



The *Boesenbergia rotunda* R2R3-MYB Transcription Factor BrMYB2 Regulates Flavonoid Biosynthesis in *Nicotiana tabacum*

Imene Tatar Caliskan¹ · Yvonne Jing Mei Liew² · Su-Ee Lau^{3,4} · Nur Kusaira Khairul Ikram⁵ · Boon Chin Tan³

Received: 15 December 2025 / Accepted: 20 March 2026
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Abstract

Flavonoids play essential roles in plant growth and development. However, their natural abundance is often low, limiting their large-scale commercial utilization. Understanding the molecular mechanisms underlying flavonoid biosynthesis is therefore critical to enhancing their production. Here, we isolated and functionally characterized *BrMYB2*, an R2R3-MYB transcriptional repressor from *Boesenbergia rotunda*. Sequence analysis revealed that *BrMYB2* encodes a 219-amino acid protein with conserved subgroup 4 MYB domains, showing 92.8% similarity to a MYB-related protein from *Zingiber officinale*. To our knowledge, this is the first report of an R2R3-MYB repressor identified in *B. rotunda*. Heterologous expression of *BrMYB2* in *Nicotiana tabacum* via *Agrobacterium*-mediated transformation led to the significant down-regulation of four key flavonoid biosynthetic genes (*PAL*, *4CL*, *CHI* and *DFR*). Correspondingly, the accumulation of several targeted flavonoids, including p-coumaric acid, cinnamic acid, pinostrobin, and anthocyanin, was markedly reduced. These findings provide new insights into the transcriptional regulation of flavonoid metabolism in *B. rotunda* and establish a foundation for metabolic engineering strategies to enhance flavonoid production for pharmaceutical and agricultural applications.

Keywords Flavonoids · Ginger · MYB · Metabolic engineering · Metabolites · Plant transformation

Introduction

Boesenbergia rotunda is a medicinal ginger belonging to the family of Zingiberaceae. This ginger contains flavonoids, such as cyclohexenyl chalcone derivatives, that hold immense potential in medicinal and nutraceutical

applications (Tan et al. 2015). Traditionally, *Boesenbergia rotunda* has been used to relieve bacterial infections, rheumatism, fever, muscle pain, peptic ulcers, and various digestive problems (Kanchanapiboon et al. 2020; Saah et al. 2021). A recent study by Liana et al. (2024) revealed that the hexane extracts of *B. rotunda* manifested encouraging multi-pharmacological characteristics, including antioxidative, anti-staphylococcal, and anti-inflammation. In addition, both hexane and ethyl acetate extracts have been reported to possess strong antioxidant, anticancer, and antibacterial activities (Sopitthummakhun et al. 2021). Its essential oil has also shown potent antibacterial properties against *Bacillus cereus* and moderate activity against the foodborne pathogens like *Escherichia coli* and *Salmonella typhi* (Tasfiyati et al. 2023). Flavonoids are a large group of naturally occurring polyphenolic compounds. In plants, the biological functions of flavonoids are very diverse, ranging from giving color and aroma to protecting plants against biotic and abiotic stresses (Karlson et al. 2022). Numerous flavonoids have been reported to have anti-oxidative, anti-inflammatory, and anti-carcinogenic properties (Aryal et al., 2019; Zahra et al., 2024; de Luna et al., 2023; Pandey et al., 2024). For example, panduratin A isolated from

Communicated by Barry Flinn.

✉ Boon Chin Tan
boonchin@um.edu.my

- ¹ Institute for Advanced Studies, Universiti Malaya, 50306 Kuala Lumpur, Malaysia
- ² Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
- ³ Centre for Research in Biotechnology for Agriculture (CEBAR), Universiti Malaya, 50306 Kuala Lumpur, Malaysia
- ⁴ UM Agroforestry, Universiti Malaya, 50306 Kuala Lumpur, Malaysia
- ⁵ Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

B. rotunda poses inhibition against dengue 2 virus (DENV-2) of NS3 protease (Kiat et al. 2006), protects human liver cells against oxidative damage (Salama et al. 2013), prevents aging in human skin fibroblasts (Shim et al. 2008), and protects autophagy in melanoma cells (Lai et al. 2018). Another hydroxyanduratin A was reported to exhibit anti-HIV-1 protease activity during viral replication (Cheenpracha et al. 2006).

The phenylpropanoid pathway, a flavonoid biosynthetic pathway, is among the most extensively studied in plants (Dong and Lin 2021). Several key genes encoding enzymes involved in the phenylpropanoid pathway have been identified in Korean mint (Park et al. 2020), rubber tree (Meng et al. 2021), *Peucedanum praeruptorum* (Song et al. 2022), Barley (Glagoleva et al. 2022), and switchgrass (Saha et al. 2021). These flavonoid-related genes are regulated by transcription factors (TFs), which are specific proteins that interact with cis-acting elements in the promoters of target genes to regulate gene transcription (Cheong 2023).

The MYB TFs comprise one of the largest TF families in plants, characterized by the highly conserved MYB DNA-binding domain (DBD). This domain contains up to four imperfect amino acid repeats, each approximately 52 amino acids long and folding into three α -helices. The second and third helices form a helix-turn-helix (HTH) structure containing three hydrophobic residues that allow the third helix of each repeat to fit into the major groove of DNA (Caliskan et al. 2025). These repeats are designated R1, R2, and R3 due to their similarity with the animal c-MYB protein. According to their repeat number and arrangement, the MYB superfamily is classified into four subfamilies: 1R-MYB (R1/R2-MYB), 2R-MYB (R2R3-MYB), 3R-MYB (R1R2R3-MYB), and 4R-MYB (R1R2R2R1/2-MYB) (Millard et al. 2019). Among them, the R2R3-MYB subgroup is the largest subfamily (Hernández-Hernández et al. 2021) and also plays crucial roles in regulating various branches of the phenylpropanoid and flavonoid biosynthetic pathways (Wu et al. 2022). To date, numerous MYB genes have been isolated, and their functional characterization has taken place in various plant species, including *N. tabacum* (Zhang et al. 2024), *Hypericum perforatum* (Zhou et al. 2020), petunia (Chen et al. 2021), and Chinese cedar (Yang et al. 2022).

Recent studies in monocots have identified several R2R3-MYB transcription factors that regulate flavonoid and anthocyanin biosynthesis. For instance, in banana (*Musa* spp.), the R2R3-MYB TFs MaMA2, MaMA4, and MaMA8, are key regulators of this pathway. Interestingly, while MaMA4 and MaMA8 can independently induce anthocyanin accumulation during early plant development, MaMA2 requires

interaction with its bHLH partner, MusaTT8 (Thakur et al. 2026). This bHLH-dependent mechanism is facilitated by conserved R2R3 DNA-binding domains and bHLH interaction domains, which enable these MYB proteins to assemble into the MYB-bHLH-WD40 (MBW) transcriptional complex to regulate flavonoid production (Muhammad et al. 2025).

Functionally, many R2R3-MYB TFs regulate a specific branch of the flavonoid pathway. For example, AtMYB111 from *Arabidopsis* participates in salt stress responses through its regulation of flavonol metabolism (Li et al. 2019a), while MtMYB134 from *Medicago truncatula* specifically modulates flavonol biosynthesis (Naik et al. 2021). However, some R2R3-MYBs have been reported to influence multiple branches of the flavonoid pathway simultaneously. In *Freesia hybrida*, overexpression of *FhMYB5* enhanced the accumulation of both anthocyanins and proanthocyanidins (Li et al. 2019a). In addition to acting as activators, several R2R3-MYB TFs have been found to function as repressors. For instance, MaMYB4 suppresses the anthocyanin-related genes *CHS*, *ANS*, *DFR*, and *bHLH* in banana (Deng et al. 2021), while OgSH11 represses lignin-related genes in African rice by binding to the *GH2* promoter (Ning et al. 2023). These MYB proteins often act in collaboration with bHLH and WD40 proteins. Together, they constitute the MBW transcriptional complex, a master regulator of many flavonoid biosynthesis genes (Muhammad et al. 2025). Such coordinated regulatory mechanisms underpin the emergence of diverse specialized metabolic traits in plants. For example, the MdHY5–MdWRKY41–MdMYB transcriptional cascade characterized in red-fleshed apple plays a pivotal role in modulating the biosynthesis and accumulation of anthocyanins and proanthocyanidins.

