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Protein Expression of Late Elongated Hypocotyl (LHY) Homolog Genes of Teak in *Escherichia coli*

Norlia Basherudin[1], Norwati Muhammad[1], Norwati Adnan[1], Mohd Rosli Haron[1] and Norihan Mohd Saleh[2]*

[1]Genetic Unit, Biotechnology Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia

[2]Cell and Molecular Biology Department, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Expression of an isolated gene in a system that directly translates it into a protein is an important step to study the protein encoded by the gene. The isolated gene can be expressed in vivo by a heterologous system. In this study, a bacteria system was used to translate the *Tectona grandis* Late Elongated Hypocotyl (Tg-LHY) gene, which was isolated from flowering tissues of teak (*Tectona grandis*). The gene was cloned into the pET 14b vector (Novagen) and transformed into BL 21(DE3)/pLysS and Rosetta 2 expression host cells (Novagen). Rosetta 2 host cell has been found to be a good candidate to express the Tg-LHY protein from plant origin, as it recognizes the codon that was found in plant but rarely used in bacteria. The expressed protein was about an expected size, which was 90 kD. Western blot analysis using antibody against His-tag, which was fused to the Tg-LHY protein, proved that the expressed protein was Tg-LHY protein.

Keywords: Heterologous protein expression, *Tectona grandis*, LHY homolog genes

INTRODUCTION

The Late Elongated Hypocotyl (LHY) homolog gene has been isolated from flowering tissues of teak (*Tectona grandis*) using subtractive hybridization technique, and was named *Tectona grandis* Late Elongated Hypocotyl (Tg-LHY) (Norlia *et al.*, 2006). This gene has similarities to LHY genes from a few plant species. It is 57% identical to LHY of *Castanea sativa*, 52% identical to *Phaseolus vulgaris* and 43% identical to *Arabidopsis thaliana* (Ramos *et al.*, 2005; Kaldis *et al.*, 2003; Schaffer *et al.*, 1998). LHY gene has been reported as one of the important genes in plant circadian clock system (Schaffer *et al.*, 1998). Other genes involved in plant circadian clock oscillation are Circadian Clock Associate 1 (CCA1) and Timing of CAB Expression 1 (TOC1) (Carre, 2002). In *Arabidopsis*, a reciprocal regulation between TOC1 and LHY/CCA1 led to oscillation of circadian clock system (Alabadi, 2001). Circadian clock systems are complex signalling networks that allow organisms to adjust cellular and physiological activities in anticipation of periodic changes in the environment. Circadian clock governs many plant processes including movement of organs like leaves and petals, hypocotyl elongation, stomata opening, expression of several genes and flowering time (Jarillo *et al.*, 2004).

It has been suggested that circadian rhythmicity depends on clock protein. In *Arabidopsis*, the most circadian clock plant studied, light was shown to modulate expression of LHY at the translation level which coincides with expression of LHY mRNA at dawn (Kim *et al.*, 2003). This simultaneous translation induction and transcription repression of LHY expression are thought to play a role in narrowing the peak of LHY protein synthesis at dawn and increasing the robustness and accuracy of circadian oscillator.

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* Corresponding Author

The expression of LHY gene in flowering tissue of teak suggested the involvement of the circadian clock system in flower development of teak. In *Arabidopsis*, which is a long day plant, the long photoperiod detected by the circadian clock led to early flower development (Blazque, 2000). Teak, which is planted in neutral day places like Malaysia, photoperiod might not be the environmental factor detected by its circadian clock system towards flower development. Comparison between five years temperature data and the reproductive cycle of teak at Mata Ayer, Perlis suggest that temperature might be the environmental factor detected by the circadian clock system of teak (Norlia, 2007). To further investigate the assumption, Tg-LHY protein in correlation with the temperature would be an indicator for the environmental factor that was detected by circadian clock system of teak.

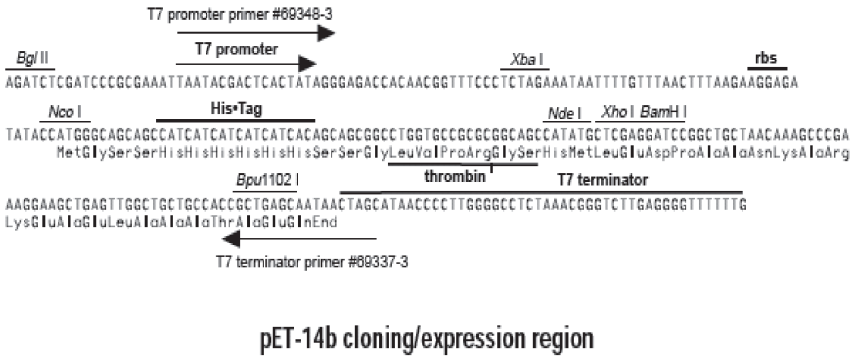
The objective of this study was to isolate the Tg-LHY protein, which will be useful in preparing the Tg-LHY specific antibodies for further Tg-LHY protein analysis in flowering tissues of teak. In this paper we report the cloning and expressing of Tg-LHY protein in a bacteria system.

