

Isolation and Characterization of Chalcone Synthase (*CHS*) Gene in Variegated-Flower of *Dendrobium 'Enobi'* and *Phalaenopsis* Hybrid Orchids

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ABSTRACT

Variegated flowers, characterized by the presence of different colors in flowers, have high economic and aesthetic values. The main pigment in the orchid's purple flowers is anthocyanin, while the chalcone synthase (*CHS*) gene is the key to anthocyanin biosynthesis. Analysis of the *CHS* gene can reveal some changes, including mutations, in the process of color patterning in flowers. This study aims to determine the structure of the *CHS* gene related to color patterning in *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid with variegated flowers. The methods applied in this study are floral morphology observation, DNA isolation, *CHS* gene amplification, anthocyanin measurement, and bioinformatic analysis. Morphologically, the variegated pattern has appeared since the flowers were still in the bud on both orchids. Based on the anthocyanin content analysis, the difference in the genus is not directly related to the differences in the flower's anthocyanin content. In addition, the purple zone in the *D. 'Enobi'* and *Phalaenopsis* hybrid has a longer fragment of *CHS* than the white zone. Our analysis suggested several mutations in the white zone and differences in the type and location of several conserved domain proteins. Mutations at the *CHS* gene fragment might cause decreased anthocyanin pigment formation in the white region.

1. Introduction

Orchids are highly cultivated because they are beautiful and precious. Orchids have a unique flower shape and bright green leaves, giving them high economic value. Variegation is a phenotype characterized by differences in color patterns. For example, *Phalaenopsis 'Sogo Vivien'* has variegated leaves and flowers (Mursyanti *et al.* 2016). Several plant species have variegated patterns as well. This attractive character can increase the beauty and commercial value of variegated plants as ornamental plants (Mursyanti *et al.* 2016; Wang and Fu 2016).

Anthocyanins are the most common flower pigments and contribute to various colors. Several enzymes relevant to the anthocyanin biosynthetic pathway have been identified in various plant species. Several genes involved in the anthocyanin biosynthetic

pathway are chalcone synthase (*CHS*) as a key enzyme in anthocyanin biosynthesis, dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), flavonoid 3'5' hydroxylase (*F3'5'H*), flavonoid 3' hydroxylase (*F3'H*), and flavanone 3-hydroxylase (*F3H*) (Dao *et al.* 2011). Research by Chen *et al.* (2014) showed several different gene expressions in peach flowers with red and white petals. Four key genes related to flavonoid biosynthesis, such as *C4H*, *CHS*, *CHI*, and *F3H*, are expressed higher in the red petal. In a study by Akhar *et al.* (2016) showing differences in *CHS* gene expression in *Petunia* hybrid flowers, the lowest *CHS* gene expression was shown in white flowers. The *CHS* gene is expressed at the early stage of flower development (Ma *et al.* 2017). In addition, *CHS*, *C4H*, *CHI*, and *F3H* genes are highly expressed in the red zone (Chen *et al.* 2014).

The structure of the *CHS* gene in the orchid species *Phalaenopsis equestris*, which has a purple flower, has two exons and one intron with a length of 1,692 bp. There are three types of conserved domain proteins:

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PHA03247, PLN03172, and PLN03173 superfamily. The structure of the *CHS* gene in the plant model *Arabidopsis thaliana*, which has white flowers, has two exons and one intron with a length of 1,792 bp located on chromosome five. There are two types of conserved protein domains: PLN03170 and PLN03172 superfamily. The differences in the structure of the *CHS* gene in both plants with different flower colors are interesting to study because the *CHS* gene is crucial in anthocyanin biosynthesis (NCBI 2022a, 2022b). This gene has been studied in various plant species, but in orchids, it has only been studied a little (Pitakdantham *et al.* 2010).

Colors in flowers have been successfully engineered through modification of *CHS* genes, some of which are in research on *Torenia* hybrid flowers (Suzuki *et al.* 2000; Fukusaki *et al.* 2004) and Gentian flowers (Nakatsuka *et al.* 2008). The most important principle in color formation engineering on orchid flowers is to collect information about key enzymes in the anthocyanin biosynthetic pathway (Pitakdantham *et al.* 2010). Therefore, the isolation and characterization of anthocyanin pigment-forming genes like the *CHS* gene in orchids with variegated flowers are interesting topics to be studied. The variety of styles and colors of hybrid orchid flowers is huge in the market. Orchids of the genus *Dendrobium* and *Phalaenopsis* have been widely cultivated and are commonly used as parents for hybrid orchids (Comber 1990). This study used hybrid orchids from the two genera, *Dendrobium* 'Enobi' and *Phalaenopsis* hybrid. Both orchids have a variegated pattern consisting of separate purple and white colors at the edge and middle of the flower tepals. In this study, the two hybrid orchids were used because apart from the variegated phenotype, they also come from different genera and have different growth patterns. Therefore, it is possible to compare the *CHS* gene structure in the two orchids, which are taxonomically and morphologically different. The genome and gene structure between two species may differ; therefore, it is important to study the *CHS* gene structure in both orchids. Suppose there are any differences in the structure of *CHS* genes from both genera and color zones; they can be used as target gene candidates for genetic engineering within plant breeding schemes with genome editing methods (CRISPR/Cas9) with more precise and accurate targets.

2. Materials and Methods

2.1. Materials

Dendrobium 'Enobi' and *Phalaenopsis* hybrid which already have the flower buds used in this research. These orchid plants were six years old and collected from three Yogyakarta nurseries: Titi Orchid, Keiki Orchid, and Kresna Nursery. Flowers with a variegated pattern consisting of purple and white colors were used in this study. Chemical materials for DNA isolation were 3% CTAB (Himedia, USA) (1M Tris HCl, 0.5M EDTA, 3% CTAB powder, NaCl, pure water), PVP 1% (Himedia, USA), chloroform (Merck, Germany), EtOH (Merck, Germany), Na-acetate (Merck, Germany), 70% ethanol (Merck, Germany), and TE buffer pH 8. The ingredients for the PCR reaction are 2x MyTaq™ HS Red Mix (Bioline, UK), pure water, and specific primers (IDT, USA) constructed by the authors for the *ACTIN* and *CHS* genes (Table 1). *ACTIN* is a housekeeping gene that serves as a positive control for PCR reactions, indicating the DNA quality used (Bao *et al.* 2016; Zhao *et al.* 2012).

