Unveiling phenylpropanoid regulation: the role of DzMYB activator and repressor in durian (Durio zibethinus) fruit

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Research Article

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Abstract

The durian fruit has high nutritional value attributed to enriched bioactive compounds, including phenolics, carotenoids, and vitamins. While various transcription factors (TFs) govern phenylpropanoid biosynthesis, MYB TFs emerge as pivotal players in regulating key genes within this pathway. This study delves into the identification of additional candidate MYB TFs from the transcriptome database of the Monthong cultivar at five developmental/postharvest ripening stages. Candidate transcriptional activators were discerned among MYBs upregulated during the ripe stage, based on the positive correlation observed between flavonoid biosynthetic genes and flavonoid contents in ripe durian pulps. Conversely, MYBs downregulated during the ripe stage were considered candidate repressors. The study focused on a candidate MYB activator (DzMYB2) and a candidate MYB repressor (DzMYB3) for functional characterization. LC–MS/MS analysis in Nicotiana benthamiana leaves transiently expressing DzMYB2 revealed increased phenolic compound contents compared to those expressing green fluorescence protein controls, while transiently expressed DzMYB3 led to a decrease in phenolic compounds. Furthermore, it was demonstrated that DzMYB2 controls phenylpropanoid biosynthesis in durian by regulating the promoters of various biosynthetic genes, including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR). Simultaneously, DzMYB3 regulates the promoters of PAL, 4-coumaroyl-CoA ligase (4CL), CHS, and CHI, resulting in the activation and repression of gene expression, respectively. Additionally, it was discovered that DzMYB2 and DzMYB3 could bind to another TF, DzbHLH1, in the regulation of flavonoid biosynthesis. These findings enhance our understanding of the pivotal role of MYB proteins in regulating the phenylpropanoid pathway in durian pulps.

Key Message

DzMYB2 functions as an activator, while DzMYB3 acts as a repressor. They bind to promoters, interact with DzbHLH1, and influence phenolic contents, revealing roles in phenylpropanoid regulation in durian pulps.

1. Introduction

Phenylpropanoids, one of the largest classes of natural plant compounds, are synthesized from primary metabolites, phenylalanine or tyrosine, through a series of enzymatic reactions. They can be categorized into five groups based on their chemical structures: flavonoids (including anthocyanins, proanthocyanidins, flavonols, flavones, flavanones, and isoflavonoids), monolignols, phenolic acids, stilbenes, and coumarins (Liu et al., 2015). These compounds are ubiquitous in the plant world and play vital roles in plant growth. They are integral to cell walls, protect against intense light and ultraviolet (UV) radiation, serve as phytoalexins against herbivores and pathogens, and act as floral pigments to facilitate plant-pollinator interactions. Additionally, phenylpropanoids have a wide range of biological

properties that are beneficial to human health (Deng & Lu, 2017). Enzymes such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-Coumarate: CoA ligase (4CL) are involved in the general phenylpropanoid pathway. The metabolic intermediates produced in this pathway are channeled into the synthesis of flavonoids (Pratyusha & Sarada, 2022).

Phenolic acids, a type of phenolic compound, are aromatic acid molecules characterized by a benzene ring attached to one or more hydroxyl or methoxy groups. They can be divided into two primary groups based on their carbon structures: hydroxycinnamic acids (C6–C3), which include p-hydroxybenzoic, vanillic, and protocatechuic acids, and hydroxybenzoic acids (C6–C1), which include chlorogenic, ferulic, rosmarinic, and sinapic acids. These compounds have medicinal significance, exhibiting antioxidant properties and effects such as antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and vasodilatory effects (Mattila and Hellström, 2007).

Another category of phenolic compounds, flavonoids, have biological effects including anti-allergic, antiviral, and anti-inflammatory properties (David et al., 2016). They can be classified into six main subclasses based on their chemical structures: flavones, flavanones, flavanols, flavonols, isoflavones, and anthocyanins/anthocyanidins (Li et al., 2018). In plants, most flavonoids are present as glycosides, enhancing their solubility and reducing their toxicity (Brodowska, 2017). The initial enzyme in this pathway, chalcone synthase, produces chalcone scaffolds that serve as the basis for all flavonoid compounds. A set of enzymes modify the basic flavonoid structure, leading to various subclasses depending on the plant species (Falcone Ferreyra et al., 2012).

MYB transcription factors (TFs) play a pivotal role in a variety of crucial processes in higher plants, including development, differentiation, secondary metabolism, signal transduction, and mechanisms to withstand biotic and abiotic stresses (Pratyusha & Sarada, 2022). Characterized by a highly conserved MYB domain with the ability to bind DNA, MYB proteins include 1R (R1/2, R3-MYB), 2R (R2R3-MYB), 3R (R1R2R3-MYB), and 4R (R1/R2-like repeats) (Dubos et al., 2010). The first identified MYB gene was the oncogene (v-myb) found in the *avian myeloblastosis* virus (Klempnauer et al., 1982). In plants, the first characterized MYB gene encoded the anthocyanin regulatory colorless 1 (C1) protein in *Zea mays* (Paz-Ares et al., 1987). MYBs are known to be widespread in various organisms, including animals, fungi, and plants, with the N-terminal region being highly conserved and the C-terminal region exhibiting greater variability (Dubos et al., 2010).

MYB proteins function by either directly or indirectly interacting with specific DNA sequences, leading to the activation or suppression of genes. The genes responsible for producing flavonoids are differentially regulated by a complex comprising R2R3 MYB TFs, basic helix-loop-helix (bHLH) proteins, and WDR proteins (Xu et al., 2015). This collaboration, collectively referred to as the MBW complex, determines the expression of structural genes involved in flavonoid biosynthesis. Numerous studies have confirmed the role of MYB TFs in overseeing genes associated with phenylpropanoid biosynthesis, acting as activators and repressors (Liu et al., 2015; Ma & Constabel, 2019). Research on the MYB family in diverse plant species has identified that the C-terminal portion of MYB repressors encompasses conserved motifs

referred to as the TLLLFR motif and C2/EAR motif (LxLxL or DLNxxP) (Ma and Constabel, 2019). The regulation of the phenylpropanoid pathway varies across species, involving either a MYB acting alone or the formation of an MYB–bHLH dimer or an MBW complex. In *Arabidopsis thaliana*, AtMYB11, AtMYB12, and AtMYB111 can individually activate genes like chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonol synthase (FLS), collectively influencing flavonoid content (Mehrtens et al., 2005; Stracke et al., 2007; Pandey et al., 2012, 2014). In maize, the R2R3-MYB; C1 and bHLH; LC form an MYB–bHLH dimer that enhances flavonol synthesis (Bovy et al., 2002). Conversely, heterologous expression of the R2R3-MYB repressor FaMYB1 in transgenic tobacco leads to a reduction in flavonoid accumulation (Aharoni et al., 2001). Regarding phenolic acid regulation, the heterologous expression of snapdragon ROSEA1 in *Salvia miltiorrhiza* boosts the levels of both rosmarinic and salvianolic acids (Wang et al., 2013). Additionally, constitutive expression of potato genes StAN1 and StMTF1 increases chlorogenic acid content, similar to the effect observed with the expression of AtMYB12 in tobacco (Luo et al., 2008; Rommens et al., 2008; Payyavula et al., 2013). Overexpressing SmMYB39 inverts the synthesis of rosmarinic acid by acting as a repressor of both C4H and a gene encoding tyrosine aminotransferase, leading to a reduction in 4-coumaric acid, rosmarinic acid, salvianolic acid, and total phenolic content. (Zhang et al., 2013).

Durian (*Durio zibethinus* L.) is a tropical fruit that grows seasonally in several Southeast Asian nations, including Thailand, Malaysia, and Indonesia, and is often referred to as the “king of fruits.” This fruit holds significant economic importance in Thailand and is nutritionally valuable due to its abundance of bioactive compounds, such as phenolics, carotenoids, and vitamins (Gorinstein et al., 2011). Flavonoid concentrations may vary across different durian varieties and stages of ripeness. Among these, the Mon Thong cultivar demonstrated the highest flavonoid content, surpassing Chanee, Kan Yao, Puang Manee, and Kradum varieties (Toledo et al., 2018; A. Aziz and Mhd Jalil, 2019). Additionally, it was observed that ripe durians generally exhibited the highest levels of flavonoids (Arancibia-Avila et al., 2008).

DzMYB1, identified and characterized as the first durian MYB activator by Weerawanich et al. (2024), operates as a homodimer and interacts with homodimerized DzbHLH1, controlling flavonoid production in durian by influencing the promoters of multiple biosynthetic genes, including *CHI*, *CHS*, and *F3H*. However, the characterization of a DzMYB repressor remains outstanding.

