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RNAi-suppression of barley caffeic acid O-methyltransferase modifies lignin despite redundancy in the gene family

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Summary
Caffeic acid O-methyltransferase (COMT), the lignin biosynthesis gene modified in many brown-midrib high-digestibility mutants of maize and sorghum, was targeted for downregulation in the small grain temperate cereal, barley (Hordeum vulgare), to improve straw properties. Phylogenetic and expression analyses identified the barley COMT orthologue(s) expressed in stems, defining a larger gene family than in brachypodium or rice with three COMT genes expressed in lignifying tissues. RNAi significantly reduced stem COMT protein and enzyme activity, and modestly reduced stem lignin content while dramatically changing lignin structure. Lignin syringyl-to-guaiacyl ratio was reduced by ~50%, the 5-hydroxyguaiacyl (5-OH-G) unit incorporated into lignin at 10–15-fold higher levels than normal, and the amount of p-coumaric acid ester-linked to cell walls was reduced by ~50%. No brown-midrib phenotype was observed in any RNAi line despite significant COMT suppression and altered lignin. The novel COMT gene family structure in barley highlights the dynamic nature of grass genomes. Redundancy in barley COMTs may explain the absence of brown-midrib mutants in barley and wheat. The barley COMT RNAi lines nevertheless have the potential to be exploited for bioenergy applications and as animal feed.

Introduction
The properties of plant biomass are largely determined by its composition and in particular by the amount and structure of lignin. These properties influence the digestibility of crop biomass as animal feed (Gressel and Zilberstein, 2003) and its potential use as a renewable raw material for an emerging biorefinery industry producing biochemicals and biofuels (Gomez et al., 2008; Halpin et al., 2010; US-DOE, 2006). The lignin content of plant biomass is negatively correlated with saccharification, the enzymatic release of simple sugars (Chen and Dixon, 2007; Van Acker et al., 2013), while changing the relative proportions of different lignin units is associated with changes to digestibility (Mechin et al., 2005) and saccharification after acid pretreatment (Studer et al., 2011; Van Acker et al., 2013). The possibility of optimising the content and structure of lignin in biomass to facilitate processes such as biofuel production is a very active area of current research worldwide.

In the C4 grasses maize (Zea mays) and sorghum (Sorghum bicolor), mutations in certain lignin biosynthesis genes, including caffeic acid O-methyltransferase (COMT), give rise to a phenotype of brown midribs that is associated with lower lignin content and higher digestibility (Bout and Vermerris, 2003; Vignols et al., 1995). Such bm or bmr mutants are consequently marketed in the USA as superior forage and silage cultivars and some are reported to increase bioethanol yields (Dien et al., 2009). Most research has focussed on the maize bm3 COMT mutant which seems to have the greatest digestibility and feeding value improvement (Barriere et al., 2004). Although the lignin pathway is generally better characterised in dicots than monocots (Anterola and Lewis, 2002), COMT’s main role in both types of plant appears to be to methylate 5-hydroxyconiferaldehyde on the route to the synthesis of S units (Osakabe et al., 1999). Nevertheless, COMT is considered a multifunctional enzyme: in Arabidopsis it was shown to be involved in the biosynthesis of sinapate esters (Goujon et al., 2003), it has been annotated as a flavonol OMT (Muzac et al., 2000), and Sorgghum bicolor COMT can methylate the flavones luteolin and selgin (Eudes et al., 2017).

The brown-midrib phenotype has not been associated with COMT mutations in C3 grasses such as wheat (Triticum spp.) and barley (Hordeum vulgare), the dominant sources of straw biomass in temperate world regions. Substantial surplus wheat
straw is available globally that could be used as a raw material for bioenergy (Copeland and Turley, 2008; Kim and Dale, 2004) but wheat is not a particularly tractable genetic system for research because of its large polyploid genome. In contrast, barley is an inbreeding true diploid for which substantial genetic and bioinformatic genomic resources are available (Hein et al., 2009, Mascher et al., 2017; Saisho and Takeda, 2011), and it is readily and efficiently transformed (Harwood et al., 2008). Barley is a particularly good model for polyploid wheat, diverging from a common ancestor only ~8–9 mya (Middleton et al., 2014). Apart from its use as a research model, barley is the fourth largest global cereal crop by production with ~144 million metric tonnes produced in 2014 (FAOSTAT, 2014). It is a staple food in countries such as Ethiopia, but in temperate regions is cultivated primarily for use for malting and animal feed (Siafar et al., 2002). The straw can also be used as fodder and forage but has potential for use as a raw material for biorefineries producing chemicals and second generation biofuels. Consequently, we aimed to downregulate COMT in barley to demonstrate the value for agriculture and industrial biotechnology of improving straw digestibility in the small grain temperate cereals.

**Results**

**Identification of the COMT genes in barley**

BLAST searches were performed in sequence databases for a phylogenetic analysis to identify COMT genes in barley. However, COMT genes cannot be identified by phylogeny alone; the closely related genes CbCOMT1 and CbEMT of Clarkia breweri (black diamond on phylogenetic tree, Figure 1), encode O-methyltransferases with distinct substrate specificities, and only one is a COMT (Wang and Pichersky, 1999). Therefore, 13 conserved residues for COMT catalytic activity and binding/positioning of the substrates ferulic acid and 5-hydroxyconiferaldehyde (Zubieta et al., 2002) were used along with phylogenetic analysis to identify COMT genes. This approach identified three COMT genes in barley and, notably, only one in brachypodium (Figure 1, red highlighted cluster of the tree). The encoded proteins all contained 12 out of 13 of the conserved residues (isoaspartic l131 is substituted by a valine in several species) and the genes were annotated as HvCOMT1, HvCOMT2, HvCOMT3 and BdCOMT (see Figure S2 for alignment). All four genes encoded a conserved Ser123 known to be phosphorylated in poplar COMT, while only HvCOMT2 encodes Ser125, an alternative phosphorylation site in poplar (Wang et al., 2015). In the phylogenetic analysis these COMT genes clustered closely with two well characterised monocot COMT genes: the maize COMT (Zea mays; ZmCOMT) which is knocked out in the bm3 mutant due to insertions or deletions in the gene (Vignols et al., 1995) and the COMT gene from perennial yeagress (Lolium perrenne; LpOMT1) (Heath et al., 1998; Tu et al., 2010). Also in this clade was the single COMT gene in rice (Oryza sativa; OsCOMT) (Hamberger et al., 2007).