The chemical materials for electrophoresis are agarose gel powder (Nippon Gene, Japan), TBE 1X, 6X loading dye (Geneaid, Taiwan), 100 bp DNA ladder (Geneaid, Taiwan), 1 Kb DNA ladder (Geneaid, Taiwan), ddH₂O, and EtBr. The materials for analyzing anthocyanin pigments are methanol (Merck, Germany), HCl (Merck, Germany), and distilled water.

2.2. Flower Morphological Observation

Morphological observation of *D. 'Enobi'* and *Phalaenopsis* hybrid flowers included ratio measurement using ImageJ. The purple and white area was marked using ImageJ, and then the ImageJ displayed the area of the purple and white zone; the data of that area were used as a base to make the ratio of the purple and white zone of the flower. Fully bloomed flowers on both orchids were used for this observation; the fully bloomed flowers are 16

Table 1. List of primers used in this research

Primer	Sequence
<i>CHS</i> F1	5'-CCGAACGCCGTGTATCAGGCGGACT-3'
<i>CHS</i> R1	5'-CCCACTCCAACCCCTCACCGGTGGT-3'
<i>ACTIN</i> F	5'-GTATTCCCTAGGATTGTGTTGGT-3'
<i>ACTIN</i> R	5'-CAGAGTGAGAATACCTCGTTG-3'

days old in *D. 'Enobi'* and 20 days in the *Phalaenopsis* hybrid since the emergence of flower buds. Flower development stages from bud until bloom were observed and documented. Flower colors were identified with the color card matching method using the Royal Horticultural Society (RHS) Color Chart. The RHS color chart is a color chart used as a standard reference by horticulturists around the world. This color chart is usually used to identify the color of flowers, fruit, leaves and other plant organs (Post and Schlautman 2020).

2.3. Genome DNA Isolation and Gel Electrophoresis

The genome DNA was isolated based on Murray and Thompson's (1980) method, with modifications by adding 1% PVP to the CTAB to produce good and thick DNA genome bands from the flower sample. Modifications were made because the orchid contains polyphenols (Sityardi and Desrini 2021). Polyphenols can inhibit various processes as contaminants in DNA isolation (Nugroho *et al.* 2015). The first step in DNA isolation is cell wall lysis. Flower samples were separated into white, purple, and mixed zone petals. 100 mg petals were placed in a microtube and added with 500 µL CTAB 3% + PVP 1%. The second step is protein denaturation, where the chloroform is added to the sample (1:1). Then, DNA precipitated by adding EtOH and 2/10 Na-acetate to the sample. The DNA genome samples were stored in a refrigerator at -20°C. DNA genomes were visualized using 0.7% agarose gel and set at 100 volts for 23 minutes.

2.4. Polymerase Chain Reaction (PCR) and Sequencing

DNA genomes were used as a *CHS* and *ACTIN* gene amplification template in the Polymerase Chain Reaction (PCR) method. The *ACTIN* gene was used as a positive control. The reagent compositions in the PCR process are presented in Table 2.

Table 2. Reagent components for PCR reaction

Reagent component	Volume (µL)
DNA genome	1.0
2x MyTaq™ HS Red Mix	12.5
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
Pure water	9.5
Total Volume	25.0

The PCR protocol was used based on (Nikmah *et al.* 2021) with modification in annealing based on several optimization experiments. The annealing temperature for *ACTIN* primer is 51°C, while the annealing temperature for *CHS* primer in *D. 'Enobi'* and *Phalaenopsis* hybrid are 62.5°C and 55.1°C.

The PCR results were visualized with 0.7% agarose gel. The PCR results were sequenced using the Sanger Sequencing method at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT, UGM), with an Applied Biosystem 3500 Genetic Analyzer 2500 (Hitachi, Japan).

2.5. Measurement of Anthocyanin Content using Spectrophotometry

Following Hermawati (2016) and Liang (2017), the anthocyanin levels were analyzed. *D. 'Enobi'* and *Phalaenopsis* hybrid flower (sepals and petals) were separated based on color, purple and white zones, then weighed 0.015 grams for each zone for three replications. The solvent was made of methanol, 1% HCl, and distilled water in a ratio of 90:1:1 (v/v/v). The ratio of the sample to the solvent is 1:10, which is 0.015 grams per 1.5 ml of solvent. The sample was put into a 1.5 ml microtube, and 500 µL of solvent was added. The sample was ground with a micro pestle, added to the remaining 1,000 µL of solvent, and vortexed. Samples were centrifuged at 2,500 rpm for 10 minutes. The sample was transferred to a cuvette, and then the anthocyanin content was measured using spectrophotometry with absorbance wavelengths of 530 nm and 657 nm. The results of the spectrophotometry were analyzed using the formula for total anthocyanin levels (Lim *et al.* 2021):

$$\text{Anthocyanin} = A_{530} - (0.25 \times A_{657})$$

Anthocyanin concentrations were analyzed using a two-sample t-test between the purple and white zones with the help of SPSS 15.0.

2.6. Data Analysis and Bioinformatics Tools

Data analysis includes qualitative data using description analysis and quantitative analysis using some software programs. Flower color ratio measurements were analyzed using ImageJ to mark the purple and white zone area. The anthocyanin pigment content was analyzed using Microsoft

Excel 365 and SPSS 15.0 for statistical analysis. The data of amplified sequences were prepared using A Plasmid Editor (ApE) and BioEdit and were analyzed using the Basic Local Alignment Search Tool (BLAST) from NCBI. The data of the conserved domain were analyzed using the Conserved Domain NCBI Database (CDD). The data of amino acids were analyzed using the Swiss Bioinformatics Resources Portal (expasy.org) for translating the nucleotide to protein. The data of amino acid motifs were analyzed using the protein and domain database (PROSITE) by adding the protein sequences analyzed from expasy.org.