Despite a few studies that supported the role of MYB TFs in flavonoid biosynthesis, little is known about this pivotal transcriptional regulation of the flavonoid biosynthetic pathways in ginger. In *B. rotunda*, MYB TFs have been identified as the second most abundantly regulated group in phenylalanine-treated cell suspension culture, exhibiting both activation and repression effects on the panduratin A biosynthesis pathway (Md-Mustafa et al. 2014). The present study aimed to isolate and characterize the full-length cDNA of *BrMYB2* from *B. rotunda*. Given that *BrMYB2* is predicted to function as a transcriptional repressor in the phenylpropanoid pathway, its cDNA was cloned and expressed in *N. tabacum* via the *Agrobacterium*-mediated transformation to evaluate its regulatory effects in planta. The flavonoid profiles of *BrMYB2*-expressing transgenic *N. tabacum* were subsequently analyzed using high performance liquid chromatography.

Materials and Methods

Plant Material

Field grown rhizomes of *B. rotunda* obtained from a local market at Chow Kit, Kuala Lumpur, Malaysia, were washed thoroughly under running tap water for 20 min before surface sterilized using 20% (v/v) Clorox (commercial bleach with 5.25% w/v sodium hypochlorite) for 20 min and 70% (v/v) ethanol for 1 min. The shoot buds induced from rhizomes were sectioned into small pieces (1 mm cross-section) and cultured on callus induction media [Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 1.0 mg L⁻¹ α -naphthaleneacetic acid (NAA), 1.0 mg L⁻¹ 6-benzyladenine (BA), 1.0 mg L⁻¹ indole-3-acetic acid (IAA), 3% (w/v) sucrose, and 0.2% (w/v) Gelrite™ (Duchefa, Netherlands)] (Yusuf et al. 2011). The established callus was subcultured every four weeks on propagation medium [MS basal medium supplemented with 3 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 3% (w/v) sucrose, and 0.2% (w/v) Gelrite™] (Liew et al. 2020). These calli were used to establish cell suspension cultures according to Liew et al. (2020). The cell suspension cultures were subcultured by transferring old cells to a new liquid MS medium supplemented with 1 mg L⁻¹ 2,4-D, 680 μ M L-glutamine, and 2% (w/v) sucrose in a ratio of 1:4 every two weeks. All cultures were maintained at 25 $\pm$ 2 °C under a 16-h photoperiod with a light intensity of 31.4 μ mol m⁻² s⁻¹ provided by cool fluorescent lamps in a growth room.

Tobacco (*N. tabacum*) seeds were surface sterilized and cultured on a Petri dish containing solid MS medium [MS salts, 3% (w/v) sucrose, and 0.2% (w/v) Gelrite™] (Burklew et al. 2012). All seeds were incubated at room temperature in a growth room under a 16-h day and 8-h night cycle. The regenerated shoots were transferred to culture jars containing solid MS media supplemented with 3% (w/v) sucrose.

Bioinformatics and Molecular Evolution Analysis of BrMYBs

The protein sequences of the two *BrMYBs*, retrieved from Md-Mustafa (2017) were analyzed using a suite of online bioinformatics tools to elucidate their structural and functional characteristics. PSIPRED software (<http://bioinf.cs.ucl.ac.uk/psipred/>) was employed to predict the secondary structure elements of the proteins. ExPASy-Prosite (<http://prosite.expasy.org>) was used to identify conserved motifs and functional signatures within the sequences. MEGA 11 software facilitated phylogenetic analysis, and MEME software (<http://meme.nbcr.net/meme/>) was used to discover conserved motifs within the sequences.

RNA Extraction

The established *B. rotunda* cells were filtered through Whatman filter paper and snap-frozen in liquid nitrogen. Total RNA was extracted according to Md-Mustafa et al. (2014). About 500 mg of *B. rotunda* cell suspension cultures were ground into fine powder in the presence of liquid nitrogen. The powder was transferred into a 2 mL microcentrifuge tube containing 1 mL of pre-heated CTAB extraction buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP), 2 mM sodium chloride (NaCl), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 100 mM tris-hydrochloride (Tris-HCl, pH 8), and 2% (v/v) β -mercaptoethanol (Sigma-Aldrich, US)], mixed well, and heated at 65 °C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1; v/v) was then added to the mixture and vortexed for few seconds before centrifuged at 10,621 $\times$ g for 15 min in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was transferred into a new microcentrifuge tube. These steps were repeated 2–3 times. Subsequently, 0.1 volume of 3 M sodium acetate and 3 volumes of chilled absolute ethanol were added to the supernatant and inverted a few times. The mixture was kept at -80 °C for 2–3 days before centrifuged at 10,621 $\times$ g for 30 min at 4 °C. The pellet was washed with 1 mL of cold 70% (v/v) ethanol and centrifuged at 10,621 $\times$ g for 5 min. The pellet was dissolved with 20–50 μ L nuclease-free water.

Isolation of cDNA of BrMYB2

The cDNA of *BrMYB2* was isolated using a pair of primers (forward: 5'-ATGGGGAGGTCGCCGTGCT-3' and reverse: 5'-CTAGTTGAGTTCTATGTTTTGAGGC-3'). One-step reverse transcription PCR (RT-PCR) was performed using a one-step RT-PCR kit (Qiagen, Netherlands) with the following conditions: 50 °C for 30 min and 94 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; followed by an extension for 10 min at 72 °C. The PCR product was purified using the Monarch® PCR & DNA Cleanup Kit (NEB, USA) and cloned into a TOPO TA-cloning vector (Thermo Fisher Scientific, USA) prior to sequencing.

Subcellular Localization

The full-length cDNA sequence of *BrMYB2* was amplified using primers (forward: 5'-GAGCACTAGTATGGG GAGGTCG-3' and reverse: 5'-GATGCACTAGTGTG AGTTCTATGT-3') and ligated into the pCambia1304-GFP vector to create a CaMV35S: BrMYB2-GFP construct. The construct was transformed into onion epidermal cells

using *Agrobacterium*-mediated transformation according to the method described by Xu et al. (2014). All samples were examined under an inverted microscope (Olympus IX-HOS, Japan).

Construction of BrMYB2 Expression Vector

The coding region of *BrMYB2* together with CaMV35S promoter and NOS terminator was synthesized (Next-Gen, Malaysia) and ligated into a plant expression vector, pCAMBIA1304. The integration of the *BrMYB2* gene into the pCAMBIA1304 was confirmed by PCR amplification using the same primers and PCR conditions described earlier. The constructed plasmid, pCAMBIA1304-*BrMYB2*, was introduced into *Agrobacterium tumefaciens* LBA4404 using a heat shock method (Liew et al. 2020).

Nicotiana tabacum Transformation

The vector harboring the gene of interest (pCAMBIA1304-*BrMYB2*) and the empty vector serving as a mock control (pCAMBIA1304) were separately introduced into *N. tabacum* via *Agrobacterium*-mediated transformation (Zoolkefli et al. 2020). The leaves of *N. tabacum* were cut into small pieces (2 cm × 2 cm) and pre-cultured on MS medium containing 1 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA in dark condition for 3 days. About 2 mL of *Agrobacterium* (OD₆₀₀ = 1.2) was diluted with 13 mL of yeast extract broth (YEB) to achieve a 7.5-fold dilution before adding 100 µM acetosyringone. The leaf explants were infected with *Agrobacterium* cultures for 20 min under shaking condition of 80 rpm. The explants were then blot dried and cultured on MS medium containing 1 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA for 3 days in the dark. The explants were rinsed with sterile distilled water containing 100 mg L⁻¹ carbenicillin and 200 mg L⁻¹ cefotaxime to remove excessive *Agrobacterium*. The explants were then transferred to shoot regeneration medium [MS salts, 3% (w/v) sucrose, 0.2% (w/v) Gelrite™, 1 m L⁻¹ BA, 0.1 mg L⁻¹ NAA, 24 mg L⁻¹ hygromycin B, 100 mg L⁻¹ carbenicillin, and 200 mg L⁻¹ cefotaxime]. The cultures were transferred to fresh medium every 10 days. After 2 months, leaves from all hygromycin-positive T₀ transgenic *N. tabacum* lines were harvested for PCR analysis.

