Identification of Terminal Flowering1 (TFL1) Genes Associated with the Teak (Tectona grandis) Floral Development Regulation Using RNA-seq

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ABSTRACT

Teak is woody plants; a member of the Lamiaceae family. Teak is a plant that has a very high quality timber. Teak has constraints due to low reproductive rates and slow growth of the wood after entering the reproductive phase. Teak genetic engineering efforts by delaying flowering time was facing difficulties due to the lack of information about the role of genes regulating flowering identity in teak. Teak has indeterminate inflorescence same as the model plant Arabidopsis. In Arabidopsis, the role of Terminal Flowering 1 (TFL1) gene as a member of the Floral Meristem Identity (FMI) in regulating the vegetative to generative transition is by down regulation, so that, the downstream of the FMI genes up-regulation which resulted in the development towards the formation of flowers. In teak, this mechanism is not well known. The development of NGS technology-transcriptome analysis has allowed us to identify specific interest genes from non-model plant rapidly and cheaply relative. To determine the activity of the interest genes in silico can be undertaken with RNA-seq and QRT-PCR analysis approaches. In this study, it is identified that, TFL1 genes in teak with NGS transcriptome analysis approach that is annotated with S. lycopersicum. The TFL1 genes obtained from EST teak derived from vegetative and generative shoots buds RNA. The TFL1 genes activities on the tissues are done with RNA-seq analysis approach in order to obtain Digitally Gene Expression (DGE) of TFL1. The TFL1 gene activity was then validated in silico by QRT-PCR analysis. The results of the analysis showed that the TFL1-14 gene activity equivalent to the TFL1 gene activity in the model plant.

Key words: Terminal flowering 1, NGS-transcriptome analysis, DEG, QRT-PCR, EST, floral meristem identity genes

INTRODUCTION

Teak normally begin flowering at the age of 6-8 years after planting but in the artificial forest is reported to have early flowering at the age of two years after planting (Norwati et al., 2011; Khanduri, 2012). At the beginning of flowering teak controlled by genetic and environmental factors (Palupi et al., 2010). Early flowering in the terminal causes the main axis forking in the first
Fig. 1: Teak inflorescence, including, (a) Shoots generative apex (Generative Apical Bud/AB), (b) Lateral generative shoots (Generative Lateral Bud/LB2) and (c) Lateral generative shoots (Generative Lateral Bud/LB4)

Year of flowering and forking on other shoots occur in the next flowering season (Palupi et al., 2010; Norwati et al., 2011; Khanduri, 2012). Figure 1 is shows the forking form of teak flowering. Forking in the major axis at the earliest stage of the inflorescence can reduce the growth of timber which can damage the quality of the wood (Widiyanto et al., 2009; Palupi et al., 2010; Norwati et al., 2011; Khanduri, 2012).

Flowering reduces vegetative growth rate due to the utilization of energy for the flowering process (Widiyanto et al., 2009). Molecular biology approaches becomes important to implement to understand the function and interaction of genes involved in the flowering process in teak (Ansari et al., 2012). Understanding the role of each gene in regulating flowering in teak will facilitate the conduct of engineering to improve the quality of teak (Widiyanto et al., 2009; Palupi et al., 2010; Norwati et al., 2011; Ansari et al., 2012).

