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RESEARCH PAPER

Light, the circadian clock, and sugar perception in the control of lignin biosynthesis

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Abstract

Experiments were undertaken to investigate some of the mechanisms that may function to regulate lignin biosynthesis (lignification) in *Arabidopsis thaliana*. Northern blot analyses revealed that several genes encoding enzymes involved in the synthesis of lignin monomers displayed significant changes in transcript abundance over a diurnal cycle. Northern blot analysis also suggested that some of the changes in diurnal transcript abundance were likely to be attributable to circadian regulation, whereas others were likely to be attributable to light perception. Comparison of circadian changes in transcript abundance of lignin biosynthetic genes between wild-type plants and the *sex1* mutant, which is impaired in starch turnover, suggested that carbon availability related to starch turnover might determine the capacity to synthesize lignins. This hypothesis was supported by the observation that the *sex1* mutant accumulated fewer lignins than wild-type plants. Consistent with the relationship between carbon availability and lignin accumulation, analysis of dark-grown wild-type *A. thaliana* seedlings uncovered a role for sugars in the regulation of lignin biosynthesis. Analysis of lignin accumulation, as determined by qualitative changes in phloroglucinol staining, suggested that metabolizable sugars positively influence the abundance of lignins. Transcriptome analysis supports the hypothesis that sugars are not merely a source of carbon skeletons for lignification, but they also function as a signal to enhance the capacity to synthesize lignins.

Key words: Carbohydrate, circadian, diurnal, lignification, lignin, sugar.

Introduction

Lignins are complex three-dimensional polymers that are embedded in specialized plant cell walls, such as the secondary wall of xylem cells (Sederoff *et al.*, 1999). Lignins reinforce and rigidify plant cell walls and render them impermeable to water and resistant to microbial degradation (Lewis and Yamamoto, 1990; Douglas, 1996; Ros Barcelo, 1997; Boudet, 2000). Consequently, the deposition of lignins, known as lignification, is an important process in the differentiation of plant cells that contribute to support, water transport, and defence against pathogens, including xylem cells, sclerified parenchyma, and cells involved in the hypersensitive response (Esau, 1965; Douglas, 1996; Hose *et al.*, 2001; Lauvergeat *et al.*, 2001). The spatial and temporal control of lignification is necessary to ensure that lignin deposition is properly integrated into cellular differentiation. For example, as the deposition of lignins limits plant cell wall extension,

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lignification must be regulated so that it occurs after a cell has elongated so as not to impinge on plant growth. Lignification must also be regulated as it is an energetically costly process that makes use of a large quantity of carbon (Amthor, 2003).

Lignin biosynthesis is a specialized branch of phenylpropanoid metabolism (Baucher *et al.*, 1998; Humphreys and Chapple, 2002; Boerjan *et al.*, 2003). Phenylpropanoid metabolism generates the C_6–C_3 skeletons that are used to build a diverse array of phenolic compounds, including flavonoids, stilbenes, coumarins, lignans, and lignins (Douglas, 1996). Between 30% and 40% of organic carbon is thought to be channelled into phenylpropanoid metabolism in plants (van Heerden *et al.*, 1996). As anywhere between 10% and 40% of plant dry weight can be attributed to lignins (Sederoff *et al.*, 1999), it is obvious that much of the carbon that is fed into phenylpropanoid metabolism may be diverted to lignin biosynthesis. This is particularly true in lignifying cells, such as xylem, where the diversion of phenylpropane units into lignins is effectively qualitative. As plants do not possess a mechanism to degrade lignins (Lewis and Yamamoto, 1990; Trojanowski, 2001; Perez *et al.*, 2002), lignification represents a significant, non-recoverable investment of carbon and energy. Consequently, it is likely that plants monitor resource availability and regulate lignification so that the requirements for carbon skeletons and energy to build lignins do not outpace availability. It remains to be determined how plants couple the detection of resource availability with the regulation of lignin biosynthesis.

A large body of evidence suggests that many lignin biosynthetic enzymes are regulated at the transcriptional level (see review by Anterola and Lewis, 2002; Boudet *et al.*, 2003; Rogers and Campbell, 2004). For example, the timing and localization of the accumulation of transcripts corresponding to many of the genes that encode lignin biosynthetic enzymes shows a strong correlation with the deposition of lignins (Hauffe *et al.*, 1993; Feuillet *et al.*, 1995; Ehlting *et al.*, 1999; Chen *et al.*, 2000; Christensen *et al.*, 2001; Lauvergeat *et al.*, 2001, 2002; Anterola *et al.*, 2002; Hawkins *et al.*, 2003; Raes *et al.*, 2003; Sibout *et al.*, 2003). Mutant analyses and promoter:: reporter fusion experiments have begun to elucidate the upstream signals that regulate the expression of these genes (Ferrandiz *et al.*, 2000; Lacombe *et al.*, 2000; Liljegren *et al.*, 2000; Zhong *et al.*, 2002; Cano-Delgado *et al.*, 2003; Mele *et al.*, 2003; Patzlaff *et al.*, 2003; Newman *et al.*, 2004).

Here, the diurnal changes in transcript abundance are examined for the genes that encode enzymes involved in synthesizing monolignols, the monomeric precursors used to generate lignins (Fig. 1). Findings from previously published microarray experiments, which suggested that at least a subset of these genes were under circadian control (Harmer *et al.*, 2000), are confirmed using northern blot analyses. The hypothesis that the circadian control of transcription of genes encoding lignin biosynthetic enzymes may be attributable to the daily flux of carbohydrate availability is tested. It is shown that the presence of carbohydrates has a direct bearing on the extent of lignification, and present evidence that suggests that sugar-mediated control of lignin deposition may occur through a hexokinase-independent sugar-signalling pathway.

Materials and methods

Experimental organisms and growth conditions

Wild-type *Arabidopsis thaliana* seeds (Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The *sex1* mutant was generously provided by Professor Steve Smith (University of Edinburgh). Unless otherwise indicated, plants were grown at 22 °C in a mixture of Levington's Universal soil and Vermiperl vermiculite (3:1, v:v), under long-day conditions (16/8 h light/dark) at a light intensity of 130 μmol m^{-2} s^{-1}. Seeds used to germinate plants for transcript abundance analyses, and phloroglucinol staining were sterilized and grown in liquid Murashige and Skoog (MS) medium according to published methods (Newman *et al.*, 2004).

For diurnal transcript abundance analyses, plants were grown on semi-solid MS medium in Petri plates in a 16/8 h light/dark cycle for 14 d at a light intensity of 130 μmol m^{-2} s^{-1}. At dawn on the 15th day, approximately 1 g of whole plant tissue was harvested. This collection was repeated every 4 h for 48 h, with collections during the dark taking place under a green safe light.

For the circadian transcript abundance analyses, attempts were made to reproduce published conditions that had been used for the analysis of circadian changes in transcriptome activity (Harmer *et al.*, 2000). Briefly, plants were grown on semi-solid MS medium in Petri plates and entrained to a 12/12 h light/dark cycle at a light intensity of 130 μmol m^{-2} s^{-1}. On the 14th day of entrainment, the growth conditions were switched to continuous light to facilitate examination of the circadian rhythms of transcript abundance. At dawn on the 14th day, approximately 1 g of whole plant tissue was harvested from each genotype and snap frozen in liquid nitrogen. This collection was repeated every 4 h for 48 h.

Histochemistry

Phloroglucinol staining of hand cross-sections of dark-grown hypocotyls was carried out as follows: samples were placed in a 1% phloroglucinol-HCl solution for 10 min, and mounted in 50% glycerol, 6 N HCl and observed immediately. Bright field illuminated sections were taken using a Leica DMRB microscope (Leica, Wetzlar, Germany).

Chemical analysis of lignins and carbohydrates

The chemical composition of wild-type and *sex1* rosette leaves, collected at the end of the circadian rhythm experiment, in triplicate (>20 plants/replicate), was determined using a modified Klason analysis. In brief, freeze-dried *A. thaliana* leaves were ground to pass a 40-mesh screen using a Wiley mill. The ground *A. thaliana* leaves (1 g) were then Soxhlet-extracted with 100 ml acetone for 8 h to remove extractable components, and to minimize the formation of 'pseudolignin' during lignin analysis. The total weight of extractable components was determined gravimetrically following rotary evaporation. The extracted lignocellulosic material was air-dried to remove solvent and then analysed in triplicate for sugar and lignin composition.

