

A novel durian (*Durio zibethinus* L.) homeodomain-leucine zipper (DzHD-ZIP) transcription factor 1.8 regulates the production of volatile sulfur compounds during fruit ripening

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Abstract

Durian (*Durio zibethinus* L.), an important economic fruit crop in Southeast Asia, produces volatile sulfur compounds (VSCs) during fruit ripening. Methionine γ -lyase (MGL) is the key enzyme involved in the production of VSCs like methanethiol and ethanethiol, which coincide with an increased expression level of durian *MGL* (*DzMGL*) during fruit ripening. Although VSCs in durian pulps have been widely studied, the regulation of genes involved in VSC production in fruit has never been reported. Yeast one-hybrid (Y1H) screening of durian MGL promoter (*pDzMGL*) and ca. 1,200 *Arabidopsis* transcription factors (TFs) together with transcriptomic analysis revealed candidate HD-ZIP TF families in durian. Dual-luciferase assays, in silico promoter analysis, and electrophoresis mobility shift assays were performed to confirm the interaction between the promoter and DzHD-ZIP1.8. The ethylene-responsive DzHD-ZIP1.8 localizes in the nucleus. It acts as an activator and binds to the promoter of *DzMGL* to control its expression during ripening. Here, a TF that regulates the expression of *DzMGL* in durian fruit during ripening was first identified. This study elucidates the molecular regulation of an important gene involved in VSC production, which influences consumer preferences.

1. Introduction

Aroma is one of the most important fruit qualities that determines consumer preference (Schwab et al., 2008) and results from a combination of volatile organic compounds (Rodríguez et al., 2013), which are classified into four groups: terpenoids, phenylpropanoids, fatty acid derivatives, and amino acid derivatives (Negre-Zakharov et al., 2009). Among them, volatile sulfur compounds (VSCs) especially contribute to the aroma of fruits such as melon, guava, passion fruit, kiwi, and pineapple (Mussinan and Kellan, 1994; Werkhoff et al., 1998; Preston et al., 2003; Günther et al., 2010; Gonda et al., 2013). High levels of VSCs may produce unpleasant aromas in melon and other fruits (Wyllie and Leach, 1992).

Durian (*Durio zibethinus* L.) is an edible fruit native to Southeast Asia; its strong sulfuryl aroma deters many customers. The two primary durian cultivars in Thailand are Monthong (*D. zibethinus* L. “Monthong”) and Chanee (*D. zibethinus* L. “Chanee”), whose size, pulp color, and odor vary (Wisutiamonkul et al., 2015; Pinsorn et al., 2018). Chanee exhibits a stronger odor compared to Monthong cultivar because the former contains higher levels of VSCs (unpublished data). Generally, many properties of durian fruit are studied, including nutritional composition, bioactive compounds, and ripening processes (Imsabai et al., 2002; Haruenkit et al., 2007; Arancibia-Avila et al., 2008; Leontowicz et al., 2011; Ho and Bhat, 2015). Notably, the metabolome and transcriptome of durian cv. Monthong, the most famous commercial cultivar of Thailand, were recently analyzed (Pinsorn et al., 2018; Sangpong et al., 2021).

Recently, the draft genome of durian cv. Musang King included genes involved in the fruit ripening processes such as methionine γ -lyase (*MGL*), a gene that directs VSC production (Teh et al., 2017). There are four isoforms of durian MGL: MGLa1, MGLa2, MGLb1, and MGLb2. MGLb2 is consistently expressed in durian fruit (Teh et al., 2017). *MGL* causes methionine and ethionine degrading to methanethiol and

ethanethiol, respectively (Goyer et al., 2007). Besides durian fruit, MGL also guides the production of sulfur-containing volatiles in melon fruit (Gonda et al., 2013). In *Arabidopsis*, MGL contributes not to VSC production but rather to metabolic flux under sulfur starvation (Goyer et al., 2007).

Transcription factors (TFs) regulate the expression of genes that coordinate nearly all processes in plant life such as seed germination, plant growth, development, and stress responses (Harris et al., 2011; Licausi et al., 2013; Li et al., 2019; Zhao et al., 2019). Importantly, TFs regulate the fruit ripening process by triggering ethylene production in climacteric fruit (Li et al., 2020). For instance, HD-ZIP modulates ethylene production in peach fruit by interacting with the promoters of ethylene biosynthetic genes (1-Aminocyclopropane-1-carboxylate synthase (ACS) and 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO)) (Gu et al., 2019). MADS-box TF ripening inhibitor determines tomato ripening through various pathways such as ethylene biosynthesis and response, fruit softening, aroma production, and carotenoid biosynthesis (Fujisawa et al., 2011; Fujisawa et al., 2013; Wang et al., 2014). TFs related to ripening processes in banana fruit have also been identified (Elitzur et al., 2016; Kuang et al., 2017). Aroma production in fruit is also influenced by TFs such as V-myb myeloblastosis viral oncogene homolog (MYB) proteins that produce phenylpropanoid-derived and terpene volatiles in tomato and ethylene response factor (ERF) proteins that regulate genes related to hexanal and hexenal volatiles in peach fruit (Jian et al., 2019; Wang et al., 2022). The following TF families are involved in durian fruit ripening: Dof (auxin-ethylene crosstalk), ARF (ethylene biosynthesis), ERF (ethylene biosynthesis), and CAMTA (ethylene-mediated signaling) (Khaksar et al., 2019; Khaksar and Sirikantaramas, 2020; Iqbal et al., 2021).

Although *MGL* is important for VSC production in economic fruit crops such as durian and melon (Gonda et al., 2013; Teh et al., 2017), its regulation mechanism remains unknown. Therefore, we aimed to identify TFs that control *MGL* gene expression of durian fruit and to elucidate how TFs regulate fruit ripening by performing yeast one-hybrid screening, in silico promoter analysis, gene expression analysis, dual-luciferase assay, and electrophoretic mobility shift assay (EMSA) in vitro.

2. Materials And Methods

2.1. Transcriptome and gene expression analyses

Transcriptome data from durian pulps at five different stages of Monthong cultivar [Project accession number: PRJNA683229 (mature and ripe stages) and PRJNA732556 (immature1, immature 2, and midripe stages)] were processed using OmicsBox (v1.4.1.1), which was obtained from Suntichaikamolkul et al. (2021). Three replicates from three fruits collected from independent trees were conducted for each stage. The fruits were harvested, and their pulps were extracted as described by Suntichaikamolkul et al. (2021). The HD-ZIP TF family (DzHD-ZIPs) and DzMGL genes were isolated from the transcriptome data. Gene expression and correlation analyses were visualized, and their heatmap was obtained using the Euclidean distance method with Metaboanalyst 5.0 (<https://www.metaboanalyst.ca/>; (Pang et al., 2021)).

2.2. Durian pulp samples, RNA extraction, cDNA synthesis, and reverse transcription quantitative real-time PCR (RT-qPCR)

Durian pulps used for RT-qPCR analysis were obtained from the same set used for transcriptome analysis. Fruit samples used in this study were the same as in the previous report by Khaksar et al. (2019) to study ethylene production during ripening. In brief, mature durian fruit were treated with 1-methylcyclopropene (1-MCP) and ethephon to delay and accelerate the ripening period, respectively. The naturally ripe fruit (table ripe) was used as a control; the fruits treated with 1-MCP and ethephon were harvested simultaneously when the control group ripened. Four biological replicates were studied for each stage.

The total RNA of the durian pulps was extracted using Purelink Plant RNA Reagent (Invitrogen™) following the manufacturer's instructions. Genomic DNA was digested with DNase I (Thermo Scientific™). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) according to the manufacturer's instructions.

