Journal of Experimental Botany, Vol. 62, No. 10, pp. 3545–3561, 2011
doi:10.1093/jxb/err045 Advance Access publication 14 March, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Identification of genes involved in cell wall biogenesis in grasses by differential gene expression profiling of elongating and non-elongating maize internodes

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Received 22 October 2010; Revised 20 January 2011; Accepted 1 February 2011

Abstract

Despite the economic importance of grasses as food, feed, and energy crops, little is known about the genes that control their cell wall synthesis, assembly, and remodelling. Here a detailed transcriptome analysis that allowed the identification of genes involved in grass cell wall biogenesis is provided. Differential gene expression profiling, using maize oligonucleotide arrays, was used to identify genes differentially expressed between an elongating internode, containing cells exhibiting primary cell wall synthesis, and an internode that had just ceased elongation and in which many cells were depositing secondary cell wall material. This is one of only a few studies specifically aimed at the identification of cell wall-related genes in grasses. Analysis identified new candidate genes for a role in primary and secondary cell wall biogenesis in grasses. The results suggest that many proteins involved in cell wall processes during normal development are also recruited during defence-related cell wall remodelling events. This work provides a platform for studies in which candidate genes will be functionally tested for involvement in cell wall-related processes, increasing our knowledge of cell wall biogenesis and its regulation in grasses. Since several grasses are currently being developed as lignocellulosic feedstocks for biofuel production, this improved understanding of grass cell wall biogenesis is timely, as it will facilitate the manipulation of traits favourable for sustainable food and biofuel production.

Key words: Biofuel, cell wall, defence, grasses, lignocellulose, microarray, Zea mays.

Introduction

The plant cell wall is a highly dynamic structure that, besides providing mechanical support, needs to respond to various environmental and developmental cues and fulfils important functions in signalling events, the defence against biotic and abiotic stresses, and growth. Importantly, the cell wall represents a major energy storage compartment as much of the solar energy captured by plants is photosynthetically converted into chemical energy locked into the cell wall polymers: cellulose, hemicellulose, and lignin. The energy content and portability of plant-derived biofuels, and their compatibility with the existing petroleum-based transportation infrastructure, explains the attractiveness of lignocellulosic biomass as a renewable and sustainable source of mixed sugars for fermentation to biofuels. Indeed maize stover residues and several perennial grasses, including *Miscanthus* and switchgrass (*Panicum virgatum*), are currently being developed as lignocellulosic feedstocks for biofuel production. Perennial C_4 grasses are considered superior potential feedstocks because of their efficient photosynthesis and long growing season, their ability to sequester nutrients in rhizomes at the end of the growing season, and their high water-use efficiency (Lewandowski *et al.*, 2003).

However, current conversion of lignocellulosic biomass into fermentable sugars is inefficient and costly as plant cell

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walls have evolved to resist microbial and enzymatic deconstruction, collectively known as 'biomass recalcitrance' (Himmel *et al.*, 2007). Optimizing cell wall composition and cross-linking to improve digestibility is critical for enhancing conversion efficiency and is a major breeding goal for biofuel crops. To achieve this optimization will require a detailed understanding of the dynamic architectural structure and function of the grass cell wall.

The grasses represent one of the most important families of flowering plants, the Poaceae, which includes the cereals, many important feed and forages, and more recently feedstocks for biofuel and biopower. However, relatively little is known about the genes that control cell wall synthesis, assembly, and remodelling in these monocots. Grass cell walls are distinct in composition from all other flowering plants, making a so-called type II cell wall, as opposed to the type I cell wall present in all dicots and non-grass monocots (see Carpita, 1996 for a detailed description). It has been estimated that 10% of plant genomes are devoted to cell wall-related processes and that at least a third of cell wall-related genes in grasses could have no, or few, orthologues in *Arabidopsis* (Carpita and McCann, 2008). This makes the study of a grass model system essential. Differences in cell wall-related gene family structure and expression, between *Arabidopsis* and the grasses (Penning *et al.*, 2009), underscores the need for a grass model for the functional analysis of type II cell wall biogenesis.

Genetic resources for dedicated bioenergy crops such as switchgrass and *Miscanthus* are currently limited. Maize (*Zea mays*), like switchgrass and *Miscanthus*, belongs to the panicoid subfamily of the grasses (Lawrence and Walbot, 2007). This close phylogenetic relationship, combined with the long-standing genetic tools and completed genome sequence available (Schnable *et al.*, 2009), make maize an attractive model for the identification and functional analysis of genes involved in cell wall biogenesis. Subsequently this information can be translated to the more genetically recalcitrant, and undomesticated, energy grasses.

The internodes of a maize stalk represent a developmental profile in which the successive internodes from the base to the apex become progressively younger. These internodes, therefore, provide a useful model for the identification of genes involved in cell wall synthesis, assembly, and remodelling during internode elongation and thickening. Here the identification of maize genes differentially expressed between an internode undergoing active elongation, which predominantly contained cells exhibiting primary cell wall synthesis, and an internode that had just ceased elongation and in which many cells were depositing secondary cell wall material is described. Transcriptome analysis, using maize oligonucleotide arrays, focused on genes and gene families potentially involved in cell wall biogenesis. This study confirmed the involvement of known cell wall genes and identified new candidates for a role in primary and secondary cell wall-related processes in grasses. To our knowledge this study describes the first total expression profiling in maize which specifically focuses on genes involved in cell wall biogenesis, and is one of only a few studies aimed at the identification of cell wall-related genes in grasses. The data presented provide a platform for the selection and functional analysis of candidate genes involved in grass cell wall biogenesis.

Materials and methods

Plant material and growth conditions

Maize inbred line B73 plants were grown in a greenhouse. Daylight was supplemented with overhead lighting using 400W high-pressure sodium lamps for 16 h daily. Minimum temperatures were maintained at 25 °C (day) and 18 °C (night). Internode development was assessed using the vegetative identification system (Ritchie et al., 1993). At stage V13, the plants were harvested, the leaves and leaf sheath were removed, internodes 9–13 (IN9–IN13) were excised, avoiding 1cm either side of the node, frozen in liquid nitrogen, and stored at –80°C. The average length of experimental IN9s at collection was 93.5 mm (±7.1), similar to the maximum length reached by these internodes in control plants at the silking stage. Collected IN13s showed an average length of 39.2 mm (±7.8), ~40% of their maximum size at the silking stage.

RNA isolation

IN9 and IN13 from six maize plants were ground in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy MinElute Cleanup Kit columns (Qiagen, Valencia, CA, USA) according to a protocol recommended at www.maizearray.org. Following extraction, the quality and quantity of the RNA samples was determined by absorbance measurements at 230, 260, and 280 nm. RNA integrity was evaluated on an agarose gel.

RNA labelling and microarray procedures

Maize 70-mer oligonucleotide arrays, printed at the University of Arizona, were used to determine the expression profile of RNA extracted from IN9 and IN13. Each array contained 46,128 oligos printed on a single slide. The experiment consisted of six biological replicates: three slides in which IN9 RNA was labelled with Cy3 and IN13 RNA from the same plant was labelled with Cy5; and three slides in which IN13 RNA was labelled with Cy3 and IN9 RNA with Cy5, to ensure dye balance. RNA labelling, hybridization, and scanning were conducted at the University of Arizona by the Maize Oligonucleotide Array Project using their optimized protocols.

Data normalization and analysis

The microarray data were analysed within the statistical programming language R (version 2.7.0) using the Bioconductor library limma (Smyth, 2005). Loess normalization was applied to the raw data to remove intensity-dependent dye effects. A linear model with internode and dye as main effects was fitted and the limma-specific moderated F-test (Smyth, 2004) was applied to obtain P-values for testing the internode effect. As limma models the log ratio of the two channels on the same array this analysis automatically takes the pairing of samples into account. The Benjamini–Hochberg method (Benjamini and Hochberg, 1995) was used to adjust P-values for multiple testing. Genes were selected as significant if they had an adjusted P-value <0.01 [i.e. the false discovery rate (FDR) was controlled at 1%]. As an additional criterion only genes were selected with at least a 2-fold change in the primary analysis and a minimum of a 4-fold change for further detailed analysis. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002), accession number GSE24014.

Gene annotation and functional categories

Although gene annotation for the 46K maize oligonucleotide array was provided in the GenePix Array List (GAL-file, January 2007), the 70-mer oligos which showed >4-fold differential expression were subjected to a more thorough annotation strategy by submitting the oligo probes to Blast searches using the Maize Genome Browser (Release 4a.53) (http://www.maizesequence.org) and GenBank (http://blast.ncbi.nlm.nih.gov). The closest maize accession and *Arabidopsis thaliana* homologue were identified, and the InterPro and Pfam domains contained in the predicted protein sequences were determined. Genes were assigned to functional categories using the MIPS Functional Catalogue Database (Ruepp et al., 2004).

