ROLE OF THE KNAT7 TRANSCRIPTION FACTOR IN DIURNAL REGULATION OF LIGNIN BIOSYNTHETIC GENES AND RESOURCE ALLOCATION IN *ARABIDOPSIS THALIANA*

by

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Abstract

Lignified secondary cell walls in plants represent an irreversibly deposited final metabolic sink for global fixed carbon reserves and synthesis of these walls is an energetically expensive process for the plant; hence it follows that spatial-temporal control of secondary cell wall biosynthesis must be closely co-ordinated with carbon metabolism. The function of the KNOTTED ARABIDOPSIS THALIANA 7 *(*KNAT7) homeodomain protein, a repressor of transcription and part of the multi-level network of transcription factors regulating secondary cell wall biosynthesis and deposition, was studied to investigate its potential role in diurnal regulation of a set of lignin biosynthetic genes (LBGs*)* in *Arabidopsis thaliana*. I found that in young wild-type seedlings, transcript accumulation levels of LBGs grown in long day conditions vary diurnally but most do not show a change in temporal variation over a daily light-dark cycle in the *knat7* mutant. Diurnal patterns of LBG transcript accumulation were less clear in mature wild-type inflorescence stem tissue, however diurnal expression at subjective 'dawn' was altered for some LBGs in *knat7*, providing evidence for a function for *KNAT7* as a diurnal regulator of LBG expression in actively lignifying tissue. A diurnal regulatory function for *KNAT7* is also supported by the presence of a hypocotyl elongation phenotype as well as by an altered timing to floral transition in *KNAT7* mutants, phenotypes commonly displayed by mutants defective in clock regulation. Further, evidence for *KNAT7* function as an activator of diurnal transcription of *3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHASE 1* (*DHS1)* was observed in seedlings while in stems *KNAT7* seemed to function as a repressor of LBGs. *KNAT7* temporal expression variation itself shows minimal change in seedling and stem tissue grown in longday conditions, suggesting that post transcriptional mechanisms, such as protein-protein interactions, may be important for modulating *KNAT7* functions diurnally. Overall, my data

provides support for the hypothesis that diurnal regulation of transcript abundance is a function of *KNAT7* in seedlings and mature stem tissue, which may represent a means to fine-tune resource (sucrose and starch) allocation to secondary cell wall biosynthesis coordinated with diurnal fluctuations in availability.

Preface

This thesis is original, unpublished, independent work by the author, Orpita Das.

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List of Abbreviations

cDNA	complementary deoxyribonucleic acid
CL	continuous light
Col	Columbia
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GUS	β-glucuronidase
hy	elongated hypocotyl
irx	irregular xylem
L/D	light / dark
LD	longday
LBGs	lignin biosynthetic genes
mRNA	messenger ribonucleic acid
SDS	sodium dodecyl sulphate
T-DNA	transfer DNA
PAL	phenylalanine ammonia lyase
C4H	cinnamate-4-hydroxylase
4CL	4-coumarate CoA ligase
C3H	p-coumaroyl shikimate 3-hydroxylase
HCT	hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase
CCOMT	caffeoyl CoA o-methyltransferase

CCR	cinnamoyl CoA reductase
F5H	ferulate 5-hydroxylase
COMT	caffeic acid o-methyltransferase
CAD	cinnamyl alcohol dehydrogenase
qRT-PCR	quantitative real time polymerase chain reaction
SDS	sodium dodecyl sulphate

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Dedicated to my parents Aparajita Das, Rabindranath Das

and my brother Rohit Das.

Chapter 1: **Introduction and literature review**

1.1 Introduction to biological clocks

All eukaryotes have a circadian clock; an internal time-keeping mechanism consisting of transcription-translation feedback loops, to predictively synchronize internal biological processes with the earth's 24 hour rotation around the sun. Circadian rhythms, a subset of diurnal rhythms, are oscillations in metabolism, physiology and behaviour that have a period of ~24 h. Even in the absence of exogenous time cues these rhythms persist, indicating their generation by an endogenous circadian clock (Dunlap et. al., 2004). In trees important physiological processes like cold acclimation in winter, release from dormancy in spring and photoperiodic induction of flowering are controlled by the photoperiod sensing abilities provided by the circadian clock. Hypocotyl elongation, leaf movements and stomatal closure are all examples of daily clock controlled activities (Kinmonth-Schultz et. al., 2013).

Primary metabolism is influenced by the clock. It ensures efficiency of the photosynthetic apparatus and nutrient utilization (Harmer et. al., 2000). Carbon allocation and utilization pathway response, to regular environmental changes affecting carbon availability as well as sudden carbon starvation, is clock-regulated (Stitt et. al., 2007). Dodd et. al., (2005) showed that when *Arabidopsis thaliana* (Arabidopsis) clock period matched that of the external environment the plants made more chlorophyll, had bigger rosettes, grew faster and survived better when competing for resources; hence clocks provide plants with an adaptive advantage. Circadian clocks, consisting of transcription-translation feedback loops, have in fact evolved independently in different lineages at least 4 times in the last 2 billion years (Edgar et. al., 2012).

An enrichment of clock-regulated genes in phytohormone response as well as stress-response pathways was identified by Covington et. al., (2008). Clock regulation determines

sensitivity to auxin responses in a time-of-day specific manner (Covington et. al.,2007), allows plants to anticipate likely pest attacks (Goodspeed et. al., 2012) and respond to wounding, cold stress (Pruneda-Paz et. al., 2010) and pathogen stress by induction of secondary metabolism gene expression (Nascimento and Fett-Nato, 2010). Temporal compartmentalization of maximum expression of the thousands of transcripts generated and metabolic processes performed by a cell over the day may also be a function of the clock (Harmer et. al., 2000).

Up to 35% of the Arabidopsis transcriptome shows clock regulation (Michael and McClung, 2003). More recently over half of the Arabidopsis expressed genes were shown to be diurnally regulated (Michael et. al., 2008) and/or nearly one-third of expressed genes were shown to be circadian regulated (Covington et, al., 2008). All of these data indicate the far-reaching consequences of clock regulation on co-ordination of plant gene expression, physiology, metabolism and growth.

1.1.1 Clock mechanism in plants

The molecular mechanism of the plant circadian clock can be thought to consist of 3 major parts - a self-sustaining core biochemical oscillator consisting of transcription factors and associated genes, input pathways for environmental sensing and output pathways for co-ordination of cellular and physiological responses. In Arabidopsis more than 20 core clock genes have been identified to date. The complexity of transcriptional and post-translational feedback mechanisms making up the clock in higher plants makes it difficult to identify the roles of individual genes or molecular interactions. In the most recent mathematical model of the clock, transcriptional activation is primarily the responsibility of *REVEILLE8* (*REV8*) while the rest of the clock genes are modelled as repressors to corroborate experimental evidence from the

literature (Fig. 1-1; Fogelmark and Troein, 2014). These core clock genes show peaks in mRNA and protein levels at distinct times of day (Fig. 1-2; Carre and Veflingstad, 2013; Fogelmark and Troein, 2014) and reciprocally regulate expression of other clock genes as well as downstream processes at the transcriptional and post-translational level.

LATE ELONGATED HYPOCOTYL (LHY) and a closely related MYB-like transcription factor *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* show high mRNA and protein levels in the morning and repress evening phased clock genes like *TIMING OF CAB EXPRESSION 1 (TOC1), GIGANTEA (GI), LUX ARRHYTHMO (LUX), BROTHER OF LUX ARRHYTHMO (BOA, also known as NOX), EARLY FLOWERING 3* (*ELF3*) and *ELF4* by binding to primarily the Evening Element (EE) motif. TOC1 in turn regulates *CCA1* and *LHY* and was only recently identified as a repressor (Gendron et. al., 2012; Hsu and Harmer, 2014). The *PSEUDO RESPONSE REGULATOR (PRR)* genes form a part of the second identified transcriptional feedback loop making up the core clock architecture. *PRR5, PRR7* and *PRR9* repress *CCA1* and *LHY* in a partially redundant manner and enable progression of the clock by showing peaks in transcript level consecutively over the day. Several promoter motifs have been identified to mediate clock-regulated expression at specific times of the day, for example the Morning Element (ME), CCAl-Binding Site (CBS), EE and Midnight Module (PBX/TBX/SBX; Wang et. al., 1997; Harmer et. al., 2000; Harmer and Kay, 2005; Michael et. al., 2008).

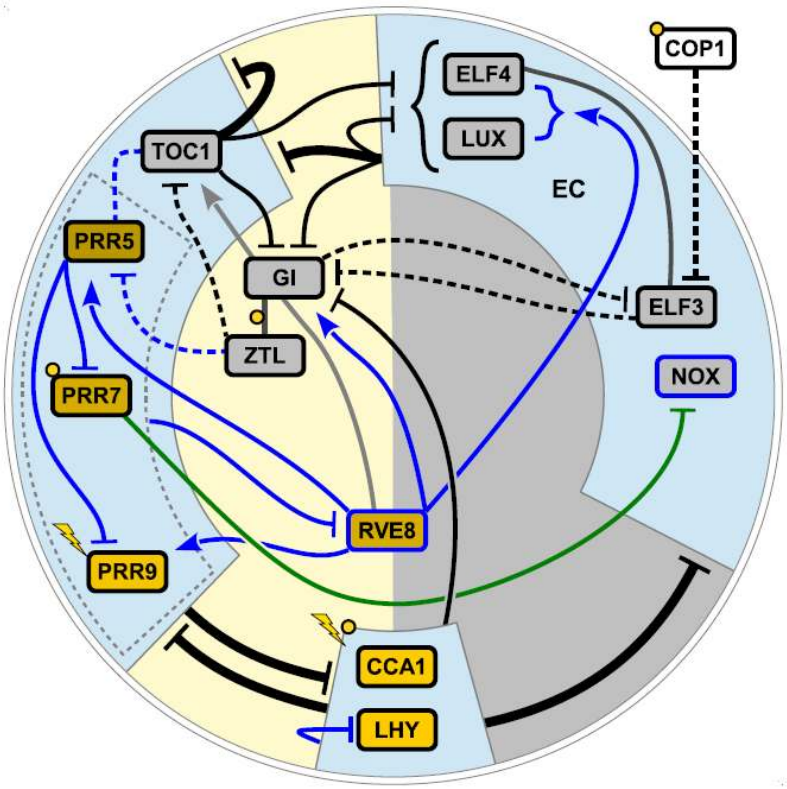

Figure 1-1 Interlocked transcription-translation feedback loops of transcription factor genes that make up the plant circadian clock.
Lightening and yellow circles indicate points at which light can provide input to the clock at the transcriptional and post-transcriptional level respectively. Solid lines indicate transcriptional regulation and dashed lines indicate protein–protein interactions, with arrows for activation and bars for repression or degradation. The green line indicates a hypothetical interaction, and the light grey line indicates an interaction that the model predicts to be extremely weak. Figure reproduced from Fogelmark & Troein, (2014)

Alternative splicing and phosphorylation of several clock genes is also observed. ZEITLUPE (ZTL), an F-box protein found exclusively in the cytoplasm, targets TOC1 and PRR5 for proteasomal degradation. GI stabilizes ZTL while CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) targets GI for degradation. A splice variant of *CCA1* lacking the MYB domain (CCA1β) can interfere with full length CCA1 (CCA1α) function (Hsu and Harmer, 2014). Cytosolic signalling molecules may also play a role in clock regulation of plant processes (Dodd et. al., 2007).

Self-sustained oscillations of mRNA or protein expression of the clock genes may be observed when plants are grown in continuous light or continuous darkness and steady temperature and environment conditions. 'Zeitgebers' like light quality, photoperiod, light intensity and temperature fluctuations provide external cues that entrain oscillations of the clock to the external environment so as to regulate physiological processes most efficiently. Diurnal patterns of transcript and protein abundance characteristic to those particular environmental conditions can be observed which may be different from circadian expression patterns.

Light determines clock oscillation but the clock also functions in the 'gating' of light input. A metaphorical gate prevents light from resetting the clock at inappropriate times of the day, allowing the circadian clock to restrict sensitivity to specific external stimuli at a particular time of the day (McWatters and Devlin, 2011). While sucrose and starch metabolism is clock regulated, rhythmic endogenous sugar signals too can provide metabolic feedback to the clock. Sucrose responsiveness of the clock is most pronounced at (is gated to) mid-day, through modulation of expression of the morning expressed gene *PRR7* (Haydon et. al., 2013).

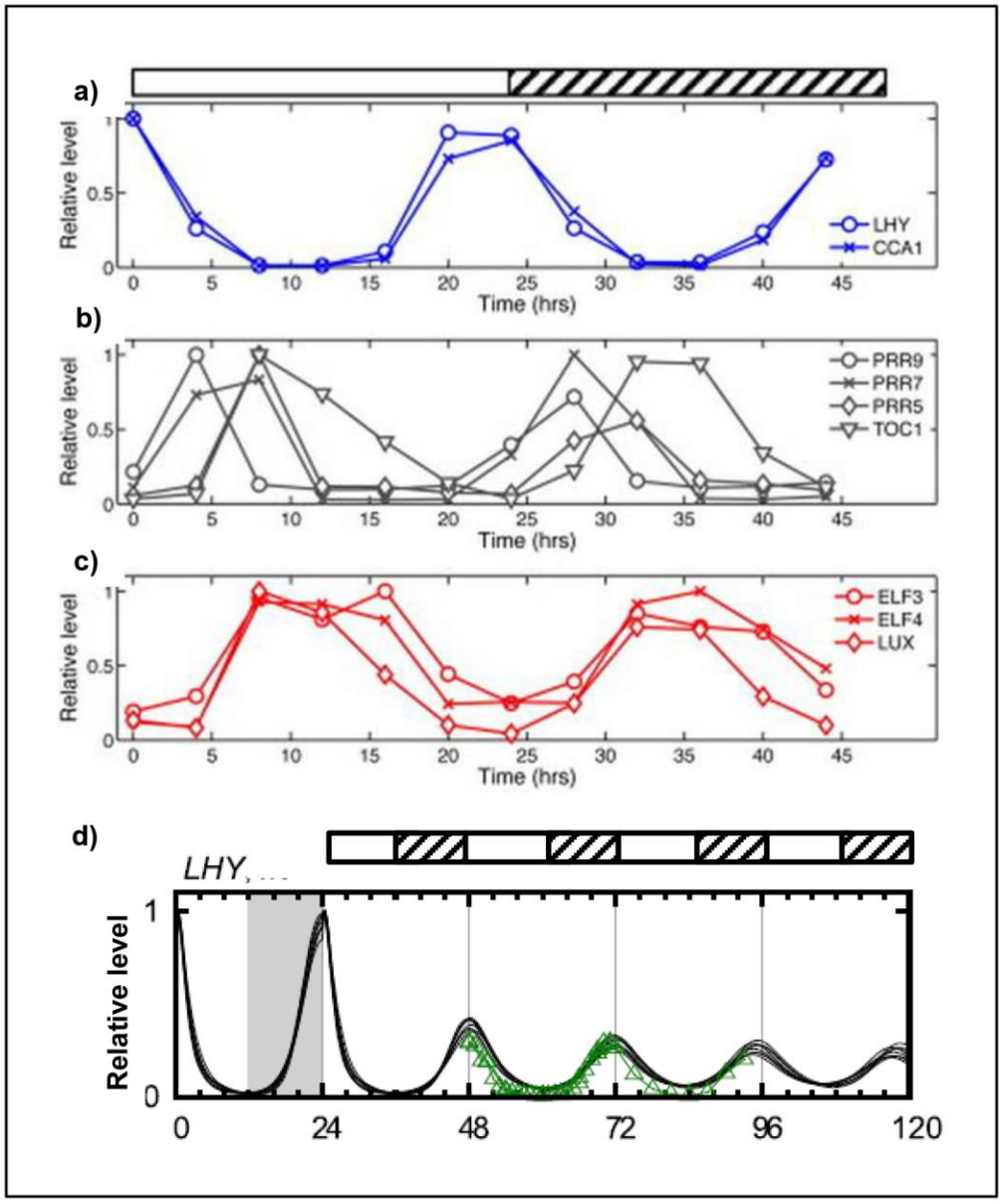

Figure 1-2 Temporal expression profiles of Arabidopsis clock genes in wild-type plants. Plants were grown under 12 h light/ 12 h dark cycles before transfer to constant light. (a), (b), (c) are reproduced from Carre and Veflingstad, (2013), data for their figure was obtained from the 'DIURNAL' database (Mockler et. al., 2007). (d) is reproduced from Fogelmark and Troein, (2014). Shaded area indicates night. Open and hatched bars above the charts indicate subjective day and subjective night, respectively.

1.1.2 Tissue, organ and species specific clock regulation

It was generally assumed that all plant cells contain identical, cell-autonomous multi-loop clocks. However, James et. al., (2008) discovered that several genes in root clocks display markedly different expression properties from those in the shoots. Of the 20 core clock genes only *CCA1, LHY, PRR5* and *PRR7* were capable of independent oscillations in Arabidopsis roots, while the rest were synchronized by a photosynthesis-related signal from the shoot. The author makes a valid observation, that metabolism is organ-specific, so clocks must be modified to support specific processes in different tissues and organs.

While roots have a simplified clock compared to the shoot, vascular-specific clocks in leaves require an additional clock gene for appropriate oscillation. *PRR3* is expressed in a vasculature specific manner and increases stability of the TOC1 core oscillator protein without affecting its transcription (Para et. al., 2007). Endo et. al., (2014) showed that morning loop clock genes and outputs were more active in mesophyll tissue while evening loop clock genes were more active in leaf vasculature. Further they showed that circadian clocks in cells of vascular tissue signal to neighbouring cell clocks in leaves; disruption of the circadian clock in the vasculature, but not the disruption of mesophyll, epidermis, stem or root clocks, affects the most well-studied and prominent clock-regulated process - timing of flower production in Arabidopsis.

Circadian systems have been shown to be well conserved between Arabidopsis, poplar and rice species (Filichkin et. al., 2011). Orthologues of Arabidopsis clock genes have been identified in chestnut trees, teak (Ibanez et. al., 2008, Norlia et. al., 2008) and several other plant species. Wood-forming tissue in eucalyptus have a functional clock and 8% of genes expressed

during xylogenesis, including those involved in carbon allocation, hormone signalling, stress response and wood formation, show diurnal expression patterns (Solomon et. al., 2010).

Mature Arabidopsis inflorescence stems, which serve as a model for angiosperm secondary cell wall and wood formation (Zhang et. al., 2011) have been little studied in terms of clock and output gene expression regulation. Elongation of the first internode of inflorescence stems shows circadian rhythmicity and is clock- regulated (Jouve et. al., 1998; Jouve et. al., 1999). Niinuma et. al., (2005) observed that stem circumnutation is clock regulated and for the first time performed a genetic analysis of the clock in 4-5cm tall inflorescence stem tissue, identifying the existence of a functional clock.

Secondary cell walls and woody biomass are major sinks for the photosynthetic resources of plants including trees, and it is of interest to further study the genetic mechanisms, like clock regulation, that may drive efficient resource allocation in its tissues.

1.2 Plant cell walls

Cell walls determine plant cell shape, functionality and collectively the plant form. All cells have a primary cell wall capable of growth and expansion, made up of cellulose and hemicellulose (predominantly xyloglucan in Arabidopsis) embedded within a matrix of pectin and proteins. When cells have ceased expansion, differentiation of certain special kinds of cells involves development of toughened secondary cell walls, made up of cellulose, hemicellulose (predominantly glucuronoxylan in Arabidopsis*)* and lignin. Cellulose is the major load-bearing component of cell walls while xyloglucan forms modifiable cross-links between the cellulose microfibrils, allowing for growth plasticity as well as mechanical strength. Several xylan deficient Arabidopsis mutants have weakened cell walls with collapsed xylem indicating the

structural role played by secondary cell wall hemicellulose. Pectin is essential for cell-adhesion and formation of the cell wall matrix. Lignin is a hydrophobic phenolic polymer that impregnates the secondary cell wall matrix, water-proofing it, providing structural support, and is in some cases associated with pathogen resistance. Lignification is considered as a signature for irreversible cell differentiation, since plant enzymes cannot degrade lignin once it has been deposited (Hao and Mohnen, 2014).

1.2.1 Lignin composition and biosynthesis

Lignin is a polyphenolic polymer that is formed from the *in muro* oxidative polymerization of the monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol that make up the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin polymer units respectively. The intermonolignol bonds are carbon–carbon bonds, condensed bonds or ethers, and the β-O-4′ ether bond is the most common linkage (Boerjan et. al., 2003).

The monolignols that polymerize in the wall to form lignin are a product of the cytosolic phenylpropanoid pathway shown in Fig. 1-3. The phenylpropanoid pathway produces many secondary metabolites involved in plant defence, structural support, and survival. Light protective anthocyanins, flavonoids that determine flower colour and rhizobacterial nitrogen fixation, coumarins and lignans are also products of the same phenylpropanoid pathway (Fraser and Chapple, 2011). The lignin biosynthetic pathway can be divided into two parts – the general phenylpropanoid pathway consisting of genes encoding *PHENYLALANINE AMMONIA LYASE (PAL), CINNAMATE-4-HYDROXYLASE (C4H), 4-COUMARATE:COA LIGASE (4CL), HYDROXYCINNAMOYL-COA SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE (HCT), P-COUMAROYL SHIKIMATE/QUINATE 3-HYDROXYLASE (C3H),*

CAFFEOYL SHIKIMATE ESTERASE (CSE) and *CAFFEOYL COA O-METHYLTRANSFERASE (CCOMT).* The monolignol specific pathway consists of *FERULATE 5-HYDROXYLASE (F5H), CAFFEIC ACID O-METHYLTRANSFERASE (COMT), CINNAMOYL COA REDUCTASE (CCR)* and *CINNAMYL ALCOHOL DEHYDROGENASE (CAD)* (Vanholme et. al., 2012). Many of these enzymes are encoded by gene families. Thirty-five genes from the above gene families can convert phenylalanine to the H, G or S monolignol subunits, but based on evolutionary analysis, promoter analysis and expression data the 'lignin toolbox' genes, identified by Raes et. al., (2005), are thought to encode the 12 enzymes specifically responsible for developmental lignification. A subset of 10 of these genes has been studied in my thesis.

