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# DIMYB113 mutation affects anthocyanin accumulation in red pericarp longan (*Dimocarpus longan* Lour.)

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## Abstract

Fruit color influences fruit quality and commodity value. Most longan (*Dimocarpus longan* Lour.) varieties have a yellowish-brown or grayish-yellow pericarp, and the discovery of red pericarp (RP) longan expanded the color varieties of longan fruit. Our previous research showed that the red pericarp of RP fruit was mainly caused by anthocyanin accumulation; however, its underlying regulatory mechanism remains unknown. Herein, DIMYB113, an R2R3-MYB transcription factor, was discovered by examining differentially expressed genes in two longan cultivars. *Dimocarpus longan* MYB (*v-myb avian myeloblastosis viral*) 113 (*DIMYB113*) expression was significantly higher in the pericarp and leaves of RP longan than in 'Shixia' longan. Sequence alignment analysis revealed two amino acid substitutions in the R3 domain between DIMYB113rp in RP longan and DIMYB113sx in 'Shixia' longan. Transient expression of *DIMYB113rp* significantly increased anthocyanin accumulation in tobacco leaves, whereas *DIMYB113sx* had negligible effect. Meanwhile, *DIMYB113* overexpression promotes anthocyanin accumulation in *Arabidopsis* and longan calli. Site-directed mutation detection revealed divergence in *DIMYB113* function when the R3 repeat 197-position base T was replaced with G, and the 317- and 318-position AT bases were replaced with GA. Our findings indicate that DIMYB113 can regulate anthocyanin production in RP longan, and three mutations in its nucleic acid sequence lead to anthocyanin accumulation, thereby developing molecular markers associated with the anthocyanin accumulation trait in RP longan. This study will facilitate early screening of longan hybrids with desirable fruit color and be significant for breeding new characteristic varieties.

**Keywords** Longan, Anthocyanin, MYB transcription factor, Site-directed mutagenesis, Functional marker

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## Introduction

Longan (*Dimocarpus longan* Lour.), native to southern China, is an evergreen fruit tree from Sapindaceae with significant nutritional and medicinal value (Shi et al. 2015; Zhu et al. 2019). Fruit appearance and color are essential indicators for evaluating and determining fruit quality and influencing fruit commodity value. Most longan varieties have a yellowish-brown or grayish-yellow pericarp; the discovery of red pericarp (RP) longan, a wild species from Southeast Asia, expanded the information on fruit color types and provided candidate materials for breeding new longan varieties. We previously demonstrated that the red color of the longan pericarp was mainly driven by anthocyanin accumulation, with cyanin-3-*O* glucoside being the primary anthocyanin component (Yi et al. 2021).

Anthocyanin is a pigment that affects fruit color formation. Several genes are required for anthocyanin production via the phenylpropanoid pathway. These genes are classified as beginning biosynthetic genes (BBGs), early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs) (Liu et al. 2014). The MBW (MYB-bHLH-WD40) complex primarily regulates these structural genes (Xu et al. 2015; Araguirang and Richter 2022). R2R3-MYBs in the MBW complex have been extensively studied in various fruit trees (Martin and Paz-Ares 1997; Stracke et al. 2001; Allan et al. 2008). Moreover, anthocyanin synthesis in apples is linked to the promoter region of *Malus domestica* MYB (*v-myb avian myeloblastosis viral*) 17 (*MdMYB17*)-specific anthocyanin synthesis (Wang et al. 2022a, b). *MdMYB90*-like directly activates anthocyanin biosynthesis genes, *Malus domestica* chalcone synthase (*MdCHS*) and *Malus domestica* Uridine diphosphate (UDP)-glucose: flavonoid 3-*O*-glycosyl-transferase (*MdUFGT*) (Sun et al. 2021). Heterologous expression of *PSMYB10.2* from plum and *v-myb avian myeloblastosis viral* 10 (*MYB10*) and *MYB110* from kiwifruit can activate genes involved in tobacco anthocyanin synthesis (Fang et al. 2021a, b; Wang et al. 2022a, b). Additionally, *LcMYB1* can activate *Litchi chinensis* Glutathione *S*-transferase (*LcGST*) expression and promote anthocyanin accumulation in vitro (Hu et al. 2016). However, whether and how the longan MYB protein regulates anthocyanin production is unclear.

Genetic mutations cause variations in anthocyanin accumulation phenotypes, which are caused by two kinds of mutations: coding and promoter region variations. Variation in the gene coding region frequently alters the three-dimensional structure of the protein, weakening or even eliminating the activity of proteins encoded by structural gene or the ability to bind to the important regulatory elements on the promoter of downstream structural genes of anthocyanin synthesis. Analysis of red and white strawberry alleles in octoploid strawberry (*Fragaria × ananassa*) revealed that in white strawberry, the coding region of

*FaMYB10* was inserted with an 8 bp ACTTATAC sequence, resulting in premature protein translation termination (Wang et al. 2020). Mutation of the G/A single nucleotide in the R3-conserved domain of turnip (*Brassica rapa* subsp. *rapa*) *Brassica rapa* MYB (*v-myb avian myeloblastosis viral*) 1a (*BrPAP1a*) leads to amino acid substitution, ultimately producing an all-white turnip mutant (Yang et al. 2021). Glutathione-*S*-transferase (GST) is an anthocyanin transporter that transports anthocyanins into vacuoles for storage and gives color to plants (Zhao et al. 2020). BSA-seq was performed on F2 populations of peaches (*Prunus persica*) with different flower colors; the 2 bp insertion or 5 bp deletion of *PpGST* in the third exon altered the reading frame and rendered the protein inactive (Lu et al. 2021).

To further elucidate the regulatory mechanism of anthocyanin biosynthesis in RP longan, an R2R3-MYB transcription factor, DIMYB113, that promotes anthocyanin accumulation was identified using transcriptomic and qPCR analyses. Subsequently, its subcellular localization was investigated, and its role in anthocyanin production was confirmed through transient transformation and over-expression. For molecular-assisted breeding, molecular markers associated with the accumulation trait of RP longan anthocyanin were developed. This study deepens our understanding of the mechanism of anthocyanin accumulation in the pericarp of RP longan, as well as the regulatory mechanism of pericarp coloration.

## Materials and methods

### Plant materials

RP longan and the main cultivar ‘Shixia’ (SX) longan were used as experimental materials. The mature leaves and pericarps of the two cultivars were harvested 95 d post-anthesis (DPA) for transcriptome sequencing analysis. Pericarp samples were collected during five developmental stages of RP (15, 35, 55, 75 and 95 DPA). RP mesocarp and seeds were sampled at 95 DPA. Furthermore, all samples were collected and preserved at −80°C.

