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The potential role of R2R3-MYB gene family in the phenylpropanoid pathway and regulatory mechanism in *Fragaria × ananassa*

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Abstract

It is common knowledge that R2R3-MYB transcription factors play significant roles in plant biological and physiological processes, especially in the phenylpropanoid metabolism pathway. The cultivated strawberry (*Fragaria × ananassa* Duch.) is an octoploid (2n = 8x = 56) species from the *Rosaceae* family and it is also an important fruit crop species. However, the function of R2R3-MYB genes in strawberry remains largely unknown. Here, based on the genome of the cultivated strawberry cv. Reikou, 66 *FanMYB* genes were found and systematically analyzed. RNA-seq analysis revealed that some *FanMYBs* exhibited tissue-specific expressions and were methyl jasmonate (MeJA)-responsive. Phylogenetic relationships and protein-protein interaction analysis suggested that 13 *FanMYBs* were likely associated with phenylpropanoid metabolism. Out of these genes, *FanMYB22*, *FanMYB36*, *FanMYB47*, *FanMYB49*, and *FanMYB63* were post-transcriptionally regulated by miR858 according to the degradome data analysis, suggesting the conservation and complex regulation network in *F. × ananassa*. Current findings provide a useful resource for future research on the function of *FanMYBs* and the regulatory mechanism of the phenylpropanoid pathway in strawberry.

Keywords: *Fragaria × ananassa*, gene expression pattern, miRNA, phylogenetic relationship, protein-protein interaction, R2R3-MYB gene family.

Introduction

A DNA-binding protein known as a transcription factor (TF) is responsible for influencing gene expression by binding to *cis*-element-specific DNA sequences. TFs are transcriptional activators or repressors in plant development, stress response, and secondary metabolism (Yang *et al*. 2012, Romani and Moreno 2021). In terms of the types of conserved domains, TFs can be grouped into various families, such as NAC, bHLH, WRKY, and MYB (Reboledo *et al*. 2022). Almost all eukaryotes possess the MYB gene family, especially plants. The MYB gene family is a large group with many different functions (Dubos *et al*. 2010).

MYB TFs have highly conserved N-terminal DNA-binding domain repeats and typically contain one, two, three, or four imperfect repeats of the MYB domain. Based on these repeats, MYB TFs are classified into R1-MYB, R2R3-MYB, R3-MYB, and R4-MYB (Dubos *et al*. 2010). The R2R3-MYBs are the most common

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Abbreviations: ANR - anthocyanidin reductase; ANS - anthocyanidin synthase; CDS - coding sequence; C4H - 4-hydroxylase; CHI - chalcone isomerase; CHS - chalcone synthase; 4CL - 4-coumarate: CoA ligase; DFR - dihydroflavonol 4-reductase; F3H - flavanone 3β-hydroxylase; FLS - flavonol synthase; FPKM - fragments per kilobase of transcript per million mapped reads; LAR - leucoanthocyanidin reductase; MeJA - methyl jasmonate; PAL - phenylalanine ammonia-lyase; RT-qPCR - real-time quantitative PCR; TF - transcription factor; UPE - unpaired energy.
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type and the largest subfamily of MYBs in plants. The number of R2R3-MYBs is unevenly distributed in plants, for example, *Arabidopsis thaliana*, *Curcuma wenyujin*, *Scutellaria baicalensis*, and *Musa acuminata* have 126, 42, 95, and 285 R2R3-MYBs, respectively (Stracke *et al*. 2001, Pucker *et al*. 2020, Wang *et al*. 2022a, Wei *et al*. 2022).

In plants, R2R3-MYBs have a variety of functions. They can participate in the primary and secondary metabolism, as well as in developmental processes and responses to biotic and abiotic stresses (Baillo *et al*. 2019, Ma and Constabel 2019). As for the secondary metabolism, there has been evidence that certain members of the R2R3-MYB family are positive regulators in the phenylpropanoid biosynthesis (Liu *et al*. 2015). For example, in *Lonicera macranthoides*, *LmMYB15* increased the accumulation of chlorogenic acid by activating the promoters of *4-coumarate: CoA ligase* (*4CL*) (Tang *et al*. 2021). In hybrid poplar, both anthocyanin and lignin biosynthesis pathways are positively regulated by *PrMYB120 via* activating transcription of *chalcone synthase* (*CHS*) and *ferulate-5 hydroxylase* (*F5H*) (Kim *et al*. 2021). When *PpMYB17* was overexpressed in *Pyrus bretschneideri* calli and *A. thaliana*, flavonoid content was increased *via* the upregulation of genes involved in the flavonoid biosynthesis, especially the *flavonol synthase* (*FLS*) (Premathilake *et al*. 2020). In addition, a number of R2R3-MYBs have been found to negatively regulate the metabolism of the phenylpropanoid compounds in plants. For instance, in *Salvia miltiorrhiza*, overexpression of *SmMYB39* significantly reduced the 4-coumaric acid, rosmarinic acid, salianolic acid B, and salianolic acid A content as well as the total phenolic content by inhibiting the transcription and enzyme activity of 4-hydroxylase (C4H) and tyrosine aminotransferase (TAT) (Zhang *et al*. 2013). *Arabidopsis* MYB4 can decrease the flavonoid content by inhibiting the gene encoding arogenate dehydratase 6 (ADT6), which catalyzes phenylalanine biosynthesis (Wang *et al*. 2020a). Great progress has been made in the research of R2R3-MYBs, but the functions of many R2R3-MYBs are still unknown.

Strawberry (*Fragaria* × *ananassa* Duch.) is an octoploid species (2n = 8x = 56) and it is an important soft fruit all over the world due to its high content of bioactive compounds, including flavonoids and anthocyanins. Other than the fruits, strawberry leaves contain a high amount of bioactive compounds with high antioxidant properties that may be useful for a variety of health-related applications (Kårlund *et al*. 2017). A number of phenolic compounds have been reported to be present in strawberry leaves, such as gallic acid derivatives, quercetin and kaempferol derivatives, catechin, *p*-coumaric acid, *p*-hydroxybenzoic acid, chlorogenic acid, as well as ellagitannins (Brčić Karačonji *et al*. 2022). These compounds are the products of the phenylpropanoid pathway. It is widely known that an array of key enzymes is involved in the biosynthesis of the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL), C4H, 4CL, CHS, chalcone isomerase (CHI), flavanone 3β-hydroxylase (F3H), FLS, dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), and anthocyanidin reductase (ANR) (Wang *et al*. 2020b). The expressions of genes encoding these key enzymes are regulated by various TFs, of which R2R3-MYBs seem to be important (Liu *et al*. 2015). Up to now, a few R2R3-MYB genes have been cloned and functionally verified in fruit development, abiotic stress response, and secondary metabolism of strawberry (Salvatierra *et al*. 2013, Medina-Puche *et al*. 2015, Castillejo *et al*. 2020, Lu *et al*. 2020, Wang *et al*. 2020b, 2021a, 2022b; Cai *et al*. 2022, Li *et al*. 2022, Liu *et al*. 2022, Luo *et al*. 2023). The R2R3-MYBs are mainly associated with petal coloration and in the pink-flowered strawberry have been identified and systematically analyzed in the cv. Camarosa genome (Liu *et al*. 2021). However, many biological processes occur during the growth of strawberry, and the genomic information of different cultivars varies quite a bit. Therefore, the identification and characterization of R2R3-MYBs in strawberry are still very important for understanding their function. In the present study, 66 members of the R2R3-MYB gene family were identified in the cv. Reikou genome. The expression profiles in different tissues and in response to methyl jasmonate (MeJA) treatments were assessed based on the published transcriptome data. Some important R2R3-MYBs in the phenylpropanoid metabolism were spotlighted based on the analysis of phylogenetic relationships, protein-protein interactions, and miRNA-mediated post-transcriptional regulation. This study can provide a foundation for the future function analysis of R2R3-MYB gene family members in strawberry.