Several other genes from barley and brachypodium clustered closely with the monocot COMT genes clade (Figure 1, blue highlighted clusters) but all lacked some of the substrate binding/positioning residues and were therefore annotated as COMT-likes (see Figure S2, Table S4). For example, HvCOMT1 (previously described by Sugimoto et al., 2003), HvCOMT2, HvCOMT3, HvCOMT4, HvCOMT7, BdCOMT1 and BdCOMT2 have an alanine (A131) substituted for the asparagine (N131) in COMT genes. Asparagine N131 is important for binding oxygenated propene side-chains on lignin pathway intermediates whereas alanine A131 is important for non-oxygenated propene side-chains such as on eugenol (Louie et al., 2010; Wang and Pichersky, 1999). HvCOMT1, HvCOMT2, HvCOMT3, HvCOMT4, HvCOMT7, BdCOMT1 and BdCOMT2 lack a catalytic histidine (H269) which functions in deprotonating the hydroxyl group. HvCOMT5, HvCOMT6 and BdCOMT3 lack several of the conserved residues. COMT genes are expected to have diverse substrates and functions distinct from those of the ‘true’ COMTs that function in lignin biosynthesis. This highlights the importance of incorporating an evaluation of COMT conserved residues in phylogenetic analysis in order to identify true COMTs that use ferulic acid and 5-hydroxyconiferaldehyde substrates in lignin biosynthesis. Previous analyses of brachypodium genes based on homology alone identified four COMTs (Dalmais et al., 2013; Wu et al., 2013) but, of these, only Bd3g16530 is identified here as a true COMT and is denoted BdCOMT (BdCOMT6 in Dalmais et al., and BdCOMT4 in Wu et al.) while the other genes, in our analysis, are COMT-likes (BdCOMT1–3). Conversely, one of our barley COMTs, HvCOMT2, was previously suggested to be a flavone-specific O-methyltransferase (Zhou et al., 2008) but has all of the conserved residues of a functioning COMT and locates to the COMT clade.

Several rice COMT-like genes (Hamberger et al., 2007) clustered in a separate clade along with three barley genes. One of these barley genes was reannotated as an N-methyltransferase HvNMT involved in gramine biosynthesis by Larsson et al. (2006) from a previous erroneous annotation as a COMT gene (Lee et al., 1997), possibly suggesting that other genes in this clade might also be NMTs.

The three barley COMT genes (HvCOMT1(7H), HvCOMT2(3H) and HvCOMT3(6H)) are located on different chromosomes (see Table S1; Method S2). Barley chromosome 7H, where HvCOMT1 is located, shares some synteny with the genomic location of OsCOMT, BdCOMT and ZmCOMT (Bennetzen and Chen, 2008; Vogel et al., 2010). HvCOMT1, 2 and 3 are homologues of wheat COMT and OMT genes previously identified (Jung et al., 2008) (monocot COMT clade, Figure 1) and this is further supported by the shared synteny of the chromosome arms from wheat and barley that the genes mapped to (Table S5). BdCOMT1 and BdCOMT2 are a tandem duplication on chromosome 2 in brachypodium and HvCOMT1 and HvCOMT2 are on the syntenic barley chromosome 1H.

**Barley COMTs have different expression patterns**

To investigate which COMT genes were expressed in barley stems, real-time PCR was performed (delta-delta Ct method) on the 2nd internode and the internode beneath the peduncle at different developmental stages. No expression was detected for HvCOMT3 across the stages (Figure S5). In the internode beneath the peduncle when the spike was half to fully emerged, the expression of HvCOMT2 was 100-fold less than it was when the flag leaf was emerging. In contrast, there was only a tenfold difference in HvCOMT1 expression across the same developmental stages. Expressed sequence tags (ESTs) in HarveEST#35 for HvCOMT1 and HvCOMT2 also come from a range of tissues while all ESTs for HvCOMT3 are from roots (Table S6). Recent RNAseq data...
(Mascher et al., 2017) confirms HvCOMT3 is predominantly expressed in roots and embryos while HvCOMT1 and HvCOMT2 are expressed in lignifying tissues including stems, roots, lemma, palea, and rachis, but to different levels. In the same dataset, none of the HvCOMT-likes are expressed in stem tissue (Figure S6).

Strategy to downregulate COMT genes in barley stems

The expression analyses indicated possible redundancy between COMT genes expressed in stems and therefore RNAi was chosen as the strategy to downregulate both COMT genes. A 634 bp fragment from HvCOMT1 with 92% identity to HvCOMT2 (and 90.4% identity to HvCOMT3) was used to form the inverted repeat sequences of the hairpin in the pIPKb007 RNAi vector under the control of the constitutive maize ubiquitin promoter. Regenerated plants were screened to identify those where COMT genes were downregulated.

To determine an appropriate tissue and developmental stage to screen, we investigated O-methylation of caffeic acid in internodes at different developmental stages in the primary transformants (Figure S3a). Although caffeic acid can be O-methylated by enzymes other than COMT, the assay reflects, at least in part, COMT enzyme activity in planta. O-methylating activity varied with developmental stage with activity increasing, levelling off and then decreasing as internodes developed (Figure S3b). The second internode was chosen as the tissue to screen in plants 6 to 8 weeks after sowing, when activity is relatively high and stable (Figure S3b).

