Analysis of AGAMOUS Gene Expression in Hibiscus rosa-sinensis L. Single Pink, Crested Peach, and Double Orange Flowers

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Abstract. Hibiscus rosa-sinensis L. crested peach and double orange types are different from single pink type regarding their additional petals (petaloid). The petaloid structure is thought to have originated from reproductive organs modification. AGAMOUS is class C gene that plays a role in the formation of androecium and gynoecium. Loss of AGAMOUS gene expression is assumed to be the cause of modifications occur in reproductive organs. Therefore, this study aims to determine the qualitative expression of AGAMOUS gene on single pink, crested peach, and double orange flowers. The expression of AGAMOUS gene was also assessed in leaf organ as a comparison. Analysis of AGAMOUS gene expression was done by isolating RNA from their androecium and gynoecium using the modified CTAB method. The RNA sample was converted to cDNA using reverse transcriptase, before further amplified by PCR technique using AG1 and AG2 primers. The AG1 PCR product produces bands of 100, 200, and 300 bp, while the PCR AG2 produces a single band of 200 bp. The analysis of sequencing results showed that the AGAMOUS gene expressed in all samples. Therefore, petaloids presents in crested peach and double orange flowers are not a result of a loss of AGAMOUS gene expression. The changes of reproductive organs into petaloids should be analyzed not only based on AGAMOUS gene expression but also should include other gene and their interactions.

1. Introduction

Hibiscus rosa-sinensis L. has varieties of flowers shape, namely single, double [1], and also intermediate forms called crested [2]. Based on the previous research on the morphological and anatomical observation, single pink flowers have no additional petals (Figure 1A). The crested peach flower has additional petals in the stamen whorl (staminodium petaloid) (Figure 1B), while double orange flower has additional petals both in stamen and pistil whorls (Figure 1C). The ovules developed normally in the ovary of the single pink flowers, but reduced in the crested peach flowers, and disappeared entirely and replaced by a structure resembling petals (petaloid) in double orange flowers. The additional petals in the stamen and pistil whorls could be presumed originated from a modified stamen and pistil.

Modification of one part of the flower organ into another form is thought to be a result of the absence of the expression of homeotic genes responsible for the formation of the flower organ. This phenomenon of organ replacement is known as homeosis which is under the control of the Homeotic genes, which plays a role in the development of 4 flower organ whorls (sepal, petal, stamen, and pistil), and so-called as the ABC model. The A-class regulates sepals in the first whorl, the A- and B-class specify petals formation in the second whorl, the B- and C-class regulate the stamens in the third whorl, and the C-class determine the pistils formation [3].
In *Arabidopsis*, the class C gene is *AGAMOUS (AG)* plays an essential role in the development of the stamens, pistils, and ovules. Loss function of *AGAMOUS* gene causes reduced or no stamen and pistils in the flowers [4]. Research on the *AGAMOUS* gene also been studied in other plants beside *Arabidopsis*, including the *Theobroma cacao* L. [5], *Lilium longiflorum* [6], and *Oryza sativa* [3]. These studies suggest that *AGAMOUS* are expressed in flower reproductive organs (stamen and pistils) and not in sepals, petals, and leaves.

Research on *AGAMOUS* gene expression of *H. rosa-sinensis* L. flower has never been done before. However, morphological studies on homeosis events in *H. rosa-sinensis* L. have been reported in single and double flowers [7]. Based on this information, research on *AGAMOUS* gene expression of *H. rosa-sinensis* L. single pink, crested peach, and double orange flowers are needed to determine whether the flower organ modification that occur in the variation of crested peach and double orange flowers are caused by the failure of class C genes expression. The results of the study are expected to provide preliminary data of homeosis events in *H. rosa-sinensis* L. which supports the anatomical and morphological studies that have been carried out.