In this study, we utilized the transcriptome database of the Monthong durian cultivar to identify specific candidate MYB activators and repressors responsible for regulating phenylpropanoid biosynthesis. These identified MYB TFs were subsequently subjected to detailed characterization. Acknowledging that phenylpropanoid biosynthesis is influenced by various TFs, MYB proteins emerge as pivotal in governing the target genes in this pathway (Anwar et al., 2019). Thus, our objective in this study is to gain a deeper understanding of the interplay between MYB activators and repressors in the biosynthesis of phenylpropanoid during durian fruit ripening.

2. Method

2.1 Plant materials

Durian fruit of the cv. Monthong *(D. zibethinus* L. “Monthong”) variety were harvested at different developmental stages from a commercial orchard in Trat, Thailand. These stages encompassed immature1 (85 days after anthesis (DAA)), immature2 (98 DAA), and mature stages (105 DAA), representing on-tree developmental stages. Postharvest ripening stages included midripe (3 days after harvest (DAH)) and ripe stages (5 DAH). The peel was meticulously removed to obtain pulp samples, which were then extracted as per the method outlined by Suntichaikamolkul et al. (2021) for gene expression analysis and transcriptome database creation (Suntichaikamolkul et al., 2021).

Seeds of *A. thaliana* and *Nicotiana benthamiana* were sown in small pots filled with peat moss and nurtured under controlled conditions: a temperature of 25°C, a light intensity of 4000 lux, and a 16-h photoperiod. After two weeks, the plants were individually relocated to separate pots and continued to be cultivated under the same conditions for a month.

Tomato seeds (*Solanum lycopersicum* cv Micro Tom) were soaked in water for three days, then sown in small pots filled with peat moss and placed under controlled environmental conditions, maintaining a temperature of 25°C, a light intensity of 4000 lux, and a 16-h photoperiod. After three weeks, the saplings were transferred to individual pots and continued to grow under these specified conditions for 1.5 months, until the fruit emerged and reached the breaker stage.

2.2 Transcriptome analysis

The transcriptome data for the durian fruit cv. Monthong at the five stages was processed using OmicsBox (v1.4.1.1). This data was sourced from the study by Suntichaikamolkul et al. (2021), with the project accession numbers being PRJNA683229 (for the mature and ripe stages) and PRJNA732556 (for the immature 1, immature 2, and midripe stages).

2.3 Transcriptome identification and expression pattern

MYB TFs, phenylpropanoid biosynthetic genes, and basic helix-loop-helix proteins (bHLHs) were extracted from the transcriptome data sourced from Weerawanich et al. (2024). The expression levels of these elements were represented using reads per kilobase of transcript per million reads mapped (RPKM) values, which provide a standardized measure of transcript expression. The gene expression across the five stages of the Monthong cultivar, as well as the tissue-specific expression pattern of ripening-associated DzMYBs in the Musang King cultivar at the ripe stage (obtained from MaGenDB; Wang et al., 2020), were visualized in a heatmap. This visualization was created using the Euclidean distance method through MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/; Pang et al., 2021).

2.4 Phylogenetic analysis

Candidate genes encoding MYB TFs from the transcriptome dataset were translated into amino acid sequences using an online tool (https://web.expasy.org/translate/). These amino acid sequences of MYB proteins from durian, along with those of reported TFs involved in phenylpropanoid biosynthesis

(Liu et al., 2015), were aligned using ClustalW in MEGA X software with default settings. A phylogenetic tree was then generated using the neighbor-joining method with 1000 bootstrap replicates in the MEGA X program (Kumar et al., 2018).

2.5 Total RNA isolation and quantitative reverse transcription PCR (RT-qPCR) analysis

Total RNA was isolated from durian frozen pulp powder using the PureLink Plant RNA reagent (Invitrogen, USA) as per the manufacturer's instructions. Any genomic DNA was eliminated using DNase I (Thermo Scientific, USA). For reverse transcription, 1 µg of total RNA was used to generate cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per the provided instructions. Specific primer sets for RT-qPCR were designed using the PrimerQuest tool (https://www.idtdna.com/pages/tools/primerquest) (Table S1). RT-qPCR was performed on the CFX96 Real-Time PCR System (Bio-Rad, USA) using Luna® Universal qPCR Master Mix (New England Biolabs, USA) and twenty-fold diluted cDNA samples. The PCR reaction mixture consisted of 1 µL of diluted cDNA, 5 µL of Luna Universal qPCR Master Mix, and 250 nM of each gene-specific primer, totaling a reaction volume of 10 µL. The PCR conditions were set as follows: 1 minute at 95°C, followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C. A melting curve was generated by heating from 65 to 95°C with increments of 0.5°C. The relative expression of each gene, normalized to the reference gene (elongation factor-1 alpha, EF-1α), was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6 Genomic DNA extraction

Genomic DNA was extracted from durian leaves using a NaCl/CTAB solution, as per the method described by Porebski et al. (1997). In brief, 50 mg of freshly ground leaf tissues were combined with 1.3 mL of CTAB solution. This mixture was heated at 60°C for 30 min and then centrifuged at 13,000× g for 30 min. DNA purification was achieved using a mixture of phenol/ chloroform/isoamyl alcohol (25/24/1, v/v/v). DNA precipitation was facilitated by adding an equal volume of 100% ethanol and centrifuging the solution at 13,000× g for 5 min. The resulting DNA pellet was dried and re-suspended in nuclease-free water.

2.7 Transient expression in plants

The coding sequences (CDS) of *DzMYB2* (XM_022901835.1) and *DzMYB3* (XM_022907326.1) were amplified from durian cDNA. After PCR, the products were purified and inserted into the pDONOR207 vector using BP Clonase II (Invitrogen, USA) per instructions. Subsequently, the CDS of DzMYB2 and DzMYB3 were transferred into the binary vector pGWB2 (Nakagawa et al., 2007) with LR Clonase II (Invitrogen, USA). The resulting construct was transformed into *A. tumefaciens* GV3101 via electroporation. A single recombinant *A. tumefaciens* colony, containing the gene of interest, was cultured in lysogeny broth supplemented with kanamycin (50 $\mu g.mL^{-1}$), rifampicin (50 $\mu g.mL^{-1}$), and gentamycin (50 $\mu g.mL^{-1}$). The culture grew for two nights at 30°C with agitation at 250 rpm, and cells

were harvested by centrifugation at 5000× g for 15 min. The pellet was re-suspended in 10 mM MMA buffer (MES–MgCl2–acetosyringone) to achieve an OD_{600} of 0.5, followed by incubation at room temperature for 3 h. These cells were used for co-infiltration with *A. tumefaciens* carrying the p19 gene (Lindbo, 2007) in a 1:1 ratio. Agrobacterium cells were injected into the interior of tomato fruit at the breaker stage and infiltrated into *N. benthamiana* leaves. Fruit

were harvested once red, typically within 3 to 5 days, and leaves after 5 days. The green fluorescence protein (*GFP*) gene served as a control. The fruit and leaves were promptly frozen in liquid nitrogen and stored at − 80°C for subsequent analysis.

2.8 LC-MS/MS analysis

The process commenced by subjecting frozen tomato fruit and *N. benthamiana* leaves to a two-step procedure involving freeze-drying followed by grinding. Subsequently, 20 mg of the resulting dried samples underwent extraction using a 1 ml extraction solvent composed of diethyl ether/methanol/water (2/5/2, v/v/v). This extraction solvent included 0.5 ppm of puerarin (for positive mode) and 0.5 ppm of camphor (for negative mode) as meticulously chosen internal standards. The samples were subjected to agitation at 1000 rpm for a precisely timed 15 min at 15°C using a shaking incubator from Eppendorf, Germany. Following this initial step, 400 µL of water was judiciously added and vortexed for an exact 30 sec. The resulting mixture underwent a carefully timed centrifugation at 12,000× g for precisely 1 min. The upper phase, carefully measured to total 200 µL, underwent a meticulous concentration process using a centrivap concentrator at a controlled temperature of 25°C for an exact 3 h until achieving complete solvent evaporation. Subsequently, the dried samples were reconstituted with a precisely formulated 10% methanol solution and meticulously filtered through a 0.22-µm microfilter before being meticulously transferred to a specially prepared vial. The subsequent analysis employed a high-quality C18(2) Luna column (100 mm × 2 mm × 2.5 µm) sourced from Phenomenex, Torrance, CA, USA. The column temperature was meticulously maintained at a controlled 40°C throughout the analytical process. A precisely formulated mixture of solvents A (water and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid) was employed with an optimized flow rate of 0.3 $mL.min^{-1}$. The gradient elution method commenced with an exact 0% B for a carefully timed 5 min, followed by a systematic increase to 20% B over a precise 3 min (held for 4 min), further raised to 25% B over an exact 1 min (held for 4 min), and then elevated to 50% B over a systematically timed 5 min. Subsequently, a linear gradient to 95% B was meticulously applied over an exact 1 min and held for a precisely timed 7 min. The entire analytical process was meticulously controlled, with a 2 µL injection volume employed, and the column temperature meticulously maintained at a controlled 35°C throughout the process. Compound detection was executed using a top-notch qTOF/MS LC-MS 9030 system from Shimadzu Corp., Kyoto, Japan, equipped with a highly efficient electrospray ionization source. The MS conditions were systematically fine-tuned for both positive and negative-ion modes, with a collision energy precisely set at 35 eV, DL temperature maintained at 250°C, and a drying gas (N_2) flow rate of 10.0 $L.min^{-1}$. The oven temperature was set at a controlled 40°C, and mass spectra were meticulously acquired within the m/z range of 50–3000.

