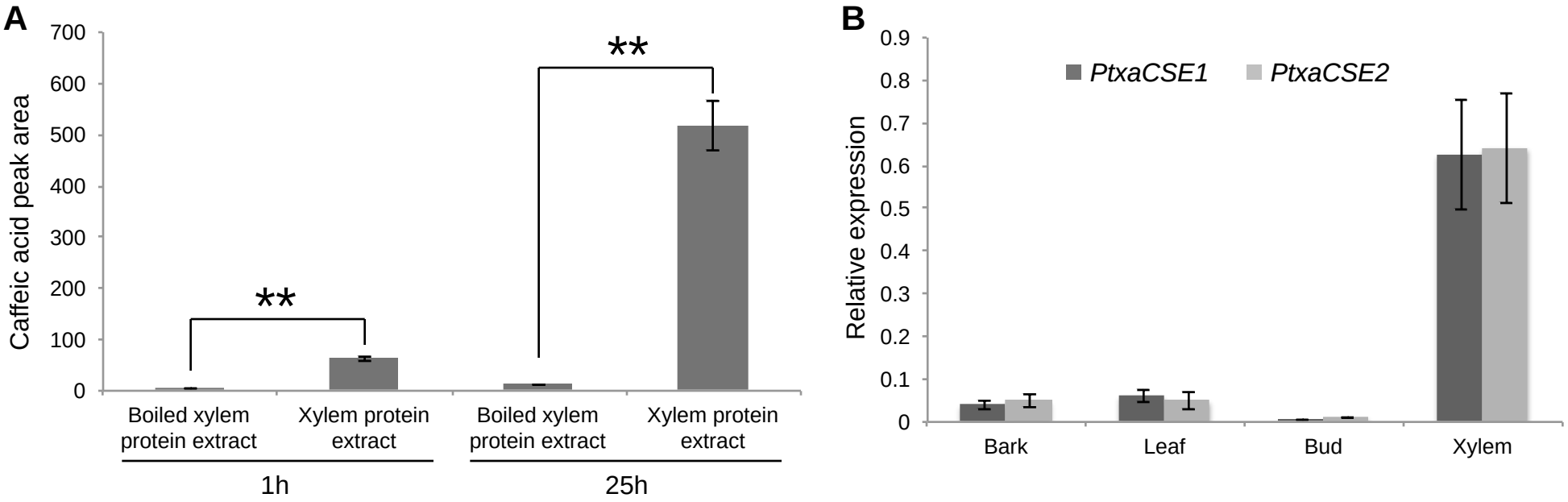
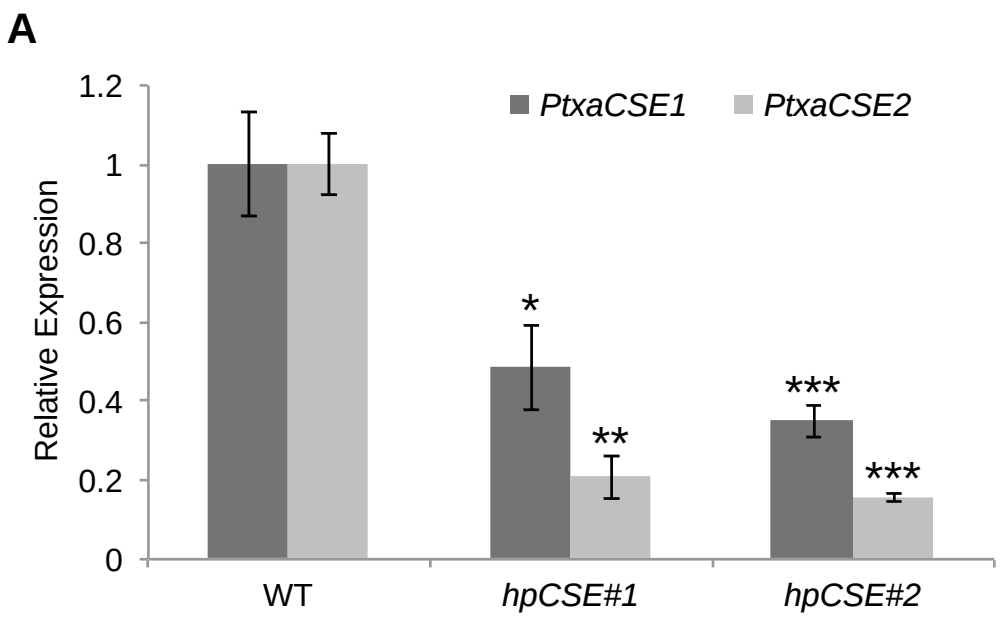
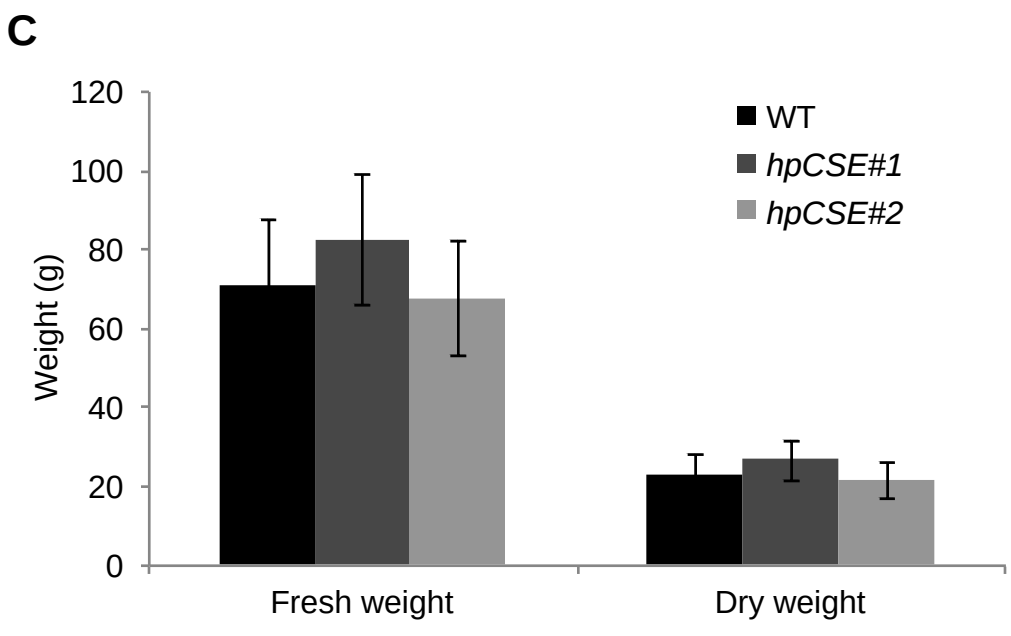


Figure 2. Poplar xylem CSE activity and *CSE* gene expression. A) CSE activity in crude poplar xylem protein extracts. Total poplar xylem protein was added to reaction buffer containing 100 µM caffeoyl shikimate, and caffeate was measured via UHPLC-MS after 1 h and 25 h. Boiled poplar xylem protein was used as negative control. Error bars indicate the standard error. Differences in caffeic acid abundance were assessed with Student's *t* test ($n = 4$; $^{**}0.01 > P > 0.001$). B) Expression analysis of *CSE* homologues in different tissues of poplar as determined via RT-qPCR. Error bars indicate the standard error (n = 4).





