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Research Article

In Vitro Culture of The Natural Orchid *Dendrobium spectabile* (Blume) Miq. and The Characteristics of its Fragment 700 bp of the *HOMEOBOX* Homologous Gene

Ana Ainina, Ahmad Yudis Mahardhika, Nuzlan Rasjid, Ni Putu Ayu Erninda Oktaviani Suputri, Endang Semiarti*

Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Sleman, D.I. Yogyakarta 55281, Indonesia

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ABSTRACT

D. spectabile is an orchid species endemic to Papua, which has the characteristic of sepals, petals, and a curly labellum, which is very attractive, so it needs to be preserved. Propagation of this plant with *in vitro* culture techniques promises plant propagation in large quantities and uniformly in a short time. In plants, the *HOMEOBOX* gene is recognized as a key regulator of gene transcription, playing a crucial role in plant organogenesis, particularly in the shoot apical meristem. This study aims to identify the optimal *in vitro* culture media for *D. spectabile* and to conduct molecular analysis of the *HOMEOBOX* gene. The methods used are: subculture, amplification, motif location analysis, physicochemical characterization, phylogenetic construction, and 2D protein sequence modeling. The results of the study showed that the subculture of *D. spectabile* on KC+IAA 20 µM media (20.67±1.76) significantly increased shoot growth. PCR with *POH1* primer successfully amplified a 700 bp *HOMEOBOX* fragment containing 2 motifs: ELK, involved in protein-to-protein interactions, and Homeobox-KN, a transcriptional regulator. Phylogenetic analysis showed a close evolutionary relationship between *D. spectabile* and *D. catenatum*. Further studies are needed to obtain the complete sequence for functional validation in *D. spectabile*.

1. Introduction

Dendrobium spectabile (Blume) Miq. is an orchid native to Papua; its uniqueness lies in its curly sepals, petals, and labellum that have high economic value as an ornamental plant. However, at the same time, native species in nature are becoming scarce due to deforestation and overexploitation. Conventionally, orchid cultivation through seeds is difficult because orchid seeds do not have endosperm as an energy reserve used for embryo growth (Jolman *et al*. 2022). *In vitro* culture is a technique for growing cells, tissues, or organs outside their natural environment (in tubes or bottles) under aseptic conditions. The advantage of *in vitro* culture is that it produces plants in large quantities and in a uniform manner. Various factors affect the success of *in vitro* orchid culture, one of which is the use of hormones.

Cytokinin hormones, such as 6-benzylaminopurine (BAP), are widely used for shoot induction. BAP concentration of 0.5 mg/L, combined with NAA, was used for *in vitro* propagation of *Cymbidium aloifolium* (Pradhan *et al.* 2016). In Brassocattleya 'Pastoral', BAP 1.0 mg/L combined with reduced concentrations of NH_4NO_3 and KNO_3 resulted in higher propagation rates (Cardoso & Ono 2011). Indole-3-Acetic Acid (IAA) plays a crucial role in orchid development,

*Corresponding Author
E-mail Address: endsemi@ugm.ac.id

influencing various aspects of its life cycle. In *Dendrobium officinale*, the IAA content plays a crucial role in the plant's vegetative development (Si *et al.* 2023). IAA plays a role against environmental stress during the vegetative phase (Casanova-Sáez *et al.* 2022). An IAA concentration of 15 µM yielded the best effect compared to concentrations of 5 µM, 10 µM, and 20 µM on *Paphiopedilum villosum* (Diengdoh *et al.* 2023).

The balance between auxin and cytokinin concentrations determines the direction of tissue differentiation. Hormonal signaling from IAA and BAP directly influences the activity of *HOMEOBOX* genes, which play a crucial role in regulating the identity and maintenance of the shoot apical meristem (SAM). Elevated levels of BAP are associated with increased expression of *STM (SHOOT MERISTEMLESS)* and *KNAT1 (KNOTTED1-LIKE* from *Arabidopsis thaliana*) genes, suggesting that cytokinin acts upstream of *HOMEOBOX* genes to modulate their expression, thereby promoting meristem activity and maintenance through hormone-regulated pathways (Rupp *et al.* 1999). The *OSH1* (*Oryza sativa homeobox1*) gene in rice functions in repressing specific hormonal pathways, such as brassinosteroid (BRs) biosynthesis, illustrating how the hormonal equilibrium governed by *HOMEOBOX* genes is critical for proper SAM function (Tsuda *et al.* 2014).

HOMEOBOX genes encode transcription factors that play a role in the regulation of growth and development in eukaryotic organisms. In plants, these genes are classified into 14 classes (Holland 2013). One of the *HOMEOBOX* families that plays a role in vegetative development is KNOX, which regulates tissue patterning in angiosperms and other green plants (Bueno *et al.* 2020). The *HOMEOBOX* gene identified in *Arabidopsis thaliana* (*KNAT1*) plays a pivotal role in shoot apical meristem formation, development, and maintenance, ensuring the retention of meristematic potential (Kuijt *et al.* 2014).