For the critical phase of data analysis, the raw data obtained from mass spectrometry underwent a rigorous conversion process to the .mzml format using the LabSolution software. Initial metabolite annotation was executed using MS-DIAL (version 4.60) from RIKEN, meticulously comparing it with an extensive public metabolomics library available at (https://prime.psc.riken.jp/compms/msdial/main.html#MSP). Subsequently, tentative peaks underwent an exhaustive annotation process, including determination of their elemental formulas and comprehensive mass spectral fragmentation, using MS-FINDER 3.5 (https://prime.psc.riken.jp/) (Lai et al., 2018). The intensity of phenolic compounds underwent a meticulous normalization process using the peak area of puerarin and camphor, deliberately added as internal standards.

2.9 Dual-luciferase reporter assay

To perform the dual-luciferase assay, a 2000-base pair segment upstream of the biosynthetic gene promoter was integrated and fused with the firefly luciferase (LUC) gene from *Photinus pyralis* within the pGreenII-0800-Luc vector. This integration was accomplished using the In-fusion HD Cloning Kit (Takara Bio Inc., Otsu, Japan) through the HindIII site. Both the *DzMYB2* and *DzMYB3* genes from durian cDNA were inserted into the pGreenII-62-SK vector, operating under the 35S promoter, employing the In-fusion HD Cloning Kit (Takara Bio Inc., Otsu, Japan) through the BamHI and HindIII sites.

To explore interactions between MYB TFs and the promoters of biosynthetic genes, the dual-luciferase reporter assay was implemented, following the protocol outlined by Hellens et al. (2005). This assay involved the co-transfection of a combination of vectors. Specifically, the pGreenII-0800-Luc vector containing each promoter (10 μg) and the pGreenII-62-SK vector containing either *DzMYB2* or *DzMYB3* (10 μg) were introduced into Arabidopsis protoplasts (5×10^4 cells) isolated from leaves. This introduction followed the procedure described by Yoo et al. (2007). To observe the combined activity of both *DzMYB2* and *DzMYB3* in the cells, *DzMYB2* and *DzMYB3* in the pGreenII-62-SK vector were co-transfected. The vector used in this assay encompasses the firefly luciferase gene under the control of the promoter of interest, while the Renilla gene (REN), driven by the constitutive 35s promoter, serves as the internal control (Hellens et al., 2005). The transfection reaction was allowed to incubate overnight, for approximately 16 h. Subsequently, the dual-luciferase activities were quantified using a Synergy H1 Plate Reader (Bio Tek, USA) equipped with luminescent detectors. Each co-transfection was replicated three times.

2.10 Yeast two hybrid (Y2H)

To unravel the protein interactions involving DzMYB2, DzMYB3, the previously documented DzMYB (DzMYB1), and the reported DzbHLH (DzbHLH1) implicated in flavonoid biosynthesis (Weerawanich et al., 2024), DzMYB1 and DzbHLH1 were strategically cloned into pGBKT7 as the bait protein. This cloning process involved fusion with the Gal4 DNA-binding domain (DNA-BD) and was executed through the In-fusion HD Cloning Kit (Takara Bio Inc., Otsu, Japan), utilizing the EcoRI and BamHI sites. Simultaneously, *DzMYB2* and *DzMYB3* were cloned into pGADT7, housing the Gal4 activation domain through the SmaI site. The cotransformation method was employed to validate the protein interactions. For the

transformation, the yeast strain Y2HGold was utilized, and the recombinant plasmid was introduced via the LiAc method. Transformants underwent selection on SD/−Leu/−Trp medium (DDO), SD/−Ade/−His/−Leu/−Trp (QDO), and SD/−Ade/−His/−Leu/−Trp supplemented with X-alpha-Gal and Aureobasidin A (QDO/X/A), following the detailed procedures outlined in the Matchmaker Gold Yeast Two-Hybrid System manual (Takara, Japan).

3. Results

3.1 DzMYB2 exhibited relatively high expression during the ripening stage, while DzMYB3 decreased expression at ripening stage

The first MYB identified in durian, DzMYB1, was reported by Weerawanich et al. (2024). This study builds upon that work by identifying additional novel MYBs potentially involved in phenylpropanoids biosynthesis. These MYB TFs were identified using transcriptome data from durian fruit cv. Monthong across five distinct stages: immature1 (IM1), immature2 (IM2), mature (M), midripe (MR), and ripe (R). From this data, 117 MYBs were found in durian pulp (Weerawanich et al., 2024). Among these, seven candidate MYB activators and repressors were randomly screened. Changes in phenolic profiles were assessed by transiently expressing these MYB genes in *N. benthamiana* leaves and comparing them to the GFP control using HPLC at 320 nm. Two MYBs were selected for further study: one that showed a significant increase (*DzMYB2*) and one that showed a significant decrease (*DzMYB3*) in expression during the ripening stage. A heat map analysis of *DzMYB2* and *DzMYB3* was conducted using the RPKM values (Fig. S1a). Previous studies (Arancibia-Avila et al., 2008) have shown that the ripe stage exhibited the highest concentration of flavonoids in durian fruit and that the high expression of the durian MYB during the ripening stage is consistent with its role as an activator (Weerawanich et al., 2024). Given this, it is suggested that DzMYB2, which exhibits high expression in durian pulp during ripening, may potentially act as a candidate activator. Conversely, DzMYB3, which has low expression during the ripening stage, may potentially function as a candidate repressor.

Furthermore, through the examination of publicly accessible transcriptomic data encompassing various durian cv. Musang King tissues (stem, root, leaf, and pulp) sourced from MaGenDB, Weerawanich et al. (2024) conducted an assessment of the expression levels of ripening-associated DzMYBs across these tissue types. It was found that DzMYB2 and DzMYB3 showed increased expression in durian pulp, while their expression was relatively low in other tissue (Fig. S1b).

The expression patterns of DzMYB2 and DzMYB3 were then validated using RT-qPCR. This confirmed their consistency with the transcriptome data, as reflected by the RPKM values (Fig. 1). This RT-qPCR validation provides strong evidence for the reliability and accuracy of the transcriptome data.

3.2 DzMYB2 was clustered with phenylpropanoid -related activators, whereas DzMYB3 grouped with phenylpropanoid-related repressors-like

In many plant species, MYB TFs have been found to act as both activators and suppressors in processes related to phenylpropanoid (Liu et al., 2015). A phylogenetic analysis was carried out on 16 plant R2R3-MYB proteins, which are associated with various functions in phenylpropanoid biosynthesis, to understand the potential roles of DzMYB2 and DzMYB3. The analysis showed that DzMYB2 is part of the MYB activator group, while DzMYB3 is closely related to the MYB repressor group (Fig. 2a). This indicates that DzMYB2 and DzMYB3 could function as an activator and repressor, respectively, in phenylpropanoid biosynthesis.

Furthermore, DzMYB2 and DzMYB3 proteins showed alignment with previously identified R2R3-MYB TFs. DzMYB2, which has an 870 bp coding sequence, encodes 290 amino acid residues. However, DzMYB3, with a 750 bp coding sequence, codes for 249 amino acid residues. As shown in Fig. 2b, both DzMYB2 and DzMYB3 have a complete and characteristic R2R3 domain, which is crucial for DNA sequence binding, and a bHLH-interacting motif for interaction with bHLH binding sites. In addition, DzMYB3 includes a C2/EAR motif (LxLxL or DLNxxP), a conserved motif linked with MYB repression, as well as a Zing finger and C4 motif located at the C-terminus.