Lignin biosynthesis takes place at least in part associated with the endoplasmic reticulum. Monolignol transport through the cytosol, across the plasma membrane, may take place through mechanisms like passive diffusion or ABCG transporter mediated active transport (Alejandro et. al., 2012). Monolignols then polymerise at the cell wall through oxidative radical coupling. One or more laccase or peroxidase enzyme may catalyse this process at the phenoxy radical of the growing lignin chain. Lignin is deposited within the carbohydrate matrix of the cell wall in the final stages of xylem cell differentiation. Lignification begins at the cell corners in the middle lamella; eventually spreading across the secondary wall towards the lumen. The process is regulated by the cell through the supply of available monomers, the chemical reactions involved, the radical-generating capacity and the conditions in the cell wall (Bonawitz and Chapple, 2010).

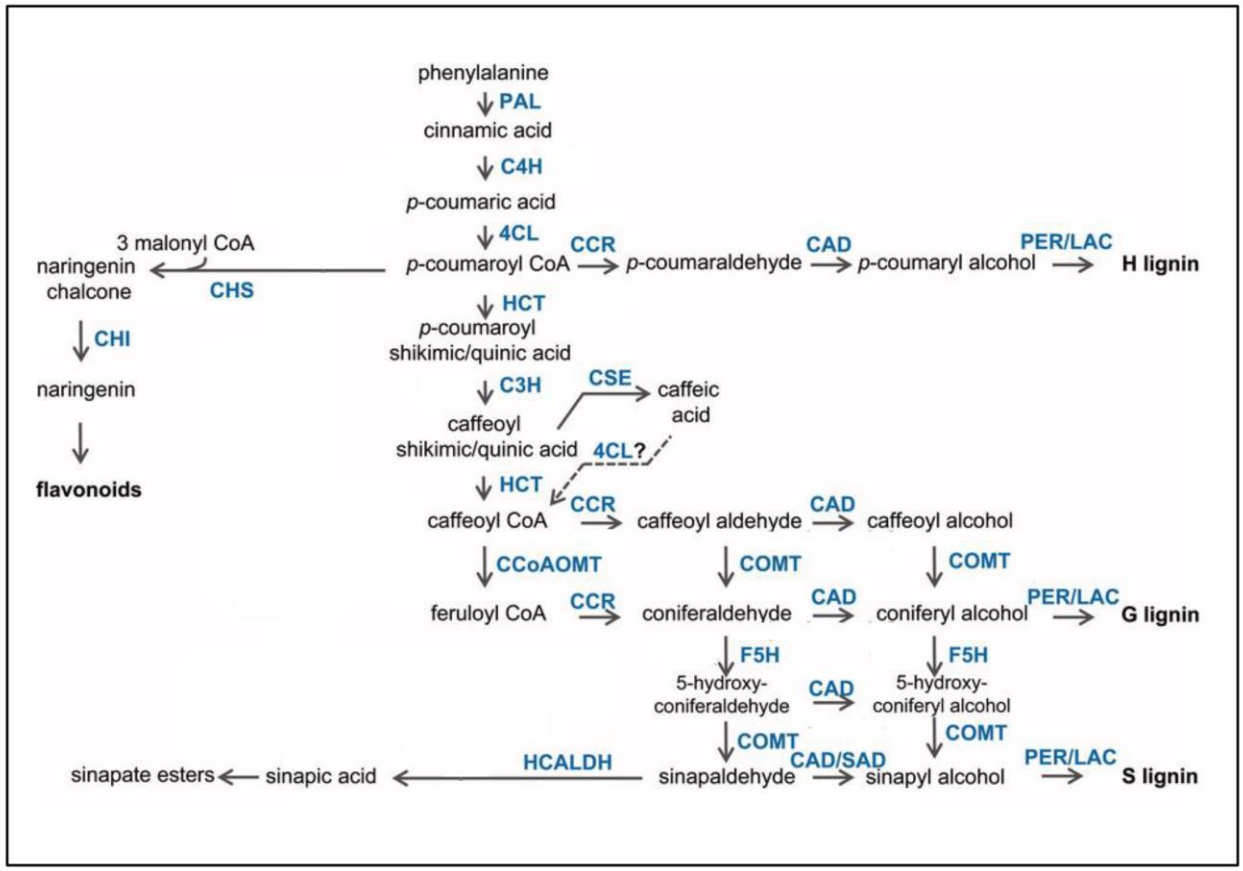

Figure 1-3 The Arabidopsis phenylpropanoid pathway.
Enzymes catalysing each step are in bold next to arrows. Dotted arrows represent predicted pathways. Figure modified from Hao and Mohnen, (2014)

1.2.2 Lignified tissue in Arabidopsis

Lignin, in the treachery elements and fibers of vascular tissue and its supportive tissue, make up a third of terrestrial woody biomass (Donaldson, 2001). Phloem fibers in stems of many dicot plants, extraxylary fibers beneath the epidermis in grass stems, sclereids in pear fruits, guard cells, root endodermis, trichomes, anther endothecium and dehiscent seed pods are some other examples of secondary cell wall containing tissues or organs (Zhong et. al., 2015).

In young Arabidopsis seedlings, appropriate spatial and temporal control of plant cell cycle and cell expansion are required so that neighbouring cells grow in a co-ordinated manner.

Procambial meristematic initials give rise to primary xylem and phloem vascular tissue. Young protoxylem elements contain secondary lignified thickenings in a helical or annular arrangement parallel to the growing axis of the cell, to allow cell expansion.

The vascular cambium in mature stems gives rise to xylem vessels, xylary fibres and phloem, while the parenchymatous tissue between the vascular bundles in Arabidopsis stems (interfascicular parenchyma) differentiates to form supportive interfascicular fibre cells. Secondary wall thickenings are formed after cell expansion ceases and initially are largely composed of cellulose and xylan (Albersheim et. al., 2010). Lignin is laid down within the matrix of the thickenings and can account for more than 25% of the dry weight of the mature wall. After lignification, vessel elements undergo cell death resulting in a toughened hollow tube made of dead cell walls. The secondary wall thickening in treachery elements has reticulated, scalariform or pitted wall thickening. Fibers on the other hand have more uniformly thickened secondary walls, except for pits where secondary wall thickening is locally prevented (Schuetz et. al., 2013). The inflorescence stem of Arabidopsis displays a gradient of secondary cell wall development at increasing distances from the shoot apical meristem (Ehlting et. al., 2005). Closer to the top of the inflorescence stem, differentiated vessel elements have thickened secondary cell walls, while interfascicular fibers have not yet formed. Further down the stem, at the same radial distance, vessels are at a more advanced stage of development as compared to fibers. At the bottom of mature inflorescence stems, active lignification of fibers takes place, while most vessels are fully differentiated and metabolically inactive (Hall and Ellis, 2013).

1.2.3 Developmental regulation of secondary cell wall formation in stems

A hierarchical network of NAC and MYB domain containing transcription factors is important for developmental regulation of secondary cell wall biosynthesis in Arabidopsis (Fig. 1-4). The NAC genes *SECONDARY WALL-ASSOCIATED NAC DOMAIN 1 (SND1), NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1), NST2, VASCULAR-RELATED NAC-DOMAIN 6 (VND6)* and *VND7* serve as the master switches for fiber and vessel cell differentiation and have tissue type specific expression. They bind to several common targets and their transcriptional activity converges at the redundantly activating MYB genes *MYB42* and *MYB83* that have a secondary wall specific expression (Kubo et. al., 2005; Zhong et. al., 2007).

MYB58, MYB63 and MYB85 specifically activate lignin biosynthesis by binding to the AC element in the promoters of *PAL, 4CL, C3H, HCT, CCOMT, CCR* and *CAD* family of genes. *F5H* contains no AC elements and is directly regulated by *SND1* (Zhong et. al., 2009). Several other MYB and NAC genes have been implicated in activating deposition of both cellulose and hemicellulose (Fig. 1-4; Schuetz et. al.,2013).

A few repressors in the network have been identified. *MYB4* can repress expression of lignin pathway genes by altering expression of *4CL, C4H* and possibly *CAD* family genes. Down-regulation *MYB4* is thought to allow lignification in response to wounding (Jin et. al., 2000). *MYB32* is also a repressor of lignin deposition and has high levels of expression in poorly lignified tissues like flowers (Preston et. al., 2004). The homeodomain transcription factor gene *BREVIPEDICLLUS (BP)* represses premature lignin deposition (Mele et. al., 2003). MYB7, MYB4 and MYB32 can repress their own expression as well as the expression of the upstream *SND1* transcription factor gene (Wang et. al., 2011).

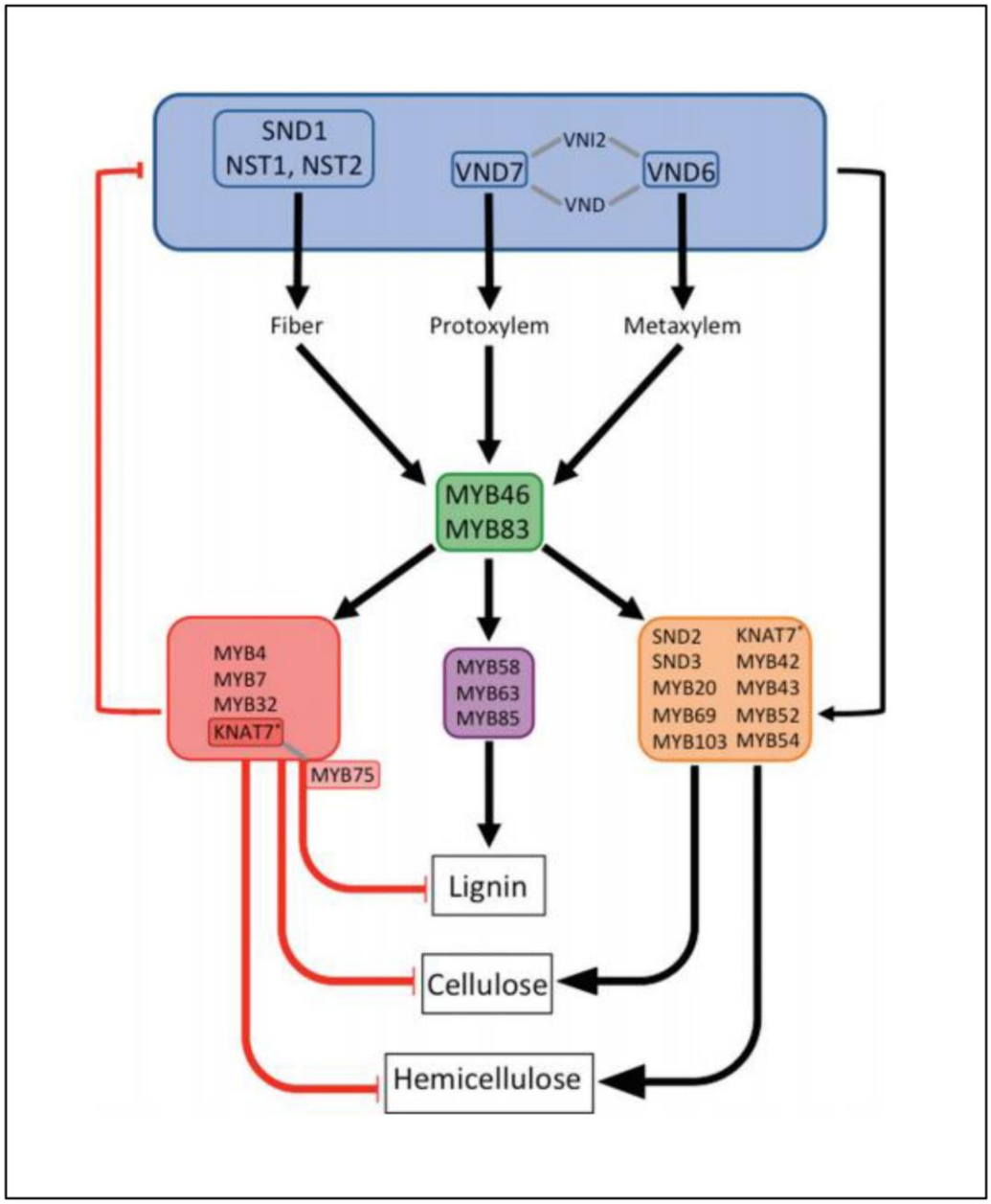

Figure 1-4 An overview of the transcriptional network regulating secondary cell wall biosynthesis in Arabidopsis.
Reproduced from Schuetz et. al., (2013)

KNOTTED ARABIDOPSIS THALIANA 7 (*KNAT7)* is another transcription factor in this regulatory network that represses secondary cell wall formation. *KNAT7* is activated by *SND1*, related *NAC* genes as well as *MYB46* and is strongly expressed in secondary wall containing tissues (Zhong et. al., 2008; Ko et. al., 2009). My thesis deals with exploring the physiological significance of this repressor transcription factor in seedling and mature stem secondary cell wall development.

1.2.4 The KNAT7 repressor complex

KNAT7 is a *KNOTTED-LIKE HOMEDOMAIN (KNOX)* gene that belongs to a plant-specific three-amino acid loop extension (TALE) superclass of homeodomain transcription factors (Hake et. al., 2004). The homeodomain in the KNAT7 protein mediates specific DNA-binding interactions while the MEINOX domain allows interactions with other TALE proteins. Class I *KNOX* genes play important roles in meristem function, tissue proliferation, control of leaf shape and hormone homeostasis in flowering plants, while the Class II clade of *KNOX* genes, formed by gene duplication in an ancestor of land plants, is thought to act in an antagonistic biochemical manner to the Class I genes, at least with respect to *KNAT3*, *KNAT4* and *KNAT5* (Fururmizo et. al., 2015). KNAT7, which belongs to the Class II clade of KNOX proteins, represses target gene expression in a protoplast transient expression system (Li et. al., 2011; Liu et. al., 2014).

Much more is known about *KNAT7* as compared to the other Class II *KNOX* genes. *KNAT7* is strongly expressed, concurrently with secondary wall formation in Arabidopsis stems and young plant hypocotyls (Brown et. al., 2005; Persson et. al., 2005; Liu et al., 2014). *KNAT7*

shows increasing expression from the top to the base of the Arabidopsis inflorescence stem (Li et. al., 2012). Expression profiling and expression pattern filtering by Ehlting et. al., (2005) identified *KNAT7* as a candidate involved in regulating secondary cell wall biosynthetic genes in fibers.

KNAT7 expression is observed in developing xylem, phloem, interfascicular fibers, cortex close to interfascicular fibers as well as in the cambial region close to the xylem in mature Arabidopsis stems (Li et. al., 2012; Liu et. al., 2014). In seedlings, strong GUS activity driven by the *KNAT7* promoter is observed in the vascular systems of roots, hypocotyl and cotyledons, and in the stele of young roots (Li et. al., 2012; Liu et. al., 2014).

The *knat7-1* knock-out allele (hence referred to as *knat7*) exhibits thickened interfascicular fiber cell walls in mature stem tissue relative to wild-type stems, while *KNAT7* overexpression mutants show the opposite phenotype in the same tissue (Li et. al., 2012; Liu et. al., 2014). This thickened fiber phenotype depends on interaction with BELL1-LIKE HOMEODOMAIN 6 (BLH6) protein as the *Pro35S:KNAT7 blh6* plants show wild-type like stem interfascicular fiber thickness. The xylem vessels in *knat7* on the other hand show a mild *irx* phenotype (collapsed, irregular shaped vessels) that is enhanced in the *blh6 knat7* background, suggesting that transcriptional regulation by *KNAT7* may be complex. A dominant transcriptional repression variant of *KNAT7* in transgenic Arabidopsis resulted in thinner interfascicular and xylary fiber cell walls, but no *irx* phenotype (Zhong et. al., 2008), which may be partly explained for by the fact that the KNAT7 protein is a repressor and a dominant repression mutant would show enhanced repression of *KNAT7* targets.

KNAT7 forms a heterodimer with BLH6 to directly repress expression of *REVOLUTA/INTERFASCICULAR FIBERLESS1* (*REV/IFL1*) in interfascicular fibers.

Interactions of KNAT7 with BLH6, as well as with OVATE FAMILY PROTEIN 1 (OFP1) and OFP4 enhance KNAT7 repression activity in protoplast transactivation studies while an activator of anthocyanin biosynthesis, MYB75, shows repression activity when it interacts with KNAT7. MYB75 and KNAT7 transcription factors together also regulate secondary cell wall formation in interfascicular fibers and the seed coat. A decrease in adherence of mucilage to seed coat in *knat7* however does not require interaction of the protein with MYB75 (Bhargava et. al., 2013).

Expression profiling of secondary cell wall biosynthetic genes has been carried out in *knat7* inflorescence stems (Liu et. al., 2014). Increased levels of the expression of two secondary cell wall cellulose synthase genes, *CELLULOSE SYNTHASE A7* (*CESA7)* and *CESA8* is observed, while primary wall *CESA* gene expression remains unchanged. *IRREGULAR XYLEM 7 (IRX7), IRX8, IRX9* and the majority of lignin biosynthesis genes also show increased expression in stem tissue and the total lignin content per gram dry weight is increased (Li et. al., 2012; Liu et. al., 2014). However the relative composition of cell wall sugars (reflecting cellulose and hemicellulose levels) does not show any difference in *knat7* stems (Li et. al., 2012). Taken together, these characteristics of the *knat7* support a role for *KNAT7* as a repressor of secondary cell wall deposition in interfascicular fibers.

Based on the phenotypes of *knat7*, KNAT7 repressor activity of the protein in protoplast systems, and its position in the developmental transcriptional regulatory network, Li et. al., (2012) suggested a role for it as part of a regulatory module for resource allocation to the secondary cell wall. *KNAT7* is also the target of *MYB61* – a resource allocation transcription factor that controls xylem quantity and quality, seed coat mucilage deposition, stomatal closure and lateral root formation (Romano et. al., 2012). The secondary cell wall transcriptional regulatory network consists of multiple feed-forward pathways that activate secondary cell wall

biosynthesis while also activating expression of a repressor. It is possible that the physiological significance of the repression activity of *KNAT7* and its interaction partners is for resource allocation.

1.3 Clock regulation of secondary metabolism

~30% of a plant's photosynthetic resources are driven into the shikimate pathway, which produces the aromatic amino acids required for protein biosynthesis, namely L-tryptophan (Trp), L-phenylalanine (Phe), and L-tyrosine (Tyr) (Maeda and Dudareva, 2012). The shikimate pathway genes are light and clock regulated. The first and the fourth enzymes for biomolecular conversion in the shikimate pathway are regulated by the circadian clock through the EE motif and the CBS promoter elements while the second and fifth enzymes in the pathway are light regulated through the PHYTOCHROME-INTERACTING FACTOR (PIF) binding G-motif promoter element. Resistance to glyphosate, which is an inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, is conferred by modulating light regulation of this pathway (Sharkhuu et. al., 2014).

Further, in a genome-wide analysis of Arabidopsis transcripts, twenty-three genes involved in phenylpropanoid secondary metabolism showed peaks in expression 20 h after dawn (Harmer et. al., 2000). Cytochrome P450 monooxygenases are likely to be rate-limiting in secondary metabolic pathways and Pan et. al., (2009) also confirmed circadian regulation of P450s in phenylpropanoid as well as carotenoid, oxylipin, glucosinolate and brassinosteroid biosynthesis pathways. Rogers et. al., (2005) used northern blots to show that lignin biosynthetic genes exhibit two different kinds of patterns of periodic transcript variation in 14-days old long day grown plants as compared to long day grown plants transferred to continuous light;

indicating evidence for light and clock regulation of lignification. They also showed that metabolizable sucrose levels play an important role in lignin biosynthetic gene regulation.

Donaldson (1992) studied seasonal variations in lignification during one growth season in radiata pine and found that the number of lignifying cells reached a maximum during the summer and was often incomplete at the onset of winter, while Grand et. al., (1979) found that light is necessary for incorporation of guaiacyl but not syringyl residues from radioactively labelled phenylalanine in poplar stems. These are some more lines of evidence for light and clock regulation of lignification in literature.

The goal of my Masters' thesis was to confirm diurnal trends in expression of selected lignin biosynthetic genes (LBGs) using quantitative measurements of transcript abundance and to test the hypothesis that *KNAT7*, a secondary wall specific transcription factor implicated in resource allocation (Li et. al., 2012), plays a role to modulate diurnal regulation of lignin biosynthesis.

1.4 Research questions and objectives

Hypothesis – *KNAT7* forms a negative regulatory loop for resource allocation to secondary cell wall biosynthesis over a 24 hour daily cycle.

Objective 1: Determine if expression of LBGs in wild-type Arabidopsis seedlings shows a free-running (circadian) and diurnal pattern of expression.

Objective 2: Determine if a diurnal pattern of expression of LBGs is observed in wild-type Arabidopsis inflorescence stems.

Objective 3: Determine whether *KNAT7* contributes to diurnal regulation of LBGs in long day conditions in seedlings and inflorescence stems.

Objective 4: Determine if *knat7* mutants show any additional phenotypes that may implicate the gene for a role of diurnal regulator.

1.5 Motivation for the research

Diurnal light rhythms and a plant's internal circadian rhythm are important signals for resource allocation and plant cell walls represent a very large sink for global fixed carbon reserves. Almost 70% of the carbon dioxide fixed by land plants is deposited as secondary cell walls (Pauly and Keegstra, 2008). A highly coordinated gene network, consisting of multiple parallel feed forward pathways is responsible for developmental regulation of secondary cell walls (Schuetz et. al., 2013). However diurnal regulation of secondary cell wall formation has not been well-studied yet.

Laying down of secondary cell walls is an energetically expensive process for the plant and appropriate spatial-temporal control of deposition is essential for plant function (Boudet, 2000). Plants lack any mechanisms for degrading lignified secondary cell walls. Under these

circumstances, a negative regulatory loop in the secondary cell wall biosynthesis network would be very important, as it would help the plant sense circadian, diurnal and sucrose availability signals to repress resource allocation to growth and development in limiting conditions.