2. Materials and Methods

Total RNA isolated from leaves, stamens, and ovules from single pink flowers; staminodium petaloid and ovules from crested peach flowers; petaloid and staminodium petaloid from modified ovules from double orange flowers. Samples from the three types of flowers were collected from the *H. rosa-sinensis* plant which grew at the University of Indonesia Depok campus and from the garden in a house in Bojong Gede. The RNA extraction was carried out using a modified CTAB method according to Zeng & Yang [8]. The isolated RNA was then reverse-transcribed by the GoScriptTM Reverse Transcription System [Promega]. The cDNA amplification of the *AGAMOUS* gene was carried out using PCR techniques and
using primers previously designed on the website of the National Center for Biotechnology Information (NCBI) namely AG1 and AG2. The primer design refers to the *Herrania umbratica* mRNA sequence which is a plant member of the Malvaceae family (LOC110424552). The cDNA amplified by the PCR technique using the GoTaq® Green Master Mix kit [Promega] with thermal cycler program as followed: 95 °C for denaturation, 54 °C for annealing, and 72 °C for polymerization.

The amplified DNA samples were separated according to their size with electrophoresis gel. The gel used is agarose gel 1% while the running solution used is TAE 1x buffer. The sample was then run at 100 V for 30 minutes [9]. After the DNA fragments are separated, the gel is cut to the desired portion of the DNA. Purification of DNA from the gel was carried out using a GenepHlowTM Gel / PCR kit [Geneaid]. Pure DNA samples were then used for sequencing Sequencing results in the form of a nucleotide base sequence, and the chromatogram was aligned with the *AGAMOUS Herrania umbratica* gene sequence using the Geneious version 11.1.4 and Bioedit version 7.0.5 software.

3. Result and Discussion

PCR results using AG1 primer at an annealing temperature of 54°C produced a variety of bands with sizes ranging from 100 to 300 bp (Figure 2), which did not show the expected amplification result of 405 bp. The band observed in lane 1 is 300 bp, whereas in lane two there is no clear band, but a smear. The bands that are visible on lanes 3, 4, 5 and 7 is only at 100 bp with a smear band on the top. PCR results in sample 6 showed three bands that were clearly visible at sizes of 100 bp, 200 bp, and 300 bp. PCR results using AG2 primer at a temperature of 54°C produced a bright band measuring around 200 bp in all samples (Figure 2), and the results were not in accordance with the prediction size of 399 bp.

The PCR result with AG1 primer used for sequencing was derived from staminode petaloid double orange flower (Figure 2 lane 6). The three bands used were 100 bp (coded A), 200 bp (coded B), and 300 bp (coded C). Meanwhile, the PCR result with AG2 primer used for sequencing came from the stamen of a single pink flower (Figure 3 lane 2). The band used is 200 bp in length and is coded D.

Sequencing results obtained were matched with *AGAMOUS Herrania umbratica* sequence using Geneious version 11.1.4 software and Bioedit version 705. Alignment of the A, B, and C bands sequences with *AGAMOUS Herrania umbratica* gene using Geneious v11.1.4 software showed a match nucleotide base sequence (Figure 4). *AGAMOUS* gene validation is supported by the alignment of the three sequences with the AG1 reverse primer using Bioedit version 705 software (Figure 5).

Alignment of the sequencing results of sample D shows a sequence match with the *AGAMOUS Herrania umbratica* gene when aligned using the Geneious version 11.1.4 software (Figure 6). The existence of AG2 reverse primer sequence on sequence D also strengthens the assumption that amplified genes are *AGAMOUS* (Figure 7). Even though the query covers still very low, the results from BLAST
sequences also verify our results that the A, B, C, and D bands showed homology to AGAMOUS *Herrania umbratica* gene, as shown in Figure 8.

![Figure 4. Alignment of the three-band sequencing results of double orange staminode petaloid samples with AGAMOUS of Herrania umbratica](image)

![Figure 5. The AG1 reverse primer attachment site in the A, B, and C band sequencing results](image)
The results of AGAMOUS gene amplification using AG1 and AG2 primers produce band size that did not match the predicted size. The sequence of genes obtained is not precisely the same; there are fragments of genes that have absolutely no match. This can affect the length of the PCR product produced, which is smaller than predicted. The incompatibility of the fragment length of the PCR results using these two primer pairs is probably due to the sequence of the AGAMOUS gene in *H. rosa-sinensis* L. only has a partial similarity to *Herrania umbratica*. The absence of *H. rosa-sinensis* L. AGAMOUS gene database makes specific primer designs difficult. The flower of *H. rosa-sinensis* L. is ephemeral; the flower has a very short bloom (one day). Aging that occurs in flowers as the age of the flower...
increases is associated with an increase in the abundance of hydrolytic gene mRNA transcripts, including nuclease. This indication raises the suspicion that the expressed AGAMOUS gene has been degraded as a result of enzyme nuclease activity [10].