Quantitative RT-PCR

Gene-specific primers were designed using Primer Express software (Applied Biosystems). Whenever possible, primer pairs spanning one or more exon–intron junction were selected. Alternatively, at least one of the primers of a pair was located in the 3'-untranslated region. Primer sequences are listed in Supplementary Table S6 available at JXB online. RNA was isolated from IN9 and IN13 as described above from three randomly selected maize plants. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, using 1 μg of total RNA and oligo(dT) primers. Based on the array data, the following reference genes were selected: cyclophilin (MZ00016819), peptidase C14 (MZ00027363), and ribosomal L11 (MZ00016094). The primer pairs for these reference genes exhibited primer efficiencies with a correlation coefficient >0.99 over a 10-fold dilution series and showed no differential expression between IN9 and IN13. Validation experiments showed that the slope of log input amount versus ΔC_T was <0.1, demonstrating that the efficiencies of target and reference were approximately equal, confirming that the comparative CT method ($\Delta\Delta C_T$) could be used for quantitation. The fold change was calculated from $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ represents ΔC_T (IN9)–ΔC_T (IN13). Quantitative RT-PCR was performed on an ABI 7500 Real Time PCR system using a SYBR Green I master mix (Applied Biosystems) with cDNA of three biological replicates. All reactions were performed in triplicate.

Histochemical staining of lignin

Maize internode sections of 10 μm thickness embedded in Agar Scientific JB-4 resin were stained for syringyl lignin using the Maule colour reaction. Sections were immersed in 1% neutral $KMnO_4$ for 3 min and rinsed in distilled water. The sections were decolorized with 3% HCl and washed thoroughly in water. Sections were mounted in concentrated NH_4OH and examined immediately by bright-field microscopy using a Leica CTR6500 fluorescence microscope.

Results and Discussion

To profile differentially the expression of genes in an actively elongating internode versus an internode that had just ceased elongation, the development of maize internodes was assessed using the vegetative identification system described by Ritchie *et al.* (1993). Based on RT-PCR data for cell wall-related genes expressed during the elongation phase, IN9 and IN13 were selected for the expression profiling experiment (data not shown). These internodes were harvested from six maize plants at 50 d after sowing, representing six biological replicates. IN9 represented a non-elongating internode, with an average length of 93.5 mm (±7.1), and IN13 represented an elongating internode with an average length of 39.2 mm (±7.8). Stem cross-sections showed that the cells within non-elongating IN9 contained significant amounts of lignin when compared with those of elongating IN13 (Fig. 1). RNA was extracted from these internodes and hybridized in a pairwise pattern to the Maize 46K Oligonucleotide microarrays, with a dye swap.

A total of 3988 oligonucleotide probes (8.6%) out of the 46,128 70-mer oligos printed on the slide exhibited >2-fold differential expression (Benjamini–Hochberg adjusted *P*-value <0.01) between IN13 and IN9 (Fig. 2; see Supplementary Table S1 at *JXB* online for a full list). The high number of differentially expressed genes is in agreement with other studies; for instance, >2000 genes were differentially expressed during stem development of *Arabidopsis* (Minic *et al.*, 2009) and >3000 in poplar (*Populus trichocarpa*) (Dharmawardhana *et al.*, 2010). A quality check of the array data is presented in Fig. 3 and Supplementary Fig. S1. The fact that points scatter close around the diagonal line in the Dye-Swap plot (Fig. 3) indicates that (i) the normalization has removed any systematic dye biases and (ii) the results are very consistent between the two dye settings. An MA-

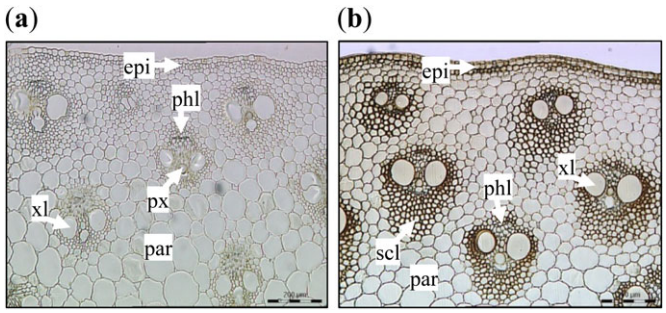

Fig. 1 Cross-section of the elongating internode IN13 (a) and the non-elongating internode IN9 (b) stained with Maule reagent. Dark coloration indicates the presence of syringyl lignin units. epi, epidermis; xl, xylem; par, parenchyma; phl, phloem; px, protoxylem; scl, sclerenchyma. Scale bar=200 μm.

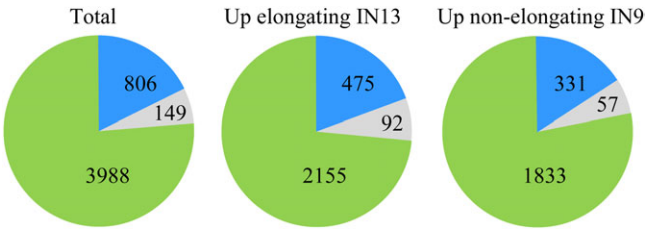

Fig. 2. The number of differentially expressed genes between the elongating internode IN13 and the non-elongating internode IN9. The green and blue areas represent the number of >2-fold (*P* <0.01) and >4-fold (*P* <0.005) differentially expressed genes, respectively. The light grey area shows the number of genes represented more than once in the >4-fold differentially expressed gene list.

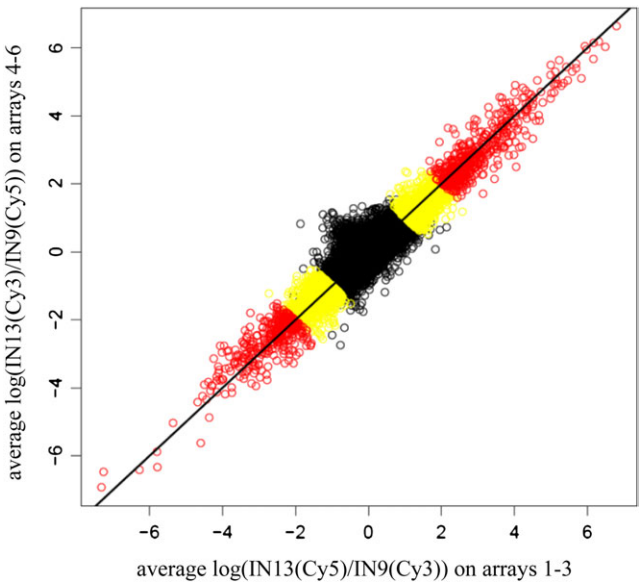

Fig. 3. Dye-swap plot depicting the log-fold change for IN13 versus IN9 from the first three arrays (IN13 on Cy5) on the *x*-axis against the corresponding log-fold change from the three dye-swapped arrays (IN13 on Cy3) on the *y*-axis. Significant genes with at least a 2-fold change are labelled in yellow and significant genes with >4-fold changes are labelled in red. A few non-significant (black coloured) spots can be seen in the >2-fold change region. These are the ones furthest away from the diagonal, which shows that the statistical test successfully eliminates genes with non-consistent changes.

plot, which indicates at which overall level of expression significant changes occur, is shown in Supplementary Fig. S1. In order to validate the differential expression profiles obtained from the microarray analysis, transcripts of 13 selected genes were analysed using quantitative real-time PCR. For all the genes tested, the expression profile was the same as obtained with the microarrays (Table 1). This confirmed the robustness of the differential expression profiles obtained between IN9 and IN13 using the maize oligonucleotide arrays.

Annotation and functional classification

Given that the existing annotation of the maize oligonucleotide array dated to January 2007, and a draft genome sequence for maize B73 was recently published (Schnable *et al.*, 2009), the gene targets for the oligo probes that showed >4-fold differential expression in the array experiment (955 oligo probes) were re-annotated. Each 70-mer oligo sequence that showed a >4-fold differential hybridization signal was analysed using BLAST in the Maize Genome Browser (Release 4a.53) to query the B73 reference genome version 1, and to identify the corresponding maize gene ID and the InterPro and Pfam domains contained in the predicted protein sequence. The closest maize accession and *A. thaliana* homologue in the National Center for Biotechnology Information (NCBI) database was also determined (Supplementary Table S2 at *JXB* online). The analysis revealed some degree of redundancy within the oligo probes, as 149 maize gene IDs (15.6%) were represented more than once in the >4-fold differentially expressed target group of genes (Fig. 2). Unless stated otherwise, further analyses and descriptions will be confined to the genes that were >4-fold differentially expressed between IN13 and IN9.

Using the MIPS Functional Catalogue Database (Ruepp *et al.*, 2004), the transcripts were functionally categorized (Fig. 4). More genes involved in carbohydrate metabolism were preferentially expressed in the elongating IN13 (n=41) compared with the non-elongating IN9 (n=18). Many genes predicted to be involved in lipid, fatty acid, and isoprenoid metabolism were also preferentially expressed in IN13 (n=30) compared with IN9 (n=6), indicating that lipid metabolic processes are important during elongation. This was confirmed by the high number of genes up-regulated in IN13 (n=21) with functions related to lipid/fatty acid transport, and which all contained a protease inhibitor/seed storage/LTP family domain. Although lipid transfer proteins (LTPs) were long thought to function as lipid carriers between intracellular organelles, LTPs are in fact small secreted proteins with a range of biological functions including defence signalling in plant systemic acquired resistance (Jung *et al.*, 2009) and the export of lipids to the cuticle (DeBono *et al.*, 2009). It has been shown that LTPs can promote cell expansion (Nieuwland *et al.*, 2005), although the mechanism of action is not clear.