3. Results

3.1. Flower Morphology

Figure 1A shows the stages of bud development of the *D. 'Enobi'* flower. On the ninth day after flower bud emergence, some purple spots appeared in the buds (marked with a red circle). After fully blooming, the *D. 'Enobi'* flower shows a ratio of white to purple area of 2:1 based on the measurement results with ImageJ, where the purple zone is located on the edge of the flower.

Figure 1B shows the stages of flower bud development in a *Phalaenopsis* hybrid. Based on the eighth day after flower bud emergence, the *Phalaenopsis* hybrid flowers appear purple since they were buds. After fully blooming, *Phalaenopsis* hybrid flowers show a ratio of purple to the white area of 1:1:1, so the purple color is slightly more dominant than the white ones. The white color is located on the edge of the flower. This pattern is the opposite of the color pattern in the *D. 'Enobi'*.

Based on the RHS color chart, the purple zone in the *D. 'Enobi'* flower is classified as N78C (deep purplish pink), while the white zone is identified as NN155C (white) (Figure 1A). The purple color in *Phalaenopsis* hybrid flowers is classified as N78A (strong reddish purple), and the white color is classified as N155B (pinkish white) (Figure 1B). Although *D. 'Enobi'* and *Phalaenopsis* hybrid have purple and white colors, they are different in the shade of flower color based on the RHS chart. *Phalaenopsis* hybrid has a deeper purple color when compared to *D. 'Enobi'*. Differences are also found in the location of the variegated pattern. *D. 'Enobi'* has a purple pattern on the flower of the flower.

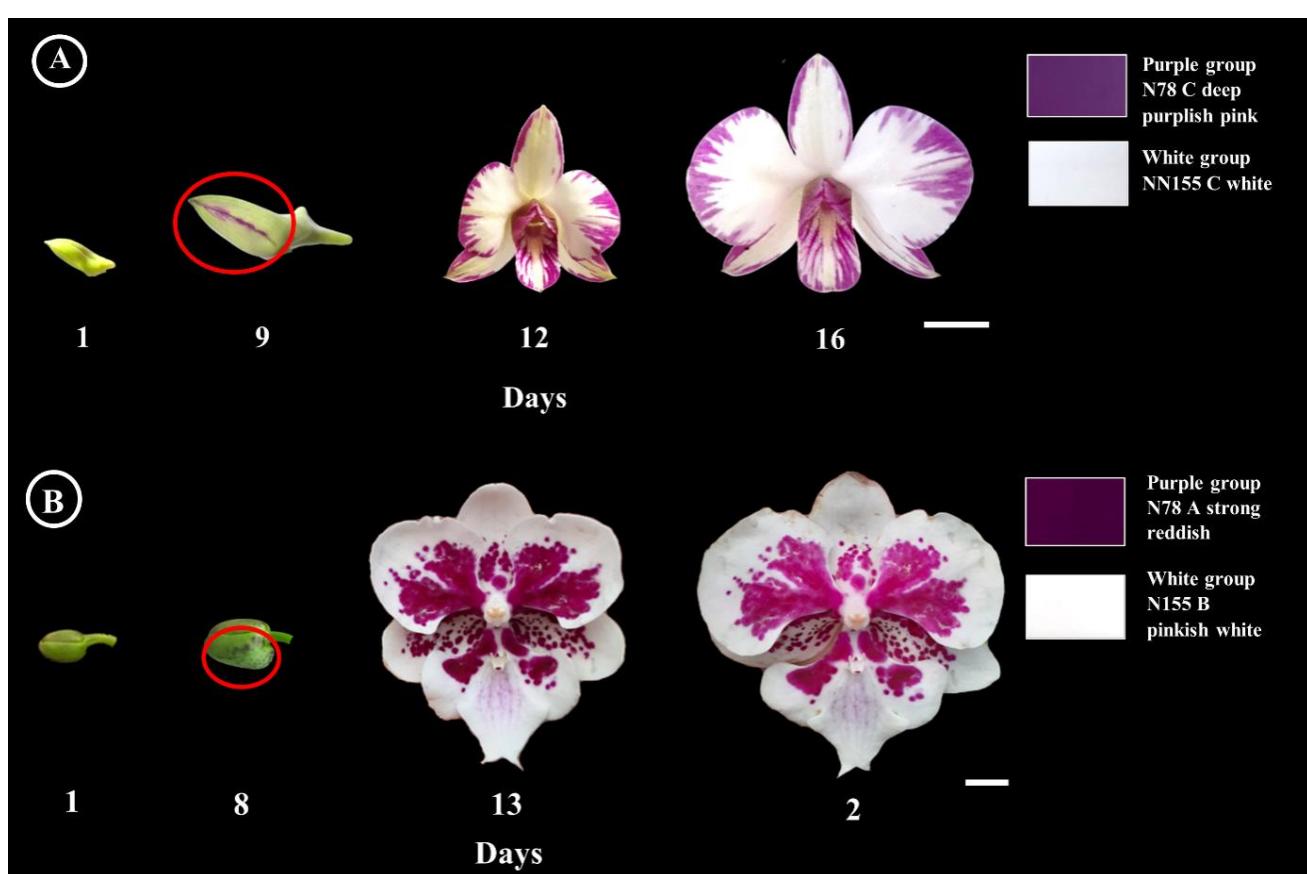


Figure 1. Flower bud development and flower color identification of *Dendrobium 'Enobi'* (A) and *Phalaenopsis* hybrid (B). The number shows the observation day. Bar: 1 cm

margins, while the *Phalaenopsis* hybrid has a purple pattern in the middle of the flower.