COMT RNAi lines have reduced COMT activity

Twenty-three independent primary transformants were assayed for reductions in COMT activity. Levels of biological variation differed between plants and this likely reflects slight differences in the developmental stage of replicate stems selected for assay. In several of the plants the activity was reduced to approximately 50% of the empty vector (EV) controls (Figure 2). In total, 12 lines were selected (11 lines with reduced activity and one line, COMTRNAi_26, which was not assayed). Southern analysis identified nine lines containing a single T-DNA locus (Figure S4) and eight of these (COMTRNAi_1, 4, 5, 9, 14, 19, 26 and 28) were taken forward to the T1 generation for detailed analyses.
COMT protein is substantially reduced in the COMT RNAi lines

To further characterise the lines, antibodies were raised against HvCOMT1 recombinant protein. Internodes from all lines showed substantial and similar reductions in COMT protein compared to the controls on western blots probed with the anti-COMT antibodies (Figure 3a). Consistent with the fact that the RNAi was expressed from a constitutive promoter, COMT protein was also substantially reduced in roots (Figure 3b). The western blot along with the enzyme assay from the primary transformants showed that COMT activity and protein were reduced in the stems of the COMT lines.

Expression of HvCOMT1 and HvCOMT2 is reduced in COMT RNAi stems

To investigate whether silencing of both HvCOMT1 and HvCOMT2 contributed to the reductions in COMT activity and
protein levels, the second internode was sampled for real-time PCR expression analysis when two nodes were present in the stem. The expression of both genes was reduced in the COMT lines compared to the controls with the expression of HvCOMT1 reduced by 20-80-fold while the reduction in HvCOMT2 expression was 5-40-fold (Figure 4a,b).

Lignin structure is dramatically changed in COMT RNAi stems

Extract-free straw from the T1 COMT lines was subjected to detailed lignin analysis. Two lines, COMTRNAi_4 and 26, had Klason lignin contents significantly lowered by 15% and 7% compared to their respective azygous controls ($P < 0.05$) (Figure 5a) while there was no significant difference in straw biomass (Figure S7). Lignin structure in the T1 COMT lines was evaluated by thioacidolysis. This analytical degradation specifically provides H, G and S thioethylated monomers from H, G and S lignin units only involved in labile β-O-4 bonds (Rolando et al., 1992), the major interunit bonds in native lignin. The yield of thioacidolysis products was significantly reduced in each of the lines by 20%–30% compared to the controls ($P < 0.05$) (Figure 5b) and the S/G ratio was significantly reduced by approximately 50% ($P < 0.05$) (Figure 5c). The reduction in S/G was accounted for by an approximate reduction of 30% in the proportion of S units in thioacidolysis products and a proportional increase of approximately 40% in G units (Table S7), consistent with COMT’s main role in the methylation of 5-hydroxyconiferaldehyde, a precursor of lignin S units. When COMT is downregulated, it is generally considered that the 5-hydroxyconiferaldehyde substrate accumulates and is reduced by cinnamyl alcohol dehydrogenase (CAD) to form 5-hydroxyconiferyl alcohol which is then incorporated into lignin as an unusual 5-OH-G unit. When subjected to thioacidolysis, the barley COMT lines released the 5-OH-G monomer at 10-15-fold higher levels compared to the wild-type control (Figure 5d) ($P < 0.05$). In addition to lignin-derived monomers, thioacidolysis provided free p-coumaric acid (pCA) and its EtSH addition product, both originating from pCA esters in the cell walls. Thioacidolysis yields of cell wall pCA from different grasses closely parallel the yields released by mild alkaline hydrolysis (Figure S8) demonstrating that they provide a true estimate of the amounts of pCA esters. In lignified grass cell walls, most pCA is ester-linked to S lignin units (Ralph et al., 1994). In the COMT lines, in agreement with the reduction in S-units, there was a significant reduction in the amount of pCA-derived thioacidolysis compounds (Figure 5e) ($P < 0.05$). By contrast to pCA units, COMT deficiency in barley did not systematically change the amount of ferulic acid (FA) and of its EtSH addition product released by thioacidolysis (Figure 5f), which suggests that cell wall-linked FA units (ester- and/or ether-linked) are not substantially affected. However, FA yields determined with thioacidolysis are an underestimate but are higher than estimates based on mild alkaline hydrolysis that breaks only the ester bonds.

NMR analysis was used to independently verify the major changes to lignin evident from thioacidolysis and to add further details. Barley lignin analysed by 2D NMR (Figure 5g,h) shows the typical dominance of G and S units (44% and 53% respectively) with minor contributions of H units (3%). As is typical of grass lignins, other aromatics are associated with the lignin component —pCA, an endunit on lignin side-chains (20% on an S + G + H = 100% basis, but over-represented due to its relaxation properties and tricin (3%), a flavone relatively recently described as a component of monocot lignins (Lan et al., 2015, 2016). A preponderance of β-aryl ether (β-O-4) units (93%) dominate with small contributions from phenylcoumaran (4%) and resinol (3%) units. In the COMT RNAi line, it is clear in the aromatic and double-bond regions of the spectra, that S units are relatively reduced and G units are increased, and H-units are essentially unchanged (Figure 5g). The spectra clearly show the benzodioxane structures (Figure 5h, structure D) that are diagnostically produced from the incorporation of the novel monolignol, 5-hydroxyconiferyl alcohol, with these structures representing some 6% in the sidechain analysis, but being
Lignin modification in barley by RNAi of COMT

(a) % Klason lignin
(b) Thioacidolysis yield
(c) S/G ratio
(d) S-G units
(e) Coumaric acid
(f) Ferulic acid
(g) EV
(h) EV

Plants with transgene
Azygotes

COMTRNAi_lines

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undetectable in the control line. The amount of tricin T was marginally reduced in the COMT line, dropping from 3% in the control to 2% in the COMT RNAi line. The level of pCA was apparently unchanged which is consistent with the thioacidolysis data’s showing no significant reduction in pCA in this particular line (COMTRNAi-Line 4), although the levels of thioacidolysis-released esterified cell wall pCA were reduced in other RNAi lines.