HOMEOBOX gene studies have been conducted on *Phalaenopsis amabilis* orchids (Rasjid *et al.* 2023). Semiarti *et al.* (2008) reported that the *Phalaenopsis orchid Homeobox1* (*POH1*) gene in *P. amabilis* partially plays a role in determining the direction of shoot growth, and also in *Vanda tricolor* (Ruben *et al.* 2022). *HOMEOBOX* gene studies on the *Dendrobium orchid* genus have been carried out on *Dendrobium* Madame Thong-In (Yu *et al*. 2000), where the KNOX domain is relatively conserved and similar to *KN1* in corn and *OSH1* in rice (Semiarti *et al.* 2014), also in *Dendrobium lineale* Rolfe (Semiarti *et al*. 2016).

Studies on *HOMEOBOX* genes in *D. spectabile* have not been reported. This study reports the protein motif of partial fragments of *HOMEOBOX* genes in *D. spectabile* compared to other plants. Further studies are needed to determine the complete structure of *HOMEOBOX* genes, which will reveal the function of the gene, particularly in the development of *D. spectabile* shoots.

2. Materials and Methods

The research was conducted at the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

2.1. *D. spectabile* Subculture

The plant material used was *D. spectabile* plantlets, grown in 10-month-old bottles at the Laboratory of Biotechnology, UGM at 20°C under bright conditions with the addition of various concentrations of IAA (0, 5, 10, 15, and 20) µM and BAP (0, 5, 10, 15, and 20) µM, independently, with three replicates for each treatment. Data on the increase in the number of shoots, plant height, number of leaves, and leaf length were analyzed using one-way ANOVA ($\alpha = 5\%$). If significant differences were obtained, the analysis would proceed to the Duncan Post Hoc Test.

2.2. Amplification of *HOMEOBOX* Gene using *POH1* (*Phalaenopsis Orchid Homeobox1*) Primers

Isolation of *D. spectabile* genomic DNA was carried out in 10-month-old control plantlets following the protocol of Murray and Thompson (1980). RNA isolation from treated plantlets was conducted using the FavorPrep™ Plant Total RNA Purification Mini Kit (100 preps), followed by cDNA synthesis with the iScript™ Select cDNA Synthesis Kit. The isolated gDNA and cDNA of *D. spectabile* were amplified using the *POH1* primer (Rasjid *et al.* 2023) to obtain the target gene, and amplified with the *ACTIN* primer as a control. The primer sequences used are shown in Table 1.

Amplification of the *HOMEOBOX* gene was performed using a pair of *POH1* primers, amplified using Bioline MyTaqTM (UK) PCR Mix. The mixture for the PCR reaction is shown in Table 2. PCR components were homogenized and then incubated in a thermal cycler

machine. PCR temperature conditions are shown in Table 3.

2.3. Amino Acid Analysis of HOMEOBOX Protein

Genomic DNA PCR was conducted to amplify the *HOMEOBOX* gene, followed by verification through electrophoresis and visualization under a UV transilluminator. The resulting PCR products were subsequently sequenced using the Sanger method, with sequencing performed by the sequencing facility PT Genetika Science. The sequences were analyzed using BioEdit software. Nucleotides were converted to amino acids with the Expasy-translate tool (https://web.expasy.org/translate/). Alignment of nucleotides and proteins was performed using MultAlin (Corpet 1988). Data mining of the *HOMEOBOX* gene was obtained from the NCBI and OrchidBase 6.0 websites. Analysis of amino acid motif distribution against homologous genes of other species using MEME Suite Tools (Bailey *et al.* 2009).

2.4. Physicochemical Characterization of HOMEOBOX Protein Structure

The physicochemical assumptions were based on the Expasy-ProtParam server (Gasteiger *et al.* 2005). Physicochemical parameters included molecular weight, aliphatic index, instability index, isoelectric point, and average hydrophobicity (GRAVY). Subcellular protein localization was predicted using CELLO v.2.5 (Yu *et al.* 2006) and PSORT (Horton *et al.* 2007). To determine the presence of signal peptide sequences and helical transmembrane regions, SignalP 4.0 and TMHMM v. 2.0 (Petersen *et al.* 2011) were used to analyze protein sequences.