3.2. Anthocyanin Contents

Based on the spectrophotometric analysis, anthocyanin content within the purple zone in *D. 'Enobi'* and *Phalaenopsis* hybrid is significantly higher when compared to the white zone (Table 3). However, when we compare the anthocyanin content of the purple zone in both orchids, the anthocyanin content is not significantly different (Table 3). Similarly, the white zone was not significantly different (Table 3). The highest anthocyanin levels were found in the purple zone of the *Phalaenopsis* hybrid ($0.1976 \mu\text{g}/\text{ml}$) (Table 3). While in the white zone, anthocyanin

Table 3. Comparison of anthocyanin content in the purple and white zone of *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid variegated flower

Sample	Zone	Anthocyanin pigment content ($\mu\text{g}/\text{ml}$)	p-value
<i>Dendrobium 'Enobi'</i>	Purple	0.1646 ± 0.139	NS
<i>Phalaenopsis</i> hybrid	Purple	0.1976 ± 0.046	
<i>Dendrobium 'Enobi'</i>	White	0.0114 ± 0.008	NS
<i>Phalaenopsis</i> hybrid	White	0.0217 ± 0.009	

NS is not significant

levels were higher in the *Phalaenopsis* hybrid as well compared to *D. 'Enobi'* ($0.0217 \mu\text{g}/\text{ml}$) (Table 3). Based on the RHS color chart, *Phalaenopsis* hybrid has a stronger purple color compared to *D. 'Enobi'*.

3.3. CHS Sequence Analysis

DNA isolation with the modification method by adding 1% PVP to the CTAB produced good and thick DNA genome bands from the flower sample. Genomic DNA has a relatively similar size between samples, which is more than 10 Kb (Figure 2). There is a single band from the PCR product of the *CHS* gene with a size of $\pm 1,300$ bp for the *D. 'Enobi'* sample and $\pm 1,000$ bp for the *Phalaenopsis* hybrid (Figure 3). As for the *ACTIN* gene PCR product, all samples showed the same size of ± 114 bp (Figure 3). Flower samples from the same species had the same size of *CHS* gene PCR products, whether there were purple, white, or mixed zones. When compared with different species, the size of the *CHS* gene PCR product was different.

Figure 4A shows that the *D. 'Enobi'* sequence length in the purple zone is longer than in the white zone. Similar results were also found in the *Phalaenopsis* hybrid. Based on Figure 4B, the *CHS* sequence isolated from the purple zone has a longer sequence than the white zone. Based on the alignment results between

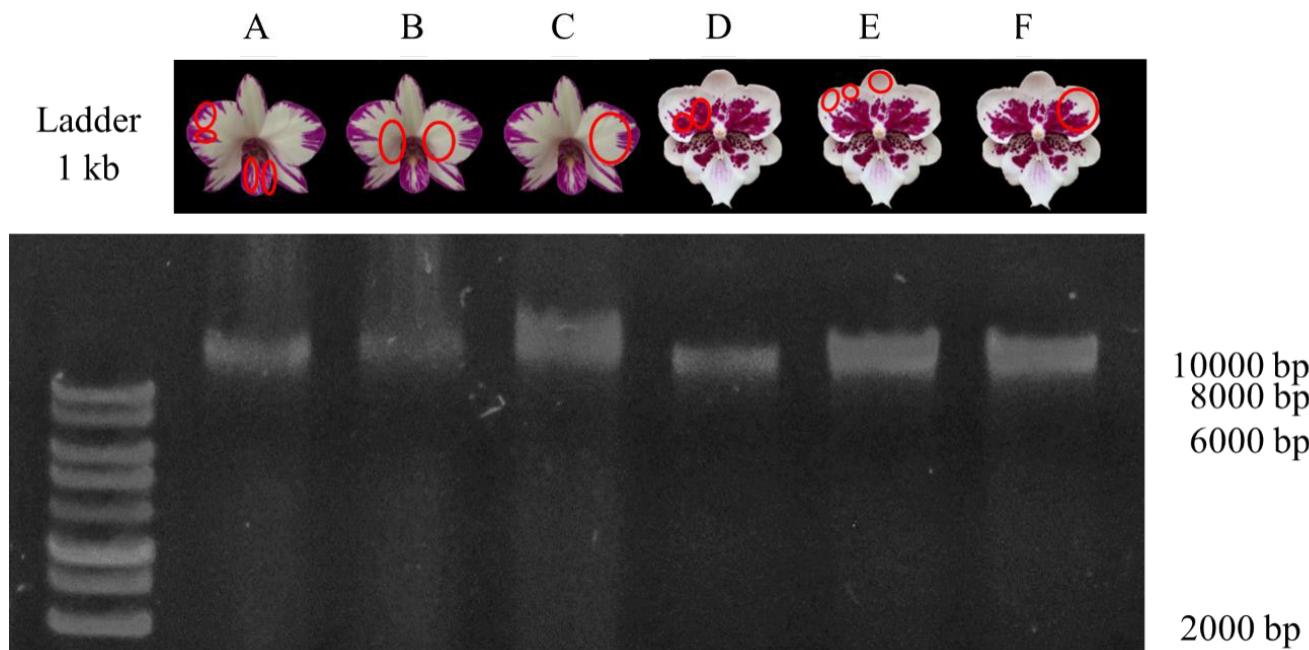


Figure 2. DNA genome visualization from *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid. (A) *D. 'Enobi'* purple zone, (B) white zone, (C) mixed, (D) *Phalaenopsis* hybrid purple zone, (E) white zone, (F) mixed

