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Isolation and Characterization of *Vanda Orchid Homeobox* Gene from *Vanda tricolor* var. Suavis Lindl. form Merapi

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ABSTRACT
Vanda tricolor var. Suavis Lindl. form Merapi is one of the crucial Indonesian orchid species. Due to natural disasters or deforestation of their natural habitat, the population of this orchid continues to decline and is threatened to be extinct. Therefore, a strategy for mass propagation of this plant as a conservation effort is needed, both *in-situ* and *ex-situ*. Mass propagation using *in vitro* culture will greatly support *ex situ* conservation. It is well known that shoot growth in plants begins with activating of the homeobox gene in the Shoot Apical Meristem (SAM), which induces the activation of related genes to regulate the growth of plant organs. Information about the homeobox gene in *V. tricolor* is necessary to support the induction of optimal shoot growth in *in vitro* culture conditions. Previous studies have discovered the homeobox gene *DOH1* in *Dendrobium* and *POH1* in *Phalaenopsis*. We assumed that *Vanda* has a homologous gene to *DOH1* and *POH1*. The objective of this study was to isolate and analyze *Vanda* homeobox gene with degenerate primers designed from *DOH1* and *POH1* sequences. Leaf from a mature *V. tricolor* was used as samples for DNA analysis. The PCR product amplified genomic DNA using primers from *DOH1* and *POH1* were analyzed by agarose gel electrophoresis. We found a similarity in the length of amplified product of *Vanda* homeobox gene using *DOH1* primer within particular showed that the PCR fragment is aligned with the N-terminal region of DOH*1*. This confirmed the conserved area and promised a high similarity in structure and functions between both genes. Amplification with *POH1* primer showed that high similarity in the length of PCR product as accumulated *POH1* transcript found in *P. amabilis* from the previous study, which showed that it was the coding region. The subsequent sequence analysis on the candidate of *DOH1* and *POH1* homologous gene in *V. tricolor* showed that the gene has 77.27% similarity with *DOH1* thus might be act as the key gene for shoot growth in *V. tricolor* orchids. The *POH1* homologous gene in *V. tricolor* showed no significant similarity with any sequence in the database, suggesting it might be a new sequence that needs further study.

Keywords: *Homeobox Gene, Homologous Gene, Vanda Orchid Homeobox, Vanda tricolor*

1. INTRODUCTION

Orchids are essential ornamental plant commodities with great value because of their high attractiveness, i.e., flowers that do not wilt quickly, long life, high productivity, good flowering season, ease of packaging, and transportation [1]. Indonesia is one of the countries with a high diversity of orchids, and because of that, it has high potential in the world's orchid market [2]. *Vanda tricolor* Lindl. var. Suavis form Merapi is an endemic orchid of Mount Merapi as well. However, this orchid is currently under threat with a declining population due to the high frequency of Mount Merapi eruptions and excessive exploitation by humans for trading [3,4].

One of the conservation efforts that is widely applied is mass propagation of the plants through *in vitro* culture. This method allows the orchid to germinate on an artificial *in vitro* medium without the role of mycorrhizae which is always necessary for nature [5]. *In vitro* culture also allows the plant to be genetically modified.

However, this treatment needs a better understanding of the gene itself. In-plant, the growth and development are regulated by a group of genes that work together to form specific proteins that play a role in the growth and development of the plant while sequentially inducing the next group of key genes for the next phase of growth. The product of the following genes group will suppress the activity of the previous phase gene pool and the cycle repeats. These genes work temporally and spatially [6]. Therefore, the genes responsible for plant growth are important to be analysed for further study manipulating plant cells in *in vitro* conditions.

Homeobox genes are transcription factors that regulate pattern formation, cell specification, or both. The role of homeobox genes in plants in regulating developmental processes is analogous to that of homeobox genes in animals. Based on conservative sequence similarity, plant homeobox genes are grouped into five groups. Those groups are *HD-ZIP, GLABRA2, KNOTTED1, PHD finger*, and *Bell1* [7,8]. *KNOTTED1-like homeobox* (*KNOX*), a group of genes encoding homeodomain transcription factors. This gene is found in higher plants and plays a role in developing and maintaining Shoot Apical Meristem (SAM) and carpel. In SAM, *KNOX* is expressed to increase the biosynthesis of cytokinin (CK) by activating *isopentenyl transferase7* (*AtIPT7*) gene and downregulating the expression of gibberellin (GA) biosynthesis gene, *GA20ox*. The result of those interactions maintains a high level of CKs while keeping GAs levels low, preventing cell differentiation and promoting cell division in SAM [9]

In *Arabidopsis*, the *KNOX* gene family consists of *SHOOT MERISTEMLESS (STM), BREVIPEDICELLUS/KNAT1 (BP/KNAT1), KNAT2*, and *KNAT6* [7]. *KNAT1* gene, in particular, takes the role in maintaining the meristematic phase. Overexpression of the *BP/KNAT1* gene can activate the formation of new SAM. It can replace the *STM* gene in SAM development if suppressed or artificially induced when *STM* function is impaired [10]. In orchids, the function of the *BP/KNAT1* gene in maintaining SAM in the meristematic stage is proven by Dwiyani et al. [11], which showed that *P.amabilis* transformants of the *BP/KNAT1* gene were able to produce multi shots than the wild type plants. The increase in the number of shoots from the transformant plant organ showed the role of the *BP/KNAT1* gene in maintaining the meristematic stage functionally, even in transformant plants.