### Complementary DNA (cDNA) library construction and RNA sequencing

RP and SX longan pericarp (SAMN16132431 and SAMN16132432) and leaf samples were used as transcriptome sequencing samples. Transcriptome sequencing was performed by Guangzhou Gene Denovo Biotechnology Co. Ltd. (Guangzhou, China). The Hisat2 (Kim et al. 2015) was used for sequence alignment on the ‘JDB’ longan genome (Genome assembly ASM2298485v1), the StringTie (Pertea et al. 2015) assembler was used to assemble and quantify the reads. The EdgeR Bioconductor package (Robinson et al. 2010) was used to identify differentially expressed genes (DEGs). The KEGG and Swiss-Prot databases were

used for annotation, TBtools and R packages clusterProfiler for analysis and visualization (Gasteiger et al. 2001; Xie et al. 2011; Chen et al. 2020; Wu et al. 2021).

#### Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of MYB amino acid sequences was performed using MAFFT software (v.7.487) (Katoh and Standley 2013). A maximum likelihood (ML) phylogenetic tree was constructed using FastTree 2.1 software with a resample value of 1,000, in which JTT (Jones et al. 1992) was the best substitution model. The sequences used for the phylogenetic tree included *AtMYB75* (AT1G56650), *AtMYB090* (At1g66390), *AtMYB113* (At1g66370), *AtMYB114* (At1g66380), *CsRUBY* (AFB73913), *AcMYB10* (MN602037), *AcMYB75* (APZ74276.1), *LcMYB1* (APP94121.1), *MdMYB10* (ACQ45201.1), *MdMYB1* (ADQ27443.1), *MdMYB110a* (JN711473), *VvMYBA1* (BAD18977), *VvMYBA2* (BAD18978), *PyMYB10* (ALN66630.1), *PcMYB10* (AFC88545.1), *FaMYB10* (ABX79947.1), *PbMYB10* (EU153577), *PdmMYB10* (EU153580), *EjMYB10* (EU153572), and *CoMYB10* (EU153571).

#### Quantitative real-time PCR (qPCR) analysis

DEG expression levels in the biosynthetic pathway of anthocyanin were analyzed using qPCR. The gene-specific primers were designed using Primer 5.0 and are listed in Table S1. The reaction system was constructed using SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was conducted on the Light-CyclerR480 system (Roche, Switzerland). The expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method.

#### Total

After weighing 0.25 g of powder from each sample, total anthocyanins were extracted in the dark at 4°C for 24 h using 10 mL of methanol: hydrochloric acid (99:1, v/v) (Tuan et al. 2015). The samples were centrifuged at 10,000 rpm for 10 min. A 1 mL supernatant was mixed with 1 mL KCL buffer (pH 1.0), and a 1 mL supernatant plus NaAc buffer (pH 4.5) was incubated at 4°C for 15 min. The absorbance was measured at 510 and 700 nm using a spectrophotometer. Three measurements were taken for each biological replicate. The relative anthocyanin content was normalized using the following formula reported by Romero et al. (2008) and Fang et al. (2021a).

#### Subcellular localization analysis

Subcellular localization was determined using fluorescent protein fusions after transient expression in onion epidermal cells (Nebenführ 2014). *Dimocarpus longan* MYB (*v-myb avian myeloblastosis viral*) *113rp* (*DlMYB113rp*) and *DlMYB113sx* were cloned and inserted into

the pCambia2300\_35S:GFP vector. A fresh onion was cut into 1 cm<sup>2</sup>, and the inner epidermal cells were placed in Murashige and Skoog (MS) medium for 2 d at 28°C in the dark. After 8 min of immersion in resuspended *Agrobacterium tumefaciens*, the cells were placed in a dark culture at 28°C for 24–72 h, and GFP expression was measured using LSM 800 laser confocal microscopy (Zeiss, Germany). A 35S:GFP vector was used as a control.

#### Transient expression of *DlMYB113* in tobacco leaves

Transient expression assays were performed in young *Nicotiana tabacum* leaves cultivated in a greenhouse, as described previously (Fang et al. 2021). The target gene-containing plant expression vector was transformed into *A. tumefaciens* and cultured in darkness at 28°C for 2 d. GV3101 bacteria were resuspended in an infiltration buffer containing 10 mM MgCl<sub>2</sub> and 100 μM acetosyringone, cultivated for 2 h in the dark, and injected into tobacco leaves.

#### Generation of *DlMYB113*-overexpressing plants

Flower-soaking transformation yielded transgenic overexpression lines of *DlMYB113rp* and *DlMYB113sx* driven by the 35S promoter (Zhang et al. 2006). Fresh *A. tumefaciens* solution (20 μL) was inoculated into 150 mL of liquid YEP medium with corresponding antibiotics, and the strain was shaken at 28°C to an OD<sub>600</sub> of approximately 1.0. The bacteria were centrifuged at room temperature for 10 min at 6000 rpm, and the supernatant was discarded. The bacteria were suspended in a 5% sucrose solution containing 0.05% Sillwet-77. *Arabidopsis thaliana* inflorescences were suspended for 30 s, incubated in the dark for 24 h, and then returned to normal conditions until seeds were harvested.

#### Generation of transgenic longan calli

The target gene-containing plant expression vector was transformed into *A. tumefaciens* GV3101, which was then incubated with longan calli for 30 min and transferred to agar-solidified MS medium and cultured for 2 d in the dark. The calli were subsequently transferred to a screening medium containing 100 μM kanamycin and 500 mg/mL Timentin.

#### Statistical analysis

Data are means ± SD of 3 biological replicates, and the asterisks indicate statistically significant differences (\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001). Bars with different letters indicate significant differences at *P* < 0.05 by ANOVA with Tukey's multiple comparison test.

## Results

### Fruit and leaf phenotype

As the picture in Fig. 1 shows, SX longan has a yellowish-brown pericarp, similar to that of most cultivars, whereas

RP longan has a dark red pericarp, which is uncommon in longan cultivars. There was no anthocyanin accumulation in SX longan cotyledons, while there was some in RP longan cotyledons. However, the aril of RP longan was similar to that of ordinary longan and appeared milk-white due to a lack of pigment accumulation (Fig. 1a, b). Moreover, SX longan leaves were green with pale yellow veins (Fig. 1c), whereas RP longan leaves were dark red on the adaxial side and green on the axial side (Fig. 1d).