DNA Extraction and PCR Amplification

The leaves of one-month-old T₀, T₁, and T₂ *N. tabacum* were sampled for DNA extraction using the CTAB precipitation method as described by Kasajima et al. (2013). The isolated DNA was amplified by PCR using both hygromycin primers

(forward: 5'-ATGCGGAGCATATACGCCCGG-3' and reverse: 5'-TTATCGGCACTTTGCATCGGC-3') and *BrMYB2*-specific primers (forward: 5'-ATGGGGAGGTCGCCG-3' and reverse: 5'-CTAGTTGAGTTCTATGTTTTGAGGC-3') under the following thermal cyclers conditions: 94 °C for 2 min, followed by 30 cycles of amplification at 94 °C for 10 s, 50 °C for 30 s, and 72 °C for 20 s; followed by an extension for 10 min at 72 °C.

Acclimatization and Seed Collection

All positive transformant plants were acclimatized in a greenhouse at the Universiti Malaya, Malaysia. The plantlets were incubated at 25 ± 2 °C under natural sunlight. T₁ seeds were collected, surface sterilized (Burklew et al. 2012), and cultured on a Petri dish containing solid MS medium [MS salts, 3% (w/v) sucrose, 0.2% (w/v) Gelrite™, and 24 mg L⁻¹ hygromycin B. The regenerated *N. tabacum* plantlets were acclimatized for T₃ seed collection. Three T₃ family lines that overexpressed *BrMYB2* gene without segregation of character were selected for further experiments.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from the *N. tabacum* leaves according to MacRae (2007). The isolated RNA was converted into cDNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan). qPCR analysis was conducted using SensiFAST SYBR® Lo-ROX Kit (Bioline, UK) and performed on ABI 7500 Real-Time PCR machine (ThermoFisher, US) under the following thermal cyclers conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 30 s in 96-well optical reaction plates (Applied Biosystems, USA). Four gene-specific primers (*4CL*, *CHS*, *CHI*, and *DFR* genes) and two pairs of reference genes (*Nt125* and *EF-1α*) were used (Supplementary Table 1). The melting curve analysis was carried out immediately after the amplification protocol. The comparative Ct method was used to determine the relative expression level. The RT-qPCR for each gene was performed with three biological replicates, with three technical replicates per biological sample.

Determination of Anthocyanin Content

Anthocyanins were extracted from *N. tabacum* inflorescences according to Stuppner et al. (2020) with minor modifications. The total anthocyanin was extracted using the pH differential method. Two buffers with pH values of 1.0 and 4.5 were prepared as follows: for the first buffer, 0.2 g of sodium chloride was dissolved in 100 mL of distilled water,

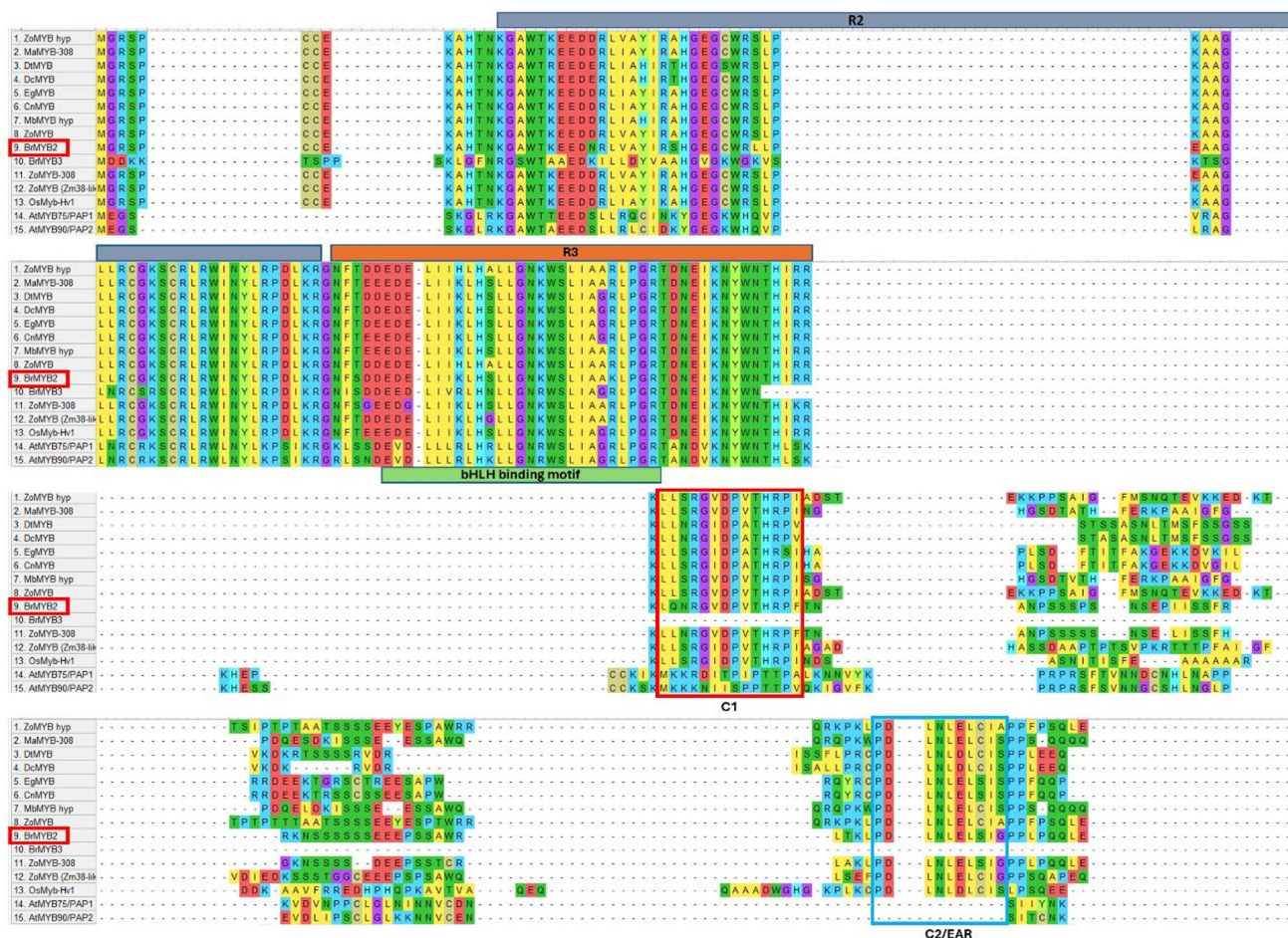


Fig. 1 Alignment by CLUSTAL W of BrMYB2 (red) and other MYB genes using the MEGA11 software. The conserved domains R2 (grey), R3 (orange), C1 (red), C2/EAR (blue), and the bHLH binding motif (green) are indicated by the labeled boxes

and the pH was adjusted to 1.0 using 0.1 N hydrochloric acid. To make the second buffer, 0.4 g of sodium acetate was dissolved in 100 mL of distilled water, and the pH was adjusted to 4.5 with 0.2 N acetic acid.