In order to observe a possible link between *KNAT7* and resource allocation, I examined LBG transcript level variation over 48 h periods in Arabidopsis wild-type and *knat7* plants by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). I was particularly interested in stem tissue, where largest amounts of secondary cell walls are deposited, *KNAT7* has highest levels of expression and the *knat7* secondary cell wall phenotypes are observed. I also used 7-day seedling tissue, since it is the most commonly studied developmental stage for diurnal/circadian rhythm studies. I predicted that if the *KNAT7* transcription factor is responsible for repression, based on resource allocation, characteristic diurnal variation in mRNA levels for LBGs (henceforth this refers to the 10 'lignin toolbox' genes selected to study) in *knat7* would be diminished, since the mutants would have lost the ability to repress LBG expression at daily expression minima.

Chapter 2: **Materials and methods**

2.1 Plant material

The *knat7-1 (knat7)* and *4CL :KNAT7 Arabidopsis thaliana* (Arabidopsis) mutants were obtained from Dr. Yuanyuan Liu and are previously described in Li et. al., (2012). Both mutants are in the A*rabidopsis ecotype* Columbia-0 (Col-0) background and this ecotype was used as the wild type throughout. The *knat7* mutant (also named SALK_ 002098) has a T-DNA insertion in the fourth intron of the *KNAT7* gene and is from the Arabidopsis Biological Resource Center (ABRC, Columbia, OH, USA, https://abrc.osu.edu/). In *4CL:KNAT7*, a parsley 4CL1 promoter drives overexpression of *KNAT7* specifically to cells with secondary wall thickening. The genotype of both mutants used in experiments was confirmed (see below).

2.2 Growth conditions

Seeds were surface-sterilized with 75% ethanol and planted on Murashige and Skoog (MS) Basal Salts media with minimal organics (Sigma) and 1% (w/v) agar (Sigma).

Plants were grown vertically (agar surface perpendicular to the ground) in wooden supports to collect samples for qRT-PCR analysis. After 3 days of cold-treatment in the dark at 4℃, seedlings were transferred to a growth chamber set at 110µmol $m^{-2}s^{-1}$ light fluence, 21℃, 70% humidity and a photoperiod of 16 h light/ 8 h dark (long day, LD or16L/8D).

Seedlings for study in continuous light were transferred to 24 h light (continuous light, CL or 24L/0D) after 7 days in 16L/8D. Aluminium foil was wrapped around simultaneously grown long day plants at night to prevent unwanted light exposure. For experiments requiring mature plants, seedlings were transferred to 2×2 inch pots containing soil (Sunshine Mix #4,

from SunGro Horticulture Canada Ltd; http://www.sungro.com) and maintained in the same growth conditions described for seedlings, in long day conditions.

2.3 Genotyping mutants

DNA was isolated from one to two young rosette leaves by grinding tissue in a 1.5 mL Eppendorf tube containing 500µl Shorty DNA extraction buffer (200mM Tris, 400mM LiCl, 25mM EDTA, 1% SDS). DNA was precipitated from the supernatant using 500µl isopropanol and the pellet was cleaned using 1ml of 70% ethanol. The pelleted DNA was suspended in 100µl distilled water.

The presence of the T-DNA insert in *knat7* was examined by semi-quantitative PCR using flanking gene specific primers (5'-AAG TTT GGG CTT GGG CTT GAC-3' and 5'-TTG CCT TGT CAT CTT CCT GTT CA-3') and T-DNA left border LBb1.3 (5'-ATT TTG CCG ATT TCG GAA C-3') to select for homozygotes. Primers used to identify presence of a *4CL: KNAT7* construct were 5'-CTA TAT ATT TGT GAG TTG GTA-3' and 5'-TCC TCT TGC GTT GGT TAA TG-3'.

2.4 Sample collection for qRT-PCR

Five seedlings each were collected in a tube, flash-frozen in liquid nitrogen and stored at -80℃ until they were used. For stem samples, at 5 weeks of age, plants with inflorescence stems with a total height of 25±1cm were identified. Stem sections 10 cm from the base of the inflorescence stems, from 3 plants, were collected in a tube, flash-frozen in liquid nitrogen and stored at -80℃ until they were used. Three biological replicate tubes were collected at 12 time

points, every four hours, over two consecutive days. The sample collected at 'dawn' (when light switches on in growth chamber) was defined as the 0 h or 24 h sample.

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol® Reagent (Life Technologies, www.lifetechnologies.com) according to the manufacturer's instructions. The concentration of RNA was measured using the absorbance at 260 nm and the quality was assessed using the A260/A280 ratio. Single-strand cDNAs were synthesized via reverse transcription using QuaniTect® Reverse Transcriptase kit (Qiagen, www.qiagen.com) that included a genomic DNA elimination step, according to manufacturer's instructions.

2.6 qRT-PCR

2.6.1 Reference gene selection

The RefGenes tool (Hruz et. al., 2011) was used to identify a set of genes that showed the least variation in expression in a set of all available light, diurnal and circadian microarray experiments in the database, as of October, 2013. The top 20 genes from this list were compared to a list of superior reference genes validated by Czechowski et. al., (2005) and Hong et. al., (2010). Based on the comparison and a survey of reference genes used by circadian biologists *UBIQUITIN-CONJUGATING ENZYME 21(AT5G25760; UBC21), PP2A (AT1G13320)* and *FBOX (AT5G15710)* were selected for further analysis of suitability. *ACTIN 2 (AT3G18780, ACT2)* a traditionally used 'housekeeping' reference gene in qRT-PCR was also included for further testing.

A set of 6 wild-type cDNA samples and 6 *knat7* cDNA samples was selected to perform qRT-PCR analysis of expression levels of each of the four genes described above. Such an analysis was performed individually for both stem and seedling samples. Fig. 2-1 indicates the threshold cycle (Ct) values for expression of these genes. The best internal reference gene would show the least amount of variation in expression levels in this set of uniformly prepared cDNA samples. Standard deviation was used as a parameter to indicate stability of expression of these genes. For stem samples, *FBOX* clearly showed the least variation in Ct value and lowest standard deviation as compared to other potential reference genes and was chosen as an internal reference for stem samples. *LATE ELONGATED HYPOCOTYL (LHY),* a gene that is reported to show robust diurnal cycling in expression (Schaffer et. al., 1998) showed clear variation in Ct values with a much larger standard deviation as compared to all the other potential reference genes. Subsequently, a similar analysis was performed for seedling samples which indicated that *FBOX* again showed the least variation in Ct value. However, *PP2A* showed a low standard deviation as well. I also was made aware of the geNorm algorithm (Vandesompele et. al., 2000; part of qbase+ software distributed by Biogazelle; www.biogazelle.com/qbaseplus) for identification of stable reference genes. Analysis using this algorithm showed *PP2A* to be the most stable reference gene. Several publications studying circadian phenomenon in plants have used *PP2A* as a reference gene hence I selected this for use for normalization of seedling qRT-PCR results.

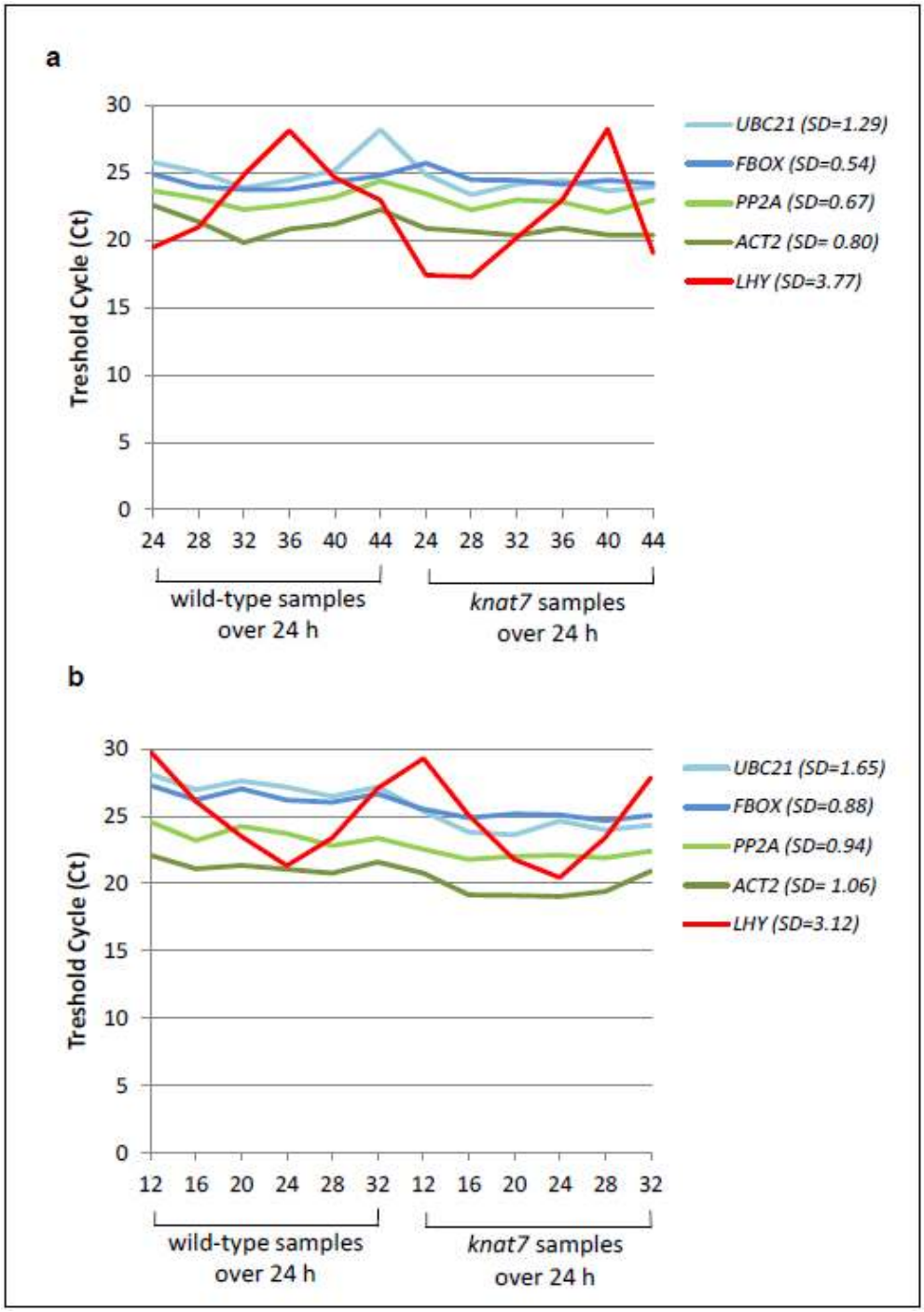

Figure 2-1 **Identification of a stable reference gene for normalization of qRT-PCR.** Threshold cycle (Ct) value from qRT-PCR assessing expression of potential reference genes *UBC21, FBOX, PP2A, ACT2* and known diurnally oscillating gene *LHY* in a set of wild-type and *knat7* a)stem samples b)seedling samples. Threshold was assigned to a region where exponentially increasing amplification in qRT-PCR was observed. SD, standard deviation of the Ct for a given gene in the twelve samples assayed.

2.6.2 Plate Setup

cDNA was diluted (1:3 for stem samples, 1:2 for seedling samples) and 1µl was used in each reaction in a 10µl q-RT-PCR reaction volume, using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.; www.bio-rad.com) following manufacturer's instructions.

qRT-PCR amplification was performed with gene-specific primers (Table 2.1). Three technical replicates of qRT-PCR were performed for each primer-sample combination. Given the large number of qRT-PCR experiments to be performed, a strategy to minimize the number of experiments and to manage for inter-run shifts in SYBR Green fluorescence measurement was required. I ensured that reactions of a gene for all 12 time points, for one set of wild-type or *knat7* biological replicates was on the same qRT-PCR plate – allowing measurement of accurate diurnal trends for that set of 12 samples. Normalization for inter-run variation was performed using an additional qRT-PCR experiment, where all biological replicates for wild-type and *knat7,* for the 0h samples were studied on the same plate.

2.6.3 Expression calculation

Fold-change of gene 1 relative to gene 2 = [2^(CT, gene 2 – CT, gene 1)]

Actual fold-change of gene 1 relative to gene 2 = [Experimental fold-change for gene 1 relative to gene 2 * Normalization factor]

Efficiency of primers used was tested using a dilution curve to ensure that it is within a 95% to 105% range. Specificity of primer binding was tested with a melt curve, at the end of every q-RT-PCR experiment. Error bars represent standard error (SE) of actual fold-changes for the three biological replicates. A student's t-test for samples with unequal variance was performed to determine statistically significant differences.

2.7 Hypocotyl length measurement and bolting study

For hypocotyl length measurements, seeds were grown on petri plates in a similar manner to the ones used for qRT-PCR. Light intensities on different shelves of a growth chamber were modified to obtain fluences of ~3, 12 and 96 µmol $m^{-2}s^{-1}$, measured using a light meter. Aluminium foil wrapped around petri dishes was used to create dark conditions. At least two plates containing both wild-type and mutant plants were treated to each of these four light conditions for 7 days. On the 8^{th} day individual hypocotyl lengths were measured using the application ImageJ (http://rsb.info.nih.gov/ij) from photographs of plates.

For flowering time analysis, plants were grown on soil at 110 µmol $m^{-2}s^{-1}$in long day photoperiod and monitored daily for bolting. When the inflorescence stem was greater than 1 cm in height, it was considered to have started 'bolting'. The number of days from transfer of seeds to conducive germination conditions, until the day of 'bolting', was counted as 'days to bolting'.

Table 2-1 Genes studied using qRT-PCR and primer pairs used.

GENE LOCUS	GENE NAME	PRIMER SEQUENCE (5'-3')
AT1G62990	*KNAT7*	*KNAT7*-F: AAATTGGTGGAGGAGACAGG
		KNAT7-R: TCCTCTTGCGTTGGTTAATG
AT1G01060	*LHY*	*LHY*-F: CCAACGAAACAGGTAAGTGG
		LHY-R: CTTGGGAACATCTTGAACCG
AT4G39980	*DHS1*	*DHS1*-F: AATACTGGCTGAAGTCCGAG
		DHS1-R: ATTCTGTCACGTTCTGACCT
AT2G37040	*PAL1*	*PAL1*-F: AAGATTGGAGCTTTCGAGGA
		PAL1-R: TCTGTTCCAAGCTCTTCCCT
AT2G30490	*C4H*	*C4H*-F: ACTGGCTTCAAGTCGGAGAT
		C4H-R: ACACGACGTTTCTCGTTCTG
AT1G51680	*4CL1*	*4CL1*-F: TCAACCCGGTGAGATTTGTA
		4CL1-R: TCGTCATCGATCAATCCAAT
AT2G40890	*C3H*	*C3H*-F: GTTGGACTTGACCGGATCTT
		C3H-R: ATTAGAGGCGTTGGAGGATG
AT5G48930	*HCT*	*HCT*-F: GCCTGCACCAAGTATGAAGA
		HCT-R: GACAGTGTTCCCATCCTCCT
AT4G34050	*CCOMT*	*CCOMT*-F: CTCAGGGAAGTGACAGCAAA
		CCOMT-R: GTGGCGAGAAGAGAGTAGCC
AT1G15950	*CCR1*	*CCR1*-F: GTGCAAAGCAGATCTTCAGG
		CCR1-R: GCCGCAGCATTAATTACAAA
AT4G36220	*F5H*	*F5H*-F: CTTCAACGTAGCGGATTTCA
		F5H-R: AGATCATTACGGGCCTTCAC
AT5G54160	*COMT*	*COMT*-F: TTCCATTGCTGCTCTTTGTC
		COMT-R: CATGGTGATTGTGGAATGGT
AT4G34230	*CAD1*	*CAD1*-F: TTGGCTGATTCGTTGGATTA
		CAD1-R: ATCACTTTCCTCCCAAGCAT
AT1G13320	*PP2A*	*PP2A*-F: TAACGTGGCCAAAATGATGC
		PP2A-R: GTTCTCCACAACCGCTTGGT
AT5G15710	*FBOX*	*FBOX*-F: TTTCGGCTGAGAGGTTCGAGT
		FBOX-R: GATTCCAAGACGTAAAGCAGATCAA
AT5G08290	*ACT2*	*ACT2*-F: CCTGAAAGGAAGTACAGTG
		ACT2-R: CTGTGAACGATTCCTGGAC
AT5G25760	*UBC21*	*UBC*-F: TTAGAGATGCAGGCATCAAGAGCGC
		UBC-R: CATATTTCTCCTGTCTTGAAATGAA

Chapter 3: **Diurnal patterns of expression of lignin biosynthetic genes in wild-type *Arabidopsis thaliana***

3.1 Introduction

Expression profiling experiments have suggested that up to one third of the genes in the Arabidopsis genome are regulated by the circadian clock (Harmer et. al., 2000; Covington et. al., 2008). Harmer et. al., (2000) identified rhythmic up- and down-regulation of expression of 23 phenylpropanoid biosynthetic genes in continuous light (CL) grown seedlings, with all having one peak in expression at 20 h after dawn. Rogers et. al., (2005) showed by northern blot analysis that 11 genes of the phenylpropanoid pathway involved in developmental lignification show rhythmic variation in diurnal expression, attributable to both light regulation and circadian clock regulation. Pan et. al., (2009) reported a circadian rhythm in expression of the rate-limiting cytochrome P450 genes (*C3H, C4H* and *F5H)* and some other genes of the monolignol branch of the phenylpropanoid pathway; a promoter analysis identified (CCA1-binding sites) CBS and morning element (ME) elements over-represented in these genes.

In this chapter my aim was to confirm diurnal and circadian rhythmicity in transcript abundance of selected lignin biosynthetic genes (LBGs) by studying LBG expression in seedlings in controlled longday (LD) and CL conditions. Temporal expression patterns identified with better accuracy and resolution in wild type (Col-0) Arabidopsis will serve as a baseline to observe any changes in LBG expression in mutant backgrounds. Further, characterisation of diurnal patterns of LBGs in tissue from mature Arabidopsis stems would provide baseline data to investigate regulation of LBGs by *KNAT7* in the tissue where prominent secondary wall defects have been observed due to loss-of-function of this transcription factor gene. I also chose

to observe diurnal and circadian patterns in conditions where seedlings were limited to their own photosynthetic capabilities (without added sucrose in the growth media), since evidence over the last few years has re-affirmed the importance of sucrose as an input as well as output to the plant circadian clock (Haydon et. al., 2013).

3.2 Results

3.2.1 Temporal expression patterns of *LHY* and *DHS1* genes in wild-type seedlings

LHY is a core clock gene responsible for maintenance of circadian oscillations in plants cells and *DHS1* encodes the first enzyme in the shikimate pathway. *DHS1* has been reported to be under circadian clock regulation by binding of clock genes to its promoter (Sharkhuu et. al., 2014). In order to validate my qRT-PCR setup, I first assayed the temporal variation in expression of these two genes from 7-8 day old seedlings grown in LD and CL conditions on agar medium without added sucrose. Steady state mRNA levels of *LHY*, assayed by qRT-PCR, showed a peak in expression at 'dawn' (0 h and 24 h; defined as the time when light switches on in the growth chamber) in LD grown seedlings and a similar pattern was observed in CL grown seedlings (Fig. 3-1). These patterns are in agreement with published literature (Schaffer et. al., 1998) and confirmed the validity of the assay system including RNA extraction, qRT-PCR setup, and the reference genes used to normalize expression from different samples.

DHS1 transcripts showed increasing abundance from dawn to end of the day in LD conditions, confirming its diurnal regulation (Fig. 3-1b). However, a largely arrhythmic pattern of expression was observed in CL for this gene (Fig. 3-1d), suggesting lack of strong circadian clock regulation in these growth conditions. This is in contrast to the findings of Sharkhuu et al., (2014), who identified a *DHS1* expression peak 1 h after dawn and an expression trough at 16 h

for LD grown plants and a peak in the middle of the day for CL grown plants. It appears that differences in growth conditions influence diurnal and clock regulated expression of *DHS1*.

3.2.2 Temporal expression patterns of lignin biosynthetic genes in wild-type seedlings

We do not yet fully understand light and the circadian clock regulation of secondary metabolism, hence it is essential to identify whether lignin biosynthetic genes show diurnal variation in expression levels in the experimental growth conditions of LD used in this study as well as test for persistence of any rhythmic expression variation in the CL growth conditions that were established in the previous section. I used qRT-PCR to assay the diurnal and circadian expression patterns of a set of 10 LBGs in 7-8 day old seedlings. One member of each of the gene families involved in developmental lignification, as described by Raes et. al., (2003), was studied.

LBGs showed consistent variations in transcript abundance over the two days expression was assayed, in both LD and CL (Figs. 3-2, 3-3, 3-4, 3-5). A peak at 4 h after dawn, drop in transcript abundance between 4-8 h after dawn and sharp drop in abundance at night (16-24 h after dawn) was observed for many LBGs in LD grown plants. *PAL1, C4H* and *4CL1* had distinctly different patterns of expression as compared to *F5H* and *COMT* in LD conditions. All five of these genes showed very clear and reproducible rhythmicity in temporal mRNA abundance as compared to the other LBGs. *PAL1*, *C4H* and *4CL1* however showed an additional up-regulation after the 8 h time point expression trough. An additional peak in expression at the end of day was observed for these three genes, not observed for *F5H* or *COMT* (Fig 3-2).

