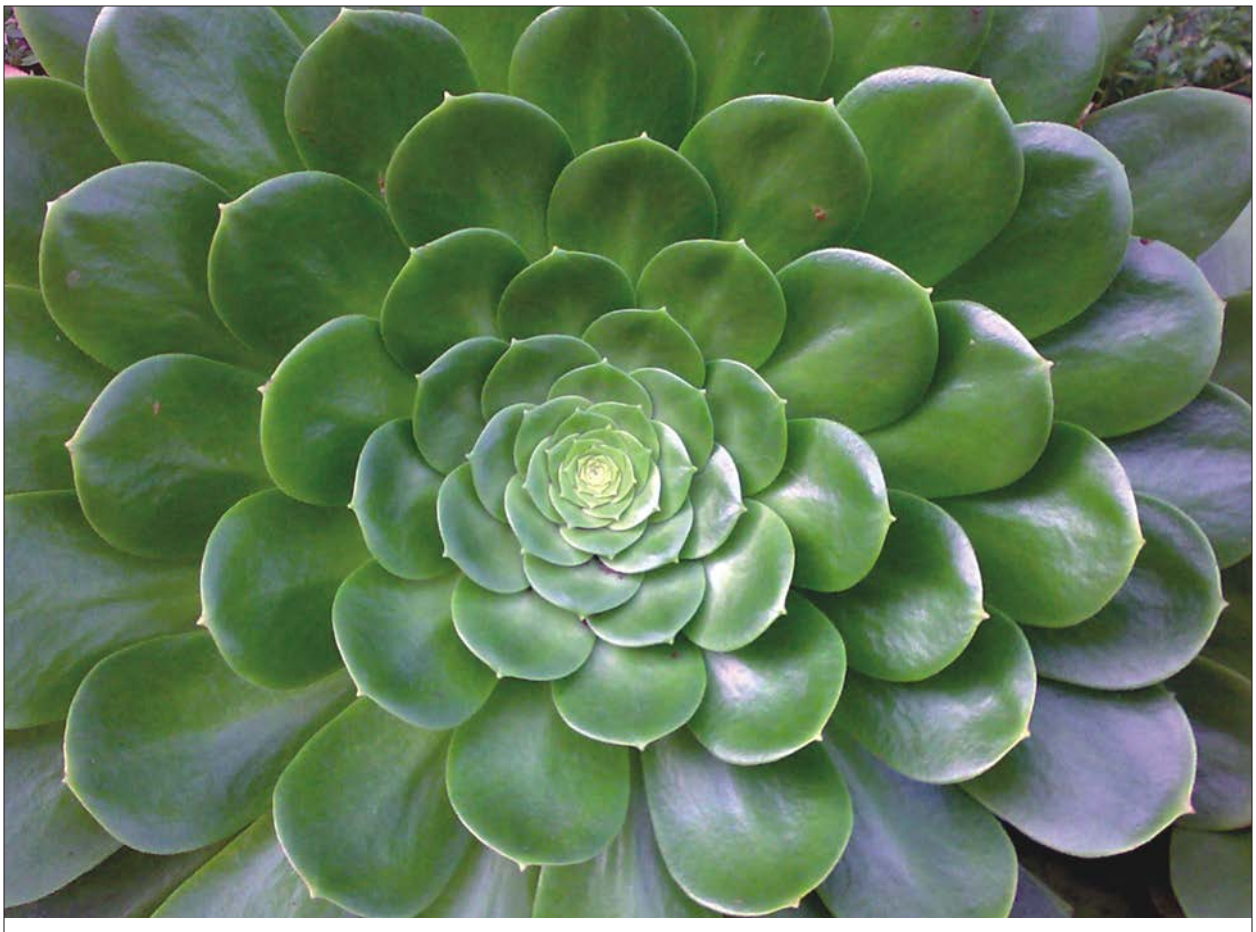

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Research Article
APETALA2 and APETALA3 Genes Expression Profiling on Floral Development of Teak (*Tectona grandis* Linn f.)

