Isolation and characterization of circadian clock genes in the biofuel plant *Pongamia* (*Millettia pinnata*)

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Abstract

The increasing human population has led to an inevitable increase in global energy demands. In the recent decades, biofuels have emerged as one of the potential solutions to the world's insatiable energy needs while reducing the high reliance on fossil fuels. *Pongamia* (*Millettia pinnata*), a nitrogen-fixing tree legume, has shown a great promise as an oil source for the production of biofuel with economical and environmental benefits. The generation of *Pongamia*-derived biofuel is dependent on the success of flowering and seed development. However, molecular control of floral initiation pathways in *Pongamia* remains largely unexplored. Photoperiod pathway has been reported to be one of the major checkpoints of plant flowering time and flower initiation. The circadian clock pathway, a part of the photoperiod pathways, is one of the key regulators of flowering time. Here, we report the identification of four *Pongamia* circadian clock genes (*ELF4*, *LCL1*, *PRR7*, and *TOC1*) through the mapping of the *Pongamia* transcriptome short-paired reads library, by using soybean circadian clock genes as the reference sequences. Furthermore, multiple alignments and phylogenetic analyses suggested that *Pongamia* clock genes are conserved among legume crops such as soybean, medicago, and garden pea. Gene expression studies highlight that *Pongamia* circadian clock genes are diurnally regulated under long- day conditions. Thus, this study reports the isolation and characterization of circadian clock genes in *Pongamia* and enhances our understanding of the molecular mechanism of flowering control in *Pongamia*.

Keywords: circadian clock; pongamia; soybean; biofuel; flowering

Introduction

A substantial increase in the human population has led to an inevitable increase in energy requirements. For example, global energy consumption rose by 2.5% in 2011 and 1.8% in 2012 [1]. As fossil fuels account for 87% of the world's energy consumption, the increased utilization resulted in the production of more harmful greenhouse gases, such as carbon dioxide (CO₂) and nitrous oxide (N₂O), which led to the observable global climate change [2]. Although the demand for crude oil rose by only 0.9% in 2012, crude oil is still the world's primary fossil fuel, accounting for 33.1% of global energy use [1]. Due to the increasing energy demand every year, oil reserves that took millions of years to form are being depleted much more rapidly, leading to the concern that the currently available sources of fossil fuels will be exhausted in the near future [3]. Accordingly, there is a pressing need to find alternative energy sources to fulfill the world's insatiable energy demand [4]. For the past decades, biofuels have emerged as one of the potential solutions to the world's growing energy needs.

Ideally, the sources of such biofuels will have minimal impact on the environment, global food supplies, and land and water use; tree legumes have been considered strong candidates as biofuel sources that meet the aforementioned criteria [5,6]. Considering the many economical and environmental benefits of tree legumes, as explained by Biswas et al. (2011; 2013) and Jensen et al. (2012) [5,6], *Pongamia* (*Millettia pinnata*, previously known as *Pongamia pinnata*) serves as an excellent feedstock (raw material) for the production of biodiesel [7]. *Pongamia* is a fast-growing, medium-sized legume tree that is drought and salt-tolerant. *Pongamia* is also perennial and has symbiotic relationships with rhizobia bacteria and mycorrhizal fungi [8,9,7,10]. Scott et al. (2008) described several characteristics of *Pongamia*'s seed oil that are considered desirable traits in the production of biodiesel. For instance, the oil consists of predominantly (~50%) mono-unsaturated oleic acid and has low amounts of saturated palmitic and stearic acids. Ideally, these characteristics are important in the generation of low cloud-point (the temperature at which dissolved solids will separate from the oil) and low pour-point (the lowest temperature at which oil will flow) fuel to be used in temperate and cold climates.

Recently, *Jatropha* spp., another oilseed candidate for biodiesel production, has caused detrimental investment loss due to unexpected lower seed production [11]. Hence, there is an obvious need to strengthen the industrial development of *Pongamia* as a sustainable biofuel source [12, 7]. As seed production is reliant upon flowering and fertilization, understanding the flowering

mechanisms that control the production of these seeds is important. Photoperiod pathway has been reported as a major control of plant flowering time [13-19]. Although the physiological properties of flowering in *Pongamia* have captured the attention of many researchers [20, 12, 21, 22], the role of the photoperiodic response of *Pongamia* in the regulation of flowering time remains largely unknown. In particular, the circadian clock, a part of the photoperiod pathway function as a system to synchronise photoperiod and other environmental cues with the internal rhythm [23]. In *Arabidopsis*, input pathways, such as light and temperature, are responsible for the entrainment of the central oscillator, which consists of a complex network of interconnected morning and evening transcriptional loops. Some of the morning genes include *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*); *LATE ELONGATED HYPOCOTYL* (*LHY*); and *PSEUDO-RESPONSE REGULATOR 5*, *7*, and *9* (*PRR5*, *PRR7*, and *PRR9*); while the evening genes consist of *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*); *GIGANTEA* (*GI*); *LUX ARRHYTHMO* (*LUX*); and *TIMING OF CAB EXPRESSION 1* (*TOC1*). Besides transcriptional regulation, the interactions between these components are also regulated through post-transcriptional, post-translational, and chromatin-remodeling mechanisms that modulate the rhythmic properties of the oscillator [24-26].

There are significant challenges in translating knowledge gained from the model plant, *Arabidopsis* to other plant species because different plants such as legumes not only display unique vegetative and floral developmental complexities but also have different requirement for the floral transition to happen. By anticipating cyclic changes of circadian rhythms mentioned above, the organism has the adaptive advantage of being able to coordinate essential developmental and physiological processes to occur at optimal times of the day, thus enhancing adaptation, fitness, and survival [27-29]. In order to trigger flower initiation at the precise time and in the right conditions, circadian clocks perceive and integrate both environmental and endogenous signals, which in turn activate mobile floral stimulus (“florigen”, *FLOWERING LOCUS T* (*FT*)) genes to induce flowering. Circadian clock genes have been reported to be responsible for natural variation across different climate and geography locations [30-32,19]. Therefore, there is a need to understand the fundamental pathways that regulate the response to the environment and flowering control to improve breeding for suitable cultivars adapted to climate changes and biofuel production.