The *N. tabacum* flowers were ground in a pestle and mortar in the presence of liquid nitrogen and freeze-dried for 2 days. 200 mg of freeze-dried flower samples were extracted in 5 mL of acidic methanol (0.1% HCL). For the pH 1.0 dilution, 0.5 mL of the sample extracts were mixed with 24.5 mL of the pH 1.0 buffer; and for the pH 4.5 dilution, 0.5 mL of the sample extracts were mixed with 24.5 mL of the pH 4.5 buffer. The samples were diluted at a ratio of 1:5 in pH 1.0 and pH 4.5 solutions. The absorbance (A) was measured at 520 nm and 700 nm, and the total anthocyanin content was measured using the following equation:

$$\text{Total anthocyanin content} \left(\frac{\text{mg}}{\text{L}} \right) = \epsilon \times l \times A \times \text{MW} \times \text{DF} \times 1000$$

where: ϵ = The molar absorptivity of cyanidin-3-O-glucoside ($26,900 \text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$); l = The optical path of the cuvette (1 cm); A = The absorbance ($A_{520 \text{ nm}} - A_{700 \text{ nm}}$) pH 1.0 - ($A_{520 \text{ nm}} - A_{700 \text{ nm}}$) pH 4.5; MW = The molecular weight of the standard cyanidin-3-O-glucoside (449.2 g mol^{-1}); DF = The dilution factor.

Isolation of Flavonoids and Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

Leaf samples from wild-type (WT) and transgenic T_3N . *tabacum* plants were collected and freeze-dried. Flavonoids were isolated according to Tan et al. (2015). About 1 g of freeze-dried leaf powder was extracted in methanol containing pinobanksin as an internal standard overnight at 4 °C. The methanolic extracts were filtered and evaporated under vacuum at 35 °C. The resultant slurry was partitioned with equal volumes of ethyl acetate (EA) and water. The

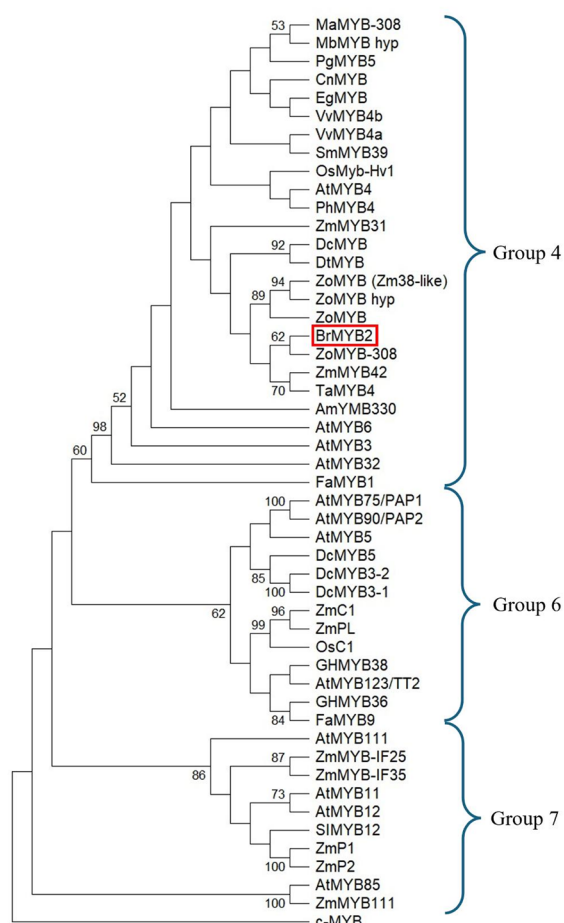


Fig. 2 Phylogenetic tree of BrMYB2 and other MYB transcription factors, based on amino acid sequences using the maximum likelihood method by the MEGA11 software. The numbers next to the nodes are bootstrap values from 1,000 replicates. The putative groups in MYB transcription factor family are indicated at the right side. c-MYB from *Homo sapiens* was used as the outgroup. All R2R3-MYB protein sequences were retrieved from GenBank database and accession numbers are as follows: *Arabidopsis thaliana* (AtMYB3; NP_564176.2), *Arabidopsis thaliana* (AtMYB4; NP_195574.1), *Arabidopsis thaliana* (AtMYB5; NP_187963.1), *Arabidopsis thaliana* (AtMYB6; NP_192684.1), *Arabidopsis thaliana* (AtMYB11; NP_191820.1), *Arabidopsis thaliana* (AtMYB12; NP_182268.1), *Arabidopsis thaliana* (AtMYB32; XP_002867097.1), *Arabidopsis thaliana* (AtMYB58; XP_002892910.1), *Arabidopsis thaliana* (AtMYB75/PAP1; NP_176057.1), *Arabidopsis thaliana* (AtMYB85; NP_567664.1), *Arabidopsis thaliana* (AtMYB90/PAP2; NP_176813.1), *Arabidopsis thaliana* (AtMYB111; NP_199744.1), *Arabidopsis thaliana* (AtMYB123/TT2; NP_198405.1), *Cocos nucifera* Myb-related protein (CnMYB; KAG1354138.1), *Daucus carota* subsp. *sativus* (DcMYB3-1; BAF49442.1), *Daucus carota* subsp. *sativus* (DcMYB3-2; XP_017220937.1), *Daucus carota* subsp. *sativus* (DcMYB5; BAF49445.1), *Dendrobium catenatum* Myb-related protein Zm38-like (DcMYB; XP_020703289.1), *Dendrobium thyrsiflorum* hypothetical protein (DtMYB; KAL0920727.1), *Eaeis guineensis* R2R3 MYB transcription factor (EgMYB; AMN15101.1), *Gossypium hirsutum* (GHMYB36; NP_001314377.1), *Gossypium hirsutum* (GHMYB38; NP_001314273.1), *Homo sapiens* (c-MYB; AAA52032.1), *Musa acuminata* AAA group Myb-related protein 308-like (MaMYB308; XP_064949658.1), *Musa bulbisiana* hypothetical protein (MybMYB hyp; THU64654.1), *Oryza sativa* (OsC1; AID46508.1), *Oryza sativa* Japonica group myb-related protein Hv1-like (OsMyb-Hv1; XP_025876044.1), *Solanum lycopersicum* (SIMYB12; NP_001234401.1), *Zea mays* (ZmC1; XP_008659144.1), *Zea mays* (ZmMYB-IF25; NP_001105937.1), *Zea mays* (ZmMYB-IF35; NP_001105092.1), *Zea mays* (ZmMYB31; NP_001105949.2), *Zea mays* (ZmMYB42; NP_001106009.2), *Zea mays* (ZmP1; NP_001278607.3), *Zea mays* (ZmP2; XP_020401761.1), *Zea mays* (ZmPL; NP_001358856.1), *Zea mays* (Zm111; AAS45643.1), *Zingiber officinale* myb-related protein 308-like (ZoMYB-308; XP_042391715.1), *Zingiber officinale* myb-related protein Zm38-like (ZoMYB Zm38-like; XP_042460828.1), *Zingiber officinale* hypothetical protein ZIOFF (ZoMYB hyp; KAG6487011.1), *Zingiber officinale* MYB protein (ZoMYB; WLQ69605.1)

EA fraction was vacuum-dried, and the resultant extract was dissolved in methanol at 30–60 μ L methanol per mg. The percentage yield of each extract was calculated using the following equation (Liana et al. 2024):

$$\% \text{ yield} = \frac{\text{weight of extract (g)}}{\text{weight of dried plant material used (g)}} \times 100$$

Prior to LC-MS analysis, the extracts were filtered through a 0.22 μ m PTFE syringe filter (German Acrodisc 13 CR). Chromatographic separation was performed on an Agilent A6490 LC-MS system equipped with a triple quadrupole mass detector and a reversed-phase Eclipse Plus C18 column (1.8 μ m, 2.1 $\times$ 50 mm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 0.2 mL min⁻¹. The gradient program was: 95% solvent A for 0 to 11 min, linearly decreased to 40% A from 11 to 13 min, further reduced to 5% A and held from 13 to 19 min, and finally returned to 95% A at 19–20 min for re-equilibration.

Mass spectra were acquired in multiple reaction monitoring (MRM) mode using positive electrospray ionization.

Analyte quantification was performed using peak-area ratios relative to the internal standard pinobanksin and a spiked calibration curve. All samples were analyzed using two biological replicates and two technical replicates, with final flavonoid concentrations expressed as ng mL⁻¹.

