Cell wall composition and lignin biosynthetic gene expression along a developmental gradient in an Australian sugarcane cultivar (#19252)

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Cell wall composition and lignin biosynthetic gene expression along a developmental gradient in an Australian sugarcane cultivar

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Sugarcane bagasse is an abundant source of lignocellulosic material for bioethanol production. Utilisation of bagasse for biofuel production would be environmentally and economically beneficial, but the recalcitrance of lignin continues to provide a challenge. Further understanding of lignin production in specific cultivars will provide a basis for modification of genomes for the production of phenotypes with improved processing characteristics. Here we evaluated the expression profile of lignin biosynthetic genes and the cell wall composition along a developmental gradient in KQ228 sugarcane. The expression levels of nine lignin biosynthesis genes were quantified in five stem sections of increasing maturity and in root tissue. Two distinct expression patterns were seen. The first saw highest gene expression in the youngest tissue, with expression decreasing as tissue matured. The second pattern saw little to no change in transcription levels across the developmental gradient. Cell wall compositional analysis of the stem sections showed total lignin content to be significantly higher in more mature tissue than in the youngest section assessed. There were no changes in structural carbohydrates across developmental sections. These gene expression and cell wall compositional patterns can be used, along with other work in grasses, to inform biotechnological approaches to crop improvement for lignocellulosic biofuel production.

Cell wall composition and lignin biosynthetic gene expression along a developmental gradient in an Australian sugarcane cultivar

Short title: Lignin biosynthesis profile in sugarcane

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Abstract

Sugarcane bagasse is an abundant source of lignocellulosic material for bioethanol production. Utilisation of bagasse for biofuel production would be environmentally and economically beneficial, but the recalcitrance of lignin continues to provide a challenge. Further understanding of lignin production in specific cultivars will provide a basis for modification of genomes for the production of phenotypes with improved processing characteristics. Here we evaluated the expression profile of lignin biosynthetic genes and the cell wall composition along a developmental gradient in KQ228 sugarcane. The expression levels of nine lignin biosynthesis genes were quantified in five stem sections of increasing maturity and in root tissue. Two distinct expression patterns were seen. The first saw highest gene expression in the youngest tissue, with expression decreasing as tissue matured. The second pattern saw little to no change in transcription levels across the developmental gradient. Cell wall compositional analysis of the stem sections showed total lignin content to be significantly higher in more mature tissue than in the youngest section assessed. There were no changes in structural carbohydrates across developmental sections. These gene expression and cell wall compositional patterns can be used, along with other work in grasses, to inform biotechnological approaches to crop improvement for lignocellulosic biofuel production.

Introduction

Sugarcane is a C4 perennial grass of high economic importance in many parts of the world (Suprasanna et al. 2011). In addition to the production of high levels of sucrose in the stem, it produces large amounts of lignocellulosic biomass that has the potential to be used for the production of bioethanol (Canilha et al. 2012). Sugarcane is a particularly attractive source of biomass for lignocellulosic biofuels production, as it is transported to a central location, thus reducing biomass transport costs that could otherwise represent a significant cost in bioethanol production. However, as with all potential lignocellulosic feedstocks, the recalcitrance of the biomass presents challenges that need to be addressed.

The deposition of the secondary cell wall is an important step in terrestrial plant development (Weng & Chapple 2010), involving the ordered deposition of cellulose and hemicellulose followed by the impregnation of lignin polymers into this polysaccharide matrix (Vogel 2008). Lignin polymers are comprised of guaiacyl (G), syringyl (S) and *p*-hydroxyl-phenyl (H) units, through oxidative polymerization of coniferyl, sinapyl and *p*-coumaryl alcohols respectively, that are produced through the lignin biosynthesis pathway (Boerjan et al. 2003; Liu 2012).

Due to the importance of lignin in structural stability and water transportation, the role and function of each gene within the lignin biosynthesis pathway is well established (Boerjan et al. 2003; Bonawitz & Chapple 2010). The relationship between lignin and efficiency of lignocellulosic bioethanol production has led to increased focus into lignin biosynthesis and manipulation, and advances the possibility of cost-competitive bioethanol being produced from lignin-altered sugarcane bagasse. Given the influence lignin has on cell wall digestibility, further understanding of control and timing of lignin deposition will be applicable for the genetic modification of plants to specifically alter lignin characteristics. This aim of this research is to

assess the expression profile of lignin biosynthetic genes and cell wall composition of a sugarcane cultivar (KQ228) along a developmental gradient to further understand the relationship between gene expression and cell wall formation and composition, with the goal of providing critical information for the biotechnological development of improved varieties of sugarcane for second generation biofuel production.