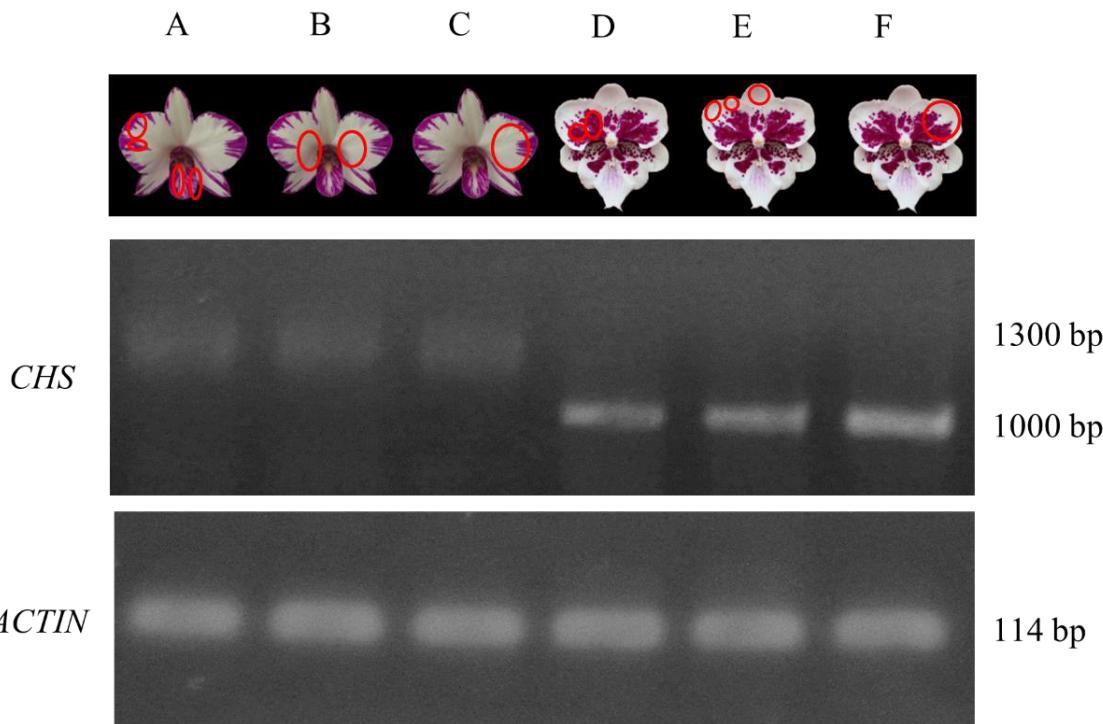


Figure 3. Detection of CHS and ACTIN genes in *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid. (A) *D. 'Enobi'* purple zone, (B) white zone, (C) mixed, (D) *Phalaenopsis* hybrid purple zone, (E) white zone, (F) mixed

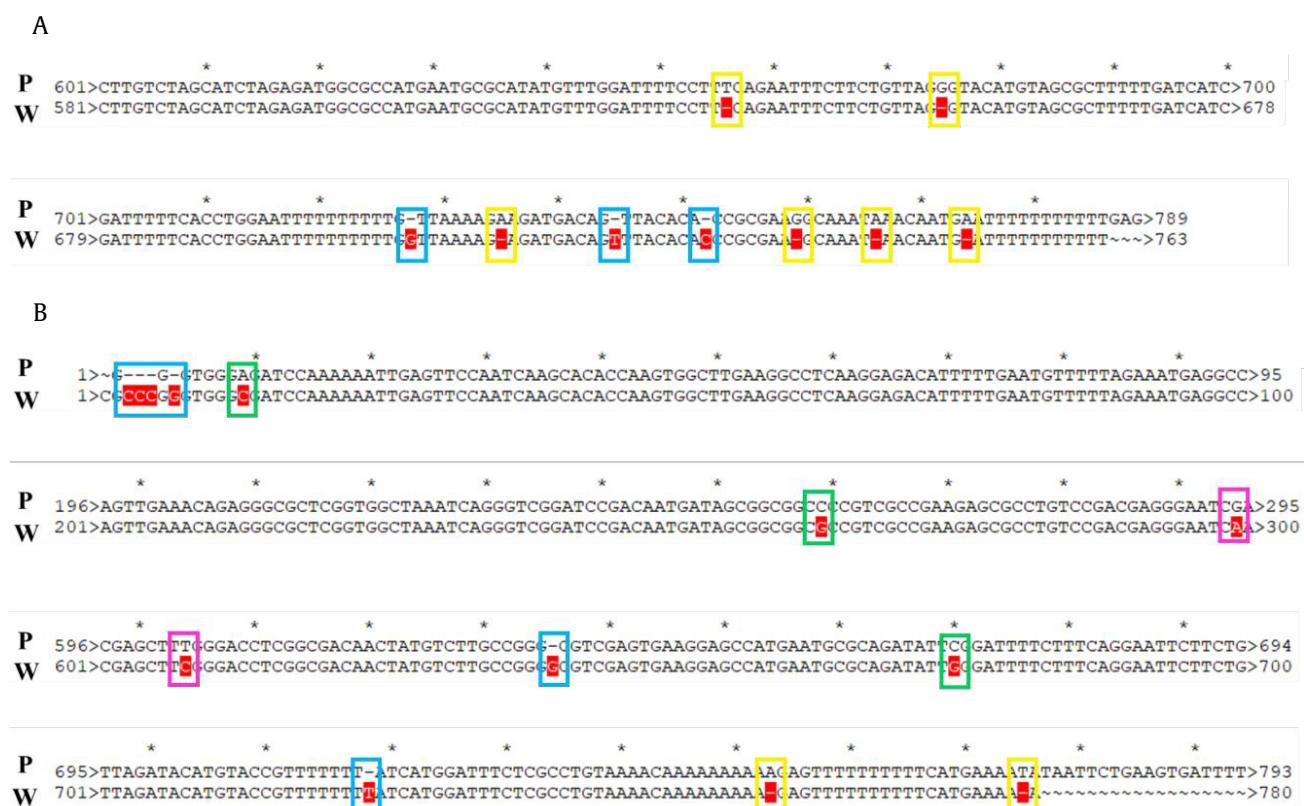


Figure 4. CHS gene sequences alignment from *Dendrobium 'Enobi'* (A) and *Phalaenopsis* hybrid (B). P: purple zone; W: white zone; mutation detected in the white zone marked with the red color; yellow: deletion sites; blue: insertion sites; green: substitution-transition sites; and pink: substitution-transversion sites

the *CHS* gene sequences in the purple and white zones, several mutation points exist in the white zone in both orchids. This causes the sequence size of the white zone to be shorter than that of the purple zone.

The mutation list detected in the white zone (Figure 4A and B) from the *CHS* gene alignment of *D. 'Enobi'* and *Phalaenopsis* hybrid is shown in Table 4. Based on the sequence alignment, nine-point mutations are detected in the white zone of *D. 'Enobi'*; there are six deletions and three insertions (Table 4). In the *Phalaenopsis* hybrid, there are thirteen-point mutations in the white zone, namely two deletions, three insertions, two substitutions (transition), and three substitutions (transversion) (Table 4).

The difference in sequence length and mutation can affect the proteins and anthocyanin biosynthetic pathway. The list of mutations found in the white zone of *D. 'Enobi'* includes six deletions and three insertions.