Extensive tissue sampling at various developmental stages in this work provided no evidence for differences in colour in the COMT RNAi lines compared to the controls in internodes, nodes, midribs, leaves or grains—even though the lignin content and structure was changed, no brown-midrib or gold hull phenotypes were evident.

**Metabolite changes in COMT RNAi lines**

In order to delve deeper into the consequences of COMT suppression at a molecular level, the bottom three internodes from two COMT RNAi lines were subjected to both transcript and metabolite profiling along with control lines. Internode phenolic metabolites were extracted and analysed via UHPLC-MS. Approximately 4924 profiled compounds had an abundance above 100 counts in at least one sample. Compounds (m/z traces) were selected for further consideration if their abundance was significantly ($P < 0.01$) different in both COMT RNAi lines compared to controls, showing at least a threefold change and an average abundance of ≥100 counts in either plant group. This generated a list of 130 m/z traces with a higher intensity in the COMT RNAi lines and six m/z traces with a lower intensity (Table S8). The 130 higher intensity m/z traces could be assigned to 108 compounds (some compounds give rise to more than one m/z trace). Based on accurate m/z, retention time and MS/MS fragmentation, we could characterize the structure of nine of the 108 compounds (Table 1, Figure S9). Four 5-hydroxyconiferyl alcohol-containing oligolignols were found to accumulate in the COMT RNAi lines; G (8-O-4)S(8-O-4)5-OH-G (compound 1), S(8-O-4)5-OH-G (compound 2), and two isomers of G(8-O-4)(5-O-4)S(8-O-4)-OH-G (compound 3 and 4). However, the m/z with the highest intensity was assigned to 5-hydroxyconiferyl alcohol linked to a hexose moiety (5-hydroxyconiferyl alcohol + hexose 1, compound 5). In addition, two other 5-hydroxyconiferyl alcohol conjugates could be structurally resolved: 5-hydroxyconiferyl alcohol + hexose 2 (compound 6) and 5-hydroxyconiferyl alcohol + acetylhexose (compound 7). Also two caffeoyl alcohol conjugates were found to accumulate in the COMT RNAi lines: caffeoyl alcohol + hexose (compound 8) and caffeoyl alcohol + acetylhexose (compound 9).

The six m/z traces with a lower intensity in COMT lines originated from six different compounds, two of which could be structurally characterized (Table 1, Figure S9). Both were oligolignols which contain only S subunits: Sox(8-O-4)S (9) and S(8-O-4) Sox(8-O-4)S.

**Transcriptome changes in COMT RNAi lines**

To evaluate the effect of COMT downregulation on gene expression in internodes, transcript profiling was performed on two COMT RNAi lines and control lines. Genes that were significantly ($P < 0.01$) differentially regulated in both COMT RNAi lines compared to controls were filtered for those showing at least a threefold change. Only four genes were substantially up-regulated in COMT RNAi lines according to these criteria (Table S9); a lectin-like receptor protein kinase, a protein of unknown function (and questionable gene model), an F-box protein, and a methyl esterase. There were 14 genes significantly down-regulated in COMT RNAi plants; HvCOMT1 itself was most reduced by 24-fold compared to controls. Other down-regulated genes included a galactan synthase, HORVU6Hr1G092840.2 encoding an OMT enzyme with unknown substrate, two zinc finger transcription factors, a F-box protein, a kinase regulator and a cyclin (Table S9). HvCOMT2 expression was reduced by 13-fold and 17-fold in the two COMT RNAi lines and would have been the second most greatly repressed gene but missed our stringent significance threshold due to variation within the controls ($P$ values of 0.13 and 0.07). As anticipated from our earlier expression analysis, expression of HvCOMT3 was not detected in control or RNAi internodes.

**Saccharification of some COMT RNAi lines is increased**

Straw from the COMT RNAi lines was subjected to saccharification without a pretreatment and after an acid pretreatment (Figure 6a,b). All RNAi lines showed a promising and consistent trend of higher saccharification compared to their corresponding azgote lines and wild-type, but the difference was only statistically significant for lines COMTRNAi_1 and 28 without a pretreatment, and for lines COMTRNAi_1 and 14 after the acid pretreatment, representing approximately 16%–20% improvements in sugar release.

**Discussion**

We show here that barley has a larger COMT gene family than brachypodium or rice suggesting COMT duplication in the barley lineage since its evolution from a common ancestor. This is consistent with the extensive gene duplication and expansion of specific gene families revealed in the barley reference sequence (Mascher et al., 2017). All three barley COMTs retain the amino acid residues essential to COMT activity and are preferentially expressed in lignifying tissues strongly suggesting that all three functions in lignin biosynthesis. Nevertheless, duplication seems to have been followed by some divergence in expression pattern, possibly reflecting subfunctionalization in different tissues or cell types (Ober, 2010). Several COMTs previously identified in wheat (Jung et al., 2008; Ma and Xu, 2008; Wang et al., 2018) are homologues of the barley COMT genes. COMT duplication events have also been noted in ryegrass (Lolium perenne) (van Parijs et al., 2015).