3.3 DzMYB2 enhanced phenolic compounds in both tomato and N. benthamiana while DzMYB3 reduced phenolic level in N. benthamiana

We conducted transient overexpression experiments to characterize DzMYB2 and DzMYB3. *DzMYB2* was overexpressed in tomato fruit and *N. benthamiana* leaves, while *DzMYB3* was overexpressed only in *N. benthamiana* leaves. We then analyzed the changes in phenolic compounds in the tomato and *N. benthamiana* samples that were transiently overexpressed with either *DzMYB2* or *DzMYB3.* This was compared to the *GFP* control using liquid chromatography-mass spectrometry (LC-MS/MS). Among all annotated metabolites in tomato fruit and *N. benthamiana* leaves identified by MS-DIAL and MS-FINDER, we observed the top four compounds with the highest intensity of phenolic compounds. In tomato fruit, we found that chlorogenic acid, kaempferol-3-*O*-rutinoside, and quercetin-3-*O*-rutinoside (rutin) accumulated to higher levels in *DzMYB2* transiently overexpressed fruit compared to the control *GFP*. However, naringenin-7-*O*-glucoside (prunin) showed no significant difference (Fig. S2). In *N. benthamiana* leaves, *DzMYB2* transiently overexpressed plants showed an increase in chlorogenic acid and feruloylquinic acid. Interestingly, naringenin-7-*O*-glucoside (prunin) and kaempferol-3-*O*-rutinoside, which were not detected in the control, were found in *DzMYB2*-infiltrated leaves. Additionally, transient overexpression of *DzMYB3* led to a decrease in chlorogenic acid (Fig. 3). The mass-to-charge ratio (m/z), retention time, and mass fragments are detailed in Table S2. These results confirm the roles of DzMYB2 and DzMYB3 in controlling phenylpropanoid biosynthesis, with DzMYB2 acting as an activator and DzMYB3 as a repressor, respectively.

3.4 DzMYB2 and DzMYB3 bind promoters of phenylpropanoid biosynthetic genes

DzMYB2 and DzMYB3 have shown the ability to increase and decrease certain phenolic acids and flavonoids, respectively, in transient plants. However, our main interest is in understanding

phenylpropanoid regulation in durian. As we are unable to conduct transient expression experiments in durian fruit, we decided to investigate the interaction between either DzMYB2 or DzMYB3 and the promoters of phenylpropanoid biosynthetic genes in durian. Our goal was to determine if our TFs, DzMYB2 and DzMYB3, could effectively bind to these promoters. The phenylpropanoid biosynthetic genes in durian were identified by Weerawanich et al. (2024), including *DzPAL1* (XM_022890420.1), *Dz4CL1* (XM_022873869.1), *DzCHS1* (XM_022905317.1), *DzCHI1* (XM_022908829.1), *DzF3H1* (XM_022910592.1), *DzDFR1* (XM_022913282.1), and *DzUFGT1* (XM_022916049.1). We examined these selected paralogs for binding activity using the luciferase reporter assay (Fig. 4a). By calculating the firefly luciferase (LUC) to Renilla luciferase (REN) signal ratio, we were able to determine the binding activity between DzMYB2 or DzMYB3 and the promoters of phenylpropanoid biosynthetic genes. According to the results from the dual-luciferase activity assay of DzMYB2, the relative LUC/REN ratio of *pPAL*, *pDzCHS*, *pDzCHI*, and *pDzDFR* was significantly higher compared to the control group without DzMYB2 TF (Fig. 4b). Conversely, for DzMYB3, the relative LUC/REN ratio of *pPAL*, *pDz4CL*, *pDzCHS*, and *pDzCHI* was significantly lower compared to the control (Fig. 4c). These findings strongly suggest that DzMYB2 could activate *pPAL*, *pDzCHS*, *pDzCHI*, and *pDzDFR*, while DzMYB3 could repress *pPAL*, *pDz4CL*, *pDzCHS*, and *pDzCHI*.

To examine the combined effect of DzMYB2 and DzMYB3, we selected the promoters of *PAL* and *CHI.* The results showed that when both the MYB activator, DzMYB2, and the MYB repressor, DzMYB3, were co-transformed, the repressor reduced the binding activity of the activator (Fig. 4d).

Figure 4. Dual-luciferase activity assay conducted in Arabidopsis protoplasts. **a** Schematic representation of the effector and reporter constructs employed in the dual-luciferase experiment. **b** DzMYB2 demonstrates binding affinity to the promoters of *DzPAL*, *DzCHI*, *DzCHS*, and *DzDFR*. **c** DzMYB3 exhibits binding affinity to the promoters of *pPAL*, *pDz4CL*, *pDzCHS*, and *pDzCHI*. **d** The cumulative effect of DzMYB2 and DzMYB3 on the *PAL* and *CHI* promoters. The relative LUC/REN ratio indicates the interaction between the promoter of each phenylpropanoid biosynthetic gene and DzMYB TFs. The LUC/REN ratio was normalized to the control, consisting of each phenylpropanoid biosynthetic gene promoter and the vector without a transcription factor (LUC/REN ratio = 1.0). An asterisk above the bars denotes a significant difference between samples (student's t-test, **: $P < 0.05$, ***: $P < 0.01$). The promoters regulated by either DzMYB2 or DzMYB3 are highlighted within purple circles. Distinct letters signify statistically significant differences between stages ($p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test).

3.5 Y2H assay confirmed the interaction between DzMYB2 and DzMYB3 with DzBHLH1

MYB TFs typically do not function in isolation; instead, they often form complexes with other proteins (Xu et al., 2015; Nemesio-Gorriz et al., 2017). Among the various groups of TFs capable of interacting with MYB TFs, bHLHs have been identified (Yue et al., 2023). Within the DzbHLHs, DzbHLH1 has been recognized for its potential to bind with the previously reported DzMYB1, a crucial contributor to

flavonoid biosynthesis (Weerawanich et al., 2024). The cotransformation method was employed to investigate the interaction with DzbHLH1. Results from Y2H demonstrated that both DzMYB2 and DzMYB3 could interact with DzbHLH1 (Fig. 5). Additionally, considering that MYBs can form heterodimers or interact with other MYBs (Wei et al., 2018), the interaction of either DzMYB2 or DzMYB3 with the reported DzMYB, DzMYB1, was observed. The findings revealed that DzMYB2 and DzMYB3 were unable to bind with DzMYB1 (Fig. 5).

Figure 5. Yeast two-hybrid assay on selective media, which includes double dropout (DDO) medium: SD/–Leu/–Trp, QDO (quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp), and QDO/X/A (quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp supplemented with X-alpha-Gal and Aureobasidin A). The yeast cultures that were transformed were spotted in 10-fold serial dilutions. After a 3-day incubation period, a photograph of the yeast colonies was taken. For the positive control, recombinant yeast containing both pGBKT-53 and pGADT7-T was used, while yeast containing pGBKT-Lam and pGADT7-T served as the negative control.

4. Discussion

While MYB TFs have been extensively identified and characterized in various plant species (Liu et al., 2015), only one durian MYB activator has been reported so far (Weerawanich et al., 2024). To better understand the regulation of phenylpropanoid biosynthesis in durian, it is important to explore additional activators and repressors. In this study, transcriptome data from the Monthong variety was used to identify a novel activator and the first repressor durian MYB, designated as DzMYB2 and DzMYB3, respectively. Both of these showed significant differences from the GFP control when transiently expressed in *N. benthamiana* leaves, making them stand out among other candidates. *DzMYB2* showed its highest expression during the ripening stage (Fig. 1), exceeding the mature stage by more than tenfold. This strongly suggests that DzMYB2 plays a crucial role in the durian ripening process. This observation aligns with the findings of Arancibia-Avila et al. (2008), which highlighted the ripe stage as the period of highest flavonoid concentration in durian pulps. The results from Weerawanich et al. (2024) also confirmed that the increased flavonoid levels are consistent with the increased expression of biosynthetic genes and TFs during ripening. Also, *DzMYB3* showed expression in the opposite direction (Fig. 1), suggesting its potential role as a candidate repressor. This is similar to other plants like apples, where the expression levels of R2R3-MYB repressors have been found to be negatively correlated with anthocyanin levels (Xu et al., 2017).