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Abstract

Background: Teak (*Tectona grandis* Linn f.) is one of valuable timber species because of the beauty, strength and durability properties. Main problem in teak improvement program is reproductive biology, a low pollen viability, high embryo abortion, low pollination success and low fruit production. Investigation carried out at the teak improvement that flowering aspects is very important. Teak development program was facing difficulties due to the lack of information about the role of genes regulating flowering. APETALA2 (AP2) and APETALA3 (AP3) genes as member of the floral organ Identity. The role of teak flowering development predicted by up and down regulation of TgAP2 and TgAP3 genes. This study was conducted to determine the gene expression profile of TgAP2 and TgAP3 in the developmental stages of teak flowering. **Materials and Methods:** In this study, TgAP2 and TgAP3 genes in teak identified from NGS transcriptome data that is annotated with *Solanum lycopersicum*. The TgAP2 and TgAP3 genes activities on the several developmental stages of teak flowering tissues are done with QRT-PCR analysis using 18S RNA as gene reference. **Results:** The highest expression level of TgAP2 on the floral development stages of 4th lateral floral buds. The TgAP3 gene expression reached the highest level on the apical floral bud stage development. Vegetative shoot as control stages, TgAP2 gene expression level reached 9 times compared to the control and TgAP3 more than 12 times. The TgAP2 and TgAP3 genes expression profile has non-significant correlation on all floral development stages. **Conclusion:** The TgAP2 and TgAP3 genes expressed with different level in all stages of the teak flowering development. The TgAP2 and TgAP3 genes have non-significant correlation expression profile in role of teak floral development. These results indicate that the TgAP2 and TgAP3 expression profiles equivalent to the general pattern in the Arabidopsis model plant.

Key words: Transcriptome, gene expression level, QRT-PCR, flowering time, floral organ identity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Teak (*Tectona grandis* Linn f.) is a woody plants member of the lamiaceae family (Palupi *et al*., 2010). Teak is well known for the quality of the wood because of the beauty, strength and durability properties (Alcantara and Veasey, 2013). Characteristic of teak flower; flowers are found in inflorescences, teak flowers are small bisexual, actinomorphic with five to seven petals. In teak inflorescences may have hundred to thousand flowers although, the number of flower is huge but seed production is low (Palupi *et al*., 2010). This low productivity is main problem in teak development (Norlia *et al*., 2008; Kokutse *et al*., 2006). These issues are related to molecular genetic processes of flowering development and can be understood through genomic studies.

Teak genomic studies on flowering molecular processes was facing difficulties due to the lack of information about the role of genes regulating flowering in teak (Widiyanto *et al*., 2009). The development of teak NGS technology-transcriptome analysis has allowed us to identify interest genes from teak rapidly and cheaply relative (Diningrat *et al*., 2015a). In Arabidopsis, the role of APETALA2 (AP2) dan APETALA3 (AP3) genes as member of the Floral Organ Identity (FOI) in regulating the floral development so that, the activity of the FOI genes regulation which resulted in the development towards the formation of flower properties; sepals, petals, stamens and carpels (Blazquez, 2000). In teak, this mechanism is not well known.

To determine the activity of the teak APETALA2 (TgAP2) dan APETALA3 (TgAP3) genes in silico can be undertaken with QRT-PCR analysis approaches. In this study, flowering development in teak can be divided into five sequential stages; (1) Vegetative shoots stage, (2) Apical floral bud stage, (3) Second lateral floral buds stage, (4) Fourth lateral floral buds stage, (5) Sixth lateral floral buds stage (Fig. 1). The TgAP2 and TgAP3 genes identified from teak NGS transcriptome data that is annotated with *S. lycopersicum*

