

**Diurnal and circadian regulation of wood
formation in *Eucalyptus* trees**

by

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DECLARATION

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree M.Sc. to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

Luke Solomon

25 April 2008

DISSERTATION SUMMARY

Wood is one of the most important products of world trade, due to its countless uses as a source of timber, fibre, and renewable energy. In addition to its economic importance, the formation of wood represents a global carbon sink which reduces the excess atmospheric CO₂ that contributes to global warming. The formation of wood or *xylogenesis* is a complex example of cell differentiation, controlled by multiple interacting environmental factors and the coordinated expression of hundreds of genes. Genomic studies have proved a valuable tool in identifying the genes associated with xylogenesis. The expression of these genes has been shown to under strict spatial regulation in a developmental-stage specific fashion. Despite recent advances in the understanding of this process, there remains much to learn about the cellular, molecular and developmental processes involved.

While the spatial regulation of wood formation has been well described, less attention has been devoted to the temporal regulation of this process. Most organisms are known to match their activities to the daily oscillation of night and day in what is known as a diurnal rhythm. A subset of these diurnal rhythms are termed circadian rhythms, and persist in the absence of environmental time cues, with a period of approximately 24 hours. Circadian rhythms are endogenous in nature, being generated by a small number of central oscillator genes, and illustrate an organism's ability to measure time. Circadian rhythms are found across a wide taxonomic spectrum, and are believed to confer an adaptive benefit, possibly due to the ability to anticipate regular changes in the external environment.

As wood formation is a major sink for the products of light driven photosynthesis, it represents a likely target for circadian control in plants. A large proportion of photosynthesis

genes themselves are known to be under circadian control, as are several cell wall formation genes. Most studies of temporal rhythms in plants, however, have used the herbaceous model species *Arabidopsis*, which does not have a woody stem. It is likely, therefore, that the circadian control of many wood formation genes remains to be discovered.

We used a spotted cDNA microarray carrying 2608 elements to quantitatively measure daily changes in transcript abundance in the wood-forming tissues of a fast growing, *Eucalyptus* hybrid. *Eucalyptus* is a large genus of tree species, many of which are of great economic importance, and are widely grown in plantations for solid timber and pulp production. We found that almost ten percent of the genes on the microarray showed significant daily changes in expression ($-\log_{10}P > 3.74$). These genes included *Eucalyptus* orthologues of the *Arabidopsis* central clock genes *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*) and *GIGANTEA* (*GI*) which cycled with a period and phase matching that seen in *Arabidopsis*. The remaining genes were involved in pathways including carbohydrate metabolism, hormone signalling, transcription regulation and wood formation. The types of genes that were seen to be diurnally influenced, suggests a role for circadian control of various important plant metabolic pathways, including aspects of carbon allocation to wood formation.

PREFACE

While the demand for wood and wood products continues to grow, rapidly disappearing natural forests are increasingly protected from commercial exploitation, as our understanding of their critical roles in climate control and sustaining biodiversity grows. To satisfy the world's growing need for sustainable and renewable forests, more wood therefore needs to be produced on less land, by cultivating elite lines of highly productive tree varieties. Forest trees are still far less domesticated than other agronomic crops, due in part to their long life spans, large size and the extended period before reproductive age is reached. For these reasons, trees stand to benefit greatly from biotechnological approaches to domestication including *in vitro* propagation, marker assisted breeding and gene transfer. Genetic modification of forest trees, along with the exploitation of naturally occurring genetic diversity are the two main strategies being employed to improve wood properties and production. Advances in either of these approaches are dependent upon a deeper understanding of the molecular functions of genes that influence the traits of interest.

Understanding the genetic regulation of wood formation or *xylogenesis* is central to tree improvement strategies. Xylogenesis is a highly ordered developmental process involving the patterned division and subsequent differentiation of secondary xylem from the vascular cambium. Wood formation is controlled by hundreds of genes that are known to be strongly spatially regulated, with substantially different transcriptomes occurring across anatomically homogenous layers surrounding the cambial meristem. Our understanding of the genetic control of wood formation is still limited. One aspect that remains largely unknown is the role played by temporal variation of gene expression in wood formation genes. Some of these gene expression changes may occur in response to environmental cues,

while others are likely to be controlled at a transcriptional level by the circadian clock. The circadian clock is an endogenous biochemical oscillator which directly and indirectly controls a large portion of diurnal gene expression in plants and other organisms. The formation of wood is a strong candidate for temporal control due to its reliance on carbon captured during the light-dependent process of photosynthesis. Furthermore, evidence already exists for the circadian control of such important wood formation processes as lignin biosynthesis.

The **aim of this M.Sc. study** was therefore to examine diurnal changes in gene expression in the wood forming xylem tissues of fast-growing commercial *Eucalyptus* hybrid clones. This was done in order to identify possible diurnal and circadian changes in gene expression, and elucidate temporal aspects of the genetic control of carbon partitioning and wood formation.

Chapter 1 of this dissertation is a review of the scientific literature regarding circadian and diurnal rhythms in plants, focussing on what is known in the model species, *Arabidopsis*, and the possible roles that biological rhythms may play in wood formation. It includes an introduction to circadian and diurnal rhythms as well as the recent history of their study, or *chronobiology*. It further describes the central role played by rhythms in regulating plant growth; their adaptive benefit and known activity in trees. Finally, it discusses the various methods of investigating circadian rhythms, including microarray technology.

Chapter 2 of this dissertation describes the sampling strategy and experimental procedure followed to identify diurnally influenced and circadianly regulated genes expressed in wood forming tissues of *Eucalyptus* trees. Significantly differentially expressed genes that may be under circadian control are identified, and the potential significance of this regulation for carbon allocation and wood formation is discussed. The expression of three central clock

genes is also investigated to determine the activity and phase of the central oscillator in woody tissues.

Finally, several conclusions, implications and future directions for this study are discussed at the end of the dissertation in a **Concluding Remarks** section.

The findings and results that comprise this dissertation represent the outcomes of a study that took place between January 2005 and November 2007 in the Department of Genetics at the University of Pretoria under the supervision of Prof. Alexander A. Myburg and co-supervision of Prof. David K. Berger. **Chapter 2** has been prepared in manuscript format for submission to a peer-reviewed research journal, and may therefore share a degree of redundancy in its introduction with **Chapter 1**. The following congress presentations were generated based on results obtained in this M.Sc. study:

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ABBREVIATIONS

ANOVA	Analysis of Variance
CCA1	<i>Circadian Clock Associated 1</i>
CTAB	Cetyl Trimethylammonium Bromide
Eg	<i>Eucalyptus grandis</i>
Et	<i>Eucalyptus tereticornis</i>
FDR	False Discovery Rate
GC	<i>Eucalyptus grandis</i> × <i>E. camaldulensis</i>
GI	<i>Gigantea</i>
GO	Gene Ontology
GU	<i>Eucalyptus grandis</i> × <i>E. urophylla</i>
LOESS	Local Estimate of Scatterplot Smoothing
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SEM	Standard Error of the Mean
Std LSM	Standardised Least Square Means
TDF	Transcript Derived Fragment
ZT	Zeitgeber Time



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CHAPTER 1

LITERATURE REVIEW

DIURNAL AND CIRCADIAN RHYTHMS IN PLANTS WITH REFERENCE TO WOOD FORMATION

1.1 INTRODUCTION

Perhaps the most obvious environmental variation faced by life on earth is the daily change from night to day, as the planet rotates on its axis. The resulting temporal switch in light availability and temperature affects almost every living thing, and leads to *diurnal* or daily rhythms in activity, behaviour and many other aspects of life. These diurnal rhythms are not always simple reactions to environmental cues such as the presence or absence of light, but may reflect evolutionary adaptation to the 24-hour rhythm of night and day. This subset of diurnal rhythms is termed *circadian* (from the Latin '*circa dies*' meaning 'about one day'), and is driven endogenously by a molecular 'clock', capable of maintaining time over several cycles without environmental input (Pittendrigh, 1960).

The concept that living things have internal mechanisms for determining the time is obvious to anyone who has experienced 'jet-lag' after a long flight, or to shift-workers trying to sleep during the day. It is also apparent that this internal clock is not exclusively restricted to humans – animals are active at particular times of day and rest at others, insects respond to the passage of time and even plants illustrate the ability to keep track of time. Plants, like other organisms are also able to keep time when removed from their natural environment with its time cues such as light and temperature (Dunlap, 1996). Take as an example plant species which close their flowers at night and open them during the day. Placed in continual light or darkness, the rhythm of opening and closing will continue in certain species over several cycles despite the lack of external indicators, indicating that the circadian clock, rather than a light response is driving this activity (van Doorn and van Meeteren, 2003).

These circadian rhythms drive more than just behavioural patterns such as sleep and daily movement, and are also known to co-ordinate multiple aspects of growth, physiology

and development with changes in the external environment (Pittendrigh, 1960). Clock directed rhythms originate at the level of gene expression, where central clock genes use negative feedback loops to cycle in transcript abundance over a 24-hour period (Roenneberg and Merrow, 2003). These genes form the ‘central oscillator’ of the circadian clock, and are able to maintain their rhythm over several cycles, even when placed into continuous light or dark environments, indicating the clock’s endogenous nature. Environmental inputs to the central oscillator are obtained mainly via phototransduction pathways (Millar, 2004), and are used to ‘fine tune’ the cycle and keep it in phase with the external light-dark cycle – analogous to resetting your watch when it loses or gains a few minutes

Circadian rhythms are fairly ubiquitous, being found across the taxonomic spectrum in most eukaryotes and in several prokaryotes (Aschoff, 1979). The adaptive benefit of circadian rhythms has been experimentally demonstrated in several different species. In the simple cyanobacteria *Synechococcus spp.*, short and long period mutants, having a circadian rhythm differing from 24 hours, have been shown to dominate in a mixed culture, if the light/dark cycle is matched to their endogenous rhythm (Ouyang et al., 1998). In the more complex model organism *Arabidopsis thaliana*, circadian mutants that have lost the ability to anticipate daily changes in the environment are demonstrably less fit than their wild-type counterparts (Green et al., 2002), mainly due to the noticeable photosynthetic advantage gained by matching the circadian period with the external dark-light cycle (Dodd et al., 2005). The mechanism for this gain in fitness is probably related to the circadian clock’s function as an internal anticipatory system.

Anticipation of rhythmic changes means that preparation for an event can be made in advance of any signal that the event has begun. This is dependent on the event being a

regular, anticipated one – as is the case for dawn and dusk; seasonal changes, tides and other natural occurrences. Internal anticipation of the external environment allows organisms to exploit temporal niches and to optimize metabolic efficiency. As an example, consider two bird species whose body temperature drops during sleep: one anticipates the dawn and begins warming up while it is still dark; at daybreak it is ready to forage. The other bird starts warming up only at the first sight of daybreak; it will waste valuable daylight time before it is ready to fly.

How does the central oscillator generate circadian rhythms? The simplest possible molecular mechanism would involve a single gene that is transcribed and then translated to protein - if the gene product interferes with the transcription of its own mRNA, it will halt transcription for as long as the protein is present in the cell. As the protein is degraded over time, the halt to transcription will end and the cycle can start over. This would be one way of creating a 24-hour oscillation in both RNA and protein levels. Evolution has created a complexity in circadian rhythms far beyond that of this simple mechanism. The circadian rhythms described thus far often involve several interconnected loops (Locke et al., 2005) consisting of numerous genes which influence each other's transcription, translation and subcellular location in many and varied ways.

As a starting point for describing and understanding circadian rhythms at a molecular level, a general simplified model for a circadian clock is pictured as consisting of three main parts (Figure 1.1). At the centre of the system is the 'central oscillator', which generates rhythm by means of a negative feedback loop. Sufficient lag to create a 24-hour cycle can be obtained by a clock protein that negatively regulates its own production either directly or indirectly. Regulation at the level of transcription, translation, post-translational modification,

and selective transport are some of the parameters that may form part of this central oscillator (Schibler and Naef, 2005).

Several criteria have been proposed that should be satisfied before a gene is considered a central oscillator component. The candidate gene should be required for output rhythms, and oscillate at the same frequency as these rhythms. It should furthermore be sensitive to environmental signals, which should cause a predictable shift in the clocks phase (Pittendrigh, 1960).

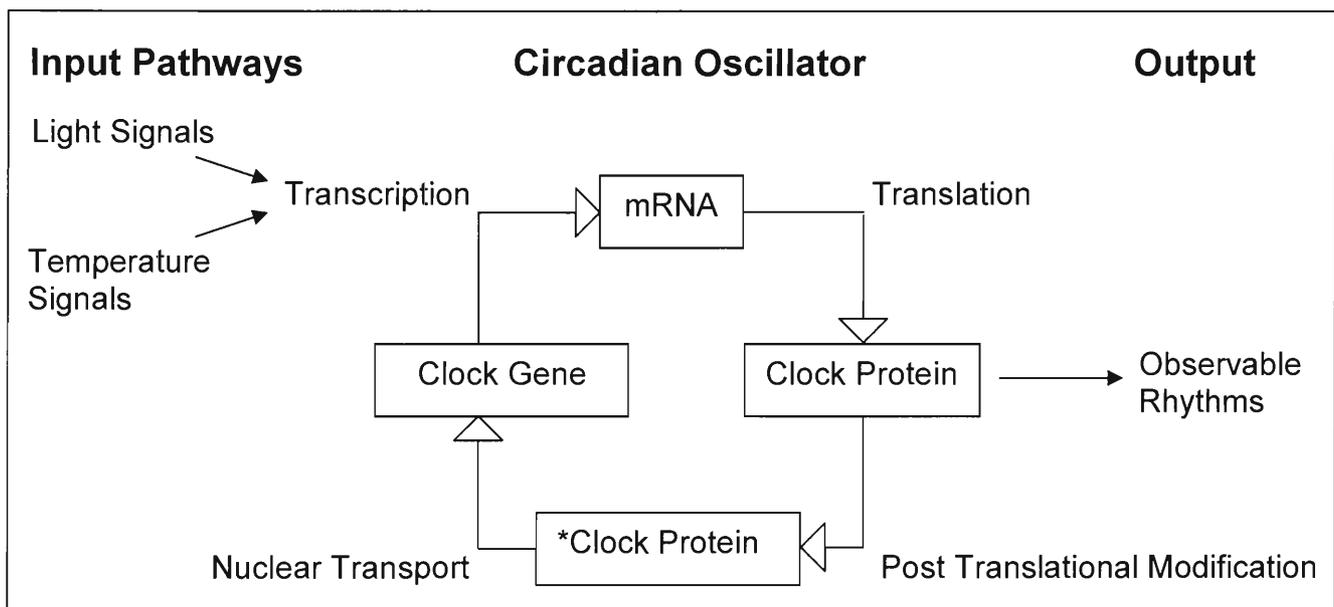


Figure 1.1. Components of the circadian clock: input signals such as light and temperature entrain the central oscillator by interacting at various stages of the feedback loop (only one example shown). The circadian oscillator consists of the autoregulatory negative feedback loop of a clock protein. The asterix indicates modification of the protein. Observable rhythms in the output pathway include clock controlled gene expression, leaf movement, enzyme activity, flowering and other processes (Anderson and Kay, 1996).

Much of the early study on circadian rhythms focussed on phase shifts, and drawing resulting ‘phase response curves’ which illustrate this effect (Devlin, 2002). In general, a light signal before dawn causes an advance in the phase of the central oscillator, while a signal after dusk causes a phase delay. Two more criteria for central oscillator components are that constant expression of the oscillator should cause arrhythmia, and the oscillator should respond rapidly to environmental signals (Dunlap, 1996; Hanks et al., 1999; Millar, 1999). An oscillator alone, however, does not form a clock. The clock must be linked to the external environment in order to be meaningfully phased, and must also control the rhythms of other processes in the organism. These ‘input’ and ‘output’ pathways complete the basic clock model.

Environmental inputs to the central oscillator form the interface between the clock and the environment, and are required in order to adjust the clock’s free running phase to ‘local time’ - a process known as *entrainment* (Salome and McClung, 2005). The endogenous nature of circadian rhythms means that organisms are in a sense detached from the environment – they do not respond directly to light, but can anticipate its arrival and prepare for it. This detachment cannot be total, otherwise circadian events would quickly fall out of synch with external events, hence the importance of entrainment. Dark/light shifts, and temperature changes are the primary clock inputs.

Light input may act at various levels, for example by light activated transcription of a clock gene, or light induced transport of a protein. Further, not only the presence and absence of light is noted, but also the quality (i.e. spectrum) can be detected by photoreceptors which are particularly sensitive to red or blue light (Millar, 2004). Environmental time cues are known as *Zeitgebers* (German for ‘time givers’) and Zeitgeber Time is commonly used in

circadian research to refer to the number of hours since the appearance of light (e.g. ZT 6 could be at midday).

The entrained oscillator controls a wide variety of processes by means of the output signal transduction pathways. These outputs include rhythms at the molecular level, such as fluctuations in calcium ion levels and gene expression (Yakir et al., 2007). At a higher level, biological processes such as leaf movement in plants, or nocturnal activity in hamsters, are brought about by circadian controlled changes at the cell and tissue level (Somers, 1999). Events that happen over time scales longer than a day, such as the induction of flowering in plants, or migration patterns in birds can also be controlled by circadian rhythms, together with changes in day length (Hotta et al., 2007). Estimates of the percentage of genes controlled as outputs of the circadian clock vary according to species, but may include almost all genes in lower species such as the prokaryote *Synechococcus* (Liu et al., 1995). In eukaryotes, estimates are typically smaller, ranging in plants from 2 to 16% of all genes (Harmer et al., 2000; Claridge-Chang et al., 2001; Schaffer et al., 2001; Duffield, 2003; Edwards et al., 2006; Covington and Harmer, 2007) but are placed by some studies as high as 36% or more (Michael and McClung, 2003).

The study of biological rhythms is termed ‘chronobiology’, and has progressed over the last century from its original descriptive nature, to a much deeper understanding of the molecular clocks that drive physiology, behaviour and gene expression in nearly every species investigated. This review looks at diurnal and circadian rhythms in general: what they are and how they work at the molecular level; and in plants in particular, with reference to the model organism *Arabidopsis thaliana*. It also looks at some of the techniques that are used to

identify circadian rhythms. Finally, it explores the possibility of circadian control of wood formation and carbon allocation in trees.

1.2 A BRIEF HISTORY OF CHRONOBIOLOGY

Chronobiology is the study of periodic or cyclic phenomena in living organisms that are also known as *biological rhythms*, focussing particularly on oscillating changes in biochemistry, physiology and behaviour. The effect of environmental periodicities including dark/light cycles, tides and seasons on living organisms have been noted since ancient times. Observers such as Aristotle, Pliny, Hippocrates and others all noted rhythmic changes in their study of living organisms. These could be attributed to either daily cycles such as day/night changes, or longer cycles, such as phases of the moon or seasonal variation (Luce, 1971; Cloudsley-Thompson, 1980; Campbell, 1988).

The first scientific experiment in the field to be recorded and published, was the work of a French astronomer Jean Jacques d'Ortous de Mairan in the early 18th century. de Mairan noted how the leaves of the heliotropic plant *Mimosa pudica*, which close at night and open during the day, continued to display this rhythm, even when the plant was moved into continual darkness in his cellar (de Mairan, 1729). This result was the first illustration that a biological rhythm could be generated endogenously, rather than as a reaction to sunlight or other environmental factors. At the time, and for many years after, it was believed that some other environmental cue, possibly related to the earth's spin could penetrate to the dark cellar and influence the plants behaviour.

In 1751 Karl Linnaeus found that many other plants also displayed temporally orchestrated events (Bunning, 1977). He used this information to design his famous 'plant

clock’, which consisted of various species of flower known to open or close their leaves or petals at specific periods. By observing which flowers were closed or open, an observer could determine the approximate time of day (Figure 1.2).



Figure 1.2. The flower clock of Linnaeus. The left half indicates flowers opening between 6 am and midday; the right side shows flowers closing between midday and 6 pm.

In 1880, Charles Darwin devoted a large portion of his book ‘The Power of Movement of Plants’ to discussing the opening and closing of plant leaves and flowers, which he described as the ‘sleep’ of plants (Darwin, 1895). Darwin performed hundreds of experiments on this system, and concluded that the plant movements were heritable and endogenous, being shaped by natural selection as an adaptation to protect the plants from radiation. Darwin also noted rhythmic behaviour in other organisms such as earthworms, which continue to venture above ground only during the subjective night even when kept in constant conditions, causing Darwin to conclude that this was a naturally selected and heritable trait developed to avoid diurnal predators (Darwin, 1881).

Darwin did not study the mechanism of these internal clocks, however, and it was only several decades after his death, in the DNA era, that it became apparent that there could be an underlying explanation for all observed biological rhythms. The first genetic evidence for the endogenous control of rhythms was found in the 1930s, when Erwin Bunning demonstrated that bean plants inherit the period length of their leaf movement (Bunning, 1935; Mitsui et al., 1986).

Bunning's work was influenced by an earlier study by Garner and Allard, which uncovered the phenomenon of *photoperiodism* by studying long-day and short-day plants, i.e. plants which flower in response to the lengthening or shortening of days respectively (Garner and Allard, 1920). The ability of a plant to measure shifts in day-length was attributed to an interaction between light-sensing and endogenous rhythm, and showed that the internal clock could influence not only daily events, but also sense seasonal changes. Bunning proposed a mechanism for photoperiodism involving a daily cycle determining how far light intrudes into its subjective 'night-time' (Bunning, 1936). So it was from the study of plants that the field of chronobiology originated, although it would soon spread to include research into the endogenous rhythms of all kinds of creatures from bacteria (Mitsui et al., 1986) to humans (Aschoff and Wever, 1962).

The term 'circadian' to describe endogenous rhythms with period of around a day length was coined by Franz Halberg, a Romanian born physician and endocrinologist who studied the effects of administering drugs at different times of day (Halberg et al., 1959). Along with Halberg, the title 'father of chronobiology' is shared with his contemporary Colin Pittendrigh, who spent most of his scientific career in Princeton and Stanford studying biological rhythms. His 1960 paper 'Circadian rhythms and the circadian organization of

living systems' (Pittendrigh, 1960) brought together for the first time all previous studies on daily rhythms from widely disparate fields, and suggested a common basis for these phenomena – that of the biological clock.

The first site of a 'circadian clock' was located in the eyes of the cockroach by Janet Harker in 1960. She also illustrated the adaptive benefit of biological rhythm by setting the two clocks in the eyes out of phase with one another – the cockroaches soon developed cancers and died (Harker, 1960). No molecular evidence for a biological clock was found, however, until the 1970s, when a clock-related *Period* gene (*Per*) was found in the fruit fly *Drosophila*, using a mutant screen for perturbed rhythms (Konopka and Benzer, 1971). The study identified three mutants displaying rhythms which differed significantly from 24-hours – one was arrhythmic, one had a longer, and the other a shorter than usual period. On investigation it was discovered that all three individuals contained mutations in the same X-linked gene. This discovery ushered in the current era of molecular studies of the circadian clock and it was the subsequent study of the *Period* gene that led to the model of clock mechanism that still stands today, namely the negative autoregulation of a gene by its protein product.

The function of the circadian clock in living organisms has been explored by many scientists, both before and after its mechanism was revealed, each study finding different ways in which biological clocks are used to co-ordinate internal functions and respond to the external environment. Bees use their internal clocks to time visits to flowers when the flower is open (Beling, 1929), birds undergoing migratory movements use their biological clock to adjust for the changing position of the sun (Kramer, 1952) and various species avoid diurnal

predation by coming out to forage at night. The adaptive benefit of circadian rhythms to plants is of particular interest to this review and will be discussed below in greater detail.

1.3 PLANT RHYTHMS

1.3.1. Diurnal regulation of plant growth

Daily (diurnal) rhythms in plant metabolism and physiology influence many pathways, including starch and sucrose metabolism, nutrient uptake, glycolysis, gluconeogenesis, amino acid and lipid metabolism, photosynthesis and others (Blasing et al., 2005). Estimates of this diurnal influence on genes in *Arabidopsis* ranges from 11–50% of the transcriptome, with more recent findings tending to the top end of that range (Schaffer et al., 2001; Blasing et al., 2005). These rhythms are induced by a variety of signals and inputs, many of which can be related directly or indirectly to the presence or absence of light. The circadian clock is responsible for the production of some diurnal rhythms, and requires the daily entrainment of light induction to its central oscillator genes to maintain time. Light is also necessary for photosynthesis, and the sugars produced during daylight hours have signalling effects causing diurnal rhythms in many genes affected by varying carbohydrate levels. Other factors influencing diurnal rhythms include hormones, water stress and nitrogen levels, amongst others (Blasing et al., 2005).

Light itself – or the absence of light, is a strong regulator of gene expression and physiology, inducing or repressing gene expression, or causing light induced protein degradation (Oliverio et al., 2007). Light's central role in generating diurnal rhythms is illustrated by the over 2500 genes in *Arabidopsis* that are regulated diurnally by the perception of light via photoreceptor molecules (Gyula et al., 2003). Photoreceptors act as

signal inputs to a complex, integrated molecular network, resulting in changing levels of transcription for affected genes, as well as protein modification and compartmentalization in the nucleus or cytoplasm (Harmer et al., 2001). The shift from light to dark has almost as large an effect on gene expression as the transition to light, with a microarray study revealing 790 ‘dark responsive’ genes which are induced in the first eight hours of a shift from constant light to dark conditions (Kim and von Arnim, 2006).

