Morphological and Molecular Characterization of Variegated-Leaf Pattern in *Dendrobium* 'Burana Green' Based on the Structure of *VAR2* Gene

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Abstract. Some plants developed variegated pattern on their leaves, where the leaves color separated to green part that indicate normal chloroplasts and white part that indicates abnormal chloroplast. *Dendrobium* 'Burana Green' is a hybrid orchid that has variegated phenotype on its leaves, where the middle part is green and surrounded by white edges. There are several factors that can cause variegated phenotypes such as transposon activity, genetic mutations linked to pigment degradation in the nucleus and plastids, and differences in pigment distribution. This study aims to investigate the variegated phenotype of *D.* 'Burana Green' through morphology, anatomy, and molecular analysis specifically on *VAR2*. Our observation showed that variegated phenotype already occured from early leaf development. The ratio between green and white part of the leaf also gradually changing with the green part eventually dominates the leaf. We also found no stomata on the white part. The chloroplast number and total chlorophyll content in the green part are significantly higher. Twenty seven mutations were detected on the *VAR2* isolated from the white part which suspected to contribute in variegated leaves phenotype.

Keywords: Anatomy-Molecular · Chlorophyll · Dendrobium 'Burana Green' · Variegated-leaf · VAR2 gene

1 Introduction

Variegated plants are plants that have color variations in leaf or flower organs. Variegation is characterized by the presence of discrete signs of various colors on organ or organism, in the form of stripes, spots, or streaks of white, cream, yellow, or other

J. Sumantyo et al. (Eds.): ICoSIA 2022, ABSR 29, pp. 524–539, 2023.
https://doi.org/10.2991/978-94-6463-122-7_50

color. Variegation in pigmented leaves originated from the formation of sectors containing normal chloroplasts or abnormal plastids, or other non-chlorophyll pigments (e.g., anthocyanins) [1, 2]. Leaf variegation can be divided into two types, namely chemical type (pigment) caused by pigment and physical (structural) type caused by the optical properties of leaf structure [3, 4]. The mechanism of leaf variegation can be divided into four types, the first is the chlorophyll type which is characterized by a deficiency of chlorophyll in the white sector of the leaf. Second is the air space type which is characterized by the presence of air spaces under the epidermis. Third, epidermis type which characterized based on the specific morphology of the adaxial epidermal cells. Fourth, pigment type which characterized by the presence of non-photosynthetic pigments covering the green color of the leaves [3, 5]. Five species have a leaf variegation mechanism of air space type, namely *Begonia aptera* Blume, *Nervilia nipponica* Makino, *Oxalis acetosella* subsp. *Griffithii*, *Smilax bracteata* C. Presl subsp. *Verruculosa*, and *Valeriana hsuii*. The chlorophyll type was found in *Selaginella picta*, the chloroplast type was found in *Paphiopedilum concolor* Pfitzer, and the epidermal type was found in *Oxalis corymbosa* DC [3]. Based on this, the airspace type is the most common type in the leaf variegation mechanism.

Leaf variegation can also occur when there are genotype differences in the green sector cell and the white sector cell that lack pigment. Cells in the green sector have a normal genotype with functional chloroplasts, while in the pigment-deficient sector consist of mutant cells with abnormal [6]. The emergence of variegation can also occur due to mutations in the *VAR1* and *VAR2* genes which will affect the process of chloroplast formation. Low temperature and high light are factors can trigger the activity of the *VAR1* and *VAR2* genes. Gene used as indicators are chloroplast-forming genes such as *VARIEGATED 2 (VAR2)*, which is encoded by FtsH. The presence of FtsH significantly affects the phenotype of the leaves. If there is a change in amino acids in areas that are important for forming chloroplasts, the green color of the leaves cannot be created and causes variegated plants. The mutations can produce patterns in leaf color, namely green, white or yellow, thus increasing its ornamental value. White leaves are caused by heteroplasty or shrinkage the lamella size structure on the plastids [7]. The existence of variegation in leaves have important biological functions, especially in adaptation to the environment. Variegation on leaves of *Hydrophyllum virginianum* can help avoid threats from herbivores [5]. *Cypripedium fargesii* has 'mildew spots' like fungi on its leaves which are used to attract insects to help pollinate [8].

Variegated-leaf is rarely found in the Orchidaceae family, although they are commonly found in *Araceae, Araliaceae, Asparagaceae, Bromeliaceae, Euphorbiaceae* and *Myrsinaceae* [1]. Some orchids that known to have motifs appeared on their leaves include *Paphiopedilum, Oeceoclades maculata, Phalaenopsis aphrodite* subsp. *formosana*, and *Phalaenopsis* 'Sogo Vivien' [2, 9, 10]. Although not all individual from those species possesed said phenotype. This study used a mutant orchid with variegated-leaves phenotype *D.* 'Burana Green' which is a hybrid orchid from *Dendrobium* 'Chittraphong' × *Dendrobium* 'Yong Kok Wah' [11].

Dendrobium 'Burana Green' has a unique phenotype that shows on its leaf coloration separated in green and white parts. This trait is known as variegation and hold great ornamental value among enthusiasts. This variegated leaf color suggests mutational

events occured in the pigment-related gene like in *P. aphrodite* subsp. *formosana* [2]. We performed this research to identify molecular changes between the green and white part of the leaf using a chloroplast gene *VAR2*. Identification of these muations is necessary to help understand leaf patterning on orchids. It could also be used to induce variegation artificially in orchid breeding program through various genetic manipulation tools such as CRISPR/*Cas9*.

2 Materials and Methods

In this research, the leaves of *D.* 'Burana Green' orchid plant with variegated patterns on the margin were used. The age of the plants were 4 years old. *D.* 'Burana Green' orchid plant obtained from Keiki Orchid Nursery, Yogyakarta. Orchids were planted on *Sphagnum* moss media, ferns, and wood charcoal media. Plants are grown in the green house of Faculty of Biology, University of Gadjah Mada with natural lights. This research was conducted from August 2021 to April 2022 at Biotechnology Laboratory, Faculty of Biology, Universitas Gadjah Mada.