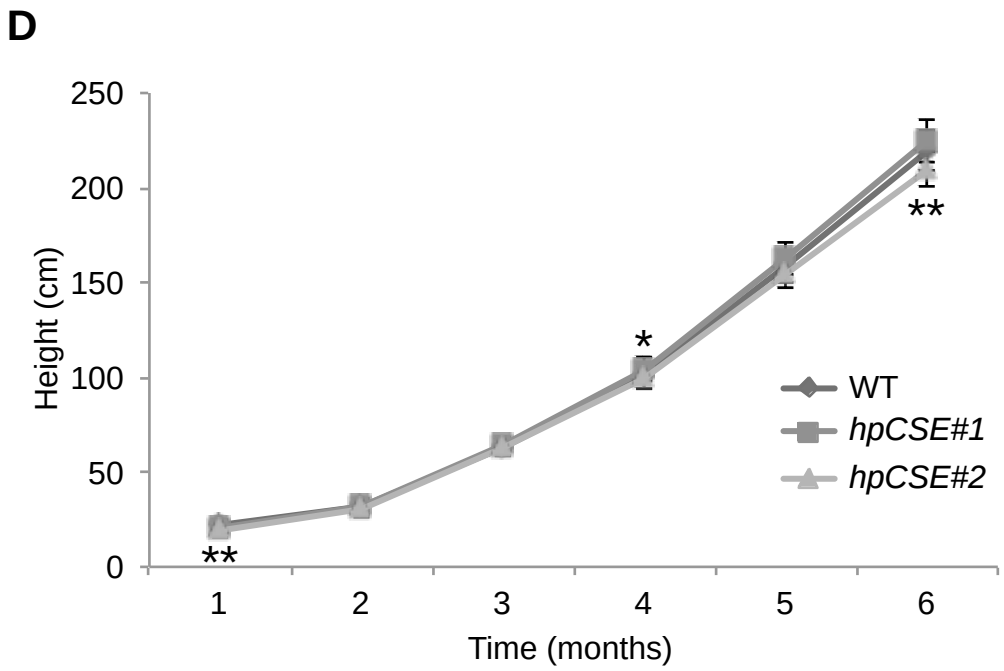


Figure 3. Residual expression of both *CSE* genes and growth analyses of poplar trees downregulated in *CSE*. A) Residual expression levels of *PtxaCSE1* and *PtxaCSE2* in the xylem of WT poplar plants and the *hpCSE* lines were determined by RT-qPCR. The expression of each *PtxaCSE* gene was normalized to that of the wild type. Error bars indicate the standard error. Differences in gene expression were assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; *** *P* < 0.001; n = 4). B) Photograph of representative plants grown for 6 months in the greenhouse. C) Fresh and dry weights of stems harvested from WT and *hpCSE* plants at the end of the 6-month growth period. No statistical difference was found among the genotypes. Error