Table 1. *POH1* and *ACTIN* primer sequences

POH1	Forward 5' - GCA GAT GAG AAA TGC GAA GG - 3' Reverse 5' - TAC ACT TGC TCA TGA AGA TGC - 3'	Amplicon 700 bp
ACTIN	Forward 5' - CAC TCG TGA GAA GAT GAC CC - 3' Reverse 5' - GTC CAT CAG GAA GCT CGT AGC - 3'	Amplicon 114 bp

Table 2. PCR components of *HOMEOBOX* gene amplification in *D. spectabile*

Materials	Composition (µl)
MyTaqTM PCR Mix Bioline	12.5
Forward Primer (10 µM)	1
Reverse primer (10 µM)	1
DNA Template (50 ng)	1
ddH_2O	Up to 25

2.5. Phylogenetic Tree Analysis of HOMEOBOX Proteins

Phylogenetic tree construction using MEGA11 (Kumar *et al.* 2018), Maximum Likelihood approach, bootstrap 1000, and Gamma 5 distribution, with JTT model.

2.6. The 2D Molecular Modeling of HOMEOBOX Protein Sequences

The selected HOMEOBOX protein sequences were predicted using SOPMA secondary structure (Sapay *et al.* 2006) to predict the structure of alpha helices, random coils, and beta turns.

3. Results

3.1. *D. spectabile* Subculture

Table 4 presents the growth and developmental responses of *D. spectabile* cultured on KC medium. The 20 µM IAA treatment produced the best results in terms of shoot number, while 10 µM BAP yielded the greatest stem height. The control treatment yielded the highest leaf number, and 10 µM BAP also produced the longest leaf length, indicating its effectiveness in promoting vegetative growth and elongation.

3.2. Amplification of The *HOMEOBOX* Gene using *POH1* Primers

The results of genomic DNA isolation from *D. spectabile* are presented in Figure 1. Based on the molecular size marker, the visualized DNA bands appeared above 10.000 bp. PCR amplification was then carried out using the *POH1* primer set, with *ACTIN* serving as the positive control (Figure 2).

Table 3. PCR conditions of *HOMEOBOX* gene amplification in *D. spectabile*

Process	Temperature (°C)	Duration time (seconds)	Number of cycles
Pre-Denaturation	95	60	35x
Denaturation	95	30	
Annealing	56	30	
Extensions	72	70	
Post-Extension	72	300	
Hold	4	~	

Table 4. Number of shoots, stem height, number of leaves, and leaf length of the plantlet *D. spectabile* on KC-basal medium for 32 weeks

Hormone	Treatments (µM)	Number of shoots	Stem height (cm)	Number of leaves	Leaf length (cm)
IAA	Control	1.00±0.58^{e}	1.36±0.09^{abc}	8.67±0.51^{a}	1.17±0.10^{bc}
	5	7.00±0.58^{cd}	1.49±0.11^{ab}	8.33±0.88^{ab}	1.58±0.06^{b}
	10	8.00±0.58^{cd}	0.89±0.11^{cd}	6.11±0.59^{abcd}	1.36±0.11^{b}
	15	16.33±1.45^{ab}	1.17±0.02^{bc}	7.11±0.62^{abc}	1.23±0.04^{bc}
	20	20.67±1.76^{a}	0.89±0.15^{cd}	6.56±0.62^{abcd}	1.06±0.23^{bc}
BAP	5	5.67±0.67^{de}	1.27±0.03^{abc}	6.89±0.80^{abcd}	1.52±0.06^{b}
	10	19.00±1.15^{a}	1.89±0.08^{a}	5.78±0.40^{bcd}	2.81±0.13^{a}
	15	11.67±0.88^{bc}	1.04±0.21^{bc}	5.11±0.29^{cd}	1.03±0.16^{bc}
	30	6.3±0.88^{d}	0.46±0.09^{d}	4.00±0.58^{d}	0.74±0.09^{c}

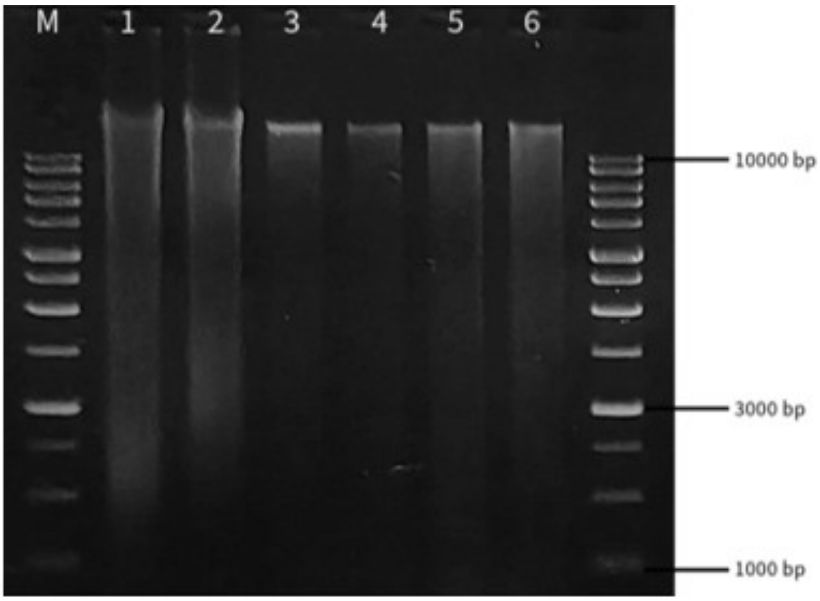

Figure 1. Genomic DNA of *D. spectabile* isolated from *in vitro* plantlets. M: 10.000 bp DNA marker; lines 1-6: genome DNA from 6 independent samples of *D. spectabile*