In this study, we have used soybean as a reference plant: unlike that of *Pongamia*, the soybean genome has been fully sequenced, and many soybean orthologs of the *Arabidopsis* circadian clock genes have been reported [33,34]. In addition, recently soybean has been focus of flowering studies [34-43] and belongs to the *phaseoloid* subfamily, is closely related to the *Pongamia millettoid* subfamily in the evolutionary tree [44]. Since the *Pongamia* genome sequence

is unavailable, we utilized bioinformatics approaches to initially identify the *Pongamia* homologs of soybean circadian clock genes. This was conducted through the mapping of the *Pongamia* transcriptome short-paired reads library (SRA046342.1) [45]. The putative *Pongamia* circadian clock genes were then isolated to determine—through protein alignment and phylogenetic analyses—the extent of conservation with other species. We also investigated the expression rhythm of putative circadian clock genes in juvenile *Pongamia* plants under favorable long-day conditions for flowering.

Materials and Methods

Plant Material and Growth Conditions

Pongamia seeds, obtained from Brisbane, Queensland, were imbibed in distilled water (1 L per 100 seeds) for 48 h at 25 °C. Seeds were then germinated and grown under long-day conditions (18 h light, 6 h dark) at 28 °C, 400 UML ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 70% humidity in Percival I-36VL growth incubators (Percival, USA).

Bioinformatics

The sequences of soybean circadian clock genes [33], sourced from the Phytozome v8.0 database (available at <http://www.phytozome.net/>) [46], were used to identify the homologs in *Pongamia*. Geneious Pro v6 software [47] was then used to map *Pongamia*'s transcriptome (SRA046342.1) short-paired reads library to the soybean reference gene sequences. Iterative fine tuning from the program was then used to improve the mapping result by obtaining more short-paired reads extending into regions where reads were previously un-mappable (Supplementary Figure S1). This fine-tuning allowed the design of primer pairs for full-length gene isolation in *Pongamia* according to the soybean reference sequences. Primers were designed using Primer3 (available at <http://frodo.wi.mit.edu/primer3>) found in Geneious Pro v6 on the *Pongamia* short-paired reads mapping results (Supplementary Table S1).

Gene isolation

Total RNA was extracted from trifoliolate leaves using TRIzol® reagent (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. The RNA samples were then treated with DNase I (Ambion, Applied Biosystem, USA) to remove genomic DNA contamination as per the manufacturer's instructions, followed by phenol-chloroform extraction to inactivate the

DNase I. First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. Reverse transcriptase polymerase chain reactions (RT-PCRs) were performed using *Taq* DNA polymerase (Invitrogen Life Technologies, USA) according to manufacturer's instructions using a Mastercycler® Nexus thermal cycler (Eppendorf, Germany). The PCR products were cloned into a pGEM®-T Easy Vector System and sequences were confirmed by sequencing (Australian Genome Research Facility, Australia).

Alignment and Phylogenetic Analysis

Amino acid sequences of circadian clock proteins of plant species were obtained by subjecting the *Arabidopsis* circadian clock proteins to tBLASTn searches on the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). Protein alignments were performed using Geneious settings (cost matrix, 65% similarity; gap open penalty, 12; and gap extension penalty, 3). The neighbor-joining method was used to build the phylogenetic tree with 1000 replicates [43]. Protein domains were then identified using the PROSITE database from ExPASy (available at <http://prosite.expasy.org/>).

DNA extraction and Southern Blot

Pongamia genomic DNA was extracted according to a modified CTAB method [48] with modification. In brief, 1g of the ground Pongamia leaf tissues in 10 mL of 2x CTAB extraction buffer (2% CTAB w/v, 75 mM Tris pH 8.0, 15 mM EDTA pH 8.0, 1.05 M NaCl, 1 % PVP, 0.6% β-mercaptoethanol) was incubated at 65°C for 1 hour. An equal volume of chloroform/isoamyl alcohol (24:1) was added and gently shaken for 10 minutes at room temperature. The mixture was centrifuged at 4,500 rpm for 15 minutes, and the aqueous phase was transferred to a new tube, before subjected for RNase A (20 mg/mL) treatment at 37°C for 30 min. 1/10 volume of 10% CTAB (10 % CTAB, 0.7 M NaCl) warmed at 55°C was added. Equal volume of chloroform:isoamyl alcohol (24:1) was added again and mixed gently. The mixture was centrifuged at 4,500rpm for 15 minutes. The aqueous phase was transferred to a new tube and genomic DNA was precipitated with 2/3 volume of ice - cold isopropanol by incubating on ice for 30 min. The mixture was then centrifuged at 4500 rpm at 15 °C for 10 min. The pellet was washed with cold 75 % ethanol, before centrifugation for 5 minutes at 10,000 rpm. The pellet containing nucleic acids obtained was dried and redissolved in 100 µL – 300 µL TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA).

Southern blot was carried out according to modified protocols of Southern et. al. 2006 [49]. 15µg of the extracted genomic DNA was digested overnight with *EcoRI*, *BamHI* and/or *HindIII* restriction enzymes. The digested DNA together with digoxigenin (DIG)-labeled DNA molecular weight marker (Roche Diagnostics Corporation, USA) were separated in 0.8% agarose gel, blotted to nitrocellulose membrane and hybridized with DIG-labeled probe of *ELF4*, *LCL1*, *PRR7* or *TOC1* gene. The probe DNA fragments were synthesized by PCR using DIG-dUTP, according to the manufacturer's instructions (Roche Diagnostics Corporation, USA). Hybridization was performed at 55 °C for 16 hours. Chemiluminescent detection of hybridization signals was observed using CDP-star according to the manufacturer's instructions (Roche Applied Science, Germany).

Tissue Sampling and Gene Expression

Gene expression studies were conducted under long-day conditions (18 h light, 6 h dark) in Percival I-36VL growth incubators (Percival, USA). The seedlings were entrained for five weeks before harvesting. Samples at seven time points after light treatment were collected in 4 h intervals across a 24 h period. Three biological replicates were obtained, and each replicate consists of two trifoliolate leaves randomly pooled together from two different plants. Total RNAs were then extracted using TRIzol[®] reagent. Reverse transcription was conducted using 1 µg of total RNA using SuperScript[™] III Reverse Transcriptase according to manufacturer's instructions. A negative control in the absence of reverse transcriptase (RT-negative) was included for each sample to ensure lack of genomic DNA contamination.