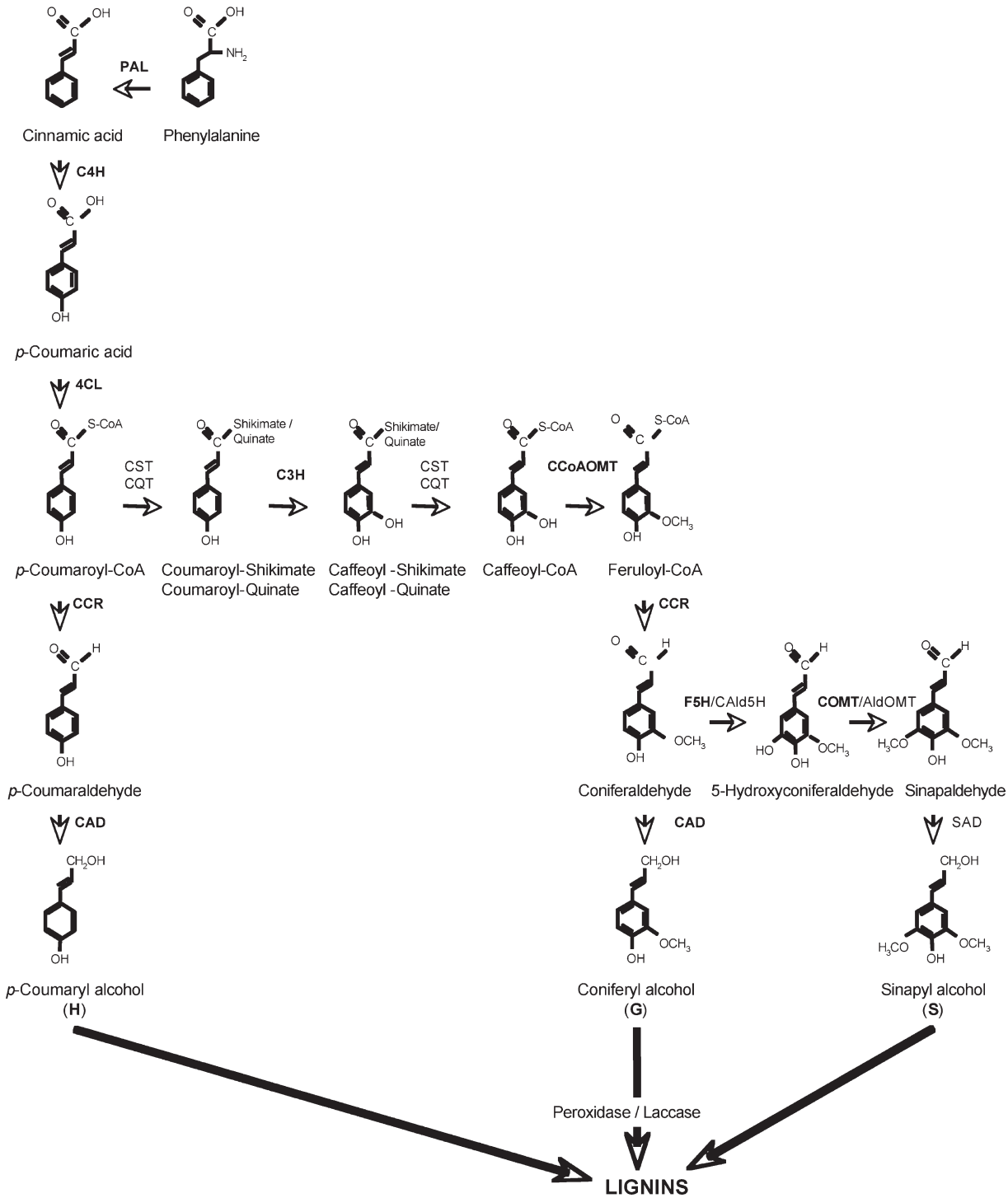

Fig. 1. A schematic representation of the monolignol biosynthetic pathway showing the enzymes encoded by the genes that were examined in this study. The pathway involves the following enzymes: phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamic acid 4-hydroxylase (C4H, EC 1.14.13.11), *p*-coumaroyl quinate/shikimate 3-hydroxylase (CSH, EC 1.14.14.1.), 4-coumarate: CoA ligase (4CL, EC 6.2.1.12), caffeoyl-CoA *O*-methyltransferase (CCoAOMT, EC 2.1.1.104), coniferyl aldehyde 5-hydroxylase/ferulate 5-hydroxylase (F5H, EC 1.14.13), caffeic acid *O*-methyltransferase (COMT, EC 2.1.1.68), hydroxycinnamoyl-CoA reductase (CCR, EC 1.2.1.44), hydroxycinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195), hydroxycinnamyl alcohol UDP-glucose glucosyltransferase (EC 2.4.1.111), coniferin β-glucosidase (EC 3.2.1.126), lignin peroxidases (EC 1.11.1.7), and laccases (EC. 1.10.3.2). The enzymes that were encoded by the genes that were examined in this study are shown in bold font. H, G, and S correspond to hydroxyphenyl, guaiacyl, and syringyl, respectively, which are the lignin subunits that are derived from the monolignols with which the letter designations are associated.

To assess lignin content, a 0.2 g sample of extracted *A. thaliana* leaves was transferred to a 15 ml reaction vial in an ice bath. A 3 ml aliquot of 72% (w/w) H_2SO_4 was added to the sample and thoroughly mixed for 1 min. The test tube was immediately transferred to a water bath maintained at 20 °C, and was subsequently mixed for 1 min every 10 min. After 2 h, the contents of each test tube were transferred to a 125 ml serum bottle, using 112 ml nanopure H_2O to rinse all the residue and acid from the reaction vial. The serum bottles (containing 115 ml H_2SO_4 at 4% w/w plus *A. thaliana* stems) were sealed with a septa and autoclaved at 121 °C for 60 min. Samples were allowed to cool, and the hydrolysates were vacuum-filtered through preweighed medium coarseness sintered-glass crucibles, and then washed with 200 ml warm (~50 °C) nanopure H_2O to remove residual acid and sugars, and dried overnight at 105 °C. The dry crucibles were weighed to determine Klason (acid-insoluble lignin) lignin gravimetrically. The filtrate was also analysed for acid-soluble lignin by absorbance at 205 nm according to TAPPI Useful Method UM250. Thioacidolysis of extracted stems was conducted according to published methods (Lapierre *et al.*, 1999), with the volumes scaled to accommodate 50 mg of starting material.

The concentration of neutral sugars in the filtrate was determined using HPLC analysis. The HPLC system (Dionex DX-500, Dionex, CA, USA) was equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS3500 autoinjector (Spectra-Physics, CA, USA). Prior to injection, samples were filtered through 0.45 μm HV filters (Millipore, MA, USA) and a 20 μl volume of sample was loaded, containing fucose as an internal standard. The column was equilibrated with 250 mM NaOH and eluted with deionized water at a flow rate of 1.0 ml min^{-1}. Detection of carbohydrates was facilitated with a post-column addition of 200 mM NaOH at 0.5 ml min^{-1}. The concentration of acid sugars in the filtrate was determined with an identical HPLC system and column, with a different elution gradient: the sugars were eluted with a gradient of 0–400 mM sodium acetate in 100 mM NaOH over 50 min, followed by a 10 min 300 mM NaOH wash. The column was then equilibrated to 100 mM NaOH prior to the next injection. The sugars were detected by pulsed amperometry with the post-column hydroxide addition.

Northern blot analysis

Total RNA was extracted using published methods (Newman *et al.*, 2004). Each pool of RNA was derived from >20 seedlings. Aliquots of 10 μg of total RNA were separated by electrophoresis in a 1.2% agarose formaldehyde gel, run in 1× MOPS buffer. RNA was then transferred onto a nylon membrane using standard capillary techniques and cross-linked under UV light (1200 μJ, Stratalinker, Stratagene, La Jolla, CA, USA). Radioactive probes were prepared using the DECAprime II DNA Labelling Kit (Ambion) and purified using the QIAquick Nucleotide Removal Kit (QIAGEN, Surrey, UK). Hybridizations were carried out using the membrane manufacturer's instruction. Membranes were exposed to a GS-525 Sample Exposure Platform (Bio-Rad, Hemel Hempstead, UK) for 2 d. The GS-525 Sample Exposure Platform was then scanned using a GS-525 Molecular Imager Laser Scanner, and the digitized image analysed using the Bio-Rad Multi-Analyst software (version 1.1). The relative amounts of transferred RNA in each lane were quantified using a Bio-Rad Fluor-S^{TM} MultiImager and Bio-Rad Multianalyst software. The RNA blots were used in northern blot analysis and hybridized at high stringency to ^{32}P labelled gene specific fragments of the respective lignin biosynthetic genes. The resulting blots were exposed on the phosphoimager and the amount of hybridizing transcripts quantified using Bio-Rad Multianalyst software. In each case, background values were also taken to account for any gradients across a blot.