Statistical Analysis

All qPCR and LC-MS data are expressed as the mean $\pm$ standard deviation. Flavonoid-related gene expression was analysed using one-way ANOVA, followed by Tukey's post hoc test. Differences in compound accumulation among wild type, control, and pCAMBIA1304-BrMYB2 transgenic plants were also assessed using one-way ANOVA with Tukey's test. Statistical significance was determined at $p < 0.05$.

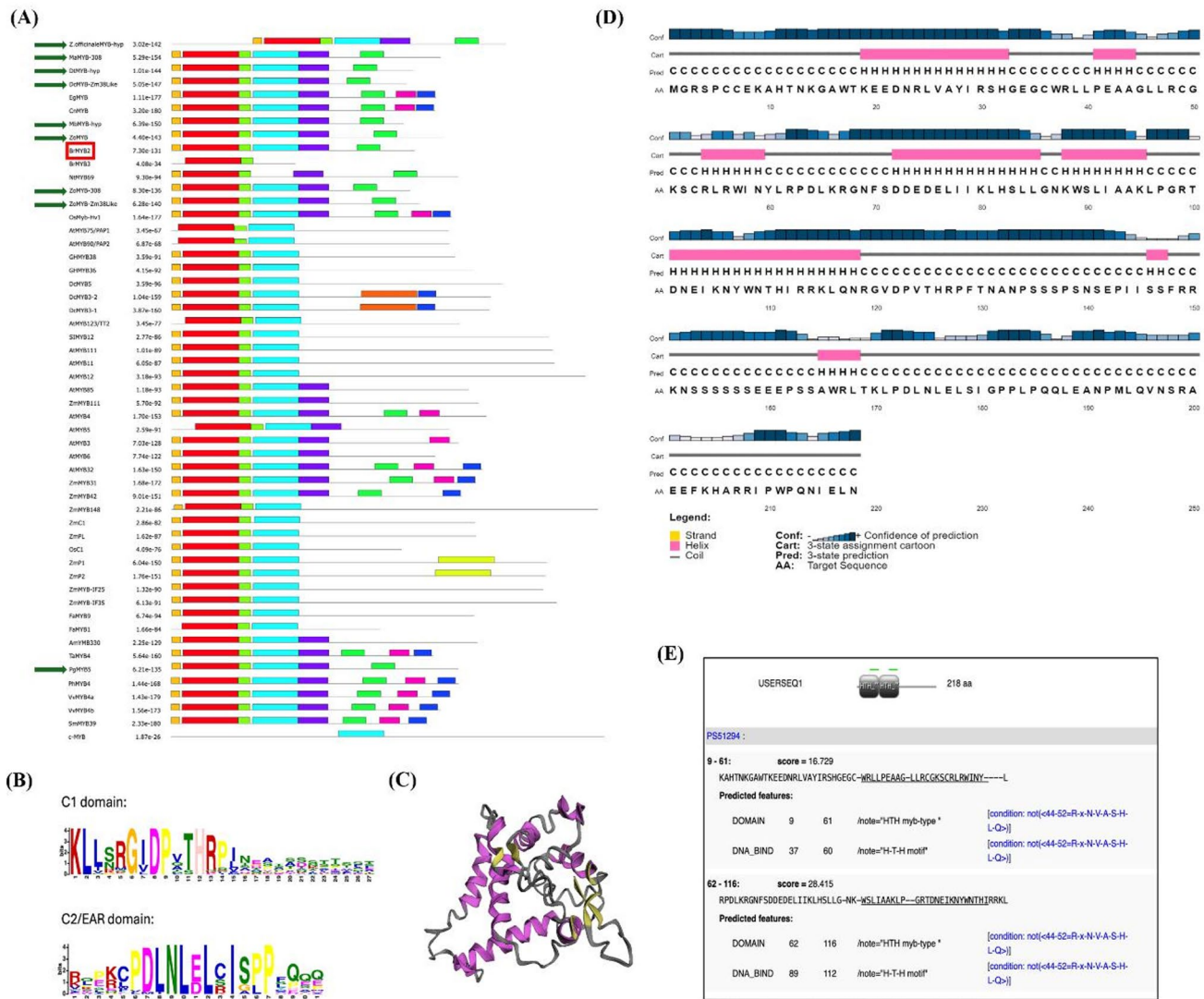


Fig. 3 (A) Motif searching by comparing different MYBs from different species using MEME (<http://meme-suite.org>); **(B)** C1 and C2/ERF-associated amphiphilic repression domain (EAR) domains found in BrMYB2. The green arrows show the MYB TFs from subgroup 4 that share the same domains with BrMYB2 which are from *Z. officinale*, *M. acuminata*, *D. thrysiflorum*, *D. catenatum*, and *P. glauca*. **(C)** The

Results

Domain Architecture and Phylogenetic Analysis of BrMYB2

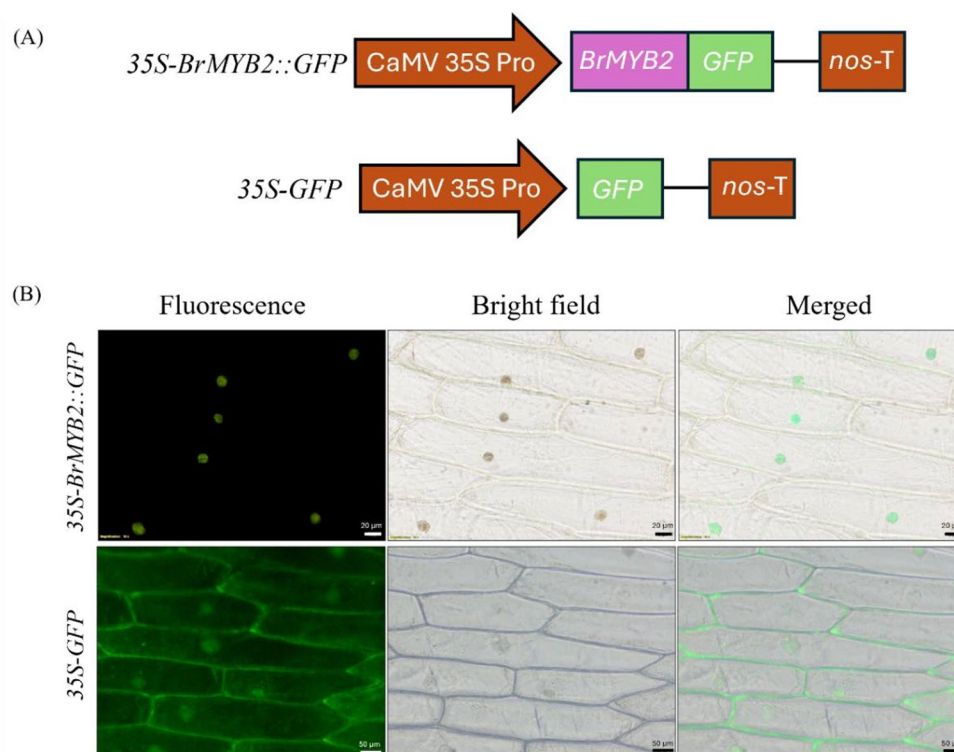
The *BrMYB2* gene open reading frame consists of 657 base pairs and encodes a protein of 218 amino acids. Sequence alignment of BrMYB2 with other MYB TFs (Fig. 1) revealed that it shares several conserved domains characteristic of MYB TFs. These include the R2 and R3 DNA-binding domains, the C1 (LxGIDPxT) and C2/EAR (LxLxL/DLNxxP) repression motifs, as well as a basic helix-loop-helix (bHLH) binding motif ((D/E)LX₂(R/K)_{x3}LX₆LX₃R).

tertiary structure of BrMYB2 predicted by SWISS-MODEL analysis (<http://swissmodel.expasy.org/>). **(D)** PSIPRED analysis shows its secondary structure, revealing the presence of α -helices mainly at the N-terminus. **(E)** The intra-domain features as predicted by Expasy-Prosite analysis (<http://prosite.expasy.org/>)

A phylogenetic tree constructed for BrMYB2 and other MYB TFs is shown in Fig. 2. BrMYB2 clustered within subgroup 4, together with MYBs from *Zingiber officinale*, *Oryza sativa*, *Zea mays* and *A. thaliana*. Members of this subgroup are typically associated with transcriptional repression of phenylpropanoid pathway genes and the modulation of secondary metabolites. Motif analysis further identified the position and distribution of conserved motifs across the MYB proteins (Fig. 3A-B), revealing that BrMYB2 contains the R2, R3, C1, and C2/EAR domains.