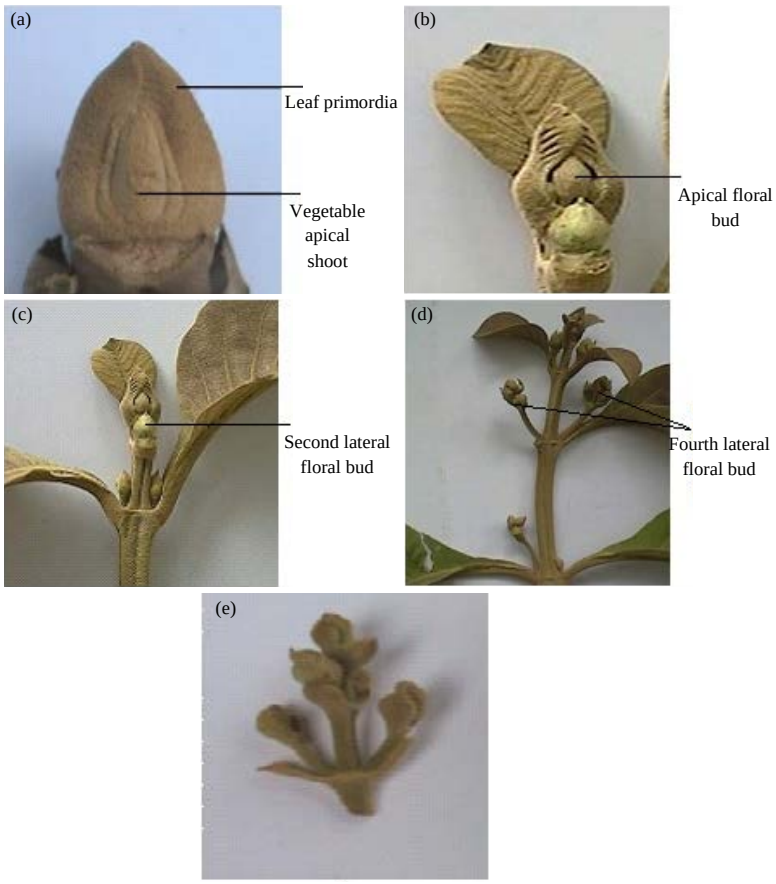

Fig. 1(a-e): Sequential flowering development stages in teak, (a) Vegetative shoots stage, (b) Apical floral bud stage, (c) Second lateral floral buds stage, (d) Fourth lateral floral buds stage and (e) Sixth lateral floral buds stage

(Diningrat *et al*., 2015b). This molecular biology studies becomes important to implement to understand the function and interaction of genes involved in the flowering process in teak. Understanding the role of TgAP2 and TgAP3 genes in regulating flowering in teak will facilitate the conduct of engineering to improve the quality of teak.

MATERIALS AND METHODS

Teak tissues materials and RNA isolation: Sequential teak flowering development tissues; vegetative shoots, apical floral bud, 2nd lateral floral buds, 4th lateral floral buds and 6th lateral floral buds were collected from a 12 years old teak plant in Institute of Technology Bandung, Indonesia for RNA isolation (Fig. 2). Those of teak tissue samples were frozen in liquid nitrogen immediately upon collection. Samples were immediately frozen at -80°C upon arrival at laboratory until use. Total RNA was obtained by using the method for RNA isolation protocol that developed by Diningrat *et al*. (2015a). Frozen tissue were ground to a fine powder under liquid nitrogen and dispersed in CTAB buffer. Following two chloroform extractions, RNA was precipitated with LiCl2, again extracted with chloroform and precipitated with ethanol. The resulting RNA pellet was re-suspended in 20-100 µL of DEPC-treated water. The RNA concentration analysis on a spectrophotometer to show a total yield of RNA sample.

Fig. 2: Twelve years old teak plant in Institute of Technology Bandung, Indonesia

Table 1: Primer sequences

Gene name	Primer sequence
TgAP2	L: TAAAACTTCGCCTTCCTGCT
	R: CCATGAGCCGCATACTGTTA
TgAP3	L: GCCCTGACAATGTCCTCACT
	R: AATCTGTCTTGCCGAGTGCT
18S rRNA	L: AATTGTTGGTCTTCAACGAGGAA
	R: AAAGGG CAGGGACGTAGTCAA

Gene expression profiles of TgAP2 and TgAP3: The TgAP2 and TgAP3 gene sequences derived from teak transcriptome data, the study result Diningrat *et al*. (2015a). Based on the sequence of TgAP2 and TgAP3 gene sequences, primer design is done by using Primer 3 software program (http://bioinfo.ut.ee/primer3-0.4.0). The parameters used are the default parameters of Primer 3. The parameters are set as follows: No. 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 (Untergasser *et al*., 2012) with the following results are shown in Table 1.

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. The QRT-PCR estimates of transcript abundance were conducted on RNA from five sequential teak flowering development tissues from teak. 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). First performed on all samples cDNA synthesis using thermo scientific revert aid first strand cDNA synthesis kit. The reaction was performed by adding reaction mix (5X reaction buffer, ribolock RNase inhibitor (20 U µL G1), 10 mM dNTP mix dan revert aid M-Mul V RT (200 U µL G1) (Thermo scientific protocol). Proceed to the RT-PCR using SYBR green and cDNA markers that have been synthesized. Optimization of PCR reactions performed with annealing temperature confirm each sample and QRT-PCR primer using the thermo scientific maxima SYBR green QRT-PCR (Thermo scientific protocol). Reaction cycle was 50 EC for 2 min, 95 EC for 10 min, 95 EC for 15 sec, reading 60 EC for 30 sec, 72 EC for 30 sec, reading the reaction cycle is