The homeobox gene in *Dendrobium* and *Phalaenopsis* has already discovered. The *Dendrobium Orchid Homeobox 1* (*DOH1*) is a homeobox gene in the class *KNOTTED1-like homeobox* in orchids isolated from *Dendrobium* Madame Thong-In [12]. *DOH1* has a vital function in maintaining the basic architecture of orchid plants through controlling the formation and development of SAM and shoot structure of plants. This can be proven by the accumulation of mRNA transcripts of *DOH1* in the meristems. Downregulation of *DOH1* activity in SAM is also required for flower transition in orchids [12]. *DOH1* homologous genes were also isolated from *P. amabilis* orchid and designed as *Phalaenopsis Orchid Homeobox 1* (*POH1*) [13]. The *POH1* gene showed 91% homology with *DOH1* and 80% with *KNAT1* in the conserved region of the *Arabidopsis* homeodomain [13]. Further study to characterize this homologous gene in another orchid is required to understand the genetic regulation in shoot development of orchids. In this study, with the assumption that *DOH1* and *POH1* are homologous with the homeobox genes in *V. tricolor*, we isolated and analyzed *Vanda Orchid Homeobox (VOH1)* gene using degenerate primers designated from *POH1* and *DOH1* conserved sequences to assist further research in this species conservation effort.

2. METHODOLOGY

2.1. Growth Observation

This research is conducted from April to October 2021 at the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada (UGM) Yogyakarta. Mature seeds of *V. tricolor* from Mount Merapi slope (Special Region of Yogyakarta) were sown on Vacin & Went medium [14]. Each embryo growth phase was observed and documented throughout June until August 2021.

2.2. Vanda tricolor Genomic DNA Isolation

The leaf of mature *V. tricolor* was cut and weighed around 1.2 grams each. Isolation of genomic DNA was carried out using the modified method of Muray & Thompson (1980) (Figure 3). The gene was amplified by PCR using *DOH1* and *POH1* primers (Table 1). We designed the primers from *DOH1 (Ascension Number: AJ276389)* isolated by Yu *et al.* [12] (Figure 1).

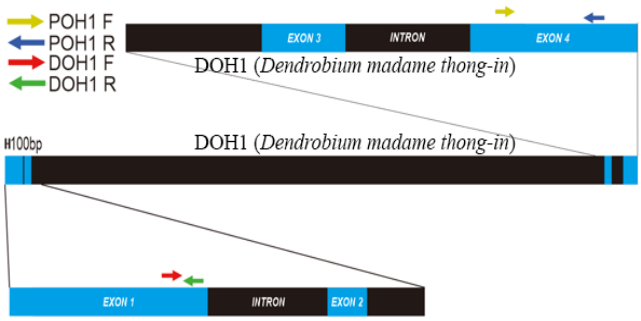

Figure 1. Primer design based on *DOH1 Dendrobium* madame Thong-in [11]. Red arrow = *DOH1* F; Green arrow = *DOH1* R; Yellow arrow = *POH1* F; Blue arrow = *POH1*.

Table 1. Primers used in *Vanda* homeobox gene amplification

Primer	Product length	Sequence		Tm	Primer length	Gc content (%)	Molecular weight (g/m)
DOH1	175bp	F	5-CACCAACGATGGATGAGATG-3	52°C	20bp	50	6170
		R	5-TCGAGAAGATGGGGATAACG-3	52°C	20bp	50	6170
POH1	932bp	F	5-GAAGAGCTCACGAGGCCAGT-3	56°C	20bp	60	6170
		R	5-CAAATAGCACCCAAACCTTTC-3	50°C	21bp	43	6303

The PCR amplifications were carried using MyTaq™ Red Mix (Bioline) by following the standard protocol using an open-source PCR Thermocycler application, OpenPCR. The result was visualized using 1% agarose gel under a UV transilluminator. Samples with multiple bands were extracted individually using Gel/ PCR DNA Fragment Extraction Kit (Geneaid) and sent to 1st Base (Apical Scientific Sdn Bhd, Malaysia) for sequencing.

