Alteration of S-adenosylhomocysteine levels affects lignin biosynthesis in switchgrass

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Summary
Methionine (Met) synthesized from aspartate is a fundamental amino acid needed to produce S-adenosylmethionine (SAM) that is an important cofactor for the methylation of monolignols. As a competitive inhibitor of SAM-dependent methylation, the effect of S-adenosylhomocysteine (SAH) on lignin biosynthesis, however, is still largely unknown in plants. Expression levels of Cystathionine γ-synthase (PvCGS) and S-adenosylhomocysteine hydrolase 1 (PvSAHH1) were down-regulated by RNAi technology, respectively, in switchgrass, a dual-purpose forage and biofuel crop. The transgenic switchgrass lines were subjected to studying the impact of SAH on lignin biosynthesis. Our results showed that down-regulation of PvCGS in switchgrass altered the accumulation of aspartate-derived and aromatic amino acids, reduced the content of SAH, enhanced lignin biosynthesis and stunted plant growth. In contrast, down-regulation of PvSAHH1 raised SAH levels in switchgrass, impaired the biosynthesis of both guaiacyl and syringyl lignins and therefore significantly increased saccharification efficiency of cell walls. This work indicates that SAH plays a crucial role in monolignol methylation in switchgrass. Genetic regulation of either PvCGS or PvSAHH1 expression in switchgrass can change intracellular SAH contents and SAM to SAH ratios and therefore affect lignin biosynthesis. Thus, our study suggests that genes involved in Met metabolism are of interest as new valuable targets for cell wall bioengineering in future.

Introduction
Plant cell walls store about 30%–40% of the annually fixed terrestrial organic carbon in the biosphere and are a rich source of fermentable sugars and polymeric substances (Zhang and Liu, 2015). Lignin is a major component of vascular plant cell walls (Boerjan et al., 2003). As a complex phenolic polymer, lignin consists mainly of hydroxycinnamyl (H), guaiacyl (G) and syringyl (S) units that are formed through an oxidative polymerization of ρ-coumaryl, coniferyl and sinapyl alcohols, respectively (Boerjan et al., 2003). Lignin linked to carbohydrate components provides rigidity, strength and hydrophobicity to cell walls (Boerjan et al., 2003). Thus, the major biological function of lignin is mechanical support, water conductivity and protection against plant pathogens. Lignin, however, has a remarkable impact on efficient pulping, forage digestion and biofuel production of lignocellulosic biomass (Ragauskas et al., 2006). Previous studies have suggested that the saccharification efficiency of cell walls is negatively correlated with lignin content (Carroll and Somerville, 2009; Chen and Dixon, 2007; Pilate et al., 2002). Thus, genetic manipulation of lignin is of great interest in the pulp and paper industry, agriculture–livestock industry and environmental protection (Bedon and Legay, 2011).

During recent decades, the structural genes and their transcriptional regulators in the lignin biosynthetic pathway have been genetically modified in many dicot and monocot plant species. The number of target genes, however, is still not sufficient to meet the needs of lignin engineering. Recently, two studies demonstrate that methylenetetrahydrofolate reductase (MTHFR) and folypolyglutamate synthase (FGPS) can significantly affect lignin biosynthesis, which may shed light on a new regulatory mechanism of lignin biosynthesis (Li et al., 2015; Tang et al., 2014). MTHFR and FGPS are involved in plant one-carbon (C1) metabolism that is essential in all organisms for the biosynthesis of methionine (Met), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), homocysteine (Hcy) and other methylated compounds (Hanson and Roje, 2001). Generally, C1 metabolism includes tetrahydrofolate (THF) and methionine (Met) cycles. The former is responsible for the transfer of C1 units, whereas the latter is for the synthesis and recycling of SAM, the activated form of Met. In the lignin biosynthetic pathway, caffeoyl CoA O-methyltransferase (CCoAOMT) and caffeic acid O-methyltransferase (COMT) are required to methylate 3- and 5-hydroxyl groups of the aromatic ring, respectively, using SAM as a cofactor and ultimately lead to the biosynthesis of G and S lignins (Boerjan et al., 2003; Roje, 2006). Moreover, many C1 metabolism genes exhibit high expression levels in well-lignified tissues in plants, implying a potential coordination between C1 metabolism and lignin biosynthesis (Scully et al., 2018; Srivastava et al., 2015; Villalobos et al., 2012).
Cystathionine γ-synthase (CGS) is the first committed enzyme for the biosynthesis of Met that can be metabolized to SAM (Figure 1). This enzyme combines O-phosphohomoserine (OPH) derived from aspartate (Asp) with the thiol group of cysteine (Cys) to form cystathionine (Galili et al., 2016). Threonine synthase (TS), however, competes with CGS for OPH that is metabolized towards threonine (Thr) and isoleucine (Ile) (Amir et al., 2002). Therefore, the biosynthesis of Met is controlled by both CGS and TS. Previous studies have indicated that the overexpression of CGS dramatically elevates the concentrations of Met and S-methylmethionine (SMM) in Arabidopsis without impacting plant growth and development (Gakière et al., 2002; Kim et al., 2002). By contrast, down-regulation of AtCGS in Arabidopsis affects Met accumulation slightly in spite of a substantial increase in OPH and a severe growth retardation (Gakière et al., 2000; Kim and Leustek, 2000). Most strikingly, either up-regulating or down-regulating CGS in the seeds of Arabidopsis can significantly increase the concentrations of Met and result in almost similar effects on the biosynthesis of other amino acids including Thr, Ile and phenylalanine (Phe) (Cohen et al., 2014, 2017a; Kim et al., 2002). In addition, the overexpression of Met-insensitive form of AtCGS increases Met levels dramatically in other dicot species including tobacco, alfalfa, soybean and azuki bean (Avraham et al., 2005; Hacham et al., 2008; Hanafy et al., 2013). However, the overexpression of StCGS does not have an effect on the biosynthesis of Met in potato (Kreft et al., 2003). Furthermore, down-regulation of CGS in potato caused a significant reduction in free Met levels, but no visible phenotypic changes (Kreft et al., 2003). Thus, the effect of CGS alteration on plant growth and development, Met and other related amino acid metabolism still remains largely unknown.