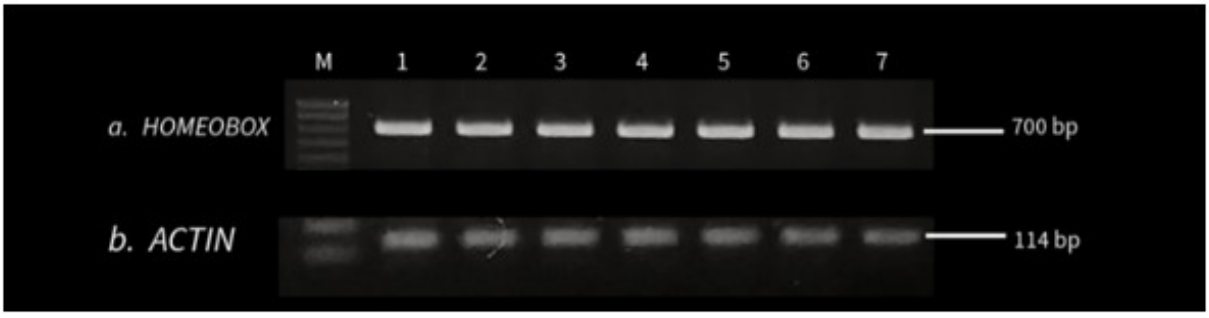

Figure 2. Amplified fragment gDNA of *D. spectabile* using *POH1* and *ACTIN* primers (a) *POH1* (b) *ACTIN*. M: DNA marker 100 bp; 1-7: fragment amplicons *HOMEOBOX* 700 bp, *ACTIN* 114 bp

3.3. Amino Acid Analysis of HOMEOBOX Protein

Alignment of HOMEOBOX protein amino acids of orchid *D. spectabile* and other plants (Figure 3) reveals the presence of a conserved HOMEOBOX protein motif shared with other plant species.

3.4. Physicochemical Characterization of HOMEOBOX Protein Structure

The physicochemical characterization parameters of HOMEOBOX proteins are shown in Table 5. Proteins with a greater number of amino acids ten to have a larger molecular weight. The molecular weight range is approximately 13,968.64 Da (*O. anthropophora*) to 38,711.77 Da (*E. pusilla*). The greater the number of amino acids, the higher the molecular weight of the protein. Most proteins have a pI below 7, meaning that these species are acidic. The Instability Index is a parameter used to estimatethe stability of a protein based on its amino acid sequence. Proteins with a high aliphatic index, such as *D. fuchsii* (73.42) and *G. conopsea* (73.63), have higher stability at extreme

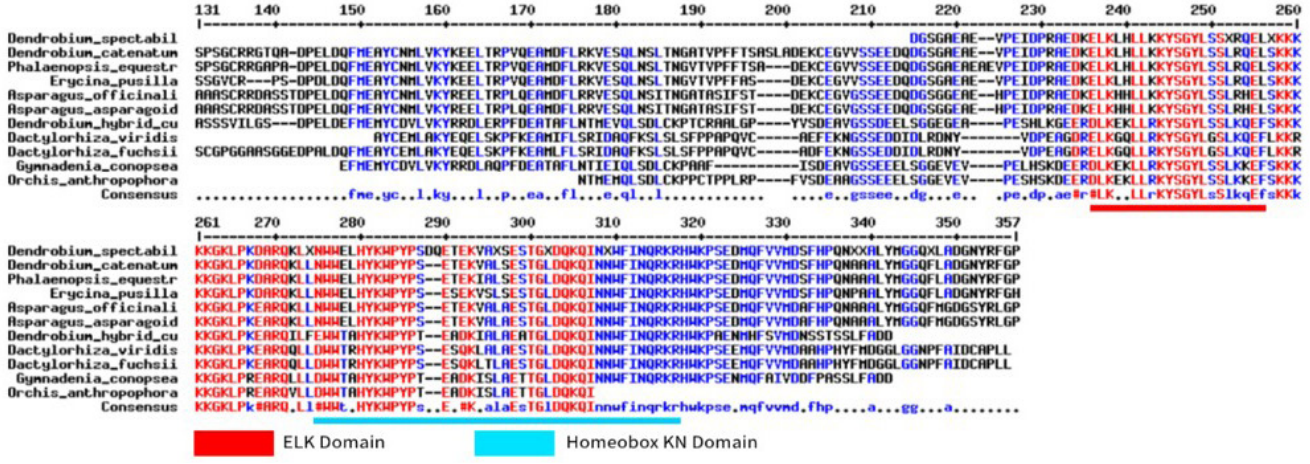

Figure 3. Amino acid alignment of *D. spectabile* HOMEOBOX protein with other plants