RT-qPCR analysis was further performed using gene-specific primers (Table S1). The PCR reaction contained 1 ng of cDNA, 5 µl of 2× SsoFast™ EvaGreen® Supermixes (Bio-Rad Laboratories Inc.), and 200 nM of each primer in a final volume of 10 µl. The RT-qPCR products were amplified as follows: enzyme activation at 98°C for 30 s, followed by 40 cycles of denaturation at 98°C for 5 s, and annealing/extension at 58°C for 5 s with a CFX95 Real-time System (Bio-Rad, USA). A melting curve was generated by heating the amplified products from 55°C to 95°C in increments of 0.5°C. The relative expression of each gene compared to the reference gene (elongation factor-1 alpha, EF-1α) was calculated using the $2^{-\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.3. Phylogenetic analysis of HD-ZIP transcription factor family

Amino acid sequences of durian HD-ZIP were obtained from the Plant Transcription Factor Database (PlantTFDB, Jin et al., 2014; Jin et al., 2015; Jin et al., 2017; Tain *et al.*, 2020). *Arabidopsis* and peach HD-ZIP amino acid sequences were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The phylogenetic tree was constructed with the neighbor-joining method using 1000 bootstrap replicates in the MEGAX program (Zuckerandl and Pauling, 1965; Felsenstein, 1985; Saitou and Nei, 1987; Kumar et al., 2016).

2.4. Genomic DNA extraction

Genomic DNA from durian leaves was extracted using NaCl/CTAB solution (Porebski et al., 1997). Briefly, freshly ground leaf tissues (50 mg) were extracted by adding 1.3 mL of CTAB solution, incubating at 60°C for 30 min, and centrifuging at 13,000 rpm for 30 min. The extracted DNA was purified with a phenol:chloroform:isoamyl alcohol mixture (25:24:1). The DNA was pelleted by adding one volume of

100% ethanol and centrifuging at 13,000 rpm for 5 min. The pellet was dried and re-suspended in nuclease-free water.

2.5. Cloning of constructs

Primers used for cloning are shown in Table S1. The *MGLb2* isoform (Duzib1.0C010G000488) was studied in yeast one-hybrid (Y1H) screening, and its promoter was cloned using promoters according to the genome of durian cv. Musang King. A bait construct was produced by amplifying the 2000-bp upstream of the start codon (full-length) of the methionine γ -lyase gene (*pDzMGL*) from genomic DNA of durian cv. Monthong and inserting it into a pTUY1H vector (Castrillo et al., 2011).

For the dual-luciferase assay, the 2000 bp region upstream of *pDzMGL* was inserted and fused with firefly, *Photinus pyralis*, luciferase (LUC) gene in the pGreenII-0800-Luc vector, which contains *Renilla reniformis* luciferase (REN) gene under the 35S promoter as internal control (Hellens et al., 2005) using the In-Fusion® HD Cloning enzyme (Takara Bio Inc.). In addition, the full-length *pDzMGL* was divided into seven short fragments (dF1–dF7, see results) and cloned into the pGreenII-0800-Luc vector. TFs of interest were cloned into the pGreenII-62-SK vector (for Agrob infiltration in *Nicotiana benthamiana* leaves) and pGWB2 (for *Arabidopsis* protoplast cells) under the 35S promoter.

For recombinant protein expression in bacteria, TFs were cloned into pDONR207 using the BP clonase enzyme (Invitrogen) without a stop codon, sub-cloned into pDESTTM24 using the LR clonase enzyme (Invitrogen), and fused with a GST tag at the C-terminal.

The subcellular location of the HD-ZIP TF was identified by fusing pDONR207 containing DzHD-ZIP1.8 coding sequences without a stop codon with green fluorescence protein (GFP) protein at the C-terminal of the pGWB5 vector. A cytosol-localized GFP was used as a control.

2.6. Yeast one-hybrid (Y1H) screening

Y1H screening was performed as described by Castrillo et al. (2011). The bait construct described in 2.5 was transformed into yeast *Saccharomyces cerevisiae* strain Y187 of mating type α . The autoactivation test of the bait was conducted by growing yeasts on synthetic defined (SD) medium without histidine containing 60 mM of 3-amino-, 2, 4-triazole (3-AT), which inhibits the product of the HIS3 gene and therefore removes false positives. A library of ca. 1,200 TFs from *Arabidopsis* was constructed by Castrillo et al. (2011), which was cloned into pDESTTM22 (Invitrogen) in a frame of Gal4 activation domain at the N-terminal and transformed into *S. cerevisiae* strain YM4271 of mating type α and served as prey for the screening. The DNA bait-TF prey interactions were identified by mating the two yeast strains and selected on SD medium with 60 mM 3-AT and without leucine, tryptophan, and histidine.

2.7. Dual-luciferase activity assay in *Arabidopsis* protoplast and *Nicotiana benthamiana* leaves

2.7.1. Dual-luciferase reporter assay in *Arabidopsis* protoplasts

Interactions between TFs and *pDzMGL* were confirmed using dual-luciferase reporter assay as described by Hellens et al. (2005). pGreenII-0800-Luc vector containing *pDzMGL* (10 µg) and pGreenII-62-SK vector containing durian TF (DzTF) (10 µg) were co-transfected into *Arabidopsis* protoplasts (5×10^4 cells) extracted from leaves as described by Yoo *et al.* (2007). The reaction was incubated overnight (~ 16 h). The dual-luciferase activities (three replicates) were measured using a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany) coupled with luminescent detectors at 560 nm for LUC and 480 nm for REN.

2.7.2. Dual-luciferase activities assay in *Nicotiana benthamiana* leaves

The interactions between each DzTF and *pDzMGL* fragment (full) and the deleted fragments (dF1-dF7) were analyzed in *N. benthamiana* leaves. Leaves of six-week-old plants were used for agroinfiltration. The pGreenII-0800-Luc vector containing *pDzMGL* (either full-length or the deleted fragments) and the pGreenII-62-SK containing each TF were transformed into *Agrobacterium tumefaciens* strain GV3101. For agroinfiltration, *A. tumefaciens* was cultured in yeast extract beef (YEB) medium containing 50 µg/mL of kanamycin, 50 µg/mL of gentamycin, and 50 µg/mL of rifampicin for two days at 28°C and shaken at 200 rpm. The cells were then harvested and re-suspended with the infiltration buffer (10 mM MES, 10 mM MgCl₂, 150 µM acetosyringone, pH 5.6). The final OD₆₀₀ at 0.3 of each construct was used for the combinations of *pDzMGL*, TF, and p19 (Lindbo, 2007). The infiltrated leaves (circle, i.d. 1 cm²) were collected at seven days post-infiltration. The leaves (three replicates) were lysed in lysis buffer (provided with the kit, Gold Biotechnology Inc.). The dual-luciferase activity of the mixture of extract (10 µl) with 40 µl of the substrates was measured according to the manufacturer's instruction (Gold Biotechnology Inc.).

2.8. Recombinant protein expression, SDS-PAGE, and Western blot analyses

The vector containing the *DzTF* gene fused with GST was transformed into *Escherichia coli* rosetta (DE3) strain containing the pRARE vector. Positive clones were selected on an LB agar plate with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) to select the positive clones which contained pDESTTM24 and pRARE vectors; one colony was cultured overnight (~ 16 h) in LB media with antibiotics. The culture (30 µL) was inoculated into fresh LB media (6 mL) with the antibiotics and shaken at 200 rpm until the OD₆₀₀ reached 0.6. The expression was induced with 1 mM of isopropyl β-D-1 thiogalactopyranoside (IPTG) and shaken at 200 rpm at 28°C overnight. The culture was collected and re-suspended in 300 µL of extraction buffer (50 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and protein inhibitor). Cells were lysed by adding lysozyme and ultrasonication. The lysate was centrifuged to separate supernatant from the pellet. The recombinant protein was confirmed by SDS-PAGE and Western blotting using primary (GST tag antibody, Novagen) and secondary (Anti-DCX

antibody produced in goat, Sigma) antibodies; its fluorescent signal (800 nm channel) was detected with a gel imaging machine coupled with GeneSys image acquisition software (Syngene).