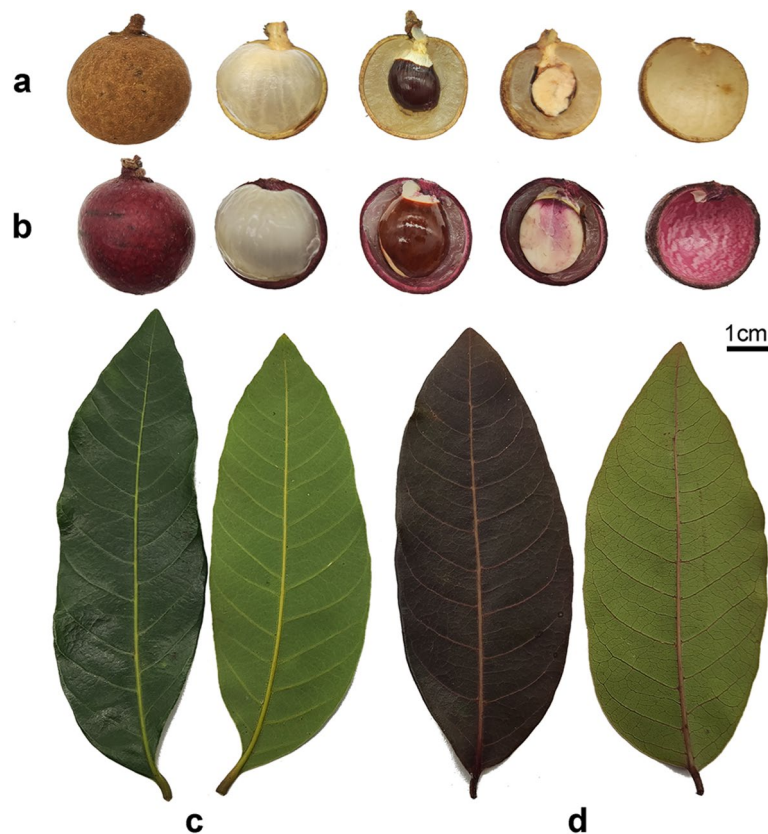
### Transcriptome sequencing and analysis

RNA-seq data from pericarp and leaf samples of two different varieties were generated. All clean reads were mapped to longan's reference genome, and most reads of RP (88.71%) and SX (93.78%) matched the reference genome. Principal component analysis (PCA) revealed that the twelve pericarp and leaf samples were then classified into four groups, each corresponding to two varieties, demonstrating that the gene expression data obtained in this study had good reproducibility (Fig. S1). In addition, 4985 DEGs were discovered in the transcriptomes of RP longan and SX longan leaf and pericarp (Fig. S2).

DEGs were annotated using gene ontology (GO) terms to help classify their function. Biological process was

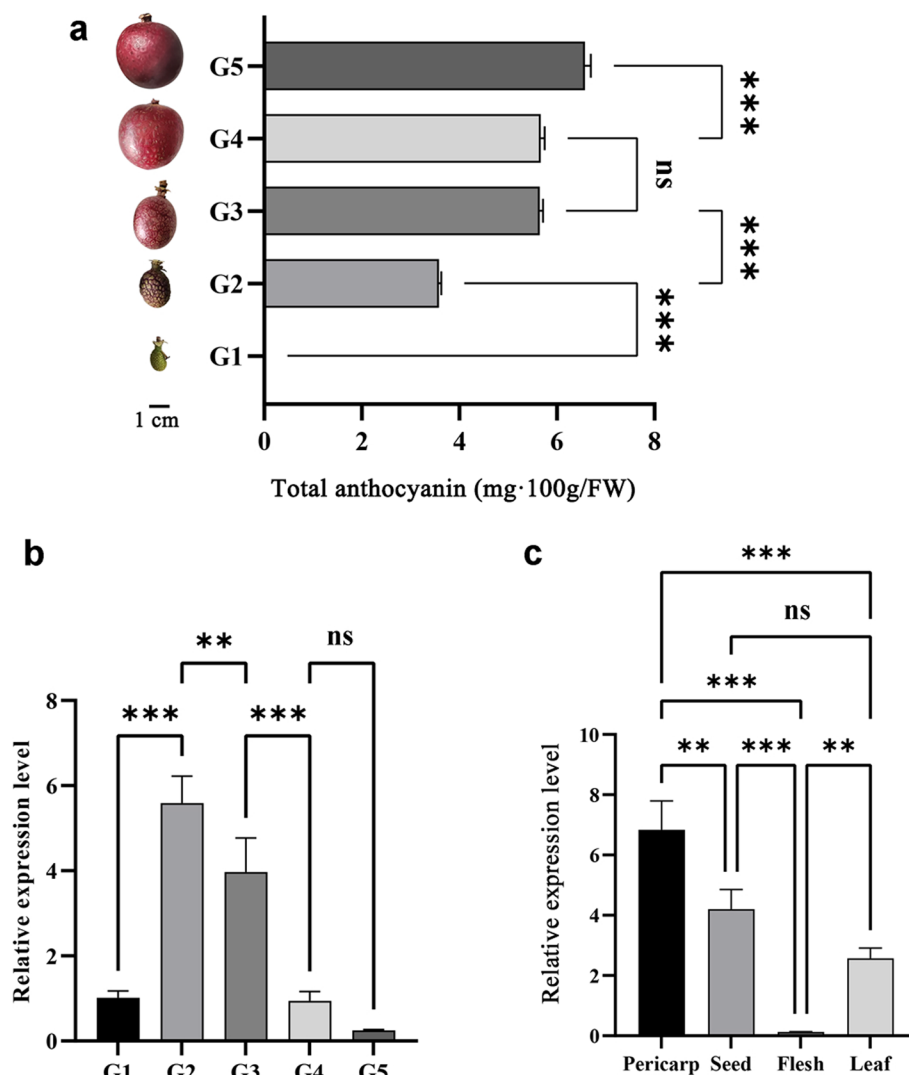
more closely related to the secondary metabolic activity occurring between the pericarp and leaf of RP and SX longans (Figs. S3 and S4). DEGs were analyzed using the eggNOG-mapper and TBtools analytic tools to identify key metabolic pathways in RP longan. The KEGG pathways ( $P < 0.05$ ) are shown in Figs. S5 and S6. The pericarp upregulated DEGs were significantly enriched in cytochrome P450, phenylpropanoid biosynthesis, flavone and flavonol biosynthesis, and biosynthesis of other secondary metabolites pathway in RP longan. The upregulated DEGs in the leaves were significantly enriched in anthocyanin biosynthesis, phenylpropanoid biosynthesis, biosynthesis of other secondary metabolites, and cytochrome P450 pathway.

Based on the Swiss-Prot database annotation information, 25 anthocyanin-related structural genes were screened from the pericarp and leaf common DEGs, including two *4-coumarate CoA ligase* (4CL), one *Phenylalanine ammonia-lyase* (PAL), two *Chalcone synthase* (CHS), one *Flavonoid 3',5'-hydroxylase* (F3'5'H), six *UFGT*, and 13 *GST* (Fig. S7). In addition, 81 transcription factors were identified from DEGs, including 24 *MYBs*, 11 *bHLH*, 18 *AP2/ERF*, nine *MADS-box*, seven *NACs*, and 10 *WRKYs* (Fig. S8).