The list of mutations found in the white zone of *Phalaenopsis* hybrid includes two deletions, six insertions, two substitutions (transitions), and three substitutions (transversions). Transition is when the base changes from a purine to another purine or pyrimidine to another pyrimidine, while transversion is when the base changes from purine to pyrimidine or from pyrimidine to purine.

Based on the sequence analysis results through the Conserved Domain Protein NCBI (Table 5), it was found that *D. 'Enobi'* in the purple and white zones had different types of conserved domain proteins. Based on the results (Table 5), *Phalaenopsis* hybrid in the purple and white zones also have different

conserved domains. Although both orchids have different conserved domain codes in the purple and white zones, they are all classified as chalcone synthase domains.

Due to the shorter nucleotide sequence size in the white zone in both orchid species due to mutations, the amino acid sequence size is also shortened in the white zone. Therefore, the amino acid sequences in the white zone appear shorter than those in the purple zone (Figure 5 A-D). The amino acid motif identified in both zones is PS00441 CHALCONE_SYNTH (chalcone and stilbene synthases active site). Both orchid species were identified as having the same amino acid motif but in different locations.

4. Discussion

The purple color pattern was observed after the first week after flower bud emergence in *D. 'Enobi'* and *Phalaenopsis* hybrid. At the beginning of flower bud emergence, flowers are dominated by green color (Figure 1). This shows that the purple color pattern on the flower tepals in both orchids began to form and can be observed after the flower buds were one week old. Whereas at the beginning of flower bud formation, the pigment is still dominated by chlorophyll green pigment. As the age of flower buds increases, the variegated pattern will become clearer because the accumulation of anthocyanins will increase (Han et al. 2006). Based on the RHS color chart, the purple zone of *Phalaenopsis* hybrid has a strong reddish-purple color (Figure 1B) compared to the purple zone of *D. 'Enobi'*, which is deep purplish pink (Figure 1A). Based on the

Table 4. Mutation list in the white zone of *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid

Sample	Nucleotide	Number of mutation	Mutation type
<i>Dendrobium 'Enobi'</i>	637, 656, 714, 738, 745, 753	6	Deletion
	707, 724, 731	3	Insertion
	768, 790	2	Deletion
	3, 4, 5, 7, 640, 723	6	Insertion
<i>Phalaenopsis</i> hybrid	299, 608	2	Substitution (Transition)
	13, 263, 674	3	Substitution (Transversion)

Table 5. The conserved domain of *CHS* from *Dendrobium 'Enobi'* and *Phalaenopsis* hybrid

Sample	Zone	Conserved domain	Accession	Description	Interval	E-value
<i>D. 'Enobi'</i>	Purple	PLN03170 superfamily	CI30450	Chalcone synthase; Provisional	2-655	5.73e-142
	White	PLN03172	CI30448	Chalcone synthase family protein; Provisional	6-722	4.03e-148
<i>Phalaenopsis</i> hybrid	Purple	PLN03170 superfamily	CI30450	Chalcone synthase; Provisional	3-731	1.03e-160
	White	PLN03173	CI30449	Chalcone synthase; Provisional	639-722	3.87e-09
		PLN03168	CI30452	Chalcone synthase; Provisional	546-722	1.01e-05
		PLN03170	CI30450	Chalcone synthase; Provisional	2-637	3.74e-139



Figure 5. Amino acid motifs in *CHS* gene. Amino acid motif in purple (A) and white zone (B) of *Dendrobium 'Enobi'*. Motif in purple (C) and white zone (D) of *Phalaenopsis* hybrid from PROSITE analysis

measurement of anthocyanin pigments (Table 3), the concentration of anthocyanin in the purple zone in *Phalaenopsis* hybrid is higher than that in *D. 'Enobi'*. This follows the results of color identification with the RHS color chart, where the *Phalaenopsis* hybrid sample has a darker purple color. When anthocyanin concentrations were compared between the two orchids in the white zone, the results obtained were also the same. This may indicate that the higher the anthocyanin content in flower, the darker the flower color will be, and vice versa. This was supported by Sangadji *et al.* (2017), anthocyanin content from flower petals with higher color intensity have higher levels of anthocyanins. Although *Phalaenopsis* hybrid has a higher anthocyanin content, the difference is not significant. Based on these statements, it can be seen that genus differences are not necessarily related to anthocyanin content in flowers.

The DNA isolation method for purple flowers, which contain anthocyanin, has been modified because the

anthocyanins contain polyphenols that can inhibit DNA amplification (Sityardi and Desrini 2021). CTAB method with modification in this research has been proven to produce a high quantity of DNA genome and PCR amplification based on the band thickness visualized on agarose gel electrophoresis (Figure 2 and 3). The thickness of the DNA band is also influenced by the concentration of DNA contained in the amplification results (Iqbal *et al.* 2016). The difference between *D. 'Enobi'* and *Phalaenopsis* hybrid has been seen in the length of the *CHS* sequence (Figure 4). *D. 'Enobi'* has a longer sequence than the *Phalaenopsis* hybrid based on band visualization on agarose gel (Figure 3). This was due to the differences in the orchid species and genus. The research by Pitakdantham *et al.* (2010) shows that the length of the *CHS* gene sequences in the genera of *Dendrobium* and *Phalaenopsis* was different.

Based on the alignment of both zones in the *D. 'Enobi'* and *Phalaenopsis* hybrid, it was found that most mutations in the *CHS* gene are caused by insertion

and deletion. Several insertions in the white zone of *D. 'Enobi'* and *Phalaenopsis* hybrid were predicted as transposons, but this still needs to be rechecked on the fully isolated *CHS* gene. Some variegated flowers are caused by the insertion of transposons into structural genes associated with anthocyanin biosynthetic pathways (Chen et al. 2014). The role of transposons in causing the variegated phenotype in flowers has been studied in *Petunia integrifolia*, *Petunia axillaris*, *Petunia hybrid*, and *Pharbitis nil* (Japanese morning glory) (Inagaki et al. 1994; Quattrocchio et al. 1999). Apart from the transposon factor, variegated flowers are usually caused by the presence of a group of colored cells derived from a single ancestral cell (ancestor) which has a somatic mutation from recessive white to a revertant pigmented allele (allele that undergoes a mutation in the form of reversion or reverse mutation) (Chen et al. 2014; Yuwono 2019).