Two of the most important causes of daily rhythms are the circadian clock and changing sugar concentrations - both of which are in turn affected by light. These factors are responsible for diurnal expression changes with the largest amplitude in *Arabidopsis* (Blasing et al., 2005). As is the case for many factors that cause diurnal changes in expression patterns, strong interactions exist between sugar sensing pathways and the circadian clock. For instance, almost a quarter of circadianly regulated gene expression patterns are reinforced by diurnal changes of sugars levels in the cell (Blasing et al., 2005). Diurnally cycling genes that are repressed or induced by sugars include transcription factors with potential wide ranging effects, as well as several protein kinases including AKIN β 1. This protein kinase is known to phosphorylate sucrose phosphate synthase (*SPS*, Sugden et al., 1999), a key enzyme regulating carbon flux into cellulose and other cell wall matrix components (Andersson-Gunneras et al., 2006). Phosphorylation of *SPS* by AKIN β 1 therefore represents a potential method for sugar influenced diurnal regulation of the sucrose synthesis pathway, including cell wall synthesis.

In addition to interactions between cellular sugar levels and circadian pathways, sugar levels also interact with light signalling pathways, leading to synergistic effects in the number of genes influenced by both pathways. A microarray study using Affymetrix technology

showed 201 genes regulated by carbon levels alone, 77 regulated by only light, and 1,247 by carbon-light interactions (Thum et al., 2004).

Hormone signalling plays a large role in generating plant diurnal rhythms, and is itself indirectly influenced by light. Abscisic acid signalling is implicated in the dark response, and is one example of a plant development process regulated by both light and hormone levels. Other hormones affecting diurnal rhythms include auxin, ethylene, brassinosteroids and cytokinin (Kim and von Arnim, 2006; Covington and Harmer, 2007). An illustration of the effect of light on the action and effectiveness of hormones is seen during hypocotyl elongation, which is controlled by plant hormones that act differently depending on whether the plant is in the light or the dark (Nozue and Maloof, 2006). The interaction between hormone signalling, light and the circadian clock in influencing diurnal patterns is illustrated well by the gene *SPINDLY* (*SPY*). *SPY* codes for a negative regulator of gibberellin signalling in *Arabidopsis* and interacts with the central clock gene *GIGANTEA* (*GI*). Through these interactions, *SPY* acts as a light-regulated promoter of hormone dependent hypocotyl elongation that is further influenced by the circadian clock (Tseng et al., 2004).

Lignin biosynthesis is an important process related to secondary cell wall formation and xylem (wood) development, and its regulation is another example of multiple levels of control involving diurnal rhythms. Experiments performed to determine the mechanisms regulating lignin biosynthesis showed that some diurnal changes in lignin gene expression are related to circadian clock control, while others are likely to be attributable to light perception and many are influenced by sugar levels in the plant (Rogers et al., 2005). Cellulose biosynthesis is also affected by light, with mutations in the cellulose synthase gene *CesA6* inhibiting hypocotyl elongation when affected *Arabidopsis* plants are grown in the dark

(Fagard et al., 2000), but not in the light (Desnos et al., 1996). The cell wall related gene *KORRIGAN* shows a similar effect (Nicol et al., 1998), and the transcription of many other cell wall genes are also affected by light (Tepperman et al., 2001)

1.3.2. The role of circadian rhythms in plants

Circadian rhythms allow for the anticipation of key events such as dawn and dusk and drive appropriate responses to the local environment. They are of particular importance to plants, due to the periodic nature of light availability and its requirement in order to sustain photosynthesis (exceptions to this relationship are found in subterranean germinating seedlings and possibly polar inhabitants). Due to the sessile nature of plants, temporal regulation of biological function takes on special significance, as they are unable to re-locate in order to avoid adverse environmental conditions, such as extreme fluctuations in temperature and light intensity. Some of the circadian rhythms in plants are evident at a macro scale, such as the sleep movements described earlier, although most involve subtle changes that are more difficult to identify. Plants are well suited to studies on circadian rhythms for several reasons, including their wide range of activities displaying 24-hour periodicity, and strong interactions between light input signals and the circadian clock in plant tissues.

Circadianly regulated plant genes can be generally assigned to one of the circadian system's three principal components: namely inputs, oscillators and outputs. The distinction between these categories is oversimplified, however, and does not always reflect reality. In plants, as in all known circadian systems, light forms the primary entraining input pathway (Gyula et al., 2003). The plant photosensory system is made up of three photoreceptor classes: phytochromes (encoded by *PHY* genes), cryptochromes (encoded by *CRY* genes) and

phototropins (encoded by *PHOT* genes) with phytochromes and cryptochromes responsible for input to the circadian oscillator in *Arabidopsis* (Somers et al., 1998). Some ‘input’ genes are also part of the clock’s ‘output’ pathways, being regulated at the level of transcription as is the case of *CRY1* and *CRY2* in *Arabidopsis*, whose expression levels peak in the late afternoon (Harmer et al., 2000). The fluctuations in *CRY* gene expression mean that the plant is more sensitive to changes in light quality at certain times of day, such as morning and evening. This modulation of the input pathway by the output pathway is known as *gating* (Devlin, 2002) and illustrates the somewhat arbitrary nature of the classification of genes in the three-component model system.

Of the three components of a circadian system, the central oscillator is the most functionally conserved across species, always consisting of gene circuit feedback loops involving positive and negative transcriptional regulators (Eriksson and Millar, 2003). Functional similarity does not extend to the sequence level, however, and is likely to be the result of convergent evolution (Dunlap, 1996). The best known central oscillator in a plant system is that of *Arabidopsis thaliana*, which is discussed in more detail in the following section.

Plants may control circadian rhythms in a strongly tissue-specific manner (Thain et al., 2002), unlike mammals, which synchronize circadian rhythms in different tissues with a single master circadian pacesetter residing in the suprachiasmatic nuclei (SCN) of the hypothalamus (Antle and Silver, 2005). Plant circadian rhythms are known which differ in phase from tissue to tissue and cells in the same leaf can be stably entrained to differing rhythms (Thain et al., 2000). It seems likely that every plant cell contains at least one oscillator, as this autonomy also extends to isolated tissue explants, and rhythms in stomatal

movement and in leaf pulvinus protoplasts have been observed in culture (Gorton et al., 1989; Mayer et al., 1997; Thain et al., 2000). It has been argued that these spatially separated copies of the circadian clock are either very weakly coupled, or even functionally independent, permitting tissue-specific specialisation of circadian timing (Thain et al., 2002).

The third component of the basic clock model is composed of the output pathways that are controlled by the central oscillator. The circadian clock controls many aspects of growth and development in the plant, from daily processes under direct clock control to circannual rhythms in bud set, leaf loss, flowering and others that are controlled in part by changes in the photoperiod (Yakir et al., 2007). Circadian rhythms may begin in the seeds of some species, where day length regulates germination (Densmore, 1997). Immediately after germination, circadian rhythms in hypocotyl elongation can be observed in many species, including *Arabidopsis* (Dowson-Day and Millar, 1999). Cotyledon and leaf movement are also under circadian control in many plant species. Leaf movement was the first circadian movement recorded (de Mairan, 1729), and one of the first to be noted in *Arabidopsis* (Engelmann et al., 1998). The revolving movement of elongating plant organs including stems and hypocotyls known as *circumnutation* has also been shown to be under circadian control (Niinuma et al., 2005).

Long term rhythms are also under clock control and can be considered circadian outputs, one of the most significant of which is reproduction. The timing of flower set is crucial to a plant's reproductive success, and is regulated by photoperiodism, so that a peak in circadian expression must coincide with sufficient light levels in order to promote flowering (Searle and Coupland, 2004). After flowering, pollination is also influenced by circadian control, with certain species opening their flowers or secreting nectar only during the hours

that their pollinators are most active (Matile, 2006). Winter dormancy is another long term process influenced by clock rhythms. The chestnut tree orthologues of two central oscillator genes in *Arabidopsis*, *LHY* and *TOC1* (see next section), which cycle as in *Arabidopsis* during the growing season, cease cycling and are expressed at a constitutively high level during dormancy in chestnut trees (Ramos et al., 2005). It is hypothesised that disruption of the clock could initialise the physiological changes that occur during entry into dormancy.

Due to their autotrophic nature, higher plants such as *Arabidopsis thaliana* have a large proportion of their genomes assigned to controlling metabolic activities (Arabidopsis Genome Initiative, 2000). Circadian output genes have been suggested to coordinate metabolic processes by either *external coincidence* (anticipation of an upcoming event, e.g. dawn), or *internal co-ordination* (temporally separating biochemically incompatible processes, (Millar, 1999). Genes involved in photosynthetic processes and the resulting fixation of carbon are often circadian output genes – unsurprisingly, given their dependence on the availability of light. Many of the light harvesting genes of *Arabidopsis* are circadian output genes, including *CAB*, which begins expression before dawn, lowering its expression at night (Millar and Kay, 1991). It has been suggested that clock control of metabolic processes is required in order to temporally separate the energy producing reactions of photosynthesis from such energy consuming processes as assimilation of nitrogen (Harmer et al., 2000). Other physiological changes regulated by circadian output genes include stomatal opening and closing, transport processes, intracellular signalling and regulation of membrane properties (Webb, 2003).

1.3.3. The *Arabidopsis* model of the circadian clock

The small size, short generation time, large number of offspring and relatively small (125 Mbp), fully sequenced genome of *Arabidopsis thaliana* makes it an ideal genetic model for identifying genes and determining their functions (Arabidopsis Genome Initiative, 2000). Even before its sequencing began in 1996, *Arabidopsis* was already an important circadian model organism for higher plants. The first published report of circadian rhythms in *Arabidopsis* described the circadian control of *CAB* gene expression (Millar and Kay, 1991). The authors of this study illustrated that *Arabidopsis* was an appropriate model organism for circadian research in higher plants, and led to *Arabidopsis* currently having the best described circadian system in plants.

In 1998, two of the genes involved in the central oscillator of the *Arabidopsis* circadian clock were described in the journal *Cell* (Schaffer et al., 1998; Wang and Tobin, 1998), setting the stage for a model of circadian control to develop. *LHY* (*Late Elongated Hypocotyl*) and *CCA1* (*Circadian Clock Associated 1*) are closely related genes that encode MYB DNA-binding proteins, and whose mRNA shows circadian oscillations in transcript abundance, with a peak expression around dawn. Constitutive over expression of either gene leads to repressed oscillations, indicating negative autoregulation of their own expression (Figure 1.3). However, knockout of the two genes did not result in arrhythmia as expected, unless both genes were knocked out together, and even then only under certain conditions (Alabadi et al., 2002). Taken together, these effects indicate that *LHY* and *CCA1* are partially redundant in function, and necessary but not sufficient for proper circadian function (Schoning et al., 2006).

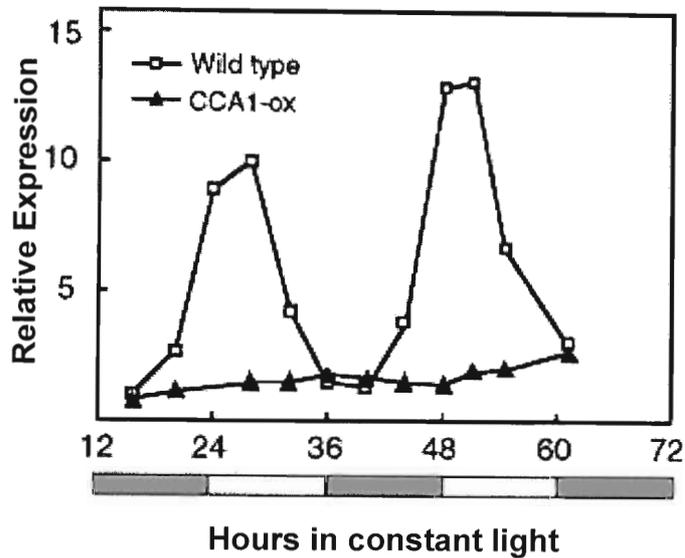


Figure 1.3. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) is a central circadian oscillator component. Its mRNA abundance fluctuates with a 24hr period in wild type *Arabidopsis*. *CCA1* over expression (CCA1-ox) leads to suppression of its circadian rhythm (Wang and Tobin, 1998).

A better picture of the central oscillator was obtained when pseudo response regulator *TIMING OF CAB EXPRESSION 1 (TOC1)* was identified (Strayer et al., 2000). Response regulators are involved in signalling cascades and have a conserved Asp residue in their receiver domains which pseudo response regulators lack. *TOC1* cycles with a phase 12 hours removed from that of *CCA1* and *LHY*, and has a positive effect on the expression of *CCA1* and *LHY*, which in turn repress the expression of *TOC1* by binding directly to its promoter (Alabadi et al., 2001). This discovery led to the first, simple model of the central oscillator in *Arabidopsis*, consisting of a single feedback loop. Accumulation of *TOC1* activates *CCA1/LHY*, whose gene products inhibit *TOC1*, leading to a drop off in *CCA1/LHY* activation and re-accumulation of *TOC1*.

TOC1 shares homology with four other pseudo response regulators (*APRR9*, 7, 5 and 3) in *Arabidopsis* (Strayer et al., 2000), forming what is known as the *APRR* quintet. The four *APRRs* are all circadianly controlled, being expressed in sequence from dawn until evening when *TOC1* is expressed. *APRR9* is also light activated through the phytochromes. The

expression of the five genes is related, although their interactions are not fully understood. It has been suggested that the quintet provides a mechanism for controlling gene expression at any phase from morning to evening (Eriksson and Millar, 2003; Zeilinger et al., 2006).

Since the first description of negative feedback loops essential for circadian rhythms, other genes have been discovered which further complicate the model and add complexity to the control of rhythmic oscillations. Several other genes whose roles recently became clearer are required for *CCA1/LHY* expression: these include *GIGANTEA* (*GI*, Fowler et al., 1999), *EARLY FLOWERING 3* and *4* (*ELF3/4*, Hicks et al., 2001; Doyle et al., 2002) and *LUX ARRHYTHMO* (*LUX*, Hazen et al., 2005).

A new model with extra feedback loops which interlock with the *TOC1/CCA1/LHY* loop was suggested (Locke et al., 2005) due to the inability of a single loop to fit all experimental data, and the belief that *TOC1* protein may activate *LHY/CCA1* expression indirectly. Two hypothetical components ‘X’ and ‘Y’ were described by mathematical modelling of the oscillator. X was inserted after *TOC1*, and was an intermediate between *TOC1* and *LHY*. Y would activate *TOC1*, and would itself be repressed by *TOC1* forming a feedback loop (Locke et al., 2005). *GI* was identified as a candidate for Y function and later experimentally shown to fulfil a component of this role (Locke et al., 2006). An additional loop involving *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), *PRR9* and *LHY/CCA1* (Salome and McClung, 2005) was also added to the model to form a circuit with three interconnecting loops (Figure 1.4).

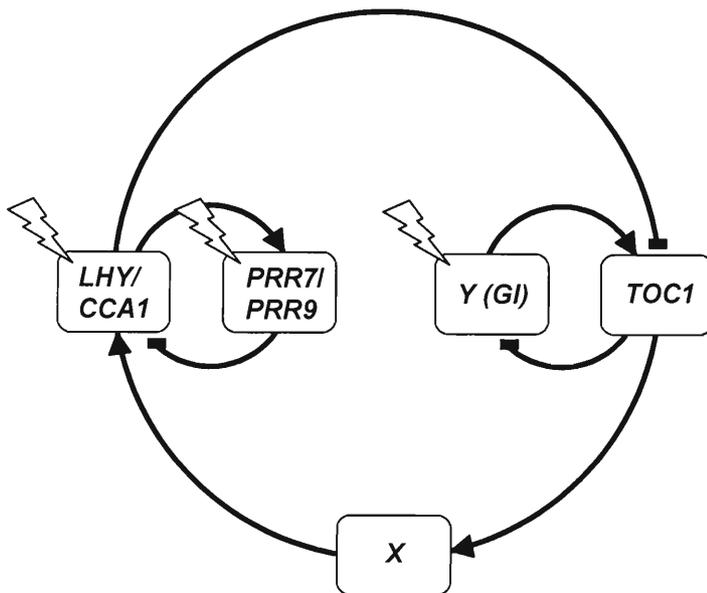


Figure 1.4. The ‘three-loop’ model of the central oscillator in *Arabidopsis*. Boxes represent genes, lines ending with arrows and rectangles represent positive and negative regulation respectively. X and Y are clock components proposed by mathematical modelling. GI fulfils a component of Y function. Flashes represent light input to the clock (Locke et al., 2006).

In order to maintain the central oscillator’s synchronisation with the external environment light acts as an input at various points in the central oscillator during entrainment (Figures 1.4 and 1.5). In *Arabidopsis* there are many light responsive proteins, of which the five phytochromes (PHY A – PHY E) covering the far red to red spectrum, and the two cryptochromes (CRY1 and CRY2) that respond to the blue to ultra violet wavelengths (Millar, 2004) are most important to the circadian clock. Several other genes interact with the light signalling pathways and are important for input to the clock (Figure 1.5). *ZEITLUPE* (*ZTL*) is a gene implicated in regulated protein degradation. It may require PHY B and CRY1 for correct function and targets TOC1 for degradation (Kevei et al., 2006). *TIME FOR COFFEE* (*TIC*) may repress light signalling (Hall et al., 2003) and functions close to the central oscillator (Ding et al., 2007).

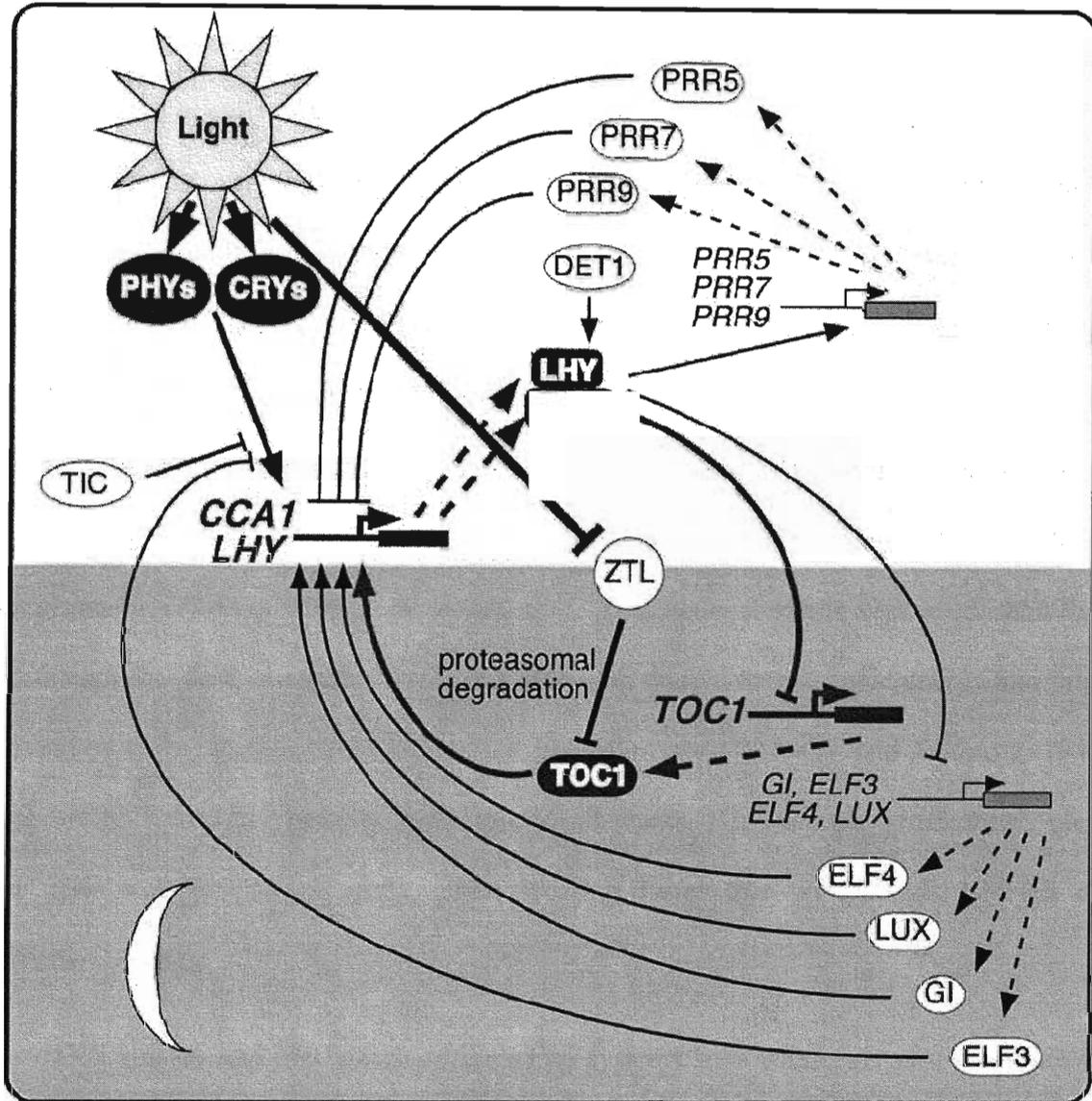


Figure 1.5. A recent view of the circadian clock in *Arabidopsis thaliana*. Genes are represented by boxes with gene names shown to the left. Proteins are represented by ovals, with their names shown inside the shape. Transcription and translation are represented by dashed lines. Solid lines indicate protein activity, with arrows representing positive action and lines ending in perpendicular dashes indicating negative action. The core *CCA1/LHY/TOC1* feedback loop is in the centre of the figure. The shaded bottom half of the figure indicates activities peaking in the subjective night, the white area indicates activities peaking during the subjective day (McClung, 2006). See text for details.

1.3.4. Adaptive benefit of plant circadian rhythms

The widespread occurrence of circadian clocks in nature, as well their independent evolution at several points on the tree of life indicates an expected adaptive benefit of circadian control. If circadian clocks are of benefit to the organisms that have acquired them, they must presumably enhance reproductive fitness in natural environments with their 24-hour cyclic conditions. Studies on plants performed in the late 40's and 50's found that tomato plants grew optimally when kept in conditions that matched that of the external environment. A plant grown in a 12-hour light, 12-hour dark cycle grew better than one kept in constant light, even though the plant in constant light was receiving double the photonic energy and might be assumed to be accumulating double the photosynthate (Withrow and Withrow, 1949). These results were not generally applicable to all plants. For example, *Arabidopsis* plants grow very well in constant light, often growing faster than in LD 12:12. (Hall and McWatters, 2005).

One way to test the benefit of circadian systems in *Arabidopsis* is to compare the fitness of central oscillator gene over expressers (*CCA1-ox*, *LHY-ox*) to wild-type plants, by measuring the number of seeds each produces, and their germinating ability (Green et al., 2002). Over expressing lines lack circadian rhythmicity, which means they can still respond to diurnal cues, but unlike wild-type plants, they cannot anticipate daily changes. Green et al. (2002) found that over expressers showed similar fitness to wild-type plants in LD 16:8 and 8:16, but illustrated lower fitness when grown in extremely short day conditions (LD 4:20), producing fewer viable seeds than wild-type plants.

More evidence of fitness enhancement by the circadian clock in *Arabidopsis* was provided by a study which showed that natural variation exists in the period, phase and

amplitude of *Arabidopsis* accessions and that the period length is correlated with the day length prevalent at the latitude of origin (Michael et al., 2003). The authors suggested that this indicates the primary role of the circadian clock is to synchronize an organism with its periodic surroundings. The large variation existing in circadian period was explored in other studies, including Swarup et al. (1999). Crossing of parental lines illustrated large amounts of genetic variation not obvious in the parental lines, implying that natural selection had favoured combinations of alleles at different gene loci so as to attain certain phenotypes. These results again indicate natural selection on the circadian clock, which implies adaptive value.

The exact nature of the advantage conferred on plants by circadian rhythms was examined in a study by Dodd et al. (2005), who compared fitness of wild-type plants with long and short period circadian mutants in varying light environments. In each case, plants whose circadian clock matched the lighting phase of the environment contained more chlorophyll, fixed more carbon, grew faster and survived better than other plants, possibly the clearest evidence yet of the adaptive benefit of circadian control in plants.

1.3.5. Diurnal and circadian rhythms in trees

Forest trees, with their long generation time, sizable genomes, and large physical size do not make ideal model organisms for genetic study (Merkle and Dean, 2000). Recent progress, however, in completing the poplar genome sequence (Tuskan et al., 2006), and the announcement by the US Department of Energy (DOE) of a genome sequencing project for *Eucalyptus grandis* (www.jgi.doe.gov/News/news_6_8_07.html), as well as other advances in *in vitro* propagation techniques and gene transfer (Merkle and Nairn, 2005) mean that trees are becoming more amenable to molecular genetic research.

A prominent rhythm observed in forest trees, and initially the most studied, is diurnal change in tree stem diameter. These changes are largely attributed to diurnal changes in the water content of the stem and have been detected since the late 19th century (MacDougal, 1924; Stewart et al., 1973; Sevanto et al., 2003). Stem diameter is normally greatest preceding dawn, with a minimum occurring in the afternoon. The amplitude of the variation is fairly small, typically around 50 – 100 μm (Downes et al., 1999; Peramaki et al., 2001; Sevanto et al., 2003), and can be detected using point dendrometers, which measure tree radius by placing an electronic displacement transducer deep into the xylem and measuring change in voltage across the two sensors as they move in relation to each other.