Materials and methods

Gene identification

Primers were designed using sequences available from the NCBI database (Table S1). Not all genes had annotated accessions available and consensus sequences were assembled from the sugarcane EST database after BLAST analysis with the equivalent maize gene as a reference sequence. The final consensus sugarcane sequences were created using only sugarcane EST sequences. Amplicons of all lignin biosynthesis genes were run on a high-resolution gel to confirm there was only one product, and then sequenced to determine primer specificity before use in qRT-PCR.

Hydroxycinnamoyl transferase (HCT) was not included in qRT-PCR analysis as at the time of this study a specific sequence could not be confidently identified. At that time, only one published accession for sugarcane HCT was found (CA210265) (Casu et al. 2007). When analyzed by BLAST it showed very close alignment with *Zea mays* anthranilate N-benzoyltransferase (NM_001153992) (Soderlund et al. 2009). Further BLAST searching in the sugarcane nucleotide and EST databases of NCBI with alternative HCT sequences from maize (AY109546, DR807341) (Barrière et al. 2007) and from MAIZEWALL (2478084.2.1_REV,

2619423.2.1) (Guillaumie et al. 2007), *Medicago sativa* L. (AJ507825) (Shadle et al. 2007), *Nicotiana benthamiana* (AJ555865) (Hoffmann et al. 2004), *Coffea arabica* (AM116757) (Salmona et al. 2008) and *Triticum aestivum* L. (CK193498, CK199765) (Bi et al. 2011) did not highlight any potential sugarcane HCT sequences, nor any conserved regions of sufficient length to design primers (standard or degenerate) for potential use in sugarcane.

Plant material, growth conditions and tissue collection

Sugarcane generated from callus (cultivar KQ228, generously provided by BSES Ltd, Meringa Queensland) was acclimatized in growth chambers before being transferred to a greenhouse. Plants were grown for nine months before being destructively harvested for analysis. Each plant was divided into five different sections (A-E) to represent increasing tissue maturity, with Section A being the youngest tissue and Section E being the most mature tissue. Within each section there were three nodes, and the topmost node was used for qRT-PCR with the remaining two nodes used for cell wall composition.

Harvesting occurred between 10am and 2pm in a single session to minimize light or circadian related fluctuations in gene expression levels (Rogers et al. 2005). For all stem analyses only internode tissue was used and node tissue was discarded. The root ball was washed to remove potting mix and buttress roots (Moore 1987) were collected from each plant for qRT-PCR analysis. Roots were included in the development of this profile to begin to gain a general understanding of overall lignin biosynthetic gene expression in this tissue.

RNA extraction and qRT-PCR

RNA was extracted from all tissue samples using Tri Reagent (Sigma). RNA concentrations were quantified with a Nanodrop 2000 spectrophotometer and 1µg of RNA was treated with

RQ1 RNase-free DNase (Promega). DNase-treated RNA was used as a template for first strand cDNA synthesis using M-MLV Reverse Transcriptase (Promega). RT negative samples were prepared by replacing reverse transcriptase with water.

qRT-PCR was optimized to attain suitable R^2 and PCR efficiency values, and primers were validated against the housekeeping primers to ensure comparable rates of product amplification (Livak & Schmittgen 2001). qRT-PCR utilized GoTaq qRT-PCR Master Mix (Promega) in a 20µL total reaction volume with 20ng of cDNA template using 10mM forward and reverse primers. Samples were prepared by a CAS1200 robot (Corbett) and analyzed using a Rotor-Gene Q (Qiagen) Relative transcript levels were quantified using delta critical threshold values (ΔCt) as previously described (Levy et al. 2004) using β-tubulin as the housekeeping gene (Rodrigues et al. 2009).

Cell wall composition

Tissue for cell wall compositional analysis was prepared as previously described (Hames et al. 2008). Dried samples were milled to pass through a 2mm screen and extracted overnight with water and ethanol respectively (Sluiter et al. 2008c). A sample of this prepared material was dried overnight at 105°C and used to determine the total solids (Sluiter et al. 2008a).