Based on the analysis of conserved domain proteins (Table 5), we found that most of the PLN superfamily was detected in the *CHS* gene sequence. PLN superfamily encoding chalcone synthase is a conserved *CHS* gene domain found in various plant species. This protein is located in the vacuole membranes, endoplasmic reticulum, and plant cell nucleus. *CHS* encodes chalcone synthase, a key enzyme involved in flavonoid biosynthesis. This protein is required to accumulate purple anthocyanins in plant organs such as roots, stems, leaves, and flowers and is also involved in regulating auxin transport and modulation of root gravitropism (NCBI 2022a, 2022b). The difference in the location and type of conserved domain protein and several mutation points located in the conserved domain protein PLN superfamily might influence differences in the structure of the *CHS* gene in the purple and white zones. It has an impact on the anthocyanin biosynthesis pathway. Phenotypic differences based on the data obtained can be seen from the significant differences in anthocyanin content in purple and white zone flowers.

Based on the analysis of amino acid motifs (Figure 5), both *D. 'Enobi'* and *Phalaenopsis* hybrid consist of chalcone synthase (*CHS*) (EC 2.3.1.74) and stilbene synthases (*STS*) (formerly known as resveratrol synthase). *CHS* and *STS* are enzymes that are commonly found in plants. *CHS* is an important enzyme in flavonoid biosynthesis, and *STS* is a key enzyme in stilbene-type phytoalexin biosynthesis. Both enzymes catalyze the addition of three molecules of malonyl-CoA to the starter CoA ester (an example is 4-coumaroyl-CoA), producing chalcone (with

CHS) or stilbene (with *STS*). *CHS* and *STS* are proteins with about 390 amino acid residues. The conserved cysteine residue is located in the center of this protein. This conserved cysteine residue is essential for the catalytic activity of both enzymes and may represent the binding site for the 4-coumaroyl-CoA group. The region around the active site of this residue is highly conserved (Schröder 1990; Lanz et al. 1991).

Based on the amino acid motifs (Figure 5), both orchids in the purple zone have longer amino acid sequences than in the white zone. The amino acid motif CHALCONE_SYNTH (chalcone and stilbene synthases active site) in the white zone in both orchids is located earlier than in the purple zone. The instability of the number of amino acids may be due to the insertion or deletion of nucleotide bases (Table 6) and the difference in the location of the amino acid motifs in the purple and white zones of *D. 'Enobi'* and *Phalaenopsis* hybrid is due to the mutation in the white zone and the difference in genus level. It could impact the regulation of anthocyanin biosynthetic pathways that might affect the absence of anthocyanin pigment in the white zone.

It can be concluded that the variegated tepals (petals and sepals) appeared very early in the buds of both *D. 'Enobi'* and *Phalaenopsis* hybrid with variegated flowers. *D. 'Enobi'* with variegated flower has a wider white area than purple, while *Phalaenopsis* hybrid has a wider purple area than white. There are some differences in the structure of the *CHS* gene in the purple and white zones of *D. 'Enobi'* and *Phalaenopsis* hybrid. The instability of the nucleotide numbers due to the presence of mutations, including eight deletions, nine insertions, and five substitutions at several points in the white zone at the conserved domain protein PLN superfamily, may cause changes in the structure of the *CHS* gene in the white zone which have an impact on the absence of anthocyanin pigments. Based on the anthocyanin content analysis of two orchids from different genera, the difference in the genus is not directly related to the differences in anthocyanin content in the flowers.