Given the redundancy in barley COMT genes, RNAi was an appropriate silencing strategy and was effective in suppressing both HvCOMT1 and HvCOMT2. Reductions in enzyme activity in the primary transformants were relatively moderate compared to reductions in HvCOMT expression and protein levels. This may reflect greater specificity of the antibodies compared to the enzyme assay where other O-methyltransferases might contribute background activity. Similarly in the maize bm3 mutant, anti-COMT antibodies could not detect residual COMT protein but enzyme activity was merely reduced (Piquemal et al., 2002).

Nevertheless, expression of HvCOMT1 and HvCOMT2 is not abolished in our barley RNAi lines, COMT protein and activity are still present, albeit greatly reduced to levels sufficient to cause significant changes to lignin content and structure.

Lignin content was reduced in two barley COMT RNAi lines by 10%–15%. This compares to reductions in Klason lignin content of 25% and 28% when COMT was suppressed in maize (Piquemal et al., 2002) and to reductions of up to 16% of acetyl bromide lignin when COMT was suppressed in perennial ryegrass (Tu et al., 2010). Comparisons are complicated, however,
because lignin content was measured at different developmental stages and by different methods in each study. Reduced thioacidolysis yields in the COMT RNAi lines are an indication of stages and by different methods in each study. Reduced

Table 1 List of structurally characterized compounds with different abundance in the internodes of COMT RNAi lines as compared to controls

| Number | m/z 1 | Name | m/z 2 | COMT RNAi lines | EV | WT |
|--------|-------|------|-------|----------------|----|----|
|        | TR | experimental | theoretical | Δppm | S.E.M. | Mean ± S.E.M. | Mean ± S.E.M. | Mean ± S.E.M. |
| 1      | 13.10 | 209.0804 | S(8-O-4)-OH-G | 209.0819 | −7.10 | 675 ± 354 | 120 ± 103 | b.d.l. | b.d.l. |
| 2      | 13.36 | 179.0692 | G(8-O-4)-OH-G | 179.0714 | −12.30 | 493 ± 199 | 110 ± 103 | b.d.l. | b.d.l. |
| 3      | 14.86 | 599.2137 | G(8-O-4)(8-O-4)-OH-G | 599.2134 | 0.60 | 15 092 ± 4356 | 3374 ± 2229 | b.d.l. | b.d.l. |
| 4      | 15.55 | 599.2132 | G(8-O-4)(8-O-4)-OH-G | 599.2134 | −1.80 | 4291 ± 1238 | 920 ± 759 | b.d.l. | b.d.l. |
| 5      | 3.73  | 357.1227 | 5-hydroxyconiferyl alcohol + hexose 1 | 357.1191 | 10.10 | 234 359 ± 49 825 | 69 993 ± 38 025 | 1107 ± 311 | 1096 ± 780 |
| 6      | 2.76  | 357.1194 | 5-hydroxyconiferyl alcohol + hexose 2 | 357.1191 | 0.80 | 947 ± 127 | 437 ± 160 | b.d.l. | b.d.l. |
| 7      | 5.83  | 399.1301 | 5-hydroxyconiferyl alcohol + acetyl hexose | 399.1297 | 0.90 | 4843 ± 1186 | 1334 ± 668 | b.d.l. | b.d.l. |
| 8      | 3.69  | 327.1087 | Caffeoyl alcohol + hexose | 327.1085 | 0.80 | 10 753 ± 790 | 4888 ± 1876 | 1006 ± 397 | 1037 ± 521 |
| 9      | 5.91  | 369.118  | Caffeoyl alcohol + acetyl hexose | 369.1191 | −3.10 | 2428 ± 355 | 901 ± 331 | b.d.l. | b.d.l. |

Compounds with reduced abundance in COMT RNAi lines

| Number | m/z 1 | Name | m/z 2 | COMT RNAi lines | EV | WT |
|--------|-------|------|-------|----------------|----|----|
|        | TR | experimental | theoretical | Δppm | S.E.M. | Mean ± S.E.M. | Mean ± S.E.M. | Mean ± S.E.M. |
| 10     | 12.53 | 433.1504 | Sox(8-O-4)S | 433.1504 | 0.00 | 1715 ± 1116 | 292 ± 382 | 3081 ± 2671 | 4395 ± 5849 |
| 11     | 14.48 | 659.2330 | S(8-O-4)(8-O-4)-SOx(8-O-4)S | 659.2346 | −2.50 | 101 ± 135 | b.d.l. | 896 ± 1142 | 1080 ± 1686 |

Compounds detected as in-source fragments as described in Figure S9. Images of these structurally characterised compounds listed above are included in Figure S9. m/z: mass difference between m/z experimental and m/z theoretical in parts per million, S.E.M.: standard error of the mean, b.d.l.: below detection limit (set at 100 counts). For full method see Method S1.
et al. (2010), in seeds of vanilla and in several cacti (Chen et al., 2012). However, our data are the first in planta evidence that the bypass-route via ckafealdehyde also occurs in grasses.

The changes described in lignin content and structure in COMT RNAi plants are likely to be beneficial for saccharification and digestibility, and moderate increases to saccharification were measured in some lines. Reduced lignin content is generally correlated with improvements in saccharification (Chen and Dixon, 2007) and downregulation or mutation of COMT has increased saccharification and/or biofuel production in switchgrass and sorghum (Dien et al., 2009; Fu et al., 2011; Saballos et al., 2008; Van Acker et al., 2013). The effect of the proportion of S units in lignin on digestibility is controversial; one study claims that the structure of lignin does not affect fermentation by ruminant microflora (Grabber et al., 2009) while another found an inverse correlation between digestibility and S lignin content (Mechin et al., 2005). Effects on saccharification are likely to depend on the pretreatment used, as reported by Studer et al. (2011). Incorporation of 5-OH-G units into lignin has been hypothesised as beneficial for saccharification; the quinone methide that forms during monomer coupling can be internally trapped by the -OH group on a 5-OH-G unit in lignin forming benzodioxane units instead of linking to polysaccharides, and that reduction in cross-linking is likely to improve the access for saccharifying enzymes (Ralph et al., 2004; Vanholme et al., 2012a,b).