2.9. Electrophoretic mobility shift assay (EMSA)

Oligonucleotide complementary probes containing a TF binding site labeled with and without fluorescent dye at 5' end (5' DY-682) were synthesized (Eurofins Genomics, Germany) (Table S1). The mixture of labeled probes (forward and reverse) was diluted to a concentration of 10 μ M with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The unlabeled probes were mixed between forward and reverse to a concentration of 50 μ M. The diluted probes were dimerized by heating at 95°C for 5 min and reducing the temperature from 95°C to 4°C by 1°C every 20 s to create double-stranded probes. After dimerization, the labeled probes were diluted 1:300 in TE buffer. The unlabeled dimerized probes were not diluted (50 μ M). The EMSA was performed using the Odyssey® EMSA buffer kit (Li-COR). The interactions between probes containing the TF binding site and the recombinant protein were prepared according to the manufacturer's instructions. The binding was detected by electrophoresis using 8% native polyacrylamide gel electrophoresis performed in TBE buffer at 4°C in the dark and compared to the control that was prepared using crude extraction of free GST protein. Fluorescent signal (700 nm channel) was detected using a gel imaging machine coupled with GeneSys image acquisition software (Syngene).

2.10. Subcellular localization of HD-ZIP transcription factors in *N. benthamiana* leaves

The pGWB5 vector containing the HD-ZIP gene fused with GFP was transformed into *A. tumefaciens* GV3101. A single colony of recombinant *A. tumefaciens* containing each construct was cultured in YEB medium containing 50 μ g/mL of kanamycin, 50 μ g/mL of gentamycin, and 50 μ g/mL of rifampicin at 28°C and shaken at 200 rpm for two nights. The cultures were harvested and re-suspended with the infiltration buffer (mentioned in 2.7.2) to an OD₆₀₀ of 1.0. The mixture of the construct at a ratio of 1:1 with *A. tumefaciens* containing the *p19* gene (Lindbo, 2007) was used for co-infiltrating into *N. benthamiana* leaves of six-week-old plants. After infiltration, plants were left for another five days. GFP signal was visualized using confocal microscopy with excitation and emission wavelengths of 488 nm and 500–800 nm, respectively.

3. Results

3.1. Yeast one-hybrid screening (Y1H) revealed transcription factor families potentially bind to durian MGL promoter

TFs in durian are rarely studied, and TFs that regulate methionine γ -lyase (*MGL*) have not yet been reported. Therefore, a library of *Arabidopsis* TFs (AtTFs) provided by Castrillo et al. (2011) containing ca. 1,200 TFs was used for Y1H screening to identify TFs that interact with the promoter of durian *MGL* (*pDzMGL*) assuming sufficient conservation and coverage. We identified 11 AtTFs including AT5G04410 (NAC domain containing protein 2), AT4G18880 (heat shock transcription factor A4A), AT4G19630

(winged-helix DNA-binding transcription factor family protein), AT1G53230 (teosinte branched 1, cycloidea and PCF transcription factor 3, TCP3), AT3G15030 (TCP family transcription factor 4, TCP4), AT5G15150 (HB-3 homeobox 3), AT5G53980 (HB52 homeobox protein 52), AT1G69780 (ATHB13 homeobox-leucine zipper protein family), AT1G79840 (HD-ZIP IV family), AT1G77640 (integrase-type DNA-binding superfamily protein), and AT1G13300 (myb-like transcription factor family protein) that possibly bind to *pDzMGL* (Fig. 1).

3.2. HD-ZIP transcription factor family was clustered based on their evolutionary relationships

In this study, we focused on the HD-ZIP family of durian (DzHD-ZIPs) because this family was mainly identified from the Y1H result (AT5G15150, AT5G53980, AT1G69780, and AT1G79840). Amino acid sequences of all DzHD-ZIP members of durian were obtained from PlantTFDB, and 116 members were identified from genome database. Phylogenetic analysis of durian, *Arabidopsis*, and peach was performed using the neighbor-joining method (Supporting Information Fig. S1). TFs were clustered into four groups. Among *Arabidopsis* HD-ZIPs (AtHD-ZIPs) from the Y1H screening, three were clustered in group 1 (AT5G15150, AT5G53980, and AT1G69780) and one was in group 4 (AT1G79840). Therefore, HD-ZIPs of group 1 and 4 were assumed to bind to the durian MGL promoter.

3.3. *DzHD-ZIP* transcription factor candidates showed high correlation with *MGL* expression level

To analyze the expression levels of TFs and *DzMGL* in durian pulps, transcriptome data of pulps at five stages of durian cv. Monthong (immature1, immature2, mature, midripe, and ripe) were obtained from Suntichaikamolkul et al. (2021). From the data, 34 DzHD-ZIP members expressed in the pulps were isolated (Fig. 2). Heat map analysis was performed based on the reads per kilobase mapped (RPKM) (Table S2), and those members were categorized according to the phylogenetic analysis (Supporting Information Fig. S1). The expression levels of those genes varied during ripening. The transcript levels of group 1 including *DzHD-ZIP1.3*, *DzHD-ZIP1.6*, *DzHD-ZIP1.7*, and *DzHD-ZIP1.8* were upregulated, while *DzHD-ZIP1.1*, *DzHD-ZIP1.2*, and *DzHD-ZIP1.9* were downregulated during ripening. The transcript levels of group 2 including *DzHD-ZIP2.1*, *DzHD-ZIP2.2*, and *DzHD-ZIP2.4* were upregulated, whereas *DzHD-ZIP2.3*, *DzHD-ZIP2.5*, and *DzHD-ZIP2.6* were downregulated during ripening. Most of *DzHD-ZIP* transcript levels in group 3 were downregulated during ripening except *HD-ZIP3.3*, whose level slightly increased. For group 4, only the transcript level of *DzHD-ZIP4.1* was unchanged, while the other genes were downregulated. A correlation analysis (Supporting Information Fig. S2) was also performed after discovering that the transcription level of *DzMGL* was dramatically upregulated during ripening (Fig. 2). Group 1 and 2 including *DzHD-ZIP1.3*, *DzHD-ZIP1.6*, *DzHD-ZIP1.7*, *DzHD-ZIP1.8*, *DzHD-ZIP2.1*, *DzHD-ZIP2.2*, and *DzHD-ZIP2.4* highly correlated with *DzMGL*. However, the members of group 2 were not suggested to be candidates based on our Y1H screening. Therefore, subsequently, we only focused on the members of group 1 in the following experiments.

3.4. DzHD-ZIP1.8 activates luciferase activity in *Arabidopsis* protoplasts

Group 1 and group 4 HD-ZIPs were isolated based on the Y1H screening (Fig. 1). However, the heatmap analysis showed that group 4 DzHD-ZIPs were downregulated during ripening (Fig. 2). Group 1, including DzHD-ZIP1.3 (XP_022741138.1), DzHD-ZIP1.6 (XP_022738207.1), DzHD-ZIP1.7 (XP_022775553.1), and DzHD-ZIP1.8 (XP_022757591.1), highly correlated with DzMGL (Supporting Information Fig. S2). Therefore, we focused on group 1 DzHD-ZIPs in this study. To confirm the interaction between the promoter and the candidate TF, a vector containing full-length *pDzMGL* fused with *LUC* was co-transfected with a vector containing the TF under 35S promoter into *Arabidopsis* protoplasts. From the dual-luciferase activity assay, the LUC/REN ratio of DzHD-ZIP1.8 was approximately two-fold higher than the control (Fig. 3).