MATERIALS AND METHODS

Primer Design and Cloning

The pET 14b plasmid vector (Novagen, USA) used in this study is a translation vector, which carries a His-Tag sequence and strong bacteriophage T7 translation signal at the 5' end of the multiple cloning site (*Fig. 1*). Cloning sites of this vector only consists of 3 restriction enzyme sites, which are *Bam H*1, *Xho* 1 and *Nde* 1 (*Fig. 1*). Therefore, primers were designed as such to include either one of these restriction enzyme sites, however the selected restriction enzyme site should not exist within the cDNA fragment. The primers designed were LHYXhof (5'-GCG CTC GAG **ATG** GAC CCT TAT TCA TCT-3') and LHYBamr (5'-GCG GGA TCC **TTA** AGT AGA AGC CTC TCC-3'), which contained restriction enzyme sites of *Xho* I and *Bam HI*, respectively (underlined bases). In designing the primer, the start codon of the gene was placed immediately after the restriction site for the 5'-primer and the stop codon was placed immediately before the restriction site for the 3'-primer (bolded bases).

PCR to amplify the full-length of Tg-LHY cDNA fragment was carried out using PE GenAmp System 9600, The program used was denaturing at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 45 sec, annealing at 60°C for 1.3 min and extension at 70°C for 10 min. The reaction was carried out by adding 2.5 µl 10X Pfx Amplification buffer (Invitrogen, USA), 1.5 µl 10 mM dNTP mixture (Fermentas, USA), 10 µM of each primer, 150 ng recombinant plasmid containing full length of Tg-LHY, 1 U Platinum Pfx DNA Polymerase and distilled water to the total volume of 25 µl. The amplified Tg-LHY fragment was then eluted and ligated into PCR 2.1 plasmid vector (Invitrogen, USA) and transformed into One Shot competent cell (Invitrogen, USA). The plasmid was digested with both restriction enzymes and the insert was sub-cloned into an expression vector, pET 14b. Recombinant plasmid was then transformed into competent DH5µ cell. The recombinant plasmids were then extracted and sequenced. The recombinant plasmids, which showed a right reading frame after sequencing, were transformed into an expression host cell, either BL 21(DE3)/pLysS or Rosetta 2 (Novagen). Both strains carry a chromosomal copy of the T7 RNA polymerase gene under the control of *lacUV5* promoter, which



pET-14b cloning/expression region

Fig. 1: Multiple cloning site of vector pET 14b (reproduced from pET 14b map, Novagen, USA)

is suitable for the production of protein from target genes cloned in pET vector. Strain Rosetta 2 has another feature that enhances the expression of eukaryotic proteins encoded by codons rarely used in *E. coli* such as AUA, AGG, AGA, CUA, CCC and GGA (Del Tito *et al.*, 1995).

Protein Induction and Denaturation

Transformed cells were incubated at 37°C with shaking at 250 rpm in Luria Bertani medium containing 50 l/ml ampicillin and 34 g/ml chloramphenicol until the OD_{600} reached 0.6. One ml of the medium containing growth cell was removed for the un-induced control. IPTG was added to the remaining cells to the final concentration of 0.4 mM. The incubation was continued until 5 hr; every one hour, 1 ml of sample was removed for time course expression analysis. The five samples collected at different time periods together with the un-induced control were centrifuged at 10,000 g for 1 min and each pellet was dissolved in 100 l of 1 X Phosphate Buffer Saline (PBS). Two hundred microlitres of 2X sample buffer (125 mM tris-HCl [pH 6.8], 4% [w/v] SDS, 5% [v/v] 2-Mercaptoethanol, 20% [v/v] glycerol and 0.1% [w/v] bromophenol blue) were added and passed through a 27-gauge needle five times to reduce the viscosity. Each sample was denatured by heating at 85°C for 5 min and stored at -20°C until further analysis.

SDS-PAGE

The expressed proteins were analysed using 12%(w/v) SDS-PAGE, which was prepared according to the Protein Electrophoresis Technical Manual of Amersham Pharmacia Biotech. The gel was stained with coomassie blue and dried in between two sheets of porous cellophane and locked into the drying frame. The framed gel was allowed to dry for two days.