COMT duplication events in barley and wheat are sufficient to explain why no brown-midrib or gold-hull mutants associated with COMT have been identified in these small grain temperate cereals. We have evidence that orange lemma mutants are the barley equivalent of maize brown-midrib and rice gold-hull but none of the orange lemma mutants we have characterised are mutants in COMT (Stephens J, Reetoo N, Daly P, Waugh R, Druka A, Lapierre C and Halpin C, unpublished). Contrary to previous reports (Dalmais et al., 2013; Wu et al., 2013), our phylogenetic analysis identified a single true COMT gene in brachypodium, suggesting that brown-midrib phenotypes might emerge if COMT was fully knocked out in this species. Various hypotheses were proposed to explain why brown-midrib phenotypes had not been seen in C3 grasses, but brachypodium plants with brown midrids (or brown-red lignified tissues) were recently described; all were plants severely suppressed or mutated in CAD (Trabucco et al., 2013; d’Yvoire et al., 2013). The existence of brachypodium plants sufficiently deficient in COMT to be expected to develop brown-midrib phenotypes has not been definitively evidenced. A mutant in the brachypodium lignin COMT has been identified but displays only moderately altered lignification and the mutant enzyme is still functional (Ho-Yue-Kuang et al., 2016). Similarly, transgenic plants overexpressing artificial microRNA designed to silence brachypodium COMT did not have significant changes to S lignin (Trabucco et al., 2013) suggesting that they were not sufficiently COMT-suppressed. Consequently, it is likely that a full knock-out of COMT in brachypodium (or other species) will be necessary before brown-midrib phenotypes are seen or their absence can reasonably prompt other explanations. In this context, it is interesting that COMT is reported to be the third most abundantly expressed gene in poplar stem-differentiating xylem, accounting for 6% of the proteome (Lin et al., 2013; Shuford et al., 2012) and its near absence is thought necessary before S lignin content is reduced (Wang et al., 2014). In barley and wheat, the difficulties in effectively silencing gene activity to

Figure 6 Saccharification of the COMT RNAi lines and controls from the T1 generation (a) without a pretreatment and (b) after an acid pretreatment. Lines marked with a * are significantly different to the azygous controls or to the wild-type where no azygous controls were available (Student’s t-test, P < 0.05).

COMT RNAi plants. Benzodioxane oligolignols have also been found in COMT-deficient poplar and Arabidopsis (Morreel et al., 2004; Vanholme et al., 2010, 2012a,b). Not all 5-hydroxyconiferyl alcohol may be used for lignification, however. Hexose and acetylhexose conjugates of 5-hydroxyconiferyl alcohol (compound 5-6 and 7, respectively) also accumulate in COMT RNAi plants and may be destined for vacular storage (Dima et al., 2015).

A striking observation is the accumulation in COMT RNAi plants of caffeoyl alcohol conjugated to hexose (compound 8) or acetyl hexose (compound 9). This suggests that either caffeoyl alcohol or caffealdehyde serve as a substrate for HvCOMT1, HvCOMT2 or both. Caffealdehyde has long been considered as an intermediate of the lignin pathway in several plant species (reviewed in Boerjan et al., 2003). A biosynthetic route to coniferaldehyde of caffeoyl-CoA → caffealdehyde → coniferaldehyde, catalysed by CCR and COMT, would bypass the more commonly described route caffeoyl-CoA → feruloyl-CoA → coniferaldehyde, catalysed by CCoAOMT and CCR. This bypass-route has been shown to be present in alfalfa (Lee et al., 2011; Parvathi et al., 2001; Zhou et al., 2010). Caffeoyl alcohol has also been found as a monomer in lignin of CCoAOMT downregulated Pinus radiata (Wagner et al., 2011), in seeds of vanilla and in several cacti (Chen et al., 2012). However, our data are the first in planta evidence that the bypass-route via caffealdehyde also occurs in grasses.

The changes described in lignin content and structure in COMT RNAi plants are likely to be beneficial for saccharification and digestibility, and moderate increases to saccharification were measured in some lines. Reduced lignin content is generally correlated with improvements in saccharification (Chen and Dixon, 2007) and downregulation or mutation of COMT has increased saccharification and/or biofuel production in switchgrass and sorghum (Dien et al., 2009; Fu et al., 2011; Saballos et al., 2008; Van Acker et al., 2013). The effect of the proportion of S units in lignin on digestibility is controversial; one study claims that the structure of lignin does not affect fermentation by ruminant microflora (Grabber et al., 2009) while another found an inverse correlation between digestibility and S lignin content (Mechin et al., 2005). Effects on saccharification are likely to depend on the pretreatment used, as reported by Studer et al. (2011). Incorporation of 5-OH-G units into lignin has been hypothesised as beneficial for saccharification; the quinone methide that forms during monomer coupling can be internally trapped by the -OH group on a 5-OH-G unit in lignin forming benzodioxane units instead of linking to polysaccharides, and that reduction in cross-linking is likely to improve the access for saccharifying enzymes (Ralph et al., 2004; Vanholme et al., 2012a,b).

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near abolition are likely to be exacerbated when more than one COMT gene needs to be suppressed. For example, our microarray data comparing the COMT RNAi lines with controls showed that, despite efficient gene downregulation, HvCOMT1 and HvCOMT2 expression could still be detected at 4% and 6%–7% of control plant values, respectively.