2.1 Observation of Anatomical Structures with Paraffin Embedding Method

The anatomical character of the third leaf *D.* 'Burana Green' from the shoot was observed using the paraffin embedding method. Sample preparation of leaf anatomy was conducted by using the paraffin embedding method, according to the protocol Ruzin (1999) with modifications. The leaf was divided, then cut at the white and green parts (0.5 cm × 1 cm), after that the samples were fixed with FAA solution (formalin: 96% ethanol: glacial acetic acid: aquadest (2:10:1:7)) for 12 h. The fixative was discarded and then replaced with a 50% ethanol solution for 1 h. After that, it was replaced with 70% ethanol. In the dehydration process, the sample was soaked in a graded ethanol solution of 80%, 90%, and 96% for 1 h. Then, the sample was soaked in an absolute ethanol solution for 12 h. During the dealcoholization process, the samples were soaked in xylol: absolute ethanol (1:3), (1:1), and (3:1) for 15 min. After that, the pure xylol 1 and 2 were soaked for 10 min. In the embedding process with paraffin, the sample was put into a petri dish containing paraffin: xylol (1:1), which was melted on a hot plate at 70 °C and transferred to pure paraffin 1, 2, 3 for 10 min. Then, the sample was placed on a paper block containing pure paraffin and dried. The paraffin block containing sample was attached to the holder and cut using a rotary microtome at a thickness of 12 μm. The sample was placed in object glass on a hot plate at of 30 °C. During the deparaffination process, paraffin is removed by the object glass placed on a hot plate at 65 °C. After melting, it was soaked in a pure xylol solution for 15 min. Then, soaked with xylol: ethanol (3:1), (1:1), (1:3), absolute ethanol, 96%, 90%, 80%, 70%, 50%, 25% ethanol, and aquadest for 1 min. Then, soaked in 1% safranin for 30 min, rinsed with aquadest, and dipped in 25%, 50%, 70%, 80%, 90%, 96% ethanol, absolute ethanol, xylol: ethanol (1:3), (1:1), (3:1), pure xylol, for 3–5 s. After that, it was mounted with Canada balsam and placed on a hotplate at 30 °C to dry. The samples were observed under a light microscope (100x).

2.2 Chlorophyll Concentration in the Green and White Parts

The third leaf of variegated-*D.* 'Burana Green' from the shoot was taken to measure chlorophyll content [12]. The white and green parts of the leaf were separated to measure the chlorophyll content in each part. The sample (15 mg) was mashed with a micropestle in a microtube, then mixed with 1.5 ml cold methanol. The samples were centrifuged at 2.500 rpm for 10 min and the supernatant was transferred to a cuvette. Absorbance at the wavelength of 665.2, 652, and 480 nm was measured using a UV-Vis Vis spectrophotometer. The content of total chlorophyll are calculated by the formula [12]:

$$\left[\text{Chlorophyll a}\right] = (16.29 \times \text{A665, 2}) - (8.54 \times \text{A652})$$
$$\left[\text{Chlorophyll b}\right] = (30.66 \times \text{A652}) - (13.58 \times \text{A665.2})$$
$$\left[\text{Total Chlorophyll}\right] = (\text{Chlorophyll a}) + (\text{Chlorophyll b})$$

2.3 DNA Isolation and Purification

Orchid gDNA isolation was carried out using a modified Murray and Thompson method (1980). The sample used in the isolation was the third leaf from the shoot of the variegated *D.* 'Burana Green'. The variegated leaf was separated to green and white parts. The sample was weighed 100 mg and grinded in a sterile microtube. The sample was mixed with 500 μl CTAB 3% + PVP 1% solution, then incubated in a waterbath at 65 °C for 30 min. After that, 500 μL (1 volume) of chloroform was added to the sample and mixed by inversion at room temperature. The samples were placed on a shaker at 100 rpm for 30 min and centrifuged (Thermo Fisher Scientific, USA) at 12.000 rpm for 10 min at room temperature. The supernatant was taken and transferred to a new 1.5 ml microtube. Then, 2 volumes of 100% cold ethanol and 0.2 volume of Na acetate (3M, pH 5.2) was added to the supernatant. Samples were incubated at -20 °C for 60 min. The sample was centrifuged at 14.000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended with 300 μl cold 70% ethanol and centrifuged at 10.000 rpm for 5 min. The supernatant was discarded again and the pellet in the tube was air-dried at room temperature for 30 min. After that, the pellets were dissolved in 30 μl of 10T 0.1E buffer pH 7.6, incubated in a waterbath at 60 °C for 5 min, and the samples were stored in the freezer (-20 °C).

The orchid gDNA purification was conducted by using PCI (Phenol: Chloroform: Isoamyl Alcohol, Sigma Aldrich, Germany) with a ratio of 25:24:1. Isolated sample was put into a 1.5 mL microtube containing a mixture of Phenol, Chloroform, Isoamyl Alcohol. The sample was shaked for 15 min and centrifuged at 10.000 rpm for 10 min at room temperature. After that, an *aqueous phase layer* will be formed and transferred to a new microtube. Then, 2 volumes of 100% cold ethanol and 0.2 volume of Na acetate (3M, pH 5.2) was added to the supernatant. The samples were inverted 6–8 times and incubated at -20 °C for 30 min. The sample was centrifuged at 14.000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended with 300 μl cold ethanol and centrifuged at 10.000 rpm for 5 min. The supernatant was discarded again and the pellet in the microtube was air-dried at room temperature for 30 min. After that, the

Table 1. Specific primer *VAR2* and *ACTIN* genes

Primer	Sequences	TM (°C)	Amplicon (bp)
VAR2 F ***VAR R***	5'GGCTGCCTCTTCTCCATGTC3' 5'TGCTTTGCTTCATCAACCCC3'	61	723
ACTIN F ***ACTIN R***	5'GTATTCCCTAGCATTGTTGGT3' 5'CAGAGTGAGAATACCTCGTTTG3'	52	114

pellets were dissolved in 25 µl of 10T 0.1E buffer pH 7.6, incubated in a water bath at 60 °C for 5 min, and the samples were stored in the freezer (-20 °C). The purified orchid's gDNA was checked using 0.7% agarose gel electrophoresis (Sigma®, Japan).