Cell wall composition was quantified by a modified acid hydrolysis method (Sluiter et al. 2008b) using 0.125g biomass, 1.5mL 72% sulfuric acid and 42mL of water. Acid soluble lignin was determined by UV-Vis spectrophotometry and acid insoluble lignin was measured gravimetrically. Cell wall carbohydrates were analyzed using High Performance Liquid Chromatography. A Waters e2695 Separations Module and Showa Denko Shodex SP-0810 sugar column (85°C) with micro-guard de-ashing columns equipped with a Waters 2414 Refractive Index Detector were employed.

Results

qRT-PCR expression profiles of lignin biosynthesis genes

Expression profiles for the nine lignin biosynthesis genes were established after qRT-PCR analysis of the five stem sections and the root tissue (Figs 1 and 2, Fig S1). The ΔCt values were normalized against Section A to allow for easier comparison of changes in expression in relation to young tissue for each gene. The raw ΔCt values show that in Section A, PAL is expressed at levels greater than the other eight lignin biosynthesis genes analyzed (Table S2). CCoAOMT, COMT and CCR also had greater expression levels in Section A than CAD, 4CL, C4H, F5H and C3H. These trends are seen across the developmental gradient (Table S2).

The genes analyzed in stem tissue fell into two general categories, those with highest expression in Section A that decreased as stem tissue matured (PAL, CCR, 4CL, COMT and CAD) (Fig 1), and those where expression showed little change across developmental stages (C3H, F5H, C4H and CCoAOMT) (Fig 2). The genes did not fall in a specific pattern based on their position within the lignin biosynthetic pathway (Fig S1).

Of the genes with highest expression in Section A (Fig 1), the trend is strongest in PAL and CCR, with expression in Section B decreased by 70% - 80% and by more than 90% in Section E. 4CL and COMT are less pronounced with expression dropping approximately 65% from Section A to Section B. Expression of 4CL and COMT decrease 65% - 75% in Sections C, D and E when compared to expression in Section A (Fig 1). ~~CAD, the final gene in this group, is the least pronounced, with a 40% decrease in expression between Section A and Section B followed by expression levelling out between 25% and 40% of Section A in Sections C, D and E (Fig 1).~~

Only PAL and COMT show a significant reduction in expression in Section B when compared to

Section A, but all five genes have significantly lower expression in Sections C, D and E relative to Section A.

The second group shows similar expression across all five sections of stem tissue analyzed (Fig 2). C4H, CCoAOMT and F5H all show no significant differences in expression levels across the five stem sections. C3H shows a significant increase in expression levels between Section A and Section B before stabilizing in Sections C, D and E.

In root tissue, C3H, CCoAOMT, F5H and CAD expression was not significantly different to any stem Section (A-E) (Figs 1 and 2, Fig S1). Expression levels of PAL and 4CL were not significantly different to Section A, but were significantly higher than Sections B-E (Fig 1). CCR and COMT showed a similar pattern being not significantly different to Sections A or B but significantly higher than expression in Sections C-E (Fig 1). Expression of C4H in root tissue was approximately 9-fold higher than in any stem section (Fig 2).

Cell wall compositional analysis

Secondary cell wall composition was quantified in the five stem sections (Table 1). Section A had significantly lower lignin levels than the more mature stem internodes, though levels appear to stabilize after Section B. This was due to lower acid insoluble lignin in Section A as there are no significant differences in acid soluble lignin levels across the five stem sections. Glucose, xylose and galactose did not vary across the developmental gradient, but arabinose levels were higher in Section A than in more mature tissue.

Discussion

Sugarcane bagasse has great potential as a lignocellulosic biofuels source, in part due to its already being moved to a centralized location for sugar production. In order to effectively produce fermentable sugars from bagasse, the challenge of cell wall recalcitrance needs to be overcome. Improved understanding of lignin biosynthesis and deposition in sugarcane will be of great value when deciding the most appropriate approaches to facilitate the development of commercial lines with increased saccharification potential. The work herein uses an economically important smut-resistant Australian sugarcane cultivar, KQ228, and assesses lignin biosynthetic gene expression and cell wall composition along a developmental gradient in an attempt to further characterize the timing and location of lignin deposition to guide attempts to improve bagasse for lignocellulosic biofuels production.