**Fig. 1** Comparison of pericarps and leaves between the two longan varieties. (a) Morphological characteristics of the fruits of 'Shixia' (SX) longan. (b) red pericarp (RP) longan. (c) Morphological characteristics of the adaxial and axial sides of SX longan. (d) RP longan leaves

**Fig. 2** Cluster analysis of anthocyanin biosynthesis-related MYB genes in different species. Cluster analysis and functional prediction of MYB in different species. The phylogenetic tree relationship between DIMYB113 and MYB proteins from other plant species *via* Fasttree. The red letters indicate DIMYB113 protein



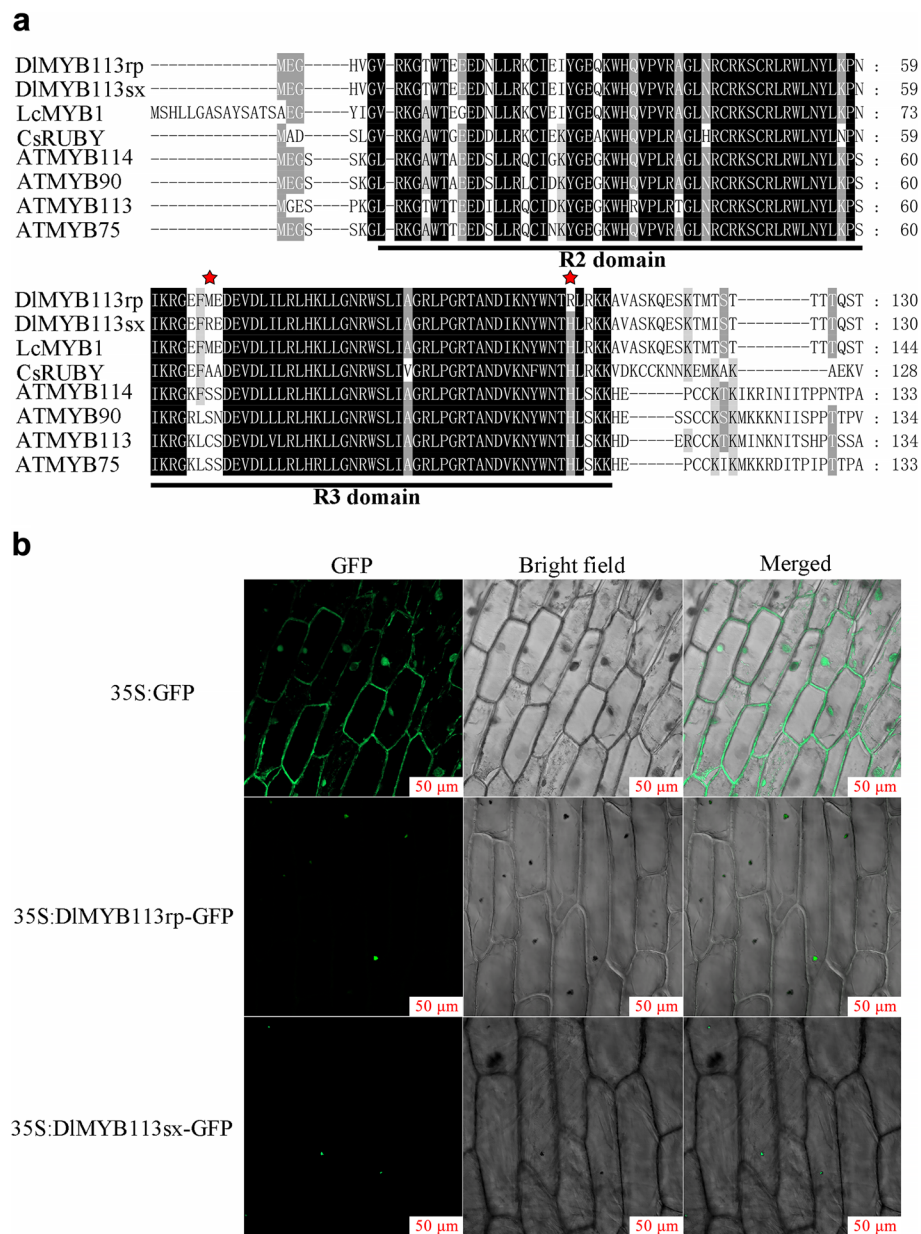
**Fig. 3** Characteristics of *v-myb avian myeloblastosis viral (MYB)* gene expression. (a) Total anthocyanin content in five developmental stages of fruits in red pericarp (RP) longan (G1–G5 stand for 15, 35, 55, 75 and 95 DPA, respectively). (b) The expression levels of *Dimocarpus longan MYB (v-myb avian myeloblastosis viral) 113 (DIMYB113)* in the pericarp during different developmental stages. (c) The expression levels of *DIMYB113* in different tissues of RP longan

sequence alignment showed that they both contained the conserved R2R3 domain (Fig. 4a). *DIMYB113* from RP longan and SX longan was designated as *DIMYB113rp* (GenBank: OQ911501) and *DIMYB113sx* (GenBank: OQ911500), respectively.

To investigate *DIMYB113* subcellular localization, recombinant (35S:*DIMYB113*:GFP) and control vectors were agroinfiltrated into onion epidermal cells. The control vector's GFP fluorescence was distributed throughout the cell, whereas 35S:*DIMYB113*:GFP vectors displayed high fluorescence signals in the nuclei of onion epidermal cells. Therefore, *DIMYB113* was likely localized and functioned in the nucleus (Fig. 4b).

#### Differences in *DIMYB113* sequence cause variation in its function

To further validate the different function of two types *DIMYB113*, *Agrobacterium tumefaciens* GV3101 containing pCAMBIA2300:35S:*DIMYB113* was transiently transformed into tobacco leaves. Transient overexpression of *DIMYB113rp* promoted anthocyanin accumulation in tobacco leaves while the difference in anthocyanin content between *DIMYB113sx* and the control was not significant (Fig. 5a, b). These results indicated that *DIMYB113rp* may regulate anthocyanin biosynthesis; however, *DIMYB113sx* did not significantly increase anthocyanin content. The inability of *DIMYB113sx* to bind to downstream target