3.5. DzHD-ZIP expression level responses to ethylene production in durian pulps

Although the transcript level of genes can be retrieved from the transcriptome data, we further validated the expression level of *DzHD-ZIP1.8* by performing RT-qPCR of durian pulps at immature 1, mature, midripe, and ripe stages. The expression level of *DzHD-ZIP1.8* increased (Fig. 4a) during ripening, which was consistent with the transcriptome data (Fig. 2). We also explored how ethylene affects ripening by analyzing the expression levels in durian fruit treated with 1-MCP and ethephon. The expression level of *DzHD-ZIP1.8* in the ethephon-treated fruit were significantly higher than those of the table ripe and 1-MCP-treated fruit (Fig. 4b).

3.6. Nuclear-localized DzHD-ZIP1.8 exhibits transcriptional capacities

Transient expression analysis showed that the GFP signal was localized in the nucleus of epidermal cells transfected with *Agrobacterium* carrying the *35S:DzHD-ZIP1.8-GFP* gene fusion construct, whereas the control cells (transformed with the empty vector, 35S:GFP) showed GFP ubiquitously distributed throughout the whole cell (Fig. 5). This GFP signal indicated that DzHD-ZIP1.8 protein localizes in the cell nucleus, confirming its transcriptional capacities.

3.7. In silico prediction and promoter deletion suggests transcription factor binding sites on *MGL* promoter

DzHD-ZIP1.8 can activate full-length *pDzMGL* in the protoplast system; therefore, full-length *pDzMGL* was used to identify TF binding sites that were predicted via PlantRegMap software (Table S3). The *Arabidopsis* TF database was used to scan the binding sites. Six of the binding sites were located on the promoter region at positions – 1565, – 1559, – 1203, – 1039, – 801, and – 767 from the start codon

(Fig. 6a). To narrow down the TF binding site on the promoter and confirm the interaction, the 5' end of *pDzMGL* was deleted (dF1–dF7), and each fragment was fused with *LUC*. Based on the prediction, fragments II, IV, and V contain the binding sites of the HD-ZIP family (Fig. 6b). Dual-luciferase activity assay in *N. benthamiana* leaves was then performed using the promoter fragment fused with *LUC* and DzHD-ZIP1.8. under 35S promoter. The LUC/REN ratio of full-length *pDzMGL* was approximately 3.5-fold higher than that of the control (Fig. 6c). Although the ratio in *Arabidopsis* protoplasts was lower (Fig. 3), it was still consistent with the *N. benthamiana* leaves. For the promoter with deleted fragment II (dF2) and fragment V (dF5), the LUC/REN ratio decreased compared to the previous adjacent fragments and the full-length promoter, suggesting that these fragments contain binding sites of DzHD-ZIP1.8. However, when the fragment IV was deleted (dF4), the ratio significantly increased compared to the dF3 fragment. Fragment IV may contain a binding site for a repressor or other proteins that regulate the promoter.

3.8. In vitro electrophoresis mobility shift assay (EMSA) confirms the direct binding of DzHD-ZIP1.8 to MGL promoter

Crude protein extract of DzHD-ZIP1.8-GST fusion was used to confirm the interactions between DzHD-ZIP1.8 and fragments II and V in vitro (Supporting Information Fig. S3). Two binding site positions were identified on each fragment based on the *in silico* predictions of fragments II and V (Fig. 6a). Position –1559 on fragment II was selected based on the number of group 1 TFs that mostly bound to this site, and position –767 on fragment V was selected because the TF predicted to bind at this site belongs to group 1 (Fig. 7a and Table S3). DNA probes containing the TF binding sites were designed and labeled with DY-682 at the 5' end (Table S1). As shown by EMSA, the bands in the second lane shifted upon addition of HD-ZIP1.8-GST with probes located on fragments II and V (Fig. 7b). Two bands shifted after incubating the crude extract with fragment II, while one band occurred in the reaction incubated with fragment V. After adding the unlabeled competitive probes, the bands of protein-bound probes could be eliminated or faded as seen in the third lane. The control reaction was prepared by using free GST protein incubated with the labeled probes as shown in the fourth lane. These findings indicated that DzHD-ZIP1.8 binds to *pDzMGL* within fragments II and V.

4. Discussion

Methionine γ -lyase (MGL) catalyzes the production of methanethiol and ethanethiol from methionine and ethionine, respectively (Goyer et al., 2007). These two VSCs generate notorious odors in ripe durian fruit (Li et al., 2017). The expression of *MGL* also highly increases during durian ripening (Fig. 2). There is only a report by Dietzen et al. (2020) regarding *Arabidopsis* MGL that is controlled by SULFUR LIMITATION1 (SLIM1) under sulfur deficiency. In addition, *Arabidopsis* growing under low sulfur conditions highly expressed MGL gene when methionine was supplied (Goyer et al., 2007). However, methanethiol could not be detected under that condition. Therefore, the understanding of *MGL* regulation mechanism related to VSC production is limited, especially in fruits.

In this study, Y1H screening was used to identify protein-DNA interactions. This technique revealed the interactions between the promoter of durian *MGL* (*pDzMGL*) and 11 *Arabidopsis* TFs belonging to NAC, winged helix, TCP, HD-ZIP, integrase-type DNA-binding, and the myb-like family (Fig. 1). HD-ZIPs belonging to group 1 (AT5G15150, AT5G53980, and AT1G69780) and group 4 (AT1G79840) were isolated from the screening. Following the Y1H results, we further explored HD-ZIP, a large TF family unique to plants. This family contains a homeodomain (HD) associated with a leucine zipper motif (ZIP). HD-ZIP is commonly divided into four groups based on phylogeny and gene structure (Ariel *et al.*, 2007). Groups 1 and 2 participate in seed germination, responses to biotic and abiotic stresses, and ripening (Agalou *et al.*, 2008; Ciarelli *et al.*, 2008; Manavella *et al.*, 2008; Stamm and Kumar 2010). Group 3 is involved in plant cell specification and differentiation, embryonic development, and the vascular system (Green *et al.*, 2005; Prigge *et al.*, 2005). Group 4 contributes to epidermal development, flavonoid biosynthesis, and osmotic tolerance (Javelle *et al.*, 2011; Chen *et al.*, 2017; Yan *et al.*, 2018; Wang *et al.*, 2020).