To examine the quantitative expression profile of *ELF4*, *LCL1*, *PRR7*, and *TOC1* genes, quantitative real time RT-PCR (qRT-PCR) was performed with 1.5 µL cDNA template in a 10 µL reaction volume using Brilliant III Ultra-Fast SYBR[®] Green QPCR Master Mix (Agilent, USA) with the Stratagene Mx3000P[™] System (Agilent, USA). The thermal profile of the reactions included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of: 95 °C for 30 s, the appropriate annealing temperature of qRT-PCR primers (Supplementary Table S1) for 30 s, and 72 °C for 30 s. The reaction was finalized by a dissociation cycle of 95 °C for 1 min, followed by a stepwise increase from 55 °C to 95 °C (with a 1 °C increase every 30 s) to obtain the melting curves of the end products. Two technical replicates were performed for each sample. Standards, a no-template control, and an RT-negative sample were included in each run.

The log scale of the cycle threshold (CT) plots with adjusted threshold values between the technical replicates was used for the qRT-PCR analysis. The expression level of each gene was

normalized against the expression of *CONS7*, a *Pongamia* housekeeping gene [50,51]. The relative transcript levels for each gene were then measured using amplification efficiency (E; assumed to be 2) and deviation of threshold cycle between the reference and target gene (ΔCT) according to the following equation:

$$\text{Relative transcript level of target gene} = (E_{\text{target}})^{\Delta CT (\text{reference}-\text{target})}$$

The average (mean $\pm$ standard error) from all three biological replicates was then used to plot the expression level of each gene at different time points.

Results

Identification and Isolation of Putative Circadian Clock Genes in Pongamia

By mapping the *Pongamia* transcriptome short paired reads library to the coding sequences of soybean clock gene homologues, the *Pongamia* counterparts were identified. Subsequently, the mapping result was improved by obtaining more short-paired reads that extended into the regions where reads were previously un-mappable via an iterative fine-tuning process (Supplementary Figure S1). Four putative *Pongamia* homologs: *ELF4*, *LCL1* (*LHY/CCA1 like-1*), *PRR7*, and *TOC1* were found to have full coverage of the soybean circadian clock genes, and those genes are known to represent either morning or evening genes in the *Arabidopsis* circadian system (Table 1) [52-56].

The mapping results showed that *ELF4*, *LCL1*, *PRR7*, and *TOC1* genes were represented in the *Pongamia* transcriptome. The full-length coding sequences of these genes were isolated from *Pongamia* leaf cDNA. Comparison of the coding sequences and their translated proteins with closely related legume species, soybean, and the model plant *Arabidopsis* (Table 2) showed that the *Pongamia* *ELF4*, *LCL1*, *PRR7*, and *TOC1* exhibit higher sequence similarity to soybean, compared to *Arabidopsis*. *PRR7* and *TOC1* show over 90% similarity at nucleotide and 87% at protein level while *ELF4* and *LCL1* show over 80% similarity at nucleotide and 75% at protein level (Table 2).

Characterization of Pongamia clock genes: Sequence and phylogenetic analyses and diurnal expression rhythms

Based on protein alignment (Error! Reference source not found.a–Error! Reference source not found.a) and phylogenetic analyses (Error! Reference source not found.b–Error! Reference source not found.b), *Pongamia* *ELF4*, *LCL1*, *PRR7*, and *TOC1* were found to be highly conserved among closely related

legume species especially soybean, as well as other plant species such as model species *Arabidopsis* and rice. In addition, southern blot analyses showed that *LCL1*, *PRR7*, and *TOC1* genes have single copy number in Pongamia genome whereas *ELF4* has two (**Error! Reference source not found.c - Error! Reference source not found.c**). Pongamia prefers long-day conditions for flowering. Our study on Pongamia *ELF4*, *LCL1*, *PRR7*, and *TOC1* showed diurnal gene expression rhythms under long-day conditions as indicated by qualitative (normal RT-PCR) and quantitative (qRT-PCR) analyses (**Error! Reference source not found.d - Error! Reference source not found.d**).

EARLY FLOWERING 4 (ELF4)

A comparison of proteins alignment showed that the Pongamia ELF4 protein possesses a highly conserved middle region; however, no known functional domain was found in this protein (**Error! Reference source not found.a**) [53,57,58]. Furthermore, the ELF4 phylogenetic tree has indicated the presence of two major clades, the ELF4 and ELF4-like clades (**Error! Reference source not found.b**). The putative Pongamia ELF4 protein, as expected, was found to belong in the ELF4 clade and is closely related with another legume (soybean, Medicago, pea) ELF4 proteins. Southern blot analysis of the ELF4 gene in Pongamia genome showed the presence of one band when digested with *BamHI*. However, two bands were observed when the Pongamia genome was digested with *EcoRI* (**Error! Reference source not found.c**). Additionally, both RT-PCR and qRT-PCR results of *ELF4* expression (**Error! Reference source not found.d**) showed that *ELF4* expression peaks at around 12 h after dawn. This is consistent with the fact that *ELF4* is an evening gene and showing similar diurnal rhythm as *AtELF4* [53,59,58,60].

LATE ELONGATED HYPOCOTYL/CIRCADIAN CLOCK ASSOCIATED 1-like 1 (LCL1)

Protein alignment of LCL1 showed that the N-terminal region is highly conserved and incorporates a Myb-type DNA binding domain (Fig. 2a) [61,62]. Several studies in *Arabidopsis* have shown that *LHY* and *CCA1* are hardly distinguishable as these genes have high sequence similarity, redundant functions, and are often co-expressed [63,64,54]. Two *LHY*- and *CCA1*-like genes, *LCL1* and *LCL2*, were found in other plants including soybean [65,33,66]. Our study shows that the putative Pongamia *LCL1* is closely related to soybean *LCL1*, but is indistinguishable from *AtLHY* and *AtCCA1* (Fig. 2b). The digestion of the Pongamia genome using *BamHI* and *HindIII* in Southern blot analysis showed the presence of only one copy number of *LCL1* gene (Fig. 2c). Moreover, the expression level of *LCL1* was found to be consistent between the RT-PCR and the qRT-PCR results (Fig. 2d), showing that *LCL1* expression peaks at 4 h after dawn, begins to decrease upon approaching dusk (8 h after dawn), and begins to rise again just before dawn (24 h after dawn). This

result is in agreement with *LCL1* being a morning gene and similar to pea, soybean and rice orthologous genes [67-69].