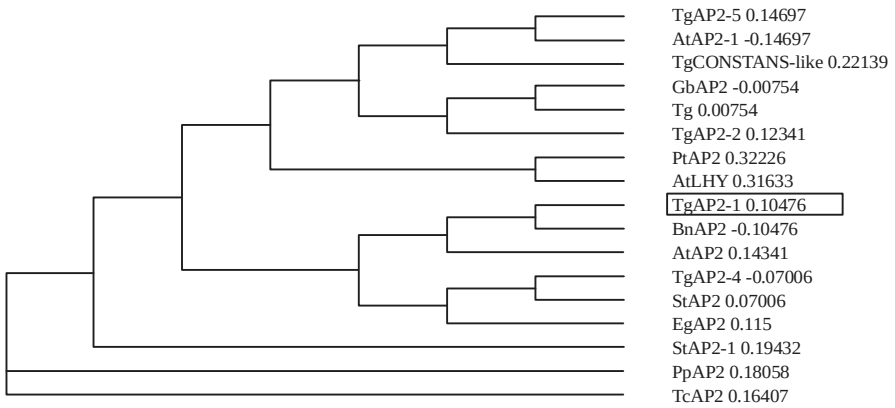

Fig. 3: Phylogram of teak TgAP2 genes results from BLASTX to nr protein sequence database NCBI (http://ncbi.nlm.nih.gov/) AtAP2-1: >gi|533708|gb|U12546.1|ATU12546 *Arabidopsis thaliana* columbia homeotic APETALA2 protein (APETALA2) mRNA, complete cds, TcAP2: >gi|590705081|ref|XM_007047275.1| *Theobroma cacao* transcription factor APETALA2 isoform 1 (TCM_000665) mRNA, complete cds, StAP2: >gi|255697189|emb|FM246879.2| *Solanum tuberosum* subsp., andigena mRNA for relative to APETALA2 1 (rap1 gene), AtAP2: >gi|22135897|gb|AY128328.1| *Arabidopsis thaliana* APETALA2 protein (At4g36920) mRNA, complete cds, StAP2-1: >gi|565388919|ref|XM_006360152.1| PREDICTED: *Solanum tuberosum* floral homeotic protein APETALA 2-like (LOC102589826), transcript variant X1, mRNA, BnAP2: >gi|315318955|gb|HQ637468.1| *Brassica napus* APETALA2 (APETALA2) mRNA, complete cds, GbAP2: >gi|816219246|gb|KP259810.1| *Gossypium barbadense* APETALA2 mRNA, complete cds, PpAP2: >gi|329565725|gb|JF683605.1| *Prunus persica* APETALA2 protein (APETALA2) mRNA, complete cds, EgAP2: >gi|848880873|ref|XM_012985357.1|, PREDICTED: *Erythranthe guttatus* floral homeotic protein APETALA 2 (LOC105961115), mRNA, Tg CONSTANS-like: >gi|662170386|gb|KF425509.1| *Tectona grandis* CONSTANS-like protein 9 mRNA, complete cds, AtLHY: >gi|334182204|ref|NM_001197953.1| *Arabidopsis thaliana* protein LHY mRNA, complete cds

repeated 40 times that at the start of the first reading and ended with a 4 EC for 10 min (Jian *et al*., 2008). Primary housekeeping genes was used 18S rRNA gene primer. The QRT-PCR results (Ct) were analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis: The TgAP2 and TgAP3 gene expression profile on each flowering development stages were analyzed by looking correlation using the Pearson and Spearman correlation coefficients. The Pearson and Spearman correlation were calculated using SPSS 18 and software Genex (http://genex.gene-quantification.info/) (Trindade *et al*., 2010).

RESULTS

Based on teak NGS-transcriptome data (Diningrat *et al*., 2015a), results from BLASTN and annotations of teak contigs against *S. lycopersicum* cds database which produces five contigs hit clicking AP2 gene. All AP2 contigs then call TgAP2 genes. The BLASTN results can be seen in Table 2. The AP2 produced had the different E-value and identity (%). It is decided to choose TgAP2-1 for further analysis because it has the lowest E-value (Diningrat *et al*., 2015b).