HD-ZIP has been shown to play various roles during ripening of many fruits. Gene silencing of group 1 HD-ZIP inhibits tomato ripening (Lin *et al.*, 2008), and HD-ZIP groups 1 and 2 stimulate genes related to cell wall degradation in banana fruit (Yang *et al.*, 2020). Group 1 HD-ZIP of tomato (VAHOX1) also acts as a repressor in fruit ripening (Li *et al.*, 2022). However, HD-ZIP involved in ripening processes of durian fruit, especially VSCs production, has never been reported. In this study, among the HD-ZIP candidates, nuclear-localized group 1 HD-ZIP (DzHD-ZIP1.8) was shown to interact with *pDzMGL* according to a dual-luciferase activity assay in protoplasts (Fig. 3). Based on gene expression analysis, ethylene-treated DzHD-ZIP1.8 was upregulated during ripening suggesting that ethylene stimulates the expression of *DzHD-ZIP1.8* and highly correlated with the *DzMGL* transcript (Supporting Information Fig. S2). In silico analysis of *pDzMGL* showed HD-ZIP binding sites on the promoter region (Table S3). Moreover, in vitro EMSA and in vivo dual-luciferase activity assay performed with promoter deletions in *N. benthamiana* leaves confirmed that DzHD-ZIP1.8 can bind at two positions on the promoter region (Figs. 6 and 7). In litchi fruit, group 1 of HD-ZIP (LcHB2 and LcHB3) positively regulates ethylene by directly by to the promoter of 1-aminocyclopropane-1-carboxylic acid oxidase and 1-aminocyclopropane-1-carboxylic acid synthase which are involved in ethylene biosynthesis (Ma *et al.*, 2019). Moreover, group 2 of HD-ZIP (PpHB.G7) was identified to regulate ripening processed by activating ethylene biosynthetic genes (Gu *et al.*, 2019). Based on previous studies, HD-ZIPs in other fruits mainly regulate ethylene biosynthetic genes. However, these findings indicate that DzHD-ZIP1.8 is a crucial TF that activates and regulates the expression of *DzMGL* which is associated with the increased in VSC production during ripening.

HD-ZIPs have been widely studied in *Arabidopsis*. HD-ZIPs and ATHB1 and -2 recognize a 9 bp pseudopalindromic sequence [CAAT(A/T)ATTG] and [CAAT(G/C)ATTG], respectively, and interact with the DNA as homodimers (Sessa *et al.*, 1993). Homodimers of HD-ZIP proteins of groups 1 and 2 as well as heterodimers of all HD-ZIP proteins of the same family were also found in rice (Meijer *et al.*, 2000). Moreover, group 1 ATHB5 recognizes similar DNA sequences but preferably binds at the central position to A/T versus G/C (Johannesson *et al.*, 2001). Sunflower HD-ZIP group 1 also binds to the sequence [CAAT(A/T)ATTG] (Palena *et al.*, 1999). According to the EMSA of the durian TFs, DzHD-ZIP1.8 can bind to the DNA probe containing the predicted HD-ZIP binding site [TTCAATTATTAGA] located on fragment II

(Fig. 7a), which has a similar core sequence, [CAATTATTA] but the nucleotide at the position 9 indicated A instead of G. Replacing a single nucleotide at positions 1, 5, or 9 decreased binding of group 1 sunflower HD-ZIP (Palena et al., 1999). The mutation sequence at positions 1 and 9 from C to T and G to A, respectively [TAAT(A/T)ATTA] can also reduce the binding of a group 1 HD-ZIP of *Medicago truncatula* (Ariel et al., 2010). The predicted binding site on fragment V [TAATCATTCAC] exhibits the 9 bp core sequence [TAATCATTC] is similar to the sequences presented above with T and C replacements at positions 1 and 9, respectively. Based on those findings, durian share the same core sequences which are 8 bp [CAATTATT] on fragment II and 7 bp [AATCATT] on fragment V.

Additionally, Y1H screening also provided other TF families such as NAC, heat shock protein, and myb-like, which supported the growth of yeast cells. NAC is one of the largest plant-specific TF families and also influences fruit ripening (Liu et al., 2022); it reportedly regulates ester volatile biosynthesis, which is related to flavor in peach fruit (Cao et al., 2021). Myb-like TFs interact with scent-related genes in flowers (Spitzer-Rimon et al., 2012). Moreover, HD-ZIP also forms complexes with others TF families to regulate various aspects in plants such as MYBs to initiate glandular secretory trichome (Xie et al., 2021), and C2H2-ZFP/WD-repeat to regulate the size of spine base in cucumber (Yang et al, 2022). According to Y1H result, not only HD-ZIP can be identified to be regulate *DzMGL* but that also other TFs may be potential candidates to bind to the promoter or form a TF complex to drive transcription during ripening. However, the functions of other TFs in durian should be investigated in future studies.

In conclusion, we identified a novel durian HD-ZIP, DzHD-ZIP1.8, which regulates the expression of *DzMGL* in durian fruit during ripening and therefore the production of VSCs. EMSA revealed at least two HD-ZIP binding sites on the promoter region of the durian *MGL* gene. These results provide the information on group 1 of HD-ZIPs that regulates VSC production, which determines fruit quality and therefore consumer preferences.

Declarations

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Conflict of interest

The authors declare no competing interests.

Author contributions

PP and SS conceived and designed the research. SS and RH supervised this work. PP performed the experiments, analyzed the data, wrote the first draft of the manuscript. PP, RH, and SS revised the manuscript.

Data availability

All data analyzed during this study are included in this published article.

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Supplementary Tables

Supplementary Tables S1-S3 are not available with this version

Figures

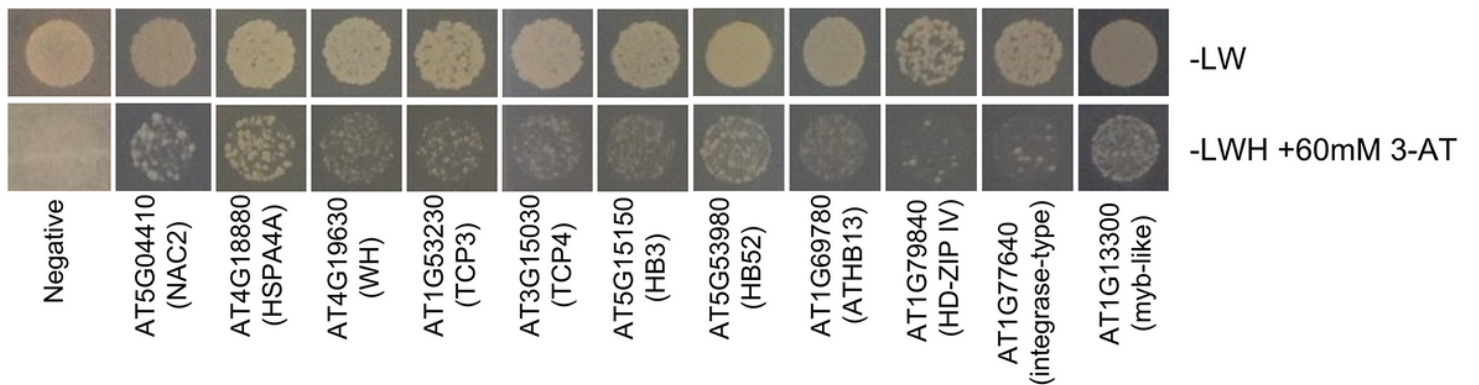


Figure 1

Yeast one-hybrid screening of durian promoter of *MGL* (*pDzMGL*) and *Arabidopsis* transcription factors (AtTFs). The upper row shows diploid yeasts growing in SD medium without leucine and tryptophan (-LW). The lower row shows the positive interaction between *pDzMGL* and AtTFs growing under SD medium with 60 mM 3-amino-, 2, 4-triazole (3-AT) and without leucine, tryptophan, and histidine (-LWH +60 mM 3-AT).