TIMING OF CAB EXPRESSION 1 (TOC1)

TOC1 is also known as PRR1, which belongs to the pseudo-response regulator (PRR) family, along with PRR3, PRR5, PRR7, and PRR9, which are known to be part of the circadian system [70,71]. The TOC1 protein includes a highly conserved pseudo-receiver (PR) domain in the N-terminus and the CONSTANS, CONSTANS-like and TOC1 (CCT) domain in the C-terminus (**Error! Reference source not found.a**). The former is known to be present in all PRR family proteins and is responsible for the transcriptional repression activity in TOC1 [72,73]. The latter, as the name suggests, is also found in CONSTANS (CO) and CONSTANS-like proteins and is responsible for DNA-binding activity [74,73]. Previous studies have found that differentiating between PRR5/PRR9 and PRR3/PRR7 is difficult, due to very close similarity [65,69]. Accordingly, three different clades of PRR1/TOC1, PRR5/PRR9, and PRR3/PRR7 were found to be present in the PRR phylogenetic tree, as shown in **Error! Reference source not found.b** and **Error! Reference source not found.b**. Furthermore, the phylogenetic analysis has determined that *Pongamia* TOC1 belongs to the PRR1/TOC1 clade and is closely related to TOC1 from other legume species (Fig. 3b). When genomic DNA is digested with *Bam*HI, *Eco*RI and *Hind*III, results from southern blot analysis showed that there is one copy of *TOC1* gene in the *Pongamia* genome (**Error! Reference source not found.c**). The expression level of *TOC1* detected by RT-PCR and qRT-PCR was found to be very low as compared to other *Pongamia* clock genes analysed in this study. Nevertheless, qRT-PCR was able to show that *TOC1* expression peaks at 20 h after dawn (**Error! Reference source not found.d**).

PSEUDO-RESPONSE REGULATOR 7 (PRR7)

Since PRR7 is part of the PRR family, it also contains a highly conserved PR domain in the N-terminus and a CCT domain in the C-terminus, as depicted in the protein alignment (**Error! Reference source not found.a**). Phylogenetic study of *Pongamia* PRR7 has placed this gene in the PRR3/PRR7 clade, showing it to be closely related to PRR3 than PRR7 (**Error! Reference source not found.b**). An analysis of the *PRR7* gene using Southern blot showed the presence of one copy when digested with *Bam*HI and *Hind*III (**Error! Reference source not found.c**). Moreover, the RT-PCR result showed that *PRR7* has similar expression levels at 8-, 12- and 16-h time points, but weaker expression at 4-h and 20-h time points. In contrast, qRT-PCR results showed that the *PRR7* expression has a broad peak around the 8-h time point, with slightly elevated expression at the 20-h time point (**Error! Reference**

source not found.d). As *PRR7* is known to be expressed in the morning in *Arabidopsis* [56,52,71], it is most likely that the peak observed at the 8-h time point corresponds to *PRR7* expression. Expression rhythms of all four *Pongamia* clock genes investigated in the present study are summarized in **Error! Reference source not found.**.

Discussion

Conservation of circadian clock genes in Pongamia

Circadian clock genes play important an role in precise integration of environmental signals required for flowering and also adaptation of plants to different geography locations (REF). However, circadian clock remains to be studied in tree legumes like *Pongamia*. Accordingly, the prerequisite for the analysis of the circadian system in *Pongamia* would be the identification of the key circadian clock genes that are responsible for the regulation of the circadian system. The mapping of the *Pongamia* transcriptome short-paired reads library [45] to the soybean circadian clock genes greatly facilitated the identification of four putative full-length *Pongamia* circadian clock genes in this study: *ELF4*, *LCL1*, *PRR7*, and *TOC1* (Table 1). Therefore, this study has demonstrated that the depth of transcriptome obtained through next generation sequencing is a reliable tool for the identification of circadian clock genes, especially in non-model organisms, such as *Pongamia*, with limited genomic information. Consequently, the transcriptome library may be used in future studies for the identification of additional *Pongamia* genes involved in the flowering pathways and also other traits to improve seed yield specially traits linked to biofuel production.

The successful isolation of putative *Pongamia* circadian clock genes has served as an initial step toward the identification of components involved in the regulation of flowering time in *Pongamia*. The clock gene *ELF4* is known to form an evening complex (EC) with *ELF3* and *LUX*, which is required for the generation of circadian rhythms and hence regulation of output pathways such as flowering [75-77]. *ELF4* belongs to a small plant kingdom-specific gene family, consists of other four gene members (*ELFL1–ELFL4*) [60,53,57]. Previous phylogenetic analysis has separated the *ELF4* gene family into two clades: (1) *ELF4* and *ELFL1*, and (2) *ELFL2*, *ELFL3*, and *ELFL4* [57], which is in agreement with the findings of this study (Fig. 1b). Consequently, *Pongamia* *ELF4* was found to be present in the *ELF4/ELF4L1* clade and clustered together with other legume homologues (soybean, medicago and pea). The Southern blot analysis of *ELF4* showed different results when digested with two different enzymes, *BamHI* and *EcoRI*, as shown in Fig. 1c. A possible explanation for this observation is that there are actually two copies of *ELF4* gene located

next to each other in the *Pongamia* genome. In fact, an *EcoRI* restriction site is present between the two *ELF4* copies, which resulted in the presence of two bands. On the other hand, *BamHI* cutting site is not present in between the two *ELF4* copies; hence only one band is obtained. Further analysis would need to be performed in order to determine which copy of *ELF4* gene that has a significant role in the *Pongamia* circadian system.