2.3. Data Analysis

The results of DNA sequencing were analyzed using BLAST [15] to determine the homology of Vanda homeobox gene with *DOH1*, *POH1*, and *KNAT1*. The phylogenetic tree was constructed using MEGA 11 software with Neighbour-Joining method. The evolutionary distances were computed using Maximum Composite Likehood Method [16,17,18].

3. RESULTS AND DISCUSSION

3.1. The growth of Vanda tricolor embryo

Each growth phase is based on embryo morphology described by Dwiyani *et al.* [19]. Here we also observed six different phases of embryo growth during observation time from June to August 2021 (12 weeks after sowing), as presented in Figure 2. Cytokinin plays a huge role during the cell division throughout Phase 1 to 6, as shown with the increased embryo size [20]. Cytokinin also plays a role in the induction and differentiation of shoot bud detected in Phase 6, where the SAM is formed [21]. The increase of Cytokinin biosynthesis is suggested to be promoted by *KNOX*, which is expressed during germination [9].

3.2. Amplification of DOH1 and POH1 homologous gene in Vanda tricolor

The results of the amplification of the *V. tricolor* genome with the *DOH1* primers showed a single specific band. The PCR product size shows a degree of similarity from *DOH1* gene in *Dendrobium* [12] and *V. tricolor* genomes. This suggests that the amplified DNA fragment is a conserved region and has high similarity with *DOH1*, which may also exhibit similar functions.

The *POH1* primer resulted in the amplification of 3 DNA fragments with a length of 900, 300, and 200 base pairs. In particular, the DNA fragments of 900 base pairs have similarities in length to accumulated *POH1* transcript found in *P. amabilis in vitro* cultivated plants [22]. These results suggested that we have successfully amplified the coding region of the *VOH1* gene.

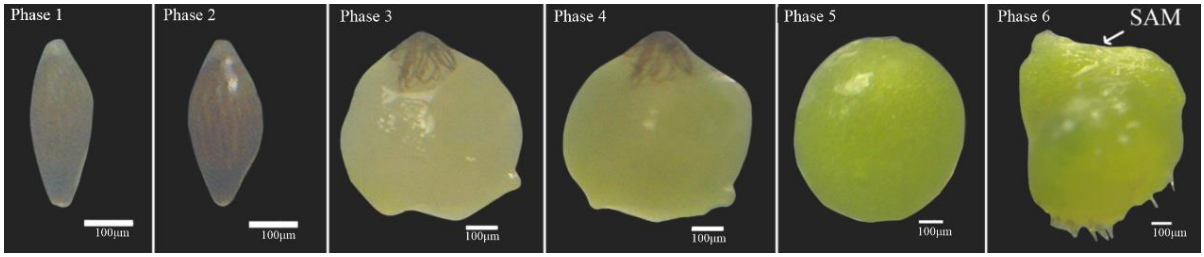

Figure 2 Growth and Development of *V. tricolor* embryo. The growth of *V. tricolor* can be divided in Six phases. Phase 1 = Orchid seed with embryo; Phase 2 = The embryo swells, streaky brown colour indicates a ruptured testa; Phase 3 = embryo is exposed with round shape, white color, testa still remains; Phase 4 = Embryo size enlarged, round shape, yellow color, testa still remains; Phase 4 = Embryo size enlarged, round shape, yellow color, testa still remains; Phase 5 = Embryo size enlarged, round shape, green colour; Phase 6= Shoot Apical Meristem (SAM) detected, green colour. Bar = 100µm.

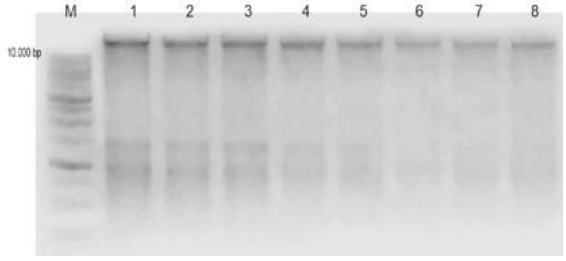

Figure 3 Visualization of genomic DNA isolated from *V. tricolor*. M. 1k bp ladder (Geneaid); 1. leaf sample 0.121g; 2. leaf sample 0.121g; 3. leaf sample 0.138g; 4. leaf sample 0.138g; 5. leaf sample 0.123g; 6. leaf sample 0.123g; 7. leaf sample 0.115g; 8. leaf

ACT4 was used as a positive control for the amplification. *ACT4* is a housekeeping gene that is the most stable reference gene for all vegetative tissue and leaves, which makes *ACT4* could be used as internal/ positive control for further amplification [23].