Whereas in our study we look only at one specific homologue for each lignin biosynthetic gene, we have provided a comparison to previous work (Bottcher et al. 2013) wherein multiple homologues are assessed (Table S1). Our work was based on available genome data at the time of the study, and despite being somewhat limited relative to Bottcher et al. (2013), provides confirmation and comparison with another economically important cultivar. In addition to this, we also examined lignin gene expression in buttress roots as this can be a key storage sink for carbon.

The trends in the stem expression data dichotomize the lignin biosynthesis genes. The two 'expression pattern groups' are genes for which expression decreases with tissue age (PAL, CCR, 4CL, COMT and CAD) or genes for which expression remains constant during maturation (C3H, F5H, C4H and CCoAOMT). As PAL catalyzes the entry of metabolites into the lignin biosynthesis pathway (Liu 2012; Weng & Chapple 2010), its high level of expression in younger

187 tissue found in this study may represent an initial metabolic flux to provide a burst of metabolites
188 for the various phenylpropanoid pathways including lignin biosynthesis. CCR functions in the
189 final stages of lignin biosynthesis and is considered a committed step, key in the production of
190 the individual lignin monomers (Vogt 2010; Weng & Chapple 2010). Given the position of CCR
191 in the lignin biosynthesis pathway, it may act as a regulating control point for directing the
192 metabolic flux into lignin monomer production (Lacombe et al. 1997). As high expression of
193 PAL in young tissue may act to stimulate metabolic flux into phenylpropanoid production, high
194 expression of CCR in young tissue may ensure a high level of metabolite commitment into lignin
195 biosynthesis, which is fundamentally important for healthy plant development (Weng & Chapple
196 2010).

197 The expression profiles of 4CL and COMT are similar to that of PAL and CCR, but they retain
198 slightly higher expression levels in more mature tissue. 4CL represents an important branch
199 where metabolites are directed either into lignin biosynthesis or to alternative phenylpropanoid
200 biosynthesis pathways (Vogt 2010; Weng & Chapple 2010). Its position also allows for direct
201 metabolite contribution into H monomer biosynthesis or redirection of metabolites for G or S
202 monomer biosynthesis. The high level of 4CL expression in young tissue may reflect its response
203 to the metabolic flux into the phenylpropanoid pathway initiated by PAL. COMT is the last of
204 two enzymes entirely responsible for the production of the S lignin monomer within the lignin
205 biosynthesis pathway (Bonawitz & Chapple 2010). The increased expression of COMT in young
206 tissue in this research may be to ensure S monomer production during the availability of the
207 initial metabolic flux.

208 The final gene showing a reduction in expression as stem tissue matures was CAD, though the
209 trend was not as strong as the previously discussed genes. CAD represents the final enzyme in

the lignin biosynthesis pathway catalyzing the production of precursor monolignols and committing them to lignin monomer synthesis (Ferrer et al. 2008). The initial high expression of CAD in young tissue may relate to the increased metabolic flux through the lignin biosynthesis pathway. Whereas overall trends between our work and previous research are the same for these genes with decreasing expression for increasing maturity, there are differences, particularly with CAD and COMT. The discrepancies between the current and published research may be a result of various experimental differences between the current research and published findings, but it is more likely that the differences arise from the differences in cultivars.

Three genes were identified with relatively consistent expression across the maturity gradient: C4H, F5H, and CCoAOMT. Results for C4H were consistent with other results (Papini-Terzi et al. 2009). For F5H, expression in the Brazilian low and high lignin cultivars was highest in intermediate aged internodes with the exception of the high lignin pith samples wherein it was highest in the mature tissue (Bottcher et al. 2013). In the 30 cultivars with varying Brix levels, F5H expression levels were higher in maturing stem tissue than in young tissue (Papini-Terzi et al. 2009). Our results for CCoAOMT closely mirrored the results of Bottcher *et al.* (Bottcher et al. 2013).

The final gene assessed in our study, C3H, had the highest expression level in Section B, immediately below the most juvenile Section A. C3H catalyzes the second aromatic hydroxylation reaction in the lignin biosynthesis pathway and is an important hub in controlling metabolic flux into G and S lignin monomer synthesis (Barriere et al. 2004; Weng & Chapple 2010). CCoAOMT, along with C3H, are hypothesized to be important control points for cell wall lignification by acting as part of the ferulate production pathway (Barriere et al. 2004). Caffeoyl CoA 3-O-methyltransferase is responsible for the 3' methylation of caffeoyl-CoA to produce

feruloyl-CoA, a key step in the production of G and S lignin monomers (Hisano et al. 2009; Raes et al. 2003). The feruloyl residues aid in cross-linking within the cell wall and may increase the resistance of the cell wall to hydrolysis by adding to its structural stability (Barriere et al. 2004; Bonawitz & Chapple 2010; Grabber 2005). The relatively steady expression of CCoAOMT and C3H within the maturing sugarcane stem may reflect their continued requirement for feruloyl residue production for ongoing cell wall lignification and not just their role in lignin monomer biosynthesis.