Earlier studies have identified that CCA1 and LHY are MYB-like transcription factors that play pivotal roles in the morning loop of the central oscillator [54,63,64]. These transcription factors belong to the REVEILLE (RVE) family that consists of 11 proteins with high sequence similarity within the MYB-like domain [78]. In the soybean, *GmLCL1* and *GmLCL2* have been identified as part of the clock central components [66,33,65]. The putative *Pongamia LCL1* and *LCL2* genes have been successfully identified in this study (Table 1). Through Southern blot analysis, only one copy of *LCL1* gene is detected in the *Pongamia* genome. Since the MYB domain is known for DNA binding [79], those conserved domain could imply an important role in the DNA-binding activity of *Pongamia LCL1*.

PRR1/TOC1, *PRR3*, *PRR5*, *PRR7*, and *PRR9* are members of *PRR* gene family, all of which are regulated by the circadian clock and have important roles in the regulation of the central oscillator [80,71,24,52]. In *Arabidopsis*, all five gene members of the *PRR* family are expressed sequentially after dawn, in the following order: *PRR9*, *PRR7*, *PRR5*, *PRR3*, and *PRR1/TOC1* [71,81,82]. Southern blot analysis of *TOC1* and *PRR7* has found that both genes have one copy in the *Pongamia* genome. Moreover, the *PRR* protein family is known to have a conserved pseudo-receiver (PR) domain at the N-terminal region and a CCT domain at the C-terminal region [70,83]. Our study also showed that the PR and CCT domains are conserved in the putative *Pongamia TOC1* and *PRR7* proteins (Fig. 3a and Fig. 4a). Due to the highly conserved PR and CCT domains, it suggests that both *Pongamia TOC1* and *PRR7* might function in a similar way as *Arabidopsis* by repressing *LCL1* expression in the central oscillator [83,71,84].

Expression patterns of Pongamia circadian clock genes

The expression profiles of putative *Pongamia ELF4*, *LCL1*, *PRR7*, and *TOC1*, under long-day conditions, were addressed by both RT-PCR and qRT-PCR analysis in this study. *Pongamia ELF4* expression peaks around 12 h after dawn, or around dusk (Fig. 1d). *Pongamia LCL1* expression peaks early in the morning, at around 4 h after dawn (Fig. 2d). The expression patterns of *Pongamia PRR7* were observed to have a broad peak near the 8-h time point (Fig. 4d). On the contrary, the

Pongamia *TOC1* expression profile showed three different peaks at 0-, 12-, and 20-h time points (Fig. 3d). The peak of *TOC1* expression at the 12-h time point (dusk) is also consistent with *TOC1* being an evening gene [84,70,55].

The morning loop genes *CCA1* and *LHY* are responsible for the activation of *PRR9*, *PRR7*, and *PRR5*, which can then form a negative feedback loop by binding to *CCA1* and *LHY* promoters, thereby inhibiting their expression [56,80]. In accordance, this study also showed that Pongamia *LCL1* and *PRR7* expressions peak together initially, but as the expression of *PRR7* increases, *LCL1* expression is repressed (Fig. 5). Moreover, *CCA1* and *LHY* from the morning loop have also been shown to form a connection to the evening loop by repressing the expression of *TOC1*, *GI*, *ELF3*, *ELF4*, and *LUX* in *Arabidopsis* [85,86,59,87]. Pongamia *LCL1* expression is anti-phase to that of Pongamia *ELF4* and *TOC1* expression (Fig. 5). Additionally, the EC comprises *ELF3*, *ELF4*, and *LUX*, which have a repressive effect on *TOC1*, *GI*, *LUX*, and *ELF4* expression [58,60]. Moreover, the EC also represses *PRR9* to form another link with the morning loop [85,88,89]. Additionally, new studies have found that *TOC1* as an evening gene is a transcriptional repressor of *CCA1* and *LHY*, rather than an activator as previously conceived [90,84,52]. The expression levels of Pongamia *LCL1* and *TOC1* are inversely related to each other (Fig. 5). Accordingly, this study has successfully shown that Pongamia *ELF4*, *LCL1*, *PRR7*, and *TOC1* are diurnally regulated in long-day conditions and show feedback regulation in their expression patterns.

In addition, future studies may involve the investigation of Pongamia *ELF4*, *LCL1*, *PRR7*, and *TOC1* expression under short-day conditions to determine whether these genes can be entrained by a different photoperiod. *ELF4*, *LCL1*, *PRR7*, and *TOC1* expression may also be studied under constant light or constant dark conditions to check if their expressions remain rhythmic in the absence of an external signal, i.e., true circadian rhythm.

Implication of the circadian clock and flowering studies on Pongamia seed production

It is well established in plants that proper matching of circadian rhythm with external cues will enhance photosynthesis and growth and thus improve survival and fitness [27,28]. In addition to this general finding, several studies have been carried out to investigate roles of the circadian clock in life cycle of plants especially crop plants such as cereals and legumes for their food and feed usage around the world. Cereals including maize, rice, and wheat accounted for most of the sources for human and animal food consumption. Maize microarray analysis has showed that ~10% of 1444 transcripts showing circadian rhythms including genes involved in photosynthesis, carbohydrate

metabolism, cell wall synthesis and flowering [91]. In barley, *Ppd-H1* was found to be a major gene that control photoperiod responsiveness in barley. Positional cloning study has identified *Ppd-H1* as a clock orthologue of *Arabidopsis* *PRR7*, and a non-photoperiod responsive allele will allow spring-sown barley to build up higher grain yield and grain weight [92]. In wheat, *PPD1* gene was also found to encode for *PRR3* or *PRR7* orthologues where *ppd-1* mutant flower 1 month later than wild type plants and *PPD1* is associated with most of the natural flowering variation in wheat [93,94]. A recent study on wheat also discovered loss-of-function in *PHYC* (*PHYTOCHROME C*) will cause altered expression of circadian and photoperiod genes and delay flowering in LD [95]. In the rice, QTL studies highlighted the importance of *Hd1*, *CONSTANCE* (*CO*) orthologue, and *Ghd7* which both are photoperiod pathways genes and colocalise with QTLs affect flowering time or number of grains per panicle [96,97]. Moreover, natural variation of cultivated rice flowering time has been shown to be associated with *Hd3a* (*FT* orthologue) promoter sequence, *Hd1* protein sequence, and *Ehd1* expression level [98,99]. Rice orthologue of clock gene *ELF3* (*OsELF3-1*) was recently discovered to be involved in promoting rice flowering under short-day by activating *Ehd1* expression [100].