2.4 Amplification on *VAR2* and *ACT* Genes

Orchid gDNA amplification for structural analysis of chloroplast gene~~s~~ was carried out with specific primers (Table 1) for the *VAR2* gene using the Bioline Kit. In addition, the gene was used as an internal control. The amplified DNA was checked using 0.7% agarose gel electrophoresis. Each PCR reaction in each sample consisted of 12.5 µl My Taq™ HS Redmix (Bioline, UK); 1 µl forward primer (10 M); 1 µL reverse primer (10 M); 1 µl DNA template (90 ng/µl); and 9.5 µl Nuclease Free Water for a total of 25 µL solution. The PCR reaction process was carried out in 35 cycles with pre-denatured conditions of 95 °C. for 1 min; Denaturation 95 °C. for 15 s; Annealing for *ACT* at 51 °C. for 15 s and *VAR2* at 54 °C for 15 s; 72 °C. extension for 10 s. The PCR products were visualized with 0.7% agarose electrophoresis gel type II in 1% TBE *buffer* with 2.5 µl EtBr staining and DNA markers 1 kb and 100 bp with a voltage of 100 V for 20 min. Then, the result was visualized with a UV transilluminator and DNA bands were documented with a digital camera. The PCR products were purified and sequenced by Sanger sequencing method.

2.5 Bionformatics Analysis

Anatomical data of variegated orchid leaves were analyzed descriptively and statistically using MS Excel 2016. Chlorophyll content data of variegated orchid leaves were analyzed using a histogram created in MS Excel 2016 and statistically using T-Test with Statistical Package for the Social Sciences (SPSS) 15.0 software. Detection of the gene encoding the chlorophyll pigment in variegated orchids was analyzed descriptively. The DNA sequence alignment of the *VAR2* gene was analyzed using ApE-A plasmid Editor v3.1.1 [13]. The conserved domain was determined using cdd stucture at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The amino acid was determined using Translate at Expasy (https://www.expasy.org/resources/translate) and Protein Blast at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [14]. Reconstruction of *a phylogenetic tree* using BLAST at NCBI (blast.ncbi.nlm.nih.gov) to see similarity and MEGA11 software (Maximum Likelihood) [15, 16].

Fig. 1. Variegated pattern on *D.* 'Burana Green' leaves. The number under the leaf indicates the age of the leaf in days. Bar: 1 cm.

3 Results and Discussions

The leaf is one of the most important organs for plants. Leaves are closely related to process photosynthesis, plant metabolism, and nutrient distribution. The green color in normal leaves is due to the presence of chloroplasts. Some leaves have white parts indicating chloroplast-forming abnormalities or called variegation [2]. It is a discrete sign of various colors on an organ or organism. The growth of *D.* 'Burana Green' leaves (Fig. 1) shows the variegated pattern that appears is marginal variegation. The leaves have formed variegated pattern at the leaf margins since the bud. On the first day, the white part on the leaves is more dominant than the green part. After some time, the green part on the leaf will get bigger, while the white part tends to remain. Marginal variegation also occurs in the leaves of *Dracaena surculose*, where its leaves have similar pattern as the leaves of *D.* 'Burana Green'. It is just that the green part in *Dracaena surculose* is on the marginal, while the white part of *D.* 'Burana Green' is in the middle [1]. The mechanism of leaf variegation can be divided into four types. The first is chlorophyll type, characterized by a deficiency of chlorophyll in the white part of the leaf. The second is the air space type, characterized by the presence of air spaces under the epidermis. The third is the epidermis type, characterized by the specific morphology of the adaxial epidermal cells. The fourth is pigment type, characterized by the presence of non-photosynthetic pigments covering the green color of the leaves [3, 5].

3.1 Comparative Anatomy of *D.* 'Burana Green' Leaf in the Green and White Parts

The adaxial and abaxial epidermis of the leaf lamina on *D.* 'Burana Green' is polygonal (Fig. 2). The size of the adaxial epidermis in the green part *D.* 'Burana Green' is an average of 5 epidermal cells, which has a length ±4.65 μm and width ±2.72 μm. The size of the abaxial epidermis is an average of 5 epidermal cells which has a length ± 5 .94 μm and width ±2.43 μm. The size of the adaxial epidermis in the white part *D.* 'Burana Green' is an average of 5 epidermal cells, which has a length ±3.38 μm and width ±2.16 μm. The abaxial epidermis size is an average of 5 epidermal cells, which has a length ±4.29 μm and width ±2.31 μm. Mesophyll parenchyma of the lamina *D.* 'Burana Green' in the green and white parts have the same character, namely mesophyll with a homogeneous type. The stomata position of *D.* 'Burana Green' (Fig. 2C) based on the location of stoma in the green part of leaf surface is amphistomatic because