Structural prediction using SWISS-MODEL revealed that BrMYB2 possesses helix–turn–helix (HTH) structures and α -helices in its N-terminal region (Fig. 3C). These

Fig. 4 Subcellular localization of the BrMYB2 protein in onion epidermal cells. **(A)** Schematic diagram of the *35S-BrMYB2::GFP* fusion construct and the *35S-GFP* empty vector control. **(B)** Transient expression of *35S-BrMYB2::GFP* and *35S-GFP* in onion epidermal cells. The fluorescent signal of the BrMYB2-GFP fusion protein is localized to a spherical structure characteristic of the nucleus, whereas the free GFP control signal is distributed throughout the cell



structural features are typical of MYB TFs. Further analysis using Expasy-Prosit confirmed the presence of two HTH structures (R2 and R3 domains), which are the conserved DNA-binding domains of the R2R3-MYB group MYB TFs.

Subcellular Localization of the BrMYB2 Protein

Subcellular localization assays were performed using onion epidermal cells to determine the destination of the BrMYB2 protein. The GFP-fused *BrMYB2* exhibited a distinct green fluorescent signal concentrated within a large, spherical body morphologically characteristic of the plant cell nucleus (Fig. 4).

Transformation of *Nicotiana tabacum* Plants

To investigate its role in flavonoid biosynthesis, the isolated *BrMYB2* gene was introduced into *N. tabacum* using *Agrobacterium*-mediated transformation. Transgenic plants expressing *BrMYB2* under the control of the CaMV35S promoter were generated, and three independent homozygous T_3 lines (35 S::BrMYB2) were selected for phenotypic characterization, RT-qPCR analysis, and flavonoid quantification. The efficiency of the transformation was calculated by dividing the number of explants forming shoots per total number of explants. About 92.9% of *N. tabacum* explants were successfully transformed with pCAMBIA1304 (data not shown).

Segregation analysis showed that the transgenic T_3 progeny inherited the *BrMYB2* gene according to the Mendelian segregation pattern with a 3:1 ratio (Supplementary Table 2). The phenotypic characteristics of the WT and transgenic plants were observed (Fig. 5A). There were no significant differences between the WT and transgenic lines (*BrMYB2*-transformed and empty vector) in the vegetative parts of the plants, as evidenced by the unchanged morphology of the leaves and roots across all lines. However, the flowers of the *BrMYB2*-overexpressing plants were noted to be less pigmented compared to those of the WT and empty vector plants (Fig. 5B). Furthermore, flowers of the *N. tabacum* plants overexpressing the *BrMYB2* gene were significantly ($p < 0.05$) larger and longer compared to those of the WT and the empty vector (P3) (Fig. 6).

The hygromycin-positive transformants were sampled for PCR analysis to verify the insertion of the T-DNA from the transformed *A. tumefaciens*. A 657 bp band was detected using *BrMYB2* specific primers, whereas a 600 bp band was detected in all transgenic lines using the hygromycin primers (Supplementary Fig. 1).

BrMYB2 Reduced the Expression of Flavonoid-Related Genes in *Nicotiana tabacum*

Four flavonoid-related genes, namely *4CL*, *CHS*, *CHI* and *DFR*, were selected for qPCR analysis. The expression level of *4CL* in the transgenic line M9 showed lower expression

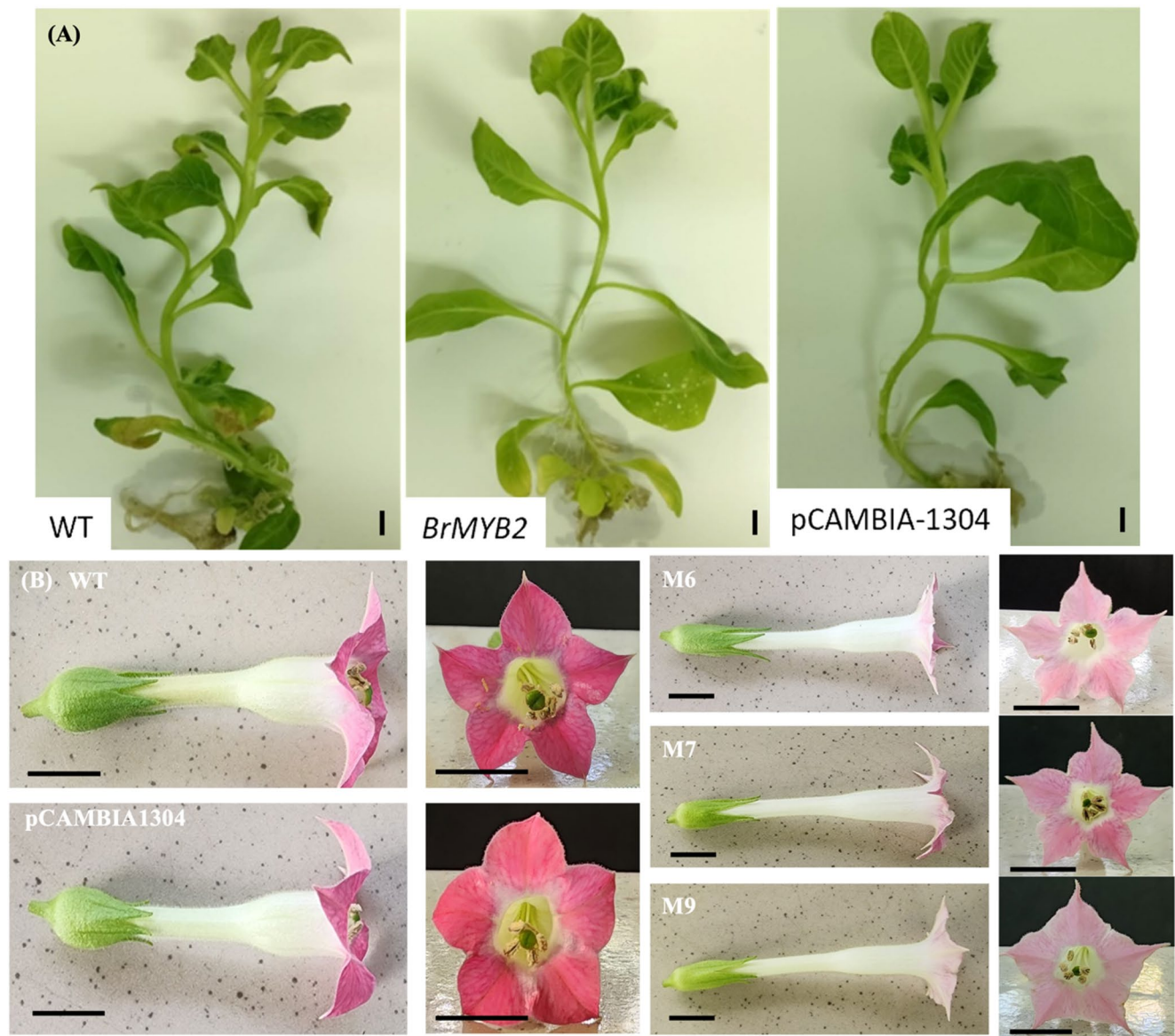


Fig. 5 (A) Three-months old wild-type and transgenic plants, and (B) A comparison of the phenotype of *Nicotiana tabacum* flowers. WT: wild-type *N. tabacum* flower; pCAMBIA1304: Flower of an empty

vector-transformed *Nicotiana tabacum* plant (P3); M6, M7, and M9: Flowers of *BrMYB2*-overexpressing *N. tabacum*. Bar = 1 cm

(8.9-fold) relative to the WT line. However, there was no significant difference between the WT and transgenic lines M6 and M7 (Fig. 7A). On the other hand, the transgenic lines generally showed lower expression levels of *CHS*, *CHI*, and *DFR*, compared to the WT line (Fig. 7B and D).