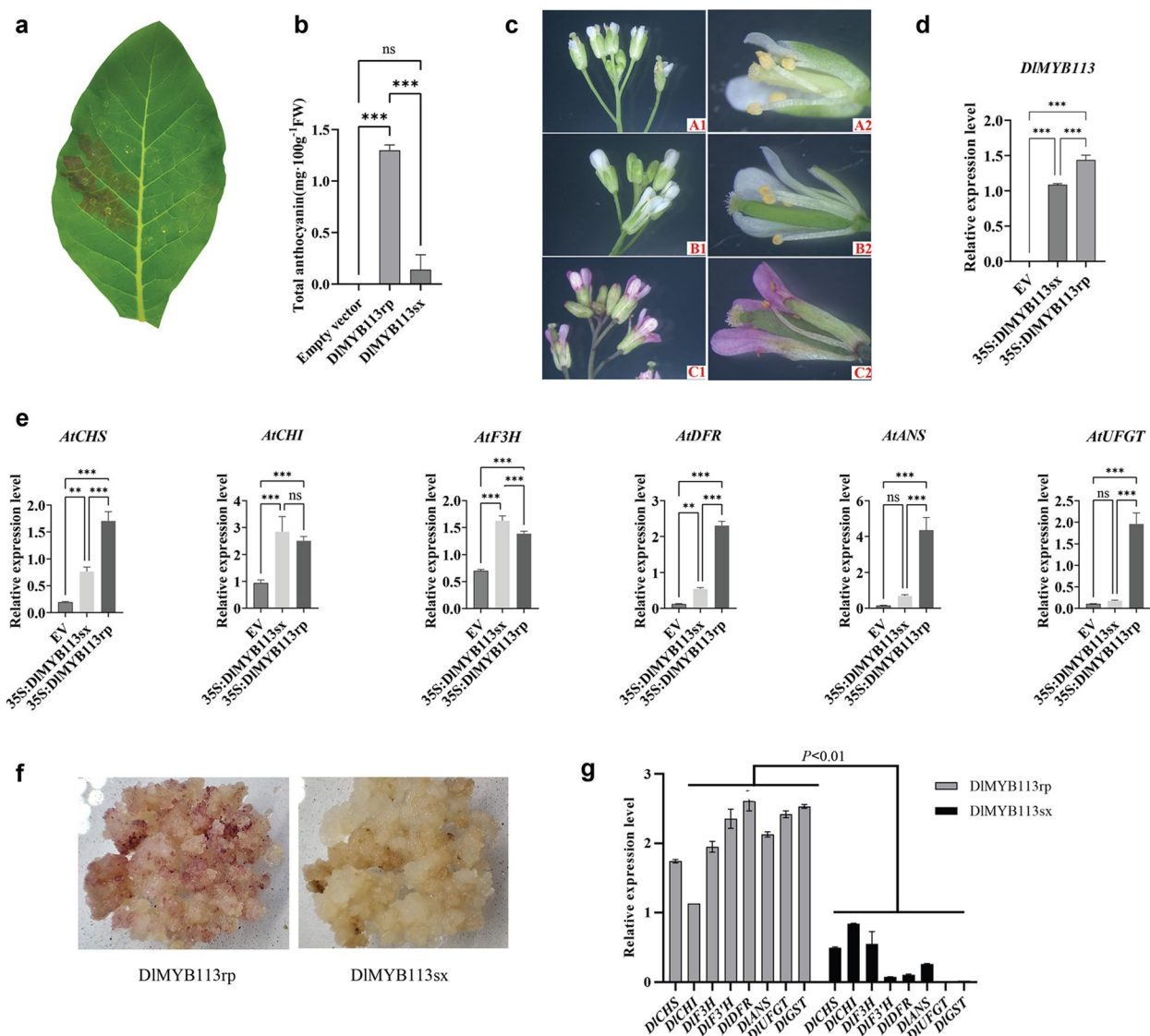


**Fig. 4** Sequence alignment and subcellular localization of DIMYB113. (a) Sequence alignment of DIMYB113, LcMYB1, and CsRUBY. The R2 and R3 domains are indicated using black lines, and the red star indicates the amino acid substitution site of DIMYB113 in the conserved domains of red pericarp (RP) longan and 'Shixia' (SX) longan. (b) Subcellular localization of DIMYB113 in onion epidermal cells. pCAMBIA2300-35s: GFP was used as a positive control

genes in tobacco may be related to the base mutation in its R3 conserved region, which may also explain why anthocyanins do not accumulate in the pericarp of SX longan.

Subsequently, the *DIMYB113rp* and *DIMYB113sx* genes were evaluated using overexpression driven by the 35S promoter in *Arabidopsis*. The 35S:*DIMYB113rp* transformants accumulated significantly more anthocyanins in petals, petioles and the anterior ends of the filaments and calyx than the control 35S:*DIMYB113sx* (Fig. 5c). *DIMYB113* was

highly expressed in 35S:*DIMYB113rp* and 35S:*DIMYB113sx* plants (Fig. 5d). Additionally, *DIMYB113* overexpression upregulated the expression of structural genes involved in anthocyanin biosynthesis, including *Arabidopsis thaliana* chalcone synthase (*AtCHS*), *Arabidopsis thaliana* chalcone isomerase (*AtCHI*), *Arabidopsis thaliana* flavanone 3-hydroxylase (*AtF3H*), *Arabidopsis thaliana* dihydroflavonol-4-reductase (*AtDFR*), *Arabidopsis thaliana* anthocyanidin synthase (*AtANS*) and *Arabidopsis thaliana*



**Fig. 5** Morphological characteristics and related gene expression of *Dimocarpus longan* MYB (*v-myb* avian myeloblastosis viral) 113 (*DIMYB113*) in tobacco leaves, *Arabidopsis* and *Dimocarpus longan* Lour. (longan) calli. **(a)** Phenotypic characteristics of *DIMYB113* transiently transformed tobacco. pCAMBIA2300-35S was used as a positive control. **(b)** Total anthocyanin content in transformed leaves of tobacco. **(c)** Phenotypic characteristics of transgenic *Arabidopsis*. A, B and C represent *Arabidopsis* flower organs transformed into empty 35S:*DIMYB113sx* and 35S:*DIMYB113rp* vectors, respectively. EV, empty vector. **(d)** The expression levels of *Dimocarpus longan* MYB (*v-myb* avian myeloblastosis viral) 113 (*DIMYB113*) in transgenic *Arabidopsis*. **(e)** Expression of structural genes involved in anthocyanin biosynthesis in *Arabidopsis* overexpressing *DIMYB113*. **(f)** Phenotypic characteristics of transgenic longan calli. **(g)** Expression of structural genes involved in anthocyanin biosynthesis in longan calli overexpressing *DIMYB113*

uridine diphosphate (UDP)-glucose: flavonoid 3-O-glycosyl-transferase (*AtUFGT*) (Fig. 5e). Therefore, *DIMYB113* can regulate anthocyanin accumulation, and the difference in sequence could be the key to anthocyanin accumulation in the RP longan pericarp.