Western Blot Analysis

Western blotting was performed in order to identify the protein fragments of expressed inserted genes using antibodies. His-Tag AP Western reagent kit and His-Tag monoclonal antibody (Novagen, USA) were used to detect the heterologous protein. Separated proteins on 12% (w/v) SDS-PAGE were electrophoretically transferred to a PVDF Western Blotting Membrane (Roche) using a semi-dry electrophoretic transfer (Biorad). Colorimetric detection of His-tag protein, encoded by pET 14b, which was fused to N-terminal of the expressed protein, was carried out according to the User Protocol of His-Tag Monoclonal Antibody supplied by the manufacturer (Novagen).

RESULTS AND DISCUSSION

Tg-LHY cDNA amplified using LHYfXho and LHYrBam primer pair, were about 2.4 kb (*Fig. 2*). The PCR fragments were ligated into the PCR 2.1 vector before digestion with *Xho* I and *Bam HI* enzyme. This was carried out to ensure complete digestion of the fragment. The digested fragments (*Fig. 3*) were then ligated into the digested pET 14b vector with similar enzymes and transformed into DH5 for recombinant clone analysis. PCR method using LHYfXho and LHYrBam primers was carried out to screen for

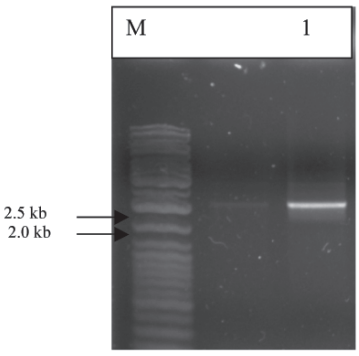


Fig. 2: PCR product of Tg-LHY cDNA using LHYfXho and LHYrBam primer pair. The amplified fragment is about 2.4 kb (1). M is DNA marker of GeneRuler Ladder Mix (Fermentas)

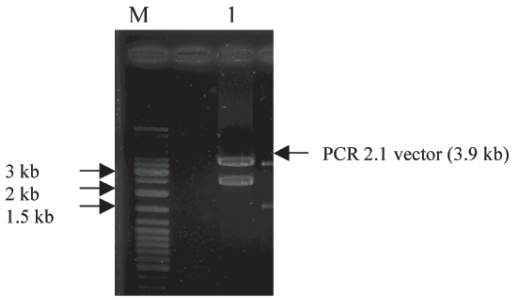


Fig. 3: Complete digestion of pET14b-TgLHY construct. (1) Tg-LHY containing Bam HI and Eco RI cutting site on its ends was digested with respective enzymes. M is DNA marker of GeneRuler Ladder Mix (Fermentas)

the presence of Tg-LHY cDNA in the recombinant plasmid (*Fig. 4*).

The positive recombinant plasmid with compatible reading frame was transformed into an expression host cell. Earlier in this experiment, BL 21(DE3)/pLysS (Novagen) host cell was used. Time course gene expression analysis showed an increase in protein synthesis at an expected size (as pointed by slunt arrow in *Fig. 5a*). The size expectation was calculated based on the assumption that the mean molecular weight of the amino acid is 110 Da, therefore the expressed protein was estimated to be about 90 kD (84 kD of Tg-LHY and 4.4 kD of His-tag protein). The size of Tg-LHY protein expressed was similar to the size of Arabidopisi LHY protein, which was reported to be about 88 kDa (Kim *et al.*, 2003). However, few other bands of expressed protein were also observed (as pointed by straight arrows in *Fig. 5a*). Based on Western analysis, the smaller protein synthesized was found to contain the His-tag protein (*Fig. 5b*). Therefore the proteins were believed to be due to premature translation termination. The phenomenon occurs as a result of differences in codon usage between the inserted gene origin and the host cell, *E. coli*. In *E. coli*, codon such as AGG, AGA, ATA, CTA, CCC, GGA and CGG are rarely used. However in Tg-LHY cDNA, about 7% of its total codons were the rare codons of *E. coli*.

Recombinant plasmid was then transformed into Rosetta 2 host cell in order to enhance the expression of eukaryotic protein that contains codons rarely used in *E. coli*. Rosetta 2 host cell is a BL21 derivative, which was engineered to contain tRNAs and able to translate a rare codon of *E. coli*. Time course protein synthesized analysis showed that after 2 hours induction with IPTG, a protein band of about 90 kD was synthesized (*Fig. 6a*). Western blot analysis against His-tag, which was fused to the N-terminal of the synthesized protein, confirmed that the bands correspond to the protein translated from the inserted genes (*Fig. 6b*).