The expression profiles of both *DzMYB2* and *DzMYB3* showed high levels in the ripe pulp while displaying lower expression in other tissues (Fig. S1b). This suggests their primary role in this tissue. However, some level of expression of *DzMYB2* and *DzMYB3* was observed in other tissues, indicating their potential involvement in functions beyond fruit-related processes. Factors such as UV light exposure and abiotic stresses like cold, salinity, and drought have been shown to influence flavonoid production in leaves (Ma et al., 2014). This expression pattern suggests that MYBs are likely involved in a wide range of physiological processes across various tissues.

The phylogenetic analysis placed DzMYB2 in a clade with phenylpropanoid activators, closely related to VvMYBPA1 and DkMYB4, which are known for their involvement in proanthocyanidin synthesis. Conversely, DzMYB3 clustered with phenylpropanoid repressors, closely associated with SmMYB39 and AmMYB308, which are associated with phenolic repression (Fig. 2a). This suggests a potential role for both DzMYB2 and DzMYB3 in phenylpropanoid biosynthesis. The alignment of the sequences of DzMYB2 and DzMYB3 with reported MYB activators and MYB repressors indicated that both DzMYB2 and DzMYB3 belong to the R2R3 MYB subclass (Fig. 2b). In the R3 domain, both DzMYB2 and DzMYB3, along with their homologous proteins, showcase a bHLH-interacting motif for bHLH binding sites, a feature that Zimmermann et al. (2004) had previously pointed out.

Repressor proteins, exemplified by DzMYB3, are distinguished by the presence of the C2 motif at the C-terminal end. This C2 motif, recognized by consensus sequences like DLNxxP or LxLxL, stands out as a prevalent transcriptional repression motif identified in plants. Its wide conservation extends to transcriptional regulators known for acting as negative regulators in diverse developmental and physiological processes across a broad spectrum of plant species (Kagale and Rozwadowski, 2011). Moreover, DzMYB3 also harbors the C4 motif and zing finger. The C4 motif, characterized by the conserved dFLGL and LDF/YRxLEMK sequences, is identified in the petunia MYB4 homolog, primarily responsible for the negative control of floral volatile benzoid/phenylpropanoid compounds (Colquhoun et al., 2011). This motif is also found in switchgrass (*Panicum virgatum*) PvMYB4, as well as in maize ZmMYB3, acting as negative regulators of the early phenylpropanoid and lignin pathways (Fornalé et al., 2010; Shen et al., 2012). Notably, despite its close relation, the snapdragon AmMYB308 does not contain the C4 motif (Tamagnone et al., 1998). Conversely, it appears that the zinc finger motif does not exhibit repressive activity. The role of the zinc finger in the regulatory region at the C-terminal end remains unclear for the R2R3-MYB subfamily four proteins (Shen et al., 2012).

Given the available data, there is a reasonable likelihood that DzMYB2 serves as a transcriptional activator for phenylpropanoid biosynthesis, while DzMYB3 is likely a transcriptional repressor. Nevertheless, additional comprehensive characterization is essential for confirmation.

The transient expression of *DzMYB2* in *N. benthamiana* leaves resulted in an elevation in the levels of phenolic acids and flavonoids, while the expression of *DzMYB3* led to a decrease in phenolic acids, consistent with our initial hypothesis (Fig. 3). Notably, not all phenolic compounds are repressed by DzMYB3, indicating its potential specificity in controlling the promoter of phenylpropanoid biosynthetic genes, resulting in the accumulation of specific compounds. We further validated this outcome in tomato fruit, our target tissue of interest, by transiently expressing *DzMYB2*. The results demonstrated an increase in phenolic compounds as well (Fig. S2), providing robust evidence that DzMYB2 functions as an activator for the phenylpropanoid pathway in both plant species and across different tissues.

As we were unable to conduct transient expression experiments in durian fruit to assess whether DzMYBs can activate phenolic compounds, our focus shifted to investigating the interaction between MYBs and phenylpropanoid biosynthetic genes in durian, as reported by Weerawanich et al. (2024). The

results revealed that DzMYB2 regulates the promoters of various biosynthetic genes, including *pDzPAL*, *pDzCHS*, *pDzCHI*, and *pDzDFR*. Concurrently, DzMYB3 regulates *pDzPAL*, *pDzC4L*, *pDzCHS*, and *pDzCHI*, highlighting the capacity of a single MYB to regulate multiple promoters (Fig. 4). This aligns with the findings for DzMYB1, which has been demonstrated to activate *pDzCHI*, *pDzCHS*, and *pDzF3H*, all of which are early biosynthetic genes in the flavonoid pathway (Weerawanich et al., 2024). These results indicated functional redundancy among durian MYB activators in controlling phenylpropanoid biosynthesis. The findings strongly support the roles of DzMYB2 as an activator and DzMYB3 as a repressor in the regulation of phenolic compounds in durian. Since both DzMYB2 and DzMYB3 regulate the same genes, we then observed their combined effect in the cell, focusing on *pDzPAL* and *pDzCHI*, key genes in the phenylpropanoid pathway. Notably, the results demonstrated that DzMYB3 could suppress the activity of DzMYB2. In other words, DzMYB2, an activator, can partially counteract the repression effect of DzMYB3, a repressor. This cooperative interaction between an activator and a repressor is a common regulatory mechanism observed in other plants, helping to maintain phenolic accumulation at an appropriate level within the cell (Zhang, 2020).

The regulation of phenylpropanoid biosynthesis is not solely dependent on MYB but also involves other TFs from three TF families: R2R3-MYB, bHLH, and WDR. R2R3-MYB TFs can function independently or as part of MBW complexes with bHLHs and WDRs. WDR proteins primarily serve as stable frameworks for interactions between proteins and DNA (Miller and Clay, 2016). The first 200 amino acids at the N-terminus of the bHLH protein are essential for interaction with the MYB TF, while the subsequent 200 amino acids enable binding with a WDR protein (Fan et al., 2021). In terms of function, the basic region is crucial for making DNA contacts, while the HLH region facilitates dimerization among bHLH proteins (Massari and Murre, 2000). However, MYB proteins play a central role in controlling the expression of target genes involved in the phenylpropanoid biosynthetic pathway (Anwar et al., 2019).

Given the potential of MYB proteins to interact with various partners, we investigated protein-protein interactions. The previously reported DzMYB1 functions as a homodimer and forms a complex with homodimerized DzbHLH1, regulating flavonoid biosynthesis in durian (Weerawanich et al., 2024). DzbHLH1 was selected for interaction studies with both DzMYB2 and DzMYB3. It was observed that both DzMYB2 and DzMYB3 have the capability to bind with DzbHLH1(Fig. 5), mirroring the interaction observed with DzMYB1. This indicates a relatively low specificity of bHLH proteins, consistent with a study by Hichri et al. (2010) where VvMYC1 interacted with MYB5a, MYB5b, MYBA1/A2, and MYBPA1 to activate flavonoid pathway gene promoters. Moreover, MYBs can form homodimers or heterodimers by interacting with another MYB (Persak and Pitzschke, 2013; Wei et al., 2018). Unfortunately, auto-activation issues with DzMYB2 and DzMYB3 in yeast prevented the observation of their dimerization. Attention was then directed to observing the heterodimerization of either DzMYB2 or DzMYB3 with DzMYB1. Results indicated that these two MYBs were unable to interact with DzMYB1 (Fig. 5), suggesting that their primary roles may not involve direct interaction with each other.

The interplay between a transcriptional activator and a repressor may involve interactions with bHLH TFs. MYB repressors within the flavonoid biosynthesis pathway have the ability to disrupt the MBW

complex by binding to bHLH co-activators. Studies with PhMYB27 in yeast three-hybrid assays have shown that MYB activators and repressors can simultaneously bind to the same bHLH cofactor (Albert et al., 2014). This suggests that the activation or repression at a promoter could depend primarily on the relative abundance of MYB activator and repressor proteins within the cells, with MYB repressors influencing gene expression directly by competing for binding sites with MYB activators (Ma et al., 2019).

Taking into account all the findings, a proposed molecular mechanism for how DzMYB2 and DzMYB3 control the presence of phenolic compounds in durian can be outlined. During various stages of fruit development, including immature and mature stages, durian pulp does not require high levels of phenolic content, unlike during ripening. At this point, the repressor, DzMYB3, is expressed and forms the MBW complex with bHLH1. Together, they bind to *pDzPAL* and *pDzCHI*, suppressing the expression of phenylpropanoid biosynthesis. As the durian fruit ripens, signals may trigger the expression of the activator, DzMYB2. DzMYB2 competes with DzMYB3 to bind with bHLH1, activating the expression of *pDzPA*L and *pDzCHI*, resulting in a high level of phenolic compounds at the ripe stage. To prevent excessive accumulation of phenolic compounds after the ripe stage, DzMYB3 may be activated by DzMYB2, providing delicate fine-tuning for balancing secondary metabolite accumulation in pulp cells (Fig. 6).