The functional category 'cell rescue, defence, and virulence' contained many differentially expressed genes, with approximately twice as many genes up-regulated in IN13 (n=40) compared with IN9 (n=18). This is consistent with the need for more defence-related genes to be expressed in fragile elongating tissues. Many of the proteins involved in cell wall-related processes might also function in cell wall remodelling during a defence response. Examples of this included the secretory plant peroxidases with 11 genes up-regulated in IN13 and six in IN9. Such class III peroxidases are involved in multiple processes including cell wall loosening, cross-linking, and responses to wounding (Passardi *et al.*, 2004) and some are believed to catalyse lignin polymerization (Fagerstedt *et al.*, 2010). Thaumatins are specifically up-regulated in IN13 (n=7). The expression of plant thaumatins is induced by environmental stress and some thaumatin-like proteins exhibit β-1,3-glucanase activity and xylanase inhibitor activity (reviewed in Liu *et al.*, 2010). Several thaumatin-like proteins were also differentially expressed in developing stems of *Arabidopsis* (Minic *et al.*, 2009), suggesting that these defence-related secretory proteins could also function in cell wall remodelling during development. The high number (n=39) of 'nucleosome assembly'-related genes preferentially expressed in IN13 is potentially surprising. Almost all of these genes encode histones (n=36). Maize internodes do not contain a vascular meristem, so involvement in secondary growth is unlikely. One explanation could be that some cells undergoing cell division were still present in IN13 samples, even though the

Table 1. Quantitative RT-PCR results for selected transcripts

Annotation	Maize gene ID	Oligo ID	Array		Q-RT-PCR[a]	
			IN9	IN13	IN9	IN13
NAC TF	GRMZM2G162739	MZ00028246	5.9	1	17.2 (11.2–26.4)	1 (0.8–1.2)
NAC TF	GRMZM2G068973	MZ00026127	6.2	1	21.0 (17.3–25.5)	1 (0.7–1.4)
NAC TF	GRMZM2G167018	MZ00022590	2.9	1	7.0 (5.6–8.7)	1 (0.8–1.2)
MYB TF	AC197146.3_FG002	MZ00030474	5.9	1	23.0 (15.7–33.5)	1 (0.4–2.5)
MYB TF	GRMZM2G037650	MZ00040639	5.3	1	10.6 (7.4–15.2)	1 (0.8–1.3)
MYB TF	GRMZM2G088524	MZ00055303	5.2	1	11.0 (9.5–12.9)	1 (0.9–1.2)
GH16/XTH	GRMZM2G004699	MZ00024711	1	42.9	1 (0.4–2.6)	274.0 (245–306)
GlD1L2	GRMZM2G049675	MZ00020346	1	6.4	1 (0.8–1.2)	16.9 (14.8–19.2)
RF2C	GRMZM2G071021	MZ00044059	1	5.3	1 (0.9–1.1)	12.8 (10.8–15.1)
GT47	GRMZM2G100143	MZ00033488	4.9	1	6.7 (4.7–9.5)	1 (0.7–1.4)
GT47	GRMZM2G059825	MZ00036858	4.1	1	4.1 (3.4–5.0)	1 (0.7–1.5)
GT43	gbIBT036881.1I[b]	MZ00056172	1	4.6	1 (0.8–1.2)	13.0 (10.4–16.4)
GT43	GRMZM2G150302	MZ00015783	2.9	1	3.3 (2.4–4.5)	1 (0.8–1.2)

[a] Values in parentheses indicate the range of fold differential expression by incorporating the standard deviation of the ΔΔCT into the fold difference calculation.
[b] No gene associated with the oligo using the maize genome browser.

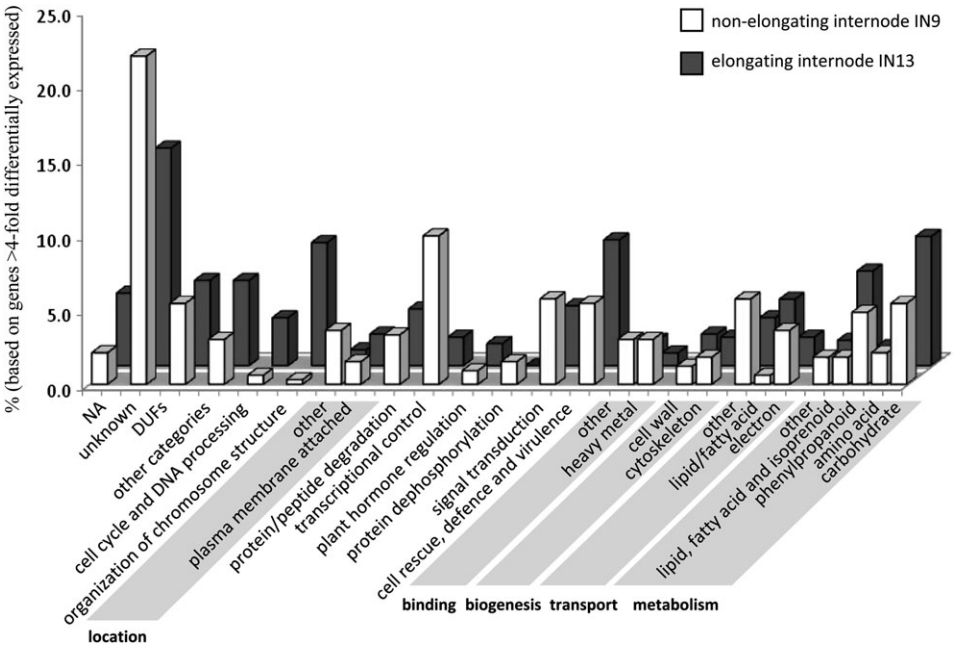

Fig. 4. Functional categories of genes showing >4-fold differential expression between the elongating internode IN13 and the non-elongating internode IN9.

1 cm basal part, containing the active intercalary meristem, was not included during sample collection.

The largest numbers of differentially expressed genes were classified as of unknown function, with n=69 and n=74 in IN13 and IN9, respectively. A third of the genes of unknown function up-regulated in IN13 contained at least one identifiable conserved domain, and the equivalent figure was 21% for IN9. For only 39% of the unknown genes >4-fold up-regulated in IN13 could an *Arabidopsis* homologue be identified based on e $<1^{-10}$; this figure was even lower for IN9 unknowns (26%), while these values were 94% and 88%, respectively, for the genes in the other functional categories.

Cell wall biogenesis is a complex process involving the action of many protein families directly involved in the synthesis of cell wall polysaccharides and the rearrangement of cell wall polymers, but also proteins with indirect involvement such as regulatory genes including transcription factors (TFs). In the remaining part of this section a more detailed analysis of selected genes and gene families with known or potential involvement in cell wall biogenesis is reported.

Carbohydrate metabolism: glycosyl transferases and hydrolases

Glycosyl transferases (GTs) constitute a large family of enzymes involved in the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates. Several genes encoding GTs showed >4-fold differential expression, including GT1, GT2, GT8, GT31, GT43, and GT47 family members (Table 2). Mutants for several *Arabidopsis* homologues to the GTs up-regulated in IN9 have been extensively studied and show an irregular xylem (IRX) phenotype. The *Arabidopsis* homologues for the two GT2 genes preferentially expressed in IN9, IRX1 and IRX5, both encode cellulose synthases (AtCesA8 and AtCesA4) involved in secondary cell wall cellulose synthesis (Taylor *et al.*, 2003). The third maize cellulose synthase (ZmCesA12) suggested to be involved in secondary wall formation (Appenzeller *et al.*, 2004) was not >4-fold preferentially expressed in IN9, assuming that an oligo probe was available for this gene. The GT2 up-regulated in IN13 suggests involvement in primary cell wall biosynthesis. The *Arabidopsis* homologue to this GT2, AtCSLC12, is a potential β-1,4-glucan synthase involved in the synthesis of the xyloglucan backbone rather than cellulose (www.uniprot.org), supporting this hypothesis. None of the presumed primary cell wall-associated CesAs (ZmCesA1–ZmCesA9; Appenzeller *et al.*, 2004) showed >4-fold preferential expression in IN13. Upon querying the maize oligonucleotide annotation file, expression data for all but one of these CesAs could be retrieved (see Supplementary Table S3 at *JXB* online). Only ZmCesA1 was >2-fold preferentially expressed in IN13, while the remaining CesAs showed only a minor preferential expression pattern for the elongating internode. Based on phylogenetic analysis it has been suggested that some of these CesAs (ZmCesA6– ZmCesA8) are involved in primary cell wall synthesis later in development before the onset of secondary wall formation (Appenzeller *et al.*, 2004), which could explain the observed expression pattern for some of the CesAs. Experimental evidence is still needed to confirm that ZmCesA1– ZmCesA9 are indeed involved in primary cell wall cellulose synthesis. A bioinformatics approach identified the GT43, GT47, and GT61 families, and proteins containing the PF02458 domain, as the most likely candidates to encode enzymes involved in the synthesis of arabinoxylan and its side chains in the grasses (Mitchell *et al.*, 2007). It was found that genes belonging to these families were differentially expressed (Table 2 and Supplementary Table S2). For example, the orthologous GT61 gene (GRMZM2G354610) for the locus identified as the most promising candidate for a feruloyl-arabinoxylan β-1,2-xylosyl transferase in rice (Os06g27560), was 5.3-fold up-regulated in IN9 compared with IN13 (Table 2). The differentially expressed GT43 showed the highest homology to *Arabidopsis* IRX9, believed to encode a xylan synthase responsible for adding β-xylosyl residues to the nascent glucuronoxylan (Pena *et al.*, 2007). However, the maize GT43 was up-regulated in elongating tissue and therefore not involved in secondary cell wall synthesis as was the case for IRX9 in *Arabidopsis*. The *Arabidopsis* homologues of the two GT47 genes up-regulated in IN9 correspond to IRX10 and IRX10-L and are believed to be required for xylan chain elongation (Brown *et al.*, 2009) as an *irx10*/*irx10-L* double mutant exhibited a large reduction of xylan in the secondary cell walls and a severe reduction in β-(1,4) xylosyltransferase activity. The two maize GT47 genes, GRMZM2G100143 and GRMZM2G059825, preferentially expressed in the non-elongating internode, therefore represent excellent candidates for involvement in the biosynthetic process of secondary cell wall xylans in maize.