The TgAP2 genes then further analyzed by BLASTX to nr protein sequence database NCBI and phylogenetic analysis using the ClustalW2 (http://www.ebi.ac.uk/) (Diningrat *et al*., 2015b). The phylogenetic analysis results of TgAP2 gene against the NCBI nr protein sequence database showed that TgAP2-1 in one group with AP2 of *Brassica napus* and then with AP2 of *Arabidopsis thaliana* (Fig. 3). Based on these considerations, design primer of TgAP2-1 using Primer 3 software (Koressaar and Remm, 2007; Untergasser *et al*., 2012), for the analysis of gene expression of TgAP2 in the all flowering development stages of teak.

The AP3 genes hit by five contigs. The range of number of hits is from 13-37 and the E-value range is 7.37E33 upto 0.272. The greatest identities of the five TgAP3 genes are entirely 100%. The range of greatest hits with a length is from 20-25. Greatest bit scores ranged 46.429-82.353 (Table 3). Teak-D-LB2_12_L001_R1_001 (paired) contig 84546, TgAP3-1 chosen as gene for further analysis because it has the lowest

Table 2: Contigs related AP2 genes results from BLASTN to the *Solanum lycopersicum* CDS database (www.phytozome.net)

Query	Gene name	No. 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 (paired) contig 67744	TgAP2-1	47	7.30E-40	Solyc11g066770.1.1	PACid: 27295928	100	24	40.14
Teak-D-LB2_12_L001_R1_001 (paired) contig 28350	TgAP2-2	14	0.312	Solyc11g066770.1.1	PACid: 27295928	100	22	40.14
Teak-D-LB2_12_L001_R1_001 (paired) contig 35544	*TgAP2*-3	15	0.523	Solyc06g074650.2.1	PACid: 27285095	100	25	34.193
Teak-D-LB2_12_L001_R1_001 (paired) contig 46409	TgAP2-4	23	1.70E-60	Solyc11g066770.1.1	PACid: 27295928	100	20	36.175
Teak-D-LB2_12_L001_R1_001 (paired) contig 84666	TgAP2-5	55	2.29E-37	Solyc06g074650.2.1	PACid: 27285095	100	20	34.193

Table 3: Contigs related AP3 genes results from BLASTN to the *Solanum lycopersicum* CDS database (www.phytozome.net)

Query	Gene name	No. 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 (paired) contig 84546	TgAP3-1	30	7.37E33	Solyc01g079690.2.1	PACid: 27300844	100	20	82.353
Teak-D-LB2_12_L001_R1_001 (paired) contig 19432	TgAP3-2	37	6.51E07	Solyc01g006510.2.1	PACid: 27300448	100	22	76.923
Teak-D-LB2_12_L001_R1_001 (paired) contig 22749	TgAP3-3	13	0.272	Solyc07g008520.2.1	PACid: 27293705	100	25	51.852
Teak-D-LB2_12_L001_R1_001 (paired) contig 23610	TgAP3-4	23	1.42E05	Solyc04g007270.2.1	PACid: 27293433	100	20	46.429
Teak-D-LB2_12_L001_R1_001 (paired) contig 27003	TgAP3-5	13	3.80E21	Solyc07g032740.2.1	PACid: 27293637	100	24	53.333

E-value is 7.37E33. Phylogenetic analysis results showed that there was TgAP3-1 one group with AP3 of *Arabidopsis lyrata* and *Arabidopsis thaliana* (Fig. 4).

Expression profiling of genes TgAP2 and TgAP3 in the flowering development tissues is shown in Fig. 5. In the all flowering development stage tissues, TgAP2 and TgAP3 genes up-regulated experience so, it is higher than the control (vegetative stage tissues). In the apical floral bud stage, 2nd lateral floral bud stage and 6th lateral floral bud stage. The TgAP2 expression profile is lower than TgAP3 whereas on 4th lateral floral bud seen TgAP3 gene is higher experience than TgAP2. Especially, in the 4th lateral floral bud, the level of gene expression of Tg AP2 reached more than 9 times compared to the control and in the apical floral bud TgAP3 gene expression level more than 12 times.