Table 5. Physicochemical characterization of HOMEOBOX protein in various plant species

Species	Number of amino acids	Molecular weight	Isoelectric point	Instability index	Aliphatic index	GRAVY	Localization	Signal peptide	Transmembrane motif
Dendrobium spectabile	141	16511.44	8.99	34.44	51.91	-1.140	*nucleus*	0	0
Dendrobium catenatum	339	37851.56	6.14	44.13	64.51	-0.641	*nucleus*	0	0
Dendrobium hybrid cultivar	286	32017.95	5.00	34.88	71.82	-0.587	*nucleus*	0	0
Asparagus officinalis	341	38117.77	6.18	50.48	63.08	-0.727	*nucleus*	0	0
Orchis anthropophora	122	13968.84	6.21	37.71	65.57	-0.984	*nucleus*	0	0
Gymnadenia conopsea	179	20886.62	5.25	36.98	73.63	-0.737	*nucleus*	0	0
Asparagus asparagoides	341	38117.77	6.18	50.48	63.08	-0.727	*nucleus*	0	0
Erycina pusilla	344	38718.38	6.27	42.16	61.80	-0.765	*nucleus*	0	0
Phalaenopsis equestris	325	36631.21	6.05	44.67	65.78	-0.710	*nucleus*	0	0
Dactylorhiza viridis	191	22195.32	7.87	40.42	68.53	-0.740	*nucleus*	0	0
Dactylorhiza fuchsii	298	33301.07	5.93	44.05	73.42	-0.516	*nucleus*	0	0

temperatures.All species have negative GRAVY (Grand Average of Hydropathy) values, indicating that all proteins tend to be hydrophilic and soluble in water. HOMEOBOX proteins in all species are located in the nucleus, suggesting that HOMEOBOX proteins play a role in several gene regulations or processes that occur within the cell nucleus, such as transcription. All signal peptide values are 0. Signal peptides play an important role in guiding proteins into cell membranes and through secretory pathways. The transmembrane motif values for all species are 0, indicating that none of the proteins are transmembrane. This is consistent with the predicted localization in the nucleus, as transmembrane proteins are typically found in cell membranes.

3.5. Phylogenetic Tree Analysis of HOMEOBOX Proteins

The phylogenetic tree structure shows the relationships between species, as shown in Figure 4. Branch length represents the number of genetic changes that have occurred throughout evolution. Longer branches indicate more genetic differences, while the scale below (0.20) shows the evolutionary distance equivalent to a given genetic change.

3.6. 2D Molecular Structure of HOMEOBOX Protein Sequences

Protein secondary structure prediction reveals that the predicted secondary structure components include a helix (alpha helix shown in blue), a secondary structure characterized by high stability and commonly found in proteins. Coil (purple color)/random coil regions appear in some areas between helical segments. Beta sheet, detected at several positions.

4. Discussion

The results revealed a correlation between the number of shoots and plant height. In general, an increase in the number of shoots was accompanied by a decrease in stem height, indicating competition in the allocation of growth resources among vegetative organs. Treatment with IAA at concentrations of 5-20 µM led to an increase in the number of shoots from 7.00 ± 0.58 to 20.67±1.76, whereas stem height decreased from 1.49±0.11 cm to 0.89±0.15 cm. This finding aligns with Virginia and Eleni (2019), who reported that higher IAA concentrations enhanced shoot proliferation in *Satureja thymbra* under *in vitro* conditions. The hormone IAA is a class of auxins that