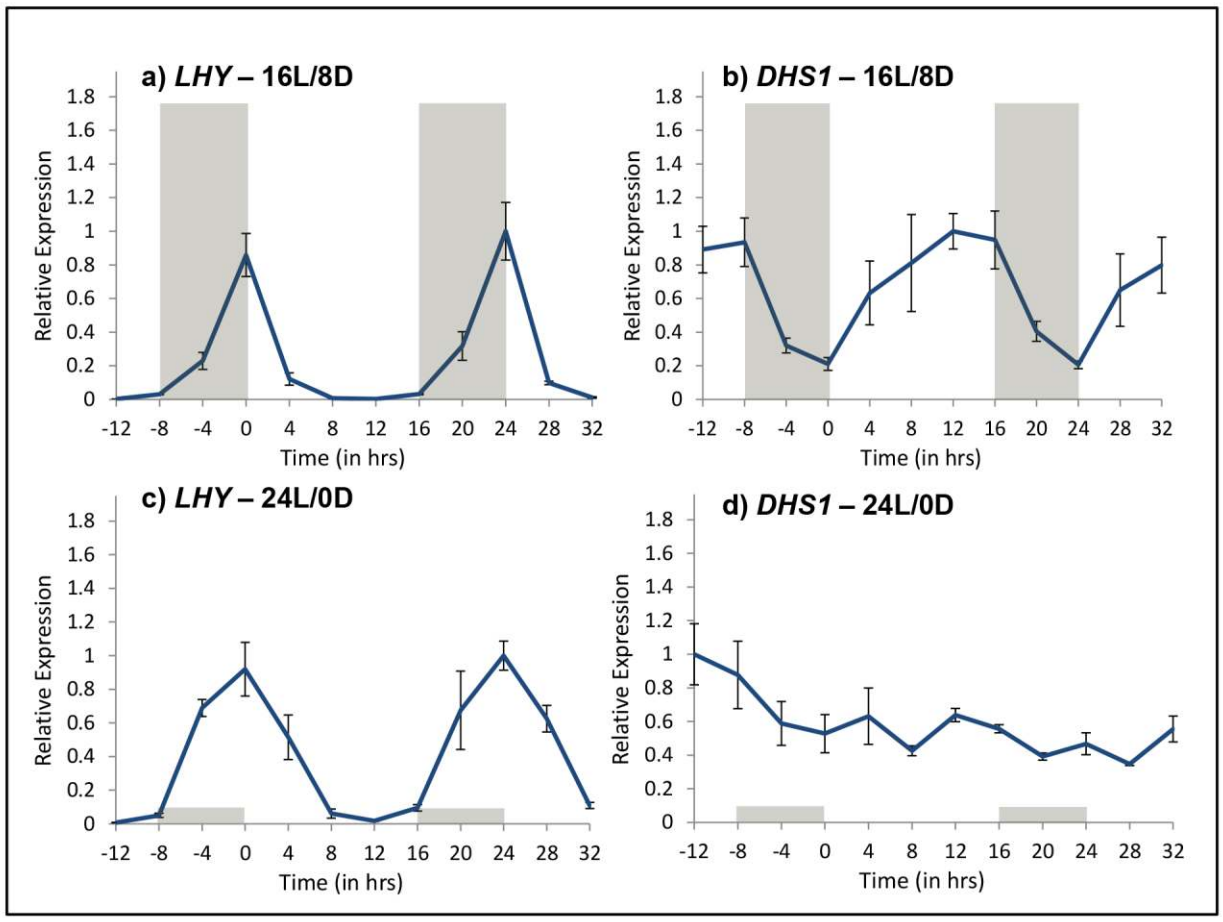

Figure 3-1 Temporal expression patterns of *LHY* and *DHS1* in wild-type seedlings grown in LD.

7-8 day old Col-0 seedlings grown on agar plates were assayed. (a), (b), LD (16L/8D entrained seedlings. (c), (d), LD-grown seedlings transferred to CL (24L/OD) at (-)8 h. Shaded regions indicate night or 'subjective' night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

When plants entrained in LD conditions were transferred to CL, an almost immediate change in the expression pattern of many LBGs was observed (Figs. 3-3, 3-5). This change was most striking for *PAL1*, *C4H*, *4CL1*, *F5H* and *COMT*, as the sharp decreases in transcript abundance of these genes at night, observed in LD, were replaced by increased or plateauing expression in the subjective nights.

LBG expression in CL seemed to be more co-regulated as compared to their diurnal patterns in LD. A drop in abundance around subjective midday and peak at subjective night was observed. No evidence of diurnal regulation of *C3H* transcript abundance was observed in both LD and CL conditions in these experimental conditions (Figs. 3-4, 3-5). In some cases high biological variability seemed to obscure apparent diurnal patterns in transcript abundance (e.g. *CCOMT* in LD; Fig. 3-4; *F5H, COMT, CAD1* in CL; Fig. 3-2, 3-4). However, overall clear rhythmicity in transcript abundance was observed for most LBGs in LD and presence of rhythmic expression variation even in CL confirmed that expression of LBGs is controlled by a circadian clock in the experimental conditions used.

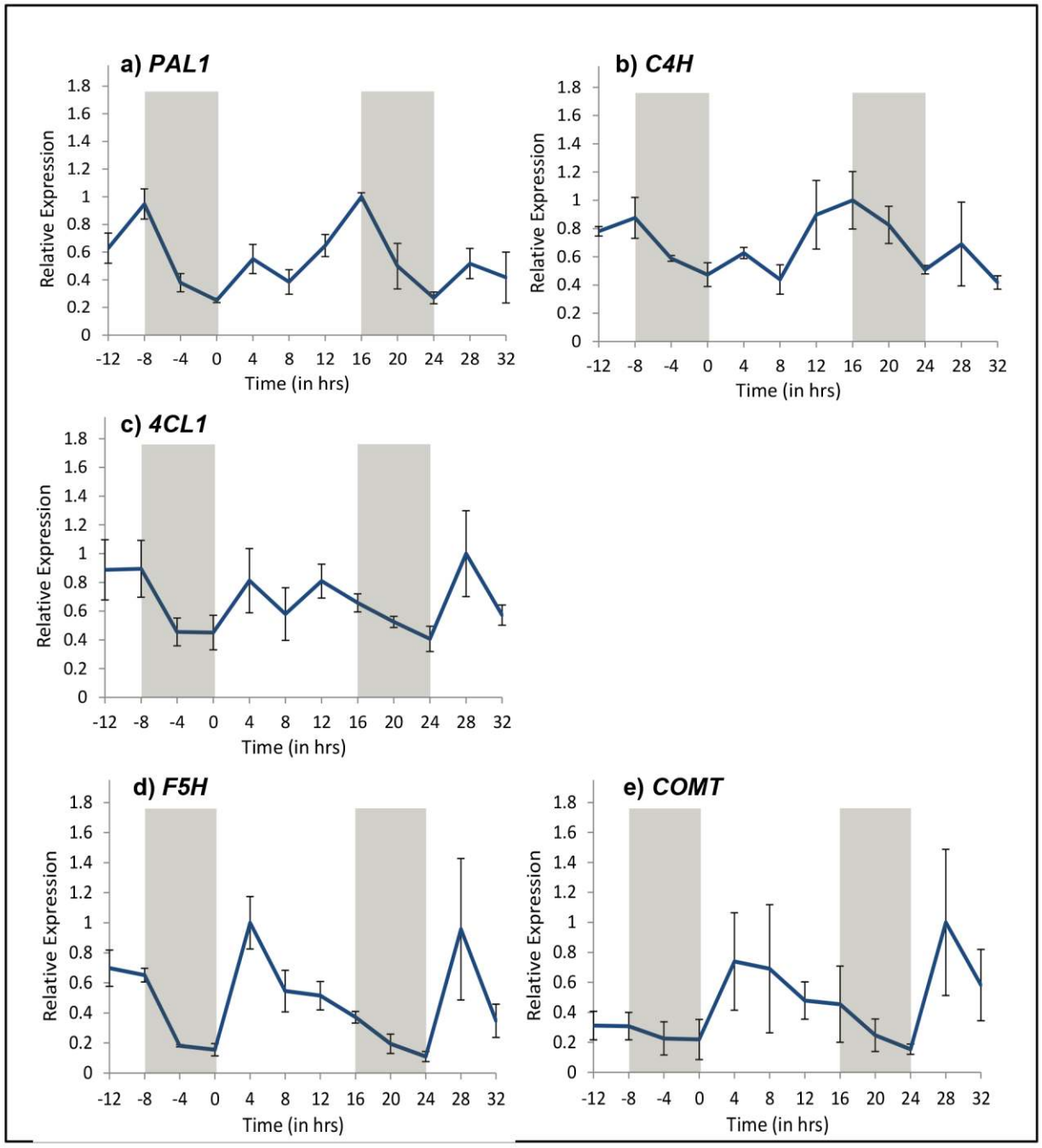

Figure 3-2 Temporal expression patterns of *PAL1, C4H, 4CL1, F5H* and *COMT* in wild-type seedlings grown in LD.

7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

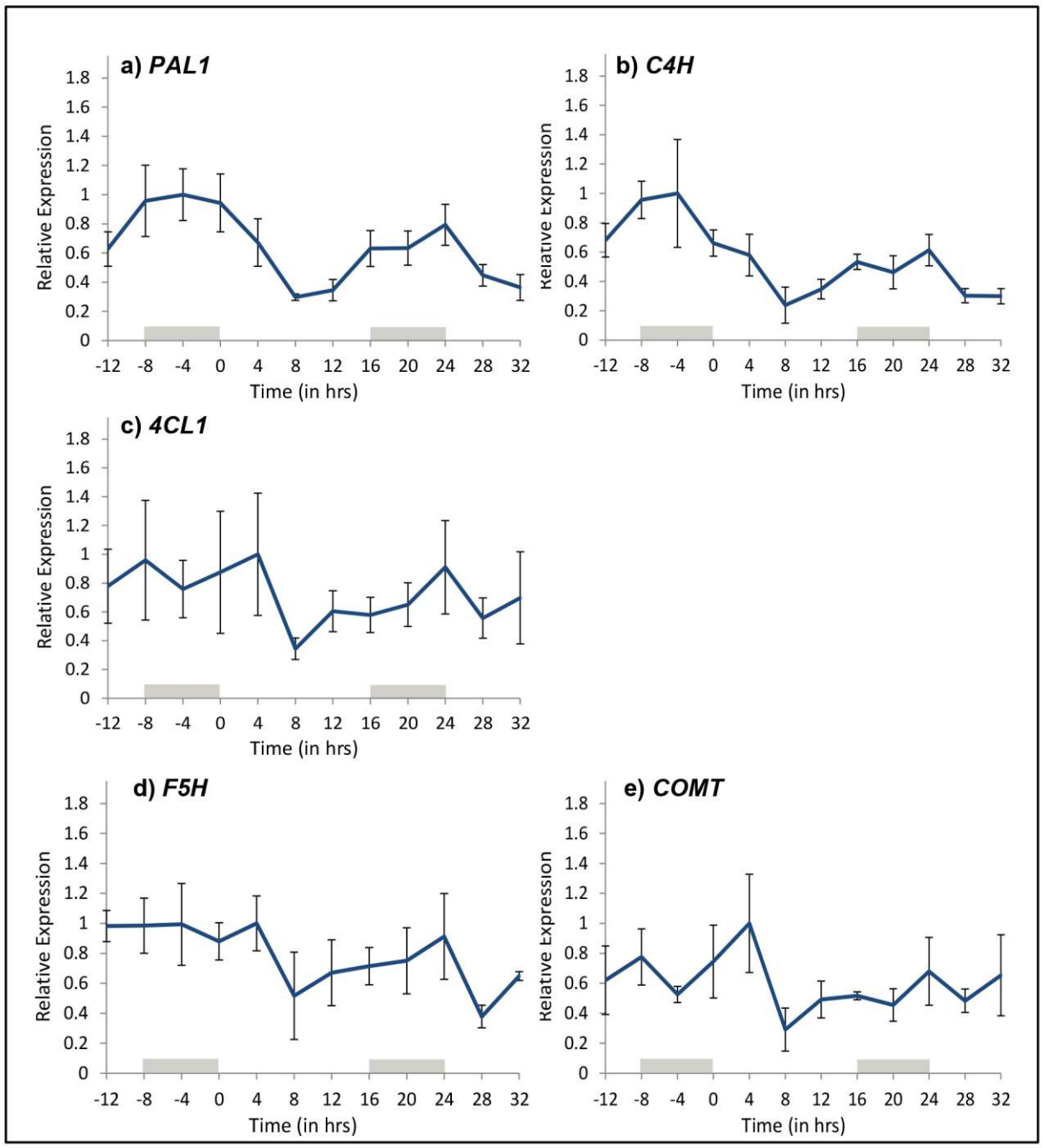

Figure 3-3 Temporal expression patterns of *PAL1, C4H, 4CL1, F5H* and *COMT* in wild-type seedlings grown in CL.

7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate 'subjective' night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

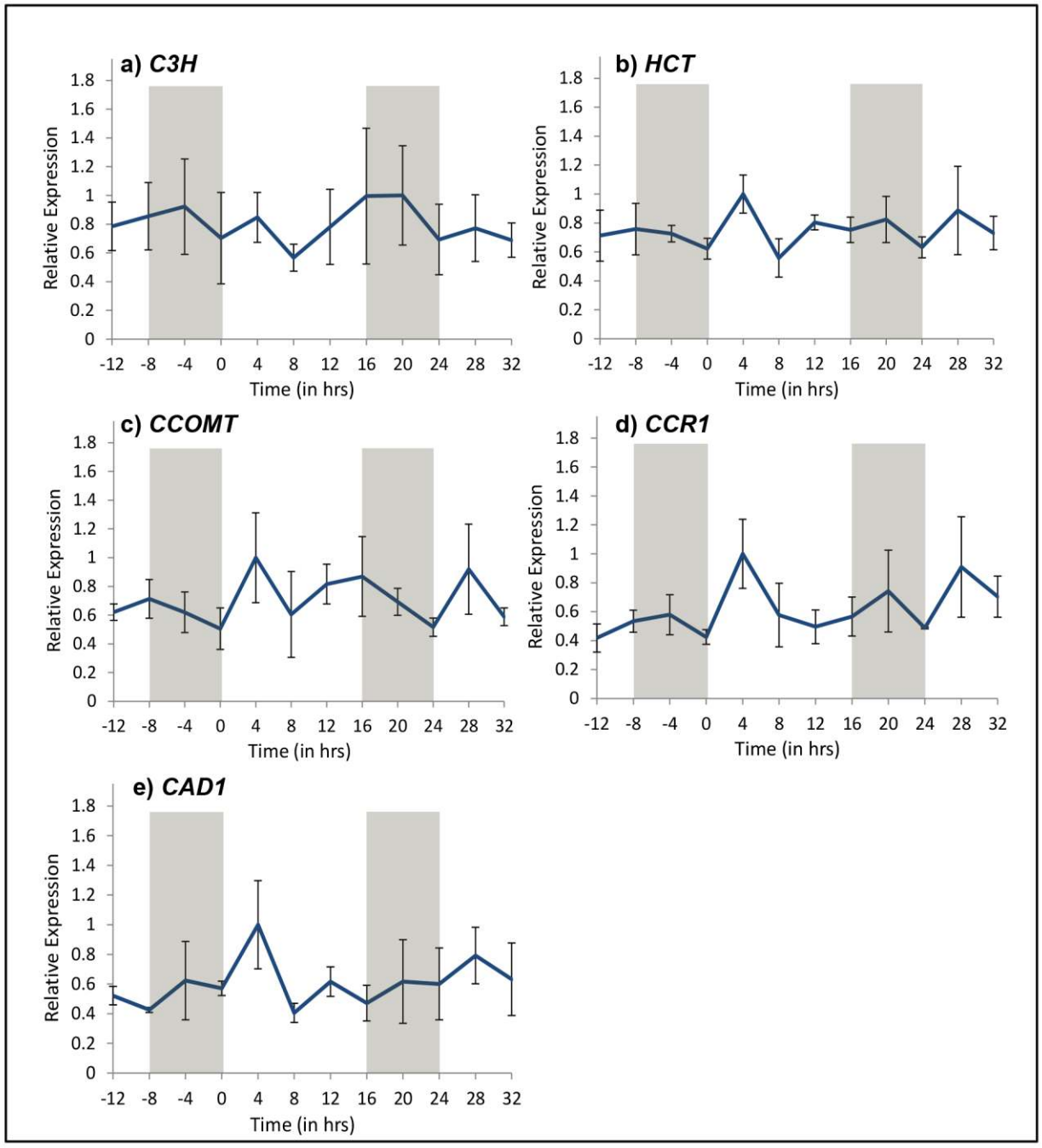

Figure 3-4 Temporal expression patterns of *C3H, HCT, CCOMT, CCR1* and *CAD1* in wild-type seedlings grown in LD.

7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

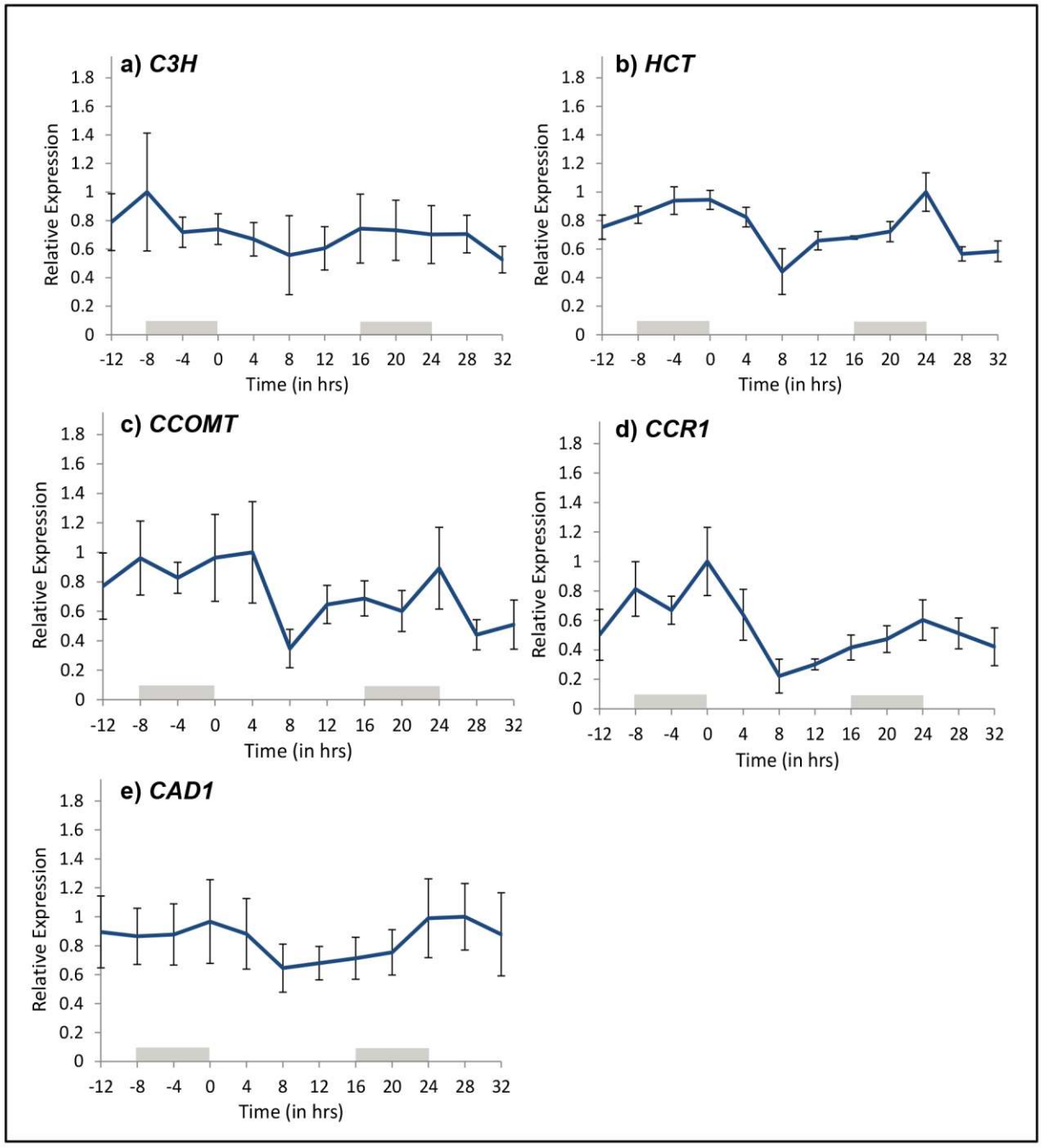

Figure 3-5 Temporal expression patterns of *C3H, HCT, CCOMT, CCR1* and *CAD1* in wild-type seedlings grown in CL.

7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate 'subjective' night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

3.2.3 Temporal expression patterns of *LHY* and *DHS1* genes in wild-type inflorescence stems

While several studies have examined diurnal and circadian rhythms in gene expression in seedlings, no studies have focused on the mature inflorescence stems where secondary wall formation and lignin deposition represents a major metabolic sink. To investigate such rhythms, I used the lower 10 cm of inflorescence stems from 5-week old Arabidopsis plants grown under LD to assay gene expression levels over a 48 h time period. Steady state mRNA levels of *LHY*, quantified by qRT-PCR, showed a peak in expression at 'dawn' while *DHS1* showed increasing levels from dawn to the end of the day in mature stem tissue (Fig. 3-6). The pattern of diurnal expression of these genes in stems showed consistent rhythmicity over the two days of the study. They had expression peaks in the same phase as in seedlings. Thus the presence of temporally varying transcript abundance of these genes indicates that these stem samples are well suited to study diurnal regulation of LGBs in stems.

3.2.4 Temporal expression patterns of lignin biosynthetic genes in wild-type inflorescence stems

I used the stem samples described above to assay expression levels of the 10 LGBs over a 48 h period. Although there were large biological variations in transcript abundance, at night LBG gene expression was generally lower by about 2-fold than expression levels over the day (Figs. 3-7, 3-8). However, it is difficult to discern consistent, rhythmic diurnal patterns in expression as seen for *LHY* in the same samples. *F5H*, one of the rate limiting genes in the monolignol specific branch of the phenylpropanoid pathway, showed the clearest diurnal pattern in expression with low levels of expression in the night and higher levels during the day.