Microarray analysis

Hybridization to the Affymetrix GeneChip microarrays (8K and ATH1, Affymetrix, Santa Clara, CA), scanning of the hybridized array, and data generation were performed at the GARNET facility at NASC (http://nasc.nott.ac.uk/) according to standard Affymetrix protocols (http://affymetrix.com). Total RNA was extracted using published methods (Newman *et al.*, 2004) from 6-d-old *A. thaliana* seedlings grown in the dark in liquid MS medium as described above. Each pool of RNA was derived from hundreds of seedlings. RNA derived from three biological replicates of each experimental condition were submitted to the facility for microarray analysis. Raw data from the microarray experiments, as well as the description of the MIAME-compliant project can be found at the following URL: http://ssbdjc2.nottingham.ac.uk/narrays/experimentpage.pl?experimentid=14. Normalization of the data was carried out using the Affymetrix Microarray Suite (MAS) software.

Using data mining procedures in a Microsoft Excel spreadsheet containing normalized data exported from the Affymetrix MAS program, lists of genes of interest and present on the chip were generated. From this dataset, new expression matrices were generated containing the mean expression values from the three replicates for each gene of interest. Using the three normalized expression values for genes of interest, a mean expression value was calculated for each gene in each 'condition' (i.e. wild type grown on medium lacking sucrose, wild type grown on sucrose-supplemented media). In order to provide an indicator of data robustness, a Mann–Whitney test was performed for each gene set. This produced a *p*-value representing the likelihood that the particular pattern of gene expression seen for each gene could occur by chance. The *p*-value for each gene set was added to the annotation file for gene function.

Results and discussion

Accumulation of transcripts corresponding to lignin biosynthetic enzymes varies over the diurnal cycle

Most analyses of the transcription of genes encoding monolignol biosynthetic enzymes have focused on single time points. These earlier studies provided excellent indicators of the relative transcript abundance for the genes encoding lignin biosynthetic enzymes at a given point in time, but may have inadvertently missed important diurnal changes in the regulation of these genes. In order to determine if there were significant diurnal changes in the accumulation of transcripts corresponding to lignin biosynthetic enzymes, northern blot analyses were used to investigate relative transcript abundance of 11 of the genes comprising the *A. thaliana* 'lignin toolbox' (Raes *et al.*, 2003) every 4 h over a 48 h time period, using RNA extracted from the rosettes of plants growing under long days (16/8 h light/dark) (Fig. 2). Changes in the accumulation of these transcripts would be predicted to affect the relative abundance of the corresponding enzymes, with concomitant impact on the channelling of the metabolites into the biosynthesis of phenylpropane units, and consequently the lignin polymer.

Several of the genes encoding monolignol biosynthetic enzymes exhibited two peaks in transcript abundance over a diurnal cycle (Fig. 2). One of these peaks occurred just prior to dawn, approximately 1 h before the growth room

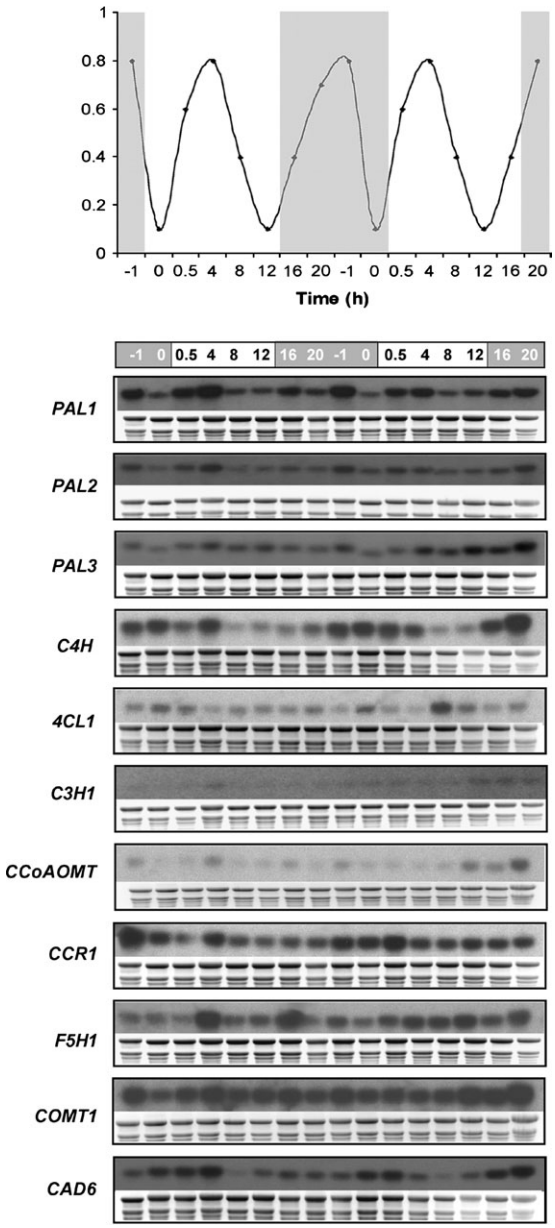

Fig. 2. Northern blot analysis of diurnal transcript abundance for 11 genes encoding enzymes involved in lignification: *PAL1*, *At2g37040*; *PAL2*, *At3g53260*; *PAL3*, *At5g04230*; *C4H*, *At2g30490*; *4CL1*, *At1g51680*; *C3H1*, *At2g40890*; *CCoAOMT1*, *At4g34050*; *CCR1*, *At1g15950*; *F5H1*, *At4g36220*; *COMT*, *At5g54160*; and *CAD6*, *At4g34230*. Grey and white boxes correspond to dark and light periods, respectively, and numbers indicate the times that the samples were collected relative to when the light period began (in hours). Upper panels for each gene show the autoradiogram for the northern blot; whereas, the lower panels show the ethidium bromide-stained membrane following transfer of RNA prior to hybridization, to show that gel loading and capillary transfer were equivalent for all RNA samples. The upper graph provides a schematic of the idealized, consensus trend in transcript abundance, indicating where peaks and troughs in transcript abundance were observed for the majority of the genes.

was illuminated. The other peak occurred in a window between 0.5 h and 4 h after the lights in the growth room were turned on. This trend was particularly prominent for transcripts corresponding to phenylalanine ammonia-lyase (*PAL1*), cinnamate 4-hydroxylase (*C4H*), hydroxycinnamoyl-CoA reductase (*CCR1*) and cinnamyl alcohol dehydrogenase (*CAD6*), but can also be observed for *PAL2 and PAL3*, hydroxycinnamate:CoA ligase (*4CL1*), and ferulate/coniferaldehyde 5-hydroxylase (*F5H1*) (Fig. 2). Consistent with the observation that transcript abundance peaked after dawn, *PAL*, *C4H*, and *4CL* have previously been shown to be light regulated. The basis for the peak in transcript abundance that was observed before dawn is not known, so further analyses were undertaken to examine the mechanisms that may be involved.

Some lignin biosynthetic genes are under circadian control

To determine if the fluctuations in transcript abundance of lignin biosynthetic genes were under circadian control, plants were entrained to a 12/12 h light/dark cycle, and then grown in continuous light for 48 h. Consistent with previous observations (Harmer *et al.*, 2000), several of the genes encoding monolignol biosynthetic enzymes exhibited circadian oscillations in transcript abundance (Fig. 3a). In particular, *C4H1*, caffeate *O*-methyltransferase (*COMT*), caffeoyl-CoA *O*-methyltransferase (*CCoAOMT1*), *CCR1*, and *CAD6* exhibited circadian fluctuations in transcript abundance. In general, transcript abundance increased from subjective dusk and peaked during the subjective night (Fig. 3a). The peak in transcript abundance that appeared to be due to light-mediated effects under diurnal conditions (Fig. 2) was not observed at subjective dawn on the second day of the circadian rhythm experiment (Fig. 3a). This suggests that the light-mediated changes in transcript abundance require a period of darkness prior to the light stimulus. By contrast, the changes in transcript abundance that took place during subjective night appeared to be independent of a light/dark stimulus, and must be either directly or indirectly under circadian control. This suggests that there are at least two factors that impinge on the abundance of transcripts corresponding to genes encoding lignin biosynthetic enzymes—light and the circadian clock.

The magnitude of circadian oscillations in transcript abundance of lignin biosynthetic genes is affected by starch turnover

Circadian changes in transcript abundance of the genes encoding lignin biosynthetic enzymes could be a direct or indirect consequence of the activity of the circadian clock. For example, it may be that circadian oscillations in other facets of metabolism have a knock-on effect influencing the abundance of lignin biosynthetic gene transcripts. As lignin biosynthesis requires large quantities of carbon skeletons, it would be advantageous for the plant if circadian changes in carbon availability functioned to inform when and where lignins were synthesized, so as to co-ordinate resource allocation with resource availability better.