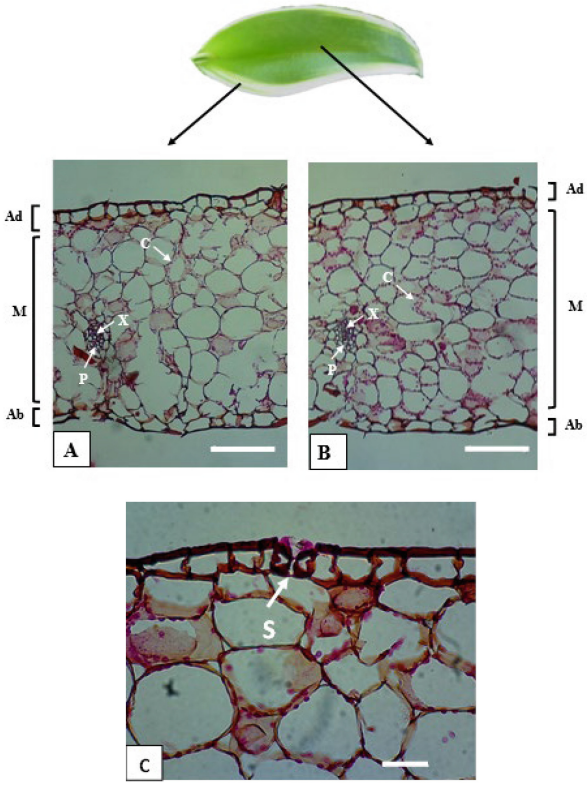


Fig. 2. Leaf anatomy of *D.* 'Burana Green' lamina by paraffin embedding method. (A) White part in variegated-leaves; (B) Green part in variegated-leaves; (C) Anomocytic stomata. The observed cell organs are adaxial epidermis (Ad), mesophyll (M), abaxial epidermis (Ab), chloroplast (C), xylem (X), phloem (P), and stomata (S). Bars A-C: 30 μm.

it's found on the abaxial and adaxial side and the stomata type is anomocytic because the guard cell is surrounded by several cells that are the same shape and size as other epidermal cells [17]. In the white part of leaf, there were no stomata found. This can happen because the process of photosynthesis in the white part does not run well due to the lack of chlorophyll present. It also affects the number of presences, size, density, and stomata distance. The larger size of the stomata will accelerate the photosynthesis process because it affects the absorption of CO_2 [18]. The higher stomata density will accelerate the transpiration process because there will be more pores to accelerate evaporation [18, 19]. The distance between the stomata will also affect the photosynthetic activity. If the stomata are too close, the evaporation from one stomata will hinder the other [20]. The function of stomata is very important for plants, that is the absorption of CO_2 in the photosynthesis process and as a barrier to water loss through transpiration [21]. Stomata that are not found in the white part is one of the effect of variegation on the leaves.

The number of chloroplast in *D.* 'Burana Green' with variegated-leaves also shows different numbers in the green and white parts (Table 2). The number of chloroplasts in

Table 2. Comparison the number of chloroplast in the green and the white parts of *D.* 'Burana Green'

Leaf	Chloroplast	
	Number per cell	Diamater (μm)
Green part	16.6 ± 3.1*	6.99 ± 0.92
White part	3.6 ± 2.17	6.1 ± 0.64

* $p < 0.05$ (significant) compare between white and green zone

the green part is four times more than in the white part. The number and distribution of chloroplasts in leaves can directly affect leaf color. Chloroplasts that have not function will cause the leaves to lose their green color and the photosynthesis process will be hampered. The level expression of key genes involved in chlorophyll biosynthesis, chloroplast development, and chloroplast division determines the color of the mutant leaves. The high green color of the leaves are influenced by the number of chloroplasts in the leaves [22, 23]. In the leaves of *D.* 'Burana Green' in green and white parts were also found chloroplast sheath cells, xylem, and phloem. Xylem and phloem in the green zone are more visible and larger, while the white part looks smaller and piled up (Fig. 2). This can happen because it is related to the photosynthesis process, which is better in the green leaf part than in the white leaf part.

3.2 Analysis of Chlorophyll Content in *D.* 'Burana Green'

Chlorophyll is a green pigment commonly found in chloroplasts. Chlorophyll content in leaves is very important on photosynthetic reactions [24]. The Optimal chlorophyll content will accelerate the process of photosynthesis and will greatly affect the results of photosynthesis.

The chlorophyll content between the green and white part of *D.* 'Burana Green' leaves were significantly different (Table 3). This results validates correlation between leaf color and chlorophyll content. The lower chlorophyll content in the white part is probably due to the inactivation of genes associated with chlorophyll degradation [25]. Micronutrient deficiencies, such as Fe deficiency or lack of sunlight can also caused low chlorophyll content [26]. This result with researches in *Cephalanthera damasonium, Hedera helix, Ardisia pusilla,* and *Scindapsus aureus* [24, 27]. Based on the measurement of chlorophyll content, the variegation of *D.* 'Burana Green' are fall in the chlorophyll type. This is due to the significant difference in chlorophyll content between the green and white parts. The difference in the distribution of functional chloroplasts between the white and green parts can lead to a variegated pattern on the leaves. There were no other color patterns besides the green and white color and no air space between the epidermis and palisade cells or between palisade cells in the white part.

Table 3. Concentration of chlorophyll in the variegated-leaves of D. 'Burana Green'

Leaf	Chlorophyll Contents (μg/ml)
Green part	12.94 ± 4.22*
White part	1.73 ± 0.32

* $p < 0.05$ (significant) compare between white and green zone

3.3 Characterization of The *VAR2* Gene-Chlorophyll Pigment Encoding

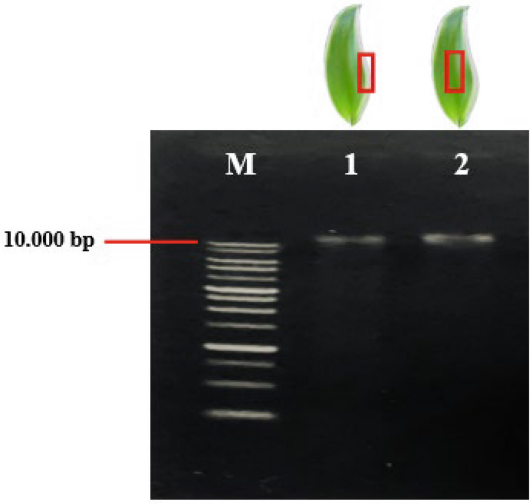
The results in Fig. 3A show that the genomic DNA from *D.* 'Burana Green' leaves were isolated and can be detected in good condition, and can be used as template for PCR analysis by using *VAR2* primers. The *VAR2* and *ACT* genes were successfully amplified from genomic DNA of *D.* 'Burana Green' (Fig. 3B). *ACT* is a housekeeping gene and commonly used as an internal control for PCR [28]. The results show a single band produced by the *VAR2* primers from both parts of the leaf. The amplicons were sequenced to reveal the sequence difference among them.