While most change in diameter can be attributed to water relations within the stem, there is also an incremental daily increase as a result of the formation and expansion of new xylem and phloem cells being formed in the cambial zone. It is not clear at this point whether there is diurnal rhythmicity in the division and growth of new cells in the cambial zone, although studies in spruce trees (*Picea abies*) showed that xylem formation is significantly correlated with mean daily temperature (Makinen et al., 2003). The dominant role of water tension in affecting stem diameter was also proven by showing that timing of stem radius increases differed from timing of new xylem formation. Xylem tissue shows a seasonal rhythm in formation which is most pronounced in temperate trees, starting in spring and ceasing during winter (Makinen et al., 2003).

Radial stem measurements are further complicated as sap flow in xylem and phloem occur in opposite directions (xylem carries water up from the roots to the leaves, phloem transports sugars down from the source organs such as leaves, to sink organs such as the stem and roots), meaning each tissue has its own changes in diameter in various situations. It is

possible to measure the changes in diameter of xylem separately from the whole stem using point dendrometers, (Sevanto et al., 2003), but for more accurate measures, such as the production of new cambial initials, small increment core sampling coupled with examination under a stereo microscope can be used (Makinen et al., 2003). A more recent study looked much more finely at diurnal influences on wood properties and discovered diurnal differences in supply of cell wall components such as glucomannan and xylan to the inner surface of the cell wall (Hosoo et al., 2006). In S₂ layer-forming tracheids, cellulose microfibrils were evident during the light period, while an amorphous glucomannan/xylan-containing material was present at night, suggesting a diurnal rhythmicity in supply of matrix components to the developing secondary cell wall.

The effect of circadian rhythms on primary metabolism can be seen in carbon acquisition, which shows both daily and seasonal changes in most tree species examined, due to the influence of diurnal photosynthesis. In loblolly pine (*Pinus taeda*), needles show pronounced seasonal changes in carbohydrate content, including starch and sugars (Yang et al., 2002). Poplar studies also show diurnal variation in xylem sap composition, with major macronutrients reaching their highest concentration during the light period and decreasing to a minimum around midnight (Siebrecht et al., 2003).

Emission of secondary compounds such as volatile organic metabolites varies diurnally in several tree species including poplar. Isoprene is one such compound, and the gene coding for isoprene synthase, displays diurnal variation in expression (Loivamaki et al., 2007). The promoter region of this gene contains circadian-determining regulatory elements. Another tree with diurnal variation in volatile emissions is *Quercus* (Oak), which shows a daily rhythm in monoterpene emission (Bertin et al., 1997). Oak trees also show diurnal

changes in pigment and carotenoid production, with antioxidants being produced at their highest levels to coincide with high light intensity, possibly to serve a protective function (Garcia-Plazaola et al., 1999).

Long term seasonal changes are influenced by diurnal and circadian rhythms in trees. Photoperiodism has a well described role in trees, where it controls seasonal changes such as flowering and winter dormancy. These traits are under strong genetic control, as bud set, for example must be induced well before the risk of the first frost. *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) are necessary for the regulation of flowering in *Arabidopsis*, cycle with a diurnal rhythm, and act between the circadian clock and control of flowering (Suarez-Lopez et al., 2001). Poplar has orthologues of both *CO* and *FT*, which behave in a similar fashion in trees as they do in *Arabidopsis*, being diurnally regulated and controlling flowering time according to day length (Bohlenius et al., 2006). Orthologues of the *Arabidopsis* central oscillator genes *TOC1* and *LHY* have been found in Chestnut (*Castanea sativa*) where their mRNA abundance cycles circadianly (Ramos et al., 2005). The discovery that the circadian expression of *CsTOC1* and *CsLHY* is disrupted during winter dormancy led to the suggestion that cessation of cycling and constant high expression of these genes in winter could play a role in signalling the induction of dormancy. This is an aspect of circadian control that was unknown in *Arabidopsis*, which is an annual plant and does not undergo dormancy.

If circadian oscillators are active in woody tissues, could they remain entrained despite the fact that light cues would not easily reach them? One possible mechanism is suggested by the fact that vascular tissues can conduct light through vessels and fibre cell walls (Sun et al., 2003). The conducted light then ‘leaks’ out of the light conducting

structures into the living tissues, with far-red light being most efficiently transferred. Far-red light can then activate phytochrome photoreceptors, which are important input signals to the central circadian oscillators (Millar, 2004).

1.3.6. Evidence for circadian control of wood formation processes

While circadian rhythms have been characterised in detail in herbaceous plants such as *Arabidopsis*, very little investigation has been undertaken in woody plants such as trees to determine whether circadian rhythms of gene expression do occur in the tissues involved in wood formation. Some evidence suggests that there is circadian control of wood formation, the process of which is outlined below.

Growth in plants occurs at specialized centres called meristems, which contain undifferentiated dividing cells. In order to gain height and increase access to sunlight, trees grow upward towards their light source at the apical meristem (*phototropism*). As their height increases, a corresponding increase in girth is required in order to stabilize the plant. This thickening of the stem and roots in areas that are no longer elongating is known as *secondary growth* and results from the activity of two lateral meristems, the vascular and cork cambium – which are themselves derived from the procambium in the apical meristem (Li et al., 2006).

The vascular cambium contains fusiform initials that are responsible for fibre and vessel cell formation, the two main cellular components of angiosperm wood. Ray initials give rise to ray cells, which transport water and nutrients horizontally across woody tissues. Fusiform initials divide periclinally (to the front and back) to produce xylem and phloem mother cells and anticlinally (to the sides) to increase the circumference of the cambium. Xylem and phloem mother cells divide again to produce xylem and phloem cells, which then differentiate in an ordered sequence of partially overlapping events (Roberts and McCann,

2000). In xylem, this process gives rise to secondary xylem or wood, and is termed *xylogenesis*.

The process of xylogenesis involves five major steps, namely: cell division; cell expansion; cell wall thickening, programmed cell death and heartwood formation. Cell identity is determined positionally - phloem cells form to the outside of the cambium, and secondary xylem to the inside (Esau, 1954). Xylem mother cells divide more often than their phloem counterparts, meaning that xylem is four to ten times more abundant than phloem, and will eventually comprise 90% of a typical tree. Xylem contains one or more major cell types, depending on the tree species. Angiosperm xylem tissues contain water transporting *vessels* and structurally supporting *fibres*, while gymnosperm xylem contains only *tracheids*, which are responsible for both functions (Plomion et al., 2001).

Plant cell walls are highly specialised and diverse structures that set plant cells apart from animal cells. All plant cells have a wall, most of which is composed of a complex mix of polysaccharides derived from the glucose precursors produced in photosynthesis. The remainder of the wall is made up of many different proteins and aromatic substances (Scheible and Pauly, 2004). Cell walls are of crucial importance to studies on wood development, as they determine the cell's shape, and are therefore directly involved in the specialization of cell types. Xylem fibres, for instance, have cell walls that are thick and strong to provide mechanical support to the stem, and contain highly specialized, lignified secondary cell walls. It is the dead cell walls of the secondary xylem - the living protoplast having disintegrated during differentiation - which forms the bulk of wood. The plant cell wall is also the primary source of cellulose, the most abundant biopolymer on Earth, and the

raw material for paper, lumber, textiles and many other products, making it the fifth most important product of world trade (Plomion et al., 2001; Cosgrove, 2005).

Various approaches have been implemented to study wood development, with advances in genomics driving new discoveries. Gene expression profiling by microarray analysis has generated many candidate genes for controlling wood formation and wood properties, with studies in *Arabidopsis* plants induced for secondary growth (Chaffey et al., 2002; Ko and Han, 2004) complemented by studies in wood forming tree tissues (Hertzberg et al., 2001; Paux et al., 2004; Schrader et al., 2004; Andersson-Gunneras et al., 2006). Expressed sequence tag (EST) sequencing approaches have yielded insights into the transcriptome of woody tissues (Allona et al., 1998; Sterky et al., 1998; Sterky et al., 2004; Pavy et al., 2005; Foucart et al., 2006), and also provided many of the gene sequences later used for production of microarrays. Other large scale genomics approaches applied to the study of wood formation include serial analysis of gene expression (SAGE, Lorenz and Dean, 2002), cDNA-amplified length polymorphism (cDNA-AFLP, Prassinis et al., 2005; Ranik et al., 2005) analysis and differential display (Cato et al., 2006).

Diurnal rhythms in gene expression in wood-forming tree tissues and the role of circadian control therein has not been studied in any detail, although certain connections can be made between what is known about the circadian control of secondary cell wall genes in *Arabidopsis*, and the likely effect of light and circadian control on wood formation genes in trees. It is likely that at least some wood formation genes would be co-ordinated in a circadian manner, as the formation of secondary xylem is a process that could conceivably benefit from circadian control. Photosynthesis is one of the most strongly circadianly controlled processes in plants, with a large proportion of genes involved in the light-

harvesting reactions of photosynthesis under circadian control (Harmer et al., 2000), peaking at midday. Wood formation is a major sink for the products of photosynthesis, as the production of wood involves sucrose being converted to glucan chains and laid down as cellulose microfibrils (Plomion et al., 2001). By producing cellulose microfibrils at the appropriate time of day, trees could increase the efficiency with which they grow.

Evidence in support of this possibility was produced in a study by Harmer et al. (2000). Although this study was performed in *Arabidopsis*, its implications can be extended to trees, as *Arabidopsis* can be induced to form secondary xylem which is analogous in its anatomy to that of a young angiosperm tree (Chaffey et al., 2002). Harmer et al. found many genes regulated by circadian rhythms, the orthologues of which may play a role in wood formation in trees. As well as photosynthetic genes, several hexose transporters were circadianly regulated (Schaffer et al., 2001). These transporters could potentially carry sugars from source to sink tissues in *Arabidopsis* and tree species. Genes involved in the channelling of sugars into various pathways were also temporally regulated, as were genes encoding enzymes implicated in starch mobilization (Harmer et al., 2000; Schaffer et al., 2001).

Several genes involved in cell wall formation are regulated by circadian rhythms, such as the auxin efflux carriers *PIN3* and *PIN7*. Auxin promotes growth in plant stems and may regulate expansins, one of which was also under circadian control. Expansins are responsible for the loosening plant cell walls during cell growth (Cosgrove, 2000). Auxin itself is also circadianly regulated at all levels (Covington and Harmer, 2007), and due to its wide ranging physiological effects, may influence wood formation in a circadian fashion. Significantly, two cellulose synthases, responsible for synthesizing cell wall polysaccharides

were upregulated late in the night, along with a dTDP-D-glucose 4, 6 hydratase homologue, which has been implicated in cell wall biosynthesis (Harmer et al., 2000).

Lignins are complex phenolic polymers found embedded in the secondary thickenings of cell walls (such as those of xylem elements which form wood) where they provide compressive strength, and play an important role in plant development (Boerjan et al., 2003). In their review on the genetic control of lignin deposition, Rogers and Campbell (2004) use the findings of Harmer to argue that circadian control of lignin deposition would provide a way to redirect carbon flux into phenylpropanoid biogenesis at the appropriate points in the diurnal cycle. Furthermore, the gene *BREVIPEDICELLUS* has been postulated to alternately repress and enhance vascular differentiation depending on day length; with the interplay between day length and circadian control providing the context for its function (Rogers and Campbell, 2004).

Northern blot analysis of several genes involved in lignin biosynthesis in *Arabidopsis* has also shown significant changes in transcript abundance over 24-hours. Several of the genes show more than one peak in expression per day, with highs immediately prior to dawn as well as around four hours after light began (Rogers et al., 2005). When transferred to constant light, circadian cycling was especially apparent in *cinnamate-4-hydroxylase* (*C4H1*), *caffeate O-methyltransferase* (*COMT*), *caffeoyl-CoA O-methyltransferase* (*CCoAOMT1*), *cinnamoyl co-A reductase* (*CCRI*), and *cinnamoyl alcohol dehydrogenase* (*CAD6*), with a general peak during the subjective night.

1.4 METHODS FOR INVESTIGATING CIRCADIAN RHYTHMS IN GENE EXPRESSION

The identification of novel plant genes that are regulated in a circadian fashion has always been a challenging area of research for chronobiologists. Early circadian research in plants was characterized by labour intensive RNA blotting and nuclear run-on assays over long time series. This type of experiment would typically reveal very few genes, and involved great effort and expense (McClung, 2006). Technological advances have greatly accelerated the rate of gene discovery, with the aid of microarrays (Harmer et al., 2000; Matsumoto, 2006; Vitalini et al., 2006); enhancer trapping (Michael and McClung, 2003) and luciferase reporter gene/promoter constructs (Millar et al., 1992; Welsh et al., 2005). Other methods which have shown their utility in circadian studies include the PCR-based techniques suppression subtractive hybridization (SSH) (Rouyer et al., 1997), qualitative real-time reverse transcription PCR (qRT-PCR) (Sugden, 2003; Shih et al., 2005), and differential display (Kreps et al., 2000).

1.4.1. Microarray

Since the first microarray experiment was unveiled in a short article in Science magazine (Schena et al., 1995) their ability to monitor the expression levels of many genes simultaneously has been put to use in studying a wide range of biological issues. The power of the technology lies in the automation and miniaturisation of what is essentially a reverse Northern blot. There are two main kind of microarrays: the first consists of printed 'spots' representing individual genes and consisting of either denatured cDNA fragments or pre-synthesised oligonucleotides (usually 60-80mers), that are fixed onto a solid surface such as a glass slide and then probed with one or more fluorescently labelled cDNA samples. By

labelling separate cDNA samples with different fluorescent dyes, the strength of fluorescence at each wavelength for a point on the slide reflects the number of copies of a specific mRNA species present in each sample. The second major system is exemplified by the Affymetrix GeneChip, and consists of small DNA fragments (usually 25mers) which are chemically synthesised *in situ* by photolithography on a coated quartz surface, allowing densities of well over 1 million, and up to 6.5 million unique features per array (Huber et al., 2006).

Microarrays are the ‘ideal tool’ for analysing circadian gene expression (McDonald and Rosbash, 2001), as profiling of gene expression over several days will reveal oscillations pointing to clock control in output and central oscillator pathways. Add to this the fact that high density printing techniques allow millions of spots to be placed on a slide and it is apparent that clock outputs can be measured in a global manner. Studies of circadian regulation have used microarrays to examine the model species *Arabidopsis thaliana* (Harmer et al., 2000; Schaffer et al., 2001; Kim and von Arnim, 2006), *Drosophila melanogaster* (McDonald and Rosbash, 2001; Etter and Ramazwami, 2002; Matsumoto, 2006) mice (Panda et al., 2002; Oishi et al., 2005) and other species including dinoflagellates (Okamoto and Hastings, 2003; Van Dolah et al., 2007) and cyanobacteria (Kucho et al., 2005).

The model plant *Arabidopsis* has been the subject of several microarray based circadian studies. Estimates for circadian control of the transcriptome vary from 2 – 16%, with the largest values being the most recent estimates (Harmer et al., 2000; Edwards et al., 2006; Covington and Harmer, 2007). These figures are significantly lower than estimates put forward by alternative techniques such as enhancer trapping studies (See 1.4.3). It has been suggested that microarray studies are liable to underestimate circadian control, due to the use

of artificial cut-offs and conservative statistical models (Duffield, 2003). Stringent measures used to ensure fewer false positives is also known to exclude genuine cycling genes, which are weakly expressed, or do not have a typical cosine pattern.

Microarray experiments generate immense quantities of data, which must be carefully analysed before conclusions about expression levels can be made. After the data have been pre-processed by filtering and normalization, the simplest method to distinguish circadian control is to create a cut-off point for change in gene expression over 24 hours. Either a two-fold threshold or an experimentally supported threshold chosen by examining fold-change in known clock genes may be used. A more sophisticated method is to analyse patterns of gene expression independently of their amplitude and look for correlation with cosine test waves having periods of 20-28 hours. Slight changes in data analysis conditions can change the number of genes considered to be circadianly regulated, introducing some measure of subjectivity into the screening process. Genes having small amplitude oscillations in expression are still harder to detect, although this can be solved to some extent by employing greater biological replication.

Another complicating factor in circadian microarray studies is the heterogeneity of samples used for profiling. Many tissues studied are composed of several cell types, or even whole body parts such as fly heads. These structures are very complex, containing various cell types and tissues, meaning expression oscillations in one tissue may be obscured if the gene is constitutively expressed in other tissues, or is circadianly regulated with a different phase in different tissues (Duffield, 2003). This problem may be resolved by the use of laser capture microscopy to isolate single cell types or even individual cells. mRNA amplification is an associated technology, which makes it possible to obtain sufficient mRNA for a

microarray study from very small amounts of starting material (Nagy et al., 2005). The biggest limitation of microarray technology is that it only measures variation in steady state mRNA levels. Not all circadian control occurs at the level of transcription, as both post-transcriptional regulation (e.g. mRNA stability) and post-translational regulation (e.g. protein phosphorylation) have been shown to play a role in circadian rhythm (Lee et al., 2001).

1.4.2. Luciferase reporter constructs

The luciferase enzyme has been widely used as a reporter of circadian gene regulation (Millar, 1995; Welsh et al., 2005). The first published example using luciferase to monitor circadian rhythms was in *Gonyaulax*, a marine dinoflagellate exhibiting natural luciferase activity with a circadian periodicity (Hastings and Sweeny, 1958). Later, exogenous luciferase genes were introduced into several disparate species including cyanobacteria, insects, plants and rodents (Welsh et al., 2005).

The first use of luciferase constructs to study plant circadian rhythms involved the use of a fragment of the *CAB 2* promoter to drive *Luc* expression in *Arabidopsis* (Millar et al., 1992). It was soon shown that this system provided a non-invasive marker for temporal regulation allowing for large scale circadian clock mutant screens (Millar, 1995). Luciferase constructs were instrumental in deletion analysis studies defining the ‘evening element’ (EE: AAAATATCT) which gives evening-specific expression to most promoters in which it is found (Michael and McClung, 2002). Luciferase constructs have also been used to elucidate the various roles of the central clock gene *GIGANTEA* (Oliverio et al., 2007).

1.4.3. Enhancer trapping

Circadian regulation does not occur only at the transcriptional level, as both post-transcriptional and post-translational regulation are known to specifically influence clock

activity (Lee et al., 2001; Brunner and Schafmeier, 2006). Steady state mRNA levels, as measured by microarrays may give misleading results when mRNA stability obscures oscillations. Enhancer trapping is therefore used to examine circadian regulation at the level of transcriptional activity. This technology involves the use of transposons to randomly insert a luciferase reporter gene without a promoter, so that when the construct is inserted upstream of a circadianly regulated promoter its fluorescence will fluctuate with a circadian rhythm (Sundaresan et al., 1995).

Enhancer trapping has been used to establish widespread circadian control of gene expression in cyanobacteria (Liu et al., 1995), and proved effective in a study on *Arabidopsis* plants, with 36% of all enhancer trap lines showing circadian rhythmicity (Michael and McClung, 2003). This study indicated a greater role for circadian control of gene expression than given by microarray studies (Edwards et al., 2006; Covington and Harmer, 2007), possibly reflecting the limitations of the microarray system and its tendency to underestimate clock control. Enhancer trapping has the benefits of avoiding total mRNA sampling which may mask tissue-specific oscillations. In addition, the luciferase assay is highly sensitive, and can be used to measure rhythms over multiple days, making reliable identification of low-amplitude rhythms possible (Michael and McClung, 2003).

1.4.4. PCR-based techniques

The problems of specificity, sensitivity and reproducibility faced by microarray-based experiments due to the many steps involved in their production, can be avoided by using various highly accurate polymerase chain reaction (PCR) based methods. In most microarray studies, an alternative form of mRNA quantification such as quantitative real-time PCR (qRT-PCR) is used with a subset of genes in order to show that their expression profiles are

in agreement with the results obtained from the microarray (Rockett and Hellman, 2004). qRT-PCR is well-suited to validating DNA array results due to its quantitative nature; the speed at which results can be obtained; and because it requires much less RNA than methods such as Northern blots (Rajeevan et al., 2001).

The use of qRT-PCR in plant studies is well-established, having been used for the detection and quantification of foreign DNA (e.g. pathogenic microorganisms); quantification of specific transcripts such as a multi-gene family; as well as confirming microarray data. A common strategy in microarray studies is to identify genes of interest (using full genome chips) and then to confirm the expression of these genes using qRT-PCR. qRT-PCR is based on recording the increase in number of a specific DNA sequence during PCR amplification by the detection of light emitted by gene specific probes, or intercalating agents. By recording the amplification in real time during the log linear phase, rather than at the saturated endpoint of the reaction, the amount of starting material can be determined accurately (Gachon et al., 2004). The high sensitivity of RT-PCR avoids a problem inherent to many microarray experiments, where highly related gene family members may cross-hybridise on the chip leading to false results.

Differential Display (DD) is a PCR based method that can be used to amplify low abundance transcripts, with 80 - 120 primer combinations sufficient to cover all transcripts in a cell (Liang and Pardee, 1992). The process involves amplification of partial cDNA sequences from mRNA subsets by reverse transcription and PCR, which are then displayed on a sequencing gel. mRNA from several samples of interest can be run side by side and compared, before individual bands are cloned and characterised. Differential display has been used to identify clock controlled genes in the African clawed frog *Xenopus laevis*

(Green and Besharse, 1996). Fluorescent differential display (FDD) represents an improvement on the original DD, allowing analysis to be performed on a DNA sequencer, allowing higher throughput. FDD has been used to identify circadian regulation in *Arabidopsis* (Kreps et al., 2000).

Suppression subtractive hybridisation (SSH) can be used to identify, clone and characterise genes that are differentially expressed between samples (Diatchenko et al., 1996). This is achieved by selectively amplifying the differentially expressed cDNA fragments and simultaneously suppressing those that are expressed constitutively. SSH is useful for functionally enriching cDNA libraries for microarray preparation (Ando et al., 2004; Van den Berg et al., 2004) and has also been used for identifying circadianly regulated genes (Diatchenko et al., 1996; Rouyer et al., 1997). Rouyer et al. used the known phase of a cycling gene *period* (*per*) to create cDNA libraries from the peak and trough of *per* expression and then subtract them to create a library enriched for *per* cDNA, and other transcripts with a similar phase. Hybridising the subtracted cDNA with both subtracted and non-subtracted cDNA revealed clones showing differences in expression between the two time points, and led to the discovery of a novel circadianly regulated gene *Crg-1*.

1.4.5. Future directions

Perhaps the next technological advance in genome-wide circadian research will emerge out of the rapid increase in sequencing potential at reduced costs. The latest generation of pyrosequencing and sequence-by-synthesis machines such as those made by 454 Life Sciences (now owned by Roche) and Solexa (now owned by Illumina) allow the tracking of nucleotide bases as they are added to a growing DNA strand. A promising method involves pyrosequencing, where the addition of a nucleotide to the DNA chain triggers the release of

pyrophosphate, a compound which induces luciferase to release a flash of light. Hundreds of thousands of growing chains are tracked by a sophisticated camera and computer software, allowing elucidation of large amounts of sequence data. 454 Sequencing has already been put to the test in several applications, including the sequencing of complete bacterial genomes and plant ESTs (Cheung et al., 2006). Solexa sequencing, which uses DNA strands attached to a glass slide and standard microarray optics to detect growth of the DNA strands has been used to generate high resolution maps of histone methylation in the human genome (Barski et al., 2007).

Soon it may be possible to obtain the entire transcriptome of tissue samples collected at different times of day, allowing the highest possible resolution of circadian control at a transcriptional level. This kind of advance does not, however, change the basic shortfall of examining rhythms at a transcriptional level only, and advances in proteomics and metabolomics will need to be integrated with transcriptome data in order to gain a fuller picture of all the roles of the circadian clock.

1.5 CONCLUSIONS

Since the development of high-throughput techniques for performing transcriptome analysis, the study of circadian rhythms has been transformed from a primarily descriptive science to an experimental one. Studies using technologies and techniques such as microarrays and promoter/reporter fusions in model organisms with sequenced genomes, are unravelling the complicated web of interactions that make up circadian regulation. Many novel genes are being shown for the first time to cycle in expression with a diurnal or circadian rhythm. Some of these genes show no homology to known proteins or have functions that remain to be

elucidated. Questions still need to be answered in well-described species such as *Arabidopsis* - where the biochemical functions of several known circadian genes are yet to be uncovered. In less well-described species there are more basic questions to answer concerning the presence, identity and influence of circadian clock genes.

Circadian rhythms have been shown to be associated with fitness, and circadian gene expression plays an important role in controlling metabolism, by temporally separating certain antagonistic chemical reactions; anticipating regular changes, and co-ordinating enzyme production with the appropriate time of day. Wood formation is a complex metabolic and developmental process, aspects of which may be under circadian control. Investigating such a possibility could lead to a better understanding of the link between primary metabolic tasks such as photosynthesis and the production of sugars, and secondary metabolism in the form of secondary cell wall thickening and xylogenesis. So far, little is known as to when these events occur in relation to each other, and how each influences and is influenced by the other. Uncovering the expression patterns of diurnal and circadianly regulated genes in trees is of interest as enhanced knowledge of wood formation is necessary for cheaper wood products and sustainable energy production through cellulosic ethanol. A high-throughput approach to studying temporal gene expression in *Eucalyptus* xylem promises to identify circadian and diurnally regulated genes involved in carbon allocation and carbohydrate metabolism in wood.