The precursors of G and S lignins are the abundant methylated products derived from phenylpropanoid and SAM biosynthetic pathways. Previous research has shown that a single-point mutation in the S-adenosylmethionine synthase 3 (SAMS3) can significantly impair both SAM and lignin accumulation in Arabidopsis (Shen et al., 2002). Moreover, disruption of THF cycle genes such as MTHFR and FPGS also affect lignin biosynthesis substantially (Li et al., 2015; Srivastava et al., 2015; Tang et al., 2014). These studies suggest that C1 metabolism may control lignin biosynthesis through regulating the metabolism of SAM, the methyl group donor involved in the methylation of monolignols. In addition, as the demethylation product of SAM, SAH competes with SAM binding, which inhibits the enzymatic activities of methyltransferases (Keating et al., 1991; Nguyen et al., 2001). An S-adenosylhomocysteine hydrolase (SAHH) is responsible for SAH hydrolysis that is crucial to maintain SAH levels in plants (Hanson and Roje, 2001). Disruption of SAHH1 in Arabidopsis impacts plant growth and development obviously (Rocha et al., 2005). However, the role of SAHH in lignin biosynthesis has yet to be investigated.

Switchgrass (Panicum virgatum L.), a dual-purpose forage and biofuel crop, is a perennial C4 tall grass native to North America (McLaughlin and Kszos, 2005). In this research, we identified the CGS gene from switchgrass and studied the effect of PvCGS on the Met cycle, Asp family amino acids and lignin biosynthesis. Our results showed that severe down-regulation of PvCGS in switchgrass resulted in growth stunting. Moreover, down-regulation of PvCGS increased lignin biosynthesis through reducing SAH contents and inducing phenylalanine and tyrosine accumulation in switchgrass. Furthermore, elevating SAH levels by down-regulation of PvSAHH1 in switchgrass enhanced its inhibition on monolignol methylation and therefore impaired the biosynthesis of both G and S lignins. Lignin alteration in SAHH-RNAi transgenic switchgrass plants improved saccharification efficiency of cell walls without biomass penalty, thus providing a potential for improving biofuel production and forage digestibility in future.

**Results**

Expression pattern of PvCGS positively correlated with those of PvSAMS and PvSAHH1 in the process of internode lignification

To study the function of CGS in switchgrass, we first identified PvCGS sequences from Panicum virgatum v4.1 genome database (Phytozome). The switchgrass genome assembly contains a pair of PvCGS genes that exist on chromosome 9 and share over 99% sequence identities to each other. The orthologs of PvCGS identified from two monocots (Zea mays

![Figure 1](image.png)
and Oryza sativa), three dicots (Arabidopsis thaliana, Nicotiana tabacum and Populus trichocarpa) and one moss (Physcomitrella patens) were employed for the phylogenetic relationship analysis. The phylogenetic tree shows that PvCGS is clustered together in a group with those from monocot species (Figure 2a). Moreover, alignment of the CGS amino acid sequences reveals high similarity between PvCGS and AtCGS (Figure S1).

The downstream enzymes of CGS in Met metabolism, SAMS and SAHH, are responsible for the biosynthesis of SAM and SAH, which are involved in the methylation of lignin monomers (Figure 1). To study the potential effects of these C1 metabolism genes on lignin biosynthesis, the expression levels of PvCGS, PvSAMS and PvSAHH1 in the process of internode lignification were detected by qRT-PCR. The tillers at three elongation stages (E2, E3 and E4) and one reproductive stage (R1) are associated with a significantly progressive lignification of cell walls. Therefore, we collected the second internode (I2) from the corresponding tillers at the above stages. The successive internodes (I2-4) and their corresponding leaf sheaths and leaf blades were dissected from the tillers harvested at the E4 stage. Our results revealed that the expression levels of PvCGS positively correlated with those of PvSAMS ($r^2 = 0.367, P < 0.05$) and PvSAHH1 ($r^2 = 0.456, P < 0.05$) (Figure 2b, Table S1). Therefore, the full-length cDNA sequence of PvCGS (Pavir.9NG556700) was isolated from switchgrass for investigating its function in Met metabolism and lignin biosynthesis.

**Down-regulation of PvCGS affected switchgrass growth and development**

To examine the function of CGS in switchgrass, we produced CGS-RNAi transgenic switchgrass plants using a single genotypic embryogenic callus line. The control plants were produced with pANIC8B empty vector that was employed as the backbone for constructing CGS-RNAi vector. Three independent positive transgenic switchgrass lines in which the transcript abundance of PvCGS was dramatically down-regulated were selected for further studies (Figure 2c). Morphological characterization of the transgenic lines showed that substantial down-regulation of PvCGS in switchgrass resulted in severe growth stunting and distinct delay in flowering (Figure 2d). Furthermore, we studied the detailed phenotype changes in CGS-RNAi transgenic switchgrass lines including plant height, internode length, internode diameter, leaf sheath length, leaf blade length and width, and flowering time (Table S2). The plant height of transgenic plants was approximately 62% shorter than that of control plants. The number of internodes was not altered in the transgenic plants although approximately 57% reduction in the internode length was

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**Figure 2** Characterization of CGS-RNAi transgenic switchgrass plants. (a) Phylogenetic tree of the CGSs in dicot and monocot plant species. A maximum likelihood tree was constructed in PhyML version 3.0 on the basis of multiple alignments of the deduce protein sequences from Panicum virgatum (PvCGS, Pavir.9NG556700), Zea mays (ZmCGS, GRMZM2G113873), Oryza sativa (OsCGS, LOC_Os03 g25940), Arabidopsis thaliana (AtCGS, AT3G01120), Nicotiana tabacum (NtCGS, mRNA, B68886_cds), Populus trichocarpa (PtCGS, POPTR_0017s12240) and Physcomitrella patens (PpCGS, Pp1s49_246V6). Bootstrap values (>50%) based on 1000 replications are indicated at nodes. The above sequence data are retrieved from Phytozone and Sol Genomics Network. (b) Correlations between expression levels of PvCGS and PvSAMS/PvSAHH. The expression levels of PvCGS, PvSAHH and PvSAMS were measured by quantitative real-time PCR. Switchgrass UBQ was used as the reference for normalization. The correlations between the expression levels of PvCGS and PvSAHH/PvSAMS were statistically significant ($P < 0.05$). Internodes, leaf sheaths and leaf blades of internode 2 (I2), internode 3 (I3) and internode 4 (I4) at E4 stage and the I2 internodes at E2, E3 and E4 stages were collected from wild-type switchgrass plants. (c) Quantitative real-time PCR analysis of PvCGS transcript abundance in the CGS-RNAi transgenic switchgrass lines. Switchgrass UBQ was used as the reference for normalization. Stems at the E4 stage were collected. Value are mean ± SE (n = 3). (d) Morphological characterization of transgenic switchgrass plants with down-regulation of CGS. The control plants (Ctrl) for CGS-RNAi transgenic lines were generated with pANIC8B empty vector.
observed. Moreover, no flowering was observed in the transgenic lines in our glasshouse conditions with 16-h light (Figure 2d, Table S2).