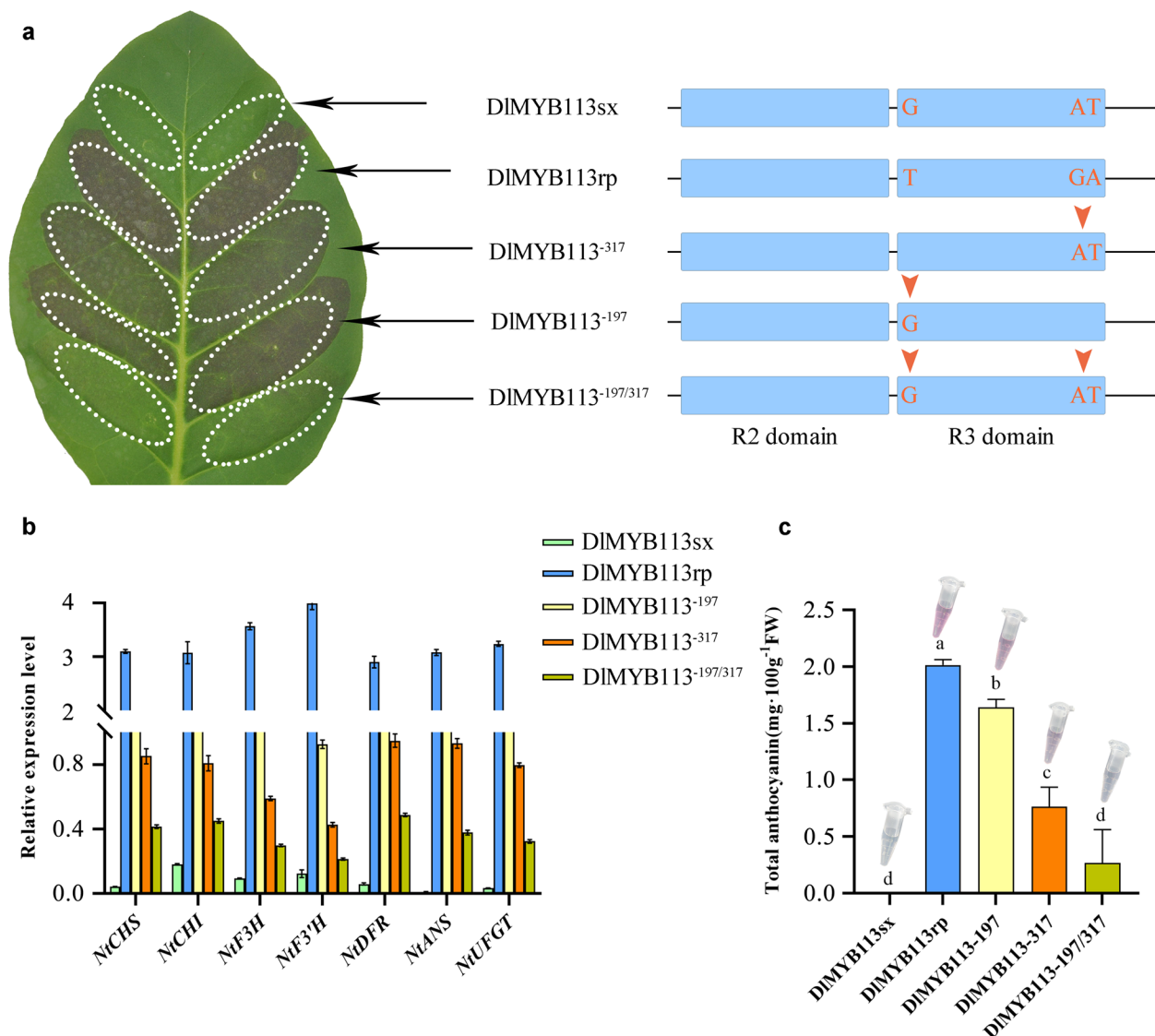
The transformation of the longan calli confirmed that anthocyanin accumulated in RP longan due to the

difference in the *DIMYB113* sequence, and *DIMYB113* was the key transcription factor regulating RP longan coloration (Fig. 5f). *DIMYB113rp* overexpression significantly increased the expression of anthocyanin biosynthesis-related structural genes (Fig. 5g), which was similar to *DIMYB113rp* overexpression in *Arabidopsis*.

### Base substitution in the conserved R3 domain resulted in function variation of DIMYB113

*DIMYB113*'s functional characterization varies due to sequence variation in the coding region. A point mutation sequence was constructed and ligated to the plant binary expression vector pCambia2300:35S to test whether amino acid substitutions in the R3 conserved structural domain of *DIMYB113* were responsible for the functional variation in its coding region. *DIMYB113rp* base T at position 197 was substituted with G

(amino acid at position 66 was replaced from methionine to arginine) and named *DIMYB113*<sup>-197</sup>. The bases AT at positions 317 and 318 in *DIMYB113rp* were replaced by GA to AT (amino acid AT position 106 was replaced from arginine to histidine) and named *DIMYB113*<sup>-317</sup>. Simultaneously, a double-mutant vector named *DIMYB113*<sup>-197/317</sup> was constructed. *DIMYB113*<sup>-197</sup> was more capable of promoting anthocyanin accumulation in tobacco leaves than *DIMYB113*<sup>-317</sup>, whereas *DIMYB113*<sup>-197/317</sup> was consistent with *DIMYB113sx*,



**Fig. 6** Phenotype and gene expression of *DIMYB113rp* site-directed mutagenesis in tobacco. **(a)** Transient phenotype of tobacco with *DIMYB113* site-directed mutations. The control groups in the model are *DIMYB113rp* and *DIMYB113sx*, and the point mutation sites are the bases indicated using orange arrows. **(b)** Tobacco anthocyanin biosynthesis-related gene expression levels in different point mutation types. **(c)** Total anthocyanin content of tobacco with different point mutation types. *NtCHS*, *Nicotiana tabacum* chalcone synthase; *NtCHI*, *Nicotiana tabacum* chalcone isomerase; *NtF3H*, *Nicotiana tabacum* flavanone 3-hydroxylase; *NtF3'H*, *Nicotiana tabacum* flavonoid 3'-hydroxylase; *NtDFR*, *Nicotiana tabacum* dihydroflavonol-4-reductase; *NtANS*, *Nicotiana tabacum* anthocyanidin synthase; *NtUGT*, *Nicotiana tabacum* uridine diphosphate (UDP)-glucose: flavonoid 3-O-glycosyl-transferase

with little anthocyanin accumulation (Fig. 6a). qPCR revealed that *DLMYB113*<sup>-197</sup>, *DLMYB113*<sup>-317</sup> and *DLMYB113*<sup>-197/317</sup> treatment showed varying degrees of significant downregulation of anthocyanin structural genes compared to *DLMYB113*rp-treated leaves and *DLMYB113*<sup>-197/317</sup> was the most significant, followed by *DLMYB113*<sup>-317</sup> (Fig. 6b). Interestingly, the trend of anthocyanin structural gene expression in tobacco leaves with point mutations was consistent with the trend of total anthocyanin content (Fig. 6c).