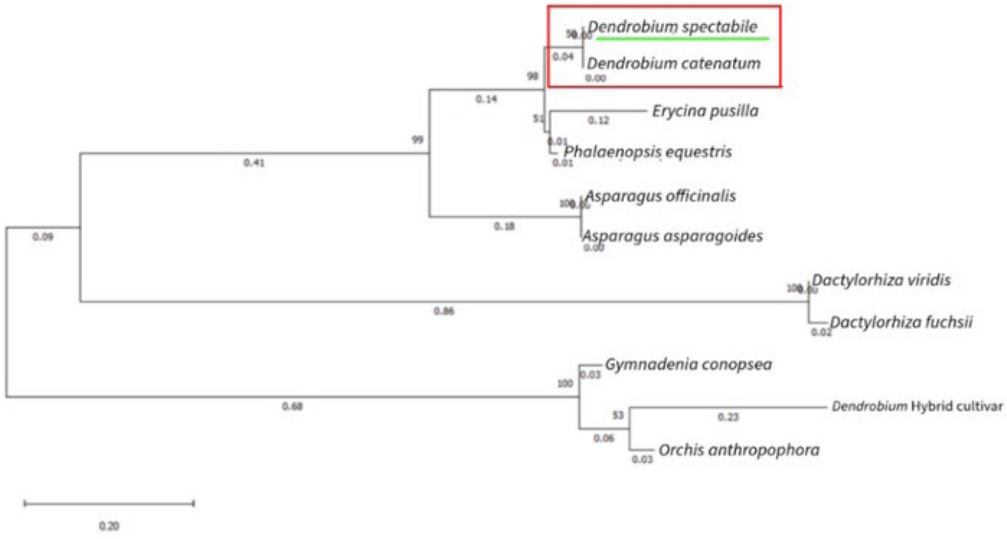

Figure 4. Phylogenetic tree of *D. spectabile* HOMEOBOX protein sequences with other plants

play a role in regulating plant growth and development. IAA is involved in the formation of plant development through polar transport pathways and signaling (Dindas *et al.* 2020). IAA functions as a signaling molecule that affects various physiological processes in plants, regulating cell division, differentiation, and senescence, while also coordinating plant responses to environmental stress (Nicastro *et al.* 2021). These results suggest that high levels of auxin preferentially promote shoot proliferation rather than stem elongation. In *Arabidopsis thaliana*, elevated auxin concentrations have been shown to enhance abscisic acid (ABA) responses through a specific signaling pathway involving the TMK1 kinase, thereby influencing the balance between shoot growth and other developmental processes that affect elongation (Yang *et al.* 2021).

The success of *D. spectabile* gDNA isolation was confirmed by visualization in Figure 1. Subsequent PCR results showed specific and clear DNA bands measuring approximately 700 bp, both from the gDNA template (Figure 2) and cDNA (Figure 5). The cDNA PCR results from the control treatment exhibited a faint amplification band, indicating low *HOMEOBOX* gene expression in untreated plant tissues, which suggests limited meristematic activity and shoot formation. In contrast, treatments with IAA at concentrations of 5-20 µM showed a gradual increase in band intensity, corresponding to increasing auxin concentration, which correlated with the shoot formation results (Table 4), where the number of shoots increased proportionally with higher IAA levels. The 10 µM BAP treatment produced a thick and intense band, reflecting high *HOMEOBOX* gene activity at this concentration, consistent with the higher number of shoots observed (Table 4). These findings confirm a functional relationship between hormonal regulation and the expression of morphogenesis-related genes, emphasizing the role of hormonal balance in modulating *HOMEOBOX* gene activity during shoot induction and meristem development. The *HOMEOBOX* gene functions as a key regulator of differentiation and organogenesis, including shoot initiation, with its activity dependent on the physiological conditions determined by endogenous hormone levels in meristematic tissues. Semiarti *et al.* (2007) reported that overexpression of the *KNAT1* gene in *Phalaenopsis amabilis* led to the production of a greater number of shoots compared to the wild type. Similarly, Lechon *et al.* (2025) demonstrated that the *STM* gene, a member of the KNOX-class I subgroup of the *HOMEOBOX* gene family, plays a crucial role in shoot development, hormonal regulation (including cytokinin and auxin), and lateral organ formation in *A. thaliana*. Furthermore, Bhattacharjee *et al.* (2015) reported that the promoter regions of *HOMEOBOX* genes in leguminous plants contain hormone-responsive elements, namely the

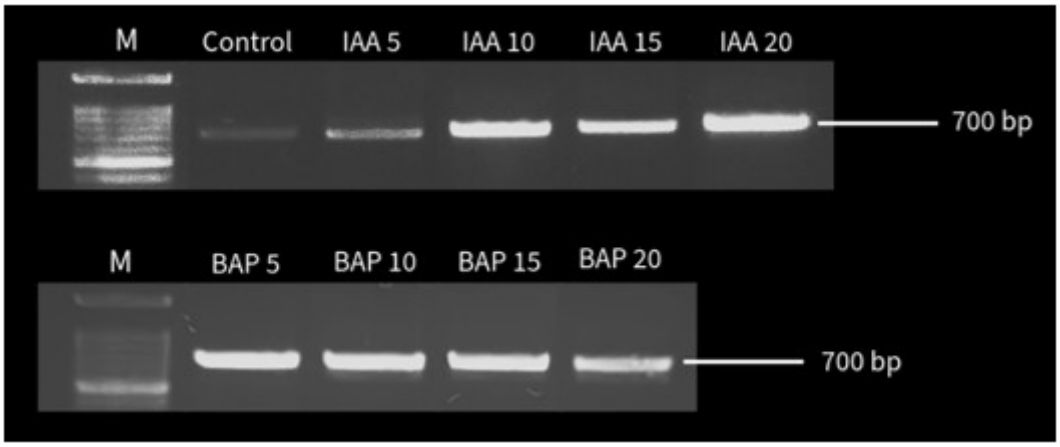

Figure 5. Amplified fragment cDNA of *D. spectabile* using *POH1* Primer. DNA marker 100 bp; sample: fragment amplicons *HOMEOBOX* 700 bp

Auxin Responsive Element (AuxRE), which mediates auxin (IAA)-induced transcription, and the Cytokinin Responsive Element (CPRE), which is associated with cytokinin (BAP) activity.