Glycosyl hydrolases (GHs) represent some of the most extensive gene families in plants, many involved in cell wall remodelling (Minic, 2008). Several GH1 β-glucosidases were highly up-regulated in elongating IN13 (Table 2). β-Glucosidases have been implicated in several functions including responses to biotic and abiotic stresses, lignification, and cell wall remodelling and metabolism (Cairns and Esen, 2010). Analysis of the five most differentially expressed β-glucosidases identified At5g44640 as the closest homologue in *Arabidopsis* and Os4bglu12 as the closest homologue in rice (see Table 2). Os4bglu12 has the highest sequence similarity to a cell wall-bound β-glucosidase containing high exoglucanase activity, consistent with a role in cell wall biogenesis (Opassiri *et al.*, 2006).

Xyloglucan endotransglucosylase/hydrolases (XTHs) are GH16 family hydrolases acting on the xyloglucan chains that cross-link the cellulose microfibrils in the cell wall, catalysing endotransglucosylase (XET) and/or xyloglucan endohydrolase activities. XTHs acting in XET mode perform molecular grafting reactions by cleaving donor xyloglucan chains and rejoining the newly formed ends (Rose *et al.*, 2002). This creates the potential ability to alter and loosen the cell wall matrix, and studies have shown strong correlations between XTH expression and cell elongation activity (Uozu *et al.*, 2000; Vissenberg *et al.*, 2000). In agreement with this, seven genes encoding XTHs showed >4-fold higher expression in IN13 compared with IN9 (Table 2). However, XTH expression does not always correlate with growth rate. XTH activity has been detected in tissues in which expansion has ceased, and involvement in the formation of secondary cell walls of vascular tissues has previously been suggested (Bourquin *et al.*, 2002). Thus, the XTH highly up-regulated in IN9 might fulfil such a secondary cell wall-related function (Table 2). The exact function of XTH in the grasses remains to be elucidated as type II cell walls contain a relatively low amount of xyloglucan. There is evidence that XTH can use a range of donor and acceptor substrates in addition to xyloglucan for its XET activity (Hrmova *et al.*, 2007), suggesting that certain XTH/XET isoforms might catalyse the formation of covalent linkages between different types of wall polysaccharides in grasses (Fincher, 2009).

Other GHs that were mostly up-regulated in IN13 and implicated in cell wall loosening during growth include members of the GH17 family, encoding putative β-1,3-glucanases, several GH35 family members, encoding β-galactosidases, and GH28 polygalacturonases (Table 2). Two

Table 2. Glycosyl transferases (GTs) and glycosyl hydrolases (GHs) >4-fold differentially expressed between IN9 and IN13

Family	Oligo ID	Signal intensity	Fold change[a]	Maize gene ID	*Arabidopsis* homologue gene	E-value	Putative annotation
GT1	MZ00015539	9.9	4.8	GRMZM2G008263	AT1G32900	0E+00	Granule bound starch synthase IIa precursor
GT1	MZ00012710	10.8	–4.6	GRMZM2G051683	AT3G16520	3E-105	Glycosyltransferase
GT2	MZ00037072	13.4	–8.0	GRMZM2G055795	AT4G18780 (IRX1)	0E+00	Cellulose synthase 11
GT2	MZ00020258	13.5	–5.5	GRMZM2G445905	AT5G44030 (IRX5)	0E+00	Cellulose synthase 10
GT2	MZ00044509	11.1	4.0	GRMZM2G074792	AT4G07960	0E+00	Putative glucosyltransferase/cellulose synthase
GT8	MZ00026735	11.4	–9.7	GRMZM2G165919	AT2G47180	3E-149	Galactinol synthase
GT8	MZ00044441	12.6	–8.3	GRMZM2G131697	AT2G47180	5E-148	Galactinol synthase
GT8	MZ00028554	9.9	4.5	GRMZM2G036918	AT2G38650	3E-137	GT8-like transferase, transferring glycosyl groups
GT31	MZ00044747	10.3	–5.3	GRMZM2G057779	AT5G57500	7E-58	Galactosyltransferase/glycosyltransferase family 31
GT31	MZ00032181	10.5	–4.5	GRMZM2G072406	AT5G57500	5E-54	Transferase, transferring glycosyl groups
GT43	MZ00056172	10.8	4.6	gbIBT036881.1I[b]	AT2G37090 (IRX9)	5E-43	GT43 [*Os03g0287800* (2e-74)]
GT47	MZ00033488	11.5	–4.9	GRMZM2G100143	AT1G27440 (IRX10)	0E+00	Secondary cell wall-related GT47 [*Os01g0926700* (0E+00)]
GT47	MZ00036858	13.5	–4.1	GRMZM2G059825	AT5G61840 (IRX10-L)	0E+00	Secondary cell wall-related GT47 [*Os01g0926700* (0E+00)]
pGH17	MZ00018424	10.0	–5.3	GRMZM2G354610	AT3G18180	9E-79	Glycosyltransferase
GH1	MZ00057235	12.7	63.6	GRMZM2G016890	AT5G44640	2E-128	Beta-D-glucosidase [*Os4bglu12* (5E-134)]
GH1	MZ00023504	11.5	60.4	GRMZM2G014844	AT5G44640	1E-135	Beta-D-glucosidase [*Os6bglu24* and *Os4bglu12* (3E-137)]
GH1	MZ00026498	12.6	34.1	GRMZM2G120962	AT5G44640	2E-137	Beta-D-glucosidase [*Os4bglu12* (6E-145)]
GH1	MZ00039183	10.8	21.1	GRMZM2G008247	AT5G44640	3E-135	Beta-D-glucosidase [*Os4bglu12* (6E-136)]
GH1	MZ00035426	10.9	17.5	refINM_001111984.1I[c]	AT5G44640	2E-128	Beta-D-glucosidase [*Os4bglu12* (6E-136)]
GH1	MZ00023721	12.5	16.8	GRMZM2G118003	AT3G18080	4E-131	Beta-D-glucosidase [*Os3bglu7* (0E+00)]
GH1	MZ00032041	11.1	–7.7	GRMZM2G457040	AT4G21760	0E+00	Beta-D-glucosidase [*Os4bglu16* (0E+00)]
GH1	MZ00016333	11.1	5.9	AC217401.3_FG002	AT3G18080	1E-176	Beta-D-glucosidase [*Os3bglu8* (0E+00)]
GH5	MZ00016790	10.1	5.0	GRMZM2G140201	AT2G20680	4E-152	Cellulase family protein
GH16	MZ00024711	12.2	42.9	GRMZM2G004699	AT5G13870	1E-131	XTH
GH16	MZ00013946	11.5	34.4	GRMZM2G180870	AT4G03210	8E-93	XTH
GH16	MZ00036398	10.9	–15.2	GRMZM2G026980	AT4G25810	2E-102	XTH
GH16	MZ00052416	9.8	6.3	GRMZM2G039919	AT1G14720	4E-75	XTH
GH16	MZ00055592	10.0	5.3	GRMZM2G030173	AT2G06850	6E-21	XTH
GH16	MZ00013272	9.9	5.1	GRMZM2G175598	AT5G57530	5E-38	XTH
GH16	MZ00004529	12.1	4.8	GRMZM2G413044	AT2G36870	5E-103	XTH
GH16	MZ00018500	11.4	4.9	GRMZM2G413006	AT5G57560	1E-108	XTH
pGH17	MZ00029303	12.7	18.7	GRMZM2G074811	AT3G13560	7E-05	Glucan endo-1,3-beta-glucosidase
GH17	MZ00028373	11.1	14.1	GRMZM2G046101	AT4G34480	4E-153	Glucan endo-1,3-beta-glucosidase
GH17	MZ00042022	11.2	12.1	GRMZM2G046459	AT2G05790	3E-161	Glycosyl hydrolase family 17 protein
GH17	MZ00037281	11.1	–7.7	GRMZM2G137535	AT4G16260	1E-85	Lichenase
GH17	MZ00028849	11.0	6.7	GRMZM2G335111	AT4G26830	6E-69	Glucan endo-1,3-beta-glucosidase
pGH17	MZ00026915	11.4	4.4	GRMZM2G447691	AT2G43670	2E-24	Glucan endo-1,3-beta-glucosidase
GH18	MZ00032065	9.7	5.1	GRMZM2G141456	AT4G19810	6E-52	Class V plant chitinase
pGH17	MZ00004170	10.1	–5.2	GRMZM2G130276	AT5G24090	3E-14	Hevamine-A/class III endochitinase
GH19	MZ00043887	10.3	–4.7	GRMZM2G145461	AT3G12500	2E-30	Chitinase
GH20	MZ00027331	10.9	7.0	GRMZM2G121514	AT1G65600	0E+00	Beta-*N*-acetylhexosaminidase/ hexosaminidase
GH28	MZ00018754	11.1	15.7	AC231180.2_FG006	AT4G23820	2E-157	Polygalacturonase
GH28	MZ00041598	11.1	4.1	GRMZM2G052844	AT4G23820	1E-163	Polygalacturonase
GH35	MZ00015016	10.0	7.7	GRMZM2G038281	AT5G63810	0E+00	Beta-galactosidase
GH35	MZ00027220	10.3	7.0	GRMZM2G127123	AT2G28470	0E+00	Beta-galactosidase
GH35	MZ00019862	11.8	4.6	GRMZM2G417455	AT4G36360	0E+00	Beta-galactosidase
GH35	MZ00039458	11.5	4.3	GRMZM2G178106	AT4G36360	0E+00	Putative galactosidase
GH36	MZ00019029	11.9	–4.0	GRMZM2G127147	AT5G20250	0E+00	Alkaline alpha galactosidase/raffinose synthase
pGH17	MZ00022215	10.6	9.6	GRMZM2G077299	AT3G10740	0E+00	Alpha-*N*-arabinofuranosidase