**Down-regulation of PvCGS affected Met cycling and amino acid accumulation**

To explore the effect of PvCGS down-regulation on Met metabolism, we measured the contents of intermediates in the Met cycle including SAM, SAH, Hcy and Met. As SAM, SAH and Hcy are fairly labile in tissue extracts, we determined their relative levels by ELISA method. Our results showed that the contents of SAH were reduced by 38.1%–40.8% in all selected transgenic lines compared with control plants. In contrast, large variations were detected in the levels of SAM and Hcy in the transgenic lines (Figure 3a, Figure S2). Finally, the dramatic reduction in SAH levels raised the ratios of SAM/SAH in all transgenic lines (Figure 3b). Furthermore, we studied the effect of PvCGS down-regulation on the accumulation of free Met and other amino acids, especially the Asp-derived ones. We randomly selected line CGSRI-1, combined with line CGSRI-3, for further amino acid profiling analysis. Our results showed that the contents of 19 of 26 amino acids detected in switchgrass stems were significantly altered in CGS-RNAi transgenic switchgrass lines (Table 1). Surprisingly, Met levels were little changed in the transgenic lines, although the levels of other Asp-derived amino acids were substantially increased, including Asp, Thr, Ile, asparagine (Asn) and lysine (Lys) (Table 1). Moreover, the transgenic lines accumulated 2.9- and 2.5-fold more phenylalanine (Phe) and tyrosine (Tyr) than control plants, respectively (Table 1).

**Transcriptome analysis of CGS-RNAi transgenic switchgrass plants**

To investigate the global impacts of down-regulation of PvCGS on free amino acid accumulation, lignin biosynthesis and plant growth, representative lines CGSRI-1 and CGSRI-3 were selected for RNA-seq analysis. Compared with control plants, a total of 847 transcripts, many of which are involved in the process of carbohydrate metabolism, the formation of precursor metabolites, and the generation of energy, were differentially expressed in transgenic lines (Figure S3). Transcript abundance of 14 genes responsible for amino acid biosynthesis was remarkably changed in transgenic lines (Table S3). Among them, the most interesting ones are those involved in the biosynthesis of Asp-derived amino acids. Closer inspection of the data revealed a fourfold increase in expression levels of asparagine synthetase (ASNS) in the transgenic lines (Table S3). In addition, transcript abundances of glutamate synthase (GOGAT), glutamate decarboxylase (GDH) and amidophosphoribosyltransferase (GPAT) were found to be threefold to sixfold reduced (Table S3).

Previous studies have suggested that CGS is a committed enzyme controlling Met biosynthesis, which could affect lignin biosynthesis by regulating SAM and SAH levels in plants (Hanson and Roje, 2001; Kim and Leustek, 2000). Thus, we examined expression levels of genes involved in Met cycling and lignin biosynthetic pathway in CGS-RNAi transgenic switchgrass lines. Our results showed that the transcript abundance of Met- and lignin-related genes, except eight class III peroxidases with fourfold to 10-fold increase, was not altered in transgenic lines compared with control plants (Table S3).

**Figure 3** The effects of CGS down-regulation on C1 metabolites and lignin biosynthesis in switchgrass. (a) The contents of SAM and SAH in control and transgenic switchgrass plants. (b) The ratios of SAM/SAH in control and transgenic switchgrass plants. The control plants (Ctrl) for CGS-RNAi transgenic lines were generated with pANIC8B empty vector. (c) Cross sections of the I2 internodes from transgenic switchgrass plants. The control plants were generated with pANIC8B empty vector. Stem at E4 stage was collected, and the different internodes were separated. Cross sections from three independent I2 internodes were used for histochemical assay. Two technical replicates were conducted. Bars = 0.1 mm. (d) G and S lignin monomer yield in CGS-RNAi transgenic switchgrass plants (Table 1).
Table 1: Amino acid profiling analysis of control and CGS-RNAi transgenic switchgrass plants

| Amino acids   | Fold change (CGS-Ri-1/Ctrl) | P-value     | Fold change (CGS-Ri-3/Ctrl) | P-value     |
|---------------|-----------------------------|-------------|-----------------------------|-------------|
| Phenylalanine | 3.19                        | 7.7E-07     | 2.58                        | 1.1E-05     |
| Tryptophan    | 3.51                        | 8.6E-05     | 3.10                        | 0.00034     |
| Creatinine    | 0.67                        | 0.01790     | 0.81                        | 0.16473     |
| Isoleucine    | 1.62                        | 0.00678     | 1.71                        | 0.00291     |
| Methionine    | 1.79                        | 0.11575     | 1.94                        | 0.06194     |
| Tyrosine      | 2.66                        | 9.2E-06     | 2.27                        | 8.0E-05     |
| Valine        | 2.56                        | 6.9E-05     | 2.20                        | 0.00047     |
| Proline       | 2.24                        | 0.00016     | 2.09                        | 0.00045     |
| Leucine       | 1.20                        | 0.52888     | 1.21                        | 0.52120     |
| Hydroxyproline| 6.18                        | 0.03141     | 7.64                        | 0.01203     |
| Threonine     | 8.53                        | 0.00154     | 8.44                        | 0.00167     |
| Alanine       | 4.62                        | 2.5E-05     | 3.36                        | 0.00065     |
| Creatine      | 0.53                        | 0.00594     | 0.54                        | 0.00722     |
| Glutamine     | 5.92                        | 0.00082     | 5.28                        | 0.00210     |
| Glutamate     | 1.30                        | 0.44510     | 2.17                        | 0.00267     |
| Glycine       | 8.18                        | 0.00015     | 6.16                        | 0.00159     |
| Serine        | 6.99                        | 5.2E-07     | 5.00                        | 1.5E-05     |
| Asparagine    | 51.38                       | 3.9E-08     | 54.48                       | 3.5E-08     |
| Citrulline    | 3.39                        | 0.35298     | 7.09                        | 0.01396     |
| Aspartate     | 3.34                        | 0.01402     | 5.34                        | 0.00025     |
| Histidine     | 3.69                        | 8.4E-05     | 3.62                        | 0.00010     |
| Arginine      | 3.68                        | 8.7E-06     | 3.96                        | 3.8E-06     |
| Lysine        | 8.90                        | 0.01069     | 10.18                       | 0.00441     |
| Ornithine     | 1.38                        | 0.86377     | 3.23                        | 0.04223     |
| Putresine     | 1.75                        | 0.03909     | 2.20                        | 0.00300     |
| Spermine      | 1.12                        | 0.62225     | 1.07                        | 0.82758     |