To our knowledge, this is the first paper that has looked at lignin biosynthetic gene expression in sugarcane buttress roots. There were no significant differences in expression levels between root tissue and the five stem sections (A-E) for C3H, CCoAOMT, F5H and CAD. Of these, C3H, CCoAOMT and F5H are all in the group with plateaued gene expression during development and may highlight the promoters of these three genes as potential biotechnological tools to drive continuous and even expression of transgenes in sugarcane stem and root tissue. The only gene with an unexpected level of expression was C4H that had approximately 9-fold higher expression in roots than in any stem section. This suggests that the C4H promoter may be useful for preferential expression of transgenes in sugarcane root tissue, however further analysis, including the functionality of this promoter in additional tissue types, such as leaves, would need to be assessed.

In addition to the assessment of lignin biosynthetic gene expression, we also examined the cell wall composition along the same developmental gradient. It is well known that the composition of the cell wall material changes as a plant matures due to secondary cell wall deposition. Following cell elongation, the secondary cell wall is formed through the deposition of cellulose and hemicellulose, followed by lignification (Vogel 2008; Weng & Chapple 2010). Within

sugarcane, rapid elongation of young internode cells precedes cell wall thickening, including lignification (Casu et al. 2007). No significant differences were seen in levels of structural carbohydrates including glucose, xylose or galactose indicating that the deposition of structural polysaccharides into the secondary cell wall had also occurred before harvesting of samples (in more juvenile tissue). This is in contrast to published findings in sugarcane (Lingle & Thomson 2012) and maize (Jung & Casler 2006). In sugarcane, cellulose peaked and then declined below internode 5, whereas hemicellulose was highest in young tissue before reducing to a steady state (Lingle & Thomson 2012). In maize, glucose content increased as tissue matured before plateauing, and hemicellulose (xylose and arabinose) decreased as tissue matured before also reaching a steady state (Jung & Casler 2006). The decrease in xylose and arabinose coincided with an increase in ferulates, and the authors suggest ferulates may be replacing the xylose and arabinose within the cell wall, hence their decrease during tissue maturation (Jung & Casler 2006).

Results suggest the lignin deposition was complete by Section B as lignin content plateaued and no differences were detected between Sections B, C, D and E. Other studies have also found that overall lignin content increased with tissue maturity in wheat and maize (Jung & Casler 2006; Ma 2007). In maize stem, lignin content decreased initially before increasing to a plateau (Jung & Casler 2006). In sugarcane, marked internodes harvested over a period of twelve weeks had increased lignin content over time (Lingle & Thomson 2012). In a second experiment, odd numbered internodes harvested at a single time point, showed lignin content increased with maturity, with the exception of a significant decrease in internode 3 (Lingle & Thomson 2012). The results of the second experiment are similar to the maize results of Jung and Casler (2006), who suggest that young maize tissue is comprised of a higher percentage of lignified protoxylem

vessels than more mature tissue, that initially results in a high lignin content in very young tissue (Jung & Casler 2006). It is likely that the Section A tissue (from internodes 2 and 3) was in what is the second zone identified in the studies by Jung and Casler (Jung & Casler 2006) and Lingle and Thomson (Lingle & Thomson 2012). This is supported by the results of Bottcher et al. (2013) who showed lower lignin levels in internode 2-4 of two sugarcane cultivars before reaching a relatively steady state lignin level for internodes 5 to 18.

The work presented herein provides a profile of lignin biosynthetic gene expression and cell wall composition for an economically important Australian sugarcane cultivar. The results support findings of previous groups and add additional information on gene expression in sugarcane buttress roots. As a key potential biofuels crop, detailed information from multiple cultivars will help to improve the understanding of lignin and cell wall formation in this species and to inform biotechnological approaches to crop improvement.