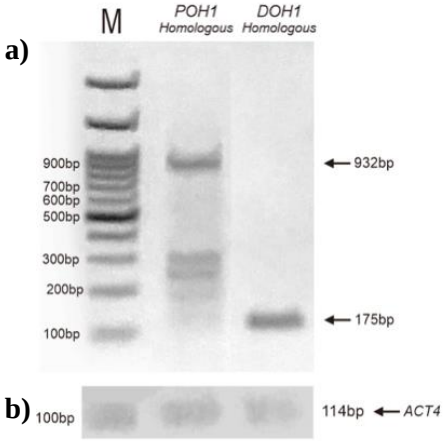

Figure 4. Amplified Fragments of *V.tricolor* genomic DNA. **a)** Amplification of *VOH1* from *V. tricolor* genomic DNA. *The amplification was conducted using DOH1 and POH1 primers in order to obtain the homologous* gene; **b)** Amplification of *ACT4* gene as internal/positive control from *V. tricolor*.(M= 100bp ladder (Geneaid).

3.3. Sequence analysis and phylogenetic tree construction of Vanda homeobox gene

We analyzed the homology of the *DOH1* homolog amplified DNA fragment with the database and found that the DNA fragment of *VOH1* gene has 77.27% similarity with *DOH1* [12]. We also constructed its phylogenetic tree (Figure 5). The phylogenetic tree showed that the *VOH1* gene belongs to the same clade with other orchids *KNOX* homologous genes such as *DOH1*, *KNOX1*, and *KN4* (Figure 5); this shows that *VOH1* genes are closely related to other orchids *KNOX* gene but not to the other *KNOX* gene in other family. The alignment of *VOH1* gene fragment with *DOH1*, in particular, showed that the PCR fragment is aligned with the N-terminal region of *DOH1* (Figure 6); this highly suggests that the amplified fragment is highly conserved. Although *Dendrobium* and *Vanda* differ in the type of stem growth, where *Dendrobium* is a sympodial plant and *Vanda* is a monopodial [5], their homeobox gene is highly similar. This result follows the result of Semiarti *et al.* [13].

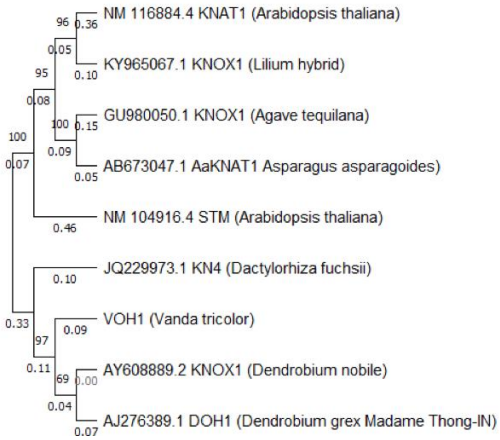

Figure 5. Phylogenetic analysis of *VOH1* gene (indicated by the asterisk) with homologous gene from other species. Genus and species are given in parentheses behind the corresponding gene. The tree was constructed using Neighbour-Joining method with 500 bootstrap replication.

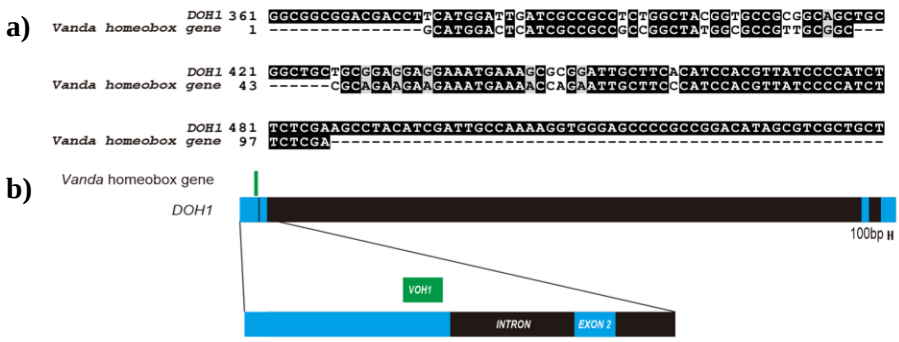

Figure 6. *VOH1* gene alignment with *DOH1*. **a)** Sequence alignment shows high similarity between *VOH1* and *DOH1* and **b)** Schematic representation for aligned *VOH1* PCR fragment with *DOH1*.

The sequence analysis of 3 DNA fragments of *POH1* homologous using BLAST resulted in no significant similarity with any sequence in the database. However, this could suggest that the sequence obtained is a new sequence that has not been registered in the database. Therefore the further study is required for this particular sequence.

The partial fragment of *VOH1* gene has been isolated from *Vanda tricolor* and has a high similarity with *DOH1*. The high similarity strongly indicates that *VOH1* gene has a similar function with *DOH1* as the key gene for shoot growth regulation in *V. tricolor*.

AUTHORS' CONTRIBUTIONS

V.R. carried out the isolation and amplification of the plant genome, also drafted the manuscript. M.D.L. and E.S. was responsible for coordinating the implementation of the research and discussion of the research results. All authors read and approved this final manuscript.

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