3.4 Mutation in *D.* 'Burana Green' Variegated-Leaf

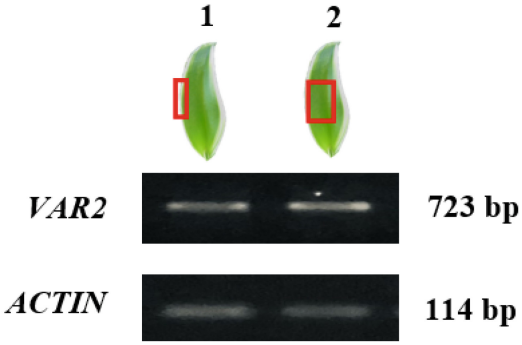
Sequence *alignment* from the *VAR2* gene of *D.* 'Burana Green' (Fig. 4) showed that the amplified length of the sequences in the green and white parts was 727 bp. Based on this alignment, several mutations were found in the white leaf part. These mutations could be one of the factors that cause variegation in leaves. The mutations found in white part *D.* 'Burana Green' are insertions, deletions, and substitutions (Table 4). Insertion is the insertion of a nucleotide into a sequence of nitrogen bases. There are ten nitrogen bases that have been inserted that are 23, 579, and 658 bases with the insertion of base C, the 496, 550, 589, 656, 663, 665 bases with the insertion of base A, the 679 bases with the insertion base T. Deletion is the loss of a nucleotide in the sequence of nitrogen bases. There are ten nitrogenous bases that have been deletion that are 32, 50, 635, 685 bases with the loss of base G, 60, 547, 659 bases with the loss of base T, the 645 bases with the loss of base C, the 632 and 648 bases with loss of base C. Substitutions are classified into transition (Ts) and transversion (Tv). The transition occurs where a purine is replaced by another purine or a pyrimidine by another pyrimidine, while transversion occurs where a purine is replaced by a pyrimidine or vice versa [29]. There are four nitrogenous bases that experience transition from T to C base that are 106, 686, 706 base, and the transition from G to T base, which is 697 base. Meanwhile, three nitrogenous bases experience C to A base transversion, which is the 611 base, the G to T base transversion, which is the 642 base, and the G to C, which is the 707 base. Based on the nucleotide sequence analysis, it can be predicted that the mutations in the white part of *D.* 'Burana Green' leaves are mostly caused by insertions and deletions.

3.5 Amino Acid Motif's in *D.* 'Burana Green' Variegated-Leaf

Mutations can cause changes in the nucleotide structure so that the motif of the amino acid sequence will change. This results in a change in function at the protein level so



(A)



(B)

Fig. 3. Electrophoregram from the leaf of the *D.* 'Burana Green' orchid. (A) gDNA; (B) Detection of *VAR2* and *ACT* genes from gDNA. Both genes were successfully amplified with no observable difference between the green and white parts. Lanes: (1) white part; (2) green part; M: 1 kb DNA ladder (Geneaid, Taiwan).

that green and white parts can be formed in variegated plants. Based on the analysis of protein motifs (Fig. 5) on *D.* 'Burana Green' leaves in the green and white parts, it can be seen that the protein domains found were FtsH protease protein (CHL00176) and FtsH_ext (pfam06480). The number of amino acids formed in the green part is 147 and the white part is 74. The amino acid FtsH protease (CHL00176) was formed in the green part at intervals of 67–214 aa, while the amino acid FtsH_ext (pfam06480) was formed at intervals of 61–140 aa. The amino acid FtsH protease (CHL00176) was formed in the

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Dendrobium 'Burana Green' sec Green.fas from 1 to 727
Alignment to
Dendrobium 'Burana Green' sec Green.fas--  Matches:727; Mismatches:0; Gaps:0; Unattempted:0
Dendrobium 'Burana Green' sec White.fas--  Matches:710; Mismatches:7; Gaps:20; Unattempted:0

                *         *         *         *         *         *         *         *         *
  1>GGCTGCCTCTTCTCCATGTCTC-ACCTCTAATGGGGTATCTAGAAAATATGGAAAAACAATTCTTACCAAGGAAATGTATGGAATGAGTCTAGCTTCCTC>99
  1>GGCTGCCTCTTCTCCATGTCTC-ACCTCTAATGGGGTATCTAGAAAATATGGAAAAACAATTCTTACCAAGGAAATGTATGGAATGAGTCTAGCTTCCTC>99
  1>GGCTGCCTCTTCTCCATGTCTCCACCTCTAATCGGGTATCTAGAAAATATCGAAAAACAACTCTTACCAAGGAAATGTATGGAATGAGTCTAGCTTCCTC>97

    *         *         *         *         *         *         *         *         *         *
100>ATCATGTCTTCCTTCACACAATGTGGTGCCTAAGCCCGTGTCTTTTACTGCCTCTTTGAACCAAAATAAGCTTTCATCAAGAAGAGAATTTCTGAAGCTG>199
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    *         *         *         *         *         *         *         *         *         *
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    *         *         *         *         *         *         *         *         *         *
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298>GGTTTTTGGAATATTTGGACAAGGATAGAGTAAAGAAAGTGGATTTATTTGAGAATGGAACCATAGCAATTGTAGAGGCTGTTTCACCTGAGCTTGGCAA>397

    *         *         *         *         *         *         *         *         *         *
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398>TCGGATTCAGAGAGTGCGTGTGCAGCTTCCAGGATTGAGTCAAGAACTTCTTCAAAAATTAAGAGAAAAAAATATTGATTTTGCAGCTCATAATGCAACA>497