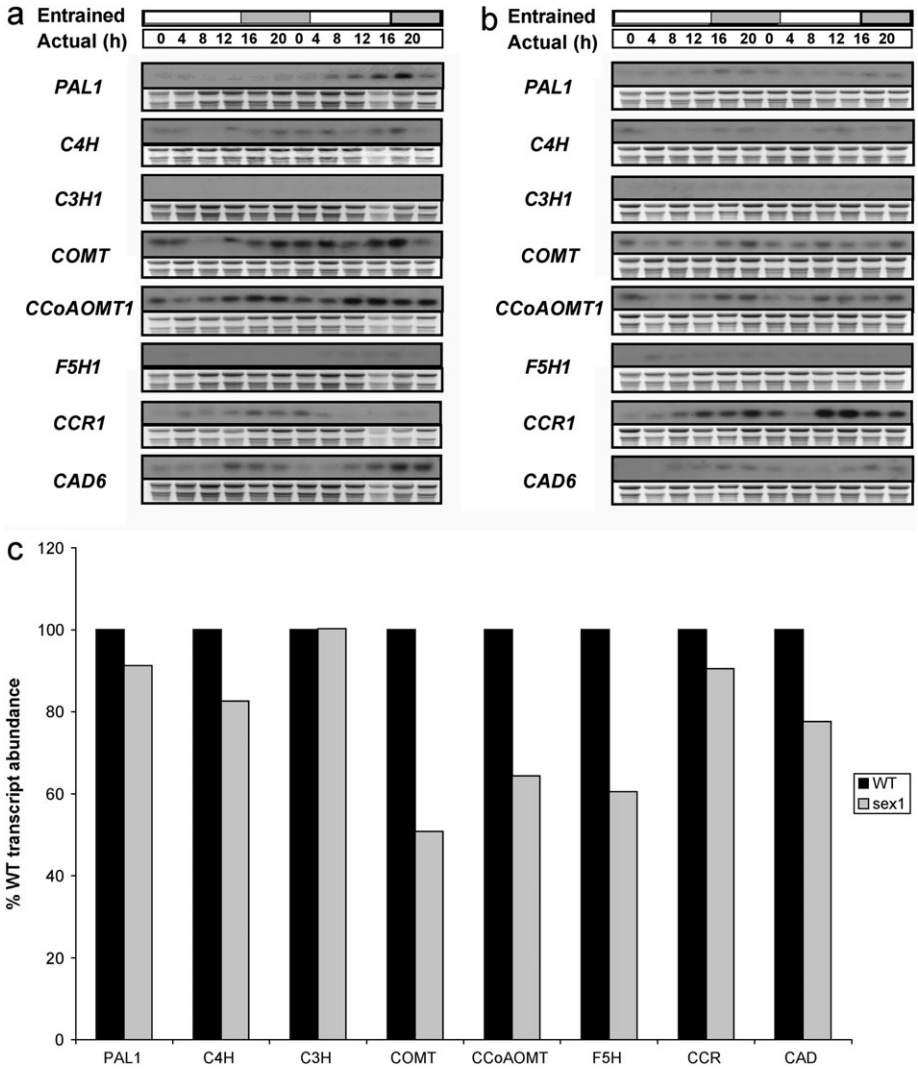

Fig. 3. Northern blot analysis of circadian transcript abundance levels of eight genes encoding enzymes involved in lignification. After entrainment to a 12/12 h light/dark cycle, plants were grown under continuous 24 h light for 48 h. The first time '0' indicates the time at which the continuous light period commenced. Grey and white boxes correspond to subjective dark and subjective light time periods, respectively. Upper panels for each gene, for each genotype, show the autoradiogram for the northern blot; whereas, the lower panels show the ethidium bromide-stained membrane following transfer of RNA prior to hybridization as per Fig. 2. The probes correspond to those indicated in Fig. 2. (a) Transcript abundance for lignin biosynthetic genes in wild-type plants. (b) Transcript abundance for lignin biosynthetic genes in the *sex1* mutant. (c) Cumulative transcript abundance over the 48 h experiment, determined by adding the normalized transcript abundance data at each time point for each gene in either wild-type plants (open bars) or the *sex1* mutant (filled bars).

Significant changes in the availability of carbon skeletons take place in a plant over the course of a day. For example, starch is generally synthesized in leaves during the day and then turned over at night, making sucrose available for anabolic processes in sink tissues, such as those making lignin. Daily fluxes in the genes controlling starch turnover are under circadian control. It may be that the circadian changes in transcript abundance of lignin biosynthetic genes are attributable to a knock-on effect created by the circadian availability of sugars derived from starch turnover. In this model, starch turnover during the evening would create a pool of soluble sugar that would be made available to sink tissues, like xylem, to synthesize carbon-rich compounds such as lignins. Starch turnover would thereby create a pool of sugars that could signal for lignin biosynthesis to begin. To test this hypothesis, circadian changes in transcript abundance of genes encoding lignin biosynthetic enzymes were investigated in the *A. thaliana* mutant *starch excess 1* (*sex1*).

The *sex1* mutant is impaired in its ability to degrade starch granules (Caspar *et al.*, 1991; Yu *et al.*, 2001; Zeeman *et al.*, 2002), which results in a reduced pool of available carbon, as well as a reduction in the amount of carbon released from starch stores, during the night. Resource availability was clearly impaired in the *sex1* mutant. Analysis of insoluble carbohydrates extracted from the *sex1*

mutant showed that most of the carbohydrate is tied up as glucose (Table 1), which is consistent with the fact that starch accumulates in the *sex1* mutant. By contrast, all other sugars liberated from the insoluble residue of the *sex1* mutant were comparatively reduced (Table 1). Similarly, there is a lower quantity of soluble acetone extractives (containing lipids, soluble sugars, and secondary metabolites) in the *sex1* mutant relative to wild-type plants. These findings suggest that the lack of starch turnover in *sex1* impinges on general carbon availability. Thus, the *sex1* mutant should facilitate the discrimination of the effect of a sudden flux of carbon from starch breakdown, from a 'true' circadian clock-controlled effect on the peak in lignin biosynthetic gene expression during the night.

The transcript abundance of genes encoding lignin biosynthetic enzymes was, in general, reduced in the *sex1* mutant relative to wild-type plants (Fig. 3b, c). Seven of the eight lignin biosynthetic genes examined in the *sex1* mutant were expressed at lower levels than those observed in wild-type plants, which supports the hypothesis that the availability of sucrose within the plant is important in the regulation of lignin biosynthesis. The only exception to this was for transcripts corresponding to caffeoyl-quinate/shikimate 3′-hydroxylase (*C3H1*) (Fig. 3b), where equivalent transcript abundance accumulation over 48 h was observed in both the *sex1* mutant and wild-type plants (Fig. 3c).

While it can be concluded that absolute and cumulative levels of transcript abundance of lignin biosynthetic genes were modulated by starch turnover (Fig. 3b, c), the circadian oscillations themselves were not. Circadian fluctuations in transcript abundance were unaffected in the *sex1* mutant for all eight genes examined (Fig. 3b). It appears that fluctuations in sucrose availability from starch breakdown may not be involved in maintaining the daily cycles of lignin biosynthetic gene expression. However, for seven of the eight genes examined, the cumulative level of transcript abundance over 48 h was reduced in the *sex1* mutant, suggesting that mechanisms such as resource availability are important in the regulation of the pathway.

Table 1. *Carbohydrate and extractives composition of insoluble leaf material from wild-type (WT) plants and the* sex1 *mutant*

Sugar quantities are expressed as nmol mg^{-1} tissue (dry weight) and acetone extractives are expressed as mg total extractives $(100\ mg)^{-1}$ tissue (dry weight).

	WT	*sex1*
Arabinose	67.30±2.81	44.32±4.72
Rhamnose	46.82±11.93	24.33±2.02
Galactose	105.18±5.59	60.63±6.53
Glucose	841.19±51.02	2070.42±216.16
Xylose	58.04±2.39	44.42±3.80
Mannose	35.98±3.11	26.21±2.38
Acetone extractives (mg $(100\ mg)^{-1}$)	17.26±0.45	10.24±2.81

Consistent with the cumulative decrease in transcript abundance of seven of the eight lignin biosynthetic genes (Fig. 3c), and with the hypothesis that decreased resource availability might impinge on lignin biosynthesis, the *sex1* mutant accumulated less lignins than wild-type plants (Table 2). The subunit composition of the lignins was also altered in the *sex1* mutant. Relative to wild-type plants, the *sex1* mutant had a higher ratio of syringyl (S):guaiacyl (G) subunits (S:G), which, in turn, resulted in an increased capacity to liberate lignin monomers by thioacidolysis (total yield) (Table 2). Furthermore, the *sex1* mutant did not accumulate *p*-hydroxyphenyl (H) subunits, where the wild type did. The latter finding is consistent with the observation that there was no decrease in the cumulative abundance of *C3H1* transcripts in *sex1*, while all other lignin biosynthetic genes exhibited a decrease in transcript abundance. That is, in the *sex1* mutant, the ratio of *C3H1* transcript accumulation relative to the other lignin biosynthetic genes is greater than that observed in wild-type plants. This change in ratio would be predicted to result in increased C3H activity relative to the other enzymes in the pathway, with a resultant increase in flux towards the synthesis of S and G subunits at the expense of H subunits. Thus, the transcript abundance data are entirely consistent with the lignin chemical analyses, providing a direct link between transcript abundance and lignin subunit composition, and showing the importance of C3H in determining the subunit composition of the lignins. Altogether, these findings support the hypothesis that diurnal changes in starch turnover affect transcript accumulation of lignin biosynthetic genes, with direct consequences on the quantity and composition of lignin polymers.