Materials and methods

Identification of *FanMYBs*: Firstly, the 78 MYB proteins from strawberry (*Fragaria* × *ananassa* Duch.) cv. Reikou were obtained from *PlantTFDB* (http://planttfdb.gao-lab.org/). Secondly, all retrieved protein sequences were carefully checked using the Conserved Domain Database on the National Center for Biotechnology Information (*NCBI*) server with default parameters (https://www.ncbi.nlm.nih.gov/). Protein sequences with two repeats in the MYB domain were recognized as members of the R2R3-MYB family. Third, the annotation and sequence length of these putative proteins were further checked using *BLASTP* analysis in *NCBI*. The erroneously predicted R2R3-MYB proteins and the seriously incomplete proteins were removed from further analyses. Finally, the CDS sequences, molecular mass, and isoelectric point values of protein sequences of candidate R2R3-MYB family members were downloaded from *PlantTFDB*.

Analysis of FanMYBs domain: The amino acid sequence of R2 and R3 repeats of R2R3-MYB proteins in *F.* × *ananassa* and *A. thaliana* were aligned with the *ClustalW* method using *MEGA5.1* software (https://www.megasoftware.net/). The sequence logos for R2 and R3 MYB repeats were created by submitting the multiple

alignment sequences to the *WebLogo* server (http://weblogo.berkeley.edu/logo.cgi).

Expression analysis of *FanMYBs*: Expression patterns of *FanMYBs* in different tissues were analyzed using the transcriptome datasets of root, runner, leaf, flower bud, and flower of octoploid strawberry downloaded from *GenBank* (accession number is SRP173045). The changes of *FanMYB* expressions in response to stress treatments were analyzed using the transcriptome datasets of leaves treated with methyl jasmonate (MeJA; accession number is SRP173045) for 0.5, 4, and 24 h. The fragments per kilobase of transcript per million mapped reads (FPKM) algorithm was used to estimate the gene expression levels using *TBtools* software (Chen *et al*. 2020). Genes with FPKM value greater than one in at least one tissue were considered to be expressed. Genes with a ratio of FPKM value between treatment and control greater than 1.5 were considered as differentially expressed genes (Low *et al*. 2017). Log2-transformed FPKM values of each *FanMYB* gene were displayed in a heatmap using *TBtools* software (Chen *et al*. 2020).

Construction of phylogenetic tree of FanMYBs involved in the phenylpropanoid pathway: A neighbor-joining (NJ) phylogenetic tree of FanMYBs and R2R3-MYBs have been confirmed to involve in secondary metabolism in strawberry and phenylpropanoid pathway in other plant species (Salvatierra *et al*. 2013, Liu *et al*. 2015, Ma and Constabel 2019, Lu *et al*. 2020, Wang *et al*. 2022b, Luo *et al*. 2023) was constructed using *MEGA 5.1* software and with 1 000 bootstrap replicates for reliability. The phylogenetic tree was displayed and annotated by the *iTOL* online tool (https://itol.embl.de/).

Construction of the protein-protein interaction network of FanMYBs: To deepen our understanding of the molecular mechanisms of *FanMYBs* expressed in at least one tissue, protein-protein interaction networks for these FanMYBs and key enzyme proteins from the phenylpropanoid pathway were constructed through the Search Tool for the Retrieval of Interacting Genes (*STRING*) database *v. 11.0* (http://www.string-db.org/). The key enzyme genes from the phenylpropanoid pathway were obtained from the cv. Reikou genome on the basis of the paralogous genes in *Fragaria vesca* using *BLASTN* analysis (Pott *et al*. 2020). Based on the edge information of *Arabidopsis* homologs, an edge information file of FanMYBs and key enzyme proteins from the phenylpropanoid pathway was created, confidence value greater than 0.700 was selected. Finally, the obtained protein-protein interaction networks were visualized using *Cytoscape* software (*v. 3.8.2*) (https://cytoscape.org/).

The miRNA-mediated post-transcriptional regulation of *FanMYBs*: The miRNA sequences from cv. Reikou were identified using *BLASTN* analysis according to the miRNA sequences that have been verified by *miR-RACE* (Han *et al*. 2014). The complementary sequences of *FanMYBs* to miRNAs were searched using *psRNATarget* (https://www.zhaolab.org/psRNATarget/analysis?function=3). The maximum expectations of 3.5 and the target accessibility-allowed maximum unpaired energy (UPE) to the target site of 30 were applied. To investigate whether these target *FanMYBs* were cleaved by miRNAs, degradome data of *F. vesca* downloaded from *GenBank* (accession number is SRP047520) (Xia *et al*. 2015) was aligned with *FanMYBs* potentially cleaved by miRNAs using *Bowtie 2* with no mismatch allowed (Langmead and Salzberg 2012).

MeJA treatments for strawberry plants *in vitro*: After disinfecting with 0.1% (m/v) $HgCl_2$, strawberry shoot tips (~0.3 mm in length) were placed on Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} thidiazuron, 1.0 mg L^{-1} 6-benzylaminopurine, and 0.1 mg L^{-1} 1-naphthylacetic acid to establish *in vitro* shoot proliferation. The explants were held in an incubator at a temperature of 23 ± 2°C, irradiance of 60 µmol m^{-2} s^{-1}, and a 16-h photoperiod. Plantlets were subcultured onto 1/2 MS medium at five-week intervals. Here, two-month-old plants were used for MeJA treatments. MeJA solution (250 µM) and distilled water with 0.25% ethanol (control) were sprayed on the aerial parts of strawberry plants until runoff. For each treatment, three plants were considered for each biological replicate, three biological replicates. After 0, 0.5, 4, and 24 h, MeJA-treated and control leaves were collected and kept at -80°C for use.

Reverse transcription-quantitative PCR (RT-qPCR) for *FanMYBs*: RT-qPCR was used to detect gene expression. *FastPure* plant total RNA isolation kit (*Vazyme*, Nanjing, China) was used to extract the total RNA. *HiScript® Q RT SuperMix* for qPCR (+ gDNA wiper) (*Vazyme*) was used for reverse transcription. *SYBR® Select Master Mix* (*2X*) (*Applied Biosystems*, USA) was chosen for PCR. Gene-specific primers were listed in Table 1 Suppl. *Actin2* was selected as the reference gene. *QuantStudio™ 5* (*Applied Biosystems*) was used to perform RT-qPCRs in a total of three biological replicates (three technical replicates for each biological one). The gene expression in the control leaves was set to 1 and the expressions in the MeJA-treated leaves were given relative to this. The relative expressions of genes were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis: All experiments were conducted in a completely randomized design. Each treatment consisted of three biological replicates. Mean values of various treatments were subjected to analysis of variance (*ANOVA*) using *SPSS 11.0*. Significant differences between treatments at $P < 0.05$ were performed.