    *         *         *         *         *         *         *         *         *         *
499>GGAAGATTCTGGCTCTCTTTTGTTTAATTTAATCGGGAATTTAGCTTTTCC-ACTCATCCTCATAGGGGGTCTATTCCTTC-TCTCTAGAAG-ATCATCT>595
499>GGAAGATTCTGGCTCTCTTTTGTTTAATTTAATCGGGAATTTAGCTTTTCC-ACTCATCCTCATAGGGGGTCTATTCCTTC-TCTCTAGAAG-ATCATCT>595
498>GGAAGATTCTGGCTCTCTTTTGTTTAATTTAATCGGGAATTTAGCTTTTCCCACTCATCCTCATAGGGGGTCTATTCCTTCCTCTCTAGAAGAATCATCT>596

    *         *         *         *         *         *         *         *         *         *
596>GGTGGTCTTGGAGGACCTGGTGGTCCCGGGTTTCCCCTTGGATTTGGTCAATCCAAAGCC-AA-GTTTC-AG-ATGGAACCTAACAC-TGGTGTGTCACT>690
596>GGTGGTCTTGGAGGACCTGGTGGTCCCGGGTTTCCCCTTGGATTTGGTCAATCCAAAGCC-AA-GTTTC-AG-ATGGAACCTAACAC-TGGTGTGTCACT>690
597>GGTGGTCTTGGAGGAACTGGTGGTCCCGGGTTTCCCCTTCGATTTGGTCATCCAAAGCCAAAGGTTCAAGAATGGAACCTAACACTTGGTGTCCACT>690

            *         *         *
691>TTTGATGGACGTTGCTGGGGGTTGATGAAGCAAAGCA>727
691>TTTGATGGACGTTGCTGGGGGTTGATGAAGCAAAGCA>727
691>TTTGATTGACGTTGCCGGGGGTTGATGAAGCAAAGCA>727
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Fig. 4. Sequence alignment from PCR results of the *D.* 'Burana Green' *VAR2* gene in the green and white parts. There are several mutations detected in the nucleotide sequences of the *VAR2* gene from the white part.

Table 4. Mutations in the white white part of *D.* 'Burana Green' variegated.leaves

Nucleotide	Number of Mutations	Mutation Type
23, 496, 550, 579, 589, 656, 658, 663, 665, 679	10	Insertion
32, 50, 60, 547, 632, 635, 645, 648, 659, 685	10	Deletion
106, 686, 697, 706	4	Substitution (Transition)
611, 642, 707	3	Substitution (Transversion)

white part the interval of 180–254 aa, while the amino acid FtsH_ext (pfam06480) was formed at the interval of 174–253 aa.

FtsH is a member of the FtsH protease superfamily. FtsH is the only membrane-bound ATP-dependent protease universally present in prokaryotes and eukaryotes. FtsH is characterized by zinc metalloprotease [30, 31]. Based on this, it can be seen that the presence of an amino acid motif in the form of FtsH greatly influences the phenotype

of the leaves. FtsH deficiency can cause varicose veins in the leaves. This is due to the inability of the *VAR2* gene due to the disturbed FtsH motif. Changes in amino acids significantly affect leaf color. The green color of the leaves indicates the presence of chloroplasts in the plant, while the white or yellow color indicates the presence of chloroplast-forming abnormalities [1, 2]. If the amino acids are changed in areas that are important for forming chloroplasts, the green color of the leaves cannot be formed and causes variegated plants.

3.6 Phylogenetic Tree in *VAR2* Gene

The result of the phylogenetic analysis for *the VAR2* gene between green and white part of *D.* 'Burana Green' leaf showed that there were 3 group groups consisting of 2 large clades, one small clade, and one outgroup (Fig. 6). Clade I consisted of 5 monocots species, that are *Musa acuminata* subsp. *Malaccensis*, *Zingiber officinale, Dioscorea cayenensis* subsp. *Rotundata, Phoenix dactylifera*, and *Elaeis guineensis.* Clade II consists of 4 species belonging to the *Orchidaceae* family that are *Phalaenopsis equestris, D. catenatum, and D.* 'Burana Green'. The species are united by the number of sequences that have resemblance. The green and white parts of *D.* 'Burana Green' in this clade has the highest bootstrap value of 100. Bootstrap analysis was used to test the validity of the *phylogenetic tree* construction. The results are considered reliable if the bootstrap value is above 90 [32]. Clade III consists of 2 dicot spesies, that are *Cucurbita pepo* and *Nelumbo nucifera.* The species used in *outgroup* is *A. thaliana* because *A. thaliana* is a species that is closely related to the group researched but is less closely related than the relationship between the species researched. Outgroup is essential to add in data analysis to determine root positions and understand the phylogenetic tree's evolution [32]. The results showed that the *VAR2* gene of *D.* 'Burana Green' was closely related to other plants belonging to the Orchidaceae family. Monocot plants will group with other monocotyledonous plants and dicotyledonous plants. Based on the relationship between alignment and phylogenetic tree in the *VAR2* gene (Fig. 4), it can be seen that if there is a change in the length of FtsH domain, it will cause a variegated color pattern on the leaves. Leaf color varies, due to abnormalities in the FtsH domain, which will cause the inability of the chloroplasts to function normally.

4 Summary

D. 'Burana Green' has a chlorophyll-type pattern with white coloration on the margin of the leaves. Histological analysis showed differences in the anatomical structure of the *D.* 'Burana Green' leaves between green and white parts. The green part has larger epidermis compared to the white. Stomata on the green part is determined to be amphistomatic and anomocytic, while no stomata observed on the white part. Xylem and phloem in the green part were more clearly visible and larger, while those in the white part were smaller and piled up. The green part of *D.* 'Burana Green' chloropasts count were 16 per cell with 6.99 ± 0.92 μm in diameter, while the white part chloroplasts count was 3 per cell with 6.1 ± 0.64 μm in diameter. Total chlorophyll contents of *D.* 'Burana Green' in the green part was 12.94 ± 4.22 μg/ml, compared to the white part with 1.73

Fig. 5. Conserved domain in D. 'Burana Green' variegated-leaf. (A) Green part; (B) White part.