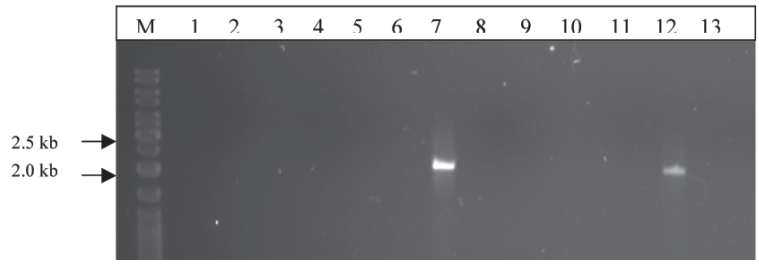


Fig. 4: PCR screening of recombinant clones containing pET14b-TgLHY. LHYfXho and LHYrBam primers were used to amplify Tg-LHY, and out of 13 clones (1 –13) screened, 2 clones (7 and 12) amplified the fragment. M is DNA marker, GeneRuler Ladder Mix (Fermentas, USA)

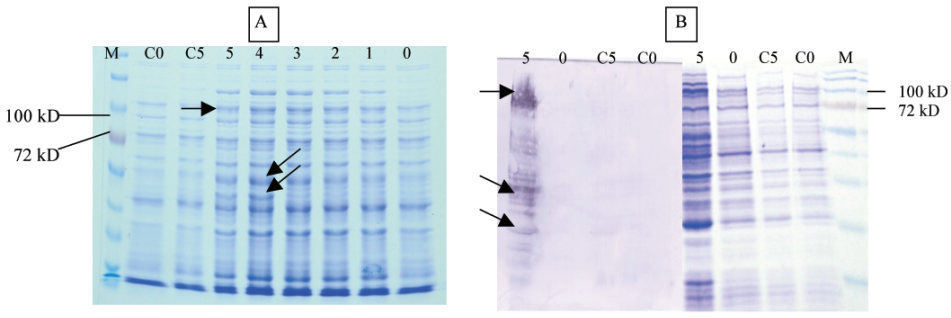


Fig. 5: SDS-PAGE analysis (A) and Western blot analysis (B) of Tg-LHY transformed into BL 21 host cell. Tg-LHY expressed protein at an expected size was marked by straight arrows and truncated expressed proteins were marked by slunt arrows. Protein loaded in the lane of SDS-PAGE were harvested before IPTG was added (0), and 1 hr (1), 2 hr (2), 3 hr (3), 4 hr (4) and 5 hr (5) after IPTG induction. The vector cell cultured (pET 14b in BL 21 host cell) was used as a control and was harvested before induction (C0) and after 5 hours induction (C5). M in both analysis is protein marker Protein prestained ladder (Crystalgen)

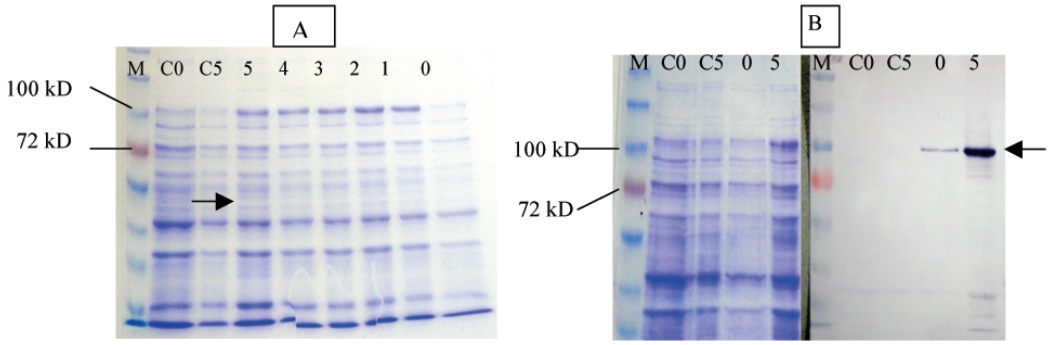


Fig. 6: SDS-PAGE analysis (A) and Western blot analysis (B) of Tg-LHY transformed into Rosetta 2 host cell. Tg-LHY expressed protein at an expected size was marked by straight arrows. Protein loaded in the lane of SDS-PAGE were harvested before IPTG was added (0), and 1 hr (1), 2 hr (2), 3 hr (3), 4 hr (4) and 5 hr (5) after IPTG induction. The vector cell cultured (pET 14b in respective host cell) was used as a control and was harvested before induction (C0) and after 5 hours induction (C5). M in both analysis is protein marker Protein prestained ladder (Crystalgen)

CONCLUSIONS

The protein of Tg-LHY gene isolated from teak was expressed in a bacteria system. However, due to the different codon usage between bacteria and plant, expression host cell that was able to recognize the rare codon should be used. In this study, Rosetta 2 has been found to be a better host cell for the expression of Tg-LHY protein, which originated from plant.

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