Legume seeds are second only to cereal crops as a source of human and animal food and include soybeans (*Glycine max*), garden peas (*Pisum sativum*), peanuts (*Arachis hypogaea*), and broad beans (*Vicia faba*). In the soybean, microarray and transcriptome analyses of developing seeds highlighted 1.8% of genes regulated by circadian rhythms, including genes involved in protein and fatty acid synthesis, lipid metabolism, and photosynthesis [101], suggesting the potential roles of the circadian clock in seed composition and development. A recent paper has showed that overexpressing *AtBBX32*, and its soybean homologues (*GmBBX52* or *GmBBX53*) increases the yield gain in soybean by affecting a wide range of yield component parameters (such as flower number, pod number, and seed number) and extending the reproductive period [102]. Interestingly, microarray analysis of the transgenic soybean overexpressing *AtBBX32* showed that most of the transcripts showed abundant changes at dawn and consistently soybean clock genes, *GmLCL2* and *GmTOC1* expression level were affected at dawn in the transgenic soybean [102] suggesting *AtBBX32* might affect grain yield through modifying the light input to circadian clock near dawn [102]. Furthermore, a strong correlation was found in circadian rhythms of soybean photoreceptor gene, *GmCRY1a* (*CRYTOCHROME 1a*) with the latitudinal distribution of soybean cultivars and the photoperiodic control of flowering time [42]. In the pea, several early or late flowering mutants were found to be encoded by circadian clock genes including *HR/ELF3*, *SN/LUX*, and *LATE1/GI* [103,89,19]. In particular, flowering time variation in global pea

germplasm was found to be controlled by a single *HR/ELF3* variant, which correlate with altered circadian rhythms and the reduced photoperiod responsiveness in the spring cultivars of pea [19].

Pongamia tree will start to flower after three to four years and flowering in the southern hemisphere occurs around November/December. After flowering, the seed takes about 10 months to mature and each seed weigh around 1.8 g with approximately 40 % oil content [12,22,7]. Pongamia is an out-crossing species through insect-mediated pollination, pollination of Pongamia mainly rely on several bee species through floral nectar sources [104]. Interestingly, nectar secretion and flower opening was found to be regulated by circadian clock to increase the maximum chances of pollination by timed to potential pollinator activity [105-107]. Hence, this paper specifically focuses on the circadian clock system, which is part of the photoperiod pathway [108,24,65,109,75], for its role as one of the key regulators of flowering time. By deciphering the molecular basis of flowering time control, we could eventually manipulate the transition from vegetation to flowering stage for shorter vegetative period and prolonged flowering stage, which will lead to the extended period of seed production in order to increase raw material of biofuel production. In addition, the identification and analyses of the genes involved in the Pongamia circadian system, as well as in other developmental pathways, has the potential to facilitate the development of Pongamia cultivars that are suitable to be grown in wide range of climates with the potential of manipulating adaptation of Pongamia plant to different photoperiod condition. This will have an implication for expanding the range of geographical areas for the large-scale production of Pongamia trees for biodiesel production. Together, extending our knowledge regarding the function of circadian clock genes and the relationship with flowering control in Pongamia is key to regulate the process of flowering and improve seed yield in order to make Pongamia a sustainable sources for biofuel production.

Acknowledgement

This work was supported by the Australian Research Council in the form of the ARC Centre of Excellence for Integrative Legume Research (CE0348212) and McKenzie Postdoctoral Fellowship by the University of Melbourne.

Figures and Tables Legends

Table 1 Mapping results of the *Pongamia* transcriptome library to the soybean circadian clock genes. The putative *Pongamia* circadian clock genes that covered the full length soybean reference genes are highlighted in dark blue and the genes that were being studied further are shown in bold letters.

Table 2 Sequences similarity of *Pongamia* nucleotides and proteins with soybean and *Arabidopsis*

Fig. 1 a ELF4 protein sequence alignment between *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Millettia pinnata* (Mp), *Medicago truncatula* (Mt) and *Pisum sativum* (Ps). The shaded regions represent 100 % similarity (black), 80 – 99 % similarity (dark grey with white font) and 60 – 79 % similarity (light grey with black font). At ELF4 (NP_565922.1), Gm ELF4b (XP_003537487.1), Mp ELF4, Mt ELF4a (AFK36717.1), Ps ELF4 (AAX47177.2). **b** Phylogenetic tree of putative *Pongamia* ELF4 protein and other plant species were constructed based on their protein alignments. The tree is drawn to given scale and the bootstrap values are shown at each node. At, *Arabidopsis thaliana* (AtELF4 = NP_565922.1, AtELFL1 = NP_180556.1, AtELFL2 = NP_565044.1, AtELFL3 = NP_565334.1, AtELFL4 = NP_564024.1); Cr, *Chlamydomonas reinhardtii* (CrELF4 = XP_001689467.1); Gm, *Glycine max* (GmELF4a = NP_001236861.1, GmELF4b = XP_003537487.1, GmELF4c = XP_003552375.1, GmELF4d = NP_001236892.1); Hv, *Hordeum vulgare* (HvELF4 = BAK04623.1); Mc, *Mesembryanthemum crystallinum* (McELF4 = AAQ73526.1); Mp, *Millettia pinnata*; Mt, *Medicago truncatula* (MtELF4a = AFK36717.1, MtELF4b = XP_003627239.1, MtELF4d = AFK49629.1) Os, *Oryza sativa* (OsELF = AAD27669.1); Ppa, *Physcomitrella patens* (PpaELF4 = XP_001774658.1); Pp, *Prunus persica* (PpELF4 = EMJ19798.1); Ps, *Pisum sativum* (PsELF4 = AAX47177.2); Pta, *Pinus taeda* (PtaELF4 = ACK56119.1); Pt, *Populus trichocarpa* (PtELF = XP_002311221.1); Sl, *Solanum lycopersicum* (SlELF = XP_004241125.1); Vv, *Vitis Vinifera* (VvELF = XP_002270733.1). **c** Southern blot analysis of *ELF4* digested with *Bam*HI and *Eco*RI. **d** Expression pattern of putative *Pongamia* *ELF4* under long day conditions (18 hours light : 6 hours dark) in a 24 hour period. qRT-PCR and RT-PCR results of *ELF4* expression normalized against *Cons7* expression; the data from three replicates have been averaged. Each time point corresponds to the number of hours after lights on, *i.e.* the number of hours after dawn.