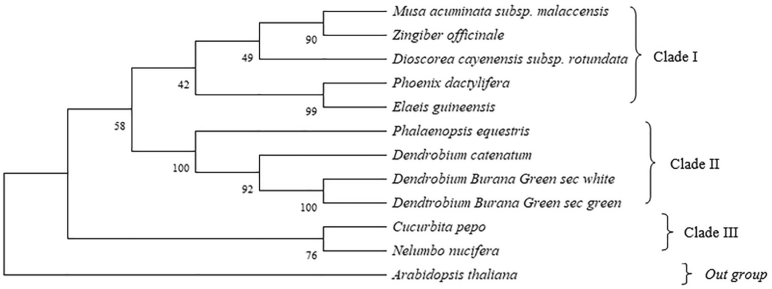

Fig. 6. *VAR2* gene phylogenetic tree in green and white parts of D. 'Burana Green' leaf using Maximum Likelihood. The *VAR2* gene of *Arabidopsis thaliana* was used as an outgroup.

± 0.32 µg/ml. The structure of the *VAR2* gene in the white part of *D.* 'Burana Green' showed 27 mutational. There were 147 amino acids formed in the green part and 74 amino acids formed in the white part.

Acknowledgments. This research was supported by a Research Grant of Final Project Recognition (Rekognisi Tugas Akhir, RTA), Universitas Gadjah Mada 2022 with Contract No: 3550/UN.1.P.III/Dit-Lit/PT.01.05/2022 at the date May 20th–November 20th 2022. The authors would like to thanks Keiki Orchid Nursery for providing *D.* 'Burana Green' variegation orchid mutant plants for this research.

Authors' Contributions. Conceived and designed the experiments: ES, SUP, and FYK. Collected the data: SUP, FYK, and AL. Analyzed the data: SUP. Wrote the paper: SUP and FYK. Conceived of the study, edited the manuscript, and approved the final manuscript: SUP, FYK, AL, MDL, and ES.

References

1. Q. Li, J. Chen, D. B. McConnell, R. J. Henny, A Simple and Effective Method for Quantifying Leaf Variegation, Horttechnology, vol. 17, 2007, pp. 285–287. DOI: https://doi.org/10.21273/HORTTECH.17.3.285
2. C. C. Tsai, Y. Wu, C. Sheue, P. Liao, Y. Chen, S. Li, J. Liu, H. Chang, W. Liu, Y. Ko, Y. Chiang, Molecular Basis Underlying Leaf Variegation of a Moth Orchid Mutant (Phalaenopsis aphrodite subsp. formosana), Front. Plant Sci, vol. 8, 2017, pp. 1–9. DOI: https://doi.org/10.3389/fpls.2017.01333
3. S.H. Pao, L.I.U. Jian-Wei, Y. Jun-Yi, P. Chesson, C.R. Sheue, Uncovering the mechanisms of novel foliar variegation patterns caused by structures and pigments, Taiwania, vol. 66, 2020, pp. 74. DOI: https://doi.org/10.6165/tai.2020.65.74
4. C.R. Sheue, S.H. Pao, L.F. Chien, P. Chesson, C.I. Peng, Natural foliar variegation without costs? The case of Begonia, Ann Bot, vol. 109, 2012, p. 1065-1074. DOI: https://doi.org/10.1093/aob/mcs025
5. J.H. Zhang, J.C. Zeng, X.M. Wang, S.F. Chen, D.C. Albach, H.Q. Li, A revised classification of leaf variegation types, Flora, vol. 272, 2020, pp. 151703. DOI: https://doi.org/10.1016/j.flora.2020.151703
6. M. Li, G. Hensel, M. Mascher, M. Melzer, N. Budhagatapalli, T. Rutten, A. Himmelbach, S. Beier, V. Korzun, J. Kumlehn, T. Börner, N. Stein, Leaf Variegation and Impaired Chloroplast Development Caused by a Truncated CCT Domain Gene in albostrians Barley, The plant cell, vol. 1, 2019, pp. 1430-1445. DOI: https://doi.org/10.1105/tpc.19.00132
7. F. Yu, S. Park, X. Liu, A. Foudree, A. Fu, M. Powikrowska, A. Khrouchtchova, P. E. Jensen, J. N. Kriger, G.R. Gray, S.R. Rodermel, SUPPRESSOR OF VARIEGATION4, a new var2 suppressor locus, encodes a pioneer protein that is required for chloroplast biogenesis, Mol. Plant, vo. 2, 2011, pp. 229-240. DOI: https://doi.org/10.1093/mp/ssq074
8. Z.X. Ren, D.Z. Li, P. Bernhardt, H. Wang, Flowers of Cypripedium fargesii (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus-infected foliage, Proc. Natl. Acad. Sci. U.S.A, vol. 108, 2011, pp. 7478–7480. DOI: https://doi.org/10.1073/pnas.1103384108
9. F.F.V.A. Barberena, Mutation in focus: first record of a wild chimeric individual for the subtribe Laeliinae (Orchidaceae), Acta bot. Bras, vol. 35, 2021, pp. 491-494. DOI: https://doi.org/10.1590/0102-33062020abb0422
10. E. Mursyanti, A. Purwantoro, S. Moeljopawiro, Semiarti, Induction of Somatic Embryogenesis through Overexpression of ATRKD4 Genes in Phalaenopsis “Sogo Vivien”, Indones. J. Biotechnol, vol. 1, 2015, pp. 42–53. DOI: https://doi.org/10.22146/ijbiotech.15276
11. Information on https://orchidroots.org/detail/information/?pid=100090213&role=pub
12. H. Croft, J.M. Chen, X. Luo, P. Bartlett, B. Chen, R.M. Staebler, Leaf chlorophyll content as a proxy for leaf photosynthetic capacity, Glob. Change Biol, vol. 23, 2017, pp. 3513-3524. DOI: https://doi.org/10.1111/gcb.13599
13. M.W. Davis, E.M. Jorgensen, ApE, a plasmid editor: a freely available DNA manipulation and visualization program, Front. Bioinfom, vol. 2, 2022, pp.1-15. DOI: https://doi.org/10.3389/fbinf.2022.818619
14. S. Duvaud, C. Gabella, F. Lisacek, H. Stockinger, V. Ioannidis, C. Durinx, C, Expasy, the Swiss Bioinformatics Resource Portal, as designed by its users, Nucleic Acids Res, vol. 49, 2021, pp. 1-12. DOI: https://doi.org/10.1093/nar/gkab225
15. G.M. Boratyn, C. Camacho, P.S. Cooper, G. Coulouris, A. Fong, N. Ma, L. Zaretskaya,, BLAST: a more efficient report with usability improvements, Nucleic Acids Res, vol. 41, 2013, pp. 29-33. DOI: https://doi.org/10.1093/nar/gkt282