bars indicate the standard deviation. D) Growth curves of the WT and the *hpCSE* lines. Height was monitored every week for a period of 6 months. The average growth in each month was used to plot the graphic. Error bars indicate the standard deviation. Differences in growth parameters between the WT and the transgenic lines were assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; WT, n = 25, *hpCSE#1*, n = 23 and *hpCSE#2*, n = 23). Asterisks above the data point represent statistical difference for line *hpCSE#1* whereas asterisks below represent statistical difference for line *hpCSE#2*.

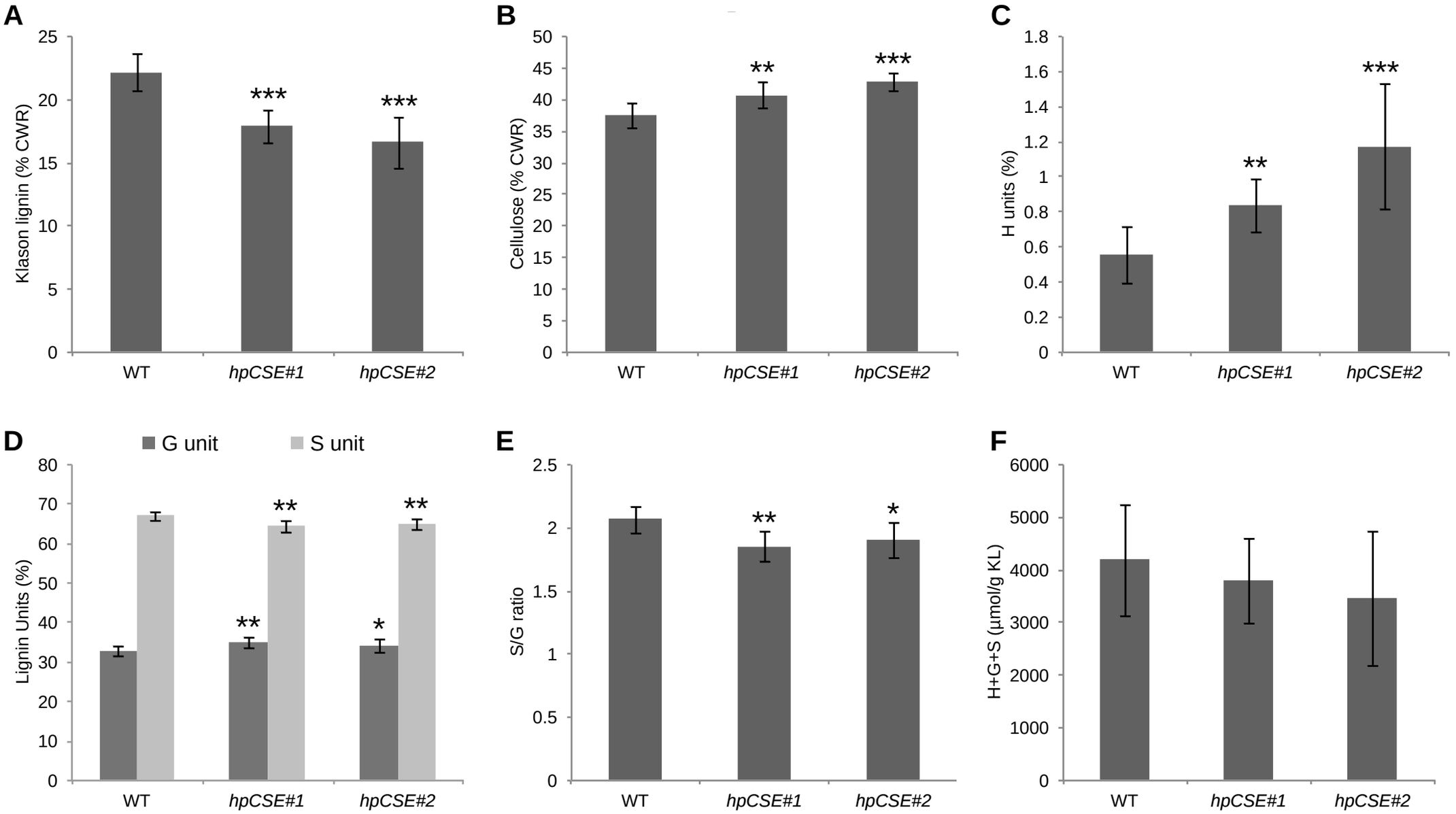
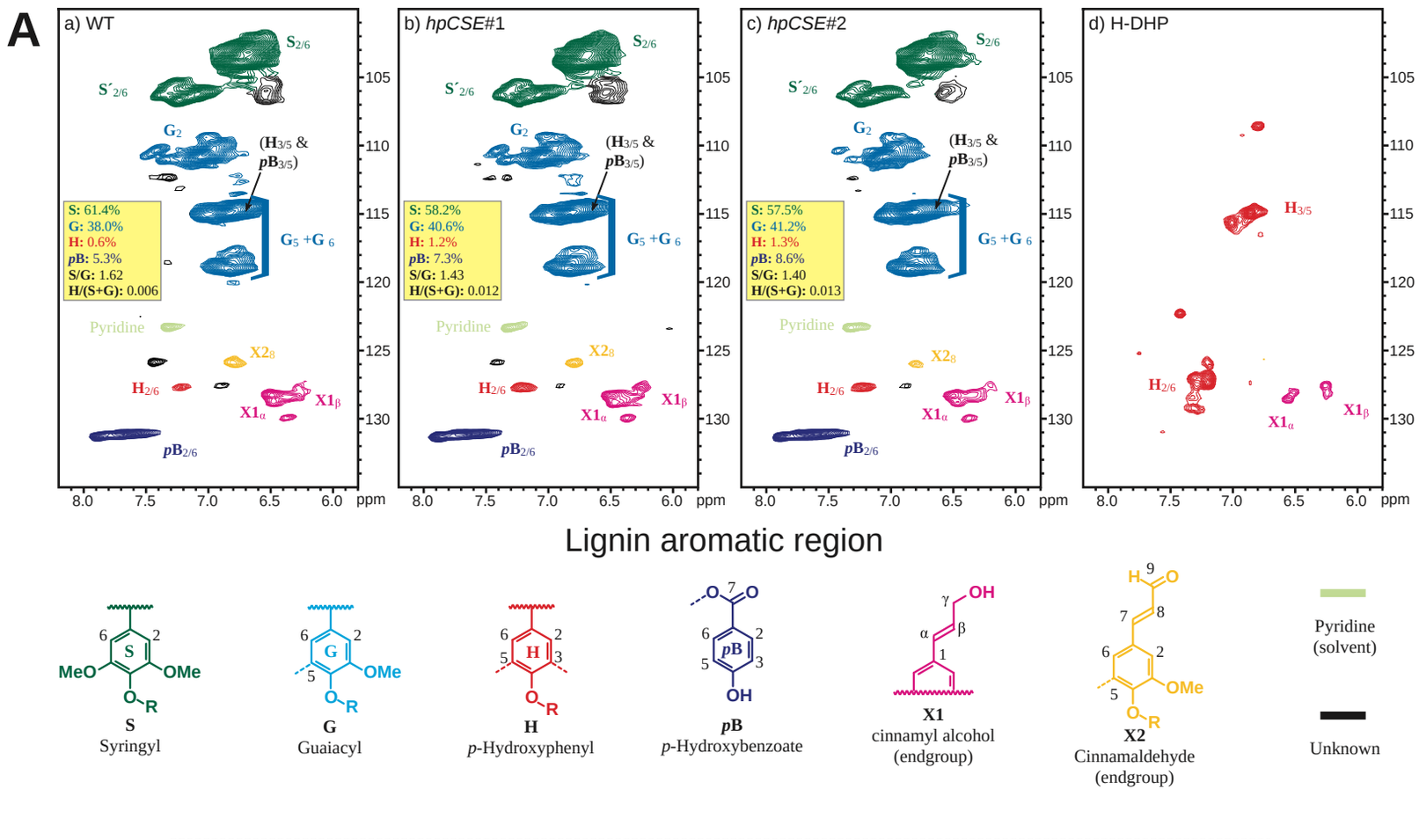


Figure 4. Lignin content and composition and cellulose content of stems from *hpCSE* lines and control plants. A) Total acid-insoluble lignin content determined by the Klason method. Data are expressed as percentage of CWR. B) Cellulose content was determined gravimetrically and is expressed as a percentage of CWR. C) Relative levels of releasable H monomers determined by thioacidolysis. D) Relative levels of releasable G and S monomers determined by thioacidolysis. E) S/G ratio determined based on thioacidolysis data. F) Total thioacidolysis yield as determined from the sum of released H, G and S monomers, expressed in µmoles per gram of Klason lignin. For all the analyses, data are means ± SD (n = 5), except for thioacidolysis yield for which standard errors are shown. Differences in cell wall parameters were assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; *** *P* < 0.001).