Flowering in teak can be divided into four sequential stages; (1) Activation of flowering time genes (flowering time genes) both by environmental and endogenous signals, (2) Activation of meristem identity genes (meristem identity genes) by some flowering time signals through various pathways that determine the identity of interest, (3) The identity of the gene activation floral organs (flower organ identity genes) by meristem identity genes that specify floral organs and (4) Activation of genes involved in organ builder of four floral organ formation (Rosli et al., 2009). These stages are equivalent to the stages that occur in the model plant (Levy and Dean, 1998b). In the model plant, TFL1 is one of the meristem identity gene that play a role in regulating the other floral meristem identity genes (Larsson et al., 1998; Levy and Dean, 1998a; Olsen et al., 2002; Jack, 2004). Down regulation of TFL1 gene will result in up-regulation of downstream genes are LFY and AP1 (Olsen et al., 2002; Ordidge et al., 2005). Up regulation of LFY and AP1 were resulting in AGL4 induced flowering organs (Olsen et al., 2002).
The question arises, whether the regulation of TFL1 in model plants also occur in teak? This research will attempt to answer that question. NGS-transcriptome analysis performed to identify genes TFL1 (Liu et al., 2013; Zhang et al., 2013). To further, analyze the gene TFL1 in teak by the application of phylogenetic analysis. Phylogenetic analysis was performed on all TFL1 genes that exist in teak. In addition, phylogenetic analysis was also performed on selected TFL1 based on the lowest E-value compared with TFL1 gene in the other plants that are the result of BLASTX to NCBI nr protein database. The RNA-seq analysis is performed to produce DEG of TFL1 on both tissues, the vegetative and generative tissues of teak (Feng et al., 2012; Mutasa-Gottgens et al., 2012). The QRT-PCR analysis was performed to validate the results of DEG, the TFL1 gene activity in vegetative and generative tissues in silico (Brunner et al., 2004; Barakat et al., 2012). In this study, it is identified that TFL1 genes in teak with NGS transcriptome analysis approach that is annotated with S. lycopersicum.

MATERIALS AND METHODS

Teak tissues materials and RNA isolation: Vegetative and generative shoot buds of teak were collected from a 12 year old teak plant in Institute of Technology Bandung, Indonesia for RNA isolation. The following vegetative tissues were sampled from vegetative apical shoots. Generative tissues were sampled from lateral (nodal) floral-Buds 2nd of generative stage shoots. Both of teak tissue samples were frozen in liquid nitrogen immediately upon collection and put in dry shipper for shipping from ITB-Indonesia to Pennsylvania State University (PSU)-USA. Samples were immediately frozen at -80°C upon arrival at PSU until use. Total RNA was obtained by using the method for RNA isolation protocol that developed by Chang et al. (1993). Frozen tissue were ground to a fine powder under liquid nitrogen and dispersed in CTAB buffer. Following 2 chloroform extractions, RNA was precipitated with LiCl2, again extracted with chloroform and precipitated with ethanol. The resulting RNA pellet was resuspended in 20-100 µL of DEPC-treated water. RNA concentration analysis on a QubitTM fluorometer (www.invitrogen.com/qubit) to show a total yield of RNA sample (Barakat et al., 2009). The RNA concentrations are 555 and 206 ng µL-1 for vegetative and generative sample, respectively. The integrity of RNA was assessed with the Agilent 6000 RNA Nano Chip Kit on 2100 Bioanalyzer (Agilent Technologies) (Barakat et al., 2012).

Paired-end cDNA library preparation and MiSeq Illumina sequencing: Total RNA of teak was extracted from the two tissues using the protocol described previously. The double-stranded cDNA was synthesized using the cDNA synthesis system using random hexamer primers (illumina) according to manufacturer’s instructions (Li et al., 2012; Lulin et al., 2012; Fu et al., 2013). The paired-end library was developed according to the protocol of the paired-end sample preparation kit (Illumina, USA) (Mizrachi et al., 2010; Lulin et al., 2012; Liu et al., 2013). The resulting library was sequenced at Penn State University using Illumina MiSeq™ 2000 (Illumina Inc., USA).

Transcript assembly and annotation: The FASTQ data file of two sequence computed with CLCbio for transcript assembly strategy (Angeloni et al., 2011; Annadurai et al., 2013). The paired-end reads were trimmed for quality score and the presence of repeated sequences >50 bp using the modified Mott-trimming algorithm present (default parameters) in CLCbio (Fu et al., 2013). We assembled de novo the Illumina-trimmed paired-end reads into transcript contigs using the software ‘CLC Genomics Workbench’ by setting minimum 95% identity, minimum 40% overlap and 200 bp as minimum contig length (Liu et al., 2013). The quality of the de novo assembly was
assessed with a local BLASTN (e-value <10^{-6}) alignment of all the contigs against S. lycopersicum (www.phytozome.com) using CLCbio workbench (Wang et al., 2010; Zhang et al., 2013). After teak TFL1 sequences obtained, then phylogenetic analysis performed on the TFL1-14 sequences to determine the TFL1 gene diversity that exist in teak. Phylogenetic analysis was also conducted to determine the teak TFL1-14 position compared with TFL1 of the other plant using BLASTX analysis approach to NCBI nr protein database. Phylogenetic analysis is performed using the ClustalW2 (http://www.ebi.ac.uk/) (Larkin et al., 2007).