[a] Positive values indicate fold higher expression in IN13 compared with IN9. Negative values indicate higher expression in IN9 compared with IN13.
[b] No gene associated with the oligo using the maize genome browser.
[c] The oligo has too low homology using the maize genome browser.
p, putative; [ ] indicates the closest rice homologue.

potential chitinases were up-regulated in IN9 while one was up-regulated in IN13. Two *Arabidopsis* chitinases, AtCTL1 and AtCTL2, are believed to be involved in secondary plant cell wall biosynthesis since mutations in these genes cause ectopic deposition of lignin (Zhong *et al.*, 2002) and increase lignin accumulation in dark-grown seedlings (Hossain *et al.*, 2010), respectively. A putative GH51 family, α-*N*-arabinofuranosidase (GRMZM2G077299), was up-regulated in IN13. GH51 enzymes catalyse the hydrolysis of terminal non-reducing α-L-arabinofuranosyl residues, although they are also capable of hydrolysing β-D-xylosyl residues and thus might be considered as bi-functional arabinofuranosidase/β-D-xylosidase enzymes. GRMZM2G077299 showed the highest homology with *Arabidopsis* At3g10740, one of the few α-*N*-arabinofuranosidases that have been studied in plants (Montes *et al.*, 2008). In grasses, the removal of arabinofuranosyl residues from arabinoxylans by α-*N*-arabinofuranosidases leads to significant changes in the physicochemical properties of the cell wall (Lee *et al.*, 2003). Arabinoxylans consist of a (1,4)-β-D-xylan backbone substituted with α-L-arabinofuranosyl units, which can be esterified with hydroxycinnamic acids, in particular ferulic acid, which may form cross-bridges between adjacent arabinoxylan chains, or with lignin, by oxidative dimerization (Hatfield *et al.*, 1999). Such esterification can significantly contribute to the recalcitrance of biomass and the conversion of cell wall polysaccharides into fermentable sugars and thus the production of biofuels. The putative maize arabinofuranosidase identified therefore represents a priority candidate for further functional analysis. Modification of endogenous arabinofuranosidase activity might provide a tool to decrease the number of α-L-arabinofuranosyl residues available for substitution with ferulic acids and thereby reduce cross-linking in the cell wall matrix.

Transcriptional control of cell wall biogenesis

Because TFs act as master regulators of cellular processes, they are predicted to be excellent candidates for modifying complex traits in crop plants. TF-based technologies are therefore likely to be a prominent part of the next generation of biotechnological crops.

It has recently been shown that a number of NAC and MYB TFs regulate the formation of secondary cell wall synthesis in *Arabidopsis* and a number of woody species including poplar, pine, and eucalyptus (Rogers and Campbell, 2004; Goicoechea *et al.*, 2005; Mitsuda *et al.*, 2007; Bomal *et al.*, 2008; Zhong *et al.*, 2008, 2010). However, to date, only a few TFs regulating cell wall-associated processes in the grasses have been identified.

A wide range of TFs, including several MYB and NAC TFs, were differentially expressed between IN9 and IN13 (Table 3). The majority of these were up-regulated in IN9, thus representing excellent candidates for further functional analysis to confirm if these genes are involved in regulating secondary cell wall biogenesis in grasses. One of the few, if not only, TF so far reported to be involved in regulating secondary cell wall synthesis in grasses is the maize MYB TF ZmMYB42 (Fornale *et al.*, 2006; Sonbol *et al.*, 2009). Overexpression of ZmMYB42 in *Arabidopsis* repressed lignin biosynthesis, reducing the lignin content of lignified tissues, and increased cell wall degradability. A close homologue of ZmMYB42, GRMZM2G419239, with 95% identity at the amino acid level, was 6-fold more highly expressed in IN9 than in IN13 (Table 3). This is somewhat surprising as it might be expected that there would be no need for repression of lignin biosynthesis in tissue undergoing secondary cell wall thickening. However, ZmMYB42 has only been studied by heterologous expression in *Arabidopsis* using the strong, and constitutively active, 35S promoter. For an accurate functional analysis, regulators of lignin biosynthetic genes should be expressed in cells actively undergoing lignification. Thus, it is uncertain whether ZmMYB42, as well as some of the other MYBs that have been studied using expression in heterologous systems driven by strong constitutive promoters, are indeed regulators of lignin biosynthesis (Zhong and Ye, 2009). The alternative interpretation is that their effects on lignin biosynthesis observed following overexpression are indirect.

Although most attention has been focused on the MYB and NAC TFs, TFs belonging to other families might also be involved in regulating cell wall biogenesis. For instance, it has been shown that WRKY TFs increase the levels of soluble and wall-bound phenolic compounds and lignin. Such increases were observed when *Medicago* WRKY genes were overexpressed in tobacco (Naoumkina *et al.*, 2008), and a grapevine WRKY TF has been shown to be involved in regulating lignification (Guillaumie *et al.*, 2010). AUX/IAA TFs have been shown to regulate the developmental processes involved in secondary growth (Oh *et al.*, 2003; Scarpella and Meijer, 2004). Moreover, it has recently been shown that transcriptional regulators including C2H2 zinc finger and AP2-EREBP TFs were more highly expressed during cell wall thickening during cotton fibre development, compared with the earlier elongation stage (Al-Ghazi *et al.*, 2009). A regulatory role for AP2-EREBPs in poplar and *Arabidopsis* secondary cell wall metabolism has also been suggested (van Raemdonck *et al.*, 2005; Lasserre *et al.*, 2008). Thus, it is possible that some of the other differentially expressed TFs listed in Table 3 are also involved in regulating secondary cell wall biogenesis in grasses. TFs specifically involved in regulating the biosynthetic pathways of cellulose and xylan synthesis remain elusive. Some of these regulatory functions might well be fulfilled by some of the other TF family members.

Many TFs are involved in regulating resistance to biotic and abiotic stress in model plants as well as in crop plants (for a review, see Century *et al.*, 2008). As the cell wall plays an intrinsic role in conferring resistance to many biotic and abiotic stresses (Bray, 2004; Fan *et al.*, 2006; Konno *et al.*, 2008; Moore *et al.*, 2008), it is possible that some of the TFs identified, in this and other studies, might be involved in regulating interacting pathways of stress-related responses and normal developmental regulation of cell wall biogenesis.

Table 3. Transcription factors >4-fold differentially expressed between IN9 and IN13