Amino acid alignment analyses (Figure 4) revealed the presence of a HOMEOBOX protein motif in *D. spectabil*e. The analysis of HOMEOBOX protein motifs in *D. spectabile* and its homologs from other orchids identified two conserved motifs: Homeobox-KN and ELK. The Homeobox-KN motif, located at the C-terminal, is crucial for homodimer formation and DNA binding (Bueno *et al.* 2020), while the upstream ELK motif facilitates DNA binding and transcriptional regulation (Jia *et al.* 2023). In *P. amabilis*, four HOMEOBOX motifs: Homeobox-KN, ELK, KNOX1, and KNOX2 have been reported (Rasjid *et al.* 2023).

KNOX transcription factors are crucial for maintaining SAM. The *KNOX* gene family is divided into two classes: KNOX1, which regulates SAM formation and maintenance (Nookaraju *et al.* 2022), and KNOX2, which contributes to secondary cell wall development (e.g., *KNAT7* and *KNAT3* in *Arabidopsis*). KNOX1 proteins interact with BELL proteins to form heterodimers that modulate gene expression (Li *et al.* 2023), while KNOX1-KNOX2 interactions influence phytohormone regulation by increasing cytokinin and decreasing auxin levels, thereby controlling SAM development. Auxin plays a key role in organogenesis, whereas cytokinin limits cell pluripotency during differentiation (Dai *et al.* 2023). Additionally, the *STM* gene, a KNOX1 member, maintains meristem pluripotency by suppressing gibberellin activity in SAM (Tan *et al.* 2022). The conservation of HOMEOBOX motifs across plant species highlights their crucial role in regulating meristems and promoting plant development. Figures 4 and 6 show that the ELK and Homeobox KN motifs are highly conserved in all plant species, indicating their function in shoot initiation (Table 6). The important role of the *HOMEOBOX* gene in regulating the vegetative growth of *D. spectabile* orchids is crucial for *in vitro* propagation efforts aimed at producing large numbers of uniform plants.

Plantlets grown on the control medium produced the highest number of leaves (8.67±0.51). This indicates that,

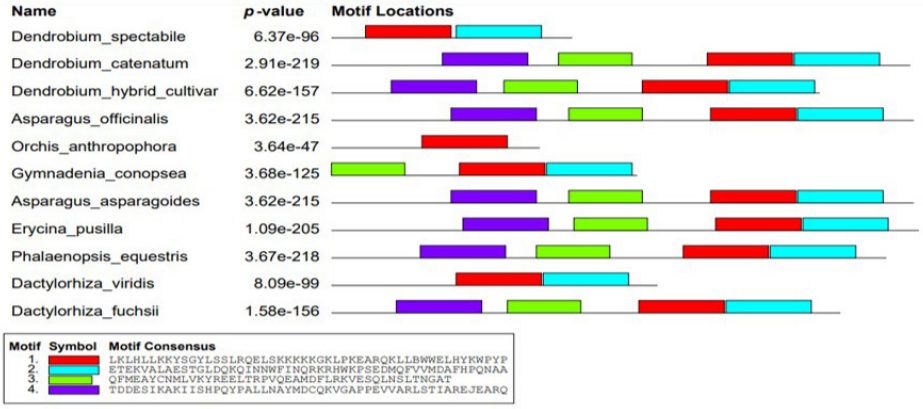

Figure 6. Amino acid motifs in HOMEOBOX proteins of *D. spectabile* and other plants

Table 6. Amino acid motif function

Motive	Symbol	Function
ELK		Interactions between proteins and localization of nuclear signals (Nookaraju *et al.* 2022)
Homeobox KN		Transcriptional regulation (Zhang *et al.* 2022)
KNOX1		Tissue proliferation and contribution to leaf shape formation (Furumizu *et al*. 2015)
KNOX2		Key regulator of secondary cell wall formation (Furumizu *et al*. 2015)

even without the addition of exogenous hormones, plant tissues possess an inherent capacity for organogenesis due to the presence of endogenous hormones. Within the SAM, hormonal signaling pathways integrate stem cell activity with the initiation of organs. Auxin transport and localized biosynthesis contribute to differential hormone accumulation across tissues, playing a key role in specific developmental processes (Wong *et al.* 2023).