Figure 6 showed a comparison of the level of TgAP2 and TgAP3 genes expression in the flowering development tissues. The TgAP2 and TgAP3 expressed in the all stage of teak flowering development. The highest level of TgAP2 gene expression at 4th lateral floral bud tissue and at the vegetative shoot tissue is the lowest expression level of TgAP2. The highest TgAP3 gene expression level at apical floral bud tissue and the lowest expression level of TgAP3 experience at vegetative shoot tissue. The TgAP2 and TgAP3 gene expression profile on each flowering development stages were analyzed by looking correlation using the Pearson and Spearman correlation coefficients. The results showed that there has non-significant correlation of profile expression between TgAP2 and TgAP3 in the role of floral organ development in teak.

DISCUSSION

In teak, flower formation occurs after vegetative growth at the age of 6-8 years (Widiyanto *et al*., 2009; Diningrat *et al*., 2013). Teak is very low reproductive rate if compared to other woody plants, this low reproductive rate is also a serious concern in the development of teak (Diningrat *et al*., 2015a). In previous study, the role of TFL1 genes in regulating the transition of vegetative to generative of teak (Diningrat *et al*., 2015b). In this study, the role of APETALA2 and APETALA3 floral organ identity genes on teak will be reported. The TgAP2 and TgAP3 genes role in regulating the teak flowering development will add information about the flowering mechanism of teak at the molecular level.

In this study, the approach to identify TgAP2 and TgAP3 genes in teak used teak NGS-transcriptome data (Diningrat *et al*., 2015b). The NGS-transcriptome of the teak sequences obtained five kinds TgAP2 gene and also five kinds TgAP3 gene which is annotated with *S. lycopersicum* (Table 2, 3). Phylogram of TgAP2 and TgAP3 genes have a homologous with AP2 and AP3 of *Arabidopsis thaliana* and *Arabidopsis lyrata* (Fig. 3, 4).

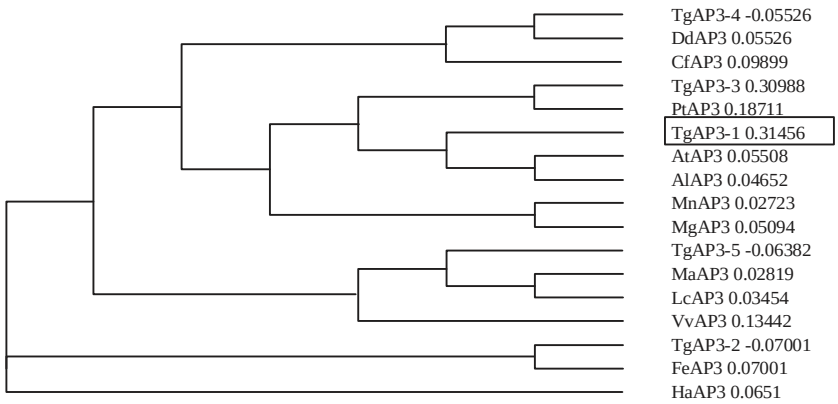

Fig. 4: Phylogram of teak TgAP3 genes results from BLASTX to nr protein sequence database NCBI (http://ncbi.nlm.nih.gov/) AlAP3: >gi|6707087|gb|AF143380.1|AF143380 *Arabidopsis lyrata* apetala3 gene, complete cds, AtAP3: >gi|600540|dbj|D21125.1|ATHAPETALA *Arabidopsis thaliana* gene for APETALA3, complete cds, MgAP3: >gi|32484005|gb|AY319685.1| *Mimulus guttatus* isolate IM62 apetala3-like protein (mAP3) gene, partial cds, FeAP3: >gi|398330373|gb|JN605355.1| *Fagopyrum esculentum* APETALA3-like protein (AP3) mRNA, complete cds, CfAP3: >gi|430763370|gb|JQ753789.1| *Cornus florida* apetala 3 (AP3) mRNA, complete cds, PtAP3: >gi|34921972|gb|AY359606.1| *Populus tomentosa* sex female APETALA3 (AP3) gene, complete cds, DdAP3: >gi|262479015|gb|GU126414.1| *Dendrobium devonianum* putative APETALA3 (AP3) mRNA, complete cds, MaAP3: >gi|395618796|gb|JQ889967.1| *Magnolia aromatica* APETALA3-like protein (AP3) mRNA, complete cds, LcAP3: >gi|395618766|gb|JQ889952.1| *Liriodendron chinense* APETALA3-like protein (AP3) mRNA, complete cds, HaAP3: >gi|27967322|gb|AY185363.1| *Helianthus annuus* MADS-box transcription factor APETALA3 (AP3) mRNA, partial cds, MnAP3: >gi|14573448|gb|AF381767.1| *Mimulus nasutus* apetala3-like protein (AP3) gene, partial cds, VvAP3: >gi|269116069|gb|GU133632.1| *Vitis vinifera* apetala3 (AP3) mRNA, complete cds

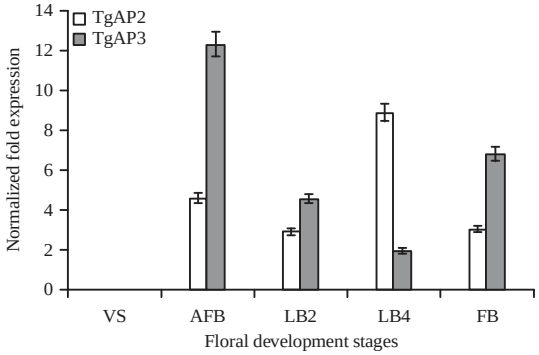

Fig. 5: Expression profiling of TgAP2 and TgAP3 genes in the flowering development tissues

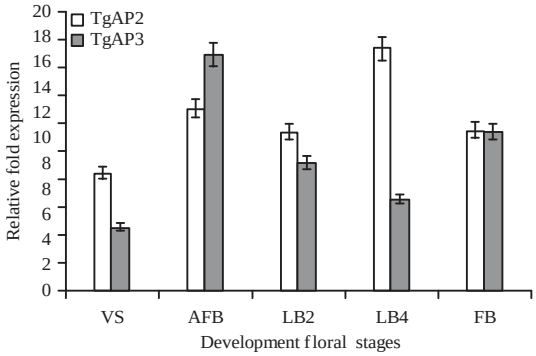

Fig. 6: Expression level of TgAP2 and TgAP3 genes in the flowering development tissues

In Arabidopsis, functions of APETALA2 (AP2) and APETALA3 (AP3)/PISTILLATA (PI) characterized in early and late stage flowers (Coen and Carpenter, 1993; Blazquez, 2000; Jack, 2004; Krizek and Fletcher, 2005). Expression of floral organ identity genes like AP2 and AP3/PI commences during early floral stages and continues for most of floral organ development (Bowman *et al*., 1991; Coen and Meyerowitz, 1991; Cseke and Podila, 2004; Ferrario *et al*., 2004). This prolonged expression implies that these genes are not only involved in the specification of organ primordia but also in the control of organ maturation during later developmental stages (Zahn *et al*., 2006).

The AP3 and PI are key floral homeotic genes play a role in positive regulation of floral homeotic genes (Putterill *et al*., 2004; Howe *et al*., 2013). Up-regulation of AP3 and PI were requirement for characterized specifying petal and stamen

identity (Park *et al*., 2004; Wellmer *et al*., 2006). Furthermore, post-transcriptional control mechanisms of AP3 and PI in the translational repression of the spatial regulator APETALA2 (AP2) will carry out the establishment of the different floral organ development (Liu *et al*., 2013).

These results in teak provide molecular evidence for an involvement of TgAP2 and TgAP3 in the control of different processes during flowering organ organogenesis and indicate that they regulate largely unique sets of genes at distinct phases of flower development. Morphological and molecular analyses have shown that flowers produced by this system can serve as a model for the upstream phase of flower development of teak after floral identity meristem genes expressed. These results indicate that the TgAP2 and TgAP3 expression profiles equivalent to the general pattern of AP2 and AP3 expression in the model plant (Arabidopsis).

Results can assume that teak TgAP2 and TgAP3 is equivalent with model but we have more than one kind of TgAP2 and TgAP3. We need further analysis to identify other TgAP2 and TgAP3 unigene existing in the teak EST database that resulted by NGS-transcriptome analysis were performed. In order to further identify TgAP2 and TgAP3 genes in teak, this study require advanced gene expression analysis including *in situ* hybridization, gene over-expression and gene silencing.

CONCLUSION

The TgAP2 and TgAP3 genes expressed with different level in all stages of the teak flowering development. The TgAP2 and TgAP3 genes have no correlation expression profile in role of teak floral development. The TgAP2 and TgAP3 has equivalent activity to the general AP2 and AP3 expression profile in the model plant. Advanced study is needed to string up the understanding about the TgAP2 and TgAP3 genes. However, the results of this study are expected to provide the basis for research on the mechanism of flowering teak.

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