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CHAPTER 2

A WOODEN CLOCK: DIURNAL AND CIRCADIAN GENE REGULATION IN *EUCALYPTUS* XYLEM

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2.1 ABSTRACT

The daily cycle of night and day affects the behaviour and physiology of almost all living things. At a molecular level, many genes show daily changes in expression levels. To determine whether such changes occur in wood forming tissues of *Eucalyptus* trees we used a cDNA microarray to examine gene expression levels at roughly four hour intervals throughout the day. Almost ten percent of genes on the microarray (217 out of 2608) showed diurnal expression profiles. Affected genes included those involved in carbohydrate metabolism, hormone signalling, transcription regulation and wood formation. *Eucalyptus* orthologues of the central clock genes *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *GIGANTEA (GI)* were expressed in differentiating xylem tissues, and cycled with a circadian rhythm. The endogenous nature of these rhythms was confirmed under controlled light conditions. The presence of a functional biological clock and extensive diurnal effects in woody tissues suggested a strong role for circadian control over xylem development and metabolism. Our study adds a temporal component to knowledge of the genetic control of wood formation processes. Spatial variation in gene expression has been well described in the woody tree stem. Less is currently known about temporal variation, which therefore has the potential to reveal novel insights into carbon allocation and xylogenesis.

2.2 INTRODUCTION

Eucalyptus is a large genus of tree species, many of which are of great economic importance, being widely grown in plantations for solid timber and pulp for the paper industry (Eldridge et al., 1994). The significance of *Eucalyptus* is likely to expand in the near future to include a role in the renewable energy economy for production of cellulosic ethanol. Understanding the

mechanisms determining the wood properties and superior growth of *Eucalyptus* tree species is therefore of interest. Many metabolic processes in trees are influenced directly or indirectly by the photoperiod, as temporal changes in light and temperature lead to *diurnal* or daily rhythms in physiology. Not all diurnal rhythms are generated in direct response to the photoperiod. A subset of diurnal rhythms are endogenously driven by the *circadian* clock, in an evolutionary adaptation to the daily rhythm of night and day (Pittendrigh, 1960). The circadian clock is an internal biochemical oscillator with a period of approximately 24-hours. It allows anticipation of daily changes and co-ordinates the internal environment with external conditions to provide an adaptive benefit (Dodd et al., 2005).

Circadian rhythms typically originate with a small number of genes connected by feedback loops, whose transcript abundance cycle over a 24-hour period (Dunlap, 1996; Zeilinger et al., 2006). These genes form the ‘central oscillator’ of the circadian clock, and are able to maintain their rhythm over several cycles, even when removed from environmental signals (Schibler and Naef, 2005). Input to the oscillator is via phototransduction pathways, which ‘fine tune’ the clock and keep it in phase with the external light-dark cycle (Millar, 2004). Output pathways in turn link the entrained clock to the gene expression rhythms and biological processes that it controls (Yakir et al., 2007).

Trees, like other plants, show both diurnal and circadian rhythms in physiology and gene expression that influence growth and development (Rogers et al., 2005; Bohlenius et al., 2006). Primary metabolism is directly influenced by diurnal changes in light availability for photosynthesis. This is reflected in one of the first diurnal rhythms to be studied in forest trees - daily changes in tree stem diameter (MacDougal, 1924; Stewart et al., 1973; Peramaki et al., 2001; Sevanto et al., 2003). Changes in stem diameter are caused primarily by changes

in water tension caused by transpiration, but are additionally affected by diurnally produced sugars that influence the solute concentration of tree sap. The sugar concentration of sap affects its flow, water movement through the stem and ultimately stem diameter (Stewart et al., 1973; Sevanto et al., 2003). The chemical composition of sap also varies predictably during the day, with macronutrient concentrations peaking in daylight and dipping at night (Siebrecht et al., 2003).

Secondary metabolic pathways in plants are also affected by daily rhythms. The production of secondary metabolites such as the volatile organic compounds monoterpene and isoprene are under both diurnal and circadian control (Pio et al., 2005; Loivamaki et al., 2007). Phenylpropanoid biosynthesis and lignin production are also temporally controlled (Harmer et al., 2000; Rogers et al., 2005). Long lasting seasonal changes, such as dormancy and flowering which occur over weeks or months are additionally controlled by the circadian clock. These processes are crucial for the survival of long-lived woody plants and are regulated by interaction between the circadian clock and changes in day length (Ramos et al., 2005; Bohlenius et al., 2006).

The circadian system in trees appears to be similar to that of the model plant *Arabidopsis thaliana*. Orthologues of *Arabidopsis* clock genes have been identified in chestnut trees (*Castanea* spp.), where their mRNA abundance cycles circadianly (Ramos et al., 2005). In *Populus*, the roles of orthologues to the *Arabidopsis* clock genes *CCA1*, *LHY* and *TIMING OF CAB 1 (TOC1)* are currently being investigated by Maria Eriksson and others (Busov and Tsai, 2007). Conservation of circadian processes also extends to transcriptional networks and temporally controlled biological processes that are conserved between *Arabidopsis* and poplar (Michael et al., 2008).

The proportion of tree genomes that show either circadian control or diurnally influenced expression is currently unknown. The best estimate of temporal control of tree genes might be gained by comparing them to *Arabidopsis*, which has the best described plant circadian system. Original estimates for circadian control of the *Arabidopsis* transcriptome were based on microarrays containing ~8000 elements and ranged from two to six percent of all genes (Harmer et al., 2000; Schaffer et al., 2001). More recent studies making use of whole-transcriptome arrays have increased that estimate to between 10 and 16% (Edwards et al., 2006; Covington and Harmer, 2007), similar to estimates in other model eukaryotes (Duffield, 2003).

Enhancer trapping offers an alternative to transcript profiling methods such as microarrays, which more directly addresses circadian regulation of transcription. Results from enhancer trap lines suggest that as much as 36% of the *Arabidopsis* transcriptome may in fact be under circadian regulation (Michael and McClung, 2003). If one includes non-circadian, diurnally influenced genes, up to half of the *Arabidopsis* transcriptome is affected by temporal variation (Schaffer et al., 2001; Blasing et al., 2005). When examined over a wide range of photo- and thermocycles, almost 90% of *Arabidopsis* genes cycle in transcript abundance (Michael et al., 2008). Temporally influenced genes do not only respond to light, as many of them are also influenced by cellular sugar concentration, hormone and nitrogen levels, as well as stress and other factors (Blasing et al., 2005).

The adaptive benefits of circadian control have been illustrated in *Arabidopsis*, where circadian mutants are demonstrably less fit than their wild-type counterparts (Green et al., 2002). This is largely due to the photosynthetic advantage gained by matching the circadian period with the external light-dark cycle (Dodd et al., 2005). In addition to photosynthesis,

many plant metabolic functions are modulated by diurnal and circadian rhythms, including starch and sucrose metabolism; nutrient uptake; amino acid and lipid metabolism; glycolysis and gluconeogenesis (Blasing et al., 2005). The temporal regulation of these functions may provide a competitive advantage due to clock controlled biology being kept in phase with external day-night cycles leading to enhanced growth (Dodd et al., 2005).

Woody plants stand to benefit even more from circadian co-ordination than herbaceous plants such as *Arabidopsis*, due to the distance between their sink and source organs, as well as their long lifespans which encompasses multiple changes of season. Wood formation and other carbon allocation pathways in trees could benefit from circadian control if temporal regulation of these processes led to enhanced energy efficiency and ultimately improved growth.

To develop new forest tree varieties as improved wood and pulp producers or economically viable bioenergy crops, a more comprehensive understanding of tree growth and development is required. An important aspect of this is describing the dynamics of carbon allocation to various sink organs of the tree; and to the different components of the plant cell wall. This study was undertaken in order to gain insight into diurnal rhythms in gene expression related to carbon allocation and cellulose/wood production in fast-growing *Eucalyptus* hybrid clones. These processes are central to the dual roles of *Eucalyptus* as a pulp/timber producer and potential bioenergy crop. The study was based on a spotted cDNA array and revealed quantitative changes in gene expression over the course of a single day. Many of the affected genes were involved in wood formation and carbon allocation processes and may reveal new insights into temporal regulation of these metabolic functions.

2.3 MATERIALS AND METHODS

2.3.1. Sampling of plant material

Tissue samples were collected for a diurnal time series extending over three days at the end of August 2004. Samples were collected from a forestry field-trial of two three-year-old hybrid *Eucalyptus* clones (*Eucalyptus grandis* × *E. camaldulensis*, ‘GC’ and *E. grandis* × *E. urophylla*, ‘GU’, Sappi Forests). The field-trial was situated near the town of KwaMbonambi in sub-tropical northern Kwazulu-Natal, South Africa. Soft, non-fibrous differentiating xylem tissue was collected by peeling the bark off the stems of standing trees, from one to two meters above ground level, and lightly scraping the exposed xylem. Stems were debarked in sections to avoid wounding-related gene expression and samples from the entire circumference of the trunk were bulked to avoid positional effects. Samples were collected at approximately four-hour intervals from one GC and one GU ramet for a total of 40 hours. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Differentiating xylem tissue was also collected from potted ramets of the same two genotypes as the field-grown trees. Potted ramets were grown outdoors in soil-filled pots until they reached approximately 1.5 m in height before being moved into a growth room under controlled light conditions. Plants were entrained to a 12 hr light / 12 hr dark cycle (LD) at a light intensity of around $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ for a total of three weeks, after which sampling began at first light, continuing for 72 hrs at six-hourly intervals. Growth conditions were switched to continuous light (LL) at dawn on the second day of sampling to allow elucidation of circadian patterns in transcript abundance. Sampling followed the model of the field-grown trees on a smaller scale: stems were severed at ground level, bark removed from approximately one centimetre to one meter above ground level and a scalpel blade used to

scrape immature xylem tissue from the stem. Tissues were immediately frozen in liquid nitrogen and stored in the same fashion as field-grown samples.

2.3.2. Experimental design, target preparation and microarray analysis

Two separate microarray experiments were performed using RNA from either GU or GC trees. A loop design was used for each experiment, linking six time points spread from 06:00 on day one to 02:00 the following day. A dye swap was incorporated to eliminate dye bias. The loop design allowed gene expression at each time point to be compared to all other time points. A total of 24 microarray slides were hybridised to complete the two loops.

Total RNA was isolated from woody tissues using the cetyl trimethylammonium bromide (CTAB) based method of Zeng and Yang (2002) with the following adaptations: for field-grown samples, 20 ml of extraction buffer were used with four grams of ground tissue; for samples from potted plants, one gram of tissue was extracted with 10 ml of buffer. Field-grown tissue samples were ground to a fine powder in liquid nitrogen using a high-speed grinder (IKA-Werke, Staufen, Germany), while the potted plant samples were ground using a pestle and mortar with liquid nitrogen. All centrifugation steps were performed at 12000 x g. Isolated total RNA was purified using the RNeasy kit (Qiagen Inc., Valencia, CA) and analysed for purity and quantity on a Nanodrop spectrophotometer (Nanodrop Technologies ND 1000, DE, USA) and on 1% agarose gels.

Aminoallyl-labelled cDNA was prepared for hybridisation to microarray slides by reverse transcription from 15 µg total RNA, using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) primed with 1 x random hexamer primers (Roche Diagnostics). cDNA was purified and labelled with either Cy3 or Cy5 dye (Amersham Biosciences, Piscataway, NJ), and excess dye molecules removed using a PCR Purification Kit (Qiagen

Inc., Valencia, CA) according to The Institute for Genome Research (TIGR) protocol #M004 (<http://pga.tigr.org/sop/M004.pdf>). Dye incorporation was measured using the ‘microarray’ function on the Nanodrop spectrophotometer. The two samples for each slide were then combined so that each contributed an equal quantity of dye. Mixed samples were dried to completion in a vacuum centrifuge (ThermoSavant VLP80, NY, NY). Dried probes were re-suspended in 60 µl of a 1 x hybridisation buffer (50% formamide, 5 x Saline Sodium Citrate (SSC), 0.5% Sodium Dodecyl Sulfate (SDS), 5 x Denhardt’s Solution, 0.5 µg/µl Poly d(A) and 0.5 µg/µl calf thymus DNA), denatured for three minutes at 95°C and cooled on ice before being applied to a pre-hybridised slide and covered with a cover-slip.

Microarray slides were pre-hybridised in a 1 x pre-hybridisation buffer (5 x SSC, 1% Bovine Serum Albumin (BSA), 0.1 x SDS) for 45 min at 42°C, rinsed twice in Ultra High Quality (UHQ) H₂O; once in isopropanol, and dried by centrifugation. Prepared slides were placed in hybridisation chambers and incubated for 18-20 hrs at 42°C in a water bath. Post-hybridisation washes were performed in three wash solutions of increasing stringency (Wash 1: [1 x SSC, 0.2% SDS at 42°C]; Wash 2: [0.1 x SSC, 0.2% SDS at room temperature]; Wash 3: [0.1% SDS at room temperature]). Washes 1 and 2 were performed once each, while Wash 3 was performed three times. Slides were dried by centrifugation and scanned within the hour.

Microarray analysis was performed on a spotted cDNA array previously described by Kirst et al. (2004). Briefly, the array consisted of 2608 cDNA fragments derived from *E. grandis* and *E. tereticornis* cDNA libraries. Spots were replica printed four times on each slide. Printed microarray slides (Corning Gap II, Corning, NY) were purchased from ArrayXpress (Raleigh, NC, USA).

2.3.3. Data treatment and statistical analysis

Slides were scanned on a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA). Laser and photomultiplier settings were adjusted to obtain a signal ratio of 1:1 between the two dyes. Image analysis was performed with GenePix Pro 5.0 (Axon Instruments, Foster City, CA). Intensities were calculated for each spot, and spots affected by dust specks or high local background were manually flagged and removed from subsequent analysis.

Raw signal intensity values were transformed (\log_2) and the four replicate spots for each target on a single slide were averaged. The transformed and averaged data were then imported to JMP Genomics V2.04 (SAS Institute, Cary, NC) for global normalisation and gene modelling. First, a mixed model analysis of variance (ANOVA, Wolfinger et al., 2001) was performed using PROC MIXED, to account for experiment-wide variation associated with ‘time’, ‘array’ and ‘dye’ effects as well as ‘array’ x ‘dye’ interaction. Residual values from this model were then used as inputs for global Local Estimate of Scatterplot Smoothing (LOESS) normalisation.

The normalised residuals were then analysed in a second, gene-specific, mixed model ANOVA using PROC MIXED, where the effect of ‘time’ on the expression of every gene was analysed individually, and least square means (LSM) estimates calculated. LSM estimates of gene expression were calculated using data from both genotypes (GU and GC) in order to identify conserved components of diurnal and circadian regulation in *Eucalyptus* trees. P-values were calculated for the effect of ‘time’ on every gene, both within each genotype, and across both genotypes. A gene was considered to be differentially expressed (diurnally responsive) if the effect of ‘time’ was significant in at least one of the time points

relative to all other time points ($-\log_{10}P > 3.74$). False Discovery Rate (FDR, $\alpha = 0.01$) was used for multiple testing correction.

2.3.4. Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis

qRT-PCR reactions were performed with the LightCycler® 480 instrument and LightCycler® 480 SYBR green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Each reaction contained 5 μ l of 2 x SYBR Green, 0.4 mM forward and reverse primers and 1 μ l template cDNA in a final volume of 10 μ l. All PCR reactions were carried out in triplicate, and amplification of a single product confirmed by melting curve analysis and agarose gel electrophoresis. No Template Controls (NTC) were included for every primer pair as an additional quality control check. Raw qPCR data quality control, gene-specific PCR efficiency correction and multiple reference gene normalisation (see below) with an adapted delta-delta-Ct method were performed using qBase software (Hellemans et al., 2007). Graphs were generated in Microsoft Excel using the 'smoothed line' option.

Gene specific primers for qRT-PCR analyses were designed using Primer Designer 4 (Scientific and Educational Software, Cary, NC). Four targets found to be differentially expressed by microarray analysis were selected for confirmation by qRT-PCR. The targets included a β -amylase (GenBank Acc: CB968031); a cinnamyl-alcohol dehydrogenase (GenBank Acc: CD669587); an endo-1, 4- β -glucanase (GenBank Acc: CB967687) and a UDP-D-glucuronic acid decarboxylase (GenBank Acc: CD669965) gene. Other genes profiled using qRT-PCR included the *Eucalyptus* cellulose synthase genes *EgCesA1-7* using previously developed primers (Ranik and Myburg, 2006), and the *Arabidopsis* central clock orthologues *EtCCA1* (*Eucalyptus tereticornis* CIRCADIAN CLOCK ASSOCIATED 1,

GenBank Acc: CD668918) and *EgGI* (*Eucalyptus grandis* GIGANTEA, GenBank Acc: DN596733). Four genes were chosen as control genes due to their constitutive expression in either the microarray results or previous studies in our lab. These included a *Eucalyptus* ADP Ribosylation Factor (GenBank Acc: AY770746); an α -*TUBULIN* (GenBank Acc: U37794); a fructose bisphosphate aldolase (GenBank Acc: CD669023) and a 26S proteasome regulatory subunit (GenBank Acc: CD668395). All primer sequences are recorded in Table S.1.

2.3.5. Gene ontology

Functional categorization of all genes on the microarray slide, as well as the diurnally responsive set was performed on TAIR (The *Arabidopsis* Information Resource: www.Arabidopsis.org/tools/bulk/go/index.jsp) using Gene Ontology (GO) slim terms for annotation. Statistical significance of enrichment was assessed using FuncAssociate (<http://llama.med.harvard.edu/Software.html>), which computes the probability of finding a certain number of significant genes given the starting list, using Fisher's Exact Test (Berriz et al., 2003).

2.4 RESULTS

2.4.1. Microarray hybridisation and expression profiling of field-grown trees

To identify diurnally influenced wood formation and carbon allocation genes, we sampled xylem at four-hourly intervals from field-grown trees. Labelled cDNA derived from total RNA from six consecutive time points was used to probe the microarray slides, which included 2608 gene fragments from *Eucalyptus* xylem, leaf, petiole, root and flower cDNA libraries (Kirst et al., 2004). Expression levels across the six time points were compared using a microarray loop design incorporating a dye swap (Figure 2.1).

After removal of low quality and manually flagged spots, normalisation and gene modelling, 217 cDNA targets were discovered to be significantly differentially expressed at one or more of the six time points. Eight out of the 217 targets were marked significant in both genotypes individually, but not when the two genotypes were considered together, indicating genotype-specific profiles. The remaining targets were significant in one (96/217) or both (111/217) of the genotypes. Std lsmean intensity values were calculated using data from both clonal genotypes in order to identify conserved rhythms. Significance levels were determined based on a False Discovery Rate of 0.01 (1% false positives), which corresponded to a negative $\log_{10}P$ -value of at least 3.74 at one or more of the six time points. No arbitrary expression level or ratio cut-off values were imposed, making the results independent of signal strength and profile amplitude. Expression ratios between the highest and lowest expression levels for the significant genes across the microarray experiment ranged from 2.7-fold to over 30-fold (results not shown).

The 217 targets represented 8.3% of the genes on the array and corresponded to 195 unique BLAST hits to *Arabidopsis* gene IDs. Thirty-six of the 195 unique gene models (18.5%, Table 2.1. marked *) are circadianly regulated in *Arabidopsis* according to a recent genome-wide study (Covington and Harmer, 2007). Peak/trough expression times for the 217 targets were distributed across all time points, with Zeitgeber Time 16 (ZT 16, 22:00) being best represented for peak expression (61 genes) and ZT 12 (18:00) having the fewest genes peaking (12 genes, Figure 2.2A,B). Zeitgeber Time measures hours since the last dark/light switch. Sunrise occurred close to 06:00 in our sampling location, and day length was close to 12 hours, with sunset occurring at around 18:00. The majority of genes on the microarray did not show significant diurnal changes in expression (Figure 2.2C).

The 217 cDNA targets found to show significant diurnal variation were clustered based on the similarity of their expression patterns (Figure 2.3A), and 10 broad gene expression clusters were defined (Figure 2.3B). Each cluster showed a distinct expression profile, containing either one or two peaks in expression.

2.4.2. Confirmation of microarray gene expression profiles by qRT-PCR

To confirm the accuracy of the microarray results, the expression levels of five genes selected from the microarray were profiled using qRT-PCR analysis. The same tissue samples profiled in the microarray experiment were used for the qRT-PCR profiling. Normalised qRT-PCR expression profiles of the five test genes closely followed those determined by the microarray analysis (Figure 2.4). The *Eucalyptus* orthologue of the *Arabidopsis* central clock oscillator gene *CCA1* was among the five genes whose microarray results were verified by qRT-PCR profiling (Figure 2.4E). Its diurnal expression pattern in *Eucalyptus* xylem closely matched that seen for *AtCCA1* in *Arabidopsis* with a morning peak in expression (Wang and Tobin, 1998), further supporting the microarray results.

2.4.3. Functional characterisation of diurnally responsive genes

The diurnally responsive gene set included representatives from many different metabolic pathways (Table 2.1). To determine which subcellular compartments and molecular functions were over-represented in the diurnally responsive set, we classified all 195 significant genes by their associated GO terms. GO terms found in the diurnally responsive genes were contrasted with the GO terms of all genes present on the microarray slide. FUNCASSOCIATE, a web-based tool used to characterise gene sets by GO attributes (Berriz et al., 2003) showed ‘response to stress’, incorporating ‘response to temperature’ (GO Attributes 0006950 and 0009266) to be significantly over-represented ($P = 1.2 \times 10^{-4}$, Figure

2.5). Included among the stress response genes were several coding for heat shock proteins (Table 2.1). ‘Cellular biosynthesis’ (GO Attribute 0044249) was also over-represented ($P = 2.4 \times 10^{-4}$).

The golgi apparatus and ‘extracellular’ compartments showed the largest proportional changes between the diurnal set and whole slide, but were not designated significant by FUNCASSOCIATE. Response to stress, along with response to abiotic/biotic stimulus were the functions with the largest proportional changes in representation, followed by electron transport/energy pathways and developmental processes (Figure 2.5).

Twenty-eight of the diurnally responsive genes are likely to be involved in starch/sucrose metabolism, including genes involved in the production and modification of cellulose and hemicellulose. Fourteen of these (7% of all diurnally influenced genes, Table 2.1) are classified as carbohydrate active enzymes, or CAZymes (Geisler-Lee et al., 2006). Of the 14 CAZymes found to be diurnally influenced in *Eucalyptus* xylem, five were glycosyl transferases (GT), eight were glycosyl hydrolases (GH), and one was a carbohydrate esterase (CE).

2.4.4. Expression profiling of central clock orthologues in *Eucalyptus* xylem

Three *Eucalyptus* genes showing homology to known *Arabidopsis* clock genes were analysed in our study. The *Eucalyptus* orthologue of the central clock gene *CCA1* (*EtCCA1*) was initially identified as diurnally expressed from the microarray results (Figure 2.6A). The cDNA represented by the microarray target (GenBank Acc: CD668918) originated from a *Eucalyptus tereticornis* flower cDNA library, and showed greatest similarity to the *CCA1* protein of *Arabidopsis* (At2g46830, BlastX $E = 1 \times 10^{-8}$).

The second central clock gene was not present on the microarray slide, but had previously been found to be expressed in *Eucalyptus grandis* xylem (Ranik et al., 2005). The sequence of the Transcript Derived Fragment (TDF, GenBank Acc: DN 596733) showed strong similarity to the central clock gene GI from *Arabidopsis* (BlastX $E = 3 \times 10^{-20}$).

The *Eucalyptus* orthologue of *Arabidopsis* clock-related gene *Zeitlupe* (*ZTL*) was also identified as a diurnally expressed gene from the microarray results. Its corresponding microarray target (GenBank Acc: CD668500) was originally from a *Eucalyptus tereticornis* capsule cDNA library, and it showed strong similarity to the *ZTL* protein of *Arabidopsis* (At5g57360, BlastX $E = 4 \times 10^{-53}$). The three *Eucalyptus* clock gene orthologues are herein referred to as *EtCCA1*, *EtZTL* and *EgGI*, to indicate their origins, although it should be noted that the corresponding *E. grandis*, *E. urophylla* and *E. camaldulensis* genes were actually profiled in the GU and GC hybrids.

To determine and confirm the activity and phase of *EtCCA1* and *EgGI* in *Eucalyptus* xylem, we profiled their expression patterns in GU and GC using qRT-PCR. Immature xylem samples from the field-grown trees and the potted ramets grown in controlled light conditions were examined. Cosine shaped fluctuations of transcript abundance were observed in the mRNA levels of both genes in light/dark conditions (LD, Figure 2.6 A,C) as well as in constant light (LL, Figure 2.6 B,D), indicating the endogenous nature of their oscillations. The expression patterns of *EtCCA1* and *EgGI* matched that of their *Arabidopsis* counterparts, with *EtCCA1* expression peaking at dawn (ZT 0) and *EgGI* peaking between midday and dusk (ZT 6-12, Wang and Tobin, 1998; Fowler et al., 1999; Alabadi et al., 2001).

EtZTL mRNA abundance showed one of the largest diurnal changes of all genes on the microarray (26-fold, results not shown). The expression fluctuation of the gene had a

cosine shaped profile which peaked at ZT 9 (15:00, Figure 2.7), approximately the same time as *EgGI*. Interestingly *ZTL* mRNA does not cycle in abundance in *Arabidopsis*, but displays circadian changes in protein abundance (Kim et al., 2003).