Results

Identification of 66 *FanMYBs*: A total of 66 full-length or near full-length members of the R2R3-MYB family in *F.* × *ananassa* were identified (Table 2 Suppl.). They were named from *FanMYB1* to *FanMYB66* based on

the Gene ID (the *MYB* genes in the cultivated strawberry previously published were usually named *FaMYB*, in order to distinguish the *FanMYB* we used). The molecular mass (Mr) of FanMYB proteins varied from 12.44 kDa (FanMYB4) to 119.63 kDa (FanMYB12), amino acid (aa) length from 108 aa (FanMYB4) to 1 063 aa (FanMYB12), and isoelectric point (pI) from 4.60 (FanMYB8) to 9.99 (FanMYB2). The number of *FanMYB* genes was less than that reported by Liu *et al*. (2021). The reasons might be the different cultivars, the different sequencing depth and coverage for sequencing, the different identification methods and only the full-length or near full-length members included.

Conservation and divergence of FanMYBs domain: To investigate the sequence characteristics of R2R3-MYB domain and the level of conservation of each residue, the sequence logos for R2 and R3 of R2R3-MYBs from *F.* × *ananassa* and *A. thaliana* were produced through multiple sequence alignment. The results revealed that the arrangement of residues in R2 and R3 of R2R3-MYBs from *F.* × *ananassa* was basically the same as that of *A. thaliana*, which all encode three α-helices (Fig. 1). For both *F.* × *ananassa* and *A. thaliana*, three highly conserved tryptophan (W) residues are shown at positions 5, 26, and 46 of R2 (Fig. 1*A*,*B*). Similarly, one phenylalanine (F) and two tryptophan (W) residues are presented at positions 5, 24, and 43 of R3 (Fig. 1*C*,*D*). Compared with the W residues at positions 24 and 43 of R3 in R2R3-MYBs from *F.* × *ananassa* and *A. thaliana*, the conservation of the F residues at position 5 is obviously lower. The other conserved residues were mostly arranged between the second and the third residues of the three conserved residues in R2 and R3. Compared with the residues between the first and the second conserved W residues in R2, the conservation of the other conserved residues between the conserved F residues and the first conserved W residues in R3 is clearly lower. Moreover, for both *F.* × *ananassa* and *A. thaliana*, highly conserved leucine (L), arginine (R), proline (P), and aspartic acid (D) residues were located in the 3' region of R2. It suggests that the amino acid arrangement in the plant R2R3-MYBs is conservative. Furthermore, compared with the R2 and R3, some of the rest residues in *F.* × *ananassa* are different

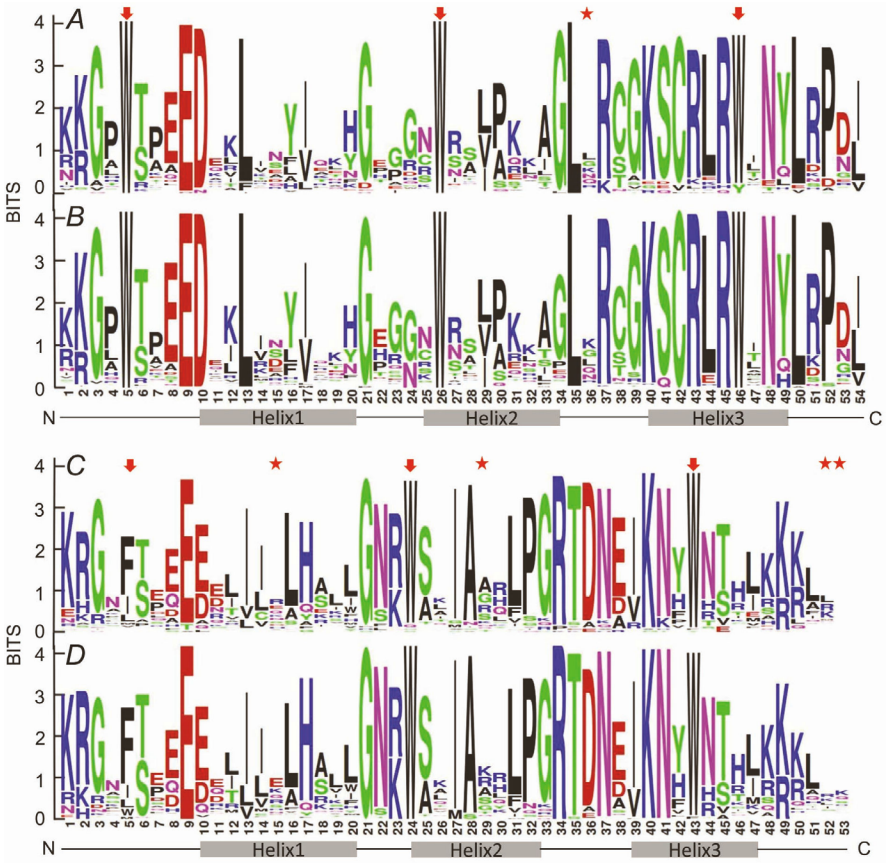

Fig. 1. Comparison of R2 and R3 sequences in R2R3-MYBs from *F.* × *ananassa* and *A. thaliana*. *A* - Sequence logos of R2 in MYBs from *F.* × *ananassa*. *B* - Sequence logos of R2 in MYBs from *A. thaliana*. *C* - Sequence logos of R3 in MYBs from *F.* × *ananassa*. *D* - Sequence logos of R3 in MYBs from *A. thaliana*. The residue positions were indicated by Arabic numerals. The positions with different residues between *S. miltiorrhiza* and *A. thaliana* are indicated by *red five-pointed stars*. Highly conserved tryptophan (W) and phenylalanine (F) residues are indicated by *red arrows*. The helices are indicated by *gray rectangles*. The overall height of each stack indicates the conservation of the amino acid residue at that position, and the bit score exhibits the relative frequency of the corresponding amino acid residue.

from *A. thaliana*, namely, the positions 36 of R2 and 15, 29, 52, and 53 of R3, showing that the MYB domain is diverse. These results are in line with the conservation and divergence of the R2R3-MYB domain in *S. miltiorrhiza* (Li and Lu 2014).

Expression patterns of *FanMYBs*: Previous studies have shown that some *MYB* genes are involved in plant developmental processes in strawberry (Castillejo *et al.* 2020, Wang *et al.* 2020b, Cai *et al.* 2022). To fully understand the role of *FanMYBs* in strawberry growth and development, the expression of *FanMYBs* in the root, runner, leaf, flower bud, and flower of the octoploid strawberry was characterized based on previously published transcriptome data, of which 57 were expressed in at least one tissue and exhibited differential expression patterns. Among the 57 *FanMYBs*, 32 exhibited tissue-specific expression (Fig. 2*A*). It includes five expressed mainly in the root, seven in the runner, three in the leaf, thirteen in the flower bud, and four in flower. A total of nine *FanMYBs* had FPKM values less than one in all the tissues analyzed, indicating that these genes might only be expressed during particular developmental stages, under particular conditions, or as pseudogenes.