The ability to modify lignin differentially in specific tissues would also have great value in lignin engineering, for example enabling the production of crops that have more digestible stems (less lignin) and roots that sequester more carbon in soil (more lignin). The kind of gene duplication and expansion events described here for barley COMTs could in some cases enable such tissue specific manipulation, if gene sequences and expression patterns have diverged sufficiently to allow individual genes expressed in specific tissues to be targeted for suppression by RNAi. Tissue specific promoters might also place appropriate limitations on RNAi expression, albeit with the complication that small silencing RNAs might move between tissues.

The advent of CRISPR-mediated targeted gene manipulation in plants offers real possibilities for more precise and effective gene manipulations. By careful selection of guide RNA sequences, several homologous genes (multiple gene family members, such as HvCOMT1 and HvCOMT2, or homoeologous genes in polyploid species) can be targeted for mutation while other closely related genes are avoided. Knock out of multiple genes in polyploid species) can be targeted for mutation while other closely related genes are avoided. Knock out of multiple COMT genes/homeologues in stems of barley and wheat might provide improved cereal straw for use as animal feed or as a feedstock for industrial processing in temperate regions of the world.

Materials and methods

Sequence retrieval and phylogenetic analysis

Barley, brachypodium and rice sequences with >40% identity to maize ZmCOMT (M73235) (Collazo et al., 1992) were retrieved from sequence databases and used for phylogenetic analysis along with published COMT genes from perennial ryegrass (Heath et al., 1998), sorghum (Bout and Vermerris, 2003), alfalfa (Zubieta et al., 2002), switchgrass (Fu et al., 2011), sugarcane (Jung et al., 2012; Selman-Housein et al., 1999), arabidopsis COMT and COMT-like genes from (Raes et al., 2003), several wheat COMT or OMT genes (Jung et al., 2008; Ma and Xu, 2008; Wang et al., 2018), and two Clarkia brewer genes (Wang and Pichersky, 1999). After importing aligned sequences into MEGA7, a maximum likelihood (ML) tree was constructed with JTT (Jones et al., 1992) + G + I as the model with five discrete gamma categories. All sites from the Gblocks-selected subset of the alignment (Figure S1) were used. Nearest-Neighbour Interchange was used as the ML heuristic method and the initial tree was made automatically. The topology of the tree was tested with 100 bootstrap replicates. Table S1 and Method S1 give more information on the genes, databases and methods used.

Examination of potential COMT genes for the presence of conserved residues for COMT function

The initial alignment (before Gblocks removal of poorly aligned regions) visualised with ESPript 3.0 (Robert and Gouet, 2014) is included as Figure S2. The sequences were examined for the presence of the conserved residues for COMT function characterised by Zubieta et al. (2002) in alfalfa (Medicago sativa) MsCOMT.

Plant materials, growth conditions and designain of internodes

Barley (H. vulgare ssp. vulgare cv. Golden Promise) was grown in a greenhouse with supplementary lighting from high pressure sodium vapour lamps. Plants for root sampling were grown in 50 : 50 sand and perlite. The internode nearest the crown greater than 1 cm long was designated the first internode, as in Tottman (1987).

Crude protein extraction and quantification

An appropriate tissue and developmental stage to assay was determined by investigating O-methylation of caffeic acid in internodes at different developmental stages (Figure S3a,b). Crude protein was extracted from 1 cm internode base by crushing in a 1.5 mL eppendorf in extraction buffer (100 mM Tris-HCl pH 7.5, 20 mM β-ME, 2% w/v PVPP, 2% w/v PEG, 1x Complete (Roche, UK)). Extracts were clarified by centrifugation and protein concentrations determined (Bradford, 1976) using the Bio-Rad reagent (Bio-Rad) and BSA standard.

Caffeic acid O-methyltransferase enzyme assay

The protocol of Fukuda and Komanine (1982) was used with modifications. Crude protein was incubated in 300 μl reactions containing 1.2 KBq S-adenosyl-L-3H-methyl-L-methionine (SAM) (Perkin Elmer, MA), 100 mM sodium ascorbate, 10 mM MgCl2, 1 mM caffeic acid (Sigma, UK) and 0.1 M potassium phosphate pH 7.5, and incubated at 30 °C for either 30 min or 3 h. The radioactive product was extracted with ethyl acetate and measured using a TriCarb 3100 TR scintillation analyser (Packard, CT).
PAGE and western blotting

Denatured crude protein was separated by SDS-PAGE on 4%–12% NuPage™ Bis-Tris precast gels (Invitrogen) (roots) or 10% homemade acrylamide gels (internodes). Proteins were electrobotted onto Amersham HyperBlot ECL nitrocellulose membranes (GE Healthcare, UK). Membranes were blocked with 5% w/v milk powder in tris buffered saline pH 7.5, 0.1% v/v Tween-20, washed, incubated with primary antibody (1 : 10 000), washed, incubated with HRP-conjugated anti-rabbit IgG (1 : 10 000) (NEB, UK), detected using LumiGLO® and Peroxide Reagents (NEB) and visualized with Amersham Hyperfilm ECL (GE Healthcare). The generation of a recombinant HvCOMT1 to raise antibodies is described in Method S3.

Klason lignin and thioacidolysis

T1 generation straw (leaves removed) were ground to pass a 0.5 mm screen. Extract-free samples were prepared by exhaustive extraction with water, then ethanol. Klason lignin was measured according to Dence (1992). Lignin structure was evaluated by thioacidolysis followed by gas chromatography-mass spectrometry (GC-MS) of lignin-derived monomers analysed as their trimethylsilyl derivatives (Lapierre et al., 1999; Rolando et al., 1992). The thioacidolysis compounds derived from p-coumaric or ferulic acid (i.e. free acid and its EtSH addition product) were also quantified to evaluate the amount of cell wall-linked p-coumaric and ferulic units.