2.4.5. Metabolic pathway analysis

The transcriptome data generated by the microarray experiment were grouped by metabolic pathway to enable visualisation of the extent of diurnal regulation of each pathway. Possible diurnal regulation of genes was observed in the carbohydrate metabolism pathways centred on starch and sucrose metabolism (Figure 2.8A). Hierarchical clustering of these genes revealed temporal co-ordination of various processes (Figure 2.8B). Genes showing high midday expression included β -amylase (At3g23920) and pyruvate kinase (At3g52990). Sucrose synthase (At5g20830) and UDP glucuronic acid decarboxylase (At2g28760), are responsible for providing carbohydrate building blocks for cell wall formation, and showed diurnal profiles with night-time peaks. High morning expression was most clearly seen in pyruvate kinase (At3g52990), which had an additional midday peak; and xyloglucan endo-transglycosylase (At1g14720), which peaked from night-time until early morning.

Co-regulation of diurnal expression was evident in key lignin biosynthesis genes, as has previously been reported in *Arabidopsis* (Harmer et al., 2000). Five lignin biosynthetic genes (Table 2.1) present on our microarray showed similar transcript abundance profiles, with daily fluctuations in mRNA abundance of up to ten fold (Figure 2.9, fold changes not shown). Two major peaks in expression occurred in this cluster, the first in the hours before dawn and the second during the mid-afternoon. Lowest expression was typically seen close to midday.

2.4.6. Diurnal and circadian regulation of cellulose synthase genes

Diurnal variation in mRNA levels was profiled for all seven known *Eucalyptus* cellulose synthase (CesA) genes (Ranik and Myburg, 2006) in both field-grown and controlled light samples from the GU and GC genotypes (Figure S.1). Temporal variation in expression was evident, with all CesA genes showing two to five fold changes from lowest to peak expression over 24-hours in the field-grown samples. CesA genes in controlled light samples showed even higher fold changes, supporting the idea that the superimposition of diurnal rhythms may result in reduced circadian signal (Michael et al., 2008). The large changes in expression level seen in the CesA genes did not, however, follow a recurring diurnal profile (Figure S.1). An exception was *EgCesA6* which peaked at dawn or dusk (GC and GU respectively, details below).

Despite lacking regular and recurring diurnal profiles, several of the CesA genes showed evidence of temporal co-regulation, when results from a single genotype and experiment (field grown or controlled light) were considered. Temporal co-regulation was clearly observed in the secondary cell wall related CesA genes (*EgCesA1-3*) whose expression profiles were highly correlated within experiments (avg $r^2 = 0.73$ in GU, 0.92 in GC, Table 2.2). In contrast, the primary cell wall related CesA genes (*EgCesA4-7*) did not share similar expression profiles (avg $r^2 = 0.22$ in GU, 0.31 in GC, Table 2.2), and correlation between primary and secondary cell wall related CesA genes was also low (avg $r^2 = 0.20$ in GU, 0.24 in GC, Table 2.2). CesA genes showed genotype-specific expression profiles, with the average r^2 value for each CesA gene in GU and GC only 0.07 (results not shown).

Growth in controlled light conditions revealed that *EgCesA6* may be under circadian control, with regularly timed peaks in expression under constant light. Interestingly, *EgCesA6*

expression profiles in the GU and GC genotypes were in opposing phases to one another (Figure 2.10), unlike the central clock oscillators *EgGI* and *EtCCA1*, which remained in phase in the two genotypes tested.

2.5 DISCUSSION

By examining changes in gene expression that occurred over a single day in the wood-forming tissues of *Eucalyptus* trees, we have identified almost 200 diurnally influenced genes where time-of-day had a significant effect on gene expression level ($-\log_{10}P > 3.74$, see Materials and Methods). A portion of these diurnally influenced genes are likely to be controlled by the circadian clock. Thirty-six of the 195 diurnally influenced genes (18.5%) show homology to genes identified as circadian in a recent genome-wide study of *Arabidopsis* (Covington and Harmer, 2007). We have also shown that the *Eucalyptus* orthologues of the central clock genes, *CCA1* and *GI* display circadian profiles in *Eucalyptus* xylem which match the profiles and phases seen in *Arabidopsis*.

Carbon levels in plants are known to fluctuate between a daily surplus and a nightly deficit (Stitt et al., 2007). Temporal variation in carbon levels is compounded by the spatial separation of carbon production and utilisation in source and sink tissues, respectively, especially in large woody plants. The daily co-ordination of carbon allocation is not well described in trees, as most temporal studies undertaken thus far have been performed in the herbaceous model plant *Arabidopsis* (Harmer et al., 2000; Rogers et al., 2005). Trees differ from herbaceous species; however, in their long life spans, as well as the large scale of their production and assimilation of photosynthate (fast-growing *Eucalyptus* plantations may add up to 100 m³ of biomass/ha/year). Circadian control of gene expression in wood-forming

tissues may be an important approach to co-ordinate xylogenesis with daily and seasonal changes that occur during a tree's lifespan. The identification of diurnally regulated genes in *Eucalyptus* will aid in elucidating the roles of biological rhythms in fast-growing plantation trees with particular reference to their functions in carbon allocation and biomass production.

Transcript profiling by microarray analysis allowed for high-throughput, quantitative analysis of changes in gene expression. Although a *Eucalyptus* genome sequencing project has been initiated, a full-genome microarray was not yet available for this study which limited the analysis to around 10% of the expected gene number in *Eucalyptus*. We attempted to minimise this limitation by focussing on the transcriptome of *Eucalyptus* xylem tissue, which was well-represented on the cDNA-microarray.

2.5.1. The central oscillator is active in wood-forming tissues of *Eucalyptus*

Recent studies in *Arabidopsis* have proposed a model for the central oscillator, consisting of interlocking, autoinhibitory feedback loops (Locke et al., 2006; Zeilinger et al., 2006). According to this model, *CCA1* represses *GI* expression, which in turn promotes *CCA1* expression via at least two intermediates, one of which is *TOC1*. The central clock in *Eucalyptus*, like that in chestnut (Ramos et al., 2005), appears to follow a similar pattern to that of *Arabidopsis*, with the homologous genes in *Eucalyptus* following the same phase as their *Arabidopsis* counterparts. The opposite expression profiles of *EtCCA1* and *EgGI* in *Eucalyptus* lends support to the existence of a similar autoinhibitory feedback loop as that seen in *Arabidopsis*.

A third gene related to the central oscillator and present on our microarray was *ZEITLUPE* (*EtZTL*). *ZTL* is implicated in regulated proteolysis that maintains robust cycling of the clock by targeting the *TOC1* protein for degradation (Mas et al., 2003). *ZTL* is

constitutively expressed in *Arabidopsis*, but displays circadian fluctuation at the protein level (Kevei et al., 2006). Recently the clock controlled protein GI was shown to bind to ZTL, conferring post-translational rhythm on ZTL protein by positively regulating its abundance (Kim et al., 2007). Our microarray results showed that *EtZTL* mRNA does oscillate in *Eucalyptus*, with a phase and peak expression very similar to that of *GI* (Figure 2.7). The fold change of *EtZTL* was furthermore one of the highest seen in this study, with a ratio of peak vs. trough expression of up to 26 fold in GU (results not shown). These results suggest that unlike in *Arabidopsis*, *Eucalyptus* ZTL is also controlled at the transcriptional level. ZTL mRNA levels have also been shown to cycle circadianly in *Mesembryanthemum crystallinum* a crassulacean acid metabolism (CAM) plant (Boxall et al., 2005).

2.5.2. Carbon allocation pathways exhibit diurnal regulation in *Eucalyptus* trees

Carbon captured during photosynthesis can be used in the originating source tissue, stored for later use, or transported to sink tissues for a variety of end uses. Regulating the allocation of carbon to these ends is necessary for optimal plant-growth under changing environmental conditions (Smith and Stitt, 2007), such as those experienced during the diurnal cycle. This may be particularly important in large woody plants where source and sink tissues are separated and rates of carbon utilization in sink tissues are extremely high. The circadian clock may be involved in regulating carbon allocation (Weise et al., 2006; Covington and Harmer, 2007), as carbon assimilation occurs only during daylight, whilst carbon-consuming growth and maintenance occur continuously. To ensure continued provision of carbon after dark, carbohydrate stores that were set aside during the day are mobilised. The plant must therefore sense sugar levels throughout the day and store enough carbon to sustain growth and metabolism during the night. Evidence of this mechanism is seen in the response of many

photosynthesis and sugar/starch metabolism genes to exogenously supplied sugars (Blasing et al., 2005).

Trehalose-6-P (Tre6P) is an intermediate product of the trehalose biosynthesis pathway that has been suggested to signal changing carbon levels (Stitt et al., 2007). Rising sugar levels are accompanied by increases in Tre6P which in turn stimulates starch biosynthesis. We found two genes with Tre6P synthase (*TPS*) domains that exhibited diurnal variation in *Eucalyptus* (At1g06410 and At4g17770, Table 2.1). The *Eucalyptus TPS* genes showed two peaks in expression, one at the end of the night/early morning, and a second during the mid-afternoon. The timing of expression appears consistent with a possible role for Tre6P in linking daily growth with starch synthesis. High early morning expression of *TPS* could serve to raise levels of Tre6P, resulting in increased starch synthesis during the daylight hours when sugars are abundant. Results from *Arabidopsis* show *TPS* to be circadianly regulated with strong expression during daylight hours, and a mid-afternoon peak (Harmer et al., 2000).

Five genes involved in the energy releasing glycolytic pathway were found to be diurnally influenced (Figure 2.8). These diurnal changes may be responsible for stimulating carbon flow into cellular energy metabolism at particular times of day. Three of the genes are involved in synthesising phosphoenolpyruvate for entry into the TCA cycle (namely, fructose bisphosphate aldolase (At2g36460 and At3g52930), phosphopyruvate hydratase (At2g36530) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, At3g04120, Table 2.1). The three genes appeared to be co-ordinately regulated in *Eucalyptus* xylem with peak transcript abundance for all three occurring at night. Temporal co-regulation of genes in common

pathways may be a mechanism employed by *Eucalyptus* to enhance the efficiency of its metabolism.

Most sugar molecules produced during the day are stored in the form of starch temporarily until required (Stitt et al., 2007). Several genes responsible for starch degradation and mobilisation are known to be under circadian control in *Arabidopsis*, providing free sugars, primarily in the form of maltose (Lu et al., 2005), for growth and metabolism when required by the plant. We found a β -amylase (At3g23920), which breaks starch down to form maltose to be most highly expressed at 11:00 (ZT 5, Figure 2.8) in *Eucalyptus*. The profile in *Eucalyptus* differed from that of its circadianly controlled *Arabidopsis* orthologue, which peaks almost twelve hours later at around 02:00 (ZT 16, Covington and Harmer, 2007).

Many discrepancies in the timing of peak expression between our data and those of *Arabidopsis* studies are likely to reflect differences between the tissues sampled in the respective studies, given that transcriptional networks are well conserved across plant species (Michael et al., 2008). The tissues sampled in *Arabidopsis* studies are primarily leaves, a source tissue, whereas we have studied *Eucalyptus* xylem, a sink tissue. Alternative timing of peak expression in some tree genes, however, may point to tree-specific adaptations to temporal environmental changes, a potential target for future studies.

2.5.3. Cell wall formation genes show diurnal expression patterns in *Eucalyptus* xylem

Carbon molecules that have been transported (mainly in the form of sucrose) to the secondary xylem are primarily allocated to the three major components of the plant cell wall, namely: cellulose, hemicellulose and lignin. Several key enzymes in these three pathways show diurnally responsive expression profiles in *Eucalyptus* and may be circadianly controlled, as are several *Arabidopsis* cell wall formation genes (Harmer et al., 2000; Rogers et al., 2005).

Sucrose synthase (*SuSy*, or *SUS*) is one of the most highly upregulated genes in cellulose-rich tissues (Andersson-Gunneras et al., 2006) where it is responsible for the conversion of sucrose to fructose and UDP-glucose. UDP-glucose is provided as the substrate to Cesa proteins for the production of cellulose. We found two *Eucalyptus SuSy* genes (At3g43190 and At5g20830, Table 2.1) to have their highest expression at 02:00, (ZT 20, Figure 2.8), possibly resulting in higher levels of cellulose production at night in *Eucalyptus* xylem.

Two main isoforms of SuSy proteins have been identified, a soluble, phosphorylated form (S-SuSy), involved in general cellular metabolism, and a particulate, non-phosphorylated form (P-SuSy), associated with the plasma membrane and linked to cellulose biosynthesis (Haigler et al., 2001). The particulate nature (and hence activity) of SuSy is affected by Ca^{2+} levels in the cell, with high Ca^{2+} concentrations favouring the P-SuSy form. Cellular Ca^{2+} levels are circadianly regulated in *Arabidopsis* (Haigler et al., 2001) and may be similarly controlled in *Eucalyptus*, with a potential peak at midnight (see 2.5.8). Increased Ca^{2+} levels occurring together with high *SuSy* transcript abundance could indicate additional temporal regulation of cellulose deposition at the level of P-SuSy activity.

The expression profiles of the seven known *Eucalyptus Cesa* genes showed large diurnal changes in expression levels (Figure S.1), consistent with the hypothesis that cellulose production is temporally regulated. The timing of expression peaks, however, was largely irregular over the three days for which expression was examined in this study. It is possible therefore that the timing of cellulose production in *Eucalyptus* may be controlled upstream of transcriptional regulation of the *Cesa* genes, either at the *Cesa* protein level or possibly at the level of *SuSy* expression and localization. The temporal co-regulation of the secondary

cell wall related Cesa genes (Table 2.2) does suggest, however, that temporal regulation of Cesa genes at a transcriptional level is important in *Eucalyptus*. Examination of the Cesa gene levels over a longer time period may therefore reveal explanations for the apparently irregular changes in their diurnal expression levels.

The exception to the lack of regularity seen in the diurnal profiles of Cesa genes is *EgCesa6* which is circadianly expressed. It is interesting to note that *EgCesa6* is closely related to *AtCesa6* (Ranik and Myburg, 2006) which has previously been noted to exhibit interaction with light levels (Fagard et al., 2000). The explanation for this relationship is hypothesised to be that phytochrome controls the expression of one or more Cesa isoforms, suggesting a connection between light, the circadian clock and at least one of the Cesa genes. The causal factor behind the opposite phasing of *EgCesa6* in the GU and GC genotypes is currently unknown. A close examination of the *EgCesa6* promoter element in each genotype could possibly reveal genotype-specific differences behind the shift in phase.

A key enzyme in the production of hemicellulose, UDP-D-glucuronic acid decarboxylase (At5g59290, Table 2.1) shared a similar expression profile with *SuSy* in our study, peaking at ZT 20 (02:00, Figure 2.8). UDP-D-glucuronic acid decarboxylase channels UDP-glucuronic acid (derived from UDP-glucose) into the biosynthesis of xylan, the most abundant hemicellulose in angiosperm wood. The co-ordinate production of hemicellulose and cellulose in woody tissues could be advantageous to the tree, as xylan's proposed function is to act as the 'glue' or cross-linking matrix holding the cellulose microfibrils together (Hayashi, 1989). Diurnal differences in the supply of hemicellulose components including xylan and glucomannan to the inner surface of the cell wall have previously been noted in the conifer *Cryptomeria japonica* (Hosoo et al., 2006). In *Cryptomeria*,

hemicelluloses appear to be supplied to the secondary cell wall during the dark period at much higher levels than during the daylight hours.

Several of the diurnally influenced genes involved in carbon allocation and cell wall formation are further categorised as carbohydrate active enzymes or CAZymes (Coutinho et al., 2003; Geisler-Lee et al., 2006). In total, 14 CAZymes were found to exhibit diurnal expression patterns (7.1% of all diurnally influenced genes, Table 2.1). CAZymes are responsible for the synthesis (glycosyltransferases), modification (carbohydrate esterases) and breakdown (glycoside hydrolases and polysaccharide lyases) of carbohydrates. In plants, these processes are of particular importance to cell wall biosynthesis, as well as signalling, starch metabolism and pathogen defence (Henrissat et al., 2001). The presence of these CAZymes in the set of diurnally influenced genes further emphasises the strong effect that diurnal and circadian rhythms may have on cell wall formation.

2.5.4. Lignin biosynthetic pathway: two diurnal peaks indicate roles in UV protection and growth

Lignin is the second most abundant component of secondary cell walls, where it is responsible for reinforcement, making the wall impermeable to water as well as resistant to microbial attack (Boerjan et al., 2003). Manipulating the lignin biosynthetic pathway is a major aim of tree biotechnology (Hu et al., 1999; Li et al., 2006) as high levels of lignin in the cell wall are undesirable for paper production as well as for the production of biofuels such as bioethanol (Houghton et al., 2006). Furthermore, quantitative trait locus (QTL) analysis has revealed lower levels of lignin biosynthesis transcripts in trees displaying superior growth (Kirst et al., 2004).

Due to the importance of lignin in the cell wall, and the large amount of carbon required for its synthesis, plants regulate the deposition of lignin both spatially and temporally (Rogers and Campbell, 2004). Many of the genes involved in lignin biosynthesis are known to be circadianly regulated in *Arabidopsis*, peaking before dawn. This occurs most noticeably with phenylalanine ammonia-lyase (*PAL*), cinnamate-4-hydroxylase (*C4H*), hydroxycinnamoyl-CoA reductase (*CCR1*) and cinnamyl alcohol dehydrogenase (*CAD*) (Harmer et al., 2000; Rogers and Campbell, 2004). Two reasons for temporal regulation of the phenylpropanoid pathway have been suggested. One involves daily production of a ‘phenolic sunscreen’ to protect plants from UV radiation; the other is to temporally separate cell growth from lignin deposition, as lignin deposition limits further cell expansion. The temporal separation of cell growth and lignin deposition is evident during hypocotyl growth in *Arabidopsis* where growth stops late at night and lignification is initiated before dawn (Dowson-Day and Millar, 1999). In the lateral meristem of *Eucalyptus* there is spatial separation between the processes of cell growth in the expansion zone and lignification in deeper xylem layers. The circadian timing of growth and lignification may therefore be most important at the edge of the expansion zone where a daily rhythm of production, expansion and lignification of new cells may occur.

Our results are consistent with the sunscreen hypothesis as well as temporal separation of growth and lignification. Genes in the lignin biosynthetic pathway showed two peaks of expression in *Eucalyptus*, as has been observed in *Arabidopsis*. It has been suggested that the night time peak in expression in *Arabidopsis* is controlled by the central oscillator, while the day time peak is affected by light levels (Rogers et al., 2005). The first peak in expression in *Eucalyptus* occurs in the mid-afternoon (ZT 9) when phenolics may be required for UV protection. The second peak occurs during the hours preceding dawn (ZT

20–24), possibly when cell expansion has ceased, allowing time for the reinforcement of new cell walls. Whether or not cambial cell division itself is co-ordinated in a circadian or diurnal fashion is a question that remains to be answered. If it is, it could conceivably synchronize cells in the cambial expansion zone to reach the lignification stage at the appropriate time of day.

2.5.5. Water transport: co-ordinating diurnal patterns of cell expansion

Aquaporins are membrane bound proteins which facilitate the transport of water into and out of plant cells. The influx of water causes the cell to swell, and is necessary for cell expansion (Uehlein and Kaldenhoff, 2006). We found two aquaporins to be diurnally expressed in *Eucalyptus* (Table 2.1). δ -TIP (Delta Tonoplast Intrinsic Protein, At3g16240) had two expression peaks at 06:00 and 22:00, while plasma membrane intrinsic protein 2B (PIP2B, At3g53420) peaked at 15:00. PIP2B is known to be circadianly regulated in *Arabidopsis* (Harmer et al., 2000; Covington and Harmer, 2007) and may be under similar control in *Eucalyptus*. The night time peak in δ -TIP expression levels could indicate circadianly controlled timing of the gene in *Eucalyptus*, with cell expansion occurring during the early night as has been suggested to take place in *Arabidopsis* (Harmer et al., 2000).

Several genes involved in lignin biosynthesis and the allocation of carbohydrate precursors to cell wall polymers are upregulated in *Eucalyptus* xylem at the end of the night (see above). This indicates that in addition to spatial separation between processes, temporal separation may exist between cell expansion controlled by proteins such as aquaporins, and secondary cell wall thickening. This temporal separation could time lignification and cell wall thickening to occur after cellular expansion has occurred, in order to reinforce the newly enlarged cells. Temporal regulation of these processes would be most critical in the

expansion zone of the cambial meristem, where there is little spatial separation between expanding cells and those undergoing lignification.

Temporal and spatial separation of cell growth and reinforcement have been hypothesised to occur in *Arabidopsis* (Harmer et al., 2000), and may be even more crucial in woody tissues where vessel and fibre precursors undergo massive expansion, and lateral growth supports large volume and weight increases, and continued transport of water and sugars between the roots and crown of the tree.

2.5.6. Diurnal cycling of transcription factors may have pathway-wide effects on gene expression

Transcription factors (TFs) may provide a link between the central clock and the various output pathways that they control. Eleven TFs were found to be diurnally influenced in *Eucalyptus* xylem (Table 2.1), including four hormone-related TFs discussed below, and one each discussed under stress response and clock genes. Diurnally influenced transcription factors included two homeobox genes (At2g22800, At2g46680), three MYB TFs including CCA1 (At2g46830, At1g66230, At5g04760), and a WRKY transcription factor (At5g22570, Table 2.1). Diurnal cycling of these transcription factors suggests the possibility of circadian control over the pathways that they influence.

MYB20 (At1g66230) is part of the large MYB gene family in plants that is known to regulate the biosynthesis of monolignols (Rogers and Campbell, 2004), and is specifically related to wood formation. MYB20 is associated with fibre formation, and is in turn regulated by SND1, a key regulator of TFs involved in secondary cell wall biosynthesis (Zhou et al., 2006). In *Eucalyptus* xylem, MYB20 expression was highest in the second half of the day,

coinciding with the peak expression of several of the cell wall biosynthesis genes found on our microarray, and may therefore be implicated in their regulation.

Several of the diurnal TFs were involved in stress-related pathways. ATHB7 (*Arabidopsis thaliana* Homeobox 7, At2g46680) is expressed during drought stress (Engstrom et al., 1996), and showed peak expression at midday in *Eucalyptus* xylem, possibly in anticipation of increased drought or water stress at this time. WRKY38 (At5g22570) is a member of the WRKY transcription factor family associated with stress response as well as plant defence and development (Mare et al., 2004). WRKY38 is also structurally similar to WRKY62, which is involved in the suppression of jasmonic acid signalling (Mao et al., 2007). Circadian control of TFs which affect hormone signalling pathways could allow for widespread influence of daily rhythms over significant portions of the *Eucalyptus* transcriptome via signalling cascades. The role of diurnal rhythms in hormone-related pathways is discussed below.

2.5.7. Plant hormones and diurnal rhythms

Plant hormones play important roles in growth, development and carbon allocation, and may represent a significant area of control for circadian rhythms. Auxin has many roles in growth and development (Woodward and Bartel, 2005) including wood formation (Demura and Fukuda, 2007). Furthermore, auxin signalling and response are known to be under circadian control in *Arabidopsis* (Covington and Harmer, 2007). We found four auxin-related genes that were diurnally regulated in *Eucalyptus*, three of which are circadianly regulated in *Arabidopsis* (Covington and Harmer, 2007). The genes included a dormancy/auxin-related protein of unknown function (At1g56220) and three auxin induced proteins (At5g56030, At1g60750, At2g04160).

One of the auxin induced proteins (At2g04160) corresponded to a subtilisin-like serine protease which has been shown to play a role in meristem activity in lateral roots (Veth-Tello et al., 2005). The auxin induced serine protease was most highly expressed at 22:00, in co-ordination with many of the wood formation genes (Table 2.1). As the lateral meristem is similar in many respects to other plant meristems (Baucher et al., 2007), an auxin induced protein with a role in meristem activity represents a possible link between cambial meristem activity and the circadian clock.

Gibberellic Acid (GA) is a plant hormone involved in wood formation, which it enhances by the stimulation of cell elongation and cambial growth, and aids indirectly by stimulating polar auxin transport (Bjorklund et al., 2007). There is evidence that GA signalling and response are affected by diurnal light cycles, and that light, GA, and auxin signalling interact (Nozue and Maloof, 2006). Gibberellin-regulated protein 4 (GASA4, At1g74670) and GASA5 (At3g02885) were diurnally influenced in *Eucalyptus*, where both peaked at ZT 16 (22:00). Cell elongation is aided by an influx of water into the cells, which is initiated by water channels such as Delta tonoplast intrinsic protein (δ -TIP, At3g16240), which exhibited a diurnal expression pattern, peaking at the same time of night (see 2.5.5).

Ethylene participates in a wide range of physiological activities including stress response, plant growth and senescence (Bleecker and Kende, 2000). Four ethylene-related genes including three members of the ethylene response factor (ERF)/AP2 transcription family exhibited diurnal expression profiles in *Eucalyptus* (Table 2.1). The three ERF/AP2 genes (At1g46768, At1g53910, At3g14230) were temporally co-expressed, with their highest expression occurring close to the dark/light switch at ZT 0. The ERF domain is known to have specific DNA binding activity to the GCC box, a sequence element involved in

ethylene-responsive gene transcription (Mitsuda et al., 2007). The increased transcription of these genes before dawn could indicate temporal regulation or gating of ethylene sensitivity in woody tissues by the circadian clock.