Transcription factor class	Oligo ID	Signal intensity	Fold change[a]	Maize gene ID	*Arabidopsis* homologue gene	E-value
NAC	MZ00023972	10.9	–7.1	GRMZM2G079632	AT5G08790/ATAF2	3E-65
NAC	MZ00026127	10.7	–6.2	GRMZM2G068973	AT5G08790/ATAF2	6E-69
NAC	MZ00028246	10.8	–5.9	GRMZM2G162739	AT5G08790/ATAF2	1E-65
NAC	MZ00035947	10.8	–4.8	GRMZM2G347043	AT5G08790/ATAF2	8E-71
NAC	MZ00027155	11.4	–4.7	GRMZM2G054252	AT2G33480	3E-29
NAC	MZ00039846	11.3	–4.3	GRMZM2G018553	AT5G08790/ATAF2	1E-68
NAC[b]	MZ00018291	9.5	–3.6	GRMZM2G123667	AT5G08790/ATAF2	4E-74
NAC[b]	MZ00022590	10.3	–2.9	GRMZM2G167018	AT1G56010/NAC021	1E-62
NAC[b]	MZ00036503	12.0	–2.9	GRMZM2G014653	AT1G01720/ATAF1	2E-92
NAC[b]	MZ00043228	11.5	–2.0	AC202396.4_FG010	AT1G01720/ATAF1	1E-07
MYB	MZ00032119	11.1	–6.0	GRMZM2G419239	AT4G38620/MYB4	2E-75
MYB	MZ00030474	11.0	–5.9	AC197146.3_FG002	AT3G28910/MYB30	3E-69
MYB	MZ00040603	10.0	–5.4	GRMZM2G037650	AT4G22680/MYB85	4E-66
MYB	MZ00055303	10.9	–5.2	GRMZM2G088524	AT5G61620	6E-30
MYB	MZ00040616	10.2	–4.2	GRMZM2G162434	AT1G08810	8E-70
AP2-EREBP	MZ00012977	11.2	–6.0	GRMZM2G069146	AT4G25470/DREB1C	7E-33
AP2-EREBP	MZ00016032	12.7	–5.6	GRMZM2G174347	AT5G44210/ERF9	2E-29
AP2-EREBP	MZ00016033	13.3	–4.9	GRMZM2G020150	AT3G15210/ERF4	9E-27
AP2-EREBP	MZ00025004	14.1	–4.5	GRMZM2G052667	AT1G72360/ERF073	2E-28
AP2-EREBP	MZ00005099	11.0	–4.5	GRMZM2G124011	AT4G25480/DREB1A	5E-30
AP2-EREBP	MZ00018542	12.3	–4.5	gb|EU955981.1|[c]	AT1G72360/ERF073	3E-29
AP2-EREBP	MZ00004814	10.2	–4.1	GRMZM2G129674	AT3G16770/RAP2-3	5E-20
AUX/IAA	MZ00029389	11.3	6.3	GRMZM2G366373	AT1G04240/IAA3	1E-40
AUX/IAA	MZ00004631	10.8	–5.8	GRMZM2G079200	AT4G14550/IAA14	4E-35
AUX/IAA	MZ00037052	12.1	–4.5	GRMZM2G115357	AT5G43700/IAA4	4E-43
AUX/IAA	MZ00024726	13.3	–4.2	GRMZM2G004696	AT1G04250/IAA17	2E-46
C2H2	MZ00029551	10.5	–5.9	GRMZM2G400714	AT5G67450/AZF1	1E-30
C2H2	MZ00021052	13.7	–4.8	GRMZM2G086835	AT1G24625/ZFP7	4E-17
C2H2	MZ00020958	10.2	–4.5	GRMZM2G035103	AT3G19580/AZF2	1E-29
C3H	MZ00037166	12.6	–9.3	GRMZM2G173124	AT2G19810	9E-70
C3H	MZ00044553	13.7	–5.7	gb|EU958052.1|[d]	AT5G58620	1E-21
bZIP	MZ00042491	9.8	5.2	GRMZM2G052102	AT3G58120/BZIP61	5E-57
bZIP	MZ00028419	11.6	–4.8	GRMZM2G073427	AT5G28770	4E-29
WRKY	MZ00042508	10.8	–10.2	ref|NM_001154079.1|[c]	AT2G38470/WRKY33	1E-89
WRKY	MZ00035864	13.0	5.1	GRMZM2G069668	AT1G29860/WRKY71	3E-19
PLATZ	MZ00029580	11.0	–4.4	GRMZM2G131280	AT4G17900	2E-75
MADS	MZ00022435	10.4	7.5	GRMZM2G137510	AT5G60910/AGL8	6E-43
HB	MZ00019195	10.1	7.5	GRMZM2G097349	AT1G69780/ATHB-13	2E-46
HB	MZ00030241	9.5	4.3	GRMZM2G106276	AT5G06710/HAT14	2E-49
HB	MZ00042036	13.2	–4.2	GRMZM2G028041	AT4G08150/KNAT1	1E-98
bHLH	MZ00033503	10.2	4.6	GRMZM2G035156	AT3G47710	2E-25
bHLH	MZ00048554	10.0	4.5	GRMZM2G180452	AT1G10120/BHLH74	2E-37

[a] Positive values indicate fold higher expression in IN13 compared with IN9. Negative values indicate higher expression in IN9 compared with IN13.
[b] Identified using the annotation file for the maize oligonucleotide array.
[c] The oligo has too low homology using the maize genome browser.
[d] No gene associated with the oligo using the maize genome browser.

Lignin synthesis

Lignin plays an important role in plant growth and development. This heterogeneous hydrophobic phenolic polymer impregnates the cellulose and hemicellulose networks, thereby strengthening the cell wall and providing a stable, waterproof coating that protects the secondary wall from physical and biological attacks. The presence of lignin is one of the main obstacles in the conversion of lignocellulosic biomass into fermentable sugars as it impedes the enzymatic digestion of the cell wall biomass (Himmel *et al.*, 2007), hence lignin modification is one of the priorities for the improvement of bioenergy feedstocks. Recently, genetic genomics approaches have been used in maize recombinant inbred lines to identify candidate genes associated with lignin content and cell wall digestibility (Shi *et al.*, 2007; Thomas *et al.*, 2010), but further investigations are needed to identify genes underlying quantitative trait loci (QTLs) involved in these

complex traits. Although lignification is spatially and temporally regulated within maize internodes, most of the lignification takes place during post-elongation secondary cell wall deposition (Jung and Casler, 2006). Lignin precursors are produced by the phenylpropanoid pathway, and many genes belonging to this pathway exhibited higher expression in IN9 (Table 4), in agreement with active secondary cell wall synthesis and lignification occurring in this internode. Because the biochemical pathways of monolignol biosynthesis are highly conserved throughout vascular plants (Xu *et al.*, 2009), the >4-fold differentially expressed gene list was extended to include potential phenylpropanoid pathway genes queried from the maize oligonucleotide annotation file showing >2-fold differential expression (Table 4).

Four putative phenylalanine ammonia-lyases (PALs), predicted to catalyse the first step in the phenylpropanoid pathway, showed a >2-fold increase in expression in IN9. Two 4-coumarate-CoA ligase (4CL) family members were >2-fold preferentially expressed in IN13 and two >2-fold preferentially expressed in IN9. The 4CL maize homologue to 4CL2 in *Arabidopsis* (At3g21240) showed the highest level of 4CL expression in the non-elongating internode, in agreement with transcriptome analysis results obtained from macroarray gene expression profiling during maize internode development (Guillaumie *et al.*, 2007). In addition, their study showed that different 4CLs exhibit different expression patterns, some being preferentially expressed in young stem tissues. Several *O*-methyltransferases (OMTs) showed >2-fold differential expression, most of these being classified as ZRP4-like OMT genes. ZRP4 is involved in suberin synthesis (Held *et al.*, 1993) and, although ZRP4-like OMTs have been associated with lignin synthesis, their involvement in the lignin pathway has not yet been firmly established. Three caffeoyl-CoA *O*-methyltransferases (CCoAOMTs), including maize CCoAOMT1 and CCoAOMT2, were >2-fold up-regulated in IN9. ZmCAD2, a cinnamyl alcohol dehydrogenase (CAD) catalysing the last step in monolignol synthesis and the activity of which is affected by the maize *brown-midrib1* mutation (*bm1*) (Halpin *et al.*, 1998), was not identified as being >2-fold differentially expressed. Likewise, the gene encoding caffeic acid *O*-methyl transferase (COMT), which is mutated in *bm3* plants (Vignols *et al.*, 1995), could not be detected as being >2-fold differentially expressed. Several putative cinnamoyl-CoA reductases (CCRs) were differentially expressed, although it is difficult to distinguish CCRs from dihydroflavonol-4-reductases (DFRs). CCR1 was the only confirmed CCR identified as being differentially expressed and exhibited the highest absolute expression level (Table 4). In agreement with expression data for ZmCCR1 showing highest expression during the period of active lignification (Pichon *et al.*, 1998), CCR1 showed 3.1-fold higher expression in the lignifying IN9 compared with the elongating IN13. Based on expression patterns that correlate with regions undergoing lignification, laccases have been proposed to be involved in the oxidative polymerization of monolignols, produced by the phenylpropanoid pathway, into lignins (Caparros-Ruiz *et al.*, 2006; Guillaumie *et al.*, 2007). The up-regulation of three laccases in the lignifying IN9 suggests an involvement in the lignification process.

Aldehyde dehydrogenase (ALDH), RF2C, was up-regulated in IN13. The closest *Arabidopsis* homologue, At3g24503/REF1, was shown to be involved in the formation of both soluble and cell wall-linked ferulate esters (Nair *et al.*, 2004). REF1 is more closely related to RF2C in maize than other *Arabidopsis* ALDHs, suggesting that RF2C, like REF1, is involved in the biosynthesis of ferulic acid, a major cell wall-esterified hydroxycinnamic acid in the grasses which impedes the hydrolysis of the cell wall biomass.

Cell wall and plasma membrane proteins

Many leucine-rich proteins (LRPs), in particular those containing extensin-like motifs, called leucine-rich extensins (LRXs), and arabinogalactan proteins (AGPs) are involved in cell wall-related processes. Several LRXs and other LRPs showed >4-fold differential expression (Table 5), most being up-regulated in the elongating IN13. A function related to cell wall biogenesis has been either shown or implicated for most of the *Arabidopsis* homologues (Table 5). For instance, AtLRX1, the closest homologue to the IN13 preferentially expressed GRMZM2G082823, is involved in regulating cell wall formation and assembly in elongating root hairs (Baumberger *et al.*, 2001). The plasma membrane/membrane-attached multicopper oxidase, GRMZM2G049693, whose *Arabidopsis* homologue, SKU5, is involved in cell wall expansion in roots (Sedbrook *et al.*, 2002), was also preferentially expressed in IN13. Fasciclin-like arabinogalactan proteins (FLAs) are a subclass of AGPs that contain putative cell adhesion domains known as fasciclin domains. Expression of some FLAs has been correlated with the onset of secondary wall cellulose synthesis in *Arabidopsis* stems (Ito *et al.*, 2005), and also with wood formation in trees (Lafarguette *et al.*, 2004; Andersson-Gunneras *et al.*, 2006), suggesting a role in secondary cell wall formation. The FLA up-regulated in IN9, EU962845, showed highest homology to *Arabidopis* AtFLA11 (Table 5). A recent study has shown that a T-DNA knockout mutant for AtFLA11 affects the tensile strength and stiffness of the stem as well as secondary cell wall composition (MacMillan *et al.*, 2010). Four FLAs were >4-fold up-regulated in IN13, suggesting that these might be involved in primary cell wall biogenesis. This hypothesis is supported by the high level of expression of the *Populus trichocarpa* homologue for AC234156.1_FG005, gw1.XIV.3668.1, in elongating internodes (Dharmawardhana *et al.*, 2010). Furthermore, based on the expression profile of FLAs during cotton fibre development and *Arabidopsis* stem development, a function in either primary cell wall development or secondary cell wall deposition has been suggested (Liu *et al.*, 2008; Minic *et al.*, 2009).