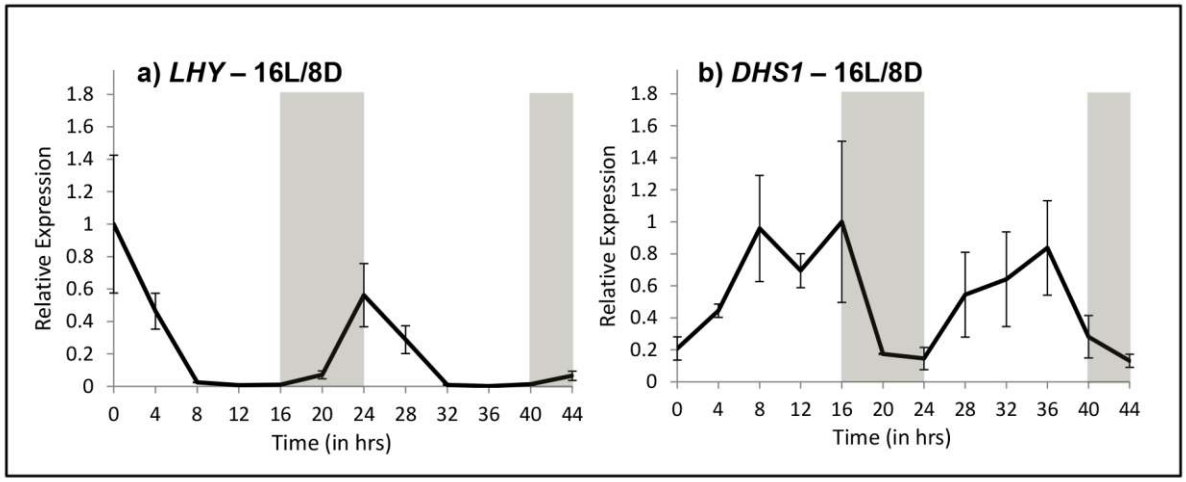

Figure 3-6 Temporal expression patterns of *LHY* and *DHS1* in wild-type inflorescence stems grown in LD.

Lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Shaded regions indicate night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

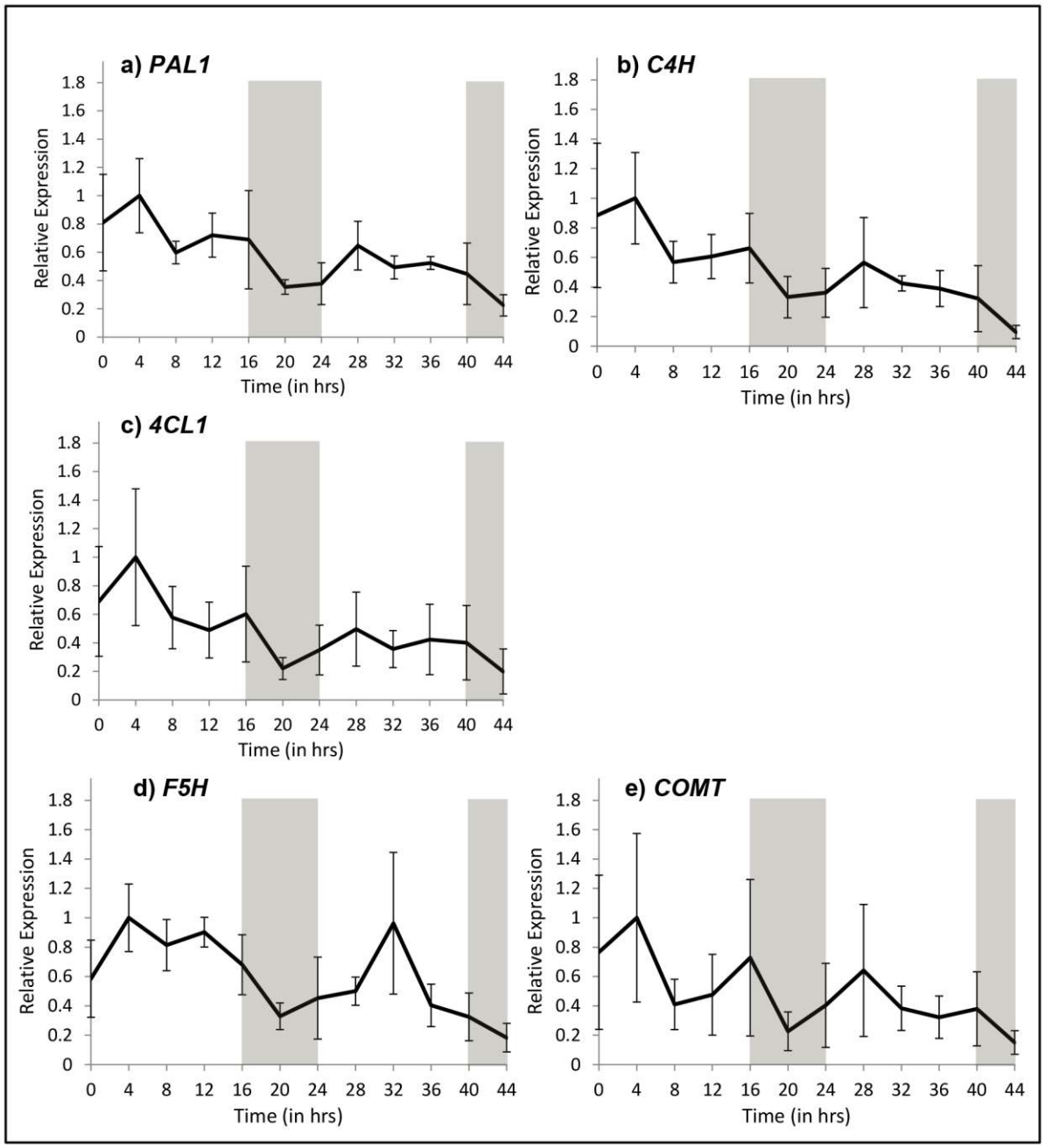

Figure 3-7 Temporal expression patterns of *PAL1, C4H, 4CL1, F5H and COMT* in wild-type inflorescence stems grown in LD.

Lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

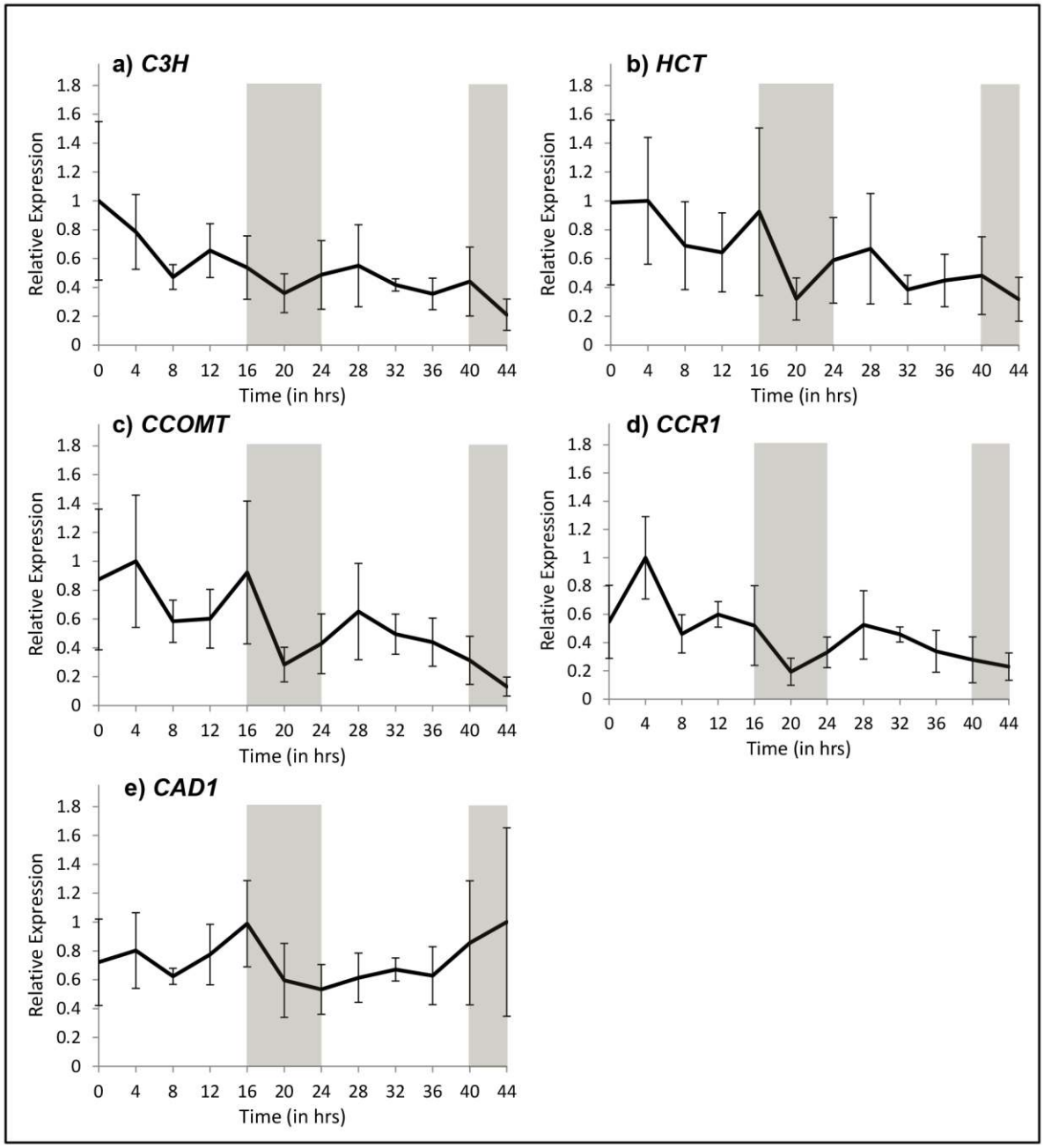

Figure 3-8 Temporal expression patterns of *C3H, HCT, CCOMT, CCR1* and *CAD1* in wild-type inflorescence stems grown in LD.

Lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Shaded regions indicate night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

3.3 Discussion

The presence of clear, reproducible variation in *LHY* expression in seedlings (Fig. 3-1) with a phase as reported in the literature, confirmed the validity of my experimental setup. Clear rhythmicity in expression in seedlings and stems indicated the presence of a functional circadian clock in the two wild-type developmental stages studied.

LHY is part of the first identified transcription- translation feedback loop of the endogenous circadian clock and has been well studied in Arabidopsis seedlings. All tissues and cell-types studied to date show cycling expression of this gene over a 24 h time period (Hsu and Harmer, 2014, Marti and Webb, 2014). Eucalyptus developing xylem has been observed to express *LHY* with a peak in transcript levels at dawn (Solomon et. al., 2010). A similar pattern in observed in the vascular tissue of leaves separated by microdissection from the rest of the leaf (Endo et. al., 2014). While *LHY* expression in secondary cell wall rich inflorescence stem bases has not been studied before it was expected that would also show similar diurnal rhythmicity.

Rhythmically expressed genes are so controlled by time-of-day specific binding of clock transcription factors at their promoter. *LHY* and *CCA1* are dawn phased repressors that function partially redundantly by binding to the EE and CBS (Michael et. al., 2008). *DHS1* is an example of a gene regulated by the clock by binding to EE and CBS at its promoter. Rhythmic variation in expression of *DHS1* was observed in seedling and stem samples in my experimental conditions (Figs. 3-1, 3-6), the phase of expression of *DHS1* in LD was not as reported (Sharkhuu et. al., 2014). This may be because these authors used different growth conditions as the ones I used: a light intensity of 22±2 µmol m^{-2} s^{-1} was used in the previous study as compared to the 110 µmol m^{-2} s^{-1} used in my experiment. Seedlings in the previous study were also grown on 2% sucrose. Light and sucrose have been shown to affect the pace of clock

regulated genes like *CHLOROPHYLL A/B-BINDING PROTEIN 2 (CAB2)* and *CAROTENOID AND CHLOROPLAST REGULATION 2 (CCR2)* (Millar, 2004, Haydon et. al., 2013). I queried the 'Diurnal' database, a web-based tool for accessing standard diurnal and circadian gene expression profiles from a collection of Arabidopsis microarray experiments (Mockler et. al., 2007) and found that in seedlings grown in conditions slightly different from my experiment, *DHS1* shows peaks in expression between 8-12 h after dawn. Hence different phases in diurnal expression of *DHS1* may be observed in different environmental conditions. It is not clear why *DHS1* showed no circadian regulation in CL grown seedlings.

As expected, most LBGs showed clear and consistent changes in expression levels over the two days of our study when studied in 7-8 day old seedlings (Figs 3-2, 3-4). Robust oscillations of LBGs, studied in seedlings, have been reported several times in the literature (Harmer et. al., 2000; Rogers et. al., 2005, Pan et. al., 2009) I also confirmed that LBGs show rhythmic up- and down- regulation of transcript abundance even in that constant environment conditions of CL (Figs. 3-3 and 3-5), indicating that an independent oscillator like the circadian clock regulates expression of the LBGs.

Most reported clock genes and clock regulated genes studied show one peak in expression over a daily cycle, so the existence of more than one peak in LBG expression is unusual but not unexpected as Rogers et. al., (2005) also found more than one peak in expression of LBGs grown in LD conditions. They observed a peak at 4 h after dawn, only in diurnal conditions, while a peak at 23 h was observed in diurnal as well as circadian conditions. I also observed a peak in LBG expression at 4 h after dawn under my experimental LD conditions, but did not include a time point corresponding to the second peak reported by Rogers et. al., (2005)

at 23 h after dawn. In my conditions a second peak at 16 h after dawn was observed for some genes in LD grown seedlings.

PAL1, C4H and *4CL1*, as part of anthocyanin biosynthesis studies, have been shown to be light induced (Das et. al., 2012) which is consistent with the observation that they show a peak after dawn in my experimental conditions. Secondary wall network analysis by an enhanced Yeast-1-Hybrid assay showed that *PAL1*, *C4H*, and *HCT* promoters may be regulated by HY5, a protein related to the phytochrome light signalling pathway (Taylor-Teeples et. al., 2014). The presence of a dark period before dawn is required to observe this peak in LBGs. This is in agreement with results from Rogers et. al., (2005). Further, I found that when plants entrained in LD are transferred to CL, a rapid response to light extension is observed clearly for genes like *PAL1, C4H, 4CL1* and *F5H.* This too may be a response to an acute light regulatory pathway.

In constant CL conditions a drop in expression of LBGs at subjective midday and a peak in expression at subjective night is observed. It is curious that even in conditions of abundant light, a drop in LBG expression takes place in the middle of the subjective day. A drop in transcript levels of several core clock genes is essential to allow expression of other reciprocally repressed transcription-translation feedback loops (McClung, 2006). The drop in expression of LBGs at 8 h after dawn may reflect rhythmicity of the clock genes regulating their expression, for example expression of a daytime repressor, like the *PRR* genes. Or it may be that rhythmicity of LBGs is a knock-on effect or default pattern related to diurnal regulation of other branches of the phenylpropanoid pathway (for example light-protective and plant defence compound related pathways). Since lignification represents an irreversible differentiation step in secondary growth, these patterns may be linked to resource availability signals, but Rogers et. al., 2005 showed that

the *sex1* mutant, that is deficient in starch turnover, shows an overall reduced level of expression of LBGs, but an unchanged diurnal pattern of expression.

We would expect that any diurnal processes regulating secondary wall formation in seedlings would be more active in inflorescence stem tissue, that deposit large amounts of carbon in its extensively lignified vascular and supportive tissue network. Yet in LD grown inflorescence stems diurnal variation in expression levels of LBGs were not clearly observed (Figs 3-7, 3-8). It is difficult to explain this result based on the current limited knowledge we have of diurnal regulation of LBGs. Developing xylem in Eucalyptus showed clear diurnal rhythmicity in expression of *C4H, C3H, CCOMT, F5H* and *CAD2* (Solomon et. al., 2010). It is possible the number of independent stem samples was not large enough to observe diurnal trends above inherent biological variation. Efforts were made during stem sample collection to minimize developmental differences in stem growth, by ensuring that stems harvested were between a narrow range of total heights at the time of collection (25 cm ± 1cm). *F5H* is considered to be one of the rate-limiting enzymes of the lignin biosynthetic pathway and functions specifically in the phenylpropanoid pathway branch for monolignol biosynthesis (Fraser and Chapple, 2011). This gene did show a clearer diurnal variation in expression as compared to the other LBGs studied.

In summary, I was able to confirm validity of my qRT-PCR methods as well as appropriateness of the tissue samples to study diurnal regulation of LBGs, laying the ground work for future studies. Clear diurnal and circadian regulated expression of LBGs was observed in seedlings and presence of an oscillating clock observed in mature Arabidopsis stems was identified based on rhythmicity of *LHY* and *DHS1* expression in this tissue; however LBGs in stems showed negligible diurnal variation in expression level.

Chapter 4: **Role for *KNAT7* in diurnal regulation of lignin biosynthetic genes in seedlings and stems**

4.1 Introduction

KNAT7 is a transcriptional regulator and a part of the complex gene network that regulates secondary cell wall biosynthesis and deposition in land plants such as Arabidopsis and poplar. Multiple feed-forward pathways of NAC and MYB master regulators direct expression of *KNAT7* as well as of genes encoding enzymes required for the biosynthesis of the cellulose, hemicellulose and lignin components of the cell wall (Schuetz, et. al., 2013). *KNAT7* is expressed co-ordinately with genes involved in secondary wall biosynthesis in young Arabidopsis seedlings as well as in inflorescence stems, with maximum levels of its expression observed at the base of the inflorescence stem (Brown et. al., 2005, Persson et. al., 2005, Ehlting et. al., 2005). *KNAT7* acts as a transcriptional repressor in transient protoplast transactivation assays (Li et. al., 2012; Liu et. al., 2014). Inflorescence stems in *knat7* loss-of-function mutants show thicker interfascicular fiber cell walls while overexpression lines show thinner walls, and the amount of lignin at the bases of mature *knat7* stems is increased (Li et. al., 2012; Liu et. al., 2014). These properties as well as the position of *KNAT7* in the secondary wall regulatory network lead to the proposal that *KNAT7* functions in a negative regulatory module to fine-tune resource allocation to secondary cell walls in the fiber cells of inflorescence stems (Li et. al., 2012; Liu et. al., 2014).

Data presented in Chapter 3 and from the literature (Harmer et. al., 2000; Rogers et. al., 2005; Pan et. al., 2009) indicate that several lignin biosynthetic genes show diurnal variations in expression levels in response to factors such as light and an internal circadian oscillator. Massive

amounts of carbon derived from photosynthesis are deposited into the secondary cell walls over the course of development. During the day photosynthate is assimilated and transported as sucrose, while in the night starch reserves are utilized in a well-regulated manner so that enough reserves are available at the end of night, in accordance with day-length (Blasing et. al., 2005). Almost 30% of these photosynthetic resources are driven into the phenylpropanoid branch of the shikimate pathway which eventually leads to biosynthesis of the monolignols incorporated into lignin (Maeda and Dudareva, 2012). In this chapter, I tested the hypothesis that a role of the *KNAT7* repressor transcription factor is the diurnal regulation of lignin biosynthetic genes in coordination with diurnally varying carbon availability over a 24 h light and dark cycle. If *KNAT7* is involved in diurnal regulation, it was expected that transcript abundance of lignin genes in seedlings and stems of a *knat7* loss-of-function mutant would show changes in diurnal expression variation as compared to wild-type expression patterns.

4.2 Results

4.2.1 Temporal expression patterns of the *KNAT7* gene in wild-type seedlings and stems

Core clock component genes in Arabidopsis show diagnostic peaks in expression once every 24 h cycle. Expression is entrained by environmental cues and persists in constant conditions such as continuous light (CL) exposure (Hsu and Harmer, 2014). I used qRT-PCR to determine if *KNAT7* temporal expression showed any such trends in wild-type Arabidopsis seedlings. In long day (LD) grown seedlings, a relatively stable pattern of *KNAT7* expression was observed over the 24 h cycle, no diagnostic peaks similar to other clock genes was observed. Transfer to CL however resulted in a peak in expression at 8 h after subjective dawn and a drop at 28 h (Fig. 4-1). Hence *KNAT7* expression levels seem to be responsive to transfer to

continuous light conditions in seedlings, suggesting that photoperiod sensing may be relevant to *KNAT7*'s physiological function.

I carried out a similar experiment under LD conditions using the lower 10cm of mature wild-type stems. Any potential diurnal trends in expression were obscured by large biological variation between samples (Fig 4-2). However, tendency towards higher expression of *KNAT7* in the day and lower expression in the nights seemed to be present consistently on both days of the experiment.