It is crucial to acknowledge the potential involvement of unidentified TFs in regulating the expression of phenylpropanoid biosynthetic genes in durian pulp. Additionally, DzMYB2 and DzMYB3 may have roles beyond their function in phenylpropanoid biosynthesis, potentially impacting other aspects of plant development. Further exploration in this area is warranted for a deeper understanding.

Conclusion

In this study, we identified *DzMYB2* with elevated expression levels during postharvest ripening and *DzMYB3* with low expression levels during postharvest ripening from the transcriptome database of Monthong as the candidate MYB activator and candidate MYB repressor, respectively. *DzMYB2* demonstrated the ability to enhance the expression of specific phenolic compounds, such as chlorogenic acid, kaempferol-3-*O*-rutinoside, and quercetin-3-*O*-rutinoside (rutin), transiently in tomato fruit. Additionally, it increased the levels of chlorogenic acid and feruloylquinic acid, as well as naringenin-7-*O*-glucoside (prunin) and kaempferol-3-*O*-rutinoside in *N. benthamiana* leaves. Conversely, *DzMYB3* reduced chlorogenic acid and feruloylquinic acid in *N. benthamiana* leaves.

We observed that DzMYB2 plays a crucial role in regulating phenylpropanoid biosynthesis in durian by modulating the promoters of various biosynthetic genes, including *PAL*, *CHS*, *CHI*, and *DFR*, leading to their activation. Simultaneously, DzMYB3 regulates *PAL*, *C4L*, *CHS*, and *CHI*. Furthermore, we demonstrated the impact of the MYB repressor, DzMYB3, on the MYB activator, DzMYB2, in terms of binding activity to *PAL* and *CHI* promoters. Additionally, our findings revealed that both DzMYB2 and DzMYB3 form complexes with homodimerized DzbHLH1 in the regulation of phenylpropanoid

biosynthesis. This study represents the first exploration of a MYB repressor responsible for regulating phenylpropanoid biosynthesis in durian.

Abbreviations

CDS Coding sequences

CHI Chalcone isomerase

CHS Chalcone synthase

DFR Dihydroflavonol reductase

EBG Early biosynthetic genes

FLS Flavonol synthase

GFP Green fluorescence protein

HLH Helix-loop-helix

MYB v-myb avian myeloblastosis viral oncogene homolog

PAL Phenylalanine ammonia-lyase

TF Transcription factor

We have not used generative artificial intelligence in the writing of this manuscript.

Declarations

Author contributions

KW and SS jointly conceived and designed the research. SS provided supervision throughout the study. KW conducted the experiments, analyzed the data, and prepared the initial draft of the manuscript. SS conducted a thorough review and contributed to the revision of the manuscript.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

Declaration of Generative AI and AI-assisted technologies in the writing process

We have not used generative artificial intelligence in the writing of this manuscript.

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Figures

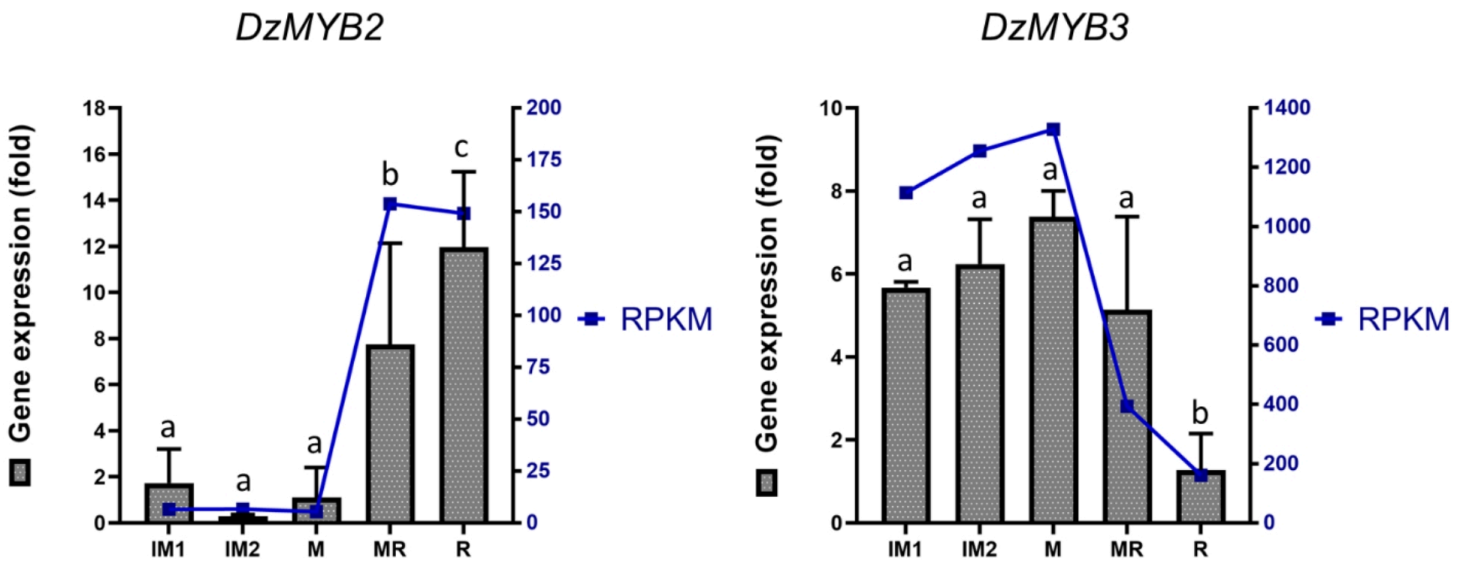

Figure 1

Fold changes in expression levels of candidate *DzMYB*s (*DzMYB2* and *DzMYB3*) determined by RT-qPCR and RPKM values from transcriptome data at five stages (immature 1; IM1, immature 2; IM2, mature; M, midripe; MR, and ripe; R) of durian fruit (Monthong cultivar). The relative gene expression levels of *DzMYB2* and *DzMYB3* were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against the EF1α transcript, with the immature 1 stage as the control for *DzMYB2* and the ripe stage as the control for *DzMYB3*. Three independent biological replicates were used. Different letters denote statistically significant differences between stages ($p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test).