Carbohydrate modulates transcript accumulation of lignin biosynthetic genes and the accumulation of lignins

As the decreases in lignin accumulation and cumulative transcript abundance of lignin biosynthetic genes observed in the *sex1* mutant might be attributable to altered carbohydrate availability, experiments were designed to test the role that carbohydrates play in regulating lignin biogenesis. Wild-type *A. thaliana* seedlings were germinated and grown in the dark for 6 d in liquid medium in the absence or presence of an exogenous carbon source. Dark-grown *A. thaliana* seedlings are etiolated, and rely completely on seed reserves or exogenous sugar as a source of carbon. Dark-grown *A. thaliana* seedlings require exogenous sucrose in order to accumulate lignins, as detected by staining with phloroglucinol-HCl (Newman *et al.*, 2004). Seedlings grown under these conditions provide a useful means to investigate the effects of carbohydrate on the control of lignin biosynthesis.

When accumulation was monitored in dark-grown seedlings using phloroglucinol-HCl, lignins were detected in

Table 2. *Chemical analysis of cell wall residue from leaves of wild-type (WT) plants and the* sex1 *mutant*

Quantities of lignins are expressed as mg lignins (100 mg)$^{-1}$ tissue (dry weight); whereas, total yield of monolignols is expressed as μmol monolignols recovered by thioacidolysis/g lignin subjected to analysis.

	Acid insoluble lignins (mg (100 mg) $^{-1}$)	Acid soluble lignins (mg (100 mg) $^{-1}$)	Monolignol ratio			Total yield of monolignols (μmol g^{-1} lignin)
			H	G	S	
WT	5.57±0.25	10.55±0.22	3.1	74.0	22.9	58.47
sex1	3.81±0.19	7.19±0.23	0	73.3	26.7	67.04

seedlings grown in Murashige-Skoog (MS) medium supplemented with sucrose, but not in seedlings grown in MS medium without sucrose (Fig. 4). Phloroglucinol staining was restricted to the xylem vessels, over the length of the hypocotyl and the root. Phloroglucinol staining was qualitative and absolute, it was either present or absent in all seedlings (>100 examined per treatment).

Sucrose may elicit its effect on the accumulation of phloroglucinol-positive material in dark-grown *A. thaliana* seedlings through a number of mechanisms. The effect was not osmotic, as growth of seedlings in the presence of mannitol or sorbitol did not result in the accumulation of phloroglucinol-positive material (Fig. 4). MS medium supplemented with raffinose also did not induce the accumulation of phloroglucinol-positive material. Raffinose, an α-galactosyl derivative of sucrose, can be translocated with sucrose in the phloem, but is not hydrolysed to produce glucose and fructose (Haritatos *et al.*, 2000). As raffinose did not result in the accumulation of phloroglucinol-positive material, this suggests that sucrose must be hydrolysed to its component hexose monomers, and that transport alone is not sufficient for lignin accumulation to occur.

As was the case with raffinose, the addition of either turanose or palatinose to the medium of dark-grown *A. thaliana* seedlings did not result in the accumulation of phloroglucinol-positive material (Fig. 4). Like sucrose, turanose and palatinose are composed of glucose and fructose, but they cannot be cleaved or transported by plant enzymes. Turanose and palatinose have been used in studies to mimic signalling by sucrose, and it has been postulated that extracellular sucrose perception involves receptors that interact with palatinose and turanose, probably through their shared fructose moiety (Loreti *et al.*, 2000; Sinha *et al.*, 2002). The absence of phloroglucinol-positive material in seedlings grown in the presence of either turanose or palatinose suggests that the induction of lignin accumulation was unlikely to involve an extracellular sucrose sensor. To investigate the potential involvement of disaccharides in the accumulation of lignins in dark-grown plants further, the effect of trehalose was examined. Trehalose is a disaccharide comprised of glucose subunits that has recently been shown to function as an important signalling molecule modulating a variety of processes in plants, including vegetative development (Elbein *et al.*, 2003; Schluepmann *et al.*, 2003, 2004; Avonce *et al.*, 2004; van Dijken *et al.*, 2004). Trehalose did not induce lignin accumulation in dark-grown seedlings, suggesting that trehalose metabolism or signalling is not involved in this process.

Consistent with the observation that the accumulation of phloroglucinol-positive material appeared to require the metabolism of sucrose, phloroglucinol-positive staining was also observed when seedlings were grown in MS medium supplemented with the metabolizable hexose monomers, glucose, and fructose, and the disaccharide maltose, but not in MS medium supplemented with the non-metabolizable analogue, 3-*O*-methylglucose (3-OMG) (Fig. 4). These findings imply that the qualitative change in lignin accumulation required metabolizable sugars, and that this was not simply an osmotic effect.

Since metabolizable carbohydrates were necessary for the accumulation of phloroglucinol-positive material in dark-grown *A. thaliana* seedlings, the potential role of hexose signalling was examined. A compelling body of evidence indicates that hexoses, particularly glucose, are not only essential sources of carbon skeletons for metabolic processes in plants, but also function as important signalling molecules (reviewed in Smeekens, 2000; Rolland *et al.*, 2002; Halford and Paul, 2003; Rook and Bevan, 2003; Gibson, 2004). Similarly, hexokinase (HXK) has been shown to function in a dual role, both as an important enzyme in primary metabolism, and as a sensor of hexose sugars that informs plant resource allocation (Harrington and Bush, 2003; Moore *et al.*, 2003). To test the hypothesis that the accumulation of phloroglucinol-positive material might be a consequence of HXK signalling, dark-grown seedlings were grown in medium supplemented with 2-deoxyglucose (2-DG) and mannose (Fig. 4). Both 2-DG and mannose can function as substrates for HXK, but are very poorly metabolized, if at all (Gibson, 2000; Pego *et al.*, 2000). The inability of 2-DG or mannose to induce the accumulation of phloroglucinol-positive material suggests that accumulation did not simply involve direct signalling via HXK. The finding that accumulation of phloroglucinol-positive material was unlikely to involve signalling through HXK was confirmed by growing plants in the dark in the presence of sugar, plus the HXK inhibitor mannoheptulose (MHL) (Jang and Sheen, 1994; Chiou and Bush, 1998). Sugar-induced accumulation of phloroglucinol-positive material was unaffected by MHL (Fig. 4), showing that HXK signalling is not involved. Given that the non-metabolizable

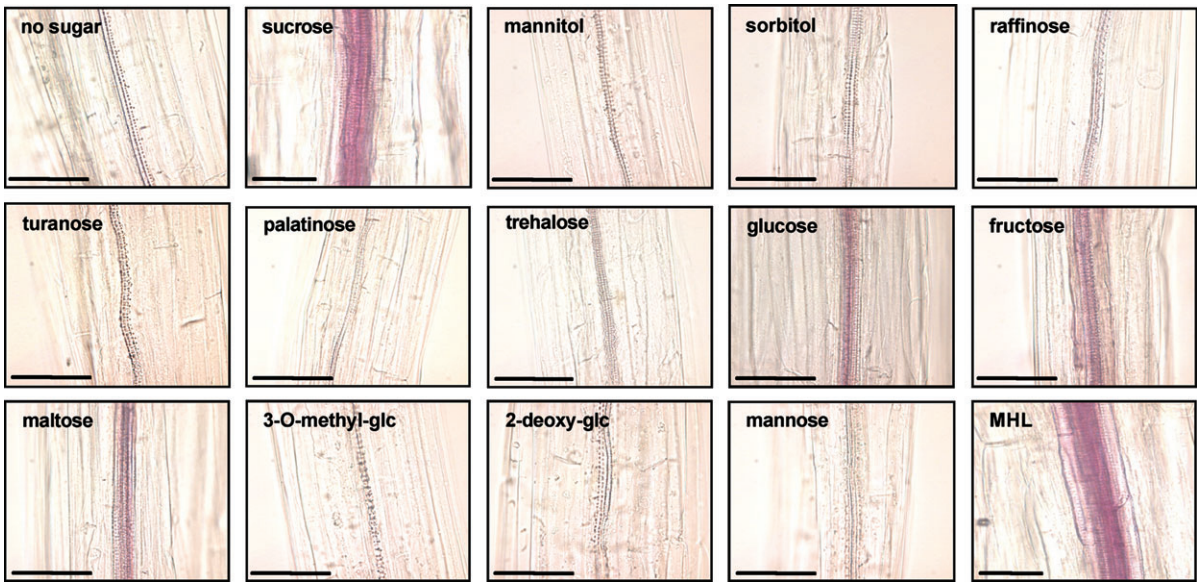

Fig. 4. Lignins in the hypocotyls of 6-d-old, dark-grown *A. thaliana* seedlings. Seedlings were germinated and grown in the dark in liquid MS medium for 6 d, and then harvested under a green safe light and stained with phloroglucinol-HCl, which stains lignins a magenta colour. Liquid MS medium contained no carbon source, and sugars were supplemented to a final concentration of 30 mM with either sucrose, glucose, fructose, maltose, turanose, palatinose, or raffinose. 2-deoxyglucose and 3-*O*-methylglucose were added to the MS medium, in the dark, to a final concentration of 30 mM, after the seeds had germinated. In the experiment with mannoheptulose (MHL), the MS medium was supplemented with 30 mM sucrose and 100 mM MHL. Scale bars=10 μm.

hexose, 3-OMG, was also unable to induce accumulation of phloroglucinol-positive material, hexose metabolism beyond HXK must be a prerequisite for accumulation to occur.