MYBs also play significant roles in plant response to stress (Li *et al.* 2019). Strawberry *FvMYB24* has been reported to improve salt tolerance in transgenic *A. thaliana* (Wang *et al.* 2021a), and *FvMYB82* was also confirmed to improve salt and cold tolerance in transgenic *A. thaliana* (Li *et al.* 2022). To investigate the roles of *FanMYBs* in response to stress, the published RNA-seq data of the octoploid strawberry leaf treated with MeJA for 0.5, 4, and 24 h were mapped to *FanMYBs*. A total of 47 *FanMYBs* were found to be expressed (Fig. 2*B*). Compared with the control, 43 *FanMYBs* were differentially expressed in at least one time-point. Among them, nine *FanMYBs* were upregulated and eighteen were downregulated with the regulation to be significant in at least one time-point. The remaining sixteen showed upregulation at one time-point and downregulation at another. It suggests that most of *FanMYBs* are MeJA-responsive.

Phylogenetic relationships and function prediction of FanMYBs: According to the phylogenetic relationships,

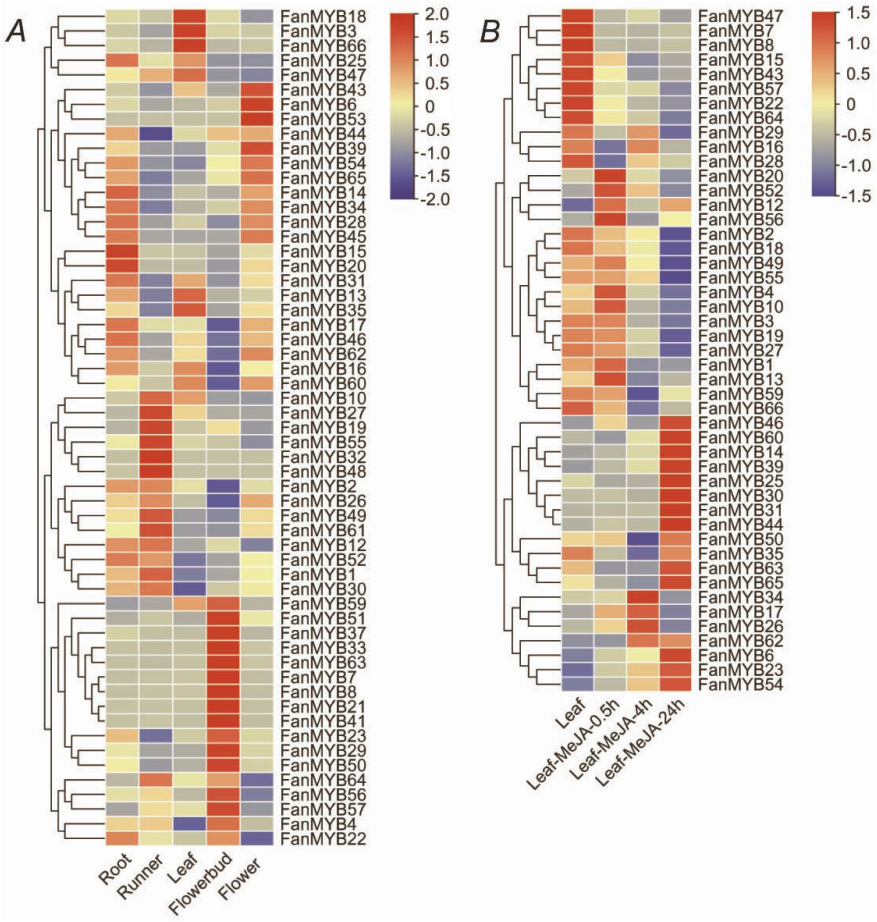

Fig. 2. Expression patterns of *FanMYBs*. *A* - Expression patterns of *FanMYBs* in different tissues. *B* - Expression patterns of *FanMYBs* under MeJA treatment. The heatmap was generated by *TBtools* software with a row scale. The *red* and *blue* colors represent the maximum and minimum values of the fragments per kilobase of transcript per million mapped reads (FPKM) which were log2-transformed, respectively.

16 members of FanMYBs were found (Fig. 3, Table 3 Suppl.). It is classified into 12 groups, named from G1 to G12. It is well-known that orthologous proteins with similar sequences might have similar functions (Zhang *et al*. 2017). Here, the proteins with amino acid sequence identity of more than 40% were considered for further analyses. The amino acid sequence identity of FanMYB13, FanMYB46, and the anthocyanin inhibitor MdMYB6 (Gao *et al*. 2011) belonging to the G1 group was 43.87% and 63.49%, respectively. Moreover, *FanMYB13* and *FanMYB46* were expressed in all the analyzed tissues (Figs. 2*A*, 6). It suggests that *FanMYB13* and *FanMYB46* might cooperatively regulate the anthocyanin biosynthesis in strawberry. *FanMYB42*, with no expression in the analyzed tissues, is very close to the eugenol regulator *FaMYB63* in strawberry fruit (Wang *et al*. 2022b) belonging to the G3 group, and the amino acid sequence similarity was 95.24%, suggesting that this gene might be involved in the secondary metabolites during strawberry fruit development. The identity of FanMYB36 and the salvianolic acid B activator SmMYB52 (Yang *et al*. 2021a) belonging to the G4 group was 44.72%, and *FanMYB36* was also not expressed in the analyzed tissues, indicating that this gene might be involved in the secondary metabolism in strawberry at specific developmental stages or under special circumstances. FanMYB47 belonging to the G6 group, is close to FaMYB9 which is positively involved in C6 volatile biosynthesis in strawberry fruit (Lu *et al*. 2020), and the amino acid sequence similarity was 95.67%, *FanMYB47* was expressed in all the analyzed tissues (Figs. 2*A*, 6), suggesting that FanMYB47 is likely to be involved in the strawberry secondary metabolism. The identity of FanMYB22 and the rutin inhibitor FtMYB13 (Zhang *et al*. 2018) belonging to the G7 group was 51.82%. *FanMYB22* was highly expressed in the root and flower bud (Figs. 2*A*, 6). The identity of FanMYB39 was close to rutin inhibitor FtMYB15 belonging to the G12 group, with 43.04% similarity. *FanMYB39* was highly expressed in the flower (Figs. 2*A*, 6). Therefore, *FanMYB22* and *FanMYB39* were probably involved in rutin biosynthesis in strawberry, in particular in root and flower bud, and flower. The identity of FanMYB25 and the anthocyanins and proanthocyanidins synthesis pathway inhibitor VvMYBC2-L1, PpMYB18, and PhMYB27 from the G11 group (Albert *et al*. 2011, Cavallini *et al*. 2015, Zhou *et al*. 2019) was 56.08, 63.53, and 43.75%, respectively. *FanMYB25* was expressed in all the analyzed tissues (Figs. 2*A*, 6). These results provide evidence that *FanMYB25* might play a role in anthocyanins and proanthocyanidins biosynthesis in strawberry. The identity of FanMYB43 and VvMYB4a, CsMYB4a, and MdMYB16 belonging to the G12 group was 69.40, 71.80, and 77.07%, respectively. *VvMYB4a* has been reported to play a key role in negatively regulating the synthesis of small-mass phenolic compounds, *e.g.*, caffeic acid, ferulic acid, caftaric acid, and chlorogenic acid (Cavallini *et al*. 2015). Overexpression of *CsMYB4a* significantly reduced the content of total lignin, rutin, chlorogenic acid, and phenylalanine in transgenic tobacco plants (Li *et al*. 2017). *MdMYB16* might be an important part of the regulatory network of the anthocyanin biosynthetic pathway (Xu *et al*. 2017). *FanMYB43* was highly expressed in the flower and leaf (Figs. 2*A*, 6). It indicated that *FanMYB43* might perform the same function in strawberry as *VvMYB4a*, *CsMYB4a*, and *MdMYB16*. However, the functions of these *FanMYBs* need to be further studied.