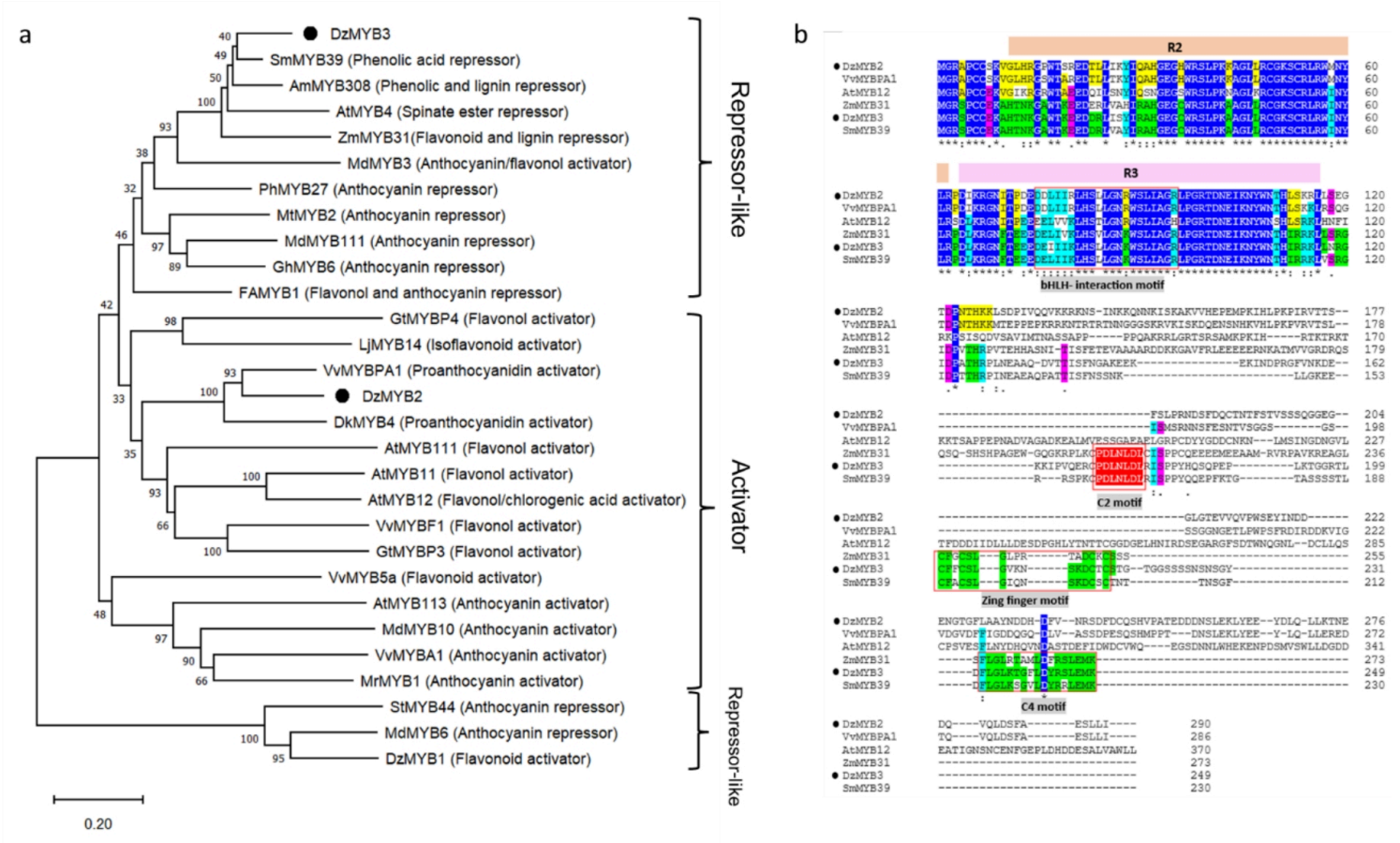

Figure 2

aPhylogenetic tree illustrating the amino acid sequences of DzMYB2 (XP_022757570.1) and DzMYB3 (XP_022763061.1). The full-length amino acid sequences were aligned with previously characterized MYBs from Arabidopsis (AtMYB4; NP_195574.1, AtMYB11; NP_191820, AtMYB12; NP_182268, AtMYB111; NP_199744, AtMYB113; NP_176811), grapevine (VvMYB5a; AAS68190, VvMYBA1; BAD18977, VvMYBF1; ACV81697, VvMYBPA1; CAJ90831), apple (MdMYB3; AEX08668, MdMYB6; AAZ20429, MdMYB10; ACQ45201; MdMYB111; ADL36754), strawberry (FaMYB1; AF401220), Japanese gentian (GtMYBP3; AB733016, GtMYBP4; BAF96934), kiwi (DkMYB4; BAI49721), potato (StMYB44; MK410942.1), lotus (LjMYB14; XP_057425335.1), petunia (PhMYB27, KF985023) Mexican cotton (GhMYB6; AAN28286), barrel medic (MtMYB2; XP_003616388), Maize (ZmMYB31; NP_001105949), Snapdragon (AmMYB308; P81393), red sage (SmMYB39; AGS55356), Chinese bayberry (MrMYB1; GQ340767), and durian (DzMYB1; XP_022757570.1). The phylogenetic tree was constructed using MEGA X software and the neighbor-joining method with 1000 bootstrap replicates. **b**Multiple sequence alignment of DzMYB2 and DzMYB3 with reported activator and repressor MYBs from other plant species, including VvMYBPA1; CAJ90831, AtMYB12; NP_182268, ZmMYB31; NP_001105949, and SmMYB39; AGS55356. The R2 and R3 conserved domains are marked in orange and pink, respectively. The bHLH interaction motif of R3, C2 repressor motif, Zing finger, and C4 motif are indicated in gray.

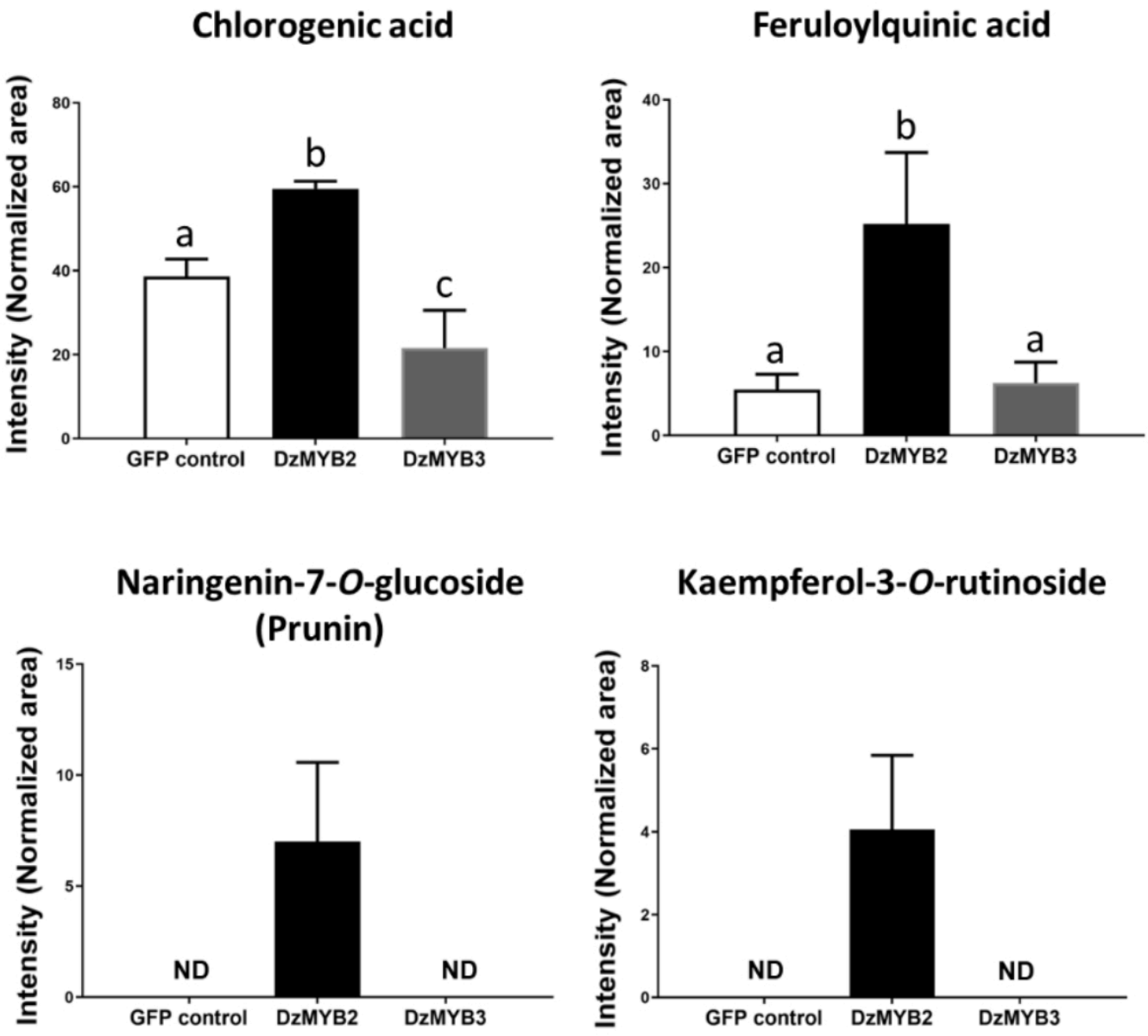

Figure 3

The intensity of phenolic compounds in transient protein expression in *Nicotiana benthamiana* leaves compared to the *GFP* control. The intensity was normalized using the peak areas of puerarin (for positive mode) and camphor (for negative mode) as internal standards. Distinct letters signify statistically significant differences between stages ($p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test).