Other hormone-related genes that were diurnally influenced include two abscisic acid-related genes (At3g15730, At4g27410) and a jasmonic acid biosynthetic pathway gene (At5g42650). Jasmonic acid is a signal of herbivore attack, and its presence results in a change in carbon flux, with rapid photosynthate export from leaf starch to the stem and roots (Babst et al., 2005). The JA biosynthesis gene showed a strong peak in expression at midday, possibly linked to changes in carbon allocation at this time. This gene is circadianly regulated in *Arabidopsis* (Covington and Harmer, 2007) indicating that temporal regulation of its expression may have adaptive benefits.

The wide range of hormone-related genes found to be diurnally regulated suggests a possible link between circadian and hormonal control of gene expression in wood forming tissues. Daily changes in sensitivity to hormones or in hormone production could have wide knock-on effects on gene expression, and may help regulate the responses of trees to their changing environments.

2.5.8. Calcium signalling may be circadianly controlled in *Eucalyptus* xylem

The concentration of cytosolic free calcium oscillates with a circadian rhythm in *Arabidopsis* and is hypothesised to modulate various stimulus-induced signalling events (Dodd et al., 2005; Dodd et al., 2006). Peak levels of free cytosolic Ca^{2+} occur regularly each day, the timing of which is determined by the length of the photoperiod (Love et al., 2004). Calcium is furthermore an important plant nutrient, known to be necessary for wood formation

(Lautner et al., 2007) and to play a role in dormancy induction in poplar (Jian et al., 1997), both circadianly regulated processes.

The possibility exists that diurnally expressed genes, which respond to calcium, or are involved in calcium signalling, may interact with circadianly controlled cellular Ca^{2+} levels. This interaction could give fine control over gene expression to processes which are influenced by calcium levels, and are also circadianly controlled. Three genes related to calcium signalling exhibited diurnal expression profiles in *Eucalyptus* (Table 2.1), and may be related to circadian signalling.

Two of the genes, a glutamate decarboxylase (GAD1, At2g02010) which senses Ca^{2+} signals (Reddy et al., 2002), and a calmodulin with a calcium binding domain (At1g62820) showed peak expression around midnight. Increased expression of these genes may reflect an increase in cytosolic free Ca^{2+} or in sensitivity to Ca^{2+} levels at this time (gating of response to Ca^{2+} levels). A second calcium binding protein (At4g27280) showed a different pattern of expression, however, with peak transcript abundance close to midday. GAD1 is a calcium-dependent calmodulin-binding protein that may be involved in directing carbon flux to the tricarboxylic acid (TCA) cycle (Zik et al., 1998). The diurnal expression of this gene may represent a link between calcium signalling and circadian control over energy producing pathways.

2.5.9. Stress response genes are over-represented amongst diurnal *Eucalyptus* genes

An area where the circadian clock may play a particularly significant role in sessile organisms such as trees is in the anticipation of and preparation for daily and seasonal stress factors. Temperature, UV radiation, water availability, and other abiotic stress factors all fluctuate in a cyclic fashion. Biotic stress factors such as herbivory could also be anticipated

according to their likely time of occurrence. A possible role for circadian control of stress processes in woody plants is pointed to by the large number of diurnally influenced stress response genes observed in our study.

Stress response genes formed the most over-represented category of diurnally influenced genes in our study ($P = 1.2 \times 10^{-4}$, Figure 2.5). Each of these are responding to, and possibly anticipating daily changes in the environment. Twenty-three unique gene models related to stress responses were diurnally influenced (Table 2.1), including six that cycle circadianly in *Arabidopsis* (Covington and Harmer, 2007). The heat shock proteins, of which there were seven, shared similar expression profiles, most peaking around midday (Table 2.1). Circadian clock control over heat shock gene transcription could direct accumulation of heat shock proteins in trees in anticipation of the hottest period of the day, thereby reducing the negative effects of high temperatures.

Galactinol synthase (Gols) is the committed enzyme in the pathway to raffinose and stachyose synthesis and may play a role in sugar transport (Taji et al., 2002). We found a galactinol synthase gene (At1g60470, Table 2.1) to be upregulated at dawn. This may be in preparation for hot dry days experienced by *Eucalyptus*, as raffinose is involved in tolerance to drought, high salinity and cold stress. Accumulated galactinol and raffinose may act as osmoprotectants in drought-stress tolerance (Taji et al., 2002).

The second most common expression pattern among the stress response genes was opposite in phase to the profile shown by the heat shock proteins, with lowest expression at midday and peak expression around midnight. This cluster was comprised of five genes that included a cold induced translation elongation factor (At1g56070) and a heat stress transcription factor (At1g46264). Other genes in this cluster are a DNA damage repair gene

(At3g12610), and two dehydration induced proteins (Early Responsive to Dehydration 15, At1g69450; and ERD4, At2g41430 which is circadian in *Arabidopsis*).

2.6 CONCLUSION

Microarray analysis of gene expression in wood-forming tissues of *Eucalyptus* revealed close to 200 unique genes with diurnal changes in transcript abundance. Many of these genes have previously been described as circadianly controlled in *Arabidopsis*, including 36 genes in a recent genome-wide study (Covington and Harmer, 2007). We also found that the central oscillator of the plant circadian clock is active in the tree stem, where it is likely to be responsible for a significant portion of the diurnal changes seen. The affected genes are involved in a wide variety of metabolic tasks, including the allocation of carbon to various end uses, such as the formation of cell walls.

These results were obtained through the study of 2608 genes or ~10% of the expected *Eucalyptus* gene number. Genetic mapping and the creation of EST databases for *Eucalyptus* (Poke et al., 2005) have increased our knowledge of this tree species, and *Eucalyptus* is scheduled to be the second tree species sequenced, after poplar (genome sequence due for completion in 2009, International *Eucalyptus* Genome Network, EUCAGEN, www.eucagen.org/). A full-genome *Eucalyptus* microarray should therefore become available in the foreseeable future, which will allow a more conclusive determination of diurnal and circadian effects in this tree, and their impact on carbon allocation and wood formation.

All studies of transcriptional regulation are limited in their usefulness by the indirect connection between transcript abundance and protein levels. Proteins with a rapid turnover rate are likely to be present in quantities similar to that of their transcripts, while long lived

proteins are less influenced by fluctuations in transcript abundance. Post-translational modifications, selective transport and various other factors all complicate inferences that can be drawn from microarray data. In general, changes in transcript abundance lead to smaller and delayed changes in enzyme activity (Blasing et al., 2005). Gene expression may also be regulated by accumulation of metabolites, meaning that diurnal changes in transcript levels over time lead to more stable changes in metabolite levels. Despite these limitations, the results obtained in this study represent a positive step toward greater understanding of the temporal control of gene expression in wood forming tissues and present many options for further study.

2.7 ACKNOWLEDGEMENTS

The authors thank Sappi for financial support and access to *Eucalyptus* clonal material. Additional financial support was supplied by the National Research Foundation of South Africa and the Technology and Human Resources for Industry Program (THRIP). The assistance of Len van Zyl with the microarray experiment is gratefully acknowledged. We also thank Chris Smith, Jason Osborne and Dahlia Nielsen of ArrayXpress for assistance with the statistical analysis.

2.8 FIGURES

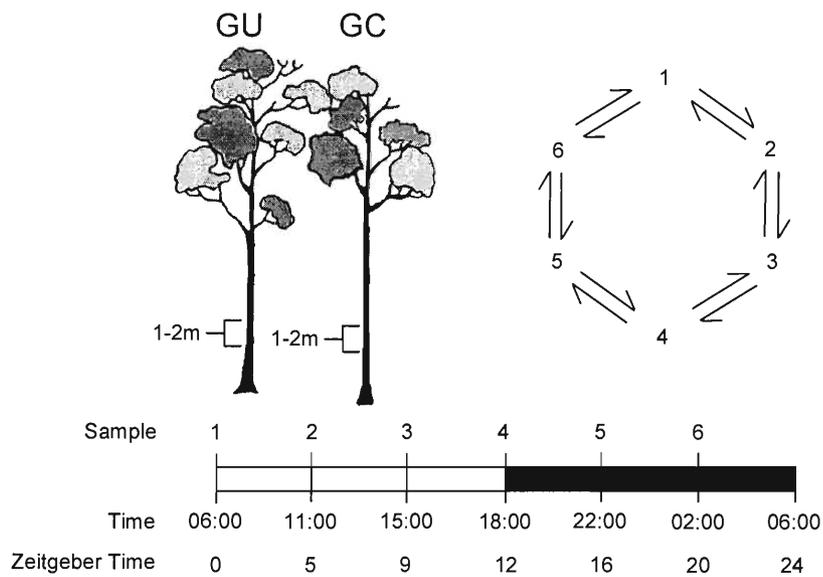


Figure 2.1. Experimental design: Immature xylem was harvested at approximately four hour intervals from the stems of three-year-old ramets of a *Eucalyptus grandis* × *E. urophylla* (GU) and an *E. grandis* × *E. camaldulensis* (GC) clone at the times indicated. Transcript profiles were compared in two separate experiments (GU and GC) using a loop design linking the six sampled time points for each genotype. Each arrow represents a microarray slide, the sample at the head of the arrow being labelled with Cy5 and the tail with Cy3.

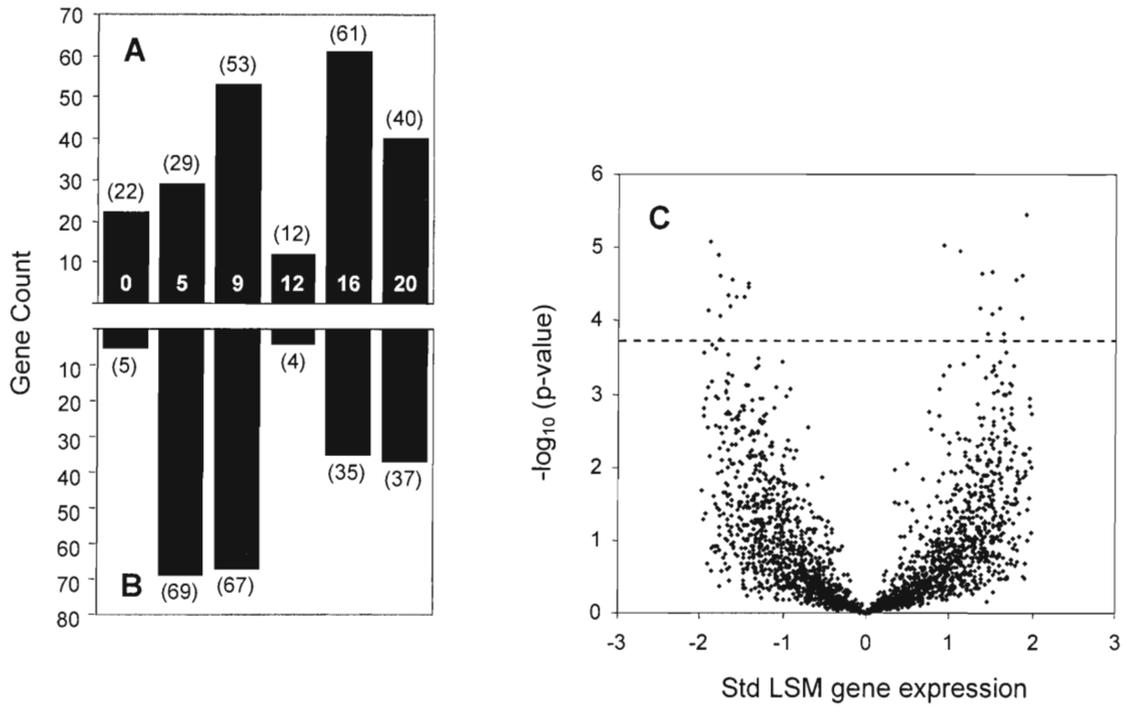


Figure 2.2. Summary of diurnally affected gene expression patterns (A) Number of genes reaching their peak expression for each sampled time point over the course of one diurnal cycle. Numbers in brackets represent the gene count for each time point. Column headings are Zeitgeber times. (B) Number of genes reaching their lowest sampled expression level over one diurnal cycle. (C) Typical volcano plot showing sample ZT 12 against all other time points. The dashed line represents the significance threshold value at $-\log_{10}P = 3.74$.

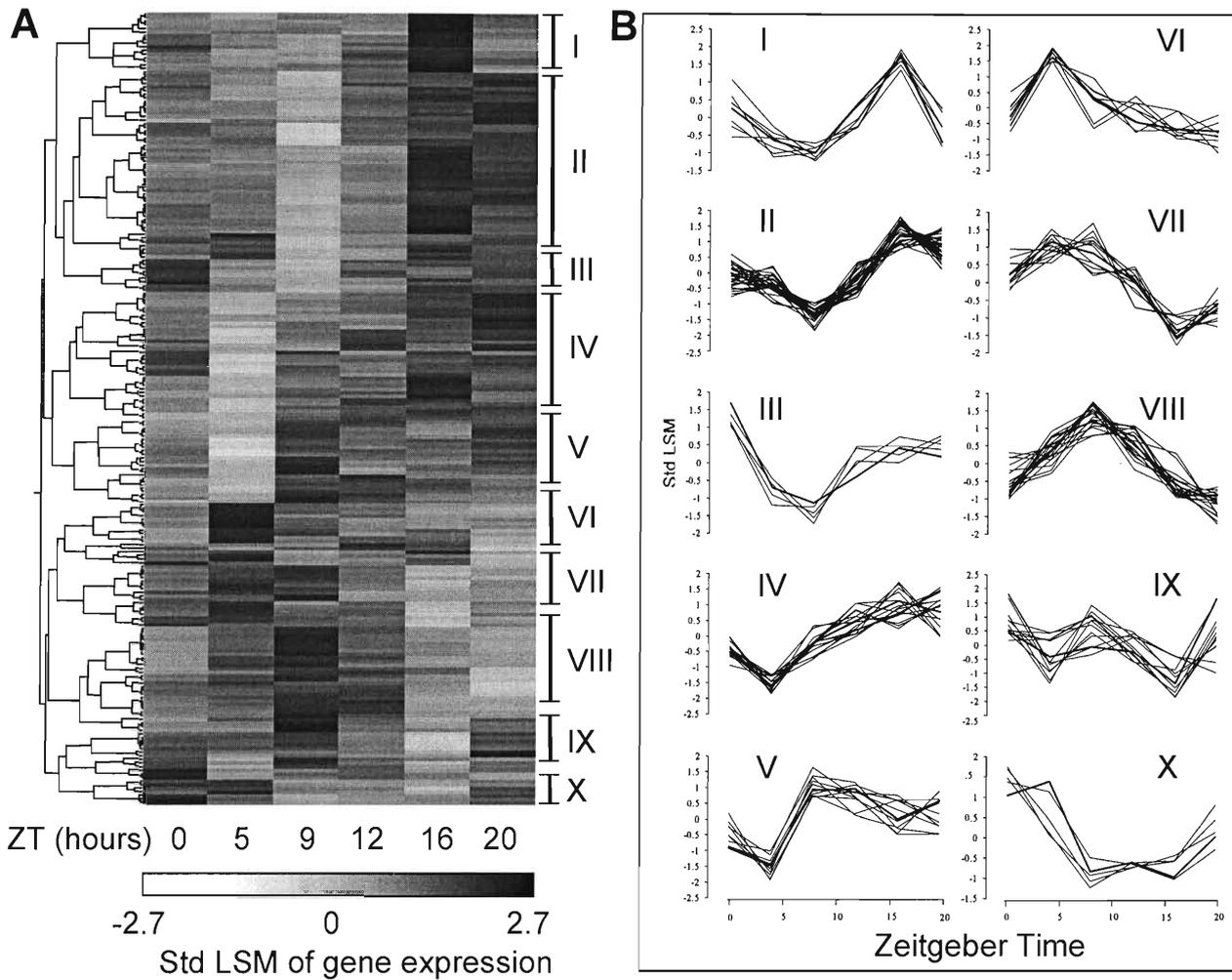


Figure 2.3. Clustering of diurnally affected microarray gene expression profiles. (A) Hierarchical clustering of 217 differentially expressed microarray elements. Relative expression levels (Std LSM: Standardized Least Square Means) are represented by a greyscale continuum, with white indicating lowest expression, and black highest. Rows represent individual microarray elements, columns represent sampling time-points. Ten groups showing similar expression profiles are indicated by vertical bars labelled ‘I’ to ‘X’ to the right of the cluster. (B) Line graphs of representative genes for clusters I – X showing the dominant profile of each group.

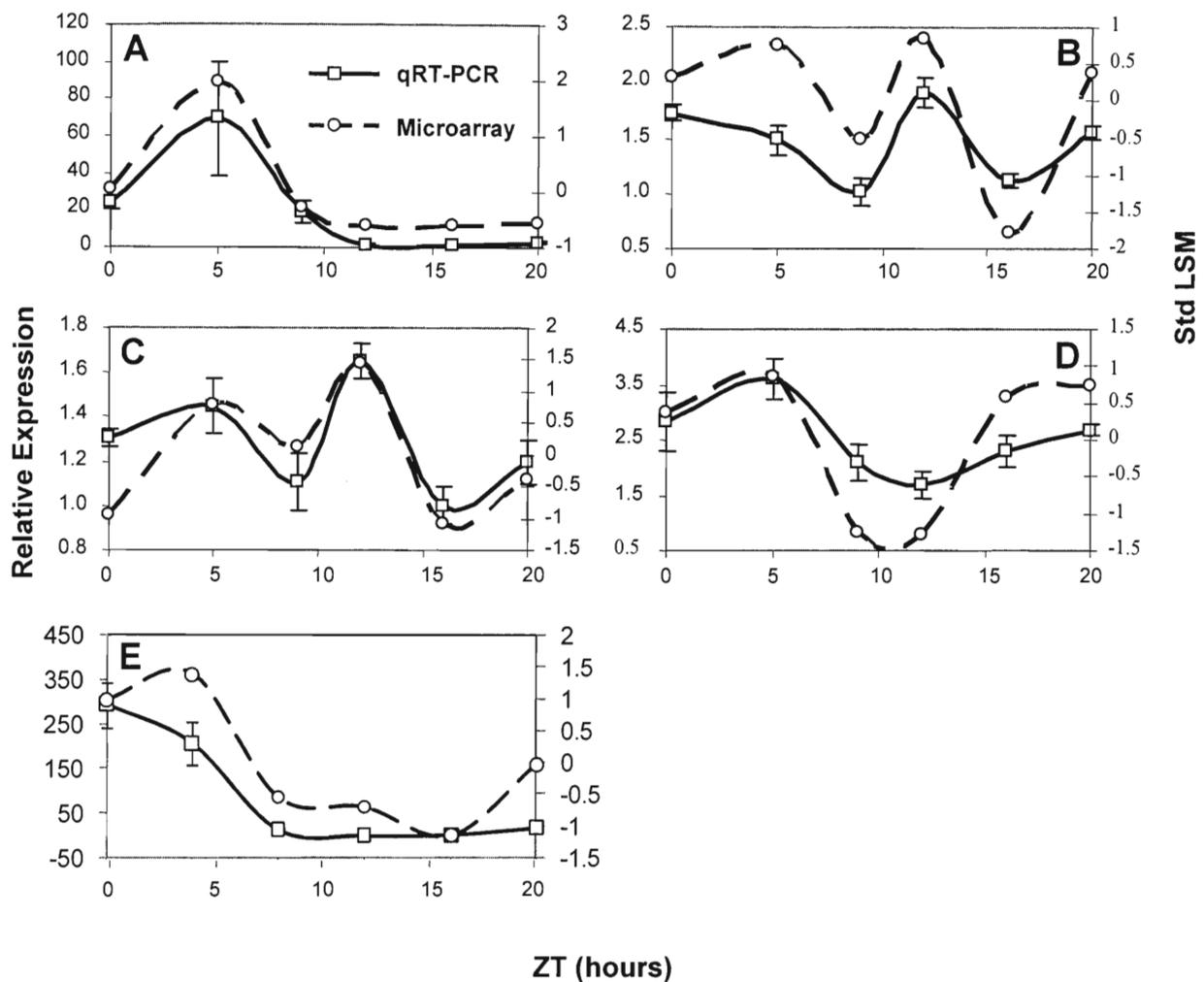


Figure 2.4. Confirmation of microarray gene expression profiles using qRT-PCR. Relative expression (primary Y-axis) refers to test gene expression relative to the geometric norm of three control genes in GU. Standard error bars represent the SEM across the three replicate reactions. Std LSM (secondary Y-axis, on right) is the standardised least square means estimate of gene expression on the GU microarray slides. (A) β -amylase, At3g23920 (B) cinnamyl-alcohol dehydrogenase, At1g72680 (C) endo-1,4- β -glucanase, At5g49720 (D) UDP-D-glucuronic acid decarboxylase, At5g59290 (E) Circadian Clock Associated 1, At2g46830 (All names are best BlastX hits of *Eucalyptus* EST sequences against *Arabidopsis*). The X-axis shows Zeitgeber Time, or hours since dawn.

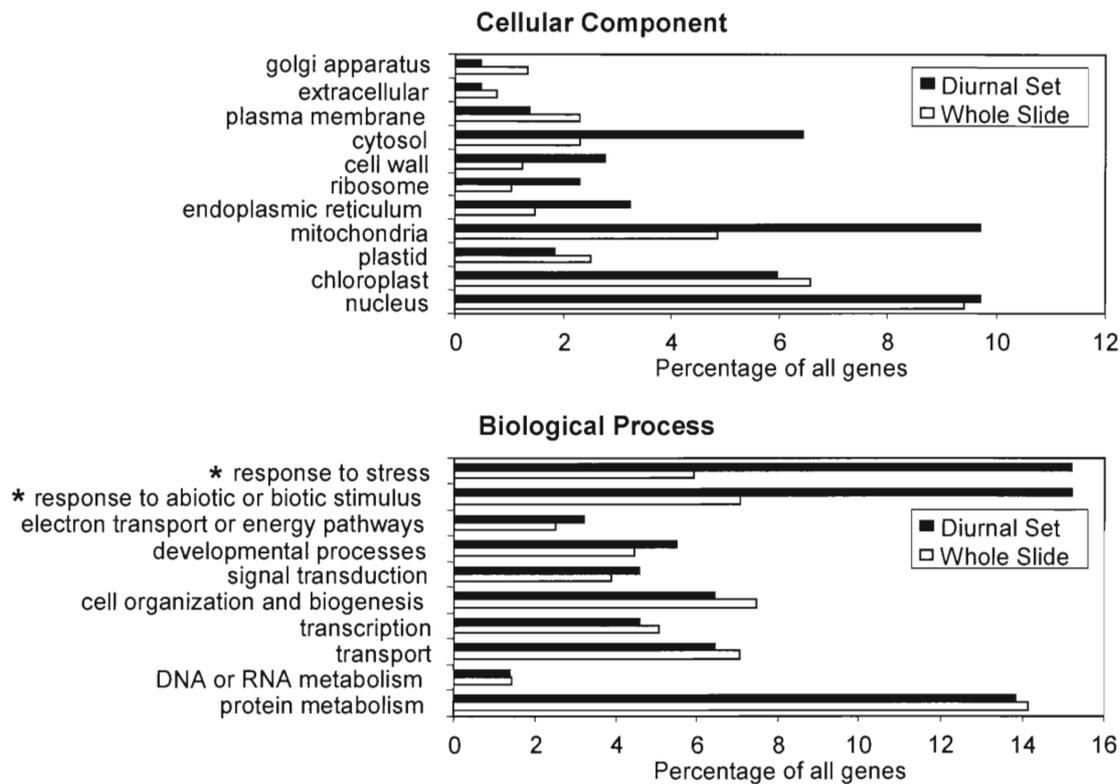


Figure 2.5. Gene ontology categories influenced by diurnal changes. Percentage of responsive genes is shown for all genes on the microarray slide, and for the diurnally responsive set of 217 microarray targets. Several very broad classes including ‘other cellular components’, and ‘other molecular functions’ were removed to enhance clarity. Categories showing the largest proportional changes are at the top of each graph.

* Significant at $P = 1.2 \times 10^{-4}$.