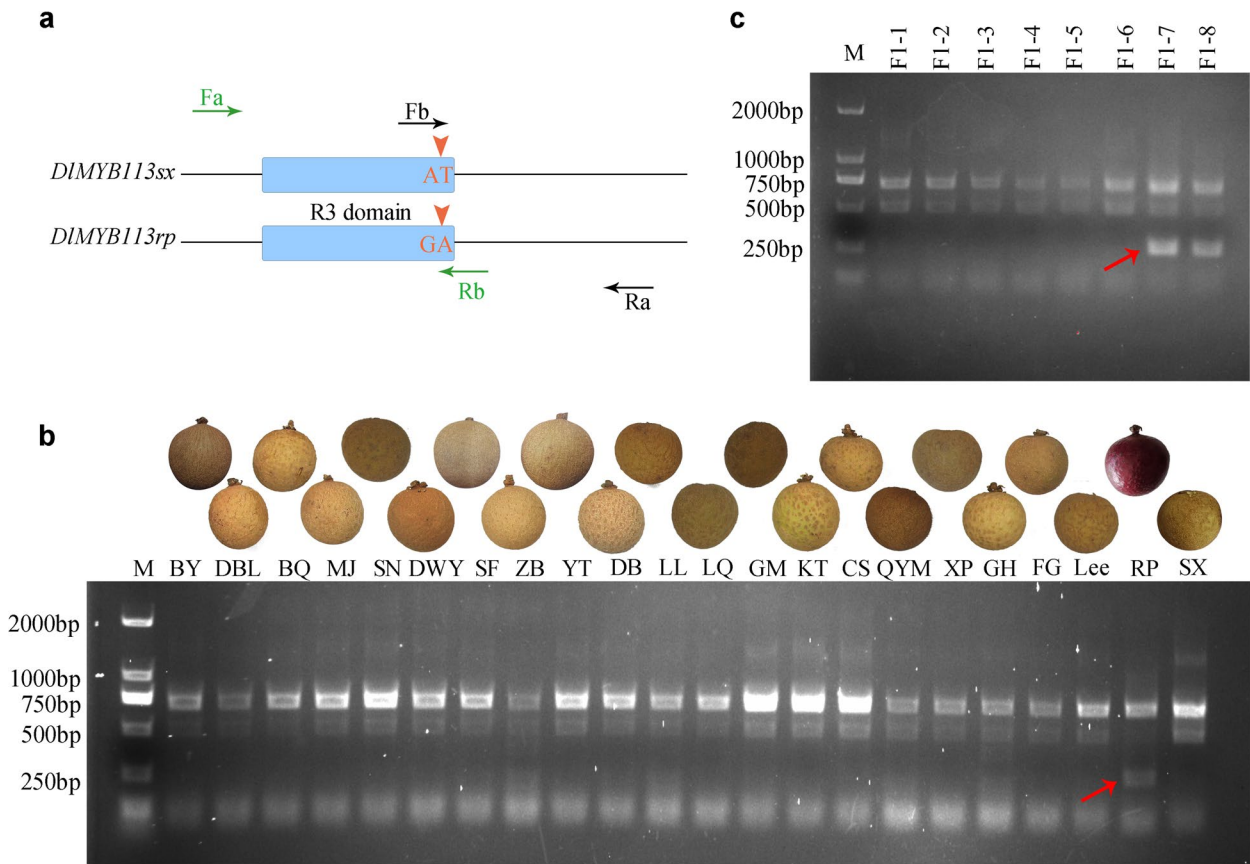
**Development of functional markers for red pericarp trait screening**

It is critical to develop screening markers for RP longan resource breeding based on *DLMYB113* mutation variations. Therefore, a *myb113arms* functional marker was developed in this study based on the double mutation site (AT/GA) of *DLMYB113*'s R3 conserved domain (Fig. 7a and Table S1). According to the Tetra-primer

ARMS-PCR primer design strategy, two bands with 716 and 253 bp in RP longan and 716 and 506 bp in SX longan could be amplified. Using the *myb113arms* functional marker to perform the Tetra-primer ARMS-PCR in 22 germplasm resources revealed that only the anthocyanin-accumulating RP longan could amplify a specific band of 253 bp, whereas the remaining 20 varieties could only amplify a band of 253 bp, as in SX longan (Fig. 7b). Using primer characteristics, the true hybrids of two longan strains (F1-7 and F1-8) were identified, revealing three band types of 716, 506, and 253 bp (Fig. 7c). This finding provides a functional marker for identifying favorable progeny in subsequent RP longan cross-breeding.

**Discussion**

Longan is an important tropical and subtropical fruit tree. Although there are numerous longan varieties, most cultivated varieties have yellowish-brown or grayish-yellow peels. Longan's relatively single and dull color



**Fig. 7** The development and utilization of *myb113arms* marker for screening various cultivars. **(a)** Primer design strategy for the functional marker *myb113arms*. Two allele-specific amplicons were generated using two pairs of primers: one (Fa and Rb) producing an amplicon representing the GA allele and the other (Fb and Ra) producing an amplicon representing the AT allele. **(b)** Tetra-primer ARMS-PCR was used to verify the functional markers of 22 longan germplasm resources (the full names of the varieties are listed in Table S2). Red arrows indicate the specific band of red pericarp (RP) longan. **(c)** Tetra-primer ARMS-PCR was used to identify the hybrid offspring of RP longan and 'Shixia' (SX) longan. Red arrows indicate the specific band of RP longan. Fa, Forward primer a; Ra, Reverse primer a; Fb, Forward primer b; Rb, Reverse primer b; *DLMYB113sx*, *Dimocarpus longan MYB (v-myb avian myeloblastosis viral) 113sx*

severely limits its diversity and market potential. The discovery of RP longan resources provided the foundation for breeding new longan varieties with bright colors. Longan is a woody fruit tree with a long breeding cycle. Elucidating the regulatory mechanism of anthocyanin synthesis in RP longan and developing red trait screening markers can facilitate early RP progeny screening and improve breeding efficiency.

The red color of red longan fruit is generated by anthocyanin accumulation, and cyanidin 3-O glucoside is the main anthocyanin component in the pericarp (Yi et al. 2021). BBGs, EBGs and LBGs regulate anthocyanin production, and most structural genes are tissue-specific (Brugliera et al. 1999; Polashock et al. 2002; Zhao et al. 2012; Gu et al. 2019). Various genes involved in the anthocyanin biosynthesis pathway were detected as DEGs between RP longan and SX longan. Two *4CL*, one *PAL*, two *CHS*, one *F3'5'H*, six *UFGT*, and 13 *GST* genes were identified (Fig. S4). These differentially structured genes in the anthocyanin synthesis pathway provide important support for elucidating the transcriptional regulatory mechanism of anthocyanin biosynthesis in RP longan.