RNA-seq analysis: Comparison of digitally gene expression (DEG-seq) between TFL1 in vegetative and generative tissues was done using RNA-seq analysis software test developed by CLCbio genomic workbench (Eveland et al., 2010; Guo et al., 2011; Barakat et al., 2012). DEG-seq analysis was used to identify TFL1 genes in transcript abundance because it integrates several statistical methods (Feng et al., 2012; Huang et al., 2012). The number of reads per contig for each TFL1 gene was compared between vegetative stage and generative tissues in teak separately (Guo et al., 2011; Pestana-Calsa et al., 2012; Sweetman et al., 2012). RNA-seq employs a random sampling model based on the read count in vegetative and generative tissues libraries and performs a hypothesis test based on that model (Mutasa-Gottgens et al., 2012). Further analysis of the DEG results should be validated by QRT-PCR (Jian et al., 2008, Barakat et al., 2012 and Zhang et al., 2013).

Validation tests of TFL1 by quantitative real-time PCR: Quantitative real-time RT-PCR (QRT-PCR) tests were conducted to determine the extent to which the number of EST reads per gene obtained by shotgun sequencing accurately reflected transcript levels in the source tissues (Brunner et al., 2004; Jian et al., 2008). The QRT-PCR estimates of transcript abundance were conducted on RNA from vegetative and generative bud tissues from teak (Heid et al., 1996). The QRT-PCRs were prepared using the SYBR Green Master Mix kit (Applied Biosystems) and run in an Applied Biorad CFX 96 Fast Real-Time PCR system with default parameters (Livak and Schmittgen, 2001). Primers were designed using Primer3 software (Koressaar and Remm, 2007). The parameters used are the default parameters of Primer3 (Untergasser et al., 2012). The parameters are set as follows: Number to return = 5, max stability = 9, max repeat mispriming = 12, pair max repeat mispriming = 24, max template mispriming = 12 and pair max template mispriming = 24. Parameters for thermodynamic also using the default parameters consisting of primer size optimum = 20 (18-27), primer tm optimum = 60 (57-63), max tm difference = 100%, primer gc minimum 20 and maksimum 80 (Http://bioinfo.ut.ee/primer3-0.4.0/ input-help.htm) (Koressaar and Remm, 2007; Untergasser et al., 2012). A gene encoding 18S rRNA was used as an endogenous standard to normalize template quantity.

The QRT-PCR analyses were performed to confirm the expression of TFL1 using in silico expression analysis (Barakat et al., 2012). For each TFL1 gene, three biological replicates and three technical replicates were performed. Statistical analyses used to estimate the significance of the differences (Livak and Schmittgen, 2001; Brunner et al., 2004; Barakat et al., 2012).

RESULTS

NGS-transcriptome analysis of vegetative and generative teak shoots: The RNA isolation was using a modified method from Chang et al. (1993) performed to isolate RNA from teak tissue of vegetative and generative shoots buds. The RNA was checked for the quality using qubits and
Table 1: Summary statistics of sequencing and de novo assembly results

| Term                        | Value     |
|-----------------------------|-----------|
| Input sequence              |           |
| Vegetative tissue           | 3,701,878 |
| Generative tissue           | 3,778,316 |
| Total bases                 | 42,435,728|
| Contigs number              | 87,365    |
| Minimum length of contigs   | 225       |
| Maximum length of contigs   | 4,361     |
| Average length of contigs   | 486       |
| N75                         | 359       |
| N50                         | 498       |
| N25                         | 805       |