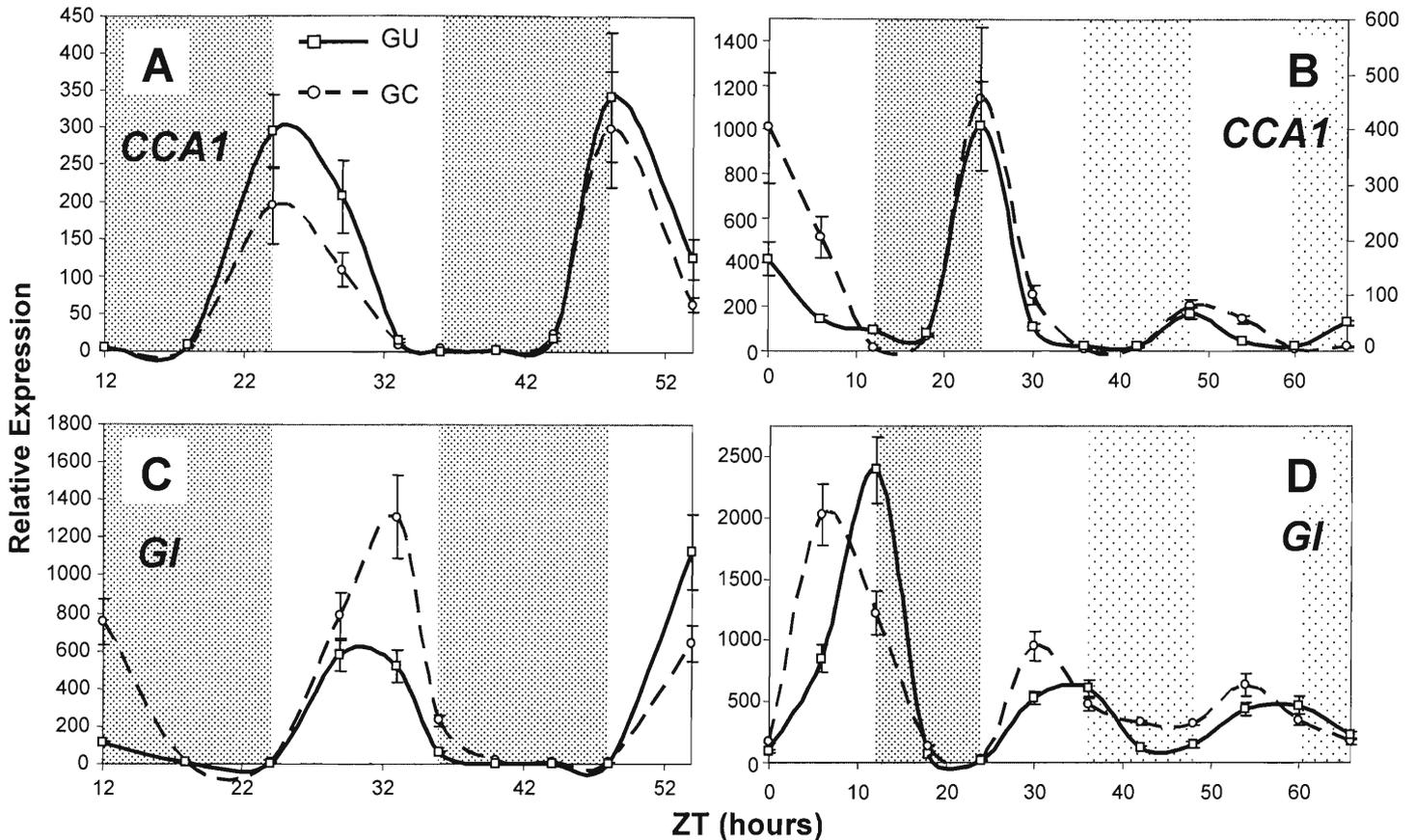


Figure 2.6. qRT-PCR expression profiling of central clock genes in *Eucalyptus* xylem. Relative expression is expressed as the ratio of the mean expression level of the gene to the geometric mean of expression in three control genes. Heavily shaded areas indicate night time and unshaded areas day time. Light shading indicates subjective night during continuous light (LL) conditions. Standard error bars represent the Standard Error of the Mean (SEM) across the three replicate reactions. (A) *CCA1* expression in immature xylem samples collected from field-grown trees. (B) *CCA1* expression in immature xylem from potted ramets entrained for three weeks in controlled light conditions. First 24 hrs are LD, next 42 hrs are LL. The secondary Y-axis on the right measures expression in GU. (C) *GI* expression in immature xylem samples from field-grown trees. (D) *GI* expression in potted ramets under controlled light. The X-axis shows Zeitgeber Time, or hours since dawn.

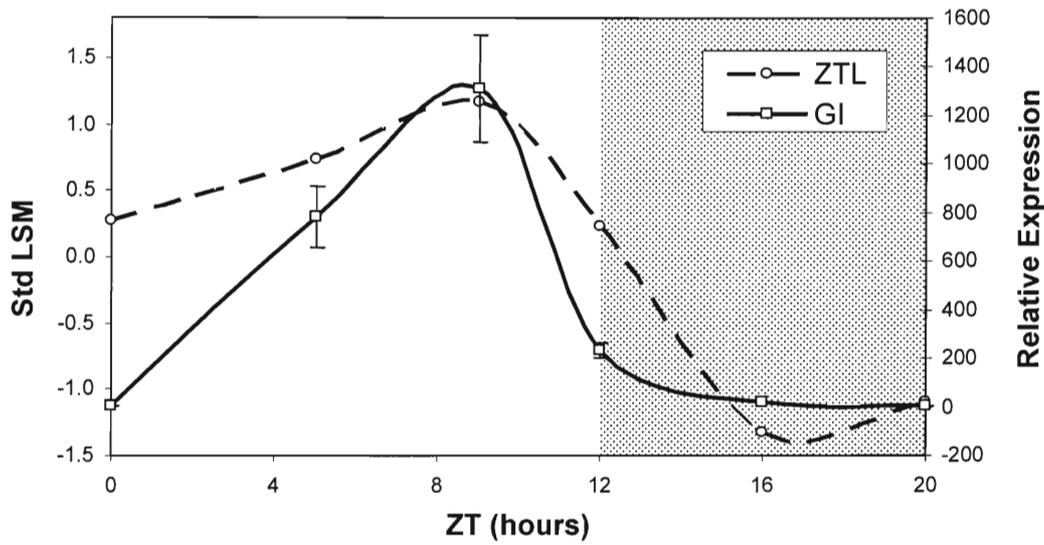
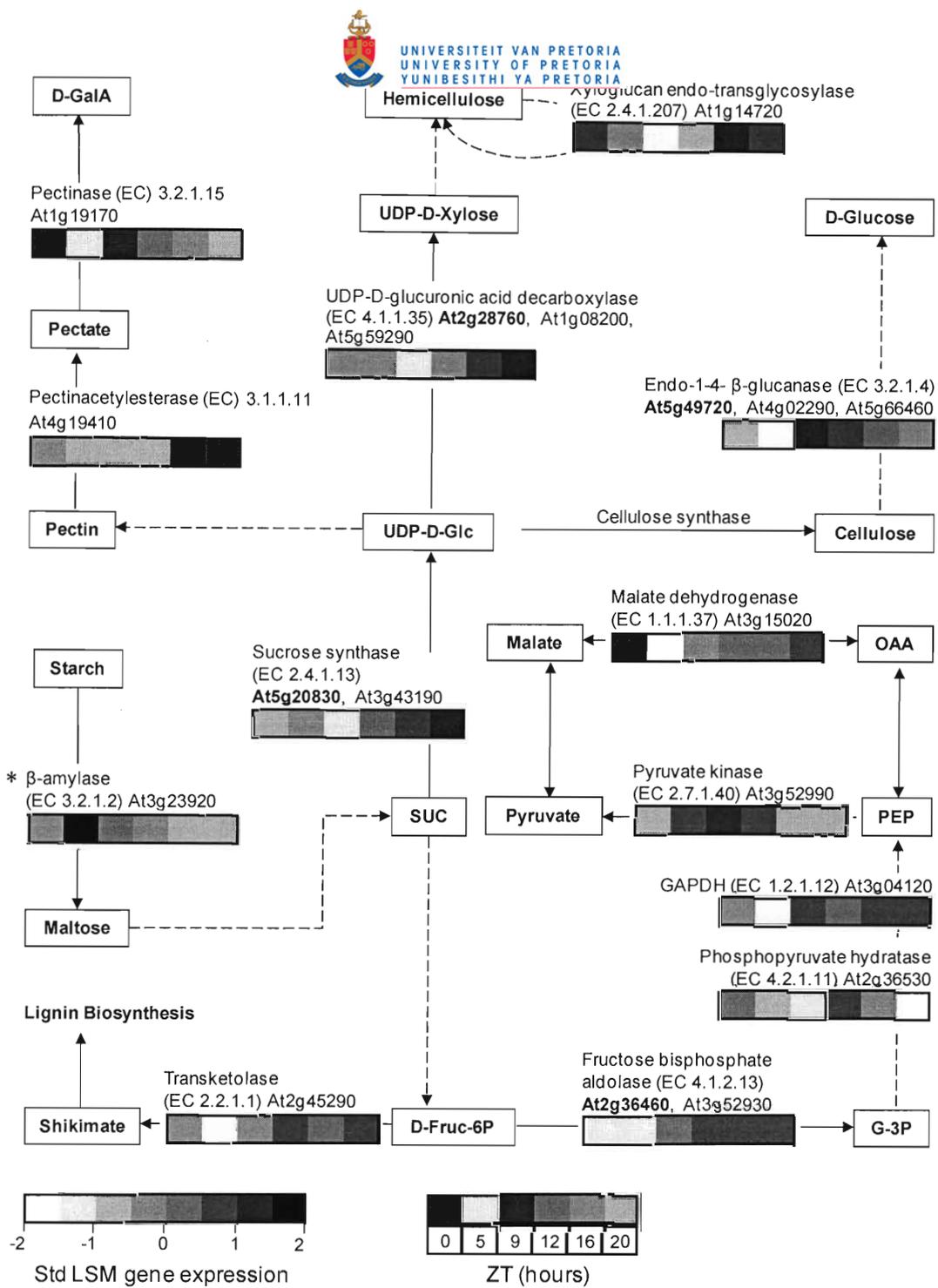


Figure 2.7. *ZTL* mRNA levels cycle in field-grown *Eucalyptus* trees with an expression profile similar to *GI*. Std LSM (Y-axis) is the standardised least square means estimate of *ZTL* gene expression on the GC microarray slides. Relative expression (secondary Y- axis) for *GI* is the ratio of the expression level of the gene measured by qRT-PCR to the geometric mean of expression in three control genes. Standard error bars represent the SEM across three replicate samples. The X-axis shows Zeitgeber Time, or hours since dawn. Shaded areas indicate night time and unshaded areas day time.



A



B

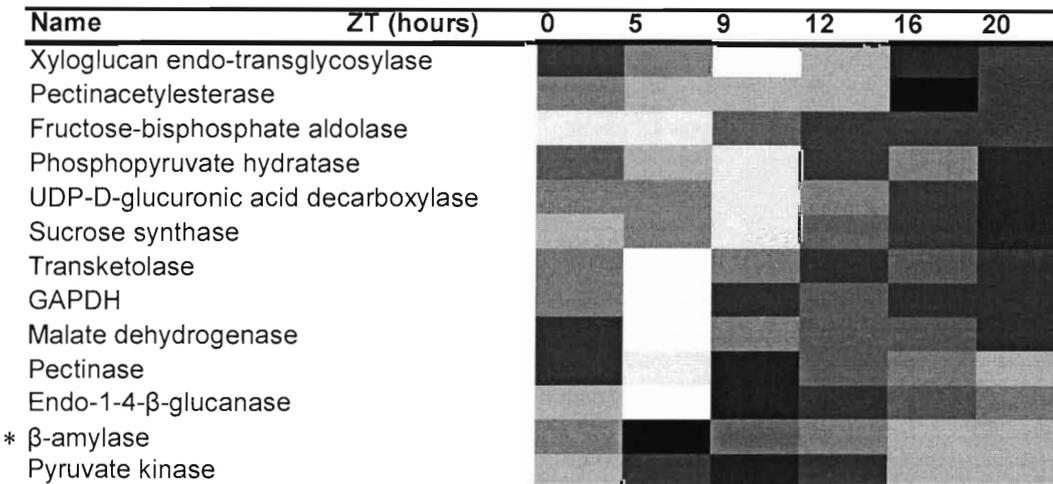


Figure 2.8. Metabolic pathway analyses of diurnally influenced carbohydrate metabolism genes in field-grown *Eucalyptus* trees. (A). Pathways are adapted from Aracyc (www.arabidopsis.org/tools/aracyc/) and KEGG Pathway (www.genome.ad.jp/kegg/). Changes in transcript abundance are represented on a greyscale continuum of standardised least square means estimates of gene expression, where white represents low expression and black, high expression. ZT = Zeitgeber Time, or hours since dawn. Gene expression is shown for six sampled time points, namely 06:00 (ZT 0); 11:00 (ZT 5); 15:00 (ZT 9); 18:00 (ZT 12); 22:00 (ZT 16) and 02:00 (ZT 20). Abbreviations: D-GalA = D-Galacturonate; UDP-D-Glc = UDP-D-Glucose; OAA = Oxaloacetate; SUC = Sucrose; PEP = Phosphoenolpyruvate; D-Fruc-6P = D-Fructose-6P; G-3P = Glyceraldehyde-3P; GAPDH = glyceraldehyde 3-phosphate dehydrogenase. Solid arrows indicate reactions occurring in a single step, dashed arrows indicate that intermediate steps have been omitted for clarity. The microarray targets with best hits to At numbers that correspond to the EC numbers for each step in the pathway are given. When more than one microarray target corresponds to a single EC number, the illustrated gene is highlighted in bold. (B) Hierarchical clustering of the same sucrose and starch metabolism genes. *Circadianly regulated (Covington and Harmer, 2007)

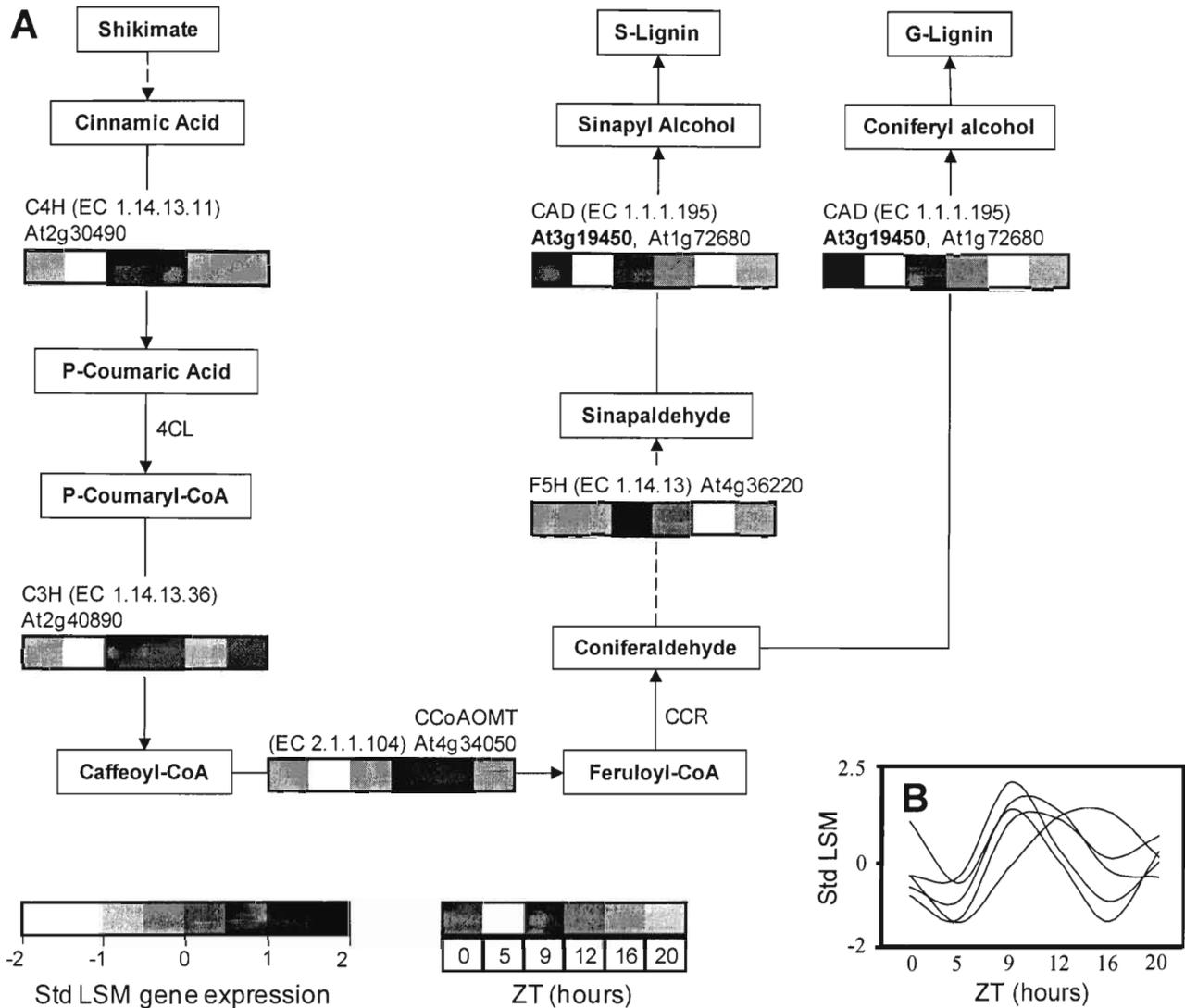


Figure 2.9. Microarray analysis reveals diurnal variation of gene expression in the lignin biosynthetic pathway in field-grown *Eucalyptus* trees. (A). Pathway adapted from KEGG Pathway (www.genome.ad.jp/kegg/). Changes in transcript abundance are represented on a greyscale continuum of standardised least square means estimates of gene expression, where white represents low expression and black, high. ZT = Zeitgeber Time, or hours since dawn. Gene expression is shown for six sampled time points, namely 06:00 (ZT 0), 11:00 (ZT 5), 15:00 (ZT 9), 18:00 (ZT 12), 22:00 (ZT 16) and 02:00 (ZT 20). Abbreviations: C4H = Cinnamate-4-Hydroxylase; CAD = Cinnamyl Alcohol Dehydrogenase; 4CL = 4-Coumarate: Coenzyme A Ligase; F5H = Ferulate-5-Hydroxylase; C3H = Cinnamate-3-Hydroxylase; CCoAOMT = Caffeoyl-CoA O-Methyltransferase; CCR = Cinnamoyl-CoA Reductase. (B) Temporal co-expression of genes related to lignin biosynthesis. Genes represented are: CCoAOMT (At4g34050), C4H (At2g30490), C3H (At2g40890), CAD (At3g19450) and F5H (At4g36220).

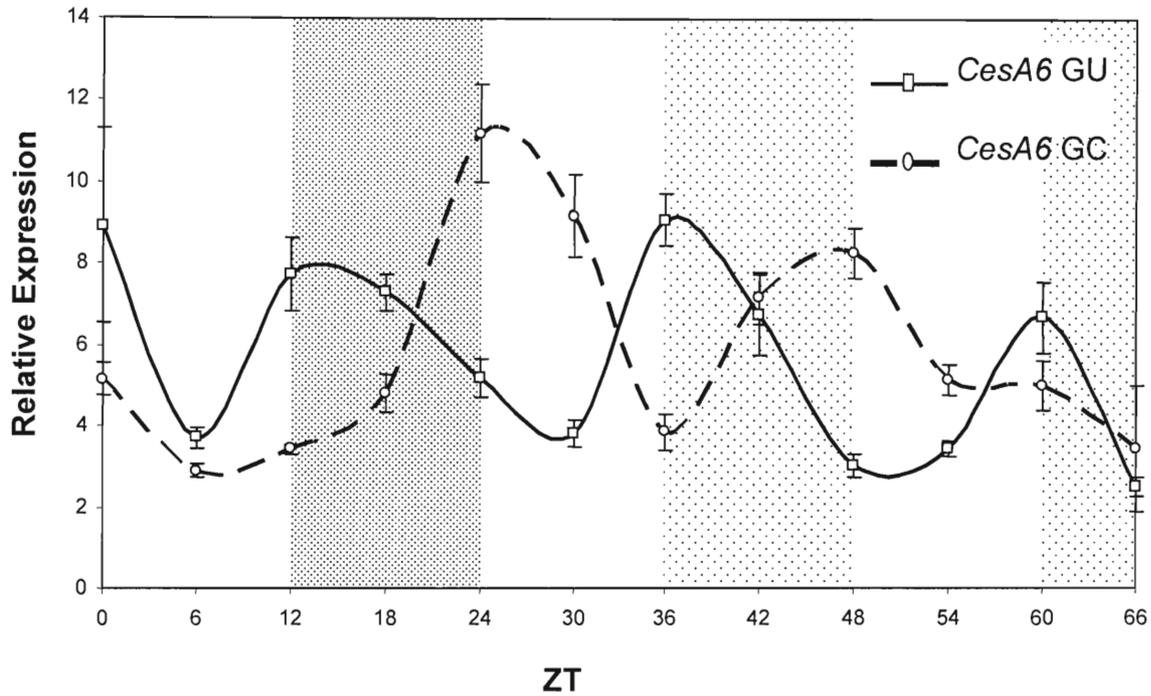


Figure 2.10. qRT-PCR analysis of circadian regulation of gene expression in *Eucalyptus cellulose synthase 6*. These results were observed in *Eucalyptus grandis* x *urophylla* and *E. grandis* x *camaldulensis* grown in controlled light conditions. Relative expression is compared to the geometric mean of expression in three control genes. Heavily shaded areas indicate night time and unshaded areas day time. Light shading represents subjective night during continuous light (LL). Standard error bars represent the SEM across three replicate samples.

2.9 TABLES

Table 2.1.

Putative identities and peak expression times (↑) of diurnally influenced *Eucalyptus* genes in pathways of interest.

At Number	Description	CAZyme Family / Category	06:00	11:00	15:00	18:00	22:00	02:00
<i>(A)</i>								
<i>Starch/sucrose metabolism</i>								
At1g06410	putative trehalose biosynthesis enzyme		↑					
At1g08200	UDP-D-glucuronic acid decarboxylase						↑	
At1g14720	xyloglucan endo-transglycosylase	GH16					↑	
At1g19170	pectinase	GH28			↑			
At1g60470	galactinol synthase	GT8/Stress	↑					
At2g21730	mannitol dehydrogenase				↑			
At2g28760	UDP-D-glucuronic acid decarboxylase							↑
At2g36460	fructose bisphosphate aldolase						↑	
At2g36530	phosphopyruvate hydratase							↑
At2g45290	transketolase							↑
At3g04120	GADPH				↑			
At3g15020	malate dehydrogenase		↑					
At3g18080	beta-glucosidase	GH1			↑			
At3g21560	UDP-glucosyltransferase *						↑	
At3g23920	β-amylase *	GH14		↑				
At3g43190	sucrose synthase	GT1						↑
At3g52930	fructose bisphosphate aldolase					↑		
At3g52990	pyruvate kinase				↑			
At3g55700	UDP-glucosyl transferase family protein	GT1			↑			
At4g02290	endo-1-4-β-glucanase	GH9		↑				



Table 2.1. Continued

At Number	Name	CAZyme Family / Category	06:00	11:00	15:00	18:00	22:00	02:00
At4g17770	putative trehalose biosynthetic enzyme				↑			
At4g19410	pectinacylesterase	CE13					↑	
At5g20830	sucrose synthase	GT1						↑
At5g49720	endo-1-4-β-glucanase	GH9			↑			
At5g59290	UDP-D-glucuronic acid decarboxylase						↑	
At5g64790	glycosyl hydrolase family 17 protein	GH17		↑				
At5g66460	endo-1-4-β-glucanase	GH5					↑	
At5g67230	glycosyl transferase family 43 protein	GT43			↑			
(B)								
Lignin biosynthesis								
At1g72680	cinnamyl-alcohol dehydrogenase (CAD)				↑			
At2g30490	cinnamate-4-hydroxylase (C4H) *				↑			
At2g40890	coumarate-3-hydroxylase (C3H) *						↑	
At3g19450	cinnamyl-alcohol dehydrogenase (CAD) *				↑			
At4g34050	caffeoyl-CoA 3-O-methyltransferase (CCoAOMT)						↑	
At4g36220	ferulate-5-hydroxylase (F5H)				↑			
(C)								
Stress response								
At1g08830	copper/zinc superoxide dismutase			↑				
At1g20030	pathogenesis-related thaumatin family protein				↑			
At1g46264	heat stress transcription factor family member	TF	↑					
At1g52560	26.5 kDa class I small heat shock protein-like						↑	
At1g54050	17.4 kDa class III heat shock protein			↑				
At1g56070	cold-induced translation elongation factor						↑	
At1g69450	early-responsive to dehydration (ERD 15)						↑	
At2g18150	peroxidase				↑			
At2g41430	dehydration-induced protein (ERD 4) *						↑	

Table 2.1. Continued

At Number	Name	CAZyme Family / Category	06:00	11:00	15:00	18:00	22:00	02:00
At3g09640	L-ascorbate peroxidase 1						↑	
At3g12580	heat shock protein 70 *				↑			
At3g12610	DNA-damage repair/tolerant protein						↑	
At4g10250	22.0 kDa small heat shock protein *				↑			
At5g12020	17.6 kDa class II heat shock protein				↑			
At5g52640	81 kDa heat shock protein *				↑			
At5g56000	heat shock protein 81-4 (HSP81-4)			↑				
At5g58070	temperature stress-induced lipocalin *						↑	
(D)								
Hormone- related								
At1g46768	ERF/AP2 transcription factor family member	TF						↑
At1g53910	similar to AP2 domain-containing protein	TF	↑					
At1g56220	dormancy/auxin associated family protein *		↑					
At1g60750	auxin-induced aldo reductase family member		↑					
At1g66340	ethylene receptor 1 (ETR1)						↑	
At1g74670	gibberellin-regulated protein 4 (GASA4) *						↑	
At2g04160	auxin-induced subtilisin-like serine protease *						↑	
At3g02885	gibberellin-regulated protein 5 (GASA5)						↑	
At3g14230	ERF/AP2 transcription factor family member	TF	↑					
At3g15730	ABA mediated stomatal movement regulator						↑	
At4g27410	NAC TF in ABA-mediated dehydration response	TF/Stress						↑
At5g42650	cytochrome p450 in the JA biosynthetic pathway *				↑			
At5g56030	auxin-induced heat shock protein 90 *	Stress		↑				
(E)								
Transcription factor								
At1g66230	putative transcription factor (MYB20)				↑			
At2g22800	homeobox-leucine zipper protein 9 (HAT9)						↑	



Table 2.1. Continued

At Number	Name	CAZyme Family / Category	06:00	11:00	15:00	18:00	22:00	02:00
At2g46680	<i>Arabidopsis thaliana</i> Homeobox 7 (ATHB7)	Stress		↑				
At5g04760	myb family transcription factor				↑			
At5g22570	WRKY transcription factor 38							↑
(F)								
<i>Calcium signalling- related</i>								
At1g62820	calmodulin, contains calcium-binding domain							↑
At2g02010	glutamate decarboxylase (GAD1)	Stress						↑
At4g27280	calcium-binding EF hand family protein			↑				
(G)								
<i>Water transport</i>								
At3g16240	δ-tonoplast intrinsic protein (δ-TIP)							↑
At3g53420	plasma membrane intrinsic protein 2B (PIP2B) *				↑			
(H)								
<i>Circadian clock- related</i>								
At2g46830	myb-related transcription factor (CCA1) *	TF	↑					
At5g57360	Zeitlupe (ZTL)				↑			

* Circadianly regulated (Covington and Harmer, 2007)

Table 2.2.