The MBW complex regulates anthocyanin production primarily in plants, and the former research indicated that MYB transcription factors may be involved in anthocyanin accumulation in RP longan (Yi et al. 2021). Many MYB transcription factors regulate anthocyanin biosynthesis by modulating structural gene transcription (Zhou et al. 2019). *LcMYB1* expression was positively correlated with anthocyanin content (Lai et al. 2014). Moreover, *LcMYB5* expression is relatively consistent in the pericarp during the coloring period of rapid anthocyanin accumulation (Lai et al. 2019). Furthermore, *Arabidopsis thaliana* AtMYB113, eggplant SmMYB113, and potato StMYB113 could bind to downstream target genes to promote anthocyanin production. In the present study, sequence analysis revealed that DIMYB113 differed between RP longan and SX longan (Fig. 4a). Three base mutations in R3's conserved domain caused the substitution of two amino acids, one within the loop and one in the third helix. The transient transformation of tobacco leaves, *Arabidopsis* and longan calli indicated that the longan pericarp coloring was caused by a sequence variation in *DIMYB113*. All these results confirmed the powerful function of *DIMYB113* in anthocyanin accumulation of RP longan.

Point mutations can produce new alleles and genetic functions, resulting in variations in the morphological structure of organisms (such as shape, size and color), which can provide important donor materials for molecular design breeding. Mutations in the

R2 and R3 domains alter the promoter target specificity and DNA binding affinity of MYB proteins (Williams and Grotewold 1997; Heppel et al. 2013). In MYB domain studies, the third helix of the R2 and R3 repeats is considered the recognition helix and binds directly to a specific cis-regulatory element (Ogata et al. 1995; Tominaga et al. 2007). However, the first two helices of the R2 and R3 repeats play a crucial role in DNA binding affinity (Jia et al. 2014). The analysis of PpMYB10.1 and PpMYB10.2 showed that the change in two amino acids, Arg/Lys<sup>66</sup> and Gly/Arg<sup>93</sup>, located in the R3 domain's second helix and loop of the R3 domain, was the cause of anthocyanin accumulation and that the mutation of the R3 domain recognition helix was not the main factor affecting anthocyanin accumulation (Zhou et al. 2018). In this study, transcriptome analysis was used to identify a potential transcription factor, DIMYB113, that regulates anthocyanin biosynthesis. Point mutations reveal that the *DIMYB113* sequence variation is one of the factors driving pericarp pigmentation; however, further research is warranted.

## Conclusions

This study compared the published transcriptomes of RP longan and SX longan pericarp with transcriptomic data on the mature leaves of RP longan and SX longan. The structural and regulatory genes involved in anthocyanin accumulation in the pericarp were investigated. Experiments using heterologous transformation further demonstrate how *DIMYB113rp*, an important regulatory gene that regulates the coloring of RP longan pericarp, promotes anthocyanin accumulation in tobacco leaves, *Arabidopsis* and longan calli. This study provides a reference for investigating the factors that control longan fruit coloration and developing molecular markers for the anthocyanin accumulation trait.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-023-00014-3>.

**Additional file 1: Fig. S1.** Principal component analysis (PCA) of transcriptome data of *Dimocarpus longan* Lour. (longan) tissue samples. (a) PCA analysis of pericarps transcriptome data. (b) PCA analysis of leaves transcriptome data. RP, red pericarp; RPL, the leaf of red pericarp longan; SX, 'Shixia'; SXL, the leaf of 'Shixia' longan **Fig. S2.** Number of differentially expressed genes (DEGs) between RP vs SX and RPL vs SXL. RP, red pericarp; RPL, the leaf of red pericarp longan; SX, 'Shixia'; SXL, the leaf of 'Shixia' longan **Fig. S3.** Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the leaf of red pericarp longan (RPL) and the leaf of 'Shixia' longan (SXL). BP, biological process; CC, cellular component; MF, molecular function **Fig. S4.** Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the leaf of red pericarp longan (RPL) and the leaf of 'Shixia' longan (SXL). BP, biological process; CC, cellular component; MF, molecular function **Fig. S5.** The function of differentially expressed genes (DEGs) is analyzed by KEGG in the leaf of red pericarp longan (RPL) and the leaf of 'Shixia' longan (SXL) **Fig. S6** The

function of differentially expressed genes (DEGs) is analyzed by KEGG in the leaf of red pericarp longan (RPL) and the leaf of 'Shixia' longan (SXL) **Fig. S7**. Heat map of structural genes related to anthocyanin biosynthesis. Red, white, blue, yellow, purple and gray represent 4-coumarate CoA ligase (4CL), Phenylalanine ammonia-lyase (PAL), Chalcone synthase (CHS), Uridine diphosphate (UDP)-glucose: flavonoid 3-O-glycosyl-transferase (UGT), Flavonoid 3',5'-hydroxylase (F3',5'H) and Glutathione S-transferase (GST) respectively **Fig. S8**. Heat map of related genes involved in the transcription regulation of anthocyanin biosynthesis. Blue, red, gray, yellow, white, green and purple represent v-myb avian myeloblastosis viral (MYB), basic Helix-Loop-Helix (bHLH), basic leucine zipper (bZIP), APETALA2/ethylene responsive factor (AP2/ERF), MCM, AG, DEFICIENS, SERUM response factor (MADS-box), the NAM/ATF1/2/CUC2 (NAC) transcription factor (NAC) and WRKY (Named for its special heptapeptide conserved sequence WRKY-GOK), respectively. RP, red pericarp; RPL, the leaf of red pericarp longan; SB, the pericarp of 'Shixia' longan; SXL, the leaf of 'Shixia' longan **Fig. S9**. Cluster analysis and functional prediction of v-myb avian myeloblastosis viral (MYB) in longan and Arabidopsis. Orange, blue, green, and purple represent the four subgroups S4, S7, S5 and S6, respectively, associated with anthocyanin biosynthesis in Arabidopsis.

**Additional file 2: Table S1.** Gene specific primers used in this study.

**Additional file 3: Table S2.** The full names and abbreviations of 22 longan germplasm resources.

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Not applicable.

## Authors' contributions

Y-H Huang, Y-Z Wei, and S-Y Shi conceived and designed the study; Y-H Huang and Y-Z Wei conducted experiments and analyzed data; L-Q Liu, D-B Yi, J-N Zhou, and X-W Hu provided the transcriptome analysis; Y-Y Lu, L-F Zhu, M-Y Chen and Y-C Wang generated transgenic plants; Y-H Huang wrote the manuscript; Y-Z Wei and S-Y Shi revised the manuscript. All authors read and approved the manuscript.

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## Availability of data and materials

The RNA-Seq raw data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra/>) under bioproject accession PRJNA663435, PRJNA943549 and PRJNA943551. All other data generated in this study are included in this published article and its supplementary information.

## Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

Authors declare that they have no conflict of interests.

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