Switchgrass stems at E4 stage were collected for profiling analysis of amino acids using LC-MS/MS. The control plants (Ctrl) were produced with pANIC8B empty vector from the same batch of experiment. Three technical replicates and two biological replicates were conducted for amino acid profiling. The mean values were used for statistical analyses. Aspartate-derived and aromatic amino acids are shown in bold font.

As down-regulation of *PvCGS* caused an apparent growth stunting, we next studied expression levels of genes involved in hormone biosynthesis and signal transduction in transgenic switchgrass lines. A total of 14 hormone-related genes were differentially altered in the transgenic lines. Among them, expression of transcripts with homology to *GA1, IAA6-like, ARF, B-ARR, AFB* and *ACO* was dramatically down-regulated (Table S3).

**Down-regulation of *PvCGS* increased both G and S lignin contents**

To study the impact of down-regulation of *PvCGS* on lignin accumulation, we first employed phloroglucinol–HCl staining to indicate the variation in lignin content in transgenic switchgrass plants. In the control internode (I2), an apparent red coloration was specially exhibited in the well-lignified tissues such as fibres, sclerenchymas and vascular bundles (Figure 3c). The structure of vascular bundles in transgenic plants, however, resembled that of control plants (Figure 3c). To further confirm the enhanced lignin biosynthesis, lignin composition of stems of transgenic and control plants was detected by the thioacidolysis method. Lignin composition analysis revealed a 14% to 45% increase in G lignin monomer yield in transgenic lines (Figure 3d). Meanwhile, S lignin monomer yield was elevated by 18%–26% in the transgenic lines as well (Figure 3d).

**SAH competitively inhibited enzymatic activities of CCoAOMT and COMT**

Previous studies have suggested that SAH is a competitive inhibitor of methyltransferases in animal and microbe cells (Keating et al., 1991; Nguyen et al., 2001). To explore the impact of SAH alteration on lignin biosynthesis, in vitro enzymatic activities of CCoAOMT and COMT in crude plant extracts from wild-type switchgrass plants were measured with various ratios of SAM/SAH. Our results revealed that both CCoAOMT and COMT activities were significantly inhibited by SAH. Moreover, the ratios of SAM/SAH rather than SAM concentrations had stronger effects on enzymatic activities of CCoAOMT and COMT (Figure 4).

**Down-regulation of *PvSAHH1* increased SAH levels, reduced lignin accumulation and increased cell wall digestibility**

To further address the role of SAH in lignin biosynthesis, we manipulated SAH contents by down-regulation of *PvSAHH1* in switchgrass. Firstly, we cloned *PvSAHH1* (Pavir.1NG430900.1) and *PvSAHH2* (Pavir.6KG019200.1) from switchgrass, which share 96% similarity in amino acids. The phylogenetic relationship analysis shows that *PvSAHH1* and *PvSAHH2* are closely related to their orthologs in maize and rice (Figure 5a). However, *PvSAHH2* is not an intact gene, in which a 133-bp deletion in the N-termini causes a frameshift mutation (Figure 5b). Thus, we selected *PvSAHH1* for further studies. Temporal and spatial expression analysis of *PvSAHH1* revealed that *PvSAHH1* was highly expressed in well-lignified tissues (Figure S4) and had a positive correlation with expressions of CCoAOMT and COMT in wild-type switchgrass (Figure S5). To reduce SAH contents in switchgrass, we next produced 30 independent positive transgenic lines using RNAi technology. The control plants were generated with pANIC8B empty vector that was employed as the backbone for constructing SAHH-RNAi vector. Among them, three transgenic lines SAHHri-1, 22 and 36 with strongly down-regulated expression levels of *PvSAHH1* and normal phenotype were selected for further investigation (Figure 5c, d).

Enzyme-linked immunosorbent assay assay of intermediates in the Met cycle showed that the contents of SAH were increased by 59%–96% in transgenic switchgrass lines compared with control plants. In contrast, no consistent changes were detected in the levels of SAM and Hcy in these transgenic lines (Figure 6a, Figure 56). As a consequence, the increased SAH levels significantly reduced the ratios of SAM/SAH in all transgenic lines (Figure 6b). Furthermore, we studied the effect of elevating SAH levels on lignin biosynthesis in switchgrass. Our results showed that both G and S lignin contents were significantly reduced in the transgenic lines (Figure 6c). In addition, given the reduced lignin biosynthesis, we further assess the effect of *PvSAHH1* down-regulation on cell wall digestibility of transgenic switchgrass plants. As expected, the enzymatic hydrolysis efficiency of cell wall polysaccharides was improved by 22%–85% in the transgenic lines compared with the control plants (Figure 6d).