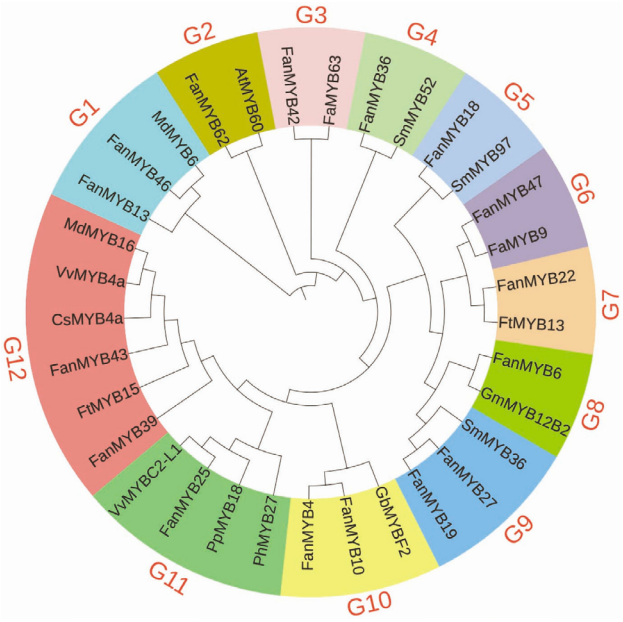

Fig. 3. Phylogenetic analysis and classification of the R2R3-MYB genes. The twelve different groups (G1 - G12) are indicated by different background colors. *Arabidopsis thaliana* AtMYB60 (AF062895), *Malus domestica* MdMYB6 (DQ074461), MdMYB16 (HM122617.1), *Fragaria* × *ananassa* FaMYB9 (JQ989281.1), FaMYB63 (MW452942.1), *Salvia miltiorrhiza* SmMYB36 (KF059390.1), SmMYB52 (KF059406.1), SmMYB97 (KF059451), *Fagopyrum tataricum* FtMYB13 (KY290579), FtMYB15 (KY290581), *Glycine max* GmMYB12B2 (JF510467), *Ginkgo biloba* GbMYBF2 (JQ068807), *Petunia hybrida* PhMYB27 (AHX24372.1), *Prunus persica* PpMYB18 (KT159234), *Vitis vinifera* VvMYBC2-L1 (NP_001268133), VvMYB4a (ABL61515), *Camellia sinensis* CsMYB4a (KY774676.1).

Interaction analysis between FanMYB proteins and proteins from the phenylpropanoid pathway in *F.* × *ananassa*: Based on the paralogous genes in *F. vesca*, 35 key enzyme genes associated with the phenylpropanoid pathway were obtained from cv. Reikou genome using *BLASTN* analysis (Table 4 Suppl.). To further uncover the potential biological functions of FanMYBs, the interaction analysis between FanMYB proteins and the 35 gene coding proteins was conducted using the *STRING* database. The results showed that six FanMYBs whose genes were expressed in at least one tissue and responded to MeJA (Figs. 2, 6), exhibited regulation relationships with the 22 phenylpropanoid pathway proteins (Fig. 4). Furthermore, FanMYB43 and FanMYB64 exhibited tight relationships with FanC4H2 which was considered to be a hub protein and partner protein with FanMYB63 and

the other 14 phenylpropanoid pathway proteins (Fig. 4*A*). FanMYB47 and FanMYB62 had a strong regulation with FanDFR3 protein which was regarded as a hub protein and partner protein with FanMYB63 and the other three phenylpropanoid pathway proteins (Fig. 4*B*). Therefore, FanMYB63 could directly interact with FanDFR3 and FanC4H2 (Fig. 4*C*). FanMYB49 had direct regulation with Fan4CL3 which was judged a hub protein and partner protein with the eight phenylpropanoid pathway proteins (Fig. 4*D*). It indicated that the six *FanMYBs* might function in the phenylpropanoid pathway. However, the functions of these genes need to be further investigated.

Analysis of miRNA targeting sites of *FanMYBs*: Through direct cleavage of target mRNAs with perfect or near-perfect complementarities, plant miRNAs play a crucial role in plant development and stress responses (Song *et al*. 2019). For example, in *A. thaliana*, miR159a/b/c, miR319a/b/c, miR828, and miR858 have been confirmed to have the complementary sites of some *AtMYBs* (Palatnik *et al*. 2007, Luo *et al*. 2012, Camargo-Ramírez *et al*. 2018). The regulatory role of miRNAs in *MYBs* also exists in other plants, such as apple, grape, and *S. miltiorrhiza* (Xia *et al*. 2012, Li and Lu 2014, Tirumalai *et al*. 2019). Here, 18 miRNAs from cv. Reikou genome (Table 5 Suppl.) were obtained using *BLASTN* analysis done according to Han *et al*. (2014). To elucidate miRNA-mediated post-transcriptional regulation of MYBs in *F.* × *ananassa*, all *FanMYBs* were searched for potential targets of the 18 miRNAs. As a result, 33 *FanMYBs* with complementary sequences to the member of MIR159/319/858 families and miR5015b.1 were found (Table 6 Suppl.). *FanMYB28*, *FanMYB45*, and *FanMYB53* were predicted as the targets of miR159, *FanMYB28*, and *FanMYB45* were also predicted as the target of miR319c, while *FanMYB53* was also the target of miR858 and miR5015b.1. The other 27 *FanMYBs* were the potential targets of miR858. The number of potential targets of *F.* × *ananassa* is less than that of apple (67) and *S. miltiorrhiza* (38) (Xia *et al*. 2012, Li and Lu 2014). Different plant species with different numbers of MYBs and only the coding regions of full-length or near full-length members of *FanMYBs* here may be the reasons, but no miR828 might be an important reason. Plant mature miRNAs usually guide RNA-induced silencing complexes (RISCs) to cleave target mRNAs at the tenth complementary nucleotide from the 5' end of