The maize britle stalk2 (ZmBK2) gene is a member of the COBRA gene family that encodes glycosylphosphatidylinositol (GPI)-anchored proteins. Several members of this family are involved in cell wall biogenesis in both dicots

Table 4. Genes involved in phenylpropanoid metabolism >2-fold differentially expressed between IN9 and IN13

Family	Oligo ID	Signal intensity	Fold change[a]	Gene ID	Arabidopsis homologue gene	E-value	Putative annotation
ALDH	MZ00039837	10.5	5.5	GRMZM2G071021	AT3G24503	0E+00	Cytosolic aldehyde dehydrogenase RF2C
CCR/DFR	MZ00000662	11.1	–21.1	gb|BT064982.1|[b]	AT1G15950	2E-125	Cinnamoyl-CoA reductase/dihydroflavonol-4-reductase
CCR/DFR	MZ00000962	10.9	–6.5	GRMZM2G001991	AT5G14700	7E-81	Dihydroflavonol-4-reductase/cinnamoyl-CoA reductase-related
CCR/DFR	MZ00027625	11.5	–5.8	GRMZM2G050076	AT2G33590	1E-58	Dihydroflavonol-4-reductase/putative cinnamoyl-CoA reductase
CCR/DFR	MZ00034340	10.1	4.0	GRMZM2G033555	AT2G33590	2E-91	Dihydroflavonol-4-reductase/putative cinnamoyl-CoA reductase
CCR/DFR	MZ00036789	11.3	4.0	GRMZM2G034069	AT2G33590	1E-100	Dihydroflavonol-4-reductase/putative cinnamoyl-CoA reductase
CCR1	MZ00015899	13.5	–3.1	GRMZM2G131205	AT1G15950	2E-134	Cinnamoyl CoA reductase1
CCR/DFR	MZ00035958	9.4	2.8	GRMZM2G034360	AT2G33590	1E-100	Dihydroflavonol-4-reductase
CCR/DFR	MZ00023228	9.9	2.4	GRMZM2G057328	AT1G15950	1E-103	Dihydroflavonol-4-reductase
CCR/DFR	MZ00012815	9.2	–2.3	gb|BT036278.1|[c]	AT2G33590	2E-85	Dihydroflavonol-4-reductase
CCR/DFR	MZ00030523	10.7	–2.2	GRMZM2G009681	AT2G33590	4E-94	Dihydroflavonol-4-reductase
OMT	MZ00044446	12.5	37.0	GRMZM2G041866	AT4G35160	4E-50	*O*-Methyltransferase ZRP4-like
OMT	MZ00016324	11.7	14.7	GRMZM2G140996	AT4G35160	7E-53	*O*-Methyltransferase ZRP4-like
OMT	MZ00042149	12.4	–14.3	GRMZM2G097297	AT4G35160	2E-49	*O*-Methyltransferase ZRP4
OMT	MZ00026069	11.3	9.7	ref|NM_001157182.1|[c]	AT4G35160	3E-25	*O*-Methyltransferase ZRP4-like
OMT	MZ00042974	13.4	6.9	GRMZM2G085924	AT4G35160	1E-26	*O*-Methyltransferase-like protein
FOMT	MZ00043408	15.1	–4.2	AC196475.3_FG004	AT5G54160	3E-128	Flavonoid *O*-methyltransferase
OMT	MZ00051351	9.7	–2.2	GRMZM2G141026	AT4G35160	4E-52	*O*-Methyltransferase ZRP4-like
CHI	MZ00036732	13.0	–18.5	GRMZM2G175076	AT5G05270	1E-57	Chalcone flavonone isomerase
CHI	MZ00026366	11.9	–2.7	GRMZM2G155329	AT3G55120	6E-73	Chalcone flavanone isomerase 1
IFR	MZ00029320	14.0	–6.4	GRMZM2G326116	AT1G32100	3E-122	Isoflavone reductase-like protein
F3'H	MZ00019364	14.6	–5.9	gb|EU971853.1|[b]	AT5G07990	1E-65	Cytochrome P450/flavonoid 3'-hydroxylase-like protein
F3'H	MZ00021196	10.7	–5.5	GRMZM2G146234	AT5G24530	2E-61	Flavanone 3-hydroxylase-like protein
F3'H	MZ00005460	11.2	–5.4	GRMZM2G160763	AT5G07990	4E-161	Cytochrome P450/ flavonoid 3-monooxygenase
CCoAOMT	MZ00000781	10.0	–4.0	GRMZM2G033952	AT4G34050	5E-81	Caffeoyl-CoA *O*-methyltransferase 1
CCoAOMT	MZ00017952	11.8	–2.4	GRMZM2G004138	AT4G34050	1E-80	Caffeoyl-CoA *O*-methyltransferase 1 like
CCoAOMT	MZ00057269	14.0	–2.0	GRMZM2G099363	AT4G34050	1E-112	Caffeoyl-CoA *O*-methyltransferase 2
PAL	MZ00014292	12.6	–4.9	GRMZM2G160541	AT3G10340	0E+00	Phenylalanine ammonia-lyase
PAL	MZ00039256	14.5	–4.1	GRMZM2G074604	AT2G37040	0E+00	Phenylalanine ammonia-lyase
PAL	MZ00025090	11.3	–2.3	GRMZM2G029048	AT2G37040	0E+00	Phenylalanine ammonia-lyase
PAL	MZ00034925	12.4	–2.2	GRMZM2G081582	AT2G37040	0E+00	Phenylalanine ammonia-lyase
4CL	MZ00016350	13.1	–3.8	gb|BT067847.1|[c]	AT3G21240	0E+00	4-Coumarate coenzyme A ligase
4CL	MZ00001892	10.4	–3.0	GRMZM2G019746	AT5G63380	1E-135	4-Coumarate coenzyme A ligase family protein
4CL	MZ00020111	10.1	2.9	GRMZM2G054013	AT1G65060	0E+00	4-Coumarate coenzyme A ligase, 4CL3
4CL	MZ00018351	10.4	2.1	GRMZM2G080663	AT5G63380	2E-70	4-Coumarate coenzyme A ligase family protein
Laccase	MZ00004658	9.8	4.8	GRMZM2G132169	AT5G05390	0E+00	Laccase/L-ascorbate oxidase
Laccase	MZ00018473	10.2	–4.1	GRMZM2G031117	AT5G05390	4E-147	Laccase 1
Laccase	MZ00004270	12.0	–4.0	GRMZM2G072780	AT5G60020	0E+00	Laccase
Laccase	MZ00055128	9.6	–2.4	GRMZM2G329311	AT3G09220	1E-68	Laccase
CAD	MZ00014812	13.9	2.4	AC234163.1_FG004	AT4G37980	6E-105	Putative cinnamyl alcohol dehydrogenase
FLS	MZ00026581	11.7	3.3	GRMZM2G152801	AT5G08640	1E-101	Flavonol synthase/flavanone 3-hydroxylase

[a] Positive values indicate fold higher expression in IN13 compared with IN9. Negative values indicate higher expression in IN9 compared with IN13.
[b] THe oligo has too low homology using the maize genome browser.
[c] No gene is associated with the oligo using the maize genome browser.

ALDH, aldehyde dehydrogenase; CAD, cinnamyl alcohol dehydrogenase, CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone flavonone isomerase; 4CL, 4-coumarate:CoA ligase; DFR, dihydroflavonol-4-reductase; FLS, flavonol synthase; FOMT, flavonoid O-methyltransferase; F3'H, flavonoid 3'-hydroxylase; IFR, isoflavone reductase; OMT, O-methyltransferase; PAL, phenylalanine ammonia-lyase.

Table 5. Selected genes encoding cell wall and plasma membrane proteins >4-fold differentially expressed between IN9 and IN13

Annotation	Oligo ID	Signal intensity	Fold change[a]	Maize gene ID	*Arabidopsis* homologue gene	E-value	Suggested function
LRR protein	MZ00019529	10.6	10.3	GRMZM2G149201	AT4G13340/LRX3	6E-127	Expression associated with elongating cells1
LRR protein	MZ00028889	9.8	5.6	GRMZM2G042181	AT4G06744	3E-74	Indirectly associated with cell wall biosynthesis2
LRR protein	MZ00046729	10.2	–5.6	GRMZM2G341410	AT3G22800/LRX6	9E-101	Potentially involved in cell wall development3
LRR protein	MZ00041948	12.5	–5.5	GRMZM2G012031	AT2G34680/AIR9	0E+00	Microtubule-Associated Protein4
LRR protein	MZ00005954	10.5	5.3	GRMZM2G342509	AT4G06744	2E-97	Indirectly associated with cell wall biosynthesis2
LRR protein	MZ00044323	11.2	5.3	GRMZM2G082823	AT1G12040/LRX1	8E-113	Cell wall formation and assembly in elongating root hairs5
LRR protein	MZ00018762	10.6	4.9	GRMZM2G022897	AT1G25570	0E+00	Unknown
LRR protein	MZ00016499	12.4	–4.0	GRMZM2G366150	AT1G15740	0E+00	Unknown
Fasciclin-like AGP	MZ00016317	11.0	15.6	AC234156.1_FG005	AT2G04780/FLA7	8E-53	Poplar homologue expressed in elongating internode6
Fasciclin-like AGP	MZ00018923	11.4	9.2	GRMZM2G301908	AT5G55730/FLA1	4E-37	Unknown
Fasciclin-like AGP	MZ00032716	12.7	-6.6	gbIEU962845.1I[b]	AT5G03170/FLA11	2E-20	Involved in secondary cell wall formation7
Fasciclin-like AGP	MZ00016177	10.6	6.0	GRMZM2G003752	AT2G45470/FLA8	3E-105	Unknown
Fasciclin-like AGP	MZ00039834	12.1	5.4	GRMZM2G084812	AT4G12730/FLA2	5E-52	Unknown
Cobra-like protein	MZ00012936	10.0	6.4	GRMZM2G167497	AT5G15630/IRX6	0E+00	Zm brittle stalk-2-like protein 7 / Unknown
Cobra-like protein	MZ00005183	12.7	–7.7	GRMZM2G109326	AT5G15630/IRX6	0E+00	Zm brittle stalk2: involved in cell wall biogenesis8
Multicopper oxidase	MZ00015277	10.1	4.5	GRMZM2G049693	AT4G12420/SKU5	0E+00	Cell wall expansion in roots9