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Discussion

Lignocellulosic biomass mainly composed of cell walls is a sustainable source utilized as animal fodder and renewable fuels. However, the digestion of cell wall polysaccharides is not easy due to the presence of lignin (Marriott et al., 2016). To date, many strategies have been employed to manipulate lignin for the improvement of cell wall digestibility (Grabber et al., 2015; Loque et al., 2015). The most straightforward way is to regulate the expression levels of structural genes involved in lignin biosynthetic pathway or their transcription factors (Bonawitz and Chapple, 2010; Zhong and Ye, 2009). A system-wide analysis of Arabidopsis mutants and transgenic sorghum lines with altered lignin biosynthesis has revealed that shikimate, phenylpropanoid and methyl donor pathways are closely coordinated (Scully et al., 2018; Vanholme et al., 2012). Recent studies also suggest a direct link between C1 metabolism and monoligol methylation (Byerrum et al., 1954; Shen et al., 2002; Srivastava et al., 2015; Tang et al., 2014). The regulatory mechanism of C1 metabolites on lignin biosynthesis, however, is still elusive. We show here that altering SAH contents by down-regulating PvCGS or PvSAHH1 in switchgrass can affect the biosynthesis of both G and S lignins. This information provided allows identification of more potential targets for the use in lignin bioengineering through altering SAH production.

Cystathionine γ-synthase is a rate-limiting enzyme in Met biosynthesis. Compared with dicot species, the function of CGS has yet to be investigated in monocots. The overexpression of CGS in Arabidopsis, tobacco, alfalfa, soybean, azuki bean and potato has no effect on plant growth and development (Avraham et al., 2005; Hacham et al., 2008; Hanafy et al., 2013; Kreft et al., 2003). In contrast, down-regulation of CGS in Arabidopsis resulted in severe growth retardation and delayed flowering (Gakière et al., 2000; Kim and Leustek, 2000). A similar phenotype has been observed in CGS-RNAi transgenic switchgrass plants. In addition, cystathionine β-lyase (CBL) is a downstream enzyme that catalyses cystathionine to form Hcy in

\[ \text{Cystathionine} \xrightarrow{\gamma\text{-Synthase}} \text{Homocysteine} \]

Figure 4 The inhibition of SAH on caffeoyl-CoA O-methyltransferase (CCoAOMT) and caffeic acid O-methyltransferase (COMT) enzyme activities. Crude enzyme extracts were prepared from the stems of wild-type switchgrass plants at the E4 stage, and the extractable CCoAOMT (a) and COMT (b) enzyme activities were assayed with 10 μM caffeoyl CoA and 10 μM caffeic acid, respectively, at different SAM and SAH concentrations. Values are means ± SE (n = 3).
The effect of CGS on lignin biosynthesis was further studied in switchgrass. Our results show that the expression levels of \( \text{PvCGS} \) are raised during the process of internode lignification, consistent with the observation of \( \text{PvSAHH1} \) in switchgrass (Figure S4) and \( \text{FPGS} \), \( \text{MTHFR} \) and \( \text{SAMS3} \) in \( \text{Arabidopsis} \) and maize (Shen et al., 2002; Srivastava et al., 2015; Tang et al., 2014). Down-regulation of \( \text{CGS} \) in switchgrass increased the contents of SAH consistently. However, the levels of SAM and Hcy were varied among the transgenic lines, suggesting that the cells temperately adjusted their biosynthetic machinery to maintain the Met levels by controlling other metabolic flux towards SAM and Hcy. Nevertheless, all transgenic switchgrass lines still exhibited the elevated ratios of SAM/SAH. Furthermore, a remarkable inhibition of SAH on both CCoAOMT and COMT activities was observed in vitro. Thus, we suspect that the reduced SAH level was one of the reasons that can improve methylation efficiency of monolignols. To further test this assumption, we produced SAHH-RNAi transgenic switchgrass lines in which SAH levels were significantly increased and the ratios of SAM/SAH were dramatically decreased. As expected, the contents of G and S lignins were reduced and, as a consequence, cell wall saccharification efficiency was improved in the transgenic switchgrass plants. Thus, our results suggest that maintenance of appropriate SAH levels is crucial for lignin biosynthesis in switchgrass.

It is notable that a sufficient substrate supply is another key factor for high product yield in monolignol methylations apart from the enhanced methyltransferase enzymatic activity. Moreover, previous research has suggested that the overexpression of \( \text{COMT} \) cannot increase lignin accumulation in \( \text{Arabidopsis} \) (Goujon et al., 2003). Therefore, we attempt to seek whether other possibilities could enhance lignin biosynthesis in CGS-RNAi transgenic switchgrass plants. Amino acid profiling analysis revealed a significant increase in the contents of \( \text{Phe} \) and \( \text{Tyr} \). Recent study indicates that both \( \text{Phe} \) and \( \text{Tyr} \) catalysed by \( \text{PAL} \) and \( \text{TAL} \) in grass species can provide precursors for lignin biosynthesis (Barros et al., 2016). Thus, the elevated levels of \( \text{Phe} \) and \( \text{Tyr} \) as...
well as the reduced SAH inhibition for CCoAOMT and COMT contribute to the significant increase in G and S lignins in CGS-RNAi transgenic switchgrass plants. The mechanism inducing the accumulation of Phe and Tyr in the CGS-down-regulated background remains largely unknown, but it deserves future investigation.

In summary, we studied the function of CGS for the first time in monocot species. Our results showed that down-regulation of CGS in switchgrass enhanced lignin biosynthesis through elevating Phe and Tyr contents and reducing SAH levels. Moreover, SAH, as a strong inhibitor of monolignol methylation reactions, plays an important role in lignin biosynthesis. Genetic manipulation of SAH can impair both G and S lignin biosynthesis and therefore improve cell wall digestibility. Thus, our work suggests that genes involved in Met metabolism are new valuable targets for cell wall bioengineering.

**Experimental procedures**

**Plant materials and growth conditions**

A lowland-type of switchgrass cultivar, Alamo (2n = 4 x =36), was employed for genetic transformation and lignin modification. Switchgrass plants were grown in the glasshouse at 26 °C with 16-h light (390 μmol/m²/s). The development stages of switchgrass in our glasshouse were identified following the criteria described by Moore et al., 1991.

**Identification and cloning of PvCGS and PvSAHH1**

*Arabidopsis thaliana* nucleic acid sequences AtCGS (AT3G01120) and AtSAHH1 (AT4G13940) were used as a query to BLAST against the *Panicum virgatum* v4.1 genome sequences (Phytozome). *PvCGS* (Pavir.9NG556700) and *PvSAHH1* (Pavir.1-NO430900) were identified as the most homologous genes. The predicted cDNA sequences of *PvCGS* and *PvSAHH1* were used to design primers for cloning the open-reading frames of *PvCGS* and *PvSAHH1*. Alignment of multiple sequences and phylogenetic tree analysis of *PvCGS*, *PvSAHH1* and their orthologs in six genomesequenced species (*Panicum virgatum*, *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, *Nicotiana tabacum* and *Populus trichocarpa*) were conducted using MEGA 5 software suite.