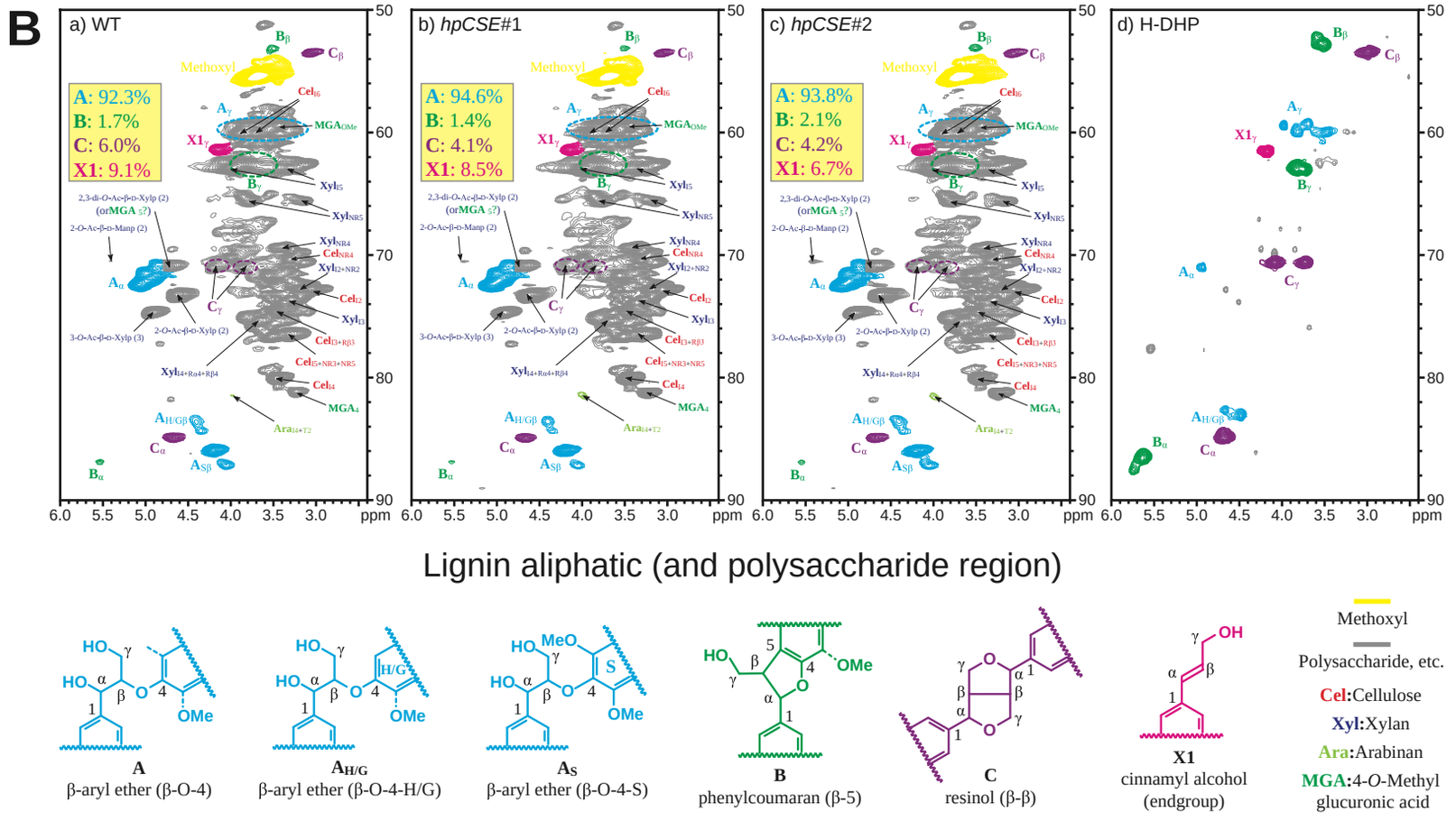


Figure 5. Structural characterization of lignin by NMR. HSQC spectra of the aromatic regions (A) and the oxygenated aliphatic regions (B) of whole cell walls from stems of WT and the two *hpCSE* lines, along with a synthetic lignin synthesized biomimetically from *p*-coumaryl alcohol to help validate the H-unit assignments (H-DHP). Integrated values for each monomeric unit H, G and S and the α-C/H correlation peaks from the major lignin interunit structures A-C are provided. The colors of the substructures shown match those of the corresponding signals in the HSQC spectra (where they are resolved).