Values of the regression (r^2) for comparisons between gene expression profiles of the seven *Eucalyptus* CesA genes. Values for GU (average of controlled light and field grown samples) are shown above the diagonal and for GC (average of controlled light and field grown samples) below the diagonal. In bold are comparisons between the secondary cell wall CesA genes which show evidence of temporal co-regulation.

	GU	CesA1	CesA2	CesA3	CesA4	CesA5	CesA6	CesA7
GC								
CesA1			0.65	0.71	0.26	0.18	0.18	0.11
CesA2		0.92		0.84	0.32	0.12	0.05	0.43
CesA3		0.91	0.92		0.26	0.10	0.06	0.32
CesA4		0.19	0.18	0.25		0.28	0.26	0.29
CesA5		0.28	0.35	0.31	0.33		0.34	0.08
CesA6		0.13	0.04	0.13	0.32	0.31		0.09
CesA7		0.32	0.40	0.29	0.19	0.55	0.15	

2.10 SUPPLEMENTARY MATERIAL

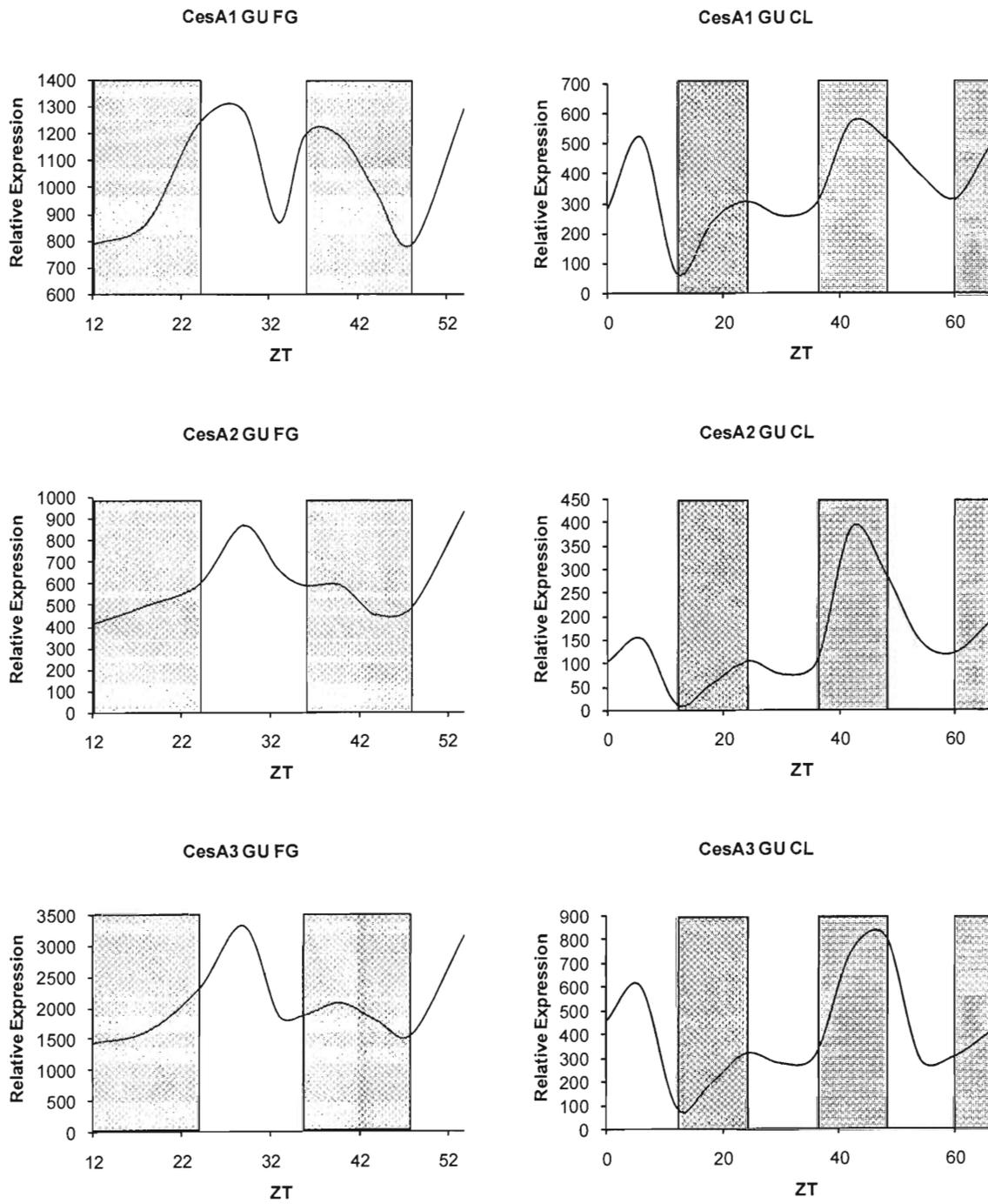


Figure S.1.

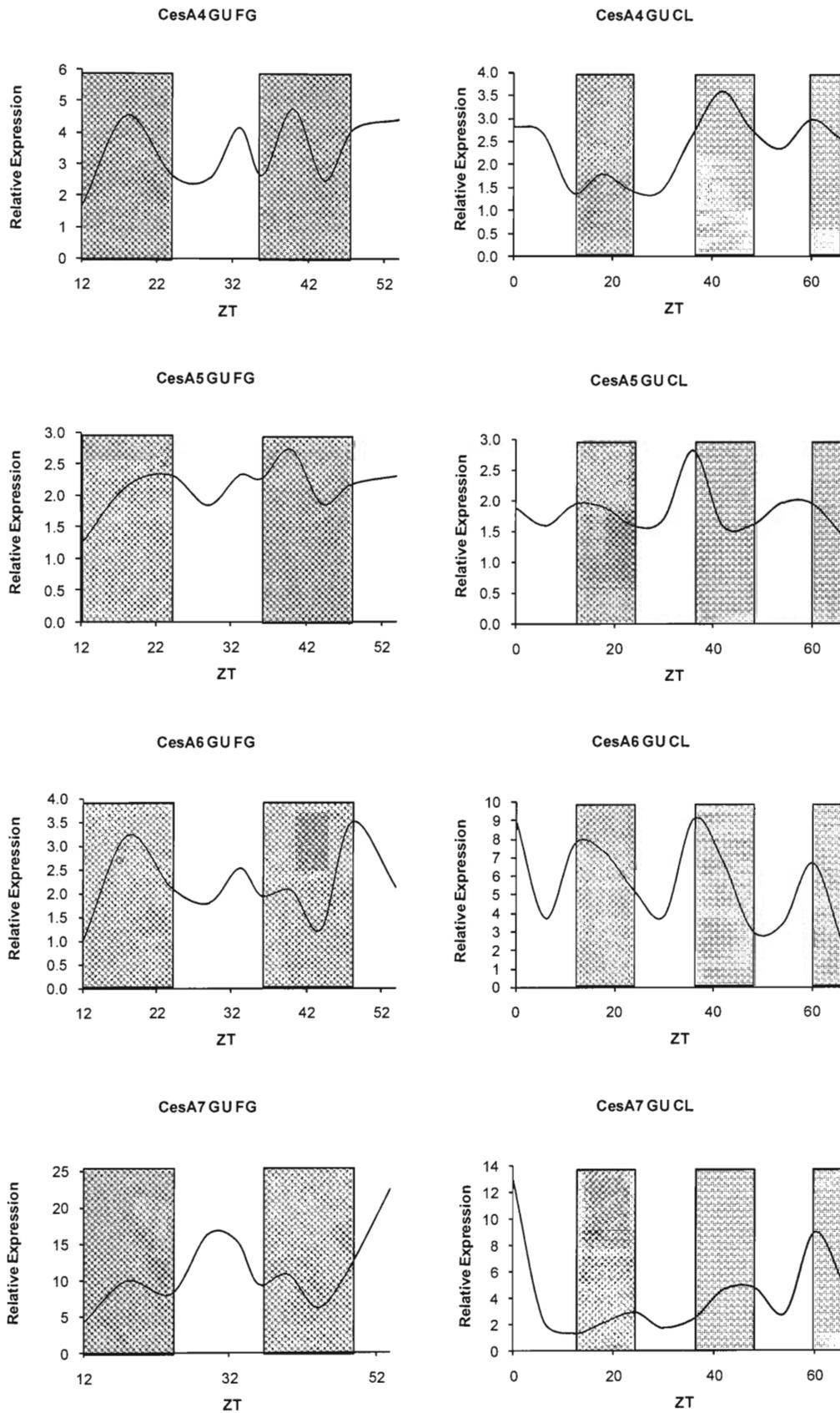


Figure S.1. (continued)

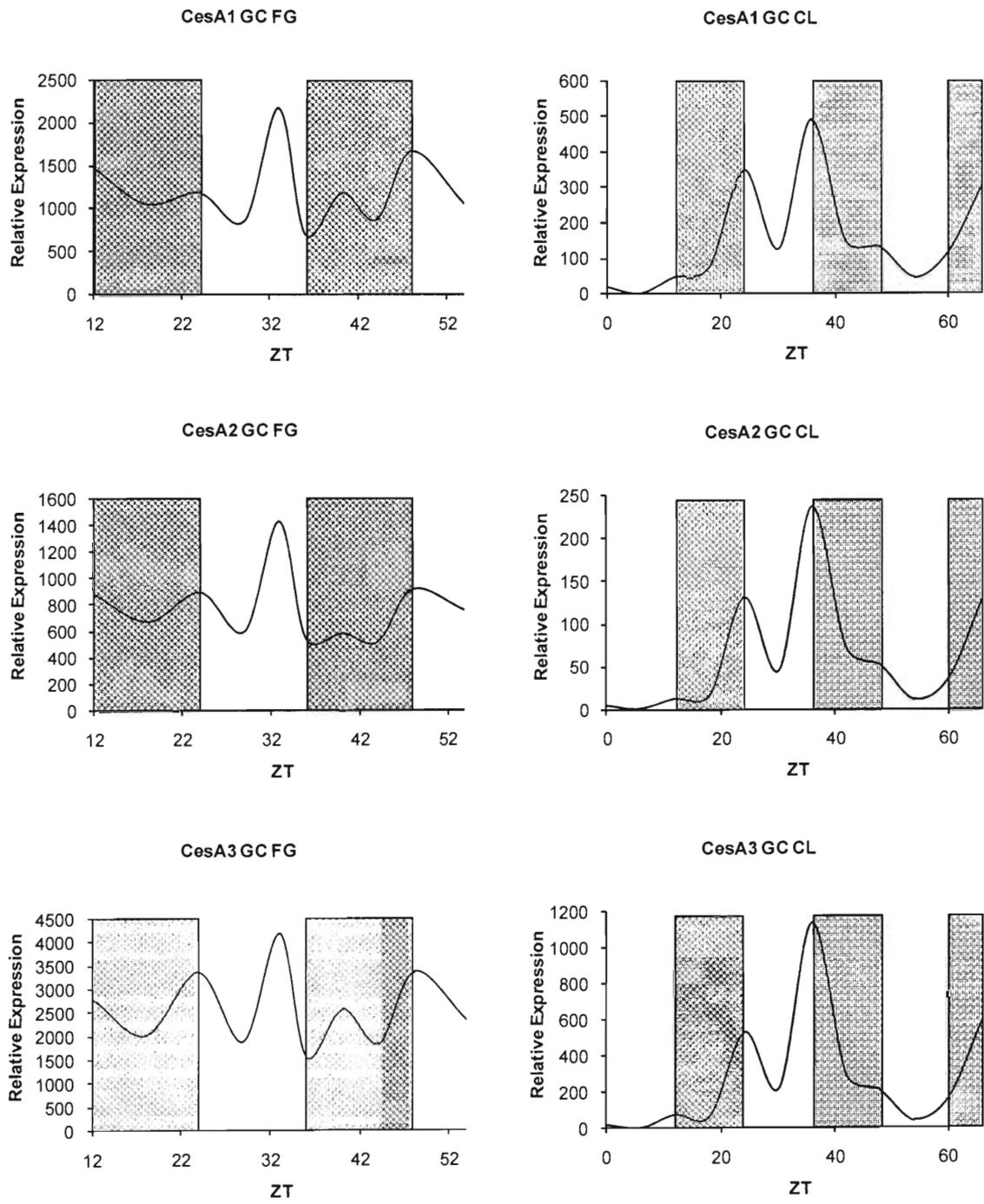


Figure S.1. (continued)

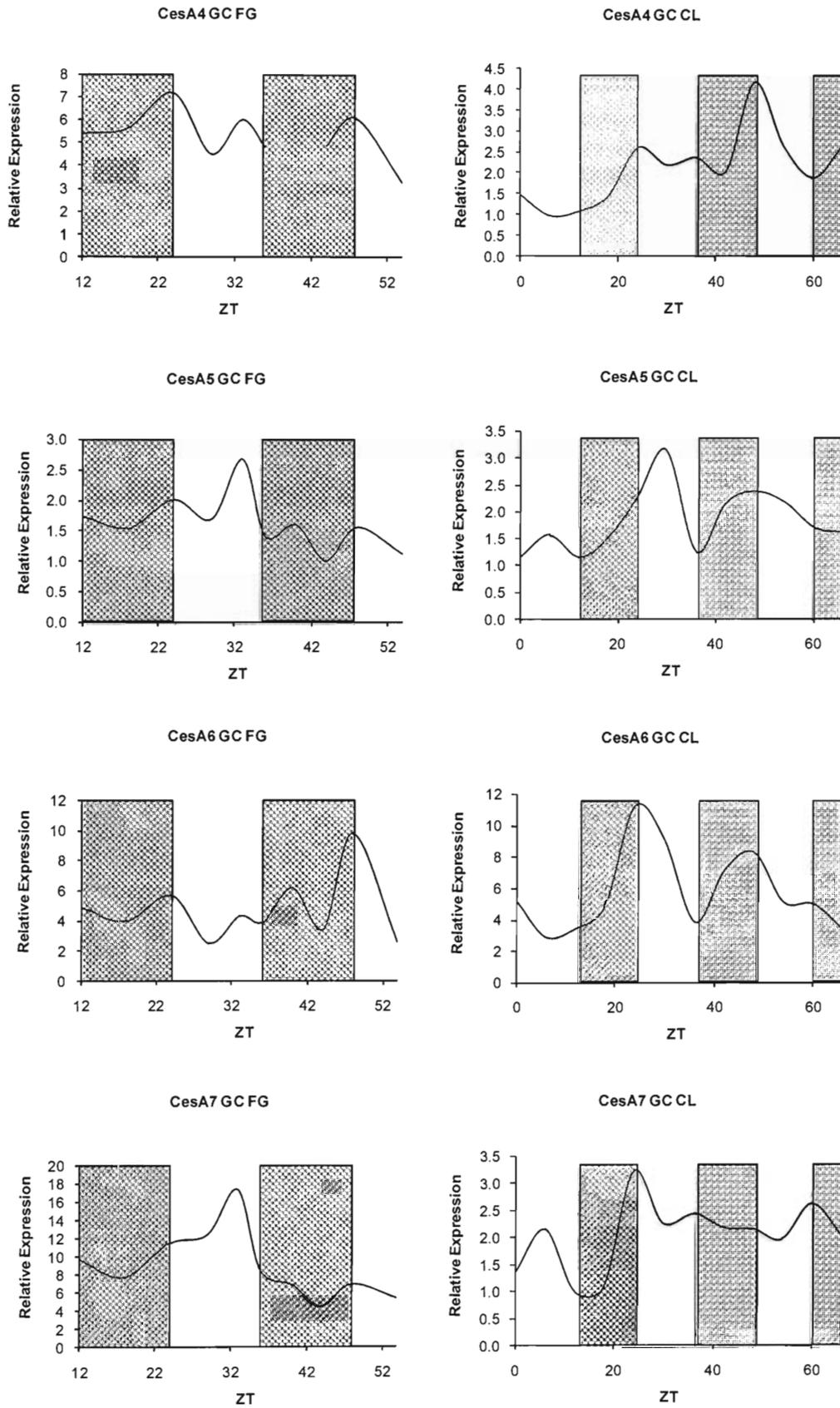


Figure S.1. (continued)

Figure S.1. qRT-PCR analysis of temporal variation in gene expression in all known *Eucalyptus* CesA genes. Results were obtained for *Eucalyptus grandis* x *urophylla* (GU) and *E. grandis* x *camaldulensis* (GC) grown in the field (FG) and under controlled light (CL) conditions. Relative expression is compared to the geometric mean of expression in three control genes. The X-axis shows Zeitgeber Time, or hours since dawn. Heavily shaded areas indicate night time and unshaded areas day time. Lightly shaded areas represent subjective night during continuous light (LL). Standard error bars are not shown. The SEM across three replicate samples averaged 13.7% of the relative expression for the entire experiment. The gap in the profile of CesA4 GC FG corresponds to a missing value.

Table S.1. Primer sequences used for qRT-PCR analysis

Gene	Genbank Acc.	At Number	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
Test Genes				
β -amylase	CB968031	At3g23920	GGACAGCGTGACGATGAATA	CCACCACACGTCCATCATAA
Cinnamyl alcohol dehydrogenase	CD669587	At1g72680	GAAGCACTGAGCCTGCTTGG	CCGGCTGCCGTGTTCGATTAT
endo 1,4- β -D-glucanase	CB967687	At5g49720	TCTGGTGGGAGGGTACTACG	GCGCTGTACTCGATGACGCT
UDP-glucuronic acid decarboxylase	CD669965	At5g59290	CCTCTGCGCAATTCCAAGTT	CCATTAGCCTGTCCACTAAG
<i>EgCesA1</i>	DQ014505	AT4G18780	TCCAAGGTTGAAGATGGCGGCATT	CGATCGCAGTGATCGATATG
<i>EgCesA2</i>	DQ014506	AT5G44030	ACATGTGATTGCTGGCCTTC	TTCTGAGACATGAGCGATGA
<i>EgCesA3</i>	DQ014507	AT5G17420	CTGTGATTGCTGCCCATGCT	CGCCACCCTGTTCCATCAAA
<i>EgCesA4</i>	DQ014508	AT5G05170	AGCCAAAGCAGAGAAAGTCA	ATAAGCGTAGAGGCCACAAA
<i>EgCesA5</i>	DQ014509	AT4G32410	GGGAAGGGTGGCAATAAGAA	AACAGGAGACTGACCGAATC
<i>EgCesA6</i>	DQ014510	AT4G39350	CCACCACCTTTGGAAGGTAT	GGCTCGTGCCTTTCAGTGTT
<i>EgCesA7</i>	Unpublished	Unpublished	GCTGCCTCTGCTGTGGAGGTAGAA	GACTAGCATCTGGCGGAACACCAC
<i>EtCCA1</i>	CD668918	At2g46830	CTGGTGCTAGTACTCCTACA	GAGCTTCCTGAGGCAGATTA
<i>EgGl</i>	DN596733	At1g22770	GATTGCTGCTCCTGAAACAC	TTTGTGCGCGATGCTGAT
Control Genes				
26S Proteasome regulatory subunit	CD668395	At5g23540	GTTGGATGTCCGGTGGTATG	GATGTAGACCTGCTCGGATG
Fructose biphosphate aldolase	CD669023	At2g01140	GAACCTTAACGCGATGAACC	TCAACATTCTCTGGCCTTCC
<i>Eucalyptus globulus</i> α -Tubulin	U37794	At4g14960	GAGCGCCTGTCTGTGGATTA	ACGGTTCGAGTTGGTGTACG
<i>Eucalyptus globulus</i> ADP ribosylation factor (<i>EgARF</i>)	AY770746	At3g62290	TTCTGGTGCCATGCTGAGAA	GATGCTGTGTTGCTCGTCTT

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3. CONCLUDING REMARKS

This study provides an initial look at diurnal patterns of gene expression in *Eucalyptus*, and is one of only a handful of such studies performed on trees. The primary aim of the study was to uncover temporally regulated wood formation genes in the woody stem of *Eucalyptus*. The existence of such regulation is likely, given that various studies have shown a large percentage of all plant genes to be circadianly controlled (Harmer et al., 2000; Edwards et al., 2006; Covington and Harmer, 2007). Few examples of circadian control in wood formation pathways exist, however, possibly because most studies on plant rhythms focus on the herbaceous species *Arabidopsis thaliana*. Only a few genes involved in wood formation processes are known to be under circadian control in *Arabidopsis* (Rogers et al., 2005). It is likely, therefore, that the circadian control of many wood formation genes remains to be discovered.

The experimental approach taken in this study allowed the detection of previously unidentified gene expression patterns, using a non model-species. The use of spotted cDNA microarrays is dependent on existing cDNA libraries and as such restricted us to less than the full transcriptome of *Eucalyptus*. It did allow, however, for the rapid, high-throughput discovery of gene expression profiles for many genes simultaneously. Using this method, we were able to identify almost two hundred diurnally influenced tree genes. Between 10 and 20% of these (40 – 80) are likely to be circadianly controlled, based on estimates of the extent of circadian control in *Arabidopsis*. The types of genes that were diurnally influenced suggest a role for circadian control of various important plant metabolic pathways, including aspects of carbon allocation as well as wood formation.

Many of the genes found to be diurnally influenced in our study correspond to known circadian genes in *Arabidopsis*. What may prove to be of particular interest, are the tree-specific diurnal profiles not seen in the herbaceous plant, as they may be related to circadian control of processes unique to trees, such as wood formation. Circadian control of these processes could occur directly via transcriptional regulation of related genes, or indirectly through transcription factors or hormone signalling. The recent discovery that the auxin synthesis and signalling pathways are under circadian control (Covington and Harmer, 2007) indicates that large portions of the transcriptome may be indirectly controlled in a circadian fashion. This is especially relevant in trees as auxin plays a multitude of roles in the developmental steps leading to the production of wood (Plomion et al., 2001). Our results provide insight into a hitherto largely unexplored level of genetic control in trees, acting in the economically important xylem tissue.

It is increasingly evident that circadian regulation of gene expression influences multiple metabolic pathways in plants. The extent of this regulation in trees could encompass wood formation and aspects of carbon allocation. The practical application of this knowledge remains uncertain to date. Given that there are circadian patterns of expression in genes that influence woods chemical properties, such as the lignin content, it is possible that altering expression profiles could have positive outcomes for industry. Other aspects of wood formation, such as cellulose content, cell wall composition and cell expansion are also likely to be influenced by circadian rhythms. Wood formation itself could potentially be manipulated, therefore, by altering of circadian promoter elements. Another possible use for emerging knowledge of circadian rhythms in plants relates to the promoter elements of circadian genes. Promoter elements that are found to bestow specific temporal profiles will allow for the exploitation of specific temporal niches. For example, transgenes could be

constructed that are active only at night; or are able to detect the subtle changes of the seasons. These could be combined with spatial control elements in order to exactly define and modulate spatiotemporal gene expression.

In the future, it seems likely that studies similar to this will be performed in other tree species including poplar. Additionally, transcriptome-wide repeats of this study will be possible once the full *Eucalyptus* genome becomes available. As more tree genomes are sequenced, comparative studies could point to adaptive benefit of circadian regulation based on functional conservation across species. Circadian clocks in different tissues of the same plant are known to be independent or only weakly linked. It would be interesting therefore, to examine circadian control in a tissue-specific manner, and identify conserved and unique expression profiles within, as well as across species. Unique temporally co-ordinated gene sets may exist in leaves, flowers or even roots. The role that they play in these organs is another topic for future research.

This M.Sc. project can be considered as a pilot study into the identification and possible function of temporal regulation of gene expression in trees. Future studies could build on these results by confirming the circadian nature of diurnal profiles with controlled light studies. The continued cycling of gene expression in an environment without time-cues is evidence of the endogenous nature of circadian rhythms. Sequencing the promoter sequences of confirmed circadian genes and subjecting them to bioinformatic analysis will reveal cis-regulatory modules in promoter elements that confer phase specific expression. This technique was recently illustrated in *Arabidopsis* by Michael et al. (2008). These promoter elements may prove valuable in controlling various transgenes targeted for cell wall modification or other molecular functions. The information obtained from this and future

studies will also aid in understanding and ultimately controlling the genetic processes leading to the formation of wood.

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