bioanalyzer. Only RNA with best RNA Integrity Number (RIN) values further analyzed using Illumina NGS-Miseq platform (Collins et al., 2008; Li et al., 2012; Liu et al., 2013). The Illumina Miseq sequencing platform generates 3,701,878 sequences for vegetative tissues and 3,778,316 sequences for generative tissues. These sequences were further analyzed, using CLC-bio workbench for trimming analysis to determine the quality of the sequence (Collins et al., 2008; Wu et al., 2010). The trimming results showed that the sequence has good quality. The following analysis also using CLC-bio workbench is de novo assembly (Annadurai et al., 2013). The results of the de novo assembly are 87,365 contigs those resulted from the combination of vegetative and generative tissue sequences. Contigs quality was also tested by trimming using CLC-bio workbench (Wu et al., 2010; Barakat et al., 2012; Annadurai et al., 2013). The trimming of the contigs result can be seen in Table 1.

*S. lycopersicum* was used for contigs annotation. Results from BLASTN and annotations of teak contigs against *S. lycopersicum* cds database which produces 14 contigs hit clicking TFL1 gene. All TFL1 contigs then we call TFL1 unigene. The BLASTN results can be seen in Table 2. The TFL1 unigene produced had the different E-value and identity (%). It is decided to choose TFL1 for further analysis because it has the lowest E-value (Huang et al., 2012; Barakat et al., 2012). TFL1 unigenes then further analyzed by phylogenetic analysis using the ClustalW2 (http://www.ebi.ac.uk/) (Larkin et al., 2007).

**TFL1 in teak:** The TFL1 genes hit by fourteen contigs. The range of number of hits is from 6-529 and the E-value range is 1.376E-07 up to 3.538. The greatest identities of the fourteenth TFL1 genes are entirely 100%. The range of greatest hits with a length is from 16-32. Greatest bit scores ranged 32.21-55.999 (Table 2). Teak-D-LB2_12_L001_R1_001 (paired) contig 81549, TFL1-14 chosen as gene for further analysis because it has the lowest E-value is 1.376E-07. Phylogenetic analysis results showed that there were ten groups of TFL1 in teak (Fig. 2).

It can be classified into three major groups of genes TFL1 namely: Major group I consists of two minor groups, TFL1-1 and TFL1-7 clustered in first minor group while TFL1-4 and TFL1-10 clustered in the second minor group and TFL1-2 and TFL1-11 clustered in the third minor group. Major group II consists of four minor groups, TFL1-3 and TFL1-12 clustered in the 1st minor group. The second minor group consists of only one member i.e., TFL1-8. TFL1-5 and TFL1-14 clustered in the 3rd minor group, while TFL1-9 being the only member of the 4th minor group of major group II. Major group III consists of only a minor group consisting of TFL1-6 and TFL1-13. TFL1-14 selection based only on the lowest E-value, if we observe the phylogenetic analysis, there is no significant difference from TFL1-14 compared with other TFL1.
Table 2: Contigs related TFL1 genes results from BLASTN to the *S. lycopersicum* CDS database (www.phytozome.com)