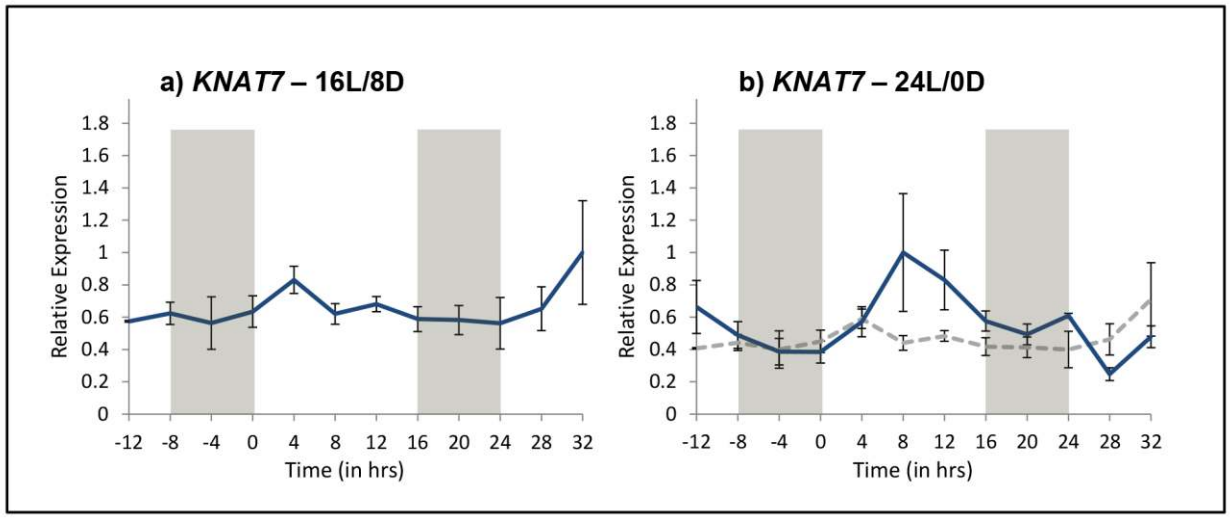

Figure 4-1 Temporal expression patterns of *KNAT7* in wild-type seedlings grown in LD and CL.
7-8 day old Col-0 seedlings grown on agar plates were assayed. (a) LD (16L/8D entrained seedlings) (b) LD-grown seedlings transferred to CL (24L/OD) at (-)8 h. Dotted lines indicate expression levels from (a) for comparison. Shaded regions indicate night or 'subjective' night. Maximum expression levels in LD samples were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

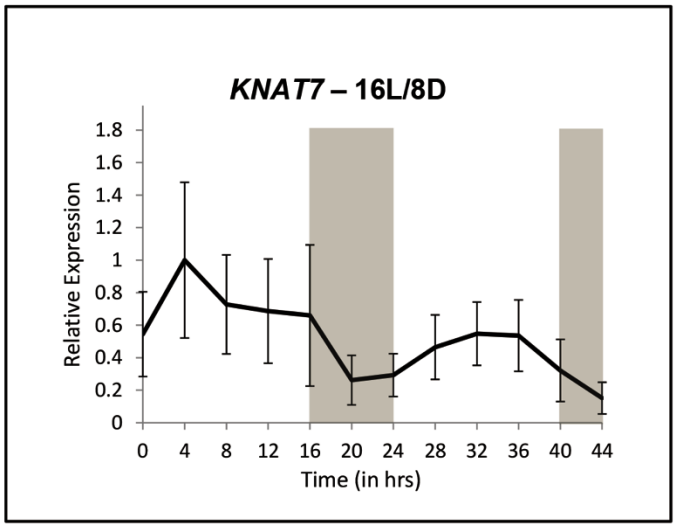

Figure 4-2 Temporal expression pattern of *KNAT7* in wild-type inflorescence stems grown in LD.
The lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Shaded regions indicate night. Maximum expression levels were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates

4.2.2 Temporal expression patterns of *LHY* and *DHS1* genes in *knat7* seedlings and stems

No change in *LHY* expression pattern was observed in *knat7* seedlings (Fig. 4-3a) or inflorescence stem bases (Fig 4-4a) relative to wild type, as expected, since all previous reports implicate *KNAT7* in the control of secondary cell wall biosynthesis, which would be downstream of clock regulation, controlled by *LHY*.

In *knat7* seedlings grown in LD conditions, *DHS1,* the evening phased, clock regulated, shikimate pathway gene, showed consistently lower expression levels during the day (4 h-16 h) on both days of study. In the night *DHS1* levels were unchanged (Fig. 4-3b). However, variability between samples made it difficult to discern consistent statistically significant differences. To gain statistical power, I pooled expression data from light time points of the first and second 24 h periods (Day 1, 4 h, 8 h, 12 h, and 16 h time points; Day 2, -12 h, -8 h, 28 h and

32 h time points) and similarly pooled data from dark time points of the first and second 24 h periods (Night 1, Night 2). The results from this are shown in Fig. 4-5a and *DHS1* consistently showed decreased expression in *knat7* in the day but not in the night, while *LHY* expression was unchanged in *knat7* after performing a similar analysis (data not shown). In *knat7* stems, no change in *DHS1* expression was observed relative to wild type levels at a $p \leq 0.05$ threshold of statistical significance (Fig 4-4b).

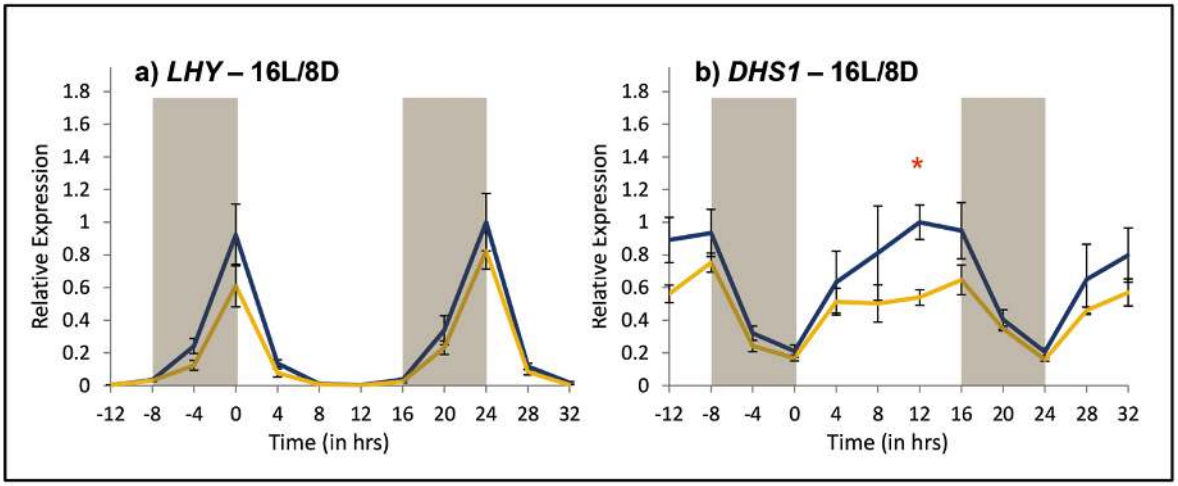

Figure 4-3 Temporal expression patterns of *LHY* and *DHS1* in wild-type and *knat7* seedlings grown in LD.
Blue line, wild-type seedlings (data from Chapter 3); yellow line, *knat7* seedlings. 7-8 day old seedlings grown on agar plates were assayed. Shaded regions indicate night. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates. Asterisk indicates a significant difference at $p \leq 0.05$.

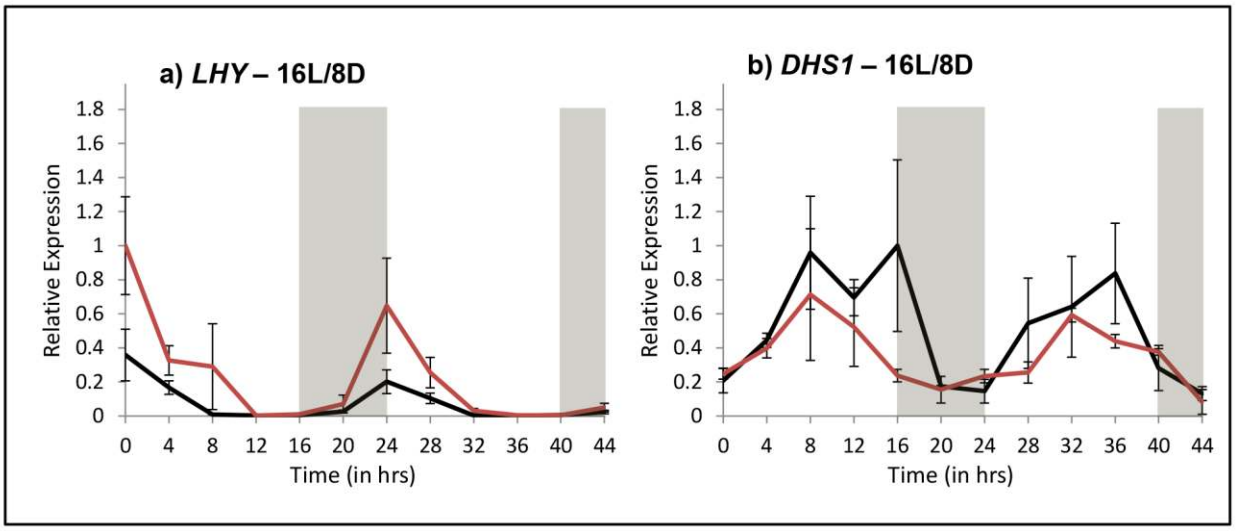

Figure 4-4 Temporal expression patterns of *LHY* and *DHS1* in wild-type and *knat7* inflorescence stems grown in LD.

Black lines, wild type (data from Chapter 3). Maroon lines, *knat7*. Lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Shaded regions indicate night. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates.

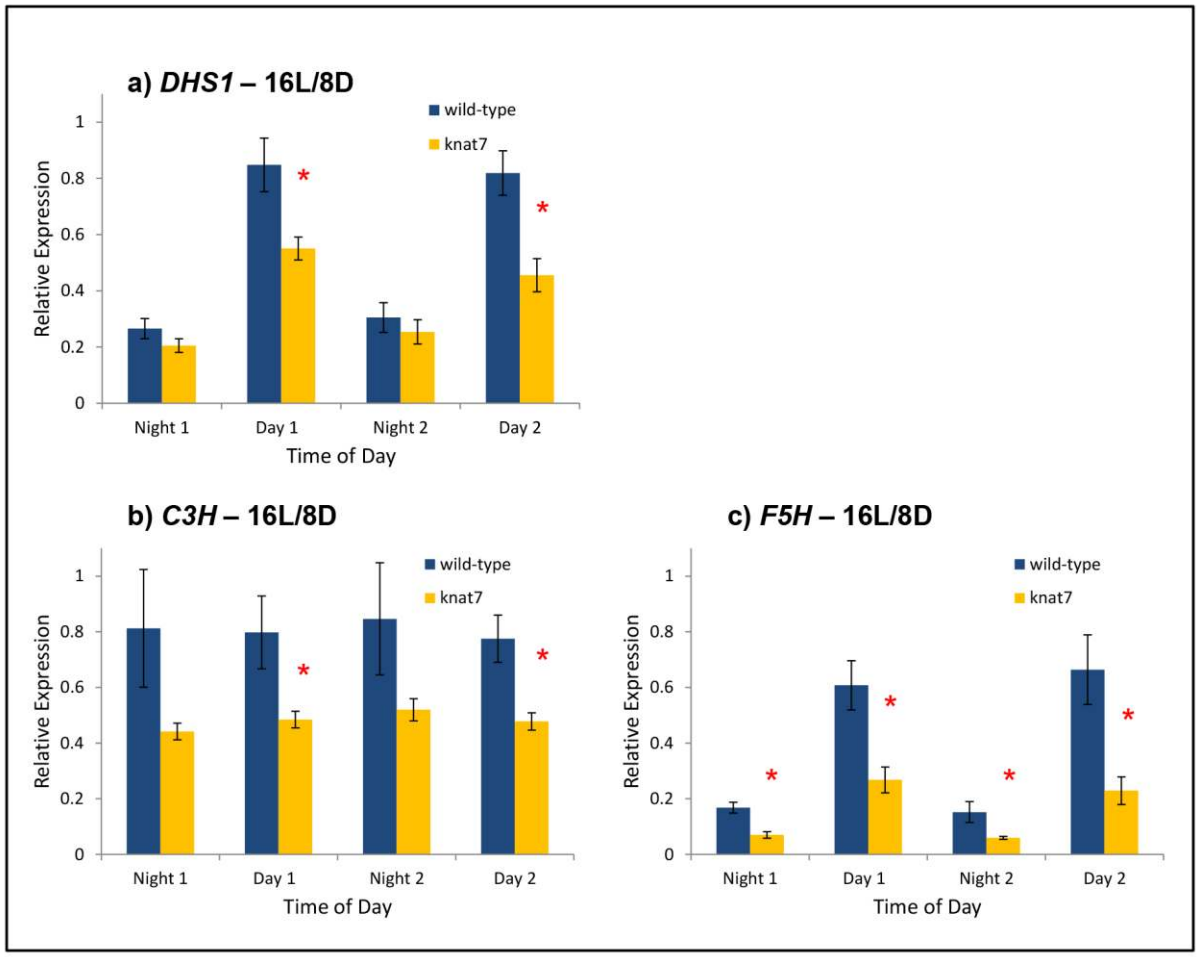

Figure 4-5 Expression of *DHS1*, *C3H* and *F5H* in the day and night in wild-type and *knat7* seedlings grown in LD.

Day 1: Data from 4 h, 8 h, 12 h, and 16 h time points were pooled; Day 2: Data from -12 h, -8 h, 28 h, 32 h time points were pooled; Night 1: Data from -4 h, 0 h time-points were pooled; Night 2: Data from 20 h, 24 h time points were pooled. 7-8 day old seedlings grown on agar plates were assayed. Bars indicate SE of 3 biological replicates at each time point. Asterisk indicates a significant difference at $p \leq 0.05$.

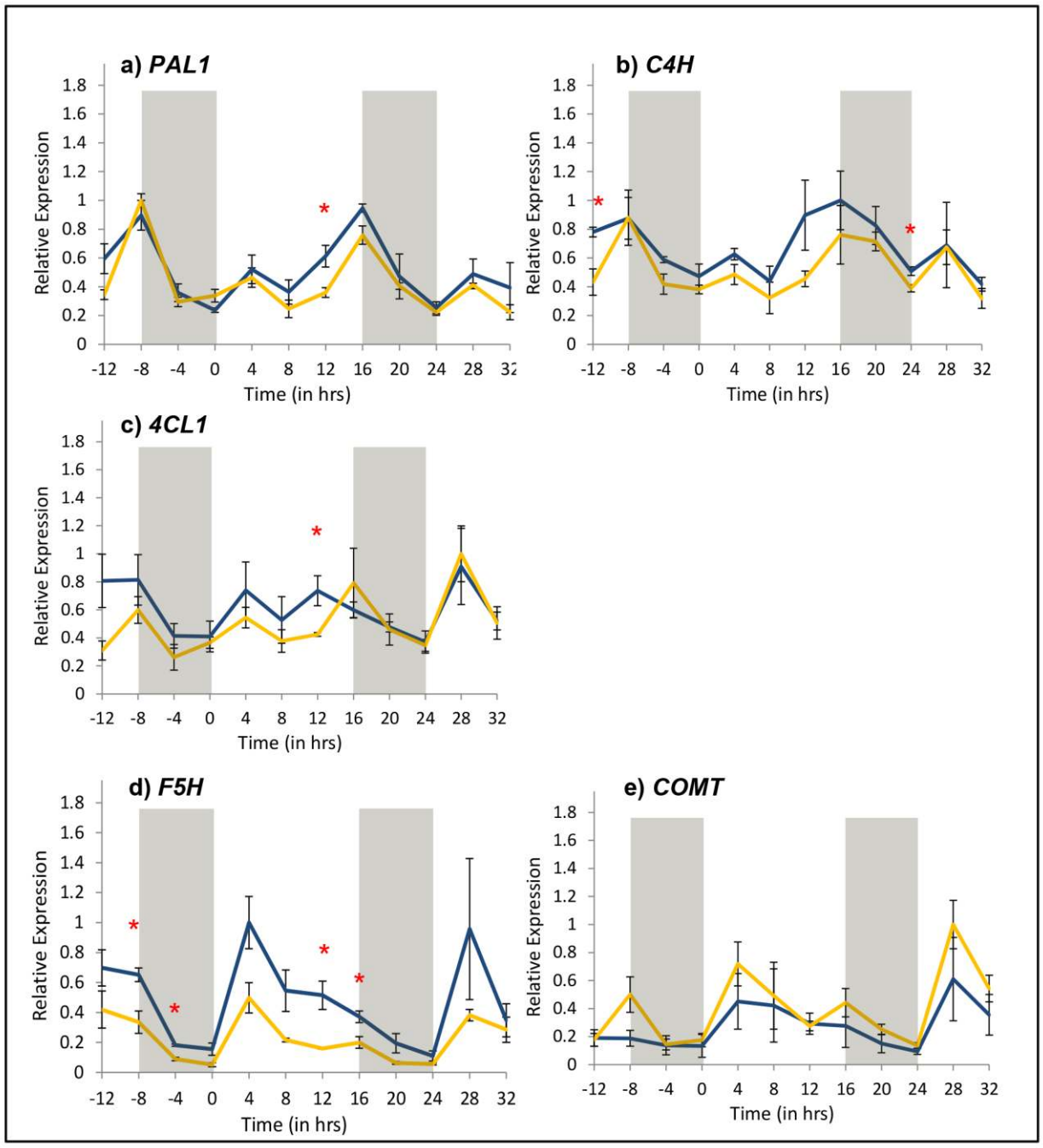

Figure 4-6 Temporal expression patterns of *PAL1, C4H, 4CL1, F5H* and *COMT* in wild-type and *knat7* seedlings grown in LD.

Blue lines, wild type (data from Chapter 3). Yellow lines, *knat7*. 7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate night. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates. Asterisk indicates a significant difference at $p \leq 0.05$.

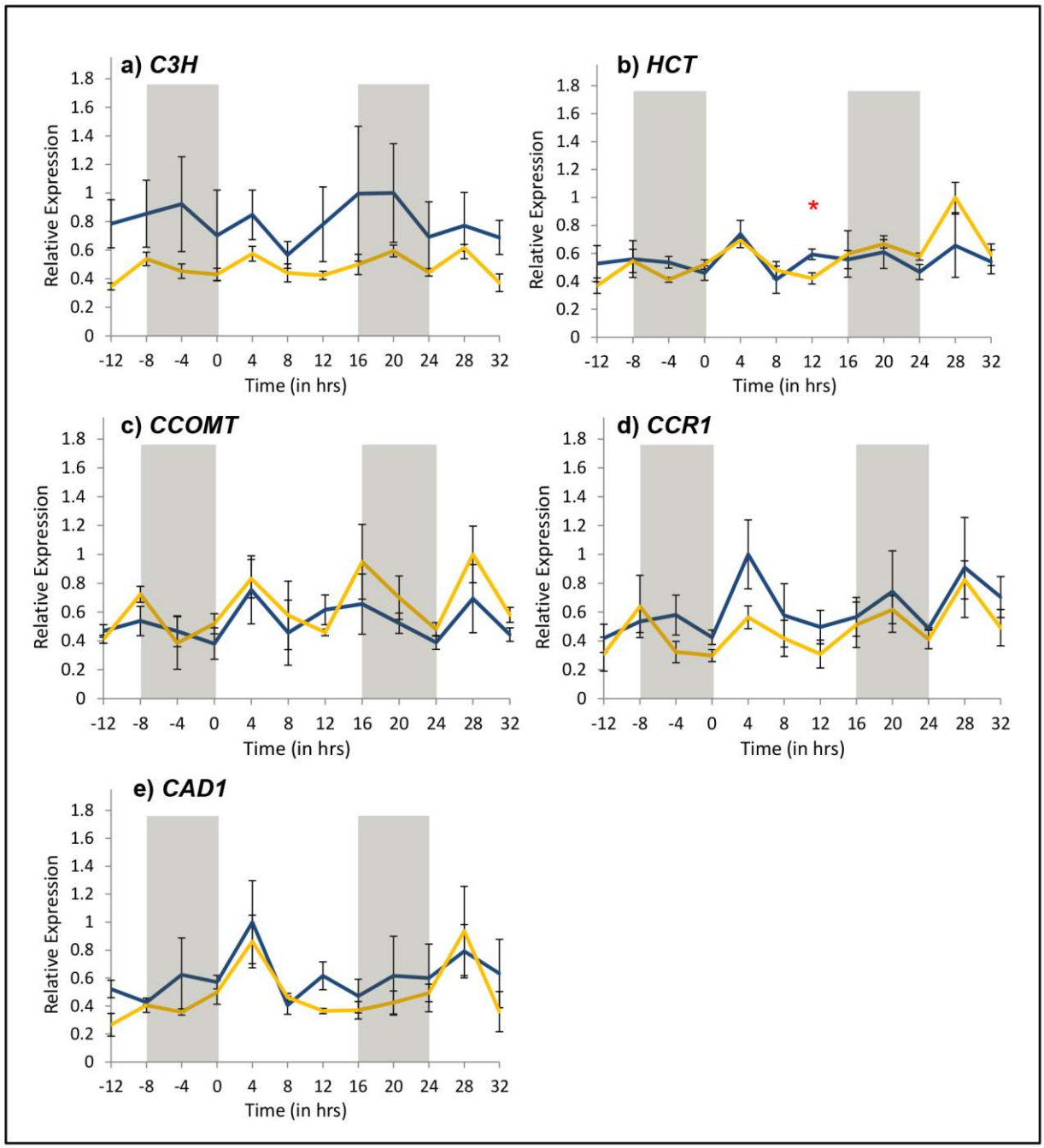

Figure 4-7 Temporal expression patterns of *C3H, HCT*, *CCOMT*, *CCR1* and *CAD1* in wild-type and *knat7* seedlings grown in LD.

*B*lue lines, wild type (data from Chapter 3). Yellow lines, *knat7*. 7-8 day old Col-0 seedlings grown on agar plates were assayed. Shaded regions indicate night. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates. Asterisk indicates a significant difference at $p \leq 0.05$.