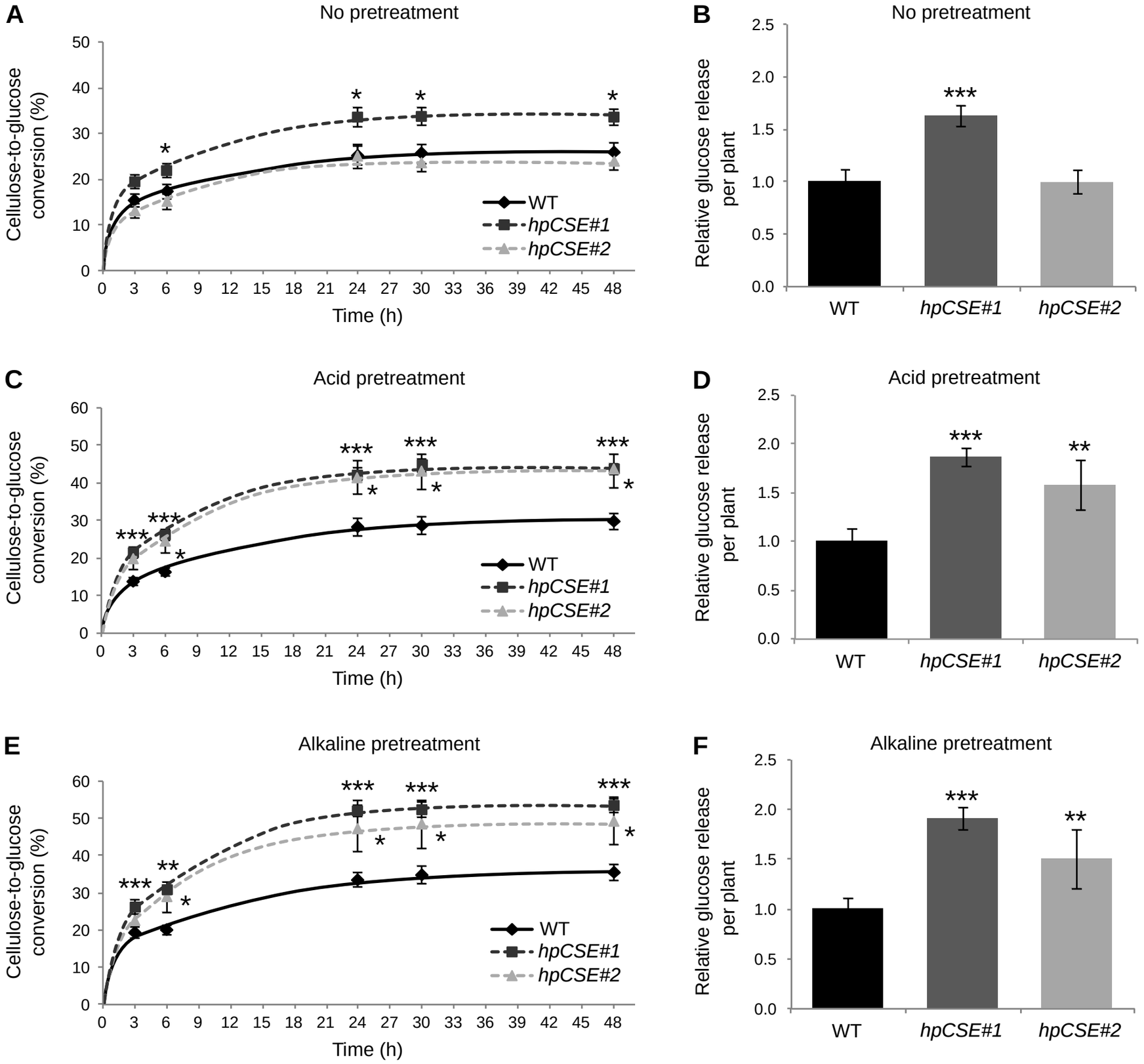


Figure 6. Saccharification efficiency of stem biomass from WT and *hpCSE* plants. Cellulose-to-glucose conversion during limited saccharification of stems from the WT (solid lines) and the *hpCSE* lines (dashed lines) without (A) and with acidic (C) and alkaline (E) pretreatments. Cellulose-to-glucose conversion was calculated based on the amounts of released glucose and the quantified amount of cellulose. Relative glucose release per plant after 48 h of saccharification without (B) and with acid (D) and alkaline (F) pretreatments, normalized to the values of the WT. Error bars indicate the standard error. Significant differences were assessed with Student's *t* test (*0.05 > *P* > 0.01; **0.01 > *P* > 0.001; *** *P* < 0.001; n = 20).

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