16. L. Derouiche, Y. Benzayed, M. Belmihoub, F. Derouiche, F, A study of genetic variants of SARS-CoV-2 using bioinformatics tools, Biodivers. Journal, vol. 6, 2022, pp. 137–148. DOI: https://doi.org/10.46325/gabj.v6i1.206
17. A. B. Sasongko, A. Fatumi, A. Indrianto, The growth improvement of Grammatophyllum scriptum (Lindl.) Bl. in vitro plantlet using photoautotrophic micropropagation system, Indones. J. Biotechnol, vol. 21, 2016, pp. 109–116. DOI: https://doi.org/10.22146/ijbiotech.27167
18. G. Wu, H. Liu, L. Hua, Q. Luo, Y. Lin, P. He, Q. Ye, Differential responses of stomata and photosynthesis to elevated temperature in two co-occurring subtropical forest tree species, Front. Plant Sci, vol. 9, 2018, pp. 467. DOI: https://doi.org/10.3389/fpls.2018.00467
19. T. Lawson, M.R. Blatt, Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency, Plant Physiol, vol. 164, 2014, pp. 1556-1570. DOI: https://doi.org/10.1104/pp.114.237107
20. C. Liu, Y. Li, L. Xu, M. Li, J. Wang, P. Yan, Stomatal arrangement pattern: a new direction to explore plant adaptation and evolution, Front. Plant Sci, vol. 12, 2021, pp. 763. DOI: https://doi.org/10.3389/fpls.2021.655255
21. T.D. Nunes, D. Zhang, M.T. Raissig, Form, development and function of grass stomata, Plant J, vol. 101, 2020, pp. 780-799. DOI: https://doi.org/10.1111/tpj.14552
22. Y. Kato, E. Miura, R. Matsushima, R, W. Sakamoto, White leaf sectors in yellow variegated2 are formed by viable cells with undifferentiated plastids, Plant Physiol, vol. 144, 2007, pp. 952–960. DOI: https://doi.org/10.1104/pp.107.099002
23. Y. Yang, X. Chen, B. Xu, Y. Li, Y. Ma, G. Wang, Phenotype and transcriptome analysis reveals chloroplast development and pigment biosynthesis together influenced the leaf color formation in mutants of Anthurium andraeanum 'Sonate', Front. Plant Sci, vol. 6, 2015, pp. 139. DOI: https://doi.org/10.3389/fpls.2015.00139
24. M. Stockel, C. Meyer, G. Gebauer, The degree of mycoheterotrophic carbon gain in green, variegated and vegetative albino individuals of Cephalanthera damasonium is related to leaf chlorophyll concentrations, New Phytol, vol. 189, 2011, pp. 790-796. DOI: https://doi.org/10.1111/j.1469-8137.2010.03510.x
25. S.C. Yuan, J.Z. Huang, F.C. Chen, Physiological and biochemical characteristics of variegated Phalaenopsis orchids, J. Hortic. Sci, vol. 65, 2019, pp. 147-164. DOI: https://doi.org/10.6964/JTSHS
26. P.N. Kamble, SP. Giri, R.S. Mane, A. Tiwana, Estimation of chlorophyll content in young and adult leaves of some selected plants, Univers. J. Environ, vol. 5, 2015, pp. 306-310. Record No.: 20173100697
27. M.D. Khalekuzzaman, K.J. Kim, H.J. Kim, H.H. Jung, H.S. Jang, Comparison of green and variegated foliage plant species based on chlorophyll fluorescence parameters under different light intensities, Pak. J. Bot, vol. 47, 2015, pp. 1709-1715. Record No.: 29660789
28. D. Zhao, J. Tao, C. Han, J. Ge, An actin gene as the internal control for gene expression analysis in herbaceous peony (Paeonia lactiflora Pall.), Afr. J. Agric. Res, vol. 7, 2012, pp. 2153–2159. DOI: https://doi.org/10.5897/AJAR11.1613
29. W. Dong, C. Xu, J. Wen, S. Zhou, Evolutionary directions of single nucleotide substitutions and structural mutations in the chloroplast genomes of the family Calycanthaceae, BMC Evol. Biol, vol. 20, 2020, pp. 1-12. DOI: https://doi.org/10.1186/s12862-020-01661-0
30. Y. Kato, T. Kouso, W. Sakamoto, Variegated tobacco leaves generated by chloroplast FtsH suppression: implication of FtsH function in the maintenance of thylakoid membranes, Plant and cell physiology, vol. 53, 2012, pp. 391-404. DOI: https://doi.org/10.1093/pcp/pcr189

31. Y. Qi, X. Wang, P. Lei, H. Li, L. Yan, J. Zhao, J. Meng, J. Shao, Li. An, F. Yu, X.X. Liu, The chloroplast metalloproteases VAR2 and EGY1 act synergistically to regulate chloroplast development in Arabidopsis, J.Biol. Chem, vol. 295, 2020, pp. 1036–1046. DOI: https://doi.org/10.1016/S0021-9258(17)49913-3
32. T. Horiike, An introduction to molecular phylogenetic analysis, Reviews in J. Agric. Sci, vol. 4, 2016, pp. 36-45. DOI: https://doi.org/10.7831/ras.4.36