The sugar-dependent accumulation of phloroglucinol-positive material could simply be a consequence of increased availability of carbon skeletons flowing through a pathway that is already active. Alternatively, sugar may function as a signal to induce lignin biosynthesis. This hypothesis was tested by examining the level of transcript abundance of the genes that comprise the *A. thaliana* 'lignin toolbox' (Raes *et al.*, 2003). In two independent experiments, 6-d-old, dark-grown seedlings were subjected to transciptome analysis using Affymetrix GeneChip microarrays. The transcript abundance found in wild-type plants grown in the dark in the presence of sucrose was compared against that found in plants grown under identical conditions (including osmotic), but without sucrose. In the first set of experiments, three biological replicates for each condition were examined using an Affymetrix GeneChip that allows the simultaneous assessment of the transcript abundance of 8.2 K genes. The second set of experiments made use of the ATH1 GeneChip, which monitored approximately 22 K genes, again in three biological replicates.

In both sets of experiments, all but two of the genes that comprised the lignin toolbox showed consistent, statistically significant increases in transcript abundance when the plants were grown in the presence of sucrose (Table 3). While absolute levels of change differed between the two experiments, using the different GeneChips, the trends were consistent. Surprisingly, two genes implicated in the control of monolignol biogenesis, *CCR* and *CAD*, did not exhibit increased transcript abundance in response to sucrose. It may be that transcripts corresponding to *CCR* and *CAD* are maintained at levels that are sufficient to produce a quantity of enzyme that is capable of handling any level of flux through the monolignol biosynthetic pathway. In this model, the modulation of transcript abundance of genes encoding upstream enzymes would control the flux into the monolignol biosynthetic pathway. Alternatively, it may be that one of the other genes encoding proteins predicted to have CCR or CAD activities function to increase flux through the latter segments of the monolignol biosynthetic pathway when the plants are grown in the presence in sucrose. Several genes encoding putative CCR or CAD isoforms have increased transcript abundance in plants grown in sucrose-supplemented medium, and these may result in increased CCR or CAD activity respectively, with a concomitant increase in monolignol biosynthetic capacity. Both models are consistent with the qualitative increases in lignin accumulation that occurred in dark-grown seedlings that were grown in medium containing sucrose. The models also imply that sucrose may indeed function as a signal to induce the activity of the lignin biosynthetic pathway, through a mechanism that does not involve direct signalling through HXK.

Conclusion

The results presented herein suggest that diurnal fluxes in lignin biosynthetic capacity may be modulated at the

Table 3. *Transcript abundance data for genes encoding putative lignin biosynthetic enzymes*

Data were obtained by transcript profiling plants using Affymetrix GeneChips (8K and ATH1) arrays. Triplicate biological replicates allowed calculation of mean transcript abundance levels (shown as expression units) for each gene in dark-grown plants grown in liquid MS medium in the absence (−) or presence (+) of 30 mM sucrose. Statistical significance of the fold changes was determined by the Mann-Whitney test. Genes that comprise the 'lignin toolbox', as described by Raes *et al.* (2003), are indicated in bold.

Description	Accession	8 K			ATH1			*P* value	MW test
		−Sucrose	+Sucrose	Fold change	−Sucrose	+Sucrose	Fold change		
Phenylalanine ammonia lyase	**At2g37040 (PAL1)**	42.1	319.8	**7.60**	**205.65**	**297.48**	**1.45**	***P*<0.05**	*
	At3g53260 (PAL2)	62.5	233.7	**3.74**	**165.72**	**229.27**	**1.38**	***P*<0.05**	*
	At5g04230 (PAL3)	**7.2**	**10.1**	**1.40**	**45.24**	**44.67**	**−1.01**	***P*>0.1**	
	At3g10340 (PAL4)	**N.A.**	**N.A.**	**N.A.**	**276.57**	**201.51**	**−1.37**	***P*<0.05**	*
Trans-cinnamate 4-hydroxylase	**At2g30490 (C4H ; CYP73A5)**	296.3	911.2	**3.08**	**891.85**	**1203.07**	**1.35**	***P*<0.05**	*
4-coumarate:CoA ligase	**At1g51680 (4CL1)**	**173.9**	**589.5**	**3.39**	**297.55**	**417.54**	**1.40**	***P*<0.05**	*
	At3g21240 (4CL2)	14.8	31.2	2.11	172.05	228.8	1.33	*P*<0.05	*
	At1g65060 (4CL3)	2.2	0.5	−4.40	47.93	73.9	1.54	*P*<0.05	*
	At3g21230 (4CL4)	11.1	1	−11.10	22.24	35.92	1.62	*P*<0.05	*
	At1g20510 (4CL-like1)	N.A.	N.A.	N.A.	43.25	55.53	1.28	*P*<0.05	*
	At1g20500 (4CL-like2)	N.A.	N.A.	N.A.	8.44	11.96	1.42	*P*>0.1	
	At1g20490 (4CL-like3)	N.A.	N.A.	N.A.	41.37	45.97	1.11	*P*>0.1	
	At1g20480 (4CL-like4)	N.A.	N.A.	N.A.	92.37	170.82	1.85	*P*<0.05	*
	At1g62940 (4CL-like5)	N.A.	N.A.	N.A.	41.82	58.41	1.40	*P*>0.1	
	At4g19010 (4CL-like6)	N.A.	N.A.	N.A.	45.47	48.94	1.08	*P*>0.1	
	At4g05160 (4CL-like7)	N.A.	N.A.	N.A.	336.8	364.31	1.08	*P*>0.1	
	At5g63380 (4CL-like8)	N.A.	N.A.	N.A.	83.53	116.58	1.40	*P*<0.05	*
	At5g38120 (4CL-like9)	N.A.	N.A.	N.A.	20.34	21.39	1.05	*P*>0.1	
Hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase	At5g48930 (HCT)	N.A.	N.A.	N.A.	703.8	758.7	1.08	*P*<0.1	
p-Coumarate 3-hydroxylase	**At2g40890 (C3H1 ; CYP98A3)**	**N.A.**	**N.A.**	**N.A.**	**337.73**	**442.26**	**1.31**	***P*<0.05**	*
	At1g74540 (C3H2 ; CYP98A8)	N.A.	N.A.	N.A.	45.85	47.6	1.04	*P*>0.1	
	At1g74550 (C3H3 ; CYP98A9)	N.A.	N.A.	N.A.	52.96	60.62	1.14	*P*>0.1	
Caffeoyl-CoA 3-*O*-methyltransferase	**At4g34050 (CCoAOMT1)**	**N.A.**	**N.A.**	**N.A.**	**1384.88**	**2011.45**	**1.45**	***P*<0.05**	*
	At1g24735 (CCoAOMT2)	N.A.	N.A.	N.A.	150.92	87.3	−1.73	*P*<0.05	*
	At3g61990 (CCoAOMT3)	N.A.	N.A.	N.A.	78.37	170.48	2.18	*P*<0.05	*
	At3g62000 (CCoAOMT4)	N.A.	N.A.	N.A.	52.68	49.34	−1.07	*P*>0.1	
	At1g67990 (CCoAOMT5)	N.A.	N.A.	N.A.	23.38	25.57	1.09	*P*>0.1	
	At1g67980 (CCoAOMT6)	N.A.	N.A.	N.A.	37.94	28.42	−1.33	*P*>0.1	
	At4g26220 (CCoAOMT7)	N.A.	N.A.	N.A.	24.22	64.22	2.65	*P*<0.05	*
Cinnamoyl-CoA reductase	**At1g15950 (CCR1)**	**N.A.**	**N.A.**	**N.A.**	**469.1**	**437.43**	**−1.07**	***P*>0.1**	
	At1g80820 (CCR2)	N.A.	N.A.	N.A.	7.8	17.71	2.27	*P*<0.1	
	At1g76470 (CCR-like1)	N.A.	N.A.	N.A.	18.78	18.35	−1.02	*P*>0.1	
	At2g02400 (CCR-like2)	64.3	37.3	−1.72	112.7	119.16	1.06	*P*>0.1	
	At2g33590 (CCR-like3)	127.1	169.2	1.33	171.25	201.12	1.17	*P*<0.1	
	At2g33600 (CCR-like4)	1	25.1	25.10	47.84	69.26	1.45	*P*<0.05	*
	At5g58490 (CCR-like5)	N.A.	N.A.	N.A.	182.12	269.09	1.48	*P*<0.05	*
	At4g30470 (CCR-like6)	N.A.	N.A.	N.A.	302.42	298.16	−1.01	*P*>0.1	
	At5g14700 (CCR-like7)	N.A.	N.A.	N.A.	24.7	22.59	−1.09	*P*>0.1	
	At2g23910 (CCR-like8)	15.1	20.7	1.37	27.84	26.84	−1.04	*P*>0.1	
Ferulate 5-hydroxylase	**At4g36220 (F5H1 ; CYP84A1)**	**29.8**	**67.1**	**2.25**	**33.53**	**37.27**	**1.11**	***P*>0.1**	
	At5g04330 (F5H2 ; CYP84A4)	N.A.	N.A.	N.A.	96.79	82.45	−1.17	*P*>0.1	
Caffeic acid *O*-methyltransferase	**At5g54160 (COMT)**	**65.5**	**120.6**	**1.84**	**988.8**	**1310.01**	**1.32**	***P*<0.05**	*
	At1g21100 (COMT-like1)	N.A.	N.A.	N.A.	58.14	49.06	−1.19	*P*>0.1	
	At1g21110 (COMT-like2)	N.A.	N.A.	N.A.	3.84	5.71	1.49	*P*>0.1	
	At1g21120 (COMT-like3)	N.A.	N.A.	N.A.	1.45	0.4	−3.63	*P*>0.1	
	At1g21130 (COMT-like4)	N.A.	N.A.	N.A.	70.76	26.32	−2.69	*P*<0.05	*
	At1g33030 (COMT-like5)	N.A.	N.A.	N.A.	37.5	33.18	−1.13	*P*>0.1	
	At1g51990 (COMT-like6)	N.A.	N.A.	N.A.	36.54	37.34	1.02	*P*>0.1	
	At1g76790 (COMT-like8)	N.A.	N.A.	N.A.	195.07	343.65	1.76	*P*<0.05	*
	At1g77530 (COMT-like10)	N.A.	N.A.	N.A.	33.12	29.28	−1.13	*P*>0.1	
	At3g53140 (COMT-like11)	N.A.	N.A.	N.A.	25.74	24.85	−1.04	*P*>0.1	
	At5g37170 (COMT-like12)	N.A.	N.A.	N.A.	22.77	20.83	−1.09	*P*>0.1	
	At5g53810 (COMT-like13)	N.A.	N.A.	N.A.	13.41	11.64	−1.15	*P*>0.1	
Cinnamyl alcohol dehydrogenase	At4g39330 (CAD1)	N.A.	N.A.	N.A.	953.87	1102.45	1.16	*P*<0.1	
	At3g19450 (CAD-2 ; CAD-C)	N.A.	N.A.	N.A.	113.29	172.03	1.52	*P*>0.1	