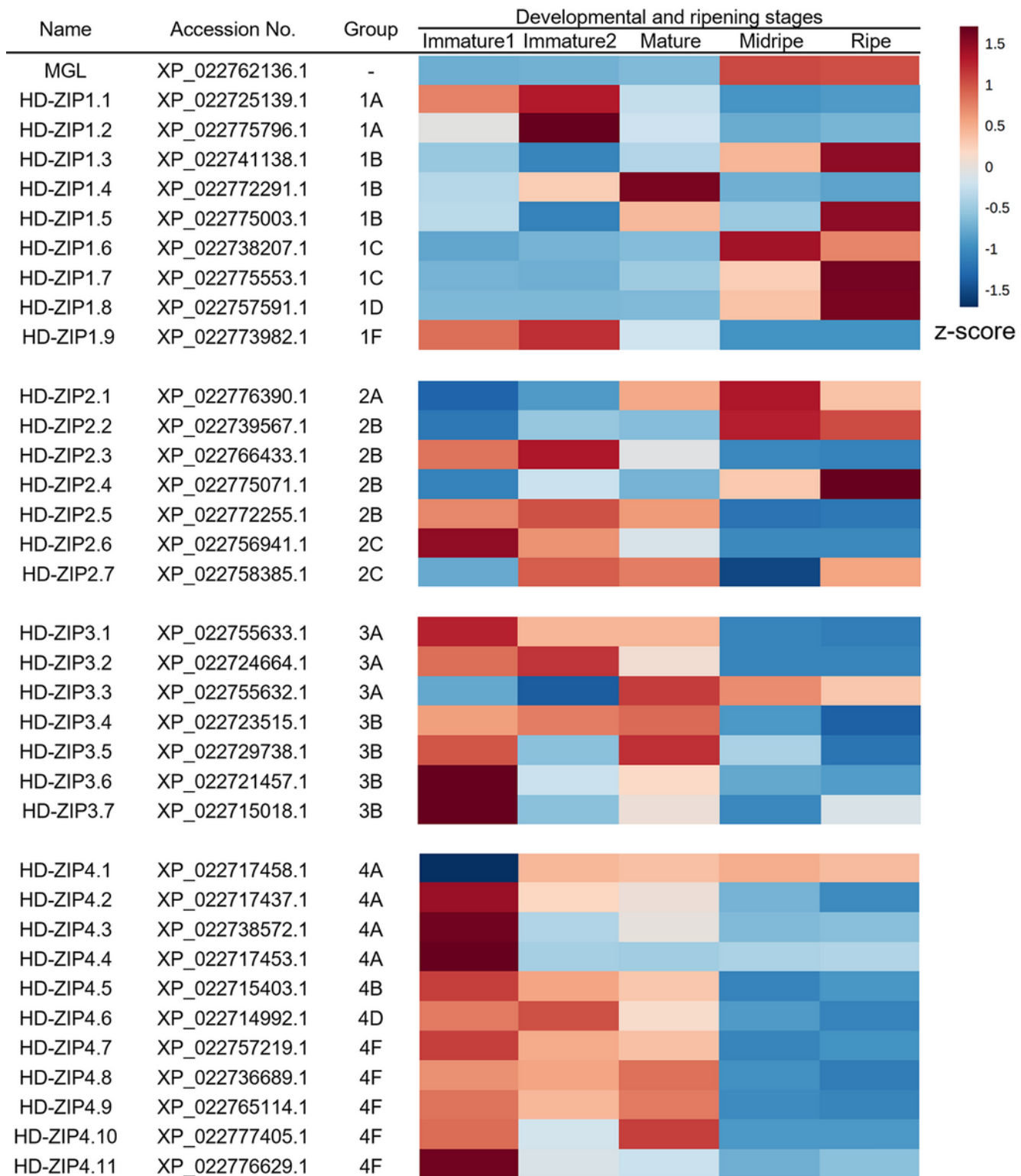


Figure 2

Heatmap analysis of *DzHD-ZIP* transcription factors and methionine g-lyase (*DzMGL*) transcript levels obtained from the transcriptome data of durian cv. Monthong at five stages (immature1, immature2, mature, midripe, and ripe).

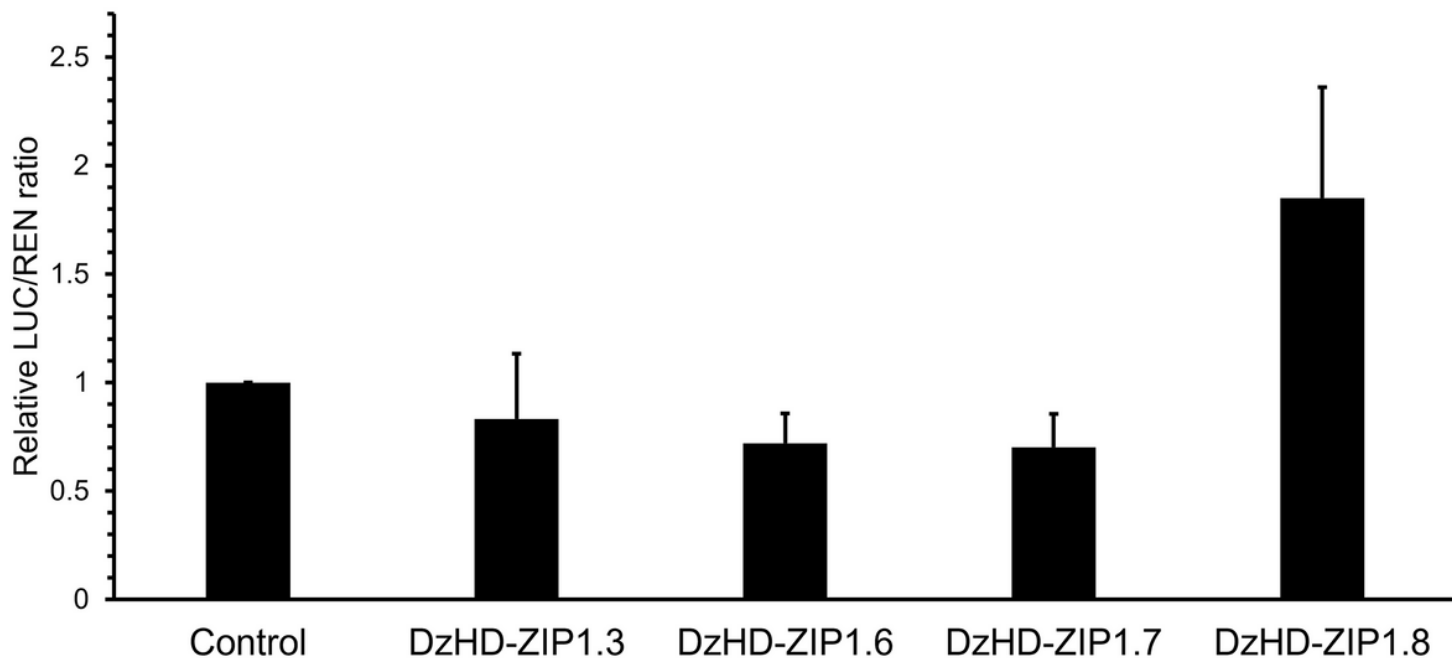


Figure 3

Dual-luciferase activity assay performed in *Arabidopsis* protoplasts. The LUC/REN ratio of the interaction between full-length *pDzMGL* and each DzHD-ZIP transcription factor was normalized to the control, which contained full-length *pDzMGL* and the vector without transcription factor (LUC/REN ratio = 1.0).