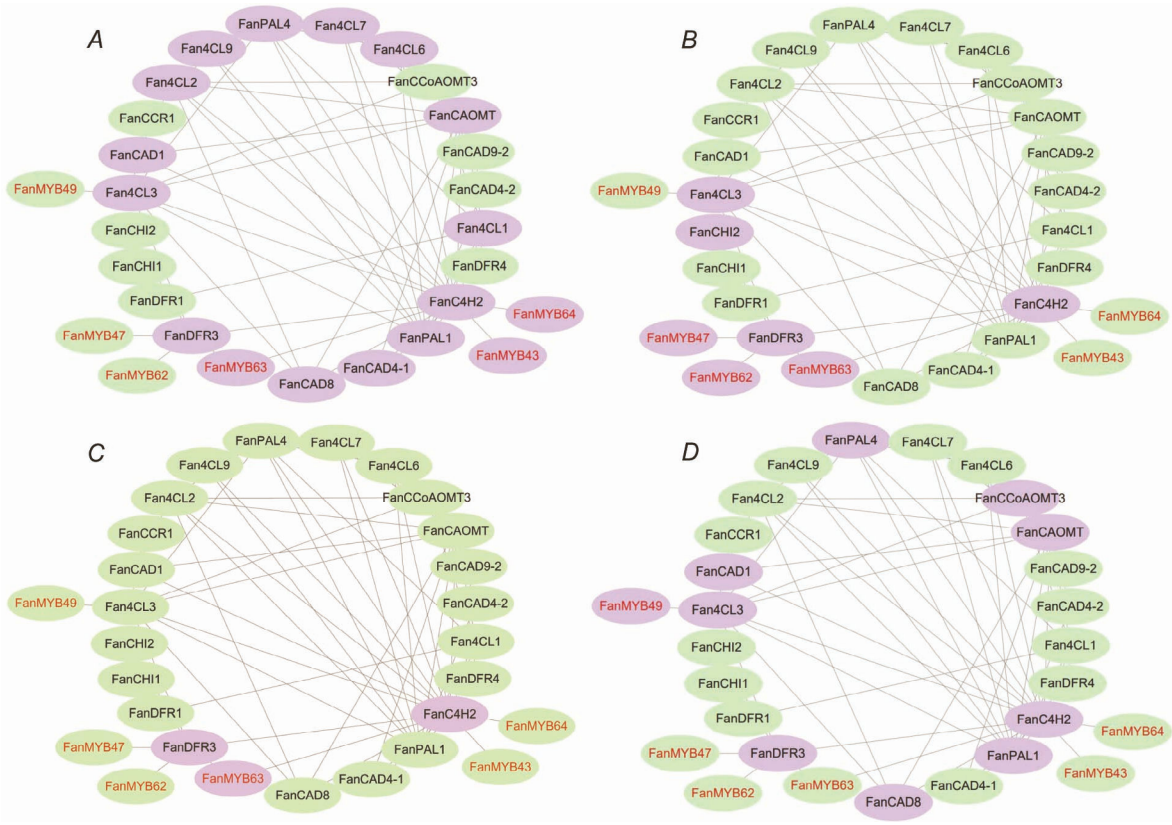

Fig. 4. Protein regulatory network between FanMYBs and proteins from the phenylpropanoid pathway in *F.* × *ananassa*. *A* - The network of cinnamate-4-hydroxylase protein (FanC4H2) and its first neighbors are in *light purple* ovals, the other proteins from the phenylpropanoid pathway are in *light green* ovals. *B* - The network of bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase protein (FanDFR3) and its first neighbors are in *light purple* ovals, the other proteins from the phenylpropanoid pathway are in *light green* ovals. *C* - The network of FanMYB63 and its first neighbors are in *light purple* ovals, the other proteins from the phenylpropanoid pathway are in *light green* ovals. *D* - The network of 4-coumarate-CoA ligase protein (Fan4CL3) and its first neighbors are in *light purple* ovals, the other proteins from the phenylpropanoid pathway are in *light green* ovals. All FanMYBs proteins are indicated by *red letters*, other proteins from the phenylpropanoid pathway in strawberry are indicated by *black letters*.

the miRNA (Llave *et al*. 2002). Therefore, strawberry degradome sequencing data sets for the potential targets' cDNA fragments resulted from miR159/319c/858/5015b.1-mediated cleavage were analyzed. The results confirmed that 12 *FanMYBs* might be the targets, including *FanMYB14/18/21/22/27/28/36/47/49/57/60/63* (Fig. 5). Except for *FanMYB36*, these genes were expressed in at least one tissue analyzed (Figs. 5, 6). It suggests that miR159/319c/858 plays important roles in strawberry tissue development *via* post-transcriptional regulation of *FanMYBs*. Additionally, according to the phylogenetic relationships and protein-protein interaction analysis mentioned above, *FanMYB22/36/47/49/63* might be involved in the phenylpropanoid pathway.

Validation of RNA-seq analysis by RT-qPCR: Based on RNA-seq results, six *FanMYBs* in response to MeJA were selected to perform RT-qPCR, including *FanMYB13/18/43/47/60/62* (Fig. 7). After MeJA treatment, the expressions of *FanMYB18*, *FanMYB43*, and *FanMYB47* in strawberry leaves were significantly downregulated while *FanMYB60* was significantly upregulated, *FanMYB13* and *FanMYB62* were firstly significantly upregulated and then downregulated but still significantly higher relative to the control. It showed that these genes had similar trends between the RT-qPCR analysis and RNA-seq analysis, which validated the RNA-seq results.