Table 3. *Continued*

Description	Accession	8 K			ATH1			*P* value	MW test
		−Sucrose	+Sucrose	Fold change	−Sucrose	+Sucrose	Fold change		
	At4g37970 (CAD3 ; CAD-A)	N.A.	N.A.	N.A.	23.11	20.85	−1.11	*P*>0.1	
	At4g37980 (CAD4 ; CAD-B1)	42.5	25.4	−1.67	52.01	57.15	1.10	*P*>0.1	
	At4g37990 (CAD5 ; CAD-B2)	79.1	67.9	−1.16	36.78	38.74	1.05	*P*>0.1	
	At4g34230 (CAD6 ; CAD-D)	**210.2**	**228.7**	**1.09**	**315.4**	**294.54**	**−1.07**	***P*>0.1**	
	At2g21890 (CAD8 ; CAD-F)	4.2	4.6	1.10	16.1	19.06	1.18	*P*>0.1	
	At1g72680 (CAD9 ; CAD-G)	N.A.	N.A.	N.A.	172.75	185.57	1.07	*P*<0.1	
	At1g09500 (CAD-like1)	3.8	1.2	−3.17	8.8	6.31	−1.39	*P*>0.1	
	At1g09480 (CAD-like2)	70.6	21.6	−3.27	26.52	26.12	−1.02	*P*>0.1	
	At1g51410 (CAD-like3)	N.A.	N.A.	N.A.	41.01	47.75	1.16	*P*>0.1	
	At5g19440 (CAD-like4)	N.A.	N.A.	N.A.	321.85	537.12	1.67	*P*>0.1	
	At1g66800 (CAD-like5)	N.A.	N.A.	N.A.	23.63	46.58	1.97	*P*>0.1	

transcriptional level by at least three different stimuli: light, the circadian clock, and available hexose carbohydrates. All three stimuli are linked through light, which functions as a signal itself, and which entrains the clock, and determines the quantity of carbohydrates that are synthesized. Nevertheless, while the three stimuli are clearly inter-related, it is also evident that the effects of each can be dissected. The circadian clock establishes a cycle that results in the accumulation of lignin toolbox transcripts through the night. The absolute abundance of these transcripts is shaped by the amount of available carbohydrate.

The link between sugar signalling and lignification is particularly interesting. Carbohydrate-mediated changes in vegetative development have been well documented in dark-grown seedlings. While wild-type *A. thaliana* seedlings are normally skotomorphogenic in the dark, those grown in the presence of exogenous sucrose undergo more extensive vegetative development, where the cotyledons open and true leaves are formed (Roldan *et al.*, 1999; Baier *et al.*, 2004). It is tempting to speculate that sucrose co-ordinates a comprehensive suite of developmental processes, including the complete differentiation of water-conducting xylem vessels, which would require lignification. There is considerable logic in using photosynthate as a signal to indicate that leaves are above the soil, and that conditions are such that investment of carbon in lignins is worthwhile, for the purpose of supporting the plant body and the differentiation of water-conducting cells.

Future studies should aim to investigate what signalling components link carbohydrate perception and lignin biosynthesis. The experimental system described herein, which makes use of dark-grown *A. thaliana* seedlings, should prove very useful for such studies, as it facilitates the examination of sugar-mediated effects in relative isolation, without the confounding inputs that are present when using plant material that has been grown for a long time in the light. Such studies should prove particularly fruitful given that the components of a non-HXK signalling pathway in plants remain undefined (Halford and Paul, 2003; Dekkers *et al.*, 2004).

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References

Amthor JS. 2003. Efficiency of lignin biosynthesis: a quantitative analysis. *Annals of Botany* **91,** 673–695.

Anterola AM, Jeon J-H, Davin LB, Lewis NG. 2002. Transcriptional control of monolignol biosynthesis in *Pinus taeda*: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. *Journal of Biological Chemistry* **277,** 18272–18280.

Anterola AM, Lewis NG. 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. *Phytochemistry* **61,** 221–294.

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G. 2004. The *Arabidopsis* trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signaling. *Plant Physiology* **136,** 3649–3659.

Baier M, Hemmann G, Holman R, Corke F, Card R, Smith C, Rook F, Bevan MW. 2004. Characterization of mutants in *Arabidopsis* showing increased sugar-specific gene expression, growth, and developmental responses. *Plant Physiology* **134,** 81–91.

Baucher M, Monties B, Van Montagu M, Boerjan W. 1998. Biosynthesis and genetic engineering of lignin. *Critical Reviews in Plant Sciences* **17,** 125–197.

Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. *Annual Review of Plant Biology* **54,** 519–546.

Boudet AM. 2000. Lignins and lignification: selected issues. *Plant Physiology and Biochemistry* **38,** 81–96.

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D. 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. *Trends in Plant Science* **8,** 576–581.

Cano-Delgado A, Penfield S, Smith C, Catley M, Bevan M. 2003. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *The Plant Journal* **34,** 351–362.

Caspar T, Lin TP, Kakefuda G, Benbow L, Preiss J, Somerville C. 1991. Mutants of *Arabidopsis* with altered regulation of starch degradation. *Plant Physiology* **95,** 1181–1188.

Chen C, Meyermans H, Burggraeve B, De Rycke RM, Inoue K, De Vleesschauwer V, Steenackers M, Van Montagu MC, Engler GJ, Boerjan WA. 2000. Cell-specific and conditional expression of caffeoyl-coenzyme A-3-*O*-methyltransferase in poplar. *Plant Physiology* **123,** 853–867.