Heterologous expression of *BrMYB2* in *N. tabacum* led to a significant suppression of anthocyanin and quercetin biosynthesis. The anthocyanin content in the transgenic lines was significantly ($p < 0.05$) lower than that of the WT line, with a range between 2.47- to 3.04-fold reduction in relation to it (Fig. 7F).

Discussion

In this study, we identified and characterized the *B. rotunda* TF *BrMYB2* and investigated its role in flavonoid biosynthesis through functional studies in *N. tabacum*. Structural predictions revealed that *BrMYB2* contains two key motifs: an HTH motif, which enables DNA binding, and a bHLH-binding motif, which facilitates interaction with bHLH partners. Together, these features suggest that *BrMYB2* can form part of the MBW complex, the central regulatory module in flavonoid biosynthesis composed of MYB, bHLH, and WD40 proteins, where MYB and bHLH directly bind

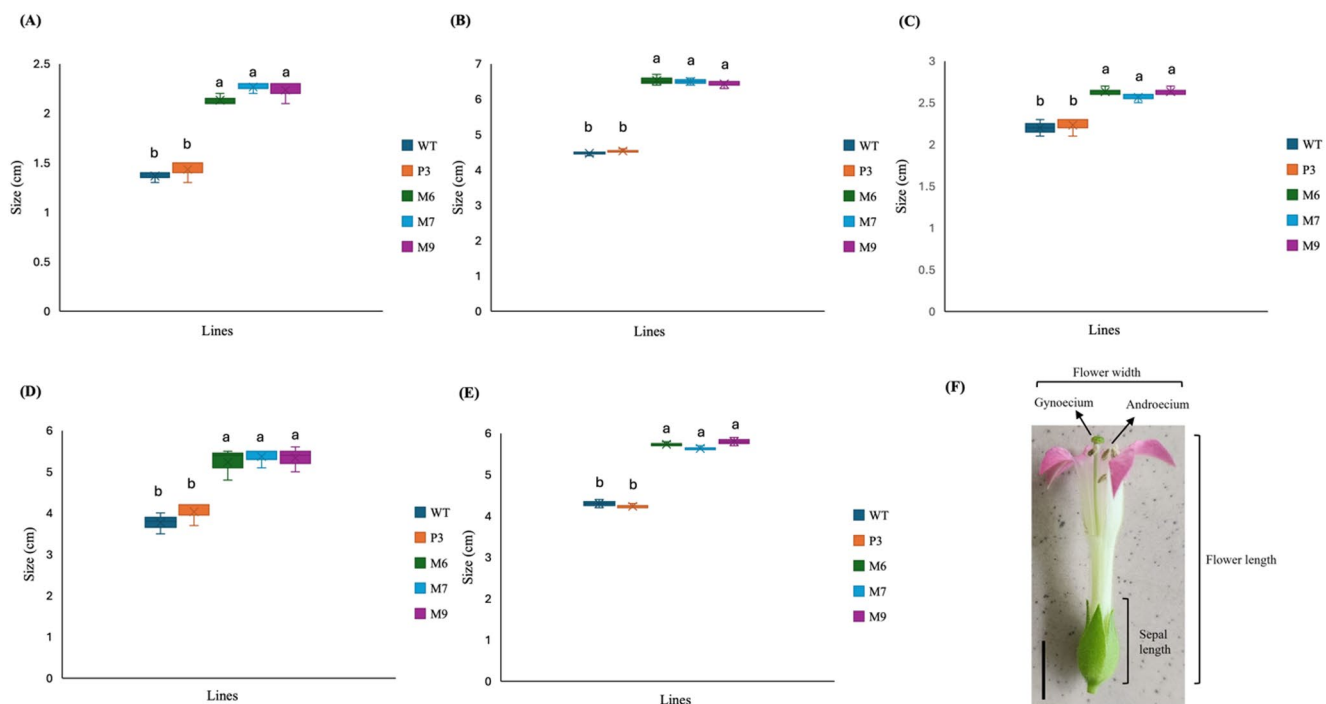


Fig. 6 Measurements of floral organs in wild-type (WT) and transgenic *Nicotiana tabacum* lines. **(A)** Sepal length; **(B)** Flower length; **(C)** Flower width; **(D)** Androecium; **(E)** Gynoecium; and **(F)** *Nicotiana tabacum* flower morphology. Bar: 1 cm. P3: Flower of an empty vec-

tor-transformed *Nicotiana tabacum* plant; M6, M7, and M9: Flowers of *BrMYB2*-overexpressing. Different letters indicate a significant difference between the lines ($p < 0.05$). Values represent mean $\pm$ SD

target gene promoters, while WD40 provides complex stability (Xu et al. 2015).

Phylogenetic and domain analyses further classified *BrMYB2* into subgroup 4 R2R3-MYBs. Members of this subgroup share conserved R2 and R3 DNA-binding domains, as well as the C1 and C2/EAR repressor motifs, and are generally known as repressors of flavonoid biosynthesis. Well-characterized examples include *AtMYB4*, *AtMYB32*, *AtMYB3*, and *AtMYB6* in *Arabidopsis thaliana* (Jin et al. 2000; Preston et al. 2004; Zhou et al. 2017; Ma and Constabel 2019), as well as *ZmMYB31* and *ZmMYB42* in *Zea mays* (Fornalé et al. 2010; Sonbol et al. 2009). This classification suggests that *BrMYB2* may exert similar repressive effects in *N. tabacum*.

To test this, *BrMYB2* was expressed in *N. tabacum* under the CaMV35S promoter. While vegetative organs of the transgenic plants appeared normal, the flowers exhibited pronounced differences. WT and empty vector control plants produced intensely pigmented flowers, whereas *BrMYB2*-overexpressing lines developed significantly paler petals, indicative of reduced anthocyanin accumulation. Consistently, flavonoid quantification showed that the transgenic lines accumulated lower levels of both anthocyanin (cyanidin-3-O-glucoside) and quercetin than WT plants. These results support the role of *BrMYB2* as a negative regulator of flavonoid biosynthesis. Interestingly, the effects of

MYB TFs on pigmentation vary across species: *RcMYB1* promotes anthocyanin accumulation in *Rosa hybrida* (He et al. 2023), while other MYBs, such as *LhMYB12* in lilies, act as repressors (Yin et al. 2020). Similarly, MYBs play dual regulatory roles in quercetin biosynthesis, acting as activators (e.g., *MrMYB12* in *M. rubra*; Cao et al. 2023) or repressors (e.g., *AtMYB12* in radish; Balasubramanian and Giriya 2024).

The broad evolutionary significance of R2R3-MYB transcription factors in regulating flavonoid metabolism is also well-documented across monocot species, though their regulatory effects can be highly context-dependent. For example, unlike the repressive function of *BrMYB2*, the R2R3-MYB TF *OsC1* in rice (*Oryza sativa*) acts as a positive regulator, activating target genes to drive anthocyanin accumulation in tissues such as the leaf sheath and grain pericarp (Upadhyaya et al. 2021). Homologous C1-type MYB regulators have also been identified in other cereal crops, including wheat (*Triticum aestivum*), where *TaC1*/*TaPL1*-like genes promote vegetative anthocyanin accumulation (Ding et al. 2025). These findings highlight the conserved role of R2R3-MYB proteins in modulating pigmentation across monocot species.