Fig. 2 a LCL1 protein alignment between *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Millettia pinnata* (Mp) and *Pisum sativum* (Ps). The region corresponding to the Myb DNA binding domain is indicated. The shaded regions represent 100 % similarity (black), 80 – 99 % similarity (dark grey with white font) and 60 – 79 % similarity (light grey with black font). At LHY (NP_001077437.1), Gm LCL1 (NP_001235187.1), Mp LCL1, Ps MYB1 (AAX33630.1). **b** Phylogenetic tree of putative *Pongamia* LCL1 protein with other plant species were constructed based on their protein alignments. The tree is drawn to given scale and the bootstrap values are shown at each node. At, *Arabidopsis thaliana* (AtCCA1 = NP_850460.1, AtLHY = NP_001077437.1); Cs, *Castanea sativa* (CsLHY = AAU20773.1); Gm, *Glycine max* (GmLCL1 = NP_001235187.1, GmLCL2 = NP_001236400.1); Lg, *Lemna gibba* (LgLHYH1 = BAD97870, LgLHYH2 = BAD97871); Lp, *Lemna paucicostata* (LpLHYH1 = BAD97866, LpLHYH2 = BAD97867); Mc, *Mesembryanthemum crystallinum* (McCCA1 = AAQ73524); Mj, *Mirabilis jalapa* (MjLHY = ACL81163); Mp, *Millettia pinnata*; Mt, *Medicago truncatula* (MtCCA1a = XP_003626618.1, MtMYBa = XP_003600632.1, MtMYBb = XP_003616176.1) Os, *Oryza sativa* (OsCCA1 = NP_001061032); Pn, *Populus nigra* (PnLHY1 = BAH09382, PnLHY2 = BAH09383); Ppa, *Physcomitrella patens* (PpaCCA1a = BAI39991, PpaCCA1b = BAI39992); Ps, *Pisum sativum*

(PsMYB1 = AAX33630.1, PsMyb2 = AAX33631.1); Pt, *Populus trichocarpa* (PtMYB = XP_002320238.1); Pv, *Phaseolus vulgaris* (PvLHY = CAD12767.2); Rc, *Ricinus communis* (RcMYB = XP_002515093.1); Sb, *Sorghum bicolor* (SbMYB = XP_002443890.1); Sl, *Solanum lycopersicum* (SLHY = XP_004248416.1); Vv, *Vitis Vinifera* (VvMYB = XP_002267720.1); Zm, *Zea mays* (ZmLHY = NP_001147482.1). **c** Southern blot analysis of *LCL1* digested with *Bam*HI and *Hind*III. **d** Expression pattern of putative *Pongamia LCL1* under long day conditions (18 hours light : 6 hours dark) in a 24 hour period. qRT-PCR and RT-PCR results of *LCL1* expression normalized against *Cons7* expression; the data from three replicates have been averaged. Each time point corresponds to the number of hours after lights on, i.e. the number of hours after dawn.

Fig. 3 a TOC1 protein alignment between *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Milletia pinnata* (Mp) and *Pisum sativum* (Ps). The regions corresponding to the PR and CCT domains are indicated. The shaded regions represent 100 % similarity (black), 80 – 99 % similarity (dark grey with white font) and 60 – 79 % similarity (light grey with black font). At PRR1 (NP_200946.1), Gm TOC1 (NP_001235202.1), Mp TOC1, Ps TOC1 (AAX47178.1). **b** Phylogenetic tree of putative *Pongamia* TOC1 protein with other PRR proteins from different plant species were constructed based on their protein alignments. The tree is drawn to given scale and the bootstrap values are shown at each node. At, *Arabidopsis thaliana* (AtPRR1 = NP_200946.1, AtPRR3 = NP_568919.1, AtPRR5 = NP_568446.1, AtPRR7 = NP_568107.1, AtPRR9 = NP_566085.1); Cs, *Castanea sativa* (CsPRR7 = ABV53463.1, CsTOC1 = AAU20772.1); Gm, *Glycine max* (GmPRR7 = XP_003536977.1, GmTOC1 = NP_001235202.1); Hv, *Hordeum vulgare* (HvPPd-H1 = AAY17586.1); Lg, *Lemna gibba* (LgPRR-H37 = BAE72700.1, LgPRR-H59 = BAE72701.1, LgPRR-H95 = BAE72702.1); Lp, *Lemna paucicostata* (LpPRR-H37 = BAE72697.1, LpPRR-H59 = BAE72698.1, LpPRR-H95 = BAE72699.1); Mp, *Milletia pinnata*; Mt, *Medicago truncatula* (MtPRR73a = XP_003606528.1); Os, *Oryza sativa* (OsPRR1 = BAD38854.1, OsPRR37 = BAD38858.1, OsPRR73 = BAD38859.1, OsPRR95 = BAD38857); Ps, *Pisum sativum* (PsPRR37 = ACU42263.2, PsTOC1 = AAX47178.1); Pt, *Populus trichocarpa* (PtPRR5 = XP_002320232.1, PtPRR7 = XP_002311123.1); Zm, *Zea mays* (ZmPRR95 = NP_001151536.1). **c** Southern blot analysis of *TOC1* digested with *Bam*HI, *Eco*RI and *Hind*III. **d** Expression pattern of putative *Pongamia TOC1* under long day conditions (18 hours light : 6 hours dark) in a 24 hour period. qRT-PCR and RT-PCR results of *TOC1* expression normalized against *Cons7* expression; the data from three replicates have been averaged. Each time point corresponds to the number of hours after lights on, i.e. the number of hours after dawn.