The PCR results of the AGAMOUS gene amplification with the AG1 and AG2 primers showed that the bands from all the samples used in this study (Fig.2 and 3) have a similar size with one of the representative bands (band A, B, C, and D) that used for sequencing. Therefore it could be assumed that the amplified bands come from the same gene. Based on sequence and homology results (Fig. 4, 6, and 8), we found that the AGAMOUS gene is expressed in all samples used in this study (leaves, stamen, and ovary of pink single flower; staminodium petaloid and ovaries of crested peach flower; staminodium petaloid and petaloid from ovary of double orange flower.

AGAMOUS is one of the MADS-box genes that play a role in flower organ formation. The AGAMOUS belongs to the class C gene and plays a role in the formation of circles 3 and 4 in flowers, namely androecium, and gynoecium [11]. Based on research by Chaidamsari et al. [5], the AGAMOUS in T. cacao is expressed on stamen, pistil, and staminode, but not in leaves, sepals, and petals. AGAMOUS gene of L. longiflorum also expressed on the flower stamen and pistils, but not on the margins and leaves [6]. Based on this information, the AGAMOUS gene should not be expressed on the leaves. Different results were found in the results of the measurement of the quantitative expression of the AGAMOUS homolog gene in Black Ash (Fraxinus nigra), FnAG, which showed that AGAMOUS mRNA transcripts were detected in leaves with relatively low intensity compared to the expression in male and female reproductive organs, that is 1 / 25.8 and 1 / 13.5 [12]. Therefore, it is necessary to analyze AGAMOUS gene expression quantitatively to find out more about the AGAMOUS gene expression on H. rosa-sinensis leaves.

Modification of stamen into staminodium petaloid in crested peach and double orange flowers and change of ovules to petaloid in double orange flowers is thought to be the result of the failure of the AGAMOUS gene expression. However, based on the results of the research that had been carried out, the AGAMOUS gene is expressed in both variations of the flower. The same results were also obtained by Prihatiningsih [13] that the AGAMOUS gene expressed in staminodium petaloid crested and double flowers. Therefore, the change in the structure of the reproductive organs of the flower to petaloid is most likely not caused by the failure of AGAMOUS gene expression but could be others. Changes in the structure of the stamen to petaloid were also observed in Canna indica. Based on research conducted on C. indica, it was concluded that the study of homeotic gene expression alone is not enough to determine the causes of changes in stamen structure to petaloid. The results of the research showed that the AGAMOUS gene was expressed on staminodium petaloid C. indica [14].

Another possibility that causes changes in the reproductive organs of flowers into petal is the presence of transposable elements (jumping gene). Transposable elements insert into the homeotic gene and cause the formation of double flower phenotypes. Research on transposable elements was carried out by Nitasaka [15] in the Japanese morning glory (Ipomoea nil) and concluded that the presence of a transposable element (jumping gene) in the homeotic gene class C DUPLICATED resulted in changes in the flower reproductive organs into perianth organs (sepals and petal) on double flowers. Another possibility is that the reproductive organs of the flower are not modified to be petal, but members of the MIR164 gene family that regulate the gene for the formation of CUC flower whorls not expressed. The loss function of MIR164 gene expression results in a flower phenotype that has more petals and fewer stamen compared to wildtype [16].

4. Conclusion
The AG1 and AG2 primers that have been designed successfully amplify the AGAMOUS gene. The PCR product sequencing results showed that the AGAMOUS gene expressed in all samples tested, including leaves. Changes in the structure of stamen and ovules into petaloid in crested peach and double orange flowers are assumed not to be caused by the failure of AGAMOUS gene expression.
Acknowledgments
This work was supported by Hibah PITTA 2018 funded by DRPM Universitas Indonesia No.2229/UN2.R3.1/HKP.05.00/2018.

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