**Gene expression pattern analysis**

Internodes, leaf sheaths and leaf blades of internode 2 (I2), internode 3 (I3) and internode 4 (I4) at the E4 stage were collected, and the I2 internodes at E2, E3, E4 and R1 stages were prepared, respectively. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the TRIZOL reagent according to the manufacturer’s supplied protocol (Thermo-Fisher Scientific) and subjected to reverse transcription with Superscript PrimeScript™RT reagent Kit (TaKaRa, Japan) after treatment with Turbo DNase I (TaKaRa, Japan). Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed to analyse the transcript abundance of *PvCGS* and *PvSAHH1* in switchgrass (Fu et al., 2012). The data were normalized against the reference genes of *PvUBQ* (GenBank accession no: HM209468). Primer pairs used for qRT–PCR are listed in Table S4.

**Generation of transgenic switchgrass plants**

A 457-bp and 480-bp cDNA fragments located in the conserved regions of *PvCGS* and *PvSAHH1*, respectively, were cloned into pANICBB, an RNAi binary vector (Mann et al., 2012). The primers used in gene cloning and RNAi vector construction were listed in Table S4. The verified recombinant constructs of pANICBB-CGSRi and pANICBB-SAHHRi were transferred into *Agrobacterium tumefaciens* strain AGL1.

A highly embryogenic callus line with single genotype generated by screening large scale of switchgrass Alamo seed-derived calli were used for *Agrobacterium*-mediated transformation following the procedure described by Xi et al. (2009). The independent positive transgenic lines were subjected to transcript abundance of endogenous *PvCGS* and *PvSAHH1* by qRT–PCR.
Characterization of plant growth and development

For morphological characterization, plant height and flowering time were measured using 6-month-old transgenic plants after cutting (Fu et al., 2012). The I2 internodes were used for measuring the internode length, internode diameter, leaf sheath length and leaf blade length and width.

RNA-seq analysis of transgenic switchgrass plants

Stems at the E4 stage were collected from control and CGS-RNAi transgenic switchgrass plants. Total RNA was extracted for RNA library preparation and deep sequencing. The genome sequences and annotated data of switchgrass were downloaded from phytozome. We aligned all reads from each sample to the reference genome of P. virgatum (v4.1) using tophat v2.1.0, allowing up to five mismatches (Trapnell et al., 2009). The expression levels of genes were measured using cufflink v2.1.1 (Trapnell et al., 2012), and the differential expression analysis between control and transgenic switchgrass plants was calculated using DEseq (Anders and Huber, 2010). The volcano plot and heatmap of differentially expressed genes were created using R package (Lawrence, 2016).

Amino acid profiling of transgenic switchgrass plants

Stems at the E4 stage were collected and homogenized in liquid nitrogen. For amino acid profiling analysis, 500 mg of powdered tissue was extracted and derivatized with diethyl ethoxymethylenemalonate as described by Cai et al. (2017). The aminonene derivatives were identified and quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The Agilent 1290 Infinity LC equipped with a Zic-HILIC column (3.5 μm, 2.1 mm × 150 mm) was employed for a high-resolution separation of amino acids. The injection volume was 2 μL. The mobile phase consisted of eluent A (25 mM HCOONH4 with 0.08% HCOOH) and eluent B (acetonitrile with 0.1% HCOOH), and separation was achieved using a linear gradient of B eluent (0–12 min, 90% B to 70% B; 12–18 min, 70% B to 50% B; 18–25 min, 50% B to 40% B; 25–30 min, 40% B) at a flow rate of 250 µL/min. The column was maintained at 40 °C. All the mass spectra were acquired using a mass selective detector (5500 QTRAP) coupled with an electrospray ionization (ESI) source. Mass spectra from positive-ion ESI were recorded over the range 50–2200 m/z. Boxplots of amino acids were generated using R package (Lawrence, 2016).

Measurement of SAM, SAH and Hcy by enzyme-linked immunosorbent assay (ELISA)

To detect the contents of SAM, SAH and Hcy, the stems of control and transgenic switchgrass plants at the E4 stage were collected. Approximately 50 mg fresh biomass was ground in phosphate buffered saline (0.01 M, pH = 7.2). After centrifuging at 2348 *g* for 15 min, the extracts were immediately used to measure the contents of SAM, SAH and Hcy according to the protocol of ELISA kit (Shanghai Enzyme-linked Biotechnology, China).

CCoAOMT and COMT enzyme activity assay

The stems of wild-type switchgrass plants were collected at the E4 stage and homogenized in liquid nitrogen. Powdered tissue (about 500 mg) was extracted for 3 h at 4 °C in extraction buffer (Liu et al., 2012) and then centrifuged at 17 900 *g* for 20 min at 4 °C. COMT and CCoAOMT activities in crude plant extracts were detected as described by Liu et al. (2012). The inhibition effect of SAH on CCoAOMT and COMT activities were determined in reactions with different ratios of SAM to SAH including 2:1, 1:1 and 1:2.

Histochemical assay

The I2 internodes were collected from the stems at the E4 stage for histochemical assay. The internode cross sections were stained with phloroglucinol–HCl reagent for lignin characterization as described previously (Chen et al., 2002). The micrographs were taken under a Nikon Microphot-FX system with a Nikon DXM 1200 colour camera (Nikon, Japan).

Thioacidolytic analysis of residual lignins

The stems were harvested at the E4 stage. The collected samples were ground in liquid nitrogen and lyophilized. Lyophilized extractive-free material was used for lignin analysis. The thioacido-lysis method was used to detect lignin composition (Lapierre et al., 1995). G and S lignins were identified and quantified by gas chromatography–mass spectrometry (GC-MS) using a Hewlett-Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column HP-1, 60 m × 0.25 mm, film thickness 0.25 μm).

Detection of saccharification efficiency

Saccharification efficiency of switchgrass cell walls was detected as described by Fu et al. (2011). The amount of fermentable sugars was measured by the phenol–sulphuric acid assay method (Dubois et al., 1956). Saccharification efficiency was determined as the ratio of sugars released by enzymatic hydrolysis to the amount of total sugars present in cell wall materials before enzymatic hydrolysis treatment.