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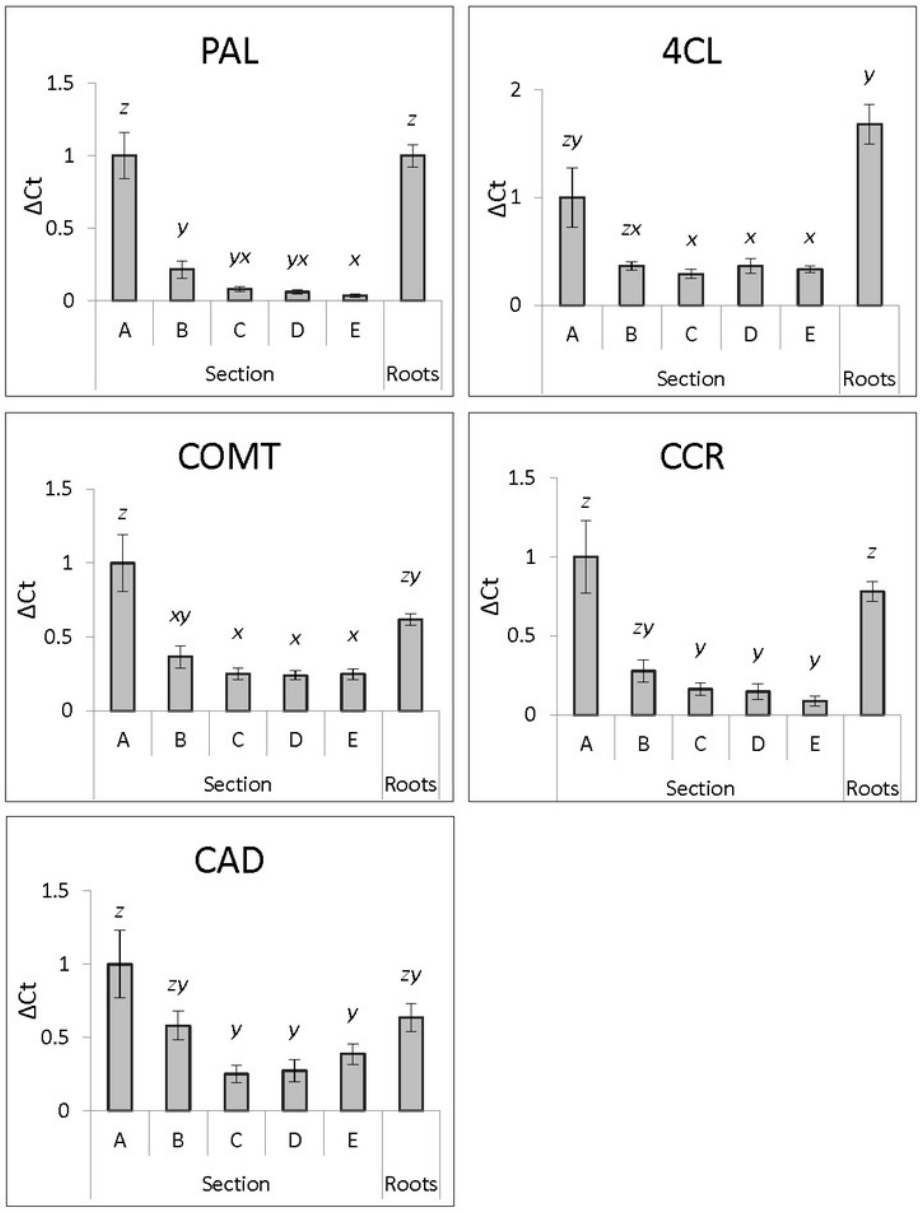
401

Figure 1

Genes with decreasing expression along developmental gradient

Genes showing highest expression in section A (young stem tissue) with decreased expression in more mature stem regions. ΔCt expression levels of lignin biosynthesis genes from the five stem sections and roots (n = 5 individual plants per tissue section) normalized against section A for each individual gene is shown with standard error of the mean. Statistical differences are noted by different letters above bars (*x*, *y* and *z*) after ANOVA analysis with Tukey post-hoc analysis ($p = 0.05$)