Chiou TJ, Bush DR. 1998. Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Sciences, USA* **95,** 4784–4788.

Christensen JH, Overney S, Rohde A, Diaz WA, Bauw G, Simon P, Van Montagu M, Boerjan W. 2001. The syringaldazine-oxidizing peroxidase PXP 3–4 from poplar xylem: cDNA isolation, characterization and expression. *Plant Molecular Biology* **47,** 581–593.

Dekkers BJ, Schuurmans JA, Smeekens SC. 2004. Glucose delays seed germination in *Arabidopsis thaliana*. *Planta* **218,** 579–588.

Douglas CJ. 1996. Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends in Plant Science* **1,** 171–178.

Ehlting J, Buttner D, Wang Q, Douglas CJ, Somssich IE, Kombrink E. 1999. Three 4-coumarate:coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. *The Plant Journal* **19,** 9–20.

Elbein AD, Pan YT, Pastuszak I, Carroll D. 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology* **13,** 17R–27R.

Esau K. 1965. *Vascular differentiation in plants*. New York: Holt Rinehart and Winston.

Ferrandiz C, Liljegren SJ, Yanofsky MF. 2000. Negative regulation of the SHATTERPROOF genes by FRUITFULL during *Arabidopsis* fruit development. *Science* **289,** 436–438.

Feuillet C, Lauvergeat V, Deswarte C, Pilate G, Boudet A, Grimapettenati J. 1995. Tissue-specific and cell-specific expression of a cinnamyl alcohol-dehydrogenase promoter in transgenic poplar plants. *Plant Molecular Biology* **27,** 651–667.

Gibson SI. 2000. Plant sugar-response pathways. Part of a complex regulatory web. *Plant Physiology* **124,** 1532–1539.

Gibson SI. 2004. Sugar and phytohormone response pathways: navigating a signalling network. *Journal of Experimental Botany* **55,** 253–264.

Halford NG, Paul MJ. 2003. Carbon metabolite sensing and signalling. *Plant Biotechnology Journal* **1,** 381–398.

Haritatos E, Medville R, Turgeon R. 2000. Minor vein structure and sugar transport in *Arabidopsis thaliana*. *Planta* **211,** 105–111.

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290,** 2110–2113.

Harrington GN, Bush DR. 2003. The bifunctional role of hexokinase in metabolism and glucose signaling. *The Plant Cell* **15,** 2493–2496.

Hauffe KD, Lee SP, Subramaniam R, Douglas CJ. 1993. Combinatorial interactions between positive and negative *cis*-acting elements control spatial patterns of 4CL-1 expression in transgenic tobacco. *The Plant Journal* **4,** 235–253.

Hawkins S, Boudet A, Grima-Pettenati J. 2003. Characterization of caffeic acid *O*-methyltransferase and cinnamyl alcohol dehydrogenase gene expression patterns by *in situ* hybridization in *Eucalyptus gunnii* Hook. plantlets. *Plant Science* **164,** 165–173.

Hose E, Clarkson DT, Steudle E, Schreiber L, Hartung W. 2001. The exodermis: a variable apoplastic barrier. *Journal of Experimental Botany* **52,** 2245–2264.

Humphreys JM, Chapple C. 2002. Rewriting the lignin roadmap. *Current Opinion in Plant Biology* **5,** 224–229.

Jang JC, Sheen J. 1994. Sugar sensing in higher plants. *The Plant Cell* **6,** 1665–1679.

Lacombe E, Van Doorsselaere J, Boerjan W, Boudet AM, Grima-Pettenati J. 2000. Characterization of *cis*-elements required for vascular expression of the cinnamoyl CoA reductase gene and for protein–DNA complex formation. *The Plant Journal* **23,** 663–676.

Lapierre C, Pollet B, Petit-Conil M, *et al*. 1999. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiology* **119,** 153–164.

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E, Roby D, Grima-Pettenati J. 2001. Two cinnamoyl-CoA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* **57,** 1187–1195.

Lauvergeat V, Rech P, Jauneau A, Guez C, Coutos-Thevenot P, Grima-Pettenati J. 2002. The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase EgCAD2 promoter is conserved among woody and herbaceous plant species. *Plant Molecular Biology* **50,** 497–509.

Lewis NG, Yamamoto E. 1990. Lignin: occurrence, biogenesis and biodegradation. *Annual Review of Plant Physiology Plant Molecular Biology* **41,** 455–496.

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF. 2000. SHATTERPROOF MADS-box genes control seed dispersal in *Arabidopsis*. *Nature* **404,** 766–770.

Loreti E, Alpi A, Perata P. 2000. Glucose and disaccharide-sensing mechanisms modulate the expression of alpha-amylase in barley embryos. *Plant Physiology* **123,** 939–948.

Mele G, Ori N, Sato Y, Hake S. 2003. The knotted1-like homeobox gene BREVIPEDICELLUS regulates cell differentiation by modulating metabolic pathways. *Genes and Development* **17,** 2088–2093.

Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T, Sheen J. 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* **300,** 332–336.

Newman LJ, Perazza DE, Juda L, Campbell MM. 2004. Involvement of the R2R3-MYB, *At*MYB61, in the ectopic lignification and dark-photomorphogenic components of the *det3* mutant phenotype. *The Plant Journal* **37,** 239–250.

Patzlaff A, McInnis S, Courtenay A, *et al*. 2003. Characterization of a pine MYB that regulates lignification. *The Plant Journal* **36,** 743–754.

Pego JV, Kortstee AJ, Huijser C, Smeekens SCM. 2000. Photosynthesis, sugars and the regulation of gene expression. *Journal of Experimental Botany* **51,** 407–416.

Perez J, Munoz-Dorado J, de la Rubia T, Martinez J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology* **5,** 53–63.

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W. 2003. Genome-wide characterization of the lignification toolbox in *Arabidopsis*. *Plant Physiology* **133,** 1051–1071.

Rogers LA, Campbell MM. 2004. The genetic control of lignin deposition during plant growth and development. *New Phytologist* **164,** 17–30.

Roldan M, Gomez-Mena C, Ruiz-Garcia L, Salinas J, Martinez-Zapater JM. 1999. Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of *Arabidopsis* in the dark. *The Plant Journal* **20,** 581–590.

Rolland F, Moore B, Sheen J. 2002. Sugar sensing and signaling in plants. *The Plant Cell* **14,** S185–S205.

Rook F, Bevan MW. 2003. Genetic approaches to understanding sugar-response pathways. *Journal of Experimental Botany* **54,** 495–501.

Ros Barcelo A. 1997. Lignification in plant cell walls. *International Review of Cytology* **176,** 87–132.

Schluepmann H, Pellny T, van Dijken A, Smeekens S, Paul M. 2003. Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **100,** 6849–6854.

Schluepmann H, van Dijken A, Aghdasi M, Wobbes B, Paul M, Smeekens S. 2004. Trehalose mediated growth inhibition of *Arabidopsis* seedlings is due to trehalose-6-phosphate accumulation. *Plant Physiology* **135,** 879–890.

Sederoff RR, MacKay JJ, Ralph J, Hatfield RD. 1999. Unexpected variation in lignin. *Current Opinion in Plant Biology* **2,** 145–152.

Sibout R, Eudes A, Pollet B, Goujon T, Mila I, Granier F, Seguin A, Lapierre C, Jouanin L. 2003. Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in *Arabidopsis*. Isolation and characterization of the corresponding mutants. *Plant Physiology* **132,** 848–860.

Sinha AK, Hofmann MG, Romer U, Kockenberger W, Elling L, Roitsch T. 2002. Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. *Plant Physiology* **128,** 1480–1489.

Smeekens S. 2000. Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **51,** 49–81.

Trojanowski J. 2001. Biological degradation of lignin. *International Biodeterioration and Biodegradation* **48,** 213–218.

van Dijken AJH, Schluepmann H, Smeekens SCM. 2004. *Arabidopsis* trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. *Plant Physiology* **135,** 969–977.

van Heerden PS, Towers GH, Lewis NG. 1996. Nitrogen metabolism in lignifying *Pinus taeda* cell cultures. *Journal of Biological Chemistry* **271,** 12350–12355.

Yu TS, Kofler H, Hausler RE, *et al*. 2001. The *Arabidopsis sex1* mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *The Plant Cell* **13,** 1907–1918.

Zeeman SC, Tiessen A, Pilling E, Kato KL, Donald AM, Smith AM. 2002. Starch synthesis in *Arabidopsis*. Granule synthesis, composition, and structure. *Plant Physiology* **129,** 516–529.

Zhong R, Kays SJ, Schroeder BP, Ye Z-H. 2002. Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *The Plant Cell* **14,** 165–179.