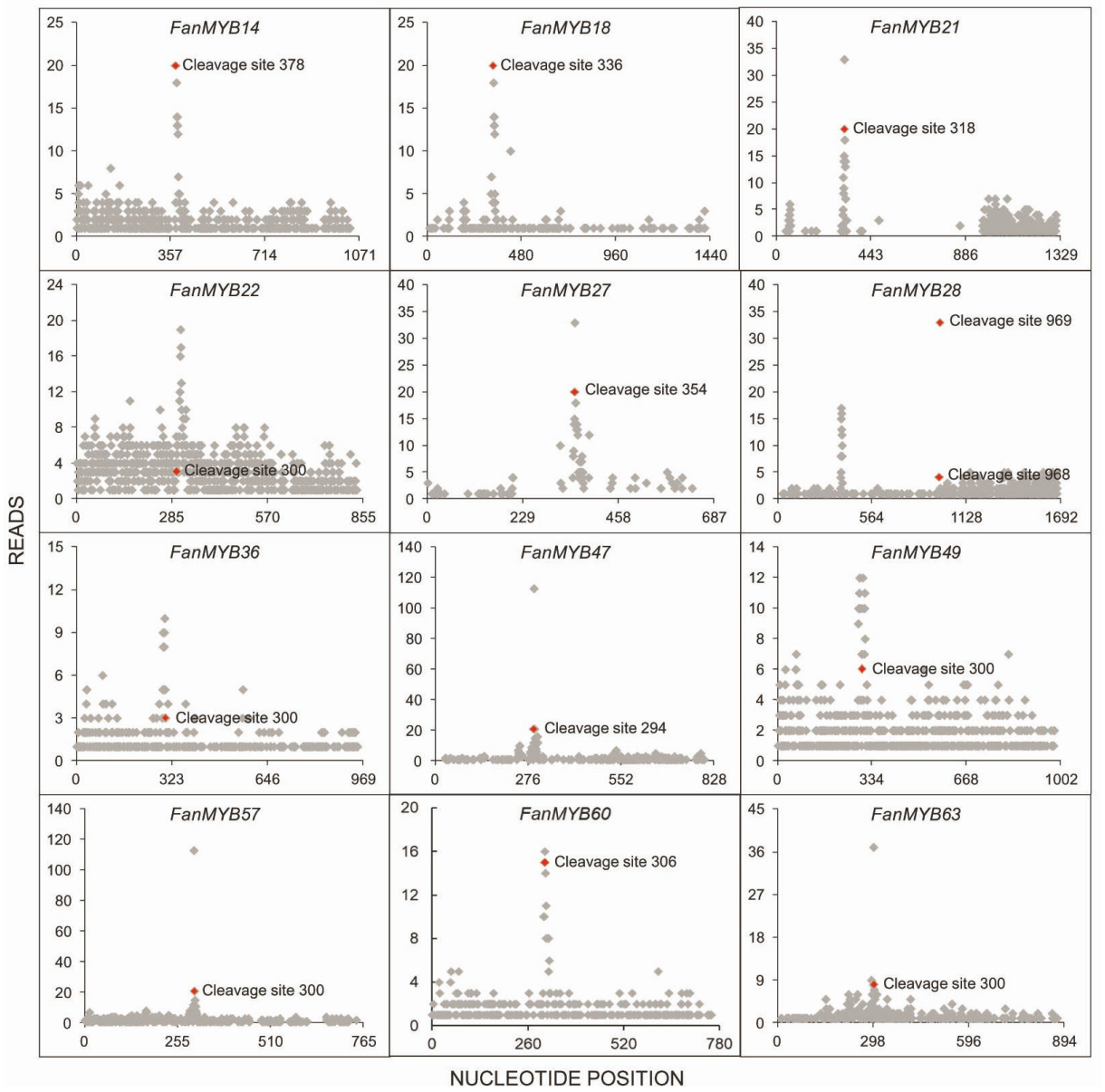

Fig. 5. Degradome analysis of miRNA-mediated cleavage of target *FanMYBs*. X-axis shows the nucleotide position of targets. Y-axis shows the reads obtained through degradome sequencing. Each *black diamond* represents a degradome fragment mapped to the potential target. The *red diamonds* indicate that the product resulted from miRNA-directed cleavage.

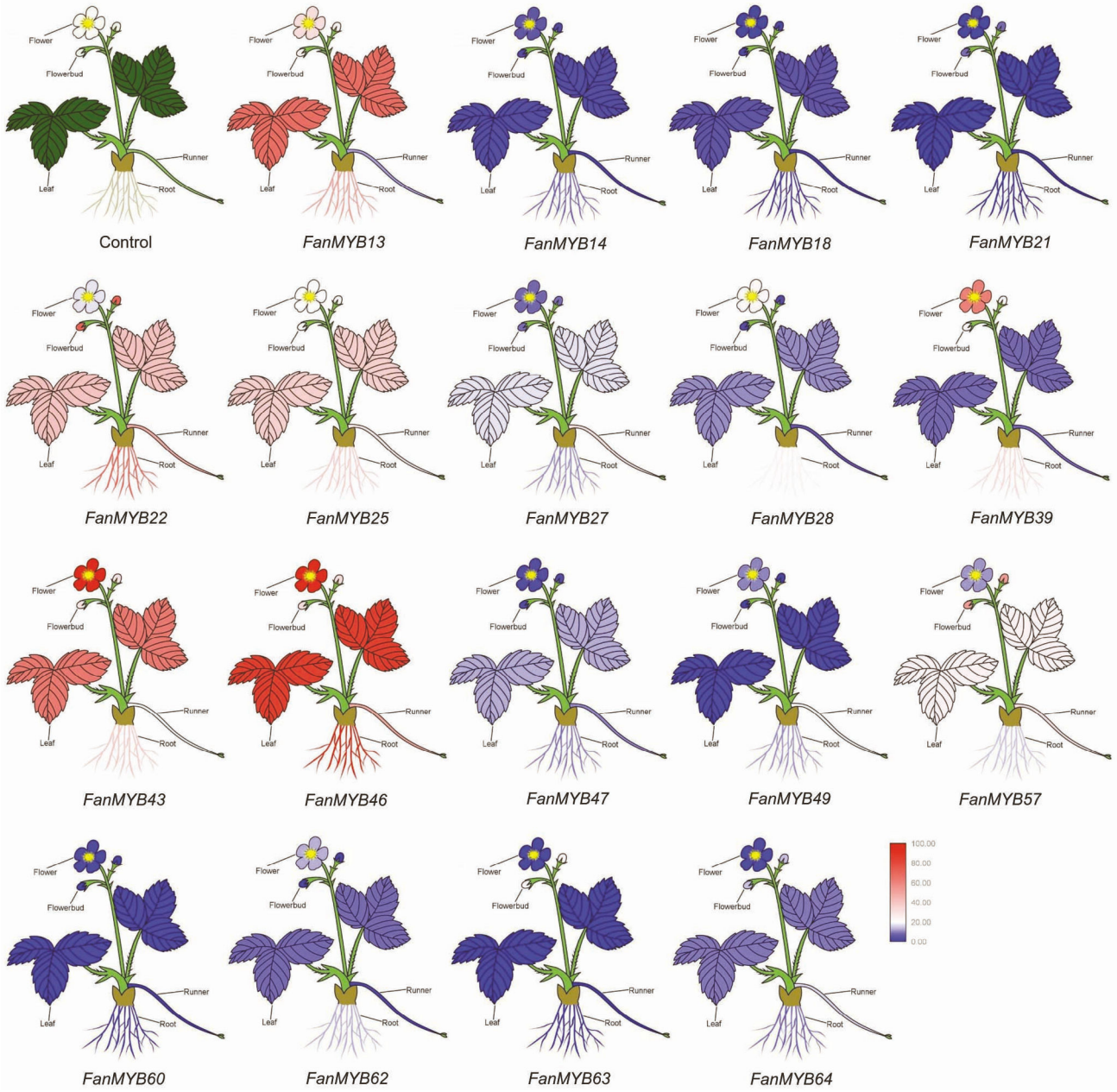

Fig. 6. The cartoon-style expression patterns of candidate *FanMYBs*. The heatmap was generated by *TBtools* software. The *red* and *blue* colors represent the maximum and minimum values of the fragments per kilobase of transcript per million mapped reads (FPKM), respectively.

Discussion

The R2R3-MYB gene family has been systematically identified in a variety of plants through genomic analysis, and the members of the R2R3-MYB gene family are known to play important roles in plant biological and physiological processes, especially in the phenylpropanoid metabolism pathway (Liu *et al*. 2015, Ma and Constabel 2019, Wu *et al*. 2022). However, the knowledge of strawberry R2R3-MYB genes' roles in phenylpropanoid metabolism need to be further investigated. In the present investigation, 66 *FanMYBs* were identified and analyzed in the *F.* × *ananassa* genome, and the roles of some important *FanMYBs* were shown.

With the help of gene expression profiles, gene regulatory roles in plant growth and development can be better understood. For instance, *EC1* (*EGG CELL 1*) genes were highly expressed in ovules and anthers, indicating their significant roles in plant flowering and reproduction in cotton (Wang *et al*. 2021b). Transcriptome profiling analysis revealed that gene expression profiles in rice-developing ovules provided evidence for the role of sporophytic tissue in female gametophyte development (Wu *et al*. 2015). Here, the expression patterns of

