RESEARCH PAPER

Two MYB transcription factors regulate flavonoid biosynthesis in pear fruit (*Pyrus bretschneideri* Rehd.)

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Abstract

Flavonoid compounds play important roles in the modern diet, and pear fruits are an excellent dietary source of these metabolites. However, information on the regulatory network of flavonoid biosynthesis in pear fruits is rare. In this work, 18 putative flavonoid-related MYB transcription factors (TFs) were screened by phylogenetic analysis and four of them were correlated with flavonoid biosynthesis patterns in pear fruits. Among these MYB-like genes, the specific functions of two novel MYB TFs, designated as *PbMYB10b* and *PbMYB9*, were further verified by both overexpression and RNAi transient assays. *PbMYB10b*, a PAP-type MYB TF with atypical motifs in its conserved region, regulated the anthocyanin and proanthocyanidin pathways by inducing the expression of *PbDFR*, but its function could be complemented by other MYB TFs. *PbMYB9*, a TT2-type MYB, not only acted as the specific activator of the proanthocyanidin pathway by activating the *PbANR* promoter, but also induced the synthesis of anthocyanins and flavonols by binding the *PbUFGT1* promoter in pear fruits. The MYBCORE-like element has been identified in both the *PbUFGT1* promoter and ANR promoters in most species, but it was not found in UFGT promoters isolated from other species. This finding was also supported by a yeast one-hybrid assay and thus enhanced the likelihood of the interaction between *PbMYB9* and the *PbUFGT1* promