Analysis of expression level of floral-identity gene (SEPALLATA) in Epicalyx of Hibiscus rosa-sinensis L.

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Abstract. Genetic identification of modified floral parts, such as epicalyx, may give new perspectives in flower development fundamentals and its applications. Research on floral-identity gene (SEPALLATA) expression level has been done in three parts of Hibiscus rosa-sinensis; they are leaves, epicalyx, and calyx. This research was conducted to observe the expression of the SEPALLATA gene in epicalyx. The expression level analysis was done qualitatively by the two-steps RT-PCR and visualized using agarose electrophoresis. Hibiscus rosa-sinensis RNA was isolated using the modified-CTAB method and continued by DNase-treatment to eliminate gDNA in the mixture. Furthermore, RNA was used to make cDNA using the Reverse Transcription method and amplified using the PCR method by specific primers. The result showed the presence of SEPALLATA amplification in epicalyx using GH7SEP1 primer, yet none on epicalyx using GHSEP1 primer. Confirmation using GH7SEP1 forward primer and GH1SEP1 reverse primer did not show any amplification. Sequencing and alignment results suggested that amplifications using GH1SEP1 or GH7SEP1 were allegedly, of which amplified from SEPALLATA gene.

Keywords: Gene expression, RT-PCR, SEPALLATA, sequencing

1. Introduction

Hibiscus rosa-sinensis L., which is noted as Kembang Sepatu in Indonesia is a species of tropical flower plant grown in Southeast Asia. This plant had been cultivated as ornamental plants over the world. As consequences, many variations among Hibiscus were gotten by crossing H. rosa-sinensis variety within H. schizopetalus from Africa [1]. Although it has varieties among its species, H. rosa-sinensis also have common features of its own. Of all features, epicalyx is the unique one found on H. rosa-sinensis flower.

Of the ABC model of flower development, floral parts, including calyx, corolla, androecium, and gynoecium, are well-defined for genetic features in its development. Another part of flower such as epicalyx, however, is not acquiring appeal. In fact, epicalyx is also played an important role in attracting pollinators, ovary protection, germination, and seed dispersal depend on plant species [2-5].

Therefore, genetic identification of epicalyx development is crucial. SEPALLATA gene is a candidate for floral development genetic identification since it is found on all floral parts. The expression of SEPALLATA gene in epicalyx may increase the perspective of the ABC model of flower development to consider the modified-out parts of calyx as floral parts as well. In contrast, a negative result of the expression of SEPALLATA gene in epicalyx may support the theory of epicalyx origin, which
development of bract [6]. Moreover, genetic identification of epicalyx can be basis of the theory of epicalyx development on evolution perspective. Thus, by gaining information about SEPALLATA gene expression in epicalyx of *H. rosa-sinensis* is crucial and it may pave the way as well as implicating the plant science.

2. Experiment materials and method

2.1. Primer design
The Primer was designed based on SEPALLATA gene sequence of *Gossypium hirsutum* (NM_001327042.1) and SEPALLATA gene sequence of *Arabidopsis thaliana* (CP002688.1). The primer used were GH1SEP1; GH7SEP1; and ATSEP1. These primers were chosen due to insufficiencies of *H. rosa-sinensis* genomic information. *Gossypium* thus, as in the same subfamilies (Malvoideae) with *Hibiscus*, was chosen as the basis of this study. *Arabidopsis thaliana* was also chosen as negative control of this study.

2.2. RNA isolation
RNA isolation was done using a modified-CTAB method adapted from Zeng et al. Modification of Zeng et al. method was decreasing the centrifugation force yet increasing the centrifugation time. In addition, spermidine was not used in the extraction buffer [7].

2.3. Two-steps RT-PCR
Amplification of RNA was done using two-steps RT-PCR. RNA was converted into cDNA using Omniscript® Reverse Transcription kit. Conversion of RNA to cDNA was done in prolonged incubation of 5 minutes pre-heating 70 °C; 5 minutes annealing 25 °C; 60 minutes extension 42 °C; and 15 minutes inactivation 70 °C, respectively. The amplification of cDNA was done using kit MyTaq™ HS Red DNA Polymerase kit. It was done in a prolonged incubation of 15 seconds denaturation 95 °C; 15 seconds annealing 54 °C; and 10 seconds extension 72 °C in 35 cycles of incubation.

2.4. Sequencing and data analysis
Sequencing was done by Macrogen. The results of sequencing were analyzed using Geneious 11.1.4 and Bioedit 7.0.5.

3. Experiment, results and discussion
The result of figure 1a shows that all samples amplified with the ATSEP1 primer did not produce any desired SEPALLATA gene product as 400 bp. Negative results of amplification of SEPALLATA gene using ATSEP1 is predicted as the consequence of incompatibility of ATSEP1 to *H. rosa-sinensis* SEPALLATA gene sequence, that may happen because of the differences sequences between SEPALLATA gene of *A. thaliana* and *H. rosa-sinensis*. Moreover, it is known that *A. thaliana* and *H. rosa-sinensis* were evolved from the different ancestor.

In contrast, amplification of SEPALLATA gene using GH1SEP1 showed a band within 400 bp length (red arrow) on lane C (calyx), yet it did not show on lane L (leaf) as well as lane E (epicalyx) (figure 1b). Meanwhile, amplification of SEPALLATA gene using GH7SEP1 showed bands within 200 bp (white arrow) (figure 1c). Those bands were found in lane C and lane E, whilst it was not found in lane L. However, GH1SEP1 and GH7SEP1 primers did not only amplify target gene and yet other genes. This prediction is due to multiple bands found in the same lane.

The amplification product of GH1SEP1 was then sequenced and aligned. The alignment result showed similarity in position 708 to 728 bp within 0.2536717 or 25.37 % likelihood (figure 2a). To be found that position which likely to SEPALLATA gene was a complement to GH1SEP1 reverse primer. Thus, it may imply that SEPALLATA gene amplified properly.
Figure 1. (a) Amplification of SEPALLATA gene using ATSEP1, (b) GH1SEP1 and (c) GH7SEP1 primers. L1 = Leaf RNA, L2 = Leaf cDNA without DNase, L3 = Leaf cDNA + DNase, E1 = Epicalyx RNA, E2 = Epicalyx cDNA without DNase, E3 = Epicalyx cDNA + DNase, C1 = Calyx RNA, C2 = Calyx cDNA without DNase, C3 = Calyx cDNA + DNase, M = Marker 100 bp; red arrow indicates 400 bp GH1SEP1 amplicon; white arrows indicate 200 bp GH7SEP1 amplicon; Agarose gel 1%; TAE buffer, 100 Volt for 30 minutes.

Figure 2. (a) Alignment of GH1SEP1 calyx sample, (b) GH7SEP1 calyx sample and (c) GH7SEP1 epicalyx sample result with G. hirsutum SEPALLATA gene.
Amplification band of GH7SEP1 from calyx (lane 3, figure 1c) and epicalyx sample (lane 2, figure 1c) were sequenced and aligned. Alignment result of calyx sample showed similarity in position 271 to 290 bp within 0.1510695 or 15.10 % likelihood (figure 2b) whereas alignment result of epicalyx sample showed similarity in position 271 to 290 bp within 0.1457219 or 14.57 % likelihood (figure 2c). The sequence complemented to GH7SEP1 reverse primer was also found. This result suggested that amplification of SEPALLATA gene using GH7SEP1 was done properly.

However, those results were contra-productive. The expression of SEPALLATA gene in epicalyx of *H. rosa-sinensis* cannot be concluded. Therefore, another trial was done using a combination of GH7SEP1 forward primer and GH1SEP1 reverse primer to figure out the contradiction. Those primers are in the very rear-ends of SEPALLATA gene, and its product is tandem so that amplification may continue from one end to another (figure 3).

Amplification of SEPALLATA gene using GH7SEP1 forward and GH1SEP1 reverse was not found any amplification bands in all samples (figure 4). Within results above, this result suggested a novel prediction of SEPALLATA gene of *H. rosa-sinensis* is different from SEPALLATA gene of other plants. This result may due to unitary pseudogenization of SEPALLATA gene in *H. rosa-sinensis* genome. It may appear because of the introduction of a premature stop codon, which results in the partial-pseudogenization SEPALLATA gene. Partial parts of SEPALLATA gene which encodes...
functional protein may be turned on, whilst the other part of \textit{SEPALLATA} gene is either completely turned off or regulated by a different promoter. As observed in AGL-6, the closely related gene to AGL-2 (\textit{SEPALLATA}), which also suggested underwent pseudogenization or neofunctionalization [8]. Thus, it is predicted either \textit{SEPALLATA} gene of \textit{H. rosa-sinensis} distinguished into different chromosomes or intact in the same chromosome yet regulated by different promoters (figure 5). To verify these hypotheses, full and short DNA fragments sequencing of \textit{SEPALLATA} gene of \textit{H. rosa-sinensis} should be done. Mismatch of short DNA fragments contig result with full sequencing may indicate that \textit{SEPALLATA} is a complex which expressed by several genes in different chromosomes or vice versa. In addition, protein expressed by \textit{SEPALLATA} gene should be checked to see whether it expresses full protein sequences as any other species or only some parts which functionally active.

\textbf{Figure 5.} (a) Prediction model of \textit{SEPALLATA} gene of \textit{H. rosa-sinensis} distinguished into different chromosomes, or (b) intact in same chromosome yet regulated by different promoters.
4. Conclusion
The amplification of SEPALLATA gene using GH1SEP1 showed a negative result in epicalyx. In contrast, amplification using GH7SEP1 showed a positive result. Another trial was done using GH7SEP1 forward primer and GH1SEP1 reverse primer, which showed negative results. These results suggested that SEPALLATA gene of H. rosa-sinensis may differ from SEPALLATA gene from other plant. SEPALLATA gene of H. rosa-sinensis is predicted for acquiring a pseudogene. Thus, it is also predicted either SEPALLATA gene of H. rosa-sinensis distinguished into different chromosomes or intact in the same chromosome yet regulated by different promoters.

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