For the leaf length parameter (Table 4), treatment with 10 µM BAP resulted in the longest leaves (2.81±0.13 cm), demonstrating a significant difference compared to the other treatments. However, the number of leaves produced was lower than in the control and IAA treatments. Increasing the BAP concentration to 20 µM resulted in shorter leaves (0.74±0.09 cm) and fewer leaves (4.00±0.58), indicating that cytokinin at 10 µM supports leaf elongation but does not significantly increase leaf number. Treatments with 5 µM and 10 µM IAA produced longer leaves (1.58±0.06 cm and 1.36±0.11 cm, respectively) compared to the control, demonstrating the role of auxin in promoting leaf cell elongation. However, at higher IAA concentrations (15 µM and 20 µM), leaf length decreased (1.23±0.04 cm and 1.06±0.23 cm, respectively), suggesting an inhibitory effect due to excess auxin. Elevated auxin concentrations are known to induce dominant cell division and leaf primordia differentiation, even in the absence of optimal elongation growth (George *et al.* 2008).

The HOMEOBOX proteins in various plant species, including *D. spectabile*, exhibit characteristic features consistent with their role as nuclear transcription factors, such as structural stability, hydrophilicity, a generally basic charge (in some species), and the absence of transmembrane domains. Specifically, in *D. spectabile*, the high isoelectric point (pI = 8.99) and low GRAVY value (–1.140) indicate strong DNA-binding affinity and high solubility, highlighting its potential significance in regulating organ development and shoot formation through the activation of target genes modulated by hormonal signals such as IAA and BAP. Negative GRAVY values denote hydrophilicity and water solubility (Sahay *et al.* 2020). Moreover, all values of zero for signal peptide and transmembrane domain prediction confirm that none of these proteins are membrane-associated (Table 6).

Figure 6 shows *D. spectabile* and *D. catenatum* forming a closely related clade, indicating a high degree of genetic similarity. *E. pusilla* and *P. equestris* also cluster closely, while the hybrid *Dendrobium* Madam Thong-in appears more genetically distinct due to hybridization. Phylogenetic trees illustrate the evolutionary relationships among species (Hirai & Iwamasa 2022).

The 2D structure of the HOMEOBOX protein sequence consists of three main elements: alpha helices, beta-turns, and random coils. The results are typically expressed as the percentage composition of each structural element within the protein sequence (Santhoshkumar & Yusuf 2020). The 2D structural profiles of *D. spectabile* and *D. catenatum* are highly similar, indicating that the DNA-binding domain of the HOMEOBOX protein is strongly conserved within the *Dendrobium* genus (Figure 7). This conservation highlights the crucial role of this domain in maintaining the fundamental mechanisms that govern plant growth regulation and morphogenesis.

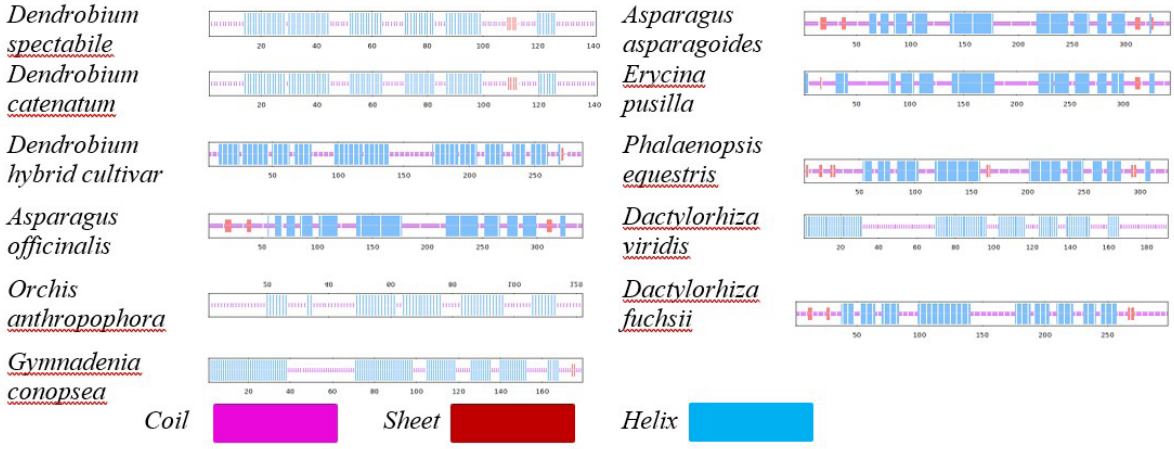

Figure 7. The 2-D protein structure analysis of the HOMEOBOX protein

This study focused on partial isolation of the *HOMEOBOX* gene; further research is needed to obtain the full-length sequence. Characterization of *HOMEOBOX* genes is crucial for overexpression studies in *D. spectabile*, facilitating functional exploration and interactions among *HOMEOBOX* class genes.

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