| Query                           | Gene name                                      | Number of hits | Lowest E-value | Accession (E-value)                  | Description (E-value)                | Greatest identity (%) | Greatest hit length | Greatest bit score |
|---------------------------------|------------------------------------------------|----------------|----------------|-------------------------------------|--------------------------------------|-----------------------|---------------------|--------------------|
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 36             | 0.047          | Solyc10g024470.1.1                  | PACid: 27318756                    | 100                   | 27                  | 38.158             |
| (paired) contig52899            | family protein (TFL1-1)                        | 6              | 3.538          | Solyc05g056660.1.1                  | PACid: 27299647                    | 100                   | 16                  | 32.100             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 31             | 0.323          | Solyc09g009560.1.1                  | PACid: 27310917                    | 100                   | 25                  | 38.158             |
| (paired) contig12806            | family protein (TFL1-3)                        | 24             | 1.929          | Solyc01g050500.1.1                  | PACid: 273102368                   | 100                   | 23                  | 32.100             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 18             | 0.123          | Solyc09g009560.1.1                  | PACid: 27310584                    | 100                   | 22                  | 36.175             |
| (paired) contig53576            | family protein (TFL1-4)                        | 16             | 0.052          | Solyc09g005000.1.1                  | PACid: 27310154                    | 100                   | 23                  | 38.158             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 7              | 0.899          | Solyc11g068660.1.1                  | PACid: 27295983                    | 100                   | 21                  | 34.193             |
| (paired) contig32476            | family protein (TFL1-5)                        | 44             | 0.119          | Solyc09g005000.1.1                  | PACid: 27310154                    | 100                   | 19                  | 36.175             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 23             | 0.046          | Solyc09g009560.1.1                  | PACid: 27310917                    | 100                   | 24                  | 38.158             |
| (paired) contig50579            | family protein (TFL1-8)                        | 20             | 0.045          | Solyc09g005000.1.1                  | PACid: 27302368                    | 100                   | 22                  | 36.175             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 21             | 0.64           | Solyc09g005000.1.1                  | PACid: 27310154                    | 100                   | 20                  | 34.193             |
| (paired) contig1004             | family protein (TFL1-9)                        | 39             | 0.593          | Solyc09g005000.1.1                  | PACid: 27310154                    | 100                   | 32                  | 55.999             |
| Teak-D-LB2_12_L001_R1_001       | PEBP (phosphatidyl ethanolamine-binding protein) | 9              | 0.214          | Solyc09g031100.1.1                  | PACid: 27289687                    | 100                   | 36                  | 39.158             |
| (paired) contig51649            | family protein (TFL1-12)                       | 529            | 1.38E-07       | Solyc11g068660.1.1                  | PACid: 27295983                    | 100                   | 21                  | 34.193             |
The other phylogenetic analysis results of TFL1 gene teak against the NCBI nr protein sequence database (Fig. 3) showed that TFL1-14 in one group with TFL1-14 is one group with TFL1 of Arabidopsis and TFL1 of sunflowers. For the analysis of gene expression of TFL1 in the vegetative to generative transition of teak, we compare the results of TFL1-14 DEG with TFL1-14 QRT-PCR results. Based on these considerations, we design primer of TFL1-14 using Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012). The primer sequence of TFL1-14 is Left Primer (L): TTCTCTTTACGGGCTTCGA, Right Primer (R): CCGACGTGACAGCTTTTGT and L: AATTGTTGGTCTTCAACGAGGA, R: AAAGGGCAGGGACGTAGTCAA for 18S. The 18S is used as a reference gene to be used for the QRT-PCR analysis.

Expression profiles TFL1-14 in the regulation of vegetative to generative transition on teak: TFL1-14 gene level expression results of the DEG and QRT-PCR analysis can be seen in Fig. 4a. These results of DEG need to be confirmed in vegetative tissues and teak generative tissue with QRT-PCR analysis (Brunner et al., 2004; Jian et al., 2008; Guenin et al., 2009; Howe et al., 2013; Barakat et al., 2012). Expression profile results of QRT-PCR analysis of TFL1-14 gene in the generative and vegetative tissue of teak can be seen in Fig. 4b. Figure 4b shows that the TFL1-14 gene expression profile results of QRT-PCR equivalent to the expression profile results of DEG analysis (Guenin et al., 2009). The TFL1-14 expression profiles in down regulation during the formation of floral organs (Fig. 4a).

DISCUSSION

Flower formation is a crucial stage of plant development, because it determines the maturity of the plant (Torti et al., 2012; Blazquez, 2000). Flowering plants that have been successful in generating flowers indicated that the plant is ready to produce offspring (Putterill et al., 2004). The next stage after the forming of flower is the formation of seeds. In teak, flower formation occurs after the age of 6-8 years (Orwa et al., 2009; Ansari et al., 2012). This is a long time and it is a serious concern in the development of teak. Teak is very low reproductive rate if compared to other woody plants that live in the same habitat. Low reproductive rate is also a serious concern in the development of teak (Orwa et al., 2009; Lyngdoh et al., 2010). In teak wood production, the reproductive stage of teak is known to inhibit the growth of wood, so that, the teak will have a long time to harvest (Widiyanto et al., 2009).
Problems in teak flowering become important to learn because it is associated with the development of teak cultivation (Rosli et al., 2009; Widiyanto et al., 2009). Flowering mechanism that occurs in teak is still very limited information (Widiyanto et al., 2009). In previous reports, we have learned about the role of LFY genes in regulating the transition of vegetative to generative of teak. In this report, we will be reported the role of other floral meristem identity genes, namely TFL1. The TFL1 role in regulating the teak transition of vegetative to generative will add information about the flowering mechanism of teak at the molecular level. This TFL1 expression profiles research on teak is expected to provide additional information on the mechanism of teak flowering.