Cell wall characterization by two-dimensional solution-state NMR

Cell walls were characterised without fractionation using two-dimensional (2D) solution-state NMR (Kim and Ralph, 2010; Mansfield et al., 2012). Straw (2-mm pieces) was pre-ground using a Mixer Mill MM400 (Retsch; 30/s vibrational frequency for 90–120 s). Samples were extracted three times with water, three times with 80% ethanol and once with acetone, then allowed to dry. The pre-ground extracted samples were ball-milled using a Fritsch Planetary micro mill Pulverisette 7 vibrating at 600 rpm with zirconium dioxide (ZrO₂) vessels containing ZrO₂ ball bearings (10 mm × 10) with 5-min milling and a 5-min cooling per milling cycle (cycle number depended on the amount of sample). The ball-milled samples were subjected to digestion (72 h × 2) to obtain ‘enzyme lignin’ (EL) by Celulysin® Cellulase, Trichoderma viridae (Calbiochem), at 35 °C in acetate buffer (pH 5.0). The EL were dissolved into DMSO-d₄/pyridine-d₅ (4 : 1) and subjected to NMR on a Bruker Biospin AVANCE-III 700 MHz spectrometer equipped with a 5-mm QCI 1H/13C/15N cryoprobe with inverse geometry (proton coil closest to the sample). 2D-1H-13C HSQC spectra were acquired using Bruker’s pulse program (hsqcetgpsip2.2). Bruker’s Topspin 3.2 (Mac) software was used to process spectra. The central DMSO peak was used as internal references (δC: 39.51, δH: 2.49 ppm).

Transcript and metabolite profiling

Five plants per line were grown for 61 days in a randomised block design. The bottom three internodes were collected, frozen and ground in liquid nitrogen, and each sample divided into two aliquots, one for transcriptome analysis and one for metabolite analysis. See Method S5 and S6 for full details.

Saccharification analyses

The same extracted sample used for lignin analysis (30 mg) was pretreated with 450 μl 1% w/v sulphuric acid in an autoclave (Astell, UK) at 121 °C for 1 h in 2 mL tubes or saccharified without pretreatment. Solids were washed three times with 1.5 mL 25 mm sodium acetate pH 4.5. Saccharifying enzyme mixture (Celluclast and Novozyme 188 (Sigma)) was prepared as described in Gomez et al. (2010). The FPU (filter paper unit) activity (65 FPU/mL) of the purified mixture was measured (Adney and Baker, 1996) along with β-glucosidase activity (95.7 CBU/mL) (Ghose, 1987). Saccharification was performed with an enzyme loading of 0.6 FPU per 30 mg of sample in 25 mm sodium acetate pH 4.5 with 0.02% w/v NaN₃ in a total volume of 1.5 mL for 72 h at 50 °C with shaking. Triplicate reactions were performed per plant. Glucose released was quantified using the GOPOD assay kit (K-GLUC) (Megazyme, Ireland) scaled for a 96-well plate and expressed as a proportion of the 30 mg extracted sample.

Statistical analysis

For most analyses, a Student’s t-test was used in Excel (Microsoft) with the option for unequal variances selected where sample sizes differed. For the metabolomics/transcriptomic experiment, model-adjusted means were used. Only metabolites/probes whose combined mean was at least threefold and significantly (P < 0.01) different from combined controls in both COMT RNAi lines was considered to be meaningfully different.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

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

Method S1 Sequence retrieval, data sources and multiple alignment

Method S2 Determination of the genomic location and evidence for expression of barley genes

Method S3 Generation and purification of a recombinant HvCOMT1 protein for antibody production

Method S4 Southern Blot

Method S5 RNA extraction and microarray processing for transcriptome analysis

Method S6 Phenolic metabolome analysis

Figure S1 The Gblocks selected parts of the original alignment used to construct the phylogenetic tree for Figure 1.

Figure S2 Alignment of the genes from the phylogenetic analysis demonstrating the absence or presence of conserved residues for COMT function.

Figure S3 Investigation of O-methylation of caffeic acid in barley internodes at different developmental stages; one of the biological replicate plants from the succession sampled at each time point is shown with the number of weeks after sowing indicated.

Figure S4 Southern blot analysis for T-DNA locus number of the COMT lines which had reduced enzyme activity.

Figure S5 The expression of HvCOMT1 and HvCOMT2 genes in internodes at different developmental stages.

Figure S6 Gene expression levels for all of the barley genes from the phylogenetic analysis for which data were available in a 16-tissue RNAseq dataset described by Mascher et al. (2017).

Figure S7 Biomass measurements of COMT RNAi lines.

Figure S8 Correlation between the amounts of p-coumaric acid (CA) released by thioacidolysis and mild alkaline hydrolysis.

Figure S9 MS-based structural elucidation of the differentially accumulating m/z traces in COMT RNAi lines as compared to empty vector and wild-type controls.

Table S1 (Excel file) Further information on the genes from the phylogenetic analysis in Figure 1

Table S2 Primer sequences used in experiments in this study

Table S3 Summary of reaction set-up and cycling conditions for real-time PCR

Table S4 The number of the conserved residues present for the binding/positioning of COMT substrates ferulic acid and 5-hydroxyconiferaldehyde, as identified by (Zubieta et al., 2002) in MsCOMT

Table S5 Demonstration of the shared synteny between the barley and wheat chromosome arms that the barley COMT genes and homologous wheat gene(s) map to

Table S6 Summary of the source tissue of ESTs for HvCOMT1, HvCOMT2 and HvCOMT3 from HarvEST #35

Table S7 Summary of lignin data from the COMT RNAi lines

Table S8 m/z traces with a different intensity in the internodes of COMT RNAi lines as compared to empty vector and wild-type controls

Table S9 Microarray transcriptome analysis of internodes from two COMT RNAi lines (COMTRNAi_4 and COMTRNAi_14) and controls (empty vector and wild-type)