PAL
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A
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zy
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Roots
Section
COMT
ΔCt
1.5
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x
x
x
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A
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C
D
E
Roots
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1.5
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CAD
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D
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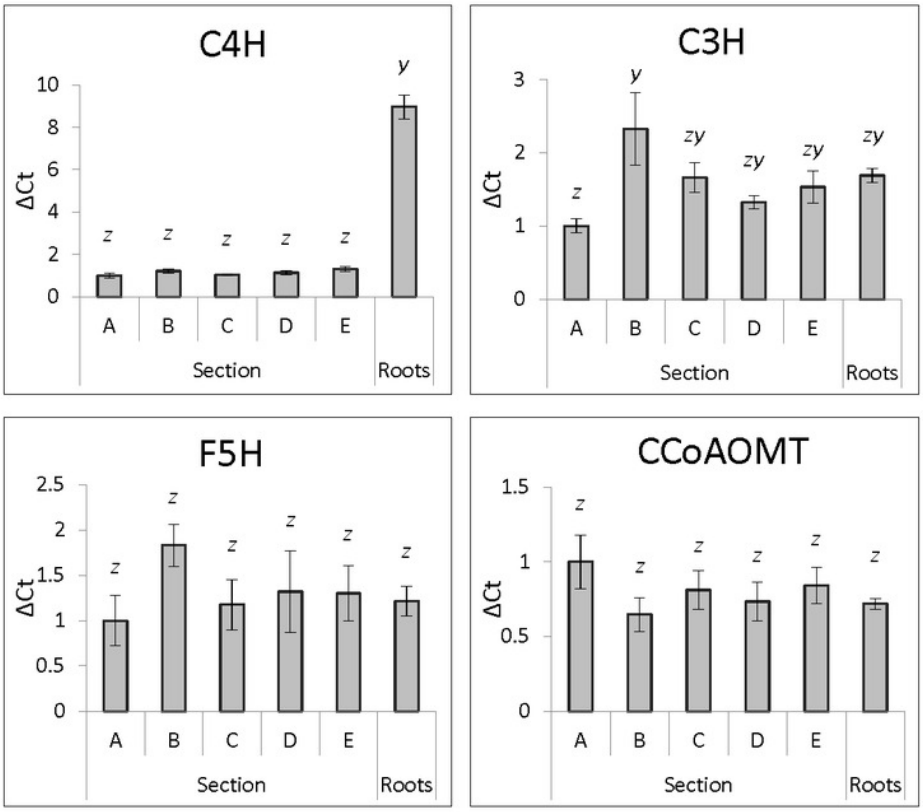
Roots
Section

Figure 2

Genes showing consistent expression along developmental gradient

ΔCt expression levels of lignin biosynthesis genes from the five stem sections and roots (n = 5 individual plants per tissue section) normalized against section A for each individual gene is shown with standard error of the mean. Statistical differences are noted by different letters above bars (*x*, *y* and *z*) after ANOVA analysis with Tukey post-hoc analysis ($p = 0.05$)


C4H
ΔCt
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8
6
4
2
0
z z z z z y
A B C D E Roots
Section
C3H
ΔCt
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2
1
0
z y zy zy zy zy
A B C D E Roots
Section
F5H
ΔCt
2.5
2
1.5
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A B C D E Roots
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CCoAOMT
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A B C D E Roots
Section

Table 1(on next page)

Accumulation of individual cell wall components in stem regions of increasing maturity

The percentage of each component of the total composition is shown with the standard error of the mean. Values in bold type are significantly different (ANOVA analysis with Tukey post-hoc analysis, $p = 0.05$) to section A for each component. No significant differences were observed between sections B, C, D or E for any component. n = 5.

1

Section	Total lignin		Acid insoluble lignin		Acid soluble lignin		Glucose		Xylose		Galactose		Arabinose	
	%	+/-	%	+/-	%	+/-	%	+/-	%	+/-	%	+/-	%	+/-
A	20.76	0.52	15.57	0.48	5.19	0.11	49.26	0.53	20.58	0.35	0.32	0.19	2.28	0.09
B	**22.35**	**0.27**	**17.14**	**0.32**	5.21	0.06	47.93	0.50	20.01	0.29	0.00	0.00	**1.63**	**0.05**
C	**23.39**	**0.13**	**18.29**	**0.10**	5.10	0.08	48.09	0.49	20.36	0.35	0.00	0.00	**1.58**	**0.04**
D	**23.24**	**0.29**	**18.08**	**0.33**	5.16	0.06	48.00	0.54	20.90	0.27	0.00	0.00	**1.58**	**0.06**
E	**22.49**	**0.24**	**17.40**	**0.30**	5.09	0.06	47.34	0.18	21.58	0.46	0.10	0.09	**1.81**	**0.12**

2