Statistical analysis

Samples were collected from three biological replicates of each transgenic line and control plant except profiling analysis of amino acids. Three technical replicates and two biological replicates were conducted for amino acid profiling. The mean values were used for statistical analyses. Data from each trait were subjected to one-way ANOVA. The significance of treatments was tested at the *p* = 0.05 level. Standard errors were provided in all tables and figures as appropriate.

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Conflict of interest

The authors declare no conflict of interest.

References

Amir, R., Hacham, Y. and Galili, G. (2002) Cystathionine gamma-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. *Trends Plant Sci.* 7, 153–156.
Anders, S. and Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biol.* 11, R106.

Avraham, T., Badani, H., Galili, S. and Amir, R. (2005) Enhanced levels of methionine and cysteine in transgenic alfalfa (*Medicago sativa* L.) plants overexpressing the Arabidopsis cystathionine gamma-synthase gene. *Plant Biotechnol. J.* 3, 71–79.

Barros, J., Serranin-Yarce, J.C., Chen, F., Baxter, D., Venables, B.J. and Dixon, R.A. (2016) Role of bifunctional ammonia-lyase in grass cell wall biosynthesis. *Nat. Plants*, 2, 16050.

Bedon, F. and Legay, S. (2011) Lignin synthesis, transcriptional regulation and potential for useful modification in plants. *CAB Rev.: Perspect. Agric., Vet. Sci., Nutri. Natural Resources*, 6, 1–28.

Boerjan, W., Ralph, J. and Baucher, M. (2003) Lignin biosynthesis. *Curr. Opin. Plant Biol.* 5, 519–546.

Bonawitz, N.D. and Chapple, C. (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annu. Rev. Genet.* 44, 337–360.

Byerum, R.U., Flikstra, J.H., Dewey, L.J. and Ball, C.D. (1954) Incorporation of formate and the methyl group of methionine into methoxyl groups of lignin. *J. Biol. Chem.* 210, 633–643.

Cai, K., Xiang, Z., Li, H., Zhao, H., Pan, W. and Lei, B. (2017) Free amino acids, biogenic amines, and ammonium profiling in tobacco from different geographical origins using microfluidic-assisted extraction followed by ultra high performance liquid chromatography. *J. Sep. Sci.* 40, 4571–4582.

Carroll, A. and Somerville, C. (2009) Cellulosic biofuels. *Annu. Rev. Plant Biol.* 60, 165–182.

Chen, F. and Dixon, R.A. (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.* 25, 759–761.

Chen, L., Auh, C., Chen, F., Cheng, X., Apte, H., Dixon, R.A. and Wang, Z. (2002) Lignin deposition and associated changes in anatomy, enzyme activity, gene expression, and ruminal degradability in stems of tall fescue at different developmental stages. *J. Agric. Food Chem.* 50, 5558–5655.

Cohen, H., Isaeli, H., Matityahu, I. and Amir, R. (2014) Seed-specific expression of a feedback-insensitive form of CYSTATHIONINE-gamma-SYNTHASE in Arabidopsis stimulates metabolic and transcriptionic responses associated with desiccation stress. *Plant Physiol.* 166, 1575–1592.

Cohen, H., Hacham, Y., Panizel, I., Rogachev, I., Aharoni, A. and Amir, R. (2017a) Repression of CYSTATHIONINE-gamma-SYNTHASE in Seeds Recruits the S-Methylmethionine Cycle. *Plant Physiol.* 174, 1322–1333.

Cohen, H., Salomon, A., Tietel, Z., Hacham, Y. and Amir, R. (2017b) The relative contribution of genes operating in the S-methylmethionine cycle to methionine metabolism in Arabidopsis seeds. *Plant Cell Rep.* 36, 731–743.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.

Fu, C.X., Mielenz, J.R., Xiao, X.R., Ge, Y.X., Hamilton, C.Y., Rodriguez, M., Cai, K., Xiang, Z., Li, H., Zhao, H., Lin, Y., Pan, W. and Lei, B. (2017) The Arabidopsis thaliana -adenosyl-L-homocysteine hydrolase gene family encodes a functional polyglyctalamylate synthase. *Plant J.* 81, 493–504.

Keating, J.N., Trimble, K.C., Mulcahy, F., Scott, J.M. and Weir, D.G. (1991) The regulation and expression of the plant enzyme indicating methionine auxotrophy and partial methionine auxotrophy and developmental abnormalities. *Plant Sci.* 151, 9–18.

Kim, J., Lee, M., Chalam, R., Martin, M.N., Leustek, T. and Boerjan, W. (2002) Constitutive overexpression of cystathionine-gamma-synthase in Arabidopsis leads to accumulation of soluble methionine and S-methylmethionine. *Plant Physiol.* 128, 95–107.

R.A. (2016) Role of bifunctional ammonia-lyase in grass cell wall biosynthesis. *Plant Biotechnology Journal* 15, 210–228.

McLaughlin, S.B. and Kszos, L.A. (2005) Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass Bioenergy* 28, 515–535.

Moore, K.J., Moser, L.E., Vogel, K.P., Waller, S.S., Johnson, B.E. and Pedersen, J.F. (1991) Describing and quantifying growth-stages of perennial forage grasses. *Agron. J.* 83, 1073–1077.

Nguyen, B.N., Yablon, S.A. and Chen, C.Y. (2001) Hypodipsic hypernatremia –phenotypic and molecular characterization of lignocellulosic biomass through plant science. *Nat. Biotechnol.* 19, 973–983.

Grabber, J.H., Santoro, N., Foster, C.E., Elumalai, S., Ralph, J. and Pan, X.J. (2015) Incorporation of flavonoid derivatives or pentagalloyl glucose into lignin enhances cell wall saccharification following mild alkaline or acidic pretreatments. *Bioenergy Res.* 8, 1391–1400.

Hacham, Y., Matityahu, I., Schuster, G. and Amir, R. (2008) Overexpression of mutated forms of aspartate kinase and cystathionine gamma-synthase in tobacco leaved resulted in the high accumulation of methionine and threonine. *Plant J.* 54, 260–271.