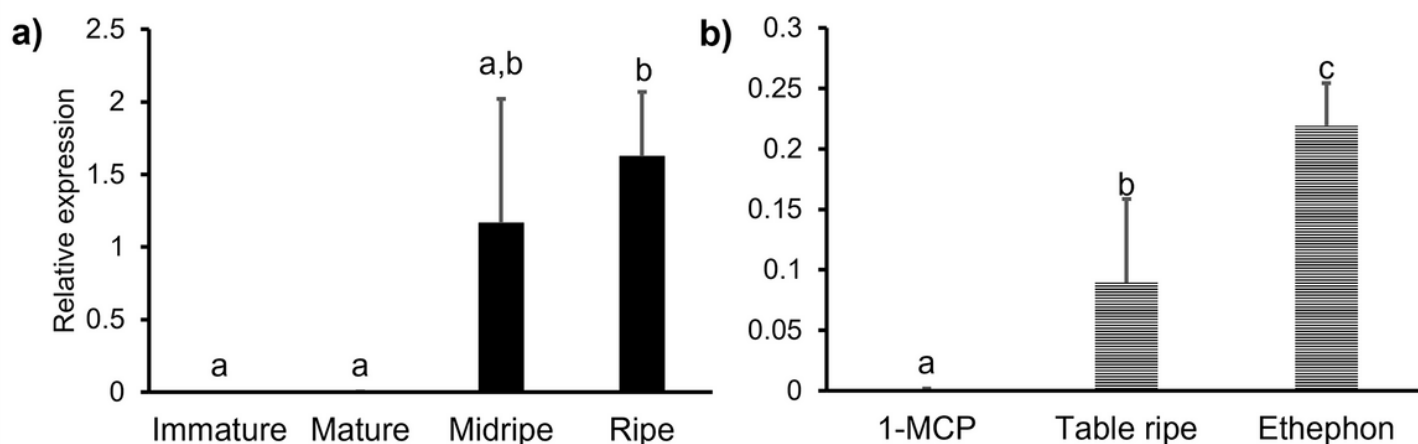


Figure 4

RT-qPCR analysis of *DzHD-ZIP1.8* in durian pulps. (a) The expression level of *DzHD-ZIP1.8* in durian pulps during ripening (immature1, mature, midripe, and ripe stages). (b) The expression level of *DzHD-ZIP1.8* in pulps of the fruit treated with 1-MCP and ethephon compared to the naturally ripe fruit (table ripe). Different lowercase letters represent values significantly different at 95% confident level.

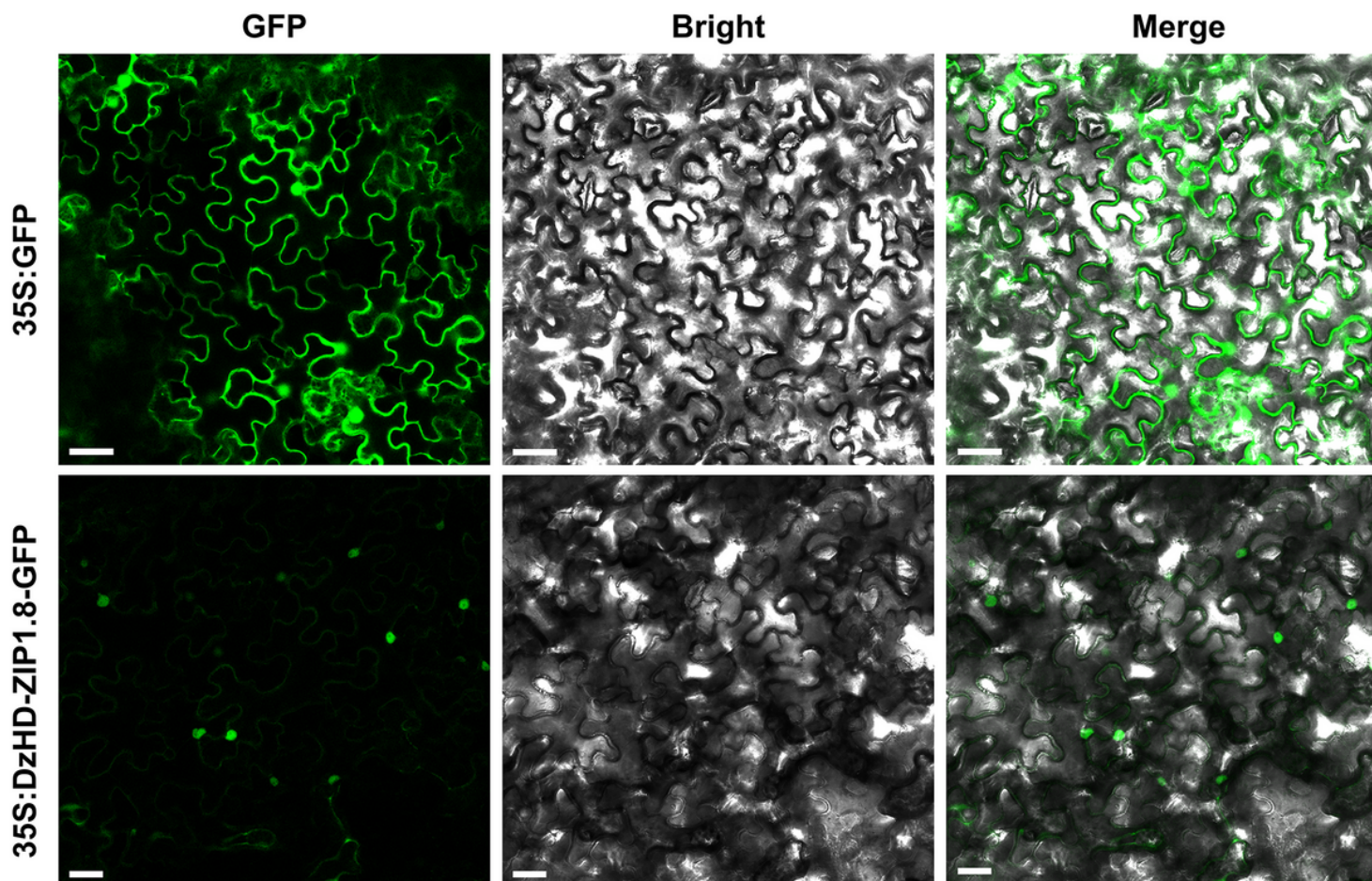


Figure 5

Subcellular localization of DzHD-ZIP1.8 in *Nicotiana benthamiana* leaves. Epidermal cells were imaged using green fluorescence (GFP), visible light (bright field), and merged light. A green fluorescent protein (35S:GFP) was used as a control. Scale bars represent 38 μm .