Fig. 4 a PRR7 protein alignment between *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Milletia pinnata* (Mp), *Medicago truncatula* (Mt) and *Pisum sativum* (Ps). The regions corresponding to the PR and CCT domains are indicated. The shaded regions represent 100 % similarity (black), 80 – 99 % similarity (dark grey with white font) and 60 – 79 % similarity (light grey with black font). At PRR7 (NP_568107.1), Gm PRR7 (XP_003536977.1), Mp PRR7, Mt PRR73a (XP_003606528.1), Ps PRR37 (ACU42263.2). **b** Phylogenetic tree of putative *Pongamia* PRR7 protein with other PRR proteins from different plant species were constructed based on their protein alignments. The tree is drawn to given scale and the bootstrap values are shown at each node. At, *Arabidopsis thaliana* (AtPRR1 = NP_200946.1, AtPRR3 = NP_568919.1, AtPRR5 = NP_568446.1, AtPRR7 = NP_568107.1, AtPRR9 = NP_566085.1); Cs, *Castanea sativa* (CsPRR7 = ABV53463.1, CsTOC1 = AAU20772.1); Gm, *Glycine max* (GmPRR7 = XP_003536977.1, GmTOC1 = NP_001235202.1); Hv, *Hordeum vulgare* (HvPPd-H1 = AAY17586.1); Lg, *Lemna gibba* (LgPRR-H37 = BAE72700.1, LgPRR-H59 = BAE72701.1, LgPRR-H95 = BAE72702.1); Lp, *Lemna paucicostata* (LpPRR-H37 = BAE72697.1, LpPRR-H59 = BAE72698.1, LpPRR-H95 = BAE72699.1); Mp, *Milletia pinnata*; Mt, *Medicago truncatula* (MtPRR73a = XP_003606528.1); Os, *Oryza sativa*

(OsPRR1 = BAD38854.1, OsPRR37 = BAD38858.1, OsPRR73 = BAD38859.1, OsPRR95 = BAD38857); Ps, *Pisum sativum* (PsPRR37 = ACU42263.2, PsTOC1 = AAX47178.1); Pt, *Populus trichocarpa* (PtPRR5 = XP_002320232.1, PtPRR7 = XP_002311123.1); Zm, *Zea mays* (ZmPRR95 = NP_001151536.1). **c** Southern blot analysis of *PRR7* digested with *BamHI* and *HindIII*. **d** Expression pattern of putative *Pongamia PRR7* under long day conditions (18 hours light : 6 hours dark) in a 24 hour period. qRT-PCR and RT-PCR results of *PRR7* expression normalized against *Cons7* expression; the data from three replicates have been averaged. Each time point corresponds to the number of hours after lights on, i.e. the number of hours after dawn.

Fig. 5 Expression pattern of putative *Pongamia ELF4*, *LCL1*, *PRR7* and *TOC1* as determined by qRT-PCR under long-day conditions (18 hours light : 6 hours dark) in a 24 hour period. To clarify the profiles, the maximum level of each transcript was taken as 1.

Tables and Figures

Table 1 Mapping results of the Pongamia Transcriptome library to the soybean circadian clock genes. The putative Pongamia circadian clock genes that covered the full-length soybean reference genes are highlighted in dark blue and the genes that were being studied further are shown in bold letters.

Circadian Clock Genes	Soybean Gene Identifiers	Coverage (%)	Reference
<i>CO</i>	Glyma08g28370	95	Jung et al. (2012)
	Glyma13g07030	45	
	Glyma18g51320	100	
	Glyma19g05170	45	
<i>ELF3</i>	Glyma04g05280	95	Jung et al. (2012)
	Glyma14g10530	65	
	Glyma17g34980	45	
<i>ELF4</i>	Glyma11g35270	100	Thakare et al. (2010)
	Glyma18g03130	100	
<i>GI</i>	Glyma10g36600	100	Thakare et al. (2010)
<i>LCL1</i>	Glyma16g01980	100	Thakare et al. (2010)
<i>LCL2</i>	Glyma03g42260	100	Thakare et al. (2010)
<i>LUX</i>	Glyma11g14490	75	Liew (unpublished data)
<i>PRR5</i>	Glyma04g40640	100	Jung et al. (2012)
	Glyma06g14150	100	
<i>PRR7</i>	Glyma10g05520	100	Jung et al. (2012)
<i>TOC1</i>	Glyma06g21120	100	Thakare et al. (2010)

1 **Table 2** Sequences similarity of Pongamia nucleotides and proteins with soybean and *Arabidopsis*

Pongamia Gene	Soybean Gene ID	Arabidopsis Gene ID	Number of Nucleotides	Nucleotides Similarity (%)		Pongamia Protein	Number of Amino Acid	Amino Acid Similarity (%)	
				Soybean	Arabidopsis			Soybean	Arabidopsis
<i>ELF4</i>	Glyma11g35270	At2g40080	354	80.2	62.6	ELF4	117	75.8	57.7
<i>LCL1</i>	Glyma16g01980	At1g01060	2305	87.9	58.4	LCL1	746	82.8	46.5
<i>PRR7</i>	Glyma10g05520	At5g02810	2436	91.3	60.0	PRR7	755	87.1	47.8
<i>TOC1</i>	Glyma06g21120	At5g61380	1692	90.2	62.2	TOC1	563	88.7	49.8

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Title:

Isolation and Characterization of Circadian Clock Genes in the Biofuel Plant Pongamia (Milletia pinnata)

Date:

2015-06-01

Citation:

Winarto, H. P., Liew, L. C., Gresshoff, P. M., Scott, P. T., Singh, M. B. & Bhalla, P. L. (2015). Isolation and Characterization of Circadian Clock Genes in the Biofuel Plant Pongamia (Milletia pinnata). BIOENERGY RESEARCH, 8 (2), pp.760-774. <https://doi.org/10.1007/s12155-014-9556-z>.

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<http://hdl.handle.net/11343/282949>