In addition to pigment reduction, *BrMYB2* expression influenced floral development. Transgenic lines M7, M6, and M9 produced flowers that were significantly longer

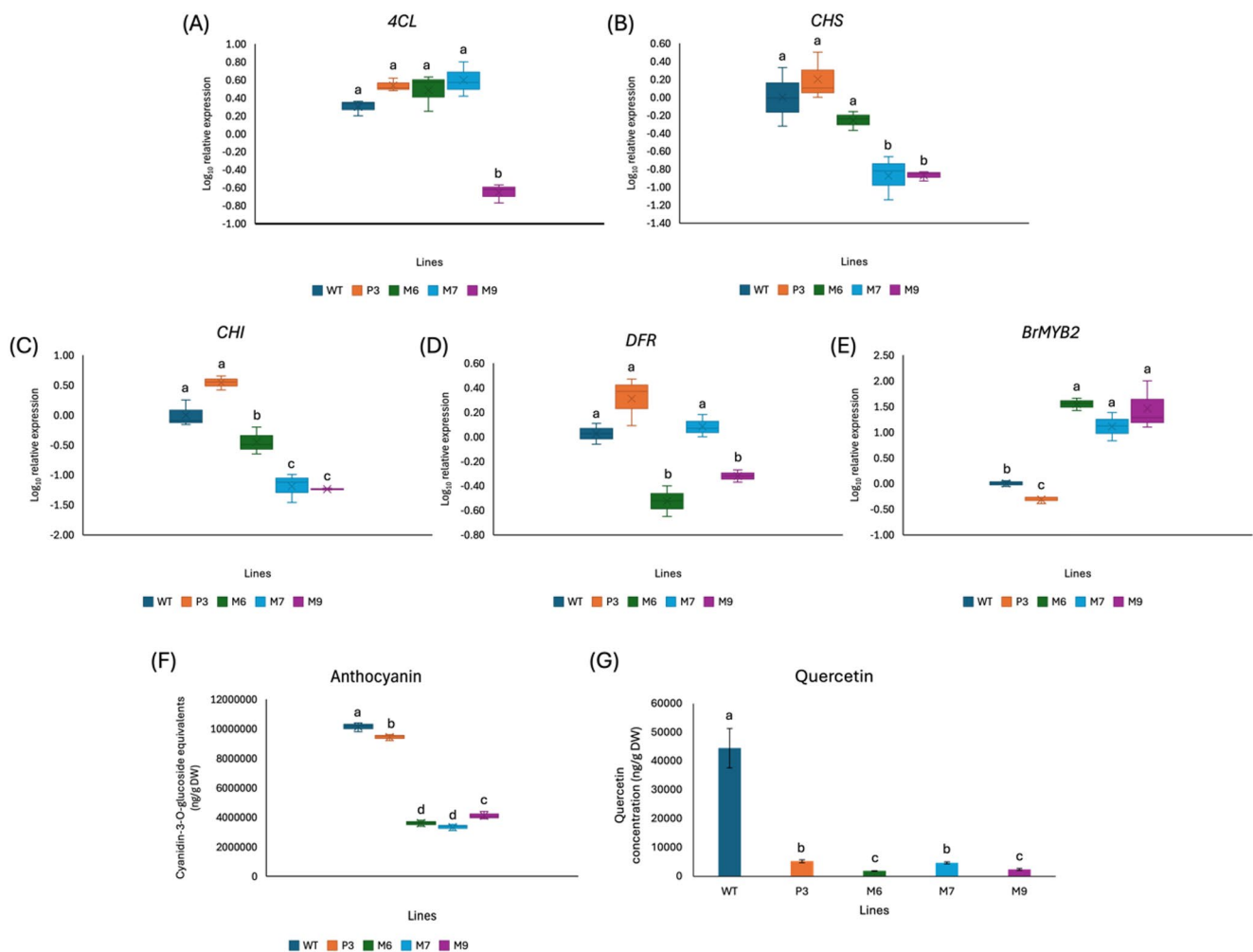


Fig. 7 The box plots of the expressions of the genes **(A)***4CL*; **(B)***CHS*; **(C)***CHI*; **(D)***DFR*, and **(E)***BrMYB2* for the wild-type (WT), empty vector plant (P3), and transgenic plants (M6, M7, and M9). The error bars indicate the standard error between the three biological replicates. **(F)** Anthocyanin content (ng g⁻¹ DW) and **(G)** Quercetin content (ng

g⁻¹ DW) in WT, *N. tabacum* transformed with empty vector (P3), and *BrMYB2*-transformed transgenic lines (M6, M7, and M9). Different letters indicate a significant difference between the lines ($p < 0.05$). Values represent mean $\pm$ SD

(6.5 cm) than those of WT and control plants (4.5 cm). This dual effect on both secondary metabolism and plant architecture is a known phenomenon among R2R3-MYBs. Comparable effects have been reported in other systems: peach MYB10.1 altered both flower size and pigmentation when overexpressed in *N. tabacum* (Rahim et al. 2019), while apple *MdMYB3* increased both petal pigmentation and flower length (Vimolmangkang et al. 2013).

BrMYB2 likely mediates these combined developmental and metabolic effects by directly binding to MYB-recognition elements (*cis*-regulatory elements) within the promoters of specific target genes. As many R2R3-MYB proteins function as core components of the MBW transcriptional complex (Albert et al. 2014), BrMYB2 may also interact with bHLH and WD40 partners to modulate these developmental processes. The observed floral elongation further suggests potential crosstalk with phytohormone signaling.

MYB transcription factors are known to influence hormonal networks, including the auxin and gibberellin pathways (Ma et al. 2025), which are critical in floral organ differentiation (He et al. 2018; Ke et al. 2021). Future studies involving promoter binding assays, protein-protein interaction analyses, and hormonal profiling will be essential to elucidate the precise mechanisms by which BrMYB2 coordinates flavonoid repression and floral elongation.

At the molecular level, RT-qPCR analysis showed that BrMYB2 downregulated several key genes in the phenylpropanoid pathway, including *CHS*, *CHI*, and *DFR*, whereas effects on *4CL* were variable across transgenic lines. This selective suppression of flavonoid-specific enzymes reinforces BrMYB2's role as a transcriptional repressor, consistent with subgroup 4 MYBs that modulate phenylpropanoid metabolism by interacting with bHLH proteins in the MBW complex via the conserved bHLH-binding motif (Su et al.

2020). Such interactions are central to flavonoid pathway regulation, as shown in poplar PdMYB118 (Wang et al. 2020a, b) and pear PyMYB10 (Cui et al. 2021). The phylogenetic proximity of BrMYB2 to *Arabidopsis* AtMYB4 and AtMYB32, both known to block MBW complex formation (Wang et al. 2020a, b), further supports this interpretation. Additional parallels include apple MdMYB305, which represses anthocyanin biosynthesis by competing for bHLH binding (Zhang et al. 2023), and *N. tabacum* NtMYB1, NtMYB2, and NtMYB4, which suppress phenylpropanoid gene expression when overexpressed (Zhang et al. 2024).

Although our molecular and metabolite analyses confirm the repressive function of BrMYB2, the contrast between unaffected vegetative tissues and altered floral structures suggests tissue-specific regulatory dynamics. Comparative profiling of leaves and floral organs was beyond the scope of this study. Future characterization of downstream gene expression and flavonoid profiles in isolated floral tissues will provide a more complete *in planta* view of BrMYB2 overexpression and may reveal distinct regulatory networks in reproductive versus vegetative tissues.

Conclusion

Our findings demonstrate that BrMYB2 from *B. rotunda* functions as a subgroup 4 MYB transcription repressor in *N. tabacum*, leading to reducing anthocyanin and quercetin accumulation, downregulation of key flavonoid biosynthetic genes, and altered flower development. The presence of conserved repression motifs suggests that BrMYB2 may interact with bHLH partners to interfere the MBW complex, thereby modulating phenylpropanoid metabolism. However, this potential interaction requires further experimental validation. These results provide valuable insights into the transcriptional regulation of flavonoid biosynthesis and highlight the potential of BrMYB2 as a molecular target for metabolic engineering of specialized metabolites.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00344-026-12178-z>.

Acknowledgements This research was supported by the Universiti Malaysia RU Grant (RU002-2025B).

Authors' Contribution Conceptualization: BCT and NKKI. Methodology and data analysis: ITC, YJML, and SEL. Writing—original draft preparation: ITC. Writing—review and editing: BCT, YJML, SEL, and NKKI. Supervision: BCT, YJML, and NKKI. Funding acquisition: BCT. All authors have read and agreed to the published version of the manuscript.

Funding Open access funding provided by The Ministry of Higher Education Malaysia and Universiti Malaysia

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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