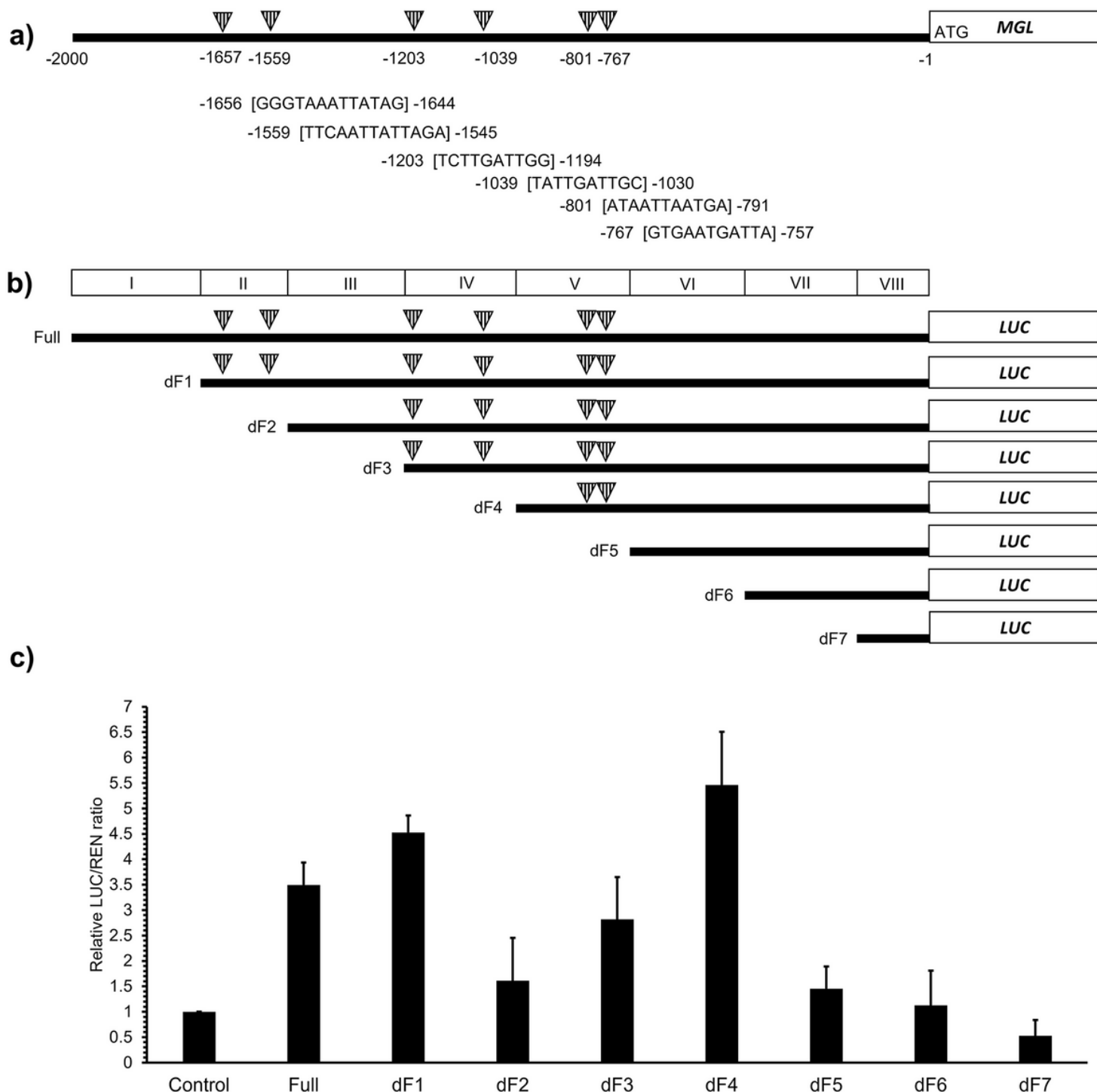


Figure 6

Promoter analysis and the effect of promoter deletion on LUC/REN ratio. **(a)** In silico promoter analysis of *pDzMGL*. Transcription factor binding sites on the promoter region were predicted with a web-based program, PlantRegMap, using its *Arabidopsis* transcription factor database. **(b)** The full-length promoter sequence was sliced into short fragments from the 5' end (dF1–dF7) and fused with *LUC*. **(c)** Dual-luciferase activity assay was performed in *Nicotiana benthamiana* leaves. LUC/REN ratios of DzHD-ZIP1.8 and promoter fragments were normalized to the control (LUC/REN ratio = 1.0).

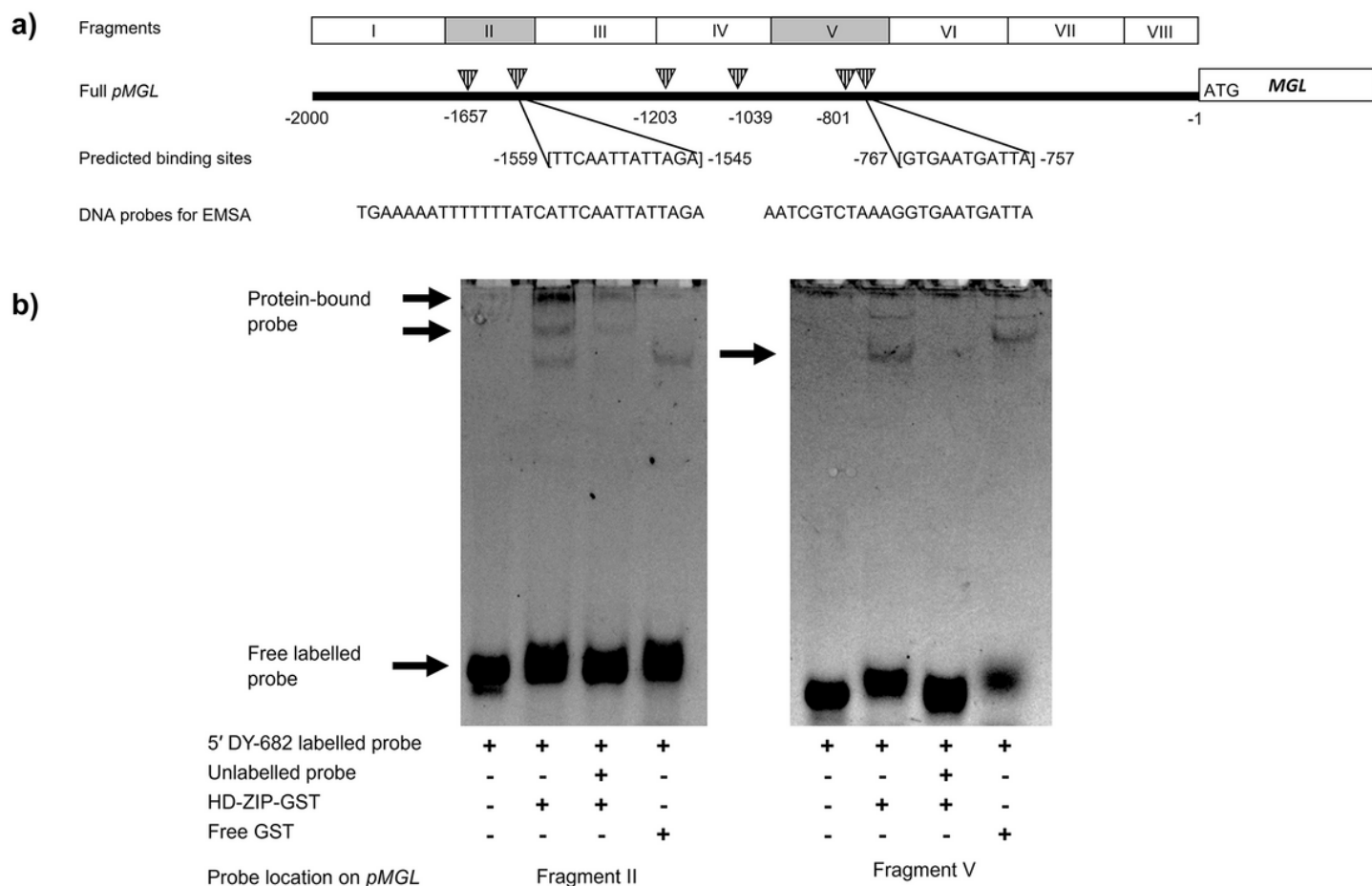


Figure 7

Electrophoresis mobility shift assay (EMSA) and probe design. **(a)** Designed probes and locations of transcription factor binding sites on the promoter of *DzMGL*. **(b)** EMSA analysis of *DzHD-ZIP1.8* and cis-element containing oligonucleotide probes. Upper arrows represent protein-bound probes. Lower arrow represents free labeled probes in lane 2. Only DY-682-labeled probes were added to lane 1, DY-682-labeled probes and proteins fused with GST tag were added to lane 2, unlabeled competitive probes were added to lane 3 together with the labeled probes, and the proteins, DY-682-labeled probes, and free GST protein were added to lane 4.

Supplementary Files

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- [Keymessage.docx](#)
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