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References

- Akhar, F.K., Bagheri, A.R., Moshtaghi, N., 2016. Analysis of chalcone synthase and chalcone isomerase gene expression in pigment production pathway at different flower colors of *Petunia hybrida*. *Journal of Cell and Molecular Research*. 8, 8-14.
- Bao, W., Qu, Y., Shan, X. and Wan, Y., 2016. Screening and validation of housekeeping genes of the root and cotyledon of *Cunninghamia lanceolata* under abiotic stresses by using quantitative real-time PCR. *International journal of molecular sciences*. 17, 1198. <https://doi.org/10.3390/ijms17081198>
- Chen, Y., Mao, Y., Liu, H., Yu, F., Li, S., Yin, T., 2014. Transcriptome analysis of differentially expressed genes relevant to variegation in peach flowers. *PLoS ONE*. 9, 1-9. <https://doi.org/10.1371/journal.pone.0090842>
- Comber, J.B., 1990. *Orchids of Java*. The Royal Botanic Garden Kew, London.
- Dao, T.T.H., Linthorst, H.J.M., Verpoorte, R., 2011. Chalcone synthase and its functions in plant resistance. *Phytochem Rev*. 10, 397-412. <https://doi.org/10.1007/s11101-011-9211-7>
- Fukusaki, E.I., Kawasaki, K., Kajiyama, S.I., An, C.I., Suzuki, K., Tanaka, Y., Kobayashi, A., 2004. Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference. *J. Biotechnol.* 111, 229-240. <https://doi.org/10.1016/j.jbiotec.2004.02.019>
- Han, Y.Y., Ming, F., Wang, J.W., Wen, J.G., Ye, M.M., Shen, D.L., 2006. Cloning and characterization of a novel chalcone synthase gene from *Phalaenopsis hybrida* orchid flowers 1. *Russ. J. Plant Physiol.* 53, 223-230. <https://doi.org/10.1134/S1021443706020129>
- Hermawati, Y., 2016. Influence of citric acid concentrate to characteristic of teak leaf anthocyanin extract with its stability test in ice cream. In: *Prosiding Seminar Nasional Pendidikan Biologi*. Malang: Universitas Muhammadiyah Malang, pp. 303.
- Inagaki, Y., Hisatomi, Y., Suzuki, T., Kasahara, K., Iida, S., 1994. Isolation of a Suppressor-mutator/Enhancer-like transposable element, Tpn1, from Japanese morning glory bearing variegated flowers. *Plant Cell*. 6, 375-383. <https://doi.org/10.1105/tpc.6.3.375>
- Iqbal, M., Buwono, I.D., Kurniawati, N., 2016. Analisis perbandingan metode isolasi DNA untuk deteksi White Spot Syndrome Virus (WSSV) pada udang Vaname (*Litopenaeus vannamei*). *Jurnal Perikanan Kelautan*. 7, 1.
- Lanz, T., Tropf, S., Marner, F.J., Schröder, J., Schröder, G., 1991. The role of cysteines in polyketide synthases. Site-directed mutagenesis of resveratrol and chalcone synthases, two key enzymes in different plant-specific pathways. *J. Biol. Chem.* 266, 9971-9976. [https://doi.org/10.1016/S0021-9258\(18\)92914-5](https://doi.org/10.1016/S0021-9258(18)92914-5)
- Liang, S., 2017. *Comprehensive Remote Sensing*. Elsevier, USA.
- Lim, S.H., Kim, D.H., Jung, J.A. and Lee, J.Y., 2021. Alternative splicing of the basic helix-loop-helix transcription factor gene CmbHLH2 affects anthocyanin biosynthesis in ray florets of *chrysanthemum (Chrysanthemum morifolium)*. *Front. Plant Sci.* 12, 669315. <https://doi.org/10.3389/fpls.2021.669315>
- Ma, Y., Xu, X., Zhang, N., Guo, J., Zang, D., 2017. cDNA cloning and expression analysis of the chalcone synthases (CHS) in *Osmanthus fragrans*. *Am. J. Mol. Biol.* 7, 41. <https://doi.org/10.4236/ajmb.2017.71004>
- Murray, M.G., Thompson, W., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8, 4321-4326. <https://doi.org/10.1093/nar/8.19.4321>
- Mursyanti, E., Purwantoro, A., Moeljopawiro, S., Semiarti, E., 2016. Micropropagation of mini orchid hybrid *Phalaenopsis "Sogo Vivien"*. *J. Tropical Biodiversity Biotechnology*. 1, 45-53. <https://doi.org/10.22146/jtbb.12933>
- Nakatsuka, T., Mishiba, K.I., Abe, Y., Kubota, A., Kakizaki, Y., Yamamura, S., Nishihara, M., 2008. Flower color modification of gentian plants by RNAi-mediated gene silencing. *Plant Biotechnol. J.* 25, 61-68. <https://doi.org/10.5511/plantbiotechnology.25.61>
- NCBI, 2022a. TT4 Chalcone and stilbene synthase family protein [*Arabidopsis thaliana* (thalecress)]. Available at: <https://www.ncbi.nlm.nih.gov/gene/831241>. [Date accessed: 30 July 2022]
- NCBI, 2022b. LOC110018302 chalcone synthase 8 [*Phalaenopsis equestris*]. Available at: <https://www.ncbi.nlm.nih.gov/gene/110018302>. [Date accessed: 25 July 2022]
- Nikmah, N., Purwestri, Y.A. and Semiarti, E., 2021. Structure analysis of genes involved in flower color formation of the orchid *Spathoglottis plicata* Blume. *International Symposium on Tropical and Subtropical Ornamentals*, 1334, 143-152. <https://doi.org/10.17660/ActaHortic.2022.1334.18>
- Nugroho, K., Terryana, R.T., Lestari, P., 2015. Optimasi metode isolasi DNA pada *Jatropha* spp. *Jurnal Agroteknologi*. 5, 16. <https://doi.org/10.24014/ja.v5i2.1350>
- Pitakdantham, W., Sutabutra, T., Chiemombat, P., Pitaksutheepong, C., 2010. Isolation and characterization of chalcone synthase gene isolated from *Dendrobium Sonia Earsakul*. *PJBS*. 13, 1000-1005. <https://doi.org/10.3923/pjbs.2010.1000.1005>
- Post, P.C., Schlautman, M.A., 2020. Measuring camellia petal color using a portable color sensor. *Horticulturae*. 6, 53. <https://doi.org/10.3390/horticulturae6030053>
- Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., Koes, R., 1999. Molecular analysis of the anthocyanin2 gene of *Petunia* and its role in the evolution of flower color. *Plant Cell*. 11, 1433-1444. <https://doi.org/10.1105/tpc.11.8.1433>
- Sangadji, I., Rijal, M., Kusuma, Y.A., 2017. Kandungan antosianin di dalam mahkota bunga beberapa tanaman hias. *Biosel: Biology Science and Education*. 6, 118-128. <https://doi.org/10.33477/bs.v6i2.163>
- Schröder, G., 1990. Stilbene and chalcone synthases: related enzymes with key functions in plant-specific pathways. *Zeitschrift für Naturforschung C*. 45, 1-8.
- Sityardi, F.S., Desrini, S., 2021. Scoping review: potential of orchid as an antifungal. *Indonesian Journal of Pharmacology and Therapy*. 2, 124.
- Suzuki, K.I., Xue, H.M., Tanaka, Y., Fukui, Y., Fukuchi-Mizutani, M., Murakami, Y., Katsumoto, Y., Tsuda, S., Kusumi, T., 2000. Flower color modifications of *Torenia hybrida* by cosuppression of anthocyanin biosynthesis genes. *Mol. Breed.* 6, 239-246. <https://doi.org/10.1023/A:1009678514695>
- Wang, D., Fu, A., 2016. The plastid terminal oxidase is a key factor balancing the redox state of thylakoid membrane, in: Lin, C., Luan, S. (Eds.), *The Enzymes*. Academic Press, Cambridge, pp. 143-171. <https://doi.org/10.1016/bs.enz.2016.09.002>
- Yuwono, T., 2019. *Biotehnologi Pertanian*. UGM Press, Sleman.
- Zhao, D., Tao, J., Han, C. and Ge, J., 2012. An actin gene as the internal control for gene expression analysis in herbaceous peony (*Paeonia lactiflora* Pall.). *African Journal of Agricultural Research*. 7, 2153-2159. <https://academicjournals.org/journal/AJAR/article-full-text-pdf/2F3EE7A39052>