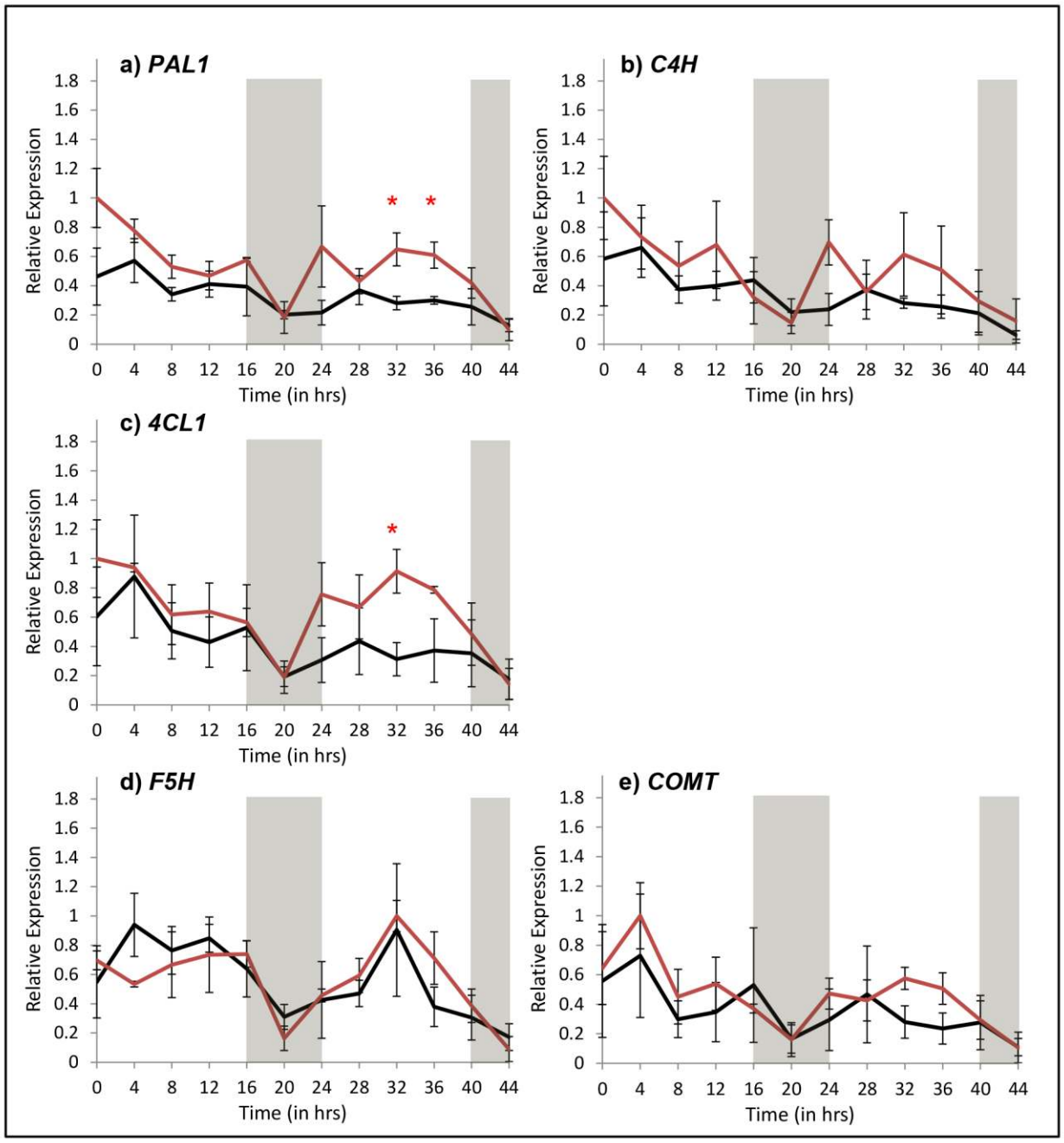

Figure 4-8 Temporal expression patterns of *PAL1, C4H, 4CL1, F5H* and *COMT* in wild-type and *knat7* inflorescence stems grown in LD.

Black line, wild type (data from Chapter 3); Maroon line, *knat7*. The lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates. Asterisks indicate a significant difference at $p \leq 0.05$.

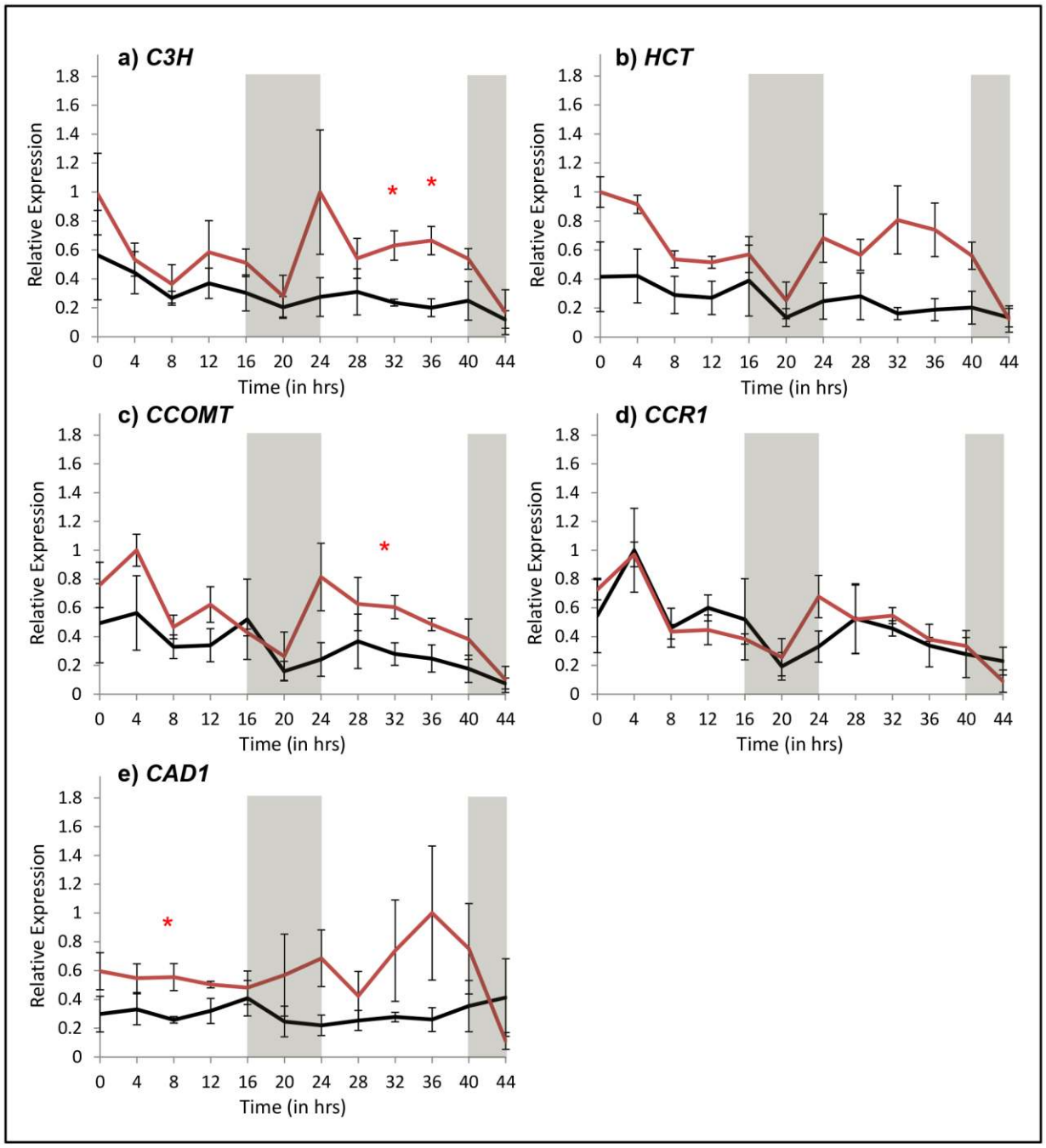

Figure 4-9 Temporal expression patterns of *C3H, HCT, CCOMT, CCR1* and *CAD1* in wild-type and *knat7* inflorescence stems grown in LD.

Black line, wild type (data from Chapter 3); Maroon line, *knat7*. The lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Maximum expression levels in wild-type were set to '1' and expression at all other time points are relative to this value. Bars indicate SE of 3 biological replicates. Asterisks indicate a significant difference at $p \leq 0.05$.

4.2.3 Temporal expression patterns of lignin biosynthetic genes in *knat7* seedlings and stems

Next I assayed LBG expression levels in LD grown *knat7* seedlings to test for changes from the wild-type diurnal patterns previously described in Figs. 3-2 and 3-4.

For most LBGs, no consistent change in diurnal expression pattern, over the two days of the study, was observed in *knat7* (Figs. 4-6, 4-7). However, *F5H* and *C3H* showed some evidence for decrease in gene expression over the diurnal cycle in *knat7* relative to wild type.

The pooling of samples strategy described for *DHS1* in the previous section was applied to LBGs. As shown in Fig. 4-5, *F5H* expression was significantly lower in *knat7* than in wild type in all four of the data pools, while *C3H* expression was significantly lower in the Day 1 and Day 2 pools than in wild type. Thus *KNAT7* does act to regulate overall expression levels of at least some LBGs in seedlings and may also play a role in diurnal regulation of expression of C3H in LD grown seedlings. Expression of *F5H* and *C3H*, two of the three rate-limiting cytochrome P450 genes, seems to be affected in *knat7* seedlings grown in LD.

Next I assayed the diurnal expression patterns of LBGs in the lower 10cm of 5-week old *knat7* inflorescence stems, where the *knat7* secondary cell wall phenotype has been described (Li et. al., 2012; Liu et. al., 2014). Relative to wild type, in *knat7*, overall expression levels of many LBGs were increased (Figs. 4-8, 4-9) consistent with earlier reports from single time-point experiments (Li et. al., 2012; Liu et. al., 2014).

Unlike results from seedlings, it was observed that temporal expression patterns of LBGs in *knat7* stems were not consistent on both days of the experiment, and variation in expression levels between biological replicate samples made it difficult to identify statistically significant diurnal changes in gene expression in *knat7* stems.

Yet, I observed that some LBGs, especially those encoding enzymes for general phenylpropanoid metabolism at the start of the pathway (*PAL1*, *C4H*, *4CL1*) and some enzymes required for G and S monolignol biosynthesis (*C3H*, *HCT*, *CCOMT*) showed larger variations in expression over the day as compared to the wild-type levels. To represent this, I pooled expression data from all time points, for a given gene, and prepared a box-plot representing the spread of the expression data over the two days of the experiment (Fig 4-10). Maximum expression of many LBGs over the two day period, represented by the top edge of the bar, and median expression value, represented by the centre of the box, was higher in *knat7* stems. Further, the difference between maximum and minimum values, represented by the range between the bars, was also greater for many LBGs in the *knat7* background, indicating that greater variation in expression of the LBGs, over the two days, occurred in the absence of *KNAT7* function. *CAD1, C3H, HCT* and *CCOMT* showed the largest increase in the range of transcript abundance values of the respective genes in *knat7* stems.

4.2.4 Temporal expression patterns of lignin biosynthetic genes in a second replicate set of *knat7* stems

In the experiments described in the previous chapter, LBG transcript levels in inflorescence stems of wild-type plants grown in LD conditions were generally at a minimum in the night, with low levels persisting at dawn. However in in *knat7* stems, many LBGs showed a trend of increased transcript levels at dawn, relative to wild type (Figs. 4-8, 4-9). This trend was also observed on the second day of the experiment and was seen in all genes except for *F5H* and *COMT*.

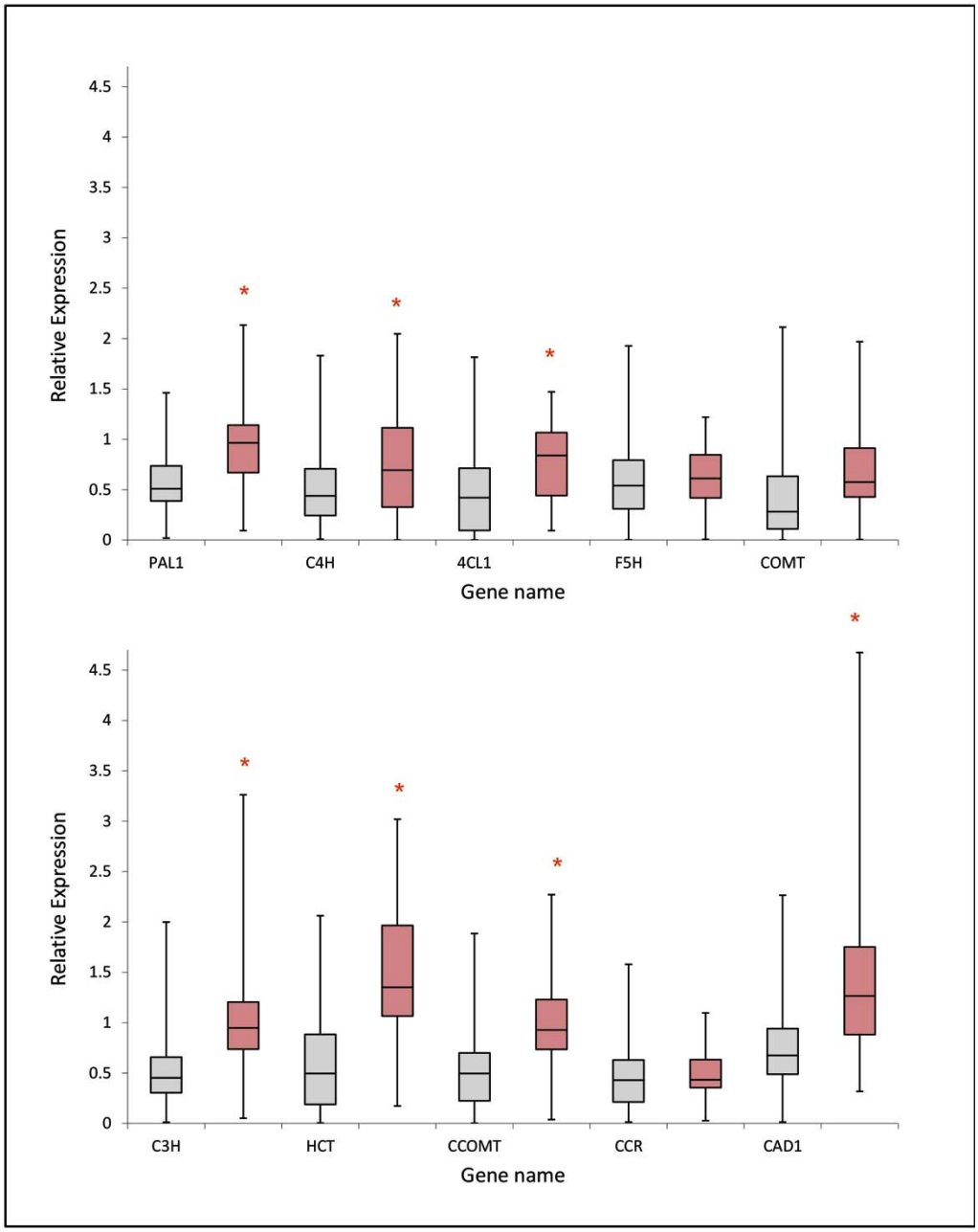

Figure 4-10 Spread of LBG expression data over 48 h in wild-type and *knat7* inflorescence stems grown in LD.

Grey, wild type; Maroon, *knat7*. The lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Expression data is the same as used in Fig 4.8, 4.9. Bars indicate maximum and minimum expression. Boxes represent upper quartile, median and lower quartile of all expression data at the 48 h time point for a given gene. Asterisk indicates a significant difference between average expression at $p \leq 0.05$ for a given gene.

Apparent biological variability made it difficult to draw definitive, statistically significant conclusions about this trend. Thus, I repeated the experiment with a second collection of stem samples and assayed expression of selected LBGs (the cytochrome P450 genes, *HCT*, *CCOMT* and *COMT*) as well as of *LHY* and *DHS1*, only at the crucial 0 h and 24 h dawn time points. The three biological replicates for each of these two time points were pooled to increase statistical power, and the average expression values in *knat7* stems were compared to wild-type levels in a combined "dawn" dataset for the two days.

Fig. 4-11 shows the results of this second experiment. Similar to the first stem collection, *LHY* and *DHS1* did not show statistically significant differences in expression levels at the dawn time-point. However, significantly increased expression was observed for *C3H, HCT, CCOMT* and *COMT* confirming trends from the first stem experiment for *C3H, HCT,* and *CCOMT*. In the first experiment analysing expression in inflorescence stems, *F5H* and *COMT* did not show a trend of higher expression at dawn relative to wild-type stems. However in this second stem experiment *F5H* showed significantly decreased expression, while *COMT* showed increased expression (Fig. 4-11). Overall, these data support a role for *KNAT7* in repressing the expression of certain LBGs during the night, suggesting that *KNAT7* may be required to ensure physiologically appropriate reduced expression levels at the end of the night. *KNAT7* does not regulate diurnal expression of all LBGs in the phenylpropanoid pathway in the same manner, a trend seen in *knat7* stems and seedlings.

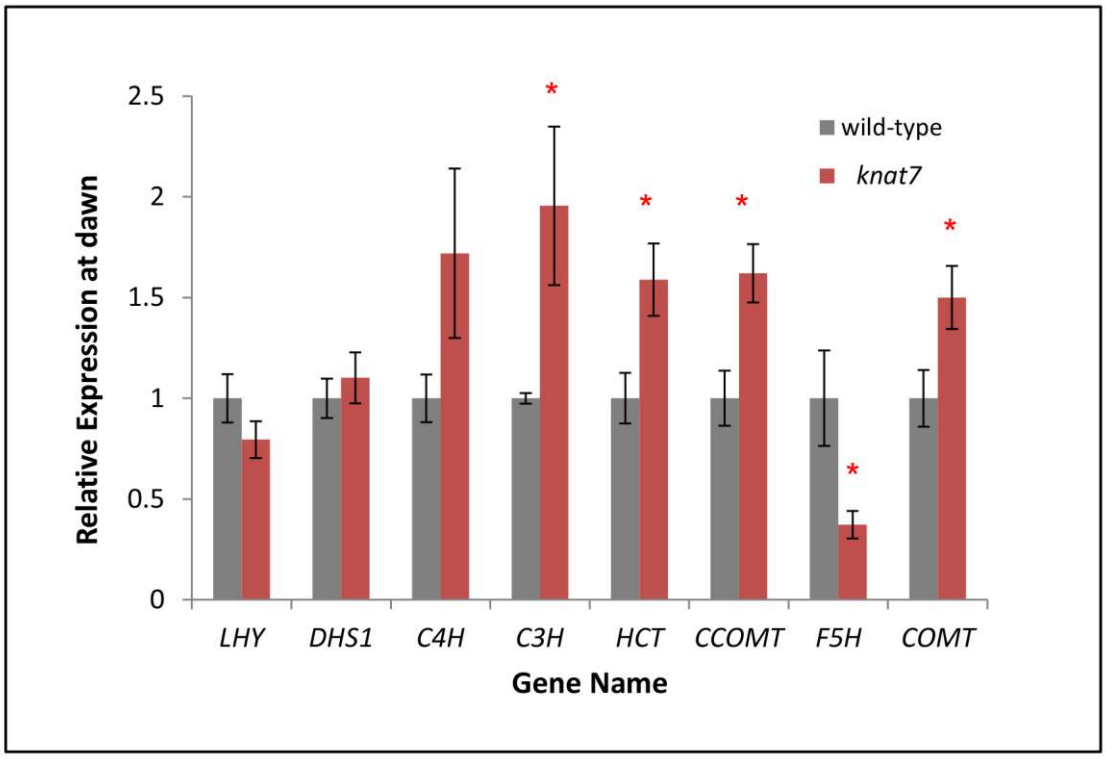

Figure 4-11 Expression of *LHY, DHS1, C4H, C3H, HCT, CCOMT, F5H* and *COMT* at dawn in a second stem collection set of wild-type and *knat7* inflorescence stems grown in LD.
Gray bars, wild type; Maroon bars, *knat7*. The lower 10cm of inflorescence stems of 5 week old plants grown on soil were assayed. Expression data from two sets of samples, at the 0 h and 24 h time points ("dawn"), were pooled. Expression levels of each gene in wild-type were set to '1' and expression in *knat7* is relative to this value. Bars indicate SE of 6 biological replicates. Asterisks indicate a significant difference at $p \leq 0.05$.

4.2.5 Phenotypic analysis of *KNAT7* mutants

My data on LBG expression in inflorescence stems in the *knat7* mutant suggest a connection between *KNAT7* and diurnal/circadian regulation. Given this apparent connection, it was of interest to determine if any clock-related phenotypes are present in the *knat7* mutant. Depending on the clock component altered, clock mutants develop either longer or shorter seedling hypocotyls relative to wild type, and exhibit either accelerated or delayed timing of floral transition, as assayed from bolting of the inflorescence stem (McClung, 2006).

To test for possible clock-related phenotypes in *knat7*, I measured hypocotyl lengths of wild-type, *knat7* and *KNAT7* overexpression (*4CL:KNAT7*) seedlings grown in the dark (0 µmol $m^{-2}s^{-1}$) and at low (3 µmol $m^{-2}s^{-1}$), medium (12 µmol $m^{-2}s^{-1}$) and high (96 µmoles $m^{-2}s^{-1}$) light fluences. These results are shown in Table 4.1. In the dark, hypocotyls of *knat7* were significantly more elongated compared to wild-type, while the overexpression line *4CL:KNAT7* showed shorter hypocotyls. A similar result was observed at the low fluence light intensity. In medium fluence light only *knat7* hypocotyls were significantly longer, and in high fluence light only *4CL:KNAT7* hypocotyls were significantly shorter than wild-type. Repetition of this experiment gave similar results (Table 4.2).

Since flowering time is also clock-controlled, I tested days to bolting (counted from the day seeds were transferred to light and temperature conducive to germination). Within the populations of plants tested, *knat7* plants bolted later than wild-type plants while *4CL:KNAT7* bolted later than both wild-type and *knat7* plants (Table 4.2).

Thus *KNAT7* mutants show altered hypocotyl growth and bolting time; phenotypes that commonly accompany mutations in genes required for clock regulation.

Table 4-1 Hypocotyl lengths in wild type *knat7* and *4CL:KNAT7* seedlings (Replicate 1).

	Hypocotyl Length (cm)			
Genotype	0 µmol $m^{-2}s^{-1}$	3 µmol $m^{-2}s^{-1}$	12 µmol $m^{-2}s^{-1}$	96 µmol $m^{-2}s^{-1}$
Wild-type	0.856 ± 0.027 [1]	0.265 ± 0.015	0.148 ± 0.006	0.130 ± 0.005
knat7	1.002 ± 0.030* [2]	0.332 ± 0.017*	0.169 ± 0.006*	0.123 ± 0.003
4CL:KNAT7	0.727 ± 0.028*	0.225 ± 0.012*	0.147 ± 0.006	0.108 ± 0.004*

[1] ± Standard Error, n = 24 hypocotyls for each condition.

[2] Asterisks indicate values significantly different from wild-type at $p \leq 0.05$.

Table 4-2 Hypocotyl lengths in wild type *knat7* and *4CL:KNAT7* seedlings (Replicate 2).

	Hypocotyl Length (cm)			
Genotype	0 µmol $m^{-2}s^{-1}$	3 µmol $m^{-2}s^{-1}$	12 µmol $m^{-2}s^{-1}$	96 µmol $m^{-2}s^{-1}$
Wild-type	1.055±0.081 [1]	0.373±0.041	0.173±0.023	0.121±0.011
knat7	1.169±0.095* [2]	0.409±0.036*	0.212±0.042*	0.144±0.013*
4CL:KNAT7	1.026±0.095	0.318±0.049*	0.145±0.016*	0.112±0.010*

[1] ± Standard Error, n = 16 hypocotyls for each condition.