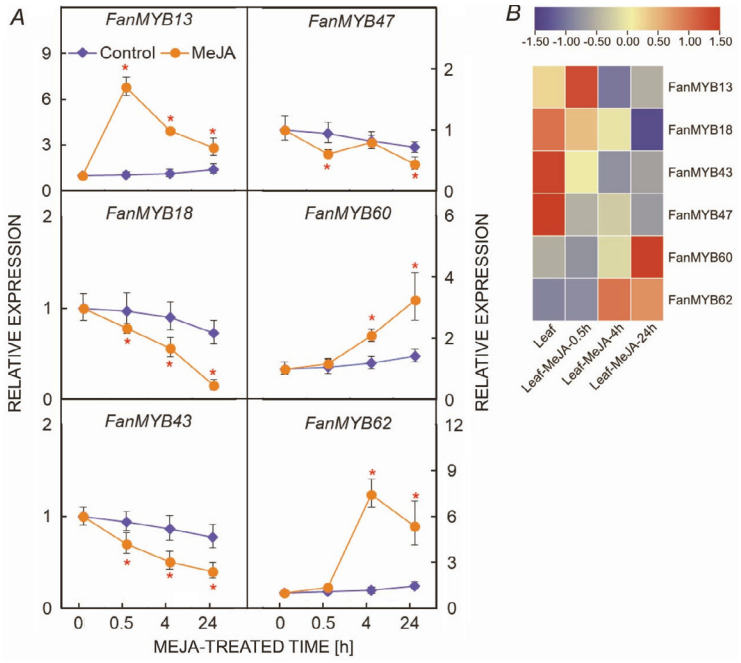

Fig. 7. The expression analysis of *FanMYBs* of strawberry leaves with MeJA treatment. *A* - RT-qPCR analysis. Data are shown as means ± SD ($n = 3$). * indicates a significant difference between control and MeJA treatment at $P < 0.05$. *B* - The heatmap was generated by *TBtools* software with a row scale. The *red* and *blue* colors represent the maximum and minimum values of the fragments per kilobase of transcript per million mapped reads (FPKM) which were log2-transformed, respectively.

FanMYBs in different tissues were investigated using online public transcriptome data; 32 out of 57 *FanMYBs* were predominantly expressed in root, runner, leaf, flower bud, or flower in *F.* × *ananassa* (Fig. 2*A*). It suggests that these *FanMYBs* are polyfunctional and redundant in strawberry plant growth and development processes. Phylogenetic analysis is considered a very important tool for investigating gene functions. During plant evolution, genes with similar functions in different species are usually strongly related to each other and are on the same branch in a phylogenetic tree (Zhang *et al.* 2015). Therefore, phylogenetic analysis could provide clues for exploring gene function. For example, based on the phylogenetic relationship of the pigeon pea CcMYB proteins and other plants R2R3-MYBs, the CcMYB proteins involved in flavonoid and lignin biosynthesis were identified (Yang *et al.* 2021b). Moreover, homologous proteins with similar sequences might have similar functions (Zhang *et al.* 2017). Here, based on the phylogenetic relationships between FanMYB proteins and other R2R3-MYB proteins involved in the phenylpropanoid pathway, the amino acid sequence similarity analysis, as well as the gene expression analysis showed that seven *FanMYBs* (*FanMYB13/22/25/39/43/46/47*) (Fig. 3, Table 3 Suppl.) might be involved in the phenylpropanoid pathway in root, runner, leaf, flower bud, and flower in *F.* × *ananassa*. Protein-protein interaction analysis is a useful tool to provide additional insights into gene functions. In *Arabidopsis*, the network of protein-protein interaction analysis implied that *DUF506* (*DOMAIN OF UNKNOWN FUNCTION 506*) genes have distinct biological functions including responses to environmental stimuli, nutrient deficiencies, and Ca^{2+} signaling (Ying 2021). Here, a regulatory network of six FanMYB proteins (FanMYB43/47/49/62/63/64) and the 22 phenylpropanoid pathway proteins were found (Fig. 4), and the six *FanMYBs* were expressed in all the analyzed tissues and in response to MeJA (Figs. 2, 6), indicating that the six *FanMYBs* might be involved in the phenylpropanoid pathway in root, runner, leaf, flower bud, and flower in *F.* × *ananassa*. Combined analysis of phylogenetic relationships and the protein regulatory network of FanMYBs, two key genes, *FanMYB43* and *FanMYB47* are likely involved in the phenylpropanoid metabolism.

It is common knowledge that miRNAs have critical roles as regulators of plant development and other physiological processes. Previous studies have confirmed that R2R3-MYB genes of *Arabidopsis* (Palatnik *et al.* 2007, Luo *et al.* 2012, Camargo-Ramírez *et al.* 2018), grape (Tirumalai *et al.* 2019), and persimmon (Yang *et al.* 2020) were the targets of some miRNAs. In strawberry fruit, *Fa-GAMYB* (1 686 bp) has been verified as a target of Fa-miR159 (Csukasi *et al.* 2012). Combining the analysis of complementary sequences of *FanMYBs* to miRNAs and degradome data, 12 *FanMYBs* were the targets of miR159/319c/858 (Fig. 5), of which 11 were mediated by miR858. MiRNA858 is well-known as a potential regulator of the phenylpropanoid pathway and plant development (Sharma *et al.* 2016). Based on the phylogenetic relationship and protein-protein interaction network of *FanMYBs*, five *FanMYBs* (*FanMYB22/36/47/49/63*) with miR858-MYB module, might be involved in the strawberry phenylpropanoid pathway. It suggests that the miRNA-MYB module is conservative and the regulation

network of the phenylpropanoid pathway is complex in *F.* × *ananassa*. Further physiological and biochemical analysis will be performed to verify the functions and regulatory mechanisms of these *FanMYBs*.

There is evidence that R2R3-MYBs take part in plant responses to stress (Zhao *et al*. 2019, Zhang *et al.* 2020, Dong *et al*. 2021, Upadhyaya *et al*. 2021). However, the function of many R2R3-MYBs in response to stress is still unknown. Especially, in strawberry, only *FvMYB24* and *FvMYB82* were reported to improve salt and cold tolerance in transgenic *Arabidopsis* plants (Wang *et al*. 2021a, Li *et al*. 2022). Jasmonates (JAs), which comprise jasmonic acid (JA) and its volatile methyl ester MeJA, play a key role in plant responses to biotic and abiotic stresses, and they strongly modulate the accumulation of secondary compounds, including alkaloids, glucosinolates, terpenoids, and phenylpropanoids in many plant species (Griffiths 2020, van der Fits and Memelink 2000). These JAs-induced accumulation of secondary metabolites are usually regulated by JAs-responsive TFs, for example, the MeJA-induced FtMYB3 negatively regulates anthocyanins and proanthocyanidins biosynthesis in Tartary buckwheat (Wang *et al*. 2022c), the MeJA-responsive SmMYB1 promotes phenolic acid biosynthesis in *S. miltiorrhiza* (Zhou *et al*. 2021), the responses to MeJA induction of GlMYB4 and GlMYB88 regulate flavonoid accumulation in *Glycyrrhiza uralensis* (Li *et al*. 2020). In the present study, based on the results of RNA-seq data, 47 *FanMYBs* were responsive to MeJA treatment (Fig. 2*B*), indicating the importance of these *FanMYBs* in strawberry stress responses and secondary metabolites.

In *F.* × *ananassa*, 66 R2R3-MYB genes were identified and systematically analyzed. The expression patterns, phylogenetic relationships, and protein-protein interaction analysis of the *FanMYB* gene family have expanded our knowledge of the genes involved in the growth, development, and phenylpropanoid metabolism in strawberry. Thirteen *FanMYBs* (*FanMYB13/22/25/36/39/42/43/46/47/49/62/63/64*) related to the phenylpropanoid metabolism were highlighted, of which five (*FanMYB22/36/47/49/63*) were post-transcriptionally regulated by miR858 based on degradome data. These data will provide valuable information for further analysis of the functions and regulatory mechanisms of these genes in strawberry.

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