![Phylogram of teak TFL1-14 genes results from BLASTX to nr protein sequence database](image)

**Fig. 3: Phylogram of teak TFL1-14 genes results from BLASTX to nr protein sequence database NCBI**
flowering. In this study, the approach used NGS-transcriptome analysis to identify TFL1 genes in teak. The results of NGS-transcriptome analysis of the teak sequences obtained fourteen kinds TFL1 unigene which is annotated with *S. lycopersicum* (Olmstead, 2005; Lyngdoh *et al*., 2010). In Arabidopsis model plant, there are 14 TFL1 alleles that have been identified ((ABRC) www.arabidopsis.org) (Ordidge *et al*., 2005). Although each allele has its own expression profile but the general profile of TFL1 expression is a gene that encodes a protein, is expressed in the cytoplasm (Ordidge *et al*., 2005; Liu *et al*., 2013). This gene controls the inflorescence meristem identity. This gene is involved in the initiation of flowering. These genes have an orthologous in Antirrhinum i.e., CENTRORADIALIS gene (CEN) (Jack, 2004; Putterill *et al*., 2004). This gene is involved in protein trafficking to the protein storage in the vacuole (Olsen *et al*., 2002). Genetic studies indicate that TFL1 acts in part by repressing the expression of LEAFY in the inflorescence strong conservation in the number, positioning and meristems (Olsen *et al*., 2002; Ordidge *et al*., 2005). Thus, down regulation of TFL1 leads to LFY expression and is one of the first steps in the genetic cascade that leads to flower formation (Olsen *et al*., 2002).

In teak, flowering was also induced by environmental and endogenous factors (Rosli *et al*., 2009; Palupi *et al*., 2010). Both of these factors interact to induce flowering. In the model plant which is induced LFY gene as a floral meristem identity (William *et al*., 2004; Widiyanto *et al*., 2009). In this study we see the expression of teak TFL1-14 unigene in vegetative and generative shoot buds to induce flowering. The results of DEG and QRT-PCR gene expression analysis showed that TFL1-14 maintained in the down regulation trend in the regulation of teak floral organ formation. These

![Fig. 4(a-b): Expressions (a) Level and (b) Profile TFL1-14 gene in the regulation of generative organ formation from vegetative shoots to generative shoot buds.](image)
results indicate that the TFL1-14 expression profiles equivalent to the general pattern of TFL1 expression in the model plant. Based on the results we can assume that teak TFL1-14 is equivalent with model but we have more than one kind of TFL1. We need further analysis to identify other TFL1 unigene existing in the teak EST database that resulted by NGS-transcriptome analysis were performed. In order to further identify TFL1-14 genes in teak, we require advanced gene expression analysis, including in situ hybridization, gene over-expression and gene silencing.

However, this result is an initial study of TFL1 the other of the floral meristem identity gene expression in the teak flowering regulation. The authors hope that the results of this study may provide a basis for further research in understanding the regulatory mechanisms of vegetative to generative transition in teak.

CONCLUSION
This study shows that (1) De novo assembly result on the outcome of NGS-Transcriptome Analysis from teak vegetative and generative shoot buds sequence produce 87,365 contigs, (2) Identification and annotations results with S. lycopersicum CDS database obtained results 14 different unigene TFL1 in teak, (3) TFL1-14 has the smallest value of the E-value was analyzed further by DEG analysis and QRT-PCR analysis, (4) DEG expression profile results of TFL1-14 in equivalent with QRT-PCR results, (5) TFL1-14 has equivalent activity to the general TFL1 expression profile in the model plant and (6) Advanced research is needed to string up the understanding about the teak TFL1 gene. However, the results of this study are expected to provide the basis for research on the mechanism of flowering teak.

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