[2] Asterisks indicate values significantly different from wild-type at $p \leq 0.05$.

Table 4-3 Days to bolting in wild type *knat7* and *4CL:KNAT7* plants.

Genotype	Days bolting
Wild-type	29.5±0.2[1]
knat7	30.6 ± 0.3*[2]
4CL :KNAT7	30.9±0.5*

[1] ± Standard Error, n = 56, 62 and 65 wild-type, *knat7* and *4CL:KNAT7* plants respectively.

[2] Asterisks indicate values significantly different from wild-type at $p \leq 0.05$.

4.3 Discussion

In this chapter I tested the hypothesis that the transcriptional repression activity of *KNAT7* modulates the rhythmic diurnal expression patterns of LBGs. My approach was to build on the results presented in Chapter 3 to test for changes in LBG expression patterns, especially changes that are consistently observed on the two days of the study, in the *knat7* mutant background relative to wild-type. I found little evidence to support this hypothesis in seedlings. However, in inflorescence stems that have a greater commitment to lignin deposition, especially in interfascicular fibers, I uncovered a possible role for *KNAT7* in repressing expression of certain LBGs in the night. Consistent with a role for *KNAT7* in clock regulation, *knat7* mutants showed hypocotyl elongation and time to bolting phenotypes that commonly accompany mutations in core clock genes.

In LD grown *knat7* seedlings, transcript levels of LBGs did not show a significantly different value at any time point consistently on both days of the study. However many showed a trend of overall lower expression level throughout the day (Figs. 4-6, 4-7). This trend in LGB

expression, and the significant decreases in expression of *C3H* and *F5H* in the *knat7* mutant (Fig. 4-5) is in contrast to the reported increase in expression of many of these genes in *knat7* inflorescence stems relative to wild type (Liu et. al., 2014).

When I assayed LBG expression in *knat7* inflorescence stems (Figs. 4-8, 4-9), however, expression levels were generally higher than wild type over the course of the diurnal cycles, consistent with the reported increases in gene expression levels in *knat7* (Liu et. al., 2014). One explanation for these results is that inflorescence stems are enriched in fiber cells, whose secondary cell walls are thicker in the *knat7* mutant (Li et. al., 2012; Liu et. al., 2014), while the vascular bundles of seedlings lack this cell type. Thus, the repression function of *KNAT7* with respect to LBG expression may target LBGs in interfascicular fiber cells and not vessels, such that the repression function is not obvious in seedlings.

In LD grown *knat7* stems, in the first stem collection, expression of many LBGs appeared to be changed in the 0 h and 24 h dawn sample, mostly genes in the general phenylpropanoid pathway and genes important for G monolignol biosynthesis (Figs. 4-8, 4-9). However because of large biological variation in the stem samples, diurnal pattern change trends were not statistically significant, even though the trend of increased expression at dawn in *knat7* was observed on both days of the study. A second set of stem samples assayed at the dawn time point, with double the number of biological replicates gave more promising results. Out of the genes *C4H, C3H, HCT, CCOMT, F5H, COMT* that were assayed *C3H, HCT*, *CCOMT and COMT* showed significantly increased expression while *F5H* showed decreased expression. Hence it appears that *KNAT7* regulates diurnal expression levels of several LBGs at the end of night in stems. Stored starch levels are depleted towards the end of night and it is possible that

KNAT7 plays a role in regulating LBG expression in co-ordination with this resource availability signal.

Another observation from assaying LBG expression in *knat7* stems was that greater variation in expression levels over the day was observed as compared to the minimal variation in LBG expression in wild-type stems (Fig. 4-10). *C3H* shows the least diurnal variation in LD grown wild-type seedlings and shows minimal diurnal variation, like the other LBGs in wild-type stems (Figs.3-4, 3-8). However, in *knat7* stems, the variation in expression values over the day was very large. In *knat7* seedlings overall lower expression of *C3H* was observed and expression was lower in pooled daytime samples but was unchanged in pooled night-time samples on both days of the study (Fig. 4.5b). In both the first and second *knat7* stem collection increased expression of *C3H* at dawn was observed. This suggests that *KNAT7* may have a more important role to play in the regulation of this gene. *F5H,* another one of the rate-limiting cytochrome P450 monooxygenase genes, shows overall decreased expression in *knat7* seedlings, overall unchanged expression in the first *knat7* stem collection and decreased expression at dawn in the second *knat7* stem collection. Clearly knockout of *KNAT7* does not affect expression of all the phenylpropanoid genes in the same manner.

Further, in both *knat7* seedlings and stems, I observed significantly different peaks in expression at 32 h and/or 36h after dawn, for some genes. Harmer et. al., 2000 showed that diurnally regulated sucrose transport and metabolism genes and starch metabolism genes show peaks in expression levels at discrete times in the day, as would be necessary for their function. Some of these genes peak at 8 h after subjective dawn in 12L/12D grown plants transferred to continuous light, while some genes peak at subjective night. The mid-day change in expression of LBGs, along with the changed expression at dawn, in LD grown *knat7* plants, may be

physiologically relevant to indicate misregulation of sucrose metabolism signals. But since the mid-day expression change is not consistently observed on both days of the study, it is possible that this may be an experimental artifact caused by perhaps a factor like unwanted light exposure during sample collection on the second night of the study.

In the *knat7* background, *LHY* expression in LD grown seedlings and stems was mostly unchanged in diurnal fluctuations in transcript abundance relative to wild-type samples grown in parallel. However an unexpected change in diurnal expression of *DHS1* was observed in the *knat7* seedling samples but not in the *knat7* stem samples. The shikimate pathway gene *DHS1* can be regulated by dawn-phased clock repressors like *LHY* and *CCA1* hence was included in this study to confirm presence of a functional clock in plant samples. While a consistent change at any particular time point was not observed in *DHS1* diurnal regulation in seedlings in *knat7* relative to wild type, I observed a decrease in *DHS1* expression in the day when data from light time points were pooled. This suggests that in seedlings, *KNAT7* positively regulates *DHS1* expression in the day.

DHS1 expression in wild-type plants is induced in response to wounding and pathogen stress (Keith et. al., 1991). DHS is the first enzyme in the shikimate pathway and is encoded by a gene family in Arabidopsis that includes *DHS1*, *DHS2* and *DHS3.* Among the three *DHS* genes, clock regulation of only *DHS1* has been studied. *DHS3* expression is highest in the second internode of the inflorescence stem, while *DHS1* has a more widespread expression with highest levels in the vegetative rosette (Arabidopsis eFP browser, Winter et. al., 2007). Based on expression profile, *DHS3* may be more important in driving carbon flux into the lignin biosynthetic pathway and is more likely than *DHS1* to be regulated by *KNAT7* with respect to secondary cell wall related functions. If *KNAT7* diurnal regulation functions extend to the

shikimate pathway this may explain why *DHS1* does not show any clear changed diurnal regulation in *knat7* stems (Fig. 4-4) even though greater diurnal effects on expression of the LBGs were observed in the stem tissue.

Vanholme et. al., 2012 found that shikimate and phenylpropanoid pathway genes are generally co-ordinately regulated at the metabolite level in mutants that result in lignin defects. Global expression profiling from top to bottom of the Arabidopsis inflorescence stem by Ehlting et. al., (2005) indicated that genes encoding enzymes of the shikimate pathway are up-regulated but that the parts of the pathway specific for other amino acids are not differentially expressed at the bottom of the stem. This provides another line of evidence to suggest that the shikimate and phenylpropanoid pathway genes may be regulated by common mechanisms. A thorough analysis of shikimate pathway genes in *KNAT7* mutants may help to identify if *KNAT7* plays a role in such a common regulatory mechanism.

The *knat7* seedlings also showed a *hy* (elongated hypocotyl) phenotype when grown in the dark or at low fluence rates. Clock gene mutants such as *cca1* and *lhy* show short hypocotyls while overexpressors of *CCA1, LHY* and *rve8, elf3, prr7, prr9* show elongated hypocotyls (Nagel and Kay, 2012). In conjunction with traits such as mis-expression of *CAB2* and *CCR2* clock output genes, this phenotype is used to characterise clock mutants. Normal hypocotyl elongation is a complex process that requires co-ordinated regulation of cell wall biosynthetic enzymes as well as cell wall modifying enzymes by light, circadian and phytohormone signalling pathways. The presence of the *hy* phenotype in the dark or only at low fluence light intensities is similar to the trends observed for the *RVE8* knock-out and overexpressor mutants (Rawat et. al., 2013). This phenotype is also similar to the trends shown by some mutants defective in phtochromeA mediated, low fluence light response (Yanovsky et. al., 2000). The role of *KNAT7* in regulation

of cell wall components apart from lignin is not clear; hence it is not clear what causes the *hy* phenotype in *knat7*. However, these results are consistent with a link between *KNAT7* and the clock.

Another complex phenotype used to characterise clock mutants is flowering time. Time to bolting is delayed in both *knat7* and the overexpressor mutant *4CL:KNAT7*. Clock mutants like *cca1, lhy, rve8, elf3* show early flowering while overexpressors of *CCA1, LHY*, *prr7, prr9* and *ztl* show delayed flowering (Nagel and Kay, 2012). Arabidopsis is a facultative long day plant, flowering more rapidly in long days than in short days. Many clock mutants lack photoperiodic sensing and hence flower at the same time in long day and short day conditions. These results further suggest a link between *KNAT7* and the clock, but bolting time phenotype of the *knat7* mutant should be studied in additional photoperiods.

If *KNAT7* is a diurnal regulator, it is possible that the expression of *KNAT7* itself may show diurnal rhythmicity. However, in LD-grown wild-type seedlings, stable expression of *KNAT7* throughout the day was observed (Fig. 4-1a). Clock related genes such as *LHY* show large diurnal variations in transcript abundance that correlate with their biological activity in modulating clock-regulated target genes. Thus, an open question is how *KNAT7* achieves its apparent function in diurnal regulation in the absence of diurnal variation in transcript abundance. The *KNOX* family genes that *KNAT7* is a part of perform their functions by interacting with specific *BLH* family protein partners (Hake et. al., 2004). KNAT7 interacts with BLH6 to regulate secondary cell wall formation and requires BLH6 interactions to repress *REV* (Liu et. al., 2014). *BLH6* turns up in a list of top 200 genes that are induced in response to light (Blasing et. al., 2005). OFP1, OFP4 and MYB75 also interact with KNAT7 (Li et. al., 2011,

Bhargava et. al., 2013). Interactions with any of these proteins may enable *DHS1* and LBG diurnal regulation.

In CL-grown wild-type seedlings I observed a change in the *KNAT7* expression pattern as compared to the LD photoperiod grown seedlings (Fig. 4-1b). It is possible that in CL the level of *KNAT7* expression as well as interactions with protein partners is important for regulating downstream processes. An analysis of *DHS1* and LBG expression in CL-grown *knat7* could shed light of the significance of *KNAT7* expression levels in CL grown seedlings.

In summary, based of differences in expression of some LBGs at dawn in *knat7* stems relative to wild type, changed expression of *DHS1* in *knat7* seedlings, and hypocotyl and bolting time phenotypes of the *knat7* mutant, a link between diurnal regulation and *KNAT7* transcriptional activity can be drawn. KNAT7 may act as either an activator or repressor and selective interactions with other transcription factor proteins and a sucrose related regulatory signal may potentially enable KNAT7 to perform its diurnal regulation functions.

Chapter 5: **Conclusion and future directions**

A hierarchical feed-forward network of several transcription factors is responsible for developmental regulation of lignin biosynthesis in secondary cell walls in Arabidopsis plants. Several LBGs have also been identified to be diurnally regulated by environmental signals like light perception and an endogenous circadian clock. The goal of my thesis was to identify whether *KNAT7*, a component of the developmental secondary wall regulatory network, may function in the diurnal regulation of the transcription of LBGs.

In Chapter 3, I identified that a clear diurnal and circadian variation in transcript abundance of LBGs in observed in wild-type seedlings (Figs. 3-2, 3-3, 3-4, 3-5). In wild-type stems, very little diurnal variation in expression levels can be observed (Figs. 3-7, 3-8). Large inherent biological variations seem to be present in these samples; however genes like *F5H* show higher expression levels during the day as compared to in the night in the wild-type stem samples.

Rhythmic cycling of the *LHY* clock gene expression in LD grown wild-type seedlings (Fig. 3-1a), with a phase and waveform as expected from literature, validated my experimental setup; persistence of rhythmic cycling of *LHY* in CL (Fig. 3-1c) confirmed the presence of a functioning endogenous clock in seedlings. *DHS1,* a clock controlled gene, showed diurnal regulation in LD indicating that the seedling clock can modulate expression of downstream targets.

I identified robust oscillations in expression of *PAL1*, *C4H*, *4CL1*, *F5H* and *COMT* in LD grown wild-type seedlings while diurnal variation in *HCT, CCOMT, CCR1* and *CAD1* was less prominent in my growth conditions. *C3H* showed the least variation in expression levels over the day. Previous reports of diurnal expression of LBGs typically identified them as co-regulated.

The greater accuracy of qRT-PCR over microarray and northern blot studies previously used (Harmer et. al., 2000; Rogers et. al., 2005) revealed finer details of the expression of the genes I analysed. For example, I identified groups of LBGs that showed similar trends in expression and these groups tended to correlate with their positions in the phenylpropanoid pathway

PAL1, C4H and *4CL1* show an additional peak in expression in LD conditions as compared to *F5H* and *COMT*. The differences in transcript abundance of the genes in the general phenylpropanoid pathway as compared to the genes specific to S monolignol synthesis may reflect the differing requirements of the many secondary metabolites that are produced by the phenylpropanoid pathway. Also, we cannot rule out that lignin biosynthetic enzyme abundance does not reflect transcript levels because of post-transcriptional processes. For example, the PAL family of enzymes in Arabidopsis was shown to be negatively regulated at the protein level via the ubiquitination–26S proteasome pathway (Zhang et. al., 2013). However the goal of my study was to characterise transcription level trends in wild-type to compare against *knat7*.

Most LBGs showed variation in transcript abundance even when transferred to CL, indicating regulation at the transcript level by an internal circadian clock, in my growth conditions, as would be expected based on earlier studies. A peak at 4 h after dawn, observed for most lignin genes in LD grown seedlings, may indicate light regulated expression based on the fact that it is not observed in CL grown wild-type seedlings and a trough in expression in the middle of the day may represent circadian regulation of expression since this peak was present in both LD and CL grown seedlings. Both these characteristics of LBG expression can be mildly observed in northern blots assay performed by Rogers et. al., (2005). The peak at 23 h after dawn observed by Rogers et. al., 2005 cannot be accounted for because of restrictions placed by my sampling frequency. In mature inflorescence stem bases, the presence of a functional circadian

clock in LD conditions was identified by observing diurnal variation in expression levels of *LHY* and *DHS1* (Fig. 3-6), in spite of low variation in LBG levels observed in the same samples.

Next, in Chapter 4, I went on to study diurnal variation in LBG expression in *knat7* seedlings and stems. I identified that in seedlings diurnal transcript abundance variations of most of the studied LBGs persisted unchanged in the *knat7* mutant (Figs. 4-6, 4-7), suggesting that *KNAT7* is not involved in diurnal regulation of LBGs in this developmental stage. Some LBGs, showed decreased levels of expression throughout the day in this developmental stage, suggesting an activation function for *KNAT7* in the regulation of LBG expression in seedlings.

Although LBGs in *knat7* seedlings did not show changed diurnal expression, decreased expression of *DHS1* (Figs. 4-3b, 4-5a), a clock output gene, was observed in *knat7* only in the daytime, providing an interesting new avenue to further investigate *KNAT7*'s diurnal regulation functions and transcriptional activation functions. No evidence for involvement of *KNAT7* with *DHS* gene expression regulation has been reported in the past. DHS1 is the first enzyme in the shikimate pathway and evidence from literature suggests that shikimate and phenylpropanoid metabolites are controlled by common regulatory pathways over development and in secondary cell wall mutants (Ehlting et. al., 2005; Vanholme et. al., 2012). It would be interesting to explore the possibilities that *KNAT7* indirectly regulates lignin biosynthesis by modulating flux down the shikimate pathway or is part of the common feedback regulatory pathway that regulates both shikimate and phenylpropanoid metabolites.

Another line of evidence linking *KNAT7* to clock regulation in seedlings is the elongated hypocotyl phenotype (Table 4.1). Light, clock and phytohormone signals control hypocotyl elongation during early photomorphogenesis by regulating expression of the *PIF4 (PHYTOCHROME INTERACTING FACTOR 4)* and *PIF5* proteins (Niva et. al., 2007). Defects

in any of these three regulatory mechanisms of targets can cause hypocotyl elongation phenotypes.

Both *knat7* and *4CL:KNAT7* also show a delay in bolting time (Table 4.2). Clock-defective mutants almost exclusively display an altered phenotype of early or late flowering time, implying a key role for the circadian clock in the photoperiodic control of flowering time (Imaizumi and Kay, 2006). A certain minimum concentration of sucrose at the shoot apical meristem of the vegetative rosette is also required to signal the transition from vegetative to reproductive growth and bolting in Arabidopsis plants (Gibson, 2005). Either of these mechanisms or a combination of the two may be disrupted in *knat7* mutants. This clock phenotype is also milder in *knat7* than in other clock mutants, perhaps double knock-out mutants disrupted in function of *KNAT7* as well as other protein-protein interacting partners may show a more prominent phenotype, indicating involvement of the partner to facilitate *KNAT7*'s diurnal functions.

Thus, *knat7* shows two common clock related phenotypes, as well as altered diurnal regulation of *DHS1*, even before it reaches the developmental stage at which most secondary cell wall phenotypes have been previously reported in *knat7*.

Since *LHY* does not show changed expression in *knat7* seedlings (Fig. 4-3a) as well as in *knat7* stems (Fig. 4-4) it is also possible that *KNAT7* acts downstream or parallel to the core clock mechanism that requires appropriate *LHY* expression, explaining milder clock related phenotypes observed.

Finally, in tissue collected from the base of the mature Arabidopsis inflorescence stem, I observed evidence of *KNAT7's* diurnal regulation functions, acting on the LBGs, that are believed to be downstream targets of *KNAT7* based on previous reports.

Altered diurnal regulation of several studied LBGs was observed at the 'dawn' time point, as compared to wild-type expression levels, in two sets of independent experiments (Figs. 4-8, 4-9, 4-11). Large variations in expression between biological replicates from stem tissue made this trend difficult to identify, but pooling of time points to increase statistical power helped to observed statistically significant increased expression levels of several LBGs at dawn. *C3H, HCT* and *CCOMT* showed the change in transcript levels at dawn, as well as overall higher expression levels over the day, as compared to the wild-type expression level. *F5H* showed either the same or decreased expression at the dawn time point.

Starch levels are depleted in the night in a precisely regulated manner, to ensure availability for metabolic activities even at the end of night (Stitt and Zeeman, 2012). Wild-type LBG expression levels, shows lower levels in the night as compared to daytime levels. Higher expression of LBGs at dawn in *knat7* stems may indicate that night-time repression in coordination with decreasing starch availability does not take place.

In the *sex1* mutant, that is defective in starch turnover, diurnal regulation of lignin genes is not affected, but overall levels are reduced (Rogers et al., 2005). Hence it appears that decreased starch levels at night do not regulate LBG diurnal abundance, but does affect its overall level. Based on this line of evidence, a proposal for *KNAT7* function in the night is that it pre-emptively blocks activation of LBG expression in the night, to prevent a draw on limited resources at this time of day from secondary cell wall containing tissue that is an irreversibly deposited final metabolic sink in cell differentiation. A well-known function of the clock is to 'gate' physiological processes so that they occur at the appropriate time of the day. The clock may regulate gating of physiologically inappropriate wall deposition through *KNAT7.*

In the absence of *KNAT7* function, it may be that the plant draws on all available resources in the night. Just like *knat7,* the *sex1* mutant shows increased expression of *C3H* in proportion to the changes in expression of other LBGs in the mutant. This suggests the possibility that *knat7* plants sense low carbon availability, even when it is available, like the *sex1* mutant. *KNAT7* acts as a repressor of secondary cell wall deposition in the interfascicular fibers of Arabidopsis stems but there is no evidence to indicate that thicker walls in *knat7* are a result of *KNAT7*'s clock related functions. The thick interfascicular fiber walls have been studied in the past in relatively higher fluence lights. Analysis of the interfascicular fiber thickenings in *sex1 knat7* double mutant and of *knat7* plants grown in low light intensities as well as studying LBGs expression in the night in the mutant stems could provide an easy means to disprove or further pursue the 'gating' theory as well as provide further evidence to investigate if thicker inflorescence cell walls are a result of disruption of diurnal regulation in *knat7*.

While there is evidence for *KNAT7*'s diurnal transcription regulation functions, the *KNAT7* transcript levels themselves do not show strong regulation in diurnal levels, in the wild-type (Figs. 4-1, 4-2). Given this finding, it is interesting to speculate how KNAT7 performs its diurnal functions. One of the few examples of clock components that do not show rhythmic expression is ZTL, a protein containing a blue-light receptor LOV domain, kelch repeats for protein-protein interactions and an F-box domain for dark-dependant targeting of TOC1 and PRR5 for proteasomal degradation (Somers et. al., 2000). Day-time activities of ZTL involve interacting with GI. Similarly for KNAT7, specific day-time and night-time protein interactions may help it perform its diurnal function.

In summary, I have established that inflorescence stems in Arabidopsis have a functional circadian clock based on variation in mRNA abundance of in this tissue. I have also shown that *KNAT7* is involved in regulating diurnal expression of LBGs at the end of night in this developmental stage. In seedlings, the diurnal regulated variation in expression of LBGs is not as clearly affected in the *knat7* mutant; however *KNAT7* regulates diurnal expression of *DHS1* in this tissue and activates overall *F5H* expression. Hypocotyl elongation phenotype and altered timing of floral transition in LD supports *KNAT7*'s role as a diurnal regulator.

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