Hanafy, M.S., Rahman, S.M., Nakamoto, Y., Fujiwara, T., Naito, S., Wakasa, K. and Ishimoto, M. (2013) Differential response of methionine metabolism in two grain legumes, soybean and azuki bean, expressing a mutated form of Arabidopsis cystathionine-gamma-synthase. *J. Plant Physiol.* 170, 338–345.

Hanson, A.D. and Roje, S. (2001) One-carbon metabolism in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 119–137.

Keating, J.N., Trimble, K.C., Mulcahy, F., Scott, J.M. and Weir, D.G. (1991) Evidence of brain methyltransferase inhibition and early brain involvement in HIV-positive patients. *Lancet.* 337, 935–939.

Kim, J. and Leustek, T. (2000) Repression of cystathionine gamma-synthase in Arabidopsis thaliana produces partial methionine auxotrophy and developmental abnormalities. *Plant Sci.* 151, 9–18.

Loque, D., Scheller, H.V. and Pauly, M. (2015) Engineering of plant cell walls for enhanced biofuel production. *Curr. Opin. Plant Biol.* 25, 151–161.

Maimann, S., Wagner, C., Kreft, O., Zeh, M., Wilmitzer, L., Hofgen, R. and Hesse, H. (2000) Transgenic potato plants reveal the indispensable role of cystathionine beta-lyase in plant growth and development. *Plant J.* 23, 747–758.

Mann, D.G., Lafayette, P.R., Abercrombie, L.L., King, Z.R., Mazaire, M., Halter, M.C., Poovaiah, C.R. et al. (2012) Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other species. *Plant Biotechnol. J.* 10, 226–236.

Marriott, P.E., Gomez, L.D. and McQueen-Mason, S.J. (2016) Unlocking the potential of lignocellulosic biomass through plant science. *New Phyto.* 209, 1366–1381.

McLaughlin, S.B. and Kszos, L.A. (2005) Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass Bioenergy* 28, 515–535.

Moore, K.J., Moser, L.E., Vogel, K.P., Waller, S.S., Johnson, B.E. and Pedersen, J.F. (1991) Describing and quantifying growth-stages of perennial forage grasses. *Agron. J.* 83, 1073–1077.

Nguyen, B.N., Yablon, S.A. and Chen, C.Y. (2001) Hypodipsic hypernatremia and diabetes insipidus following anterior communicating artery aneurysm clipping: diagnostic and therapeutic challenges in the amnestic rehabilitation patient. *Brain Inj.* 15, 975–980.

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapiere, C., Leple, J.C., Pollet, B. et al. (2002) Field and pulping performances of transgenic trees with altered lignification. *Nat. Biotechnol.* 20, 607–612.

Ragauskas, A.I., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J. et al. (2006) The path forward for biofuels and biomaterials. *Science*. 311, 484–489.

Rocha, P.S., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B. et al. (2005) The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING 1 gene codes for an S-adenosyl-L-homocysteine hydrolase
required for DNA methylation-dependent gene silencing. Plant Cell, 17, 404–417.
Roje, S. (2006) S-Adenosyl-L-methionine: Beyond the universal methyl group donor. Phytochemistry, 67, 1686–1698.
Scully, E.D., Gries, T., Palmer, N.A., Sarath, G., Funell-Harris, D.L., Baird, L., Twigg, P. et al. (2018) Overexpression of SbMyb60 in Sorghum bicolor impacts both primary and secondary metabolism. New Phytol. 217, 82–104.
Shen, B., Li, C. and Tarczynski, M.C. (2002) High free-methionine and decreased lignin content result from a mutation in the Arabidopsis S-adenosyl-L-methionine synthetase 3 gene. Plant J. 29, 371–380.
Srivastava, A.C., Chen, F., Ray, T., Pattathil, S., Pena, M.J., Avci, U., Li, H. et al. (2015) Loss of function of polypolyglutamate synthetase 1 reduces lignin content and improves cell wall digestibility in Arabidopsis. Biotechnol. Biofuels, 8, 224.
Tang, H.M., Liu, S.Z., Hill-Skinner, S., Wu, W., Reed, D., Yeh, C.T., Nettleton, D. et al. (2014) The maize brown midrib2 (bm2) gene encodes a methyleneetetrahydrofolate reductase that contributes to lignin accumulation. Plant J. 77, 380–392.
Trapnell, C., Pachter, L. and Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics, 25, 1105–1111.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H. et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.
Vilallobos, D.P., Brites, E.M., Said, E.S., Canas, R.A., Osuna, D., Van Kerckhoven, S.H.E., Bautista, R. et al. (2012) Reprogramming of gene expression during compression wood formation in pine: Coordinated modulation of S-adenosylmethionine, lignin and lignan related genes. BMC Plant Biol. 12, 100.
Xi, Y.J., Fu, C.X., Ge, Y.X., Nandakumar, R., Hisano, H., Bouton, J. and Wang, Z.Y. (2009) Agrobacterium-mediated transformation of switchgrass and inheritance of the transgenes. Bioenergy Res. 2, 275–283.
Zhang, X. and Liu, C.J. (2015) Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol. Plant, 8, 17–27.
Zhong, R. and Ye, Z.H. (2009) Transcriptional regulation of lignin biosynthesis. Plant Signal Behav. 4, 1028–1034.

Supporting information
Additional Supporting Information may be found online in the supporting information section at the end of the article:

Figure S1 Alignment of PvCGS and AtCGS amino acid sequences.
Figure S2 The contents of homocysteine (Hcy) in control and CGS-RNAi transgenic switchgrass plants.
Figure S3 Transcriptome analysis of CGS-RNAi transgenic switchgrass plants by RNA-seq.
Figure S4 Quantitative RT–PCR analysis of PvSAHH1 expression levels in different tissues.
Figure S5 Correlations between expression levels of PvSAHH1 and PvCOMT/PvCCoAMT.
Figure S6 The contents of homocysteine (Hcy) in control and SAHH-RNAi transgenic switchgrass plants.
Table S1 The temporal and spatial expression of PvCGS, PvSAMS, and PvSAHH in wild type switchgrass plants.
Table S2 Morphological characterization of CGS-RNAi transgenic switchgrass plants.
Table S3 Genes differentially expressed in CGS-RNAi transgenic switchgrass plants.
Table S4 Primers used in this study.