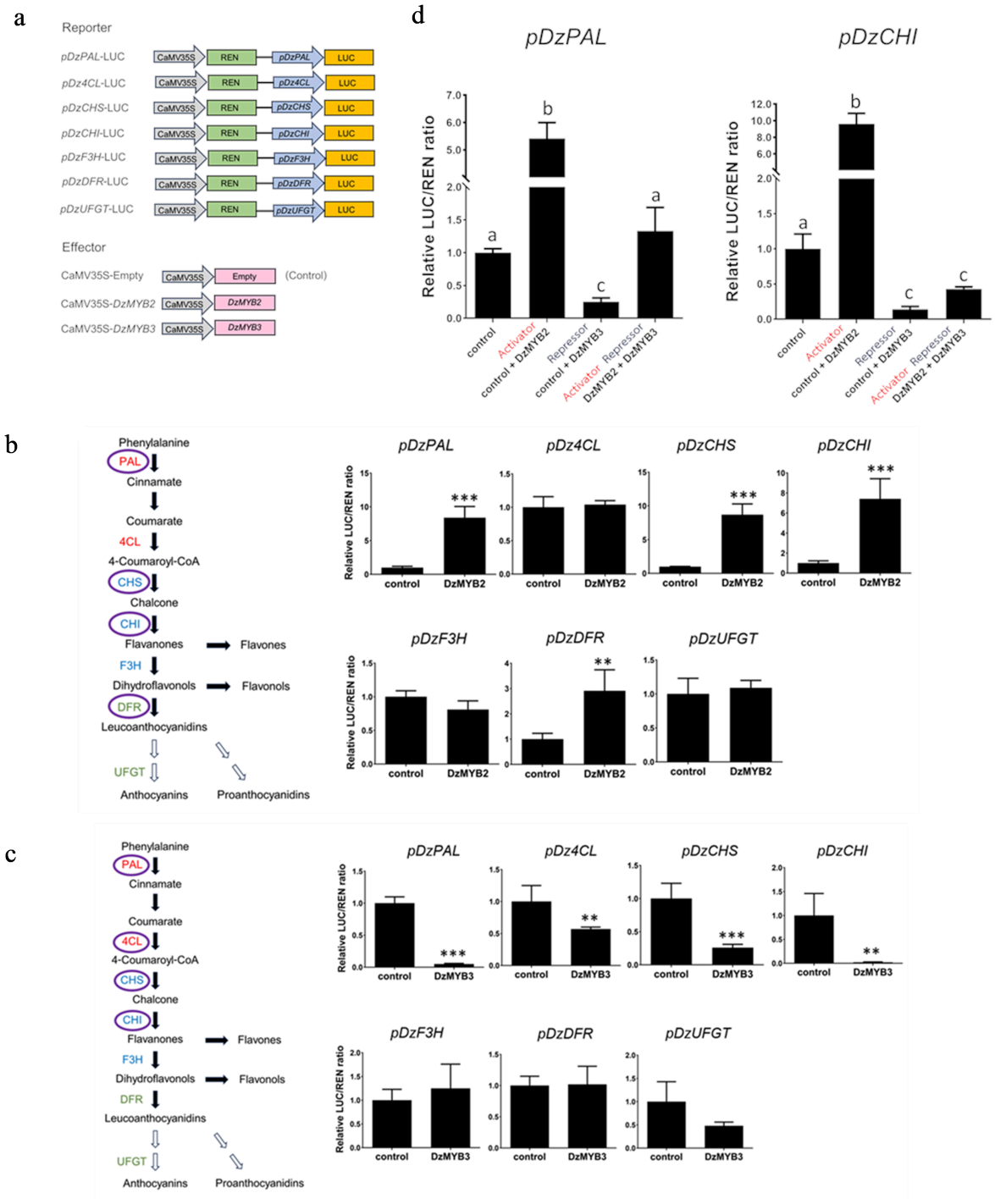

Figure 4

Dual-luciferase activity assay conducted in Arabidopsis protoplasts. **a** Schematic representation of the effector and reporter constructs employed in the dual-luciferase experiment. **b** DzMYB2 demonstrates binding affinity to the promoters of *DzPAL*, *DzCHI*, *DzCHS*, and *DzDFR*. **c** DzMYB3 exhibits binding affinity to the promoters of *pPAL*, *pDz4CL*, *pDzCHS*, and *pDzCHI*. **d** The cumulative effect of DzMYB2 and DzMYB3 on the *PAL* and *CHI*promoters. The relative LUC/REN ratio indicates the interaction between the

promoter of each phenylpropanoid biosynthetic gene and DzMYB TFs. The LUC/REN ratio was normalized to the control, consisting of each phenylpropanoid biosynthetic gene promoter and the vector without a transcription factor (LUC/REN ratio = 1.0). An asterisk above the bars denotes a significant difference between samples (student's t-test, **: P < 0.05, ***: P < 0.01). The promoters regulated by either DzMYB2 or DzMYB3 are highlighted within purple circles. Distinct letters signify statistically significant differences between stages (p < 0.05, one-way ANOVA, Tukey's multiple comparisons test).

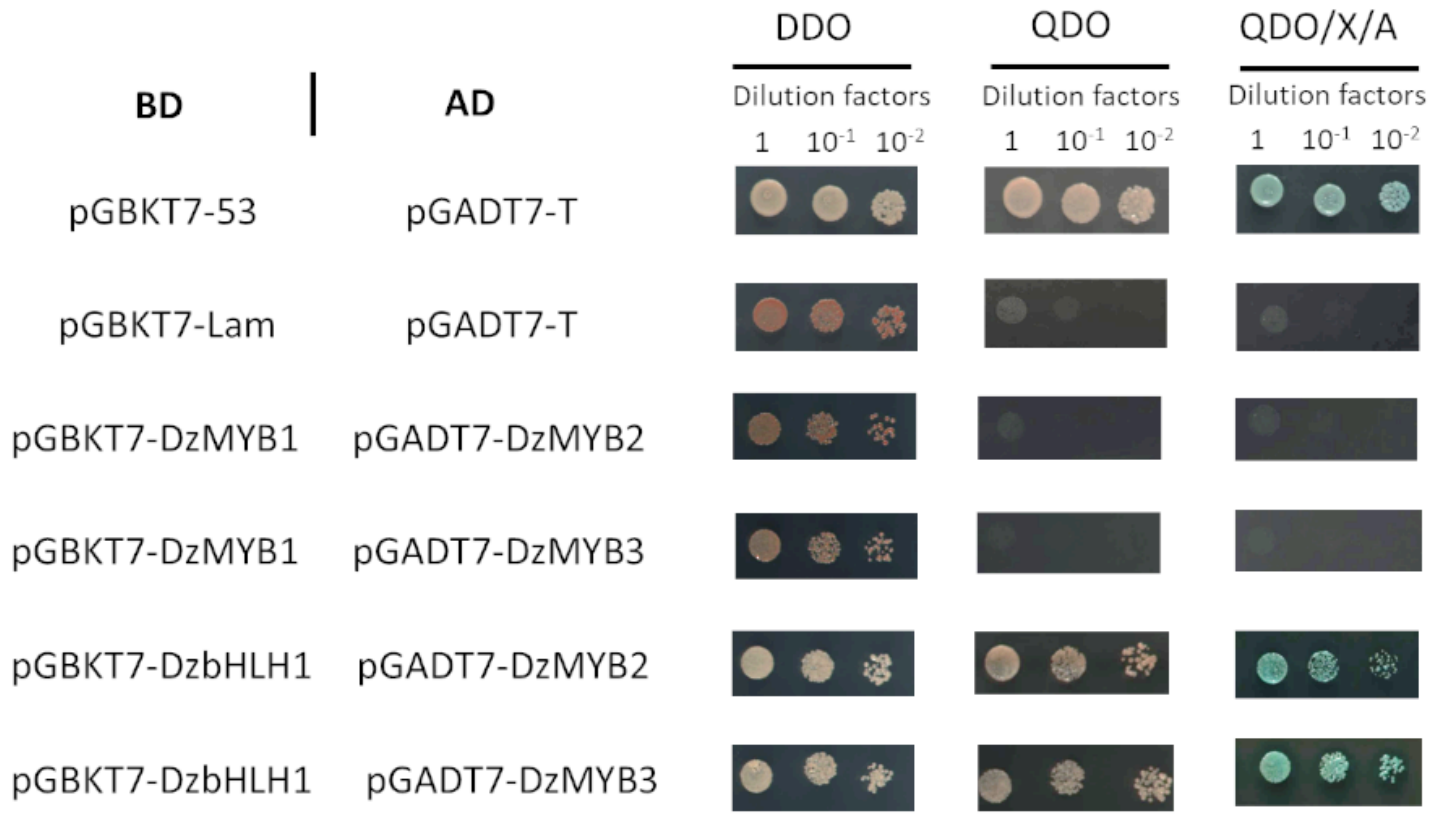

Figure 5

Yeast two-hybrid assay on selective media, which includes double dropout (DDO) medium: SD/−Leu/−Trp, QDO (quadruple dropout medium: SD/−Ade/−His/−Leu/−Trp), and QDO/X/A (quadruple dropout medium: SD/−Ade/−His/−Leu/−Trp supplemented with X-alpha-Gal and Aureobasidin A). The yeast cultures that were transformed were spotted in 10-fold serial dilutions. After a 3-day incubation period, a photograph of the yeast colonies was taken. For the positive control, recombinant yeast containing both pGBKT-53 and pGADT7-T was used, while yeast containing pGBKT-Lam and pGADT7-T served as the negative control.

Supplementary Files

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- Supplementarymaterials73.docx