[a] Positive values indicate fold higher expression in IN13 compared with IN9. Negative values indicate higher expression in IN9 compared with IN13.
[b] No gene associated with the oligo using the maize genome browser.
[1] Irshad et al., 2008; [2]Dauwe et al., 2007; [3]Diet et al., 2006; [4]Buschmann et al., 2006; [5]Baumberger et al., 2001; [6]Dharmawardhana et al., 2010; [7]Ito et al., 2005; [8]Ching et al., 2006; [9]Sedbrook et al., 2002.

and monocots. Mutations in ZmBK2 affect stalk strength in maize by interfering with the deposition of cellulose in the secondary cell wall in fibre cells (Ching *et al.*, 2006). The exact function of COBRA proteins is still unknown, although a function for BK2 in the patterning of lignin–cellulosic interactions that maintain organ flexibility has been suggested (Sindhu *et al.*, 2007). The location of COBRA proteins at the interface of the plasma membrane and the cell wall, and their association with lipid microdomains (rafts) suggest a potential function in the regulation or integration of secretion and wall assembly (Martin *et al.*, 2005). In accordance with a secondary cell wall-related function, ZmBK2 (GRMZM2G109326) was 7.7 fold up-regulated in IN9 (Table 5). Surprisingly, the ZmBK2-like gene, ZmBK2L7 (GRMZM2G167497), was 6.4-fold up-regulated in IN13, suggesting a role in primary rather than secondary cell wall biogenesis. Although phylogenetic analysis has suggested that ZmBK2 and ZmBK2L7 are co-orthologues of *Arabidopsis* AtCOBL4/ IRX6, expression analysis already suggested that ZmBK2L7 does not carry out the same function as ZmBK2 (Brady *et al.*, 2007).

Members of the receptor-like kinase (RLK) family, membrane-bound signalling molecules with an extracellular receptor domain, are good candidates for sensing and transducing the 'status' of the cell wall (Shiu and Bleecker, 2001), and it is perhaps not surprising that recently several RLKs have been implicated in cell wall integrity sensing (Kohorn *et al.*, 2006; Hématy *et al.*, 2007; Xu *et al.*, 2008; Guo *et al.*, 2009). Supplementary Table S4 at *JXB* online contains a list and analysis of the protein kinases that were >4-fold differentially expressed.

Genes specifically involved in grass cell wall biogenesis

The potential functionality of newly identified genes in grasses can currently often only be inferred from information available from studies in *Arabidopsis* due to its unique molecular genetic resources. Hence, many of the genes and gene families discussed so far contain *Arabidopsis* homologues with small E-values (Tables 2–5). However, functionality cannot always be implied from the closest homologues in *Arabidopsis*. Indeed, comparative genomics and expression analysis of cell wall-related genes and gene families between the grasses and *Arabidopsis* revealed that orthology often cannot be inferred from homology (Cao *et al.*, 2008; Penning *et al.*, 2009).

Comparative analysis of the maize genome with that of rice, sorghum, and *Arabidopsis* indicated that 71.5% of the 11 892 gene families identified in maize were shared between these grasses and *Arabidopsis*, while 17.5% (2077 gene families) appeared to be specific to the grasses (Schnable *et al.*, 2009). The differences in cell wall structure and composition between the dicots and the grasses suggest the relative contribution of specific genes and gene families involved in grass cell wall biogenesis to be even higher. Indeed it has been estimated that at least a third of cell wall-related genes of grasses could have no, or few, orthologues in *Arabidopsis*, making genetic functional analyses in a grass model system essential (Carpita and McCann, 2008).

In an attempt to identify some of the potential genes specifically involved in grass cell wall-related processes, the list of genes that showed >4-fold differential expression was filtered for maize genes with no significant *Arabidopsis* homologue based on an E-value $>1e^{-20}$ (Supplementary Table S5 at *JXB* online). The filtered list contains two jacalin-like lectin domain-containing proteins highly up-regulated in IN13. One of them, GRMZM2G402417, was one of the most highly differentially expressed genes in the experiment (105-fold). Although lectins are generally considered to play a role in defence signalling, a mannose-binding jacalin-related lectin

was involved in the regulation of rice growth and development (Jiang *et al.*, 2007). Two glycine-rich cell wall proteins (GRPs) up-regulated in IN13 and one in IN9 might be involved in cell wall sensing. Other genes up-regulated in IN13 included a number of proline-rich cell wall proteins, a putative GH17 family member, a WRKY TF, and two rapid alkalinization factor (RALF) domain-containing proteins, which are polypeptide hormones involved in regulating plant stress, growth, and development (Pearce *et al.*, 2001). A RALF domain-containing protein was also highly up-regulated in non-elongating internodes.

A number of genes encoding 'grass-specific' ABA/WDS-induced proteins were highly up-regulated in IN9. These plant defence-related proteins are induced by water deficit stress (WDS) or abscisic acid (ABA) stress and ripening. The role of these proteins at the molecular level is unclear. The plant cell wall plays an important role in conferring drought tolerance as this involves restructuring of the cell wall to allow growth at lower water content. Previous studies have shown that water deficit stress changes the expression of cell wall-related genes (Bray, 2004; Harb *et al.*, 2010), the activity of cell wall matrix enzymes (Konno *et al.*, 2008), and the accumulation of cell wall phenolics such as lignin and ferulic acid (Fan *et al.*, 2006; Hura *et al.*, 2009). It is possible that these ABA/WDS-induced proteins play a role in regulating cell wall-related processes, possibly by acting as an architectural transcriptional regulator. For example, an ABA/WDS-containing protein from *Solanum lycopersicum* (formerly *Lycopersicon esculentum*) has been shown to bind double-stranded DNA (Maskin *et al.*, 2007), and a *Vitis vinifera* ABA/WDS protein was able to interact with another TF (Saumonneau *et al.*, 2008), suggesting a recruitment function for other regulatory proteins.

Most of the genes in the filtered list without obvious *Arabidopsis* homologues were categorized as unknown (57% and 62% of the genes that were >4-fold up-regulated in IN13 and IN9, respectively). Judged by their pattern of expression, many of these genes might be involved in cell wall-related processes specific to the grasses and are therefore interesting candidates for further functional analysis.

Concluding remarks

The analysis of genes differentially expressed between an elongating internode (IN13) and a non-elongating internode (IN9) demonstrated that this profiling experiment succeeded in identifying genes associated with primary and secondary cell wall processes. A significant overlap between genes associated with cell wall biogenesis and genes implicated in responses to biotic and/or abiotic stresses was also identified. This suggests that many of the proteins involved in cell wall-related processes during normal development are also recruited during defence-related cell wall remodelling events. This is not necessarily surprising given that the plant cell wall goes through extensive remodelling and reconstruction processes to ensure an adequate defence against biotic attacks and severe environmental conditions (Sarkar *et al.*, 2009; Moura *et al.*, 2010).

Several thousand gene products are estimated to participate in the synthesis, deposition, and function of cell walls, but very few cell wall-associated genes have been identified in grasses. The data reported in this paper provide a platform for the functional testing of candidate genes for involvement in cell wall-related processes. This testing could include forward and reverse genetic-based approaches in model grasses such as again maize or *Brachypodium*. Such studies will enable a better understanding of type II cell wall biogenesis and its regulation in the grasses which is necessary to enable the manipulation of traits that contribute to biomass yield and quality. The optimization of energy crop cell walls will enable the matching of lignocellulosic feedstock chemistry to a range of biopower and biofuel end uses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. MA-plot depicting the average log-intensity on the *x*-axis against the average log-fold change on the *y*-axis.

Table S1. List of genes showing >2-fold differential expression between internode 9 and internode 13.

Table S2. List of genes showing >4-fold differential expression between internode 9 and internode 13.

Table S3. Cellulose synthase (CesA) and cellulose synthase-like (CSL) genes preferentially expressed in elongating internode 13.

Table S4. List of protein kinases >4-fold differentially expressed between internode 9 and internode 13.

Table S5. List of >4-fold differentially expressed genes without apparent *Arabidopsis* homologues.

Table S6. Primers used for quantitative RT-PCR.

Note S1. References used in the supplementary data but not listed in the main manuscript.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant on Bioenergy and Biorenewables [BBSEG00003134].

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