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ScienceDirect
South African Journal of Botany 76 (2010) 425–439
SOUTH AFRICAN JOURNAL OF BOTANY
www.elsevier.com/locate/sajb

Diurnal and circadian patterns of gene expression in the developing xylem of *Eucalyptus* trees

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Received 5 November 2009; received in revised form 2 February 2010; accepted 12 February 2010

Abstract

The daily cycle of night and day affects the physiology and behaviour of almost all living things. At a molecular level, many genes show daily changes in expression level, often providing adaptive benefit to the organism. The roles that such daily patterns of gene expression play in coordinating carbon allocation processes during wood development in trees are not fully understood. To identify genes with diurnal expression patterns in wood-forming tissues of field-grown *Eucalyptus* trees, we used a cDNA microarray to measure transcript abundance levels at roughly four-hour intervals throughout a diurnal cycle in two clonal hybrid eucalypt genotypes. Eight percent of genes on the microarray (217 out of 2608) exhibited diurnally influenced expression profiles. Affected genes included those involved in carbon allocation, hormone signalling, stress response and wood formation. *Eucalyptus* homologues of the central clock genes *Circadian Clock Associated 1 (CCA1) and GIGANTEA (GI)* were expressed in developing xylem tissues and cycled with a circadian rhythm in constant light. The presence of a functional biological clock and diurnal transcript abundance patterns during xylogenesis suggests important roles for temporal control of xylem development and metabolism in fast-growing plantation trees.
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Keywords: *Arabidopsis*; Carbon allocation; Microarray; Wood formation

1. Introduction

Many metabolic processes in plants are directly or indirectly influenced by the photoperiod, as temporal changes in light and temperature lead to diurnal rhythms in physiology (McClung, 2006). A subset of these rhythms is 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 h. It enables the anticipation of daily changes in the environment, and provides adaptive benefit in plants through enhanced photosynthesis, carbon fixation and growth (Dodd et al., 2005a,b).

Trees, like other plants, show diurnal and circadian rhythms in physiology and gene expression that influence growth and development (Rogers et al., 2005; Böhlenius et al., 2006). Primary metabolism is directly influenced by diurnal changes in the availability of light for photosynthesis. Daily rhythms in gene expression and metabolite abundance are therefore important in coordinating carbon supply and utilisation (Stitt et al., 2007). Secondary metabolic pathways are also affected by daily rhythms. This is the case for phenylpropanoid biosynthesis and lignin production which are both temporally controlled (Harmer et al., 2000; Rogers et al., 2005). Long lasting changes, such as seasonal growth, dormancy and flowering which occur over months or years are also controlled by the circadian clock. These processes are crucial for the survival and adaptation of woody perennials (Ramos et al., 2005; Böhlenius et al., 2006).

The circadian system, including transcriptional networks and temporally controlled biological processes are largely conserved between the well described *Arabidopsis* (Harmer et al., 2000;

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doi:10.1016/j.sajb.2010.02.087

Edwards et al., 2006; Covington and Harmer, 2007) and poplar systems (Michael et al., 2008). This functional conservation is likely to extend to other plants. For example, orthologues of *Arabidopsis* clock genes have been identified in chestnut trees (*Castanea* spp.) and their mRNA abundance was found to cycle circadianly (Ibañez et al., 2008). In contrast to the well described circadian system in *Arabidopsis*, much remains to be learnt about the circadian and diurnal control of tree genes, their biological function and level of regulation.

Eucalyptus tree species constitute the most extensively planted hardwood crop in the world due to their fast growth, superior wood properties and wide adaptability (Eldridge et al., 1994). Fast-growing *Eucalyptus* trees have also been earmarked as future bioenergy crops for the production of cellulosic biofuels (JGI, 2007). To develop trees with improved wood and fibre properties for bioprocessing, a 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. Woody plants stand to benefit greatly from circadian coordination, due to the distance between their sink and source organs and their long life spans which encompass multiple changes of season. Temporal coordination of wood formation and carbon allocation pathways in trees could lead to enhanced energy efficiency and improved growth.

This study was undertaken to determine the prevalence and potential roles of diurnally and circadianly regulated transcripts abundantly expressed during wood formation in *Eucalyptus* trees. The study identified 217 genes with quantitative changes in transcript abundance over the course of a diurnal cycle in field-grown trees. These genes were involved in wood formation, carbon allocation and stress responses. Their expression patterns may reveal new insights into temporal regulation of these biological processes in woody plants.

2. Materials and methods

2.1. Sampling of plant material

Tissue samples for a diurnal time series were collected over three days in early spring (August) from a field-trial of two three-year-old hybrid *Eucalyptus* clones (*Eucalyptus grandis* W. Hill ex Maid. × *E. camaldulensis* Dehnh., 'GC' and *E. grandis* × *E. urophylla* S.T. Blake, 'GU,' Sappi Forests). These hybrids are widely planted due to their superior drought and disease tolerance over pure species *E. grandis* (Eldridge et al., 1994; Retief and Stanger, 2007). The field-trial was situated near KwaMbonambi in sub-tropical northern KwaZulu-Natal, South Africa. Soft, non-fibrous differentiating xylem tissue of standing trees was collected by peeling the bark off the stems from one to two meters above ground level, and lightly scraping the exposed xylem tissues. Stems were debarked in sections to avoid wound-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 per time point, and thereafter treated as biological replicates. 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 genotypes as the field-grown trees. Potted ramets were grown outdoors 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 h light/12 h dark cycle (LD) at a light intensity of 100 μmol m^{-2} s^{-1} for three weeks, after which sampling began at 06:00 (first light), continuing for 72 h at six-hourly intervals. Growth conditions were switched to continuous light (LL) at dawn on the second day of sampling. 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 at −80 °C.

2.2. Experimental design, target preparation and microarray analysis

Two separate microarray experiments were performed using RNA extracted from each of the biological replicates (field-grown GU or GC trees). A loop design was used for each of the two replicates, linking six time points extending from 06:00 on day two to 02:00 on day three (Fig. 1). A dye swap was incorporated in each experiment as a technical replicate to eliminate dye bias. The loop design allowed transcript abundance at each time point to be compared to all other time points.

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). Potted plant samples were ground using a mortar and pestle with liquid nitrogen. All centrifugation steps were performed at 12,000 × *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 by 1% agarose gel electrophoresis.

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 × 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://compbio.dcfi.harvard.edu/docs/MicrarrayLabeling.pdf). Dye incorporation was measured using a Nanodrop spectrophotometer. The two samples for each slide were combined so that each contributed an equal quantity of dye. Mixed samples were dried in a vacuum centrifuge (ThermoSavant VLP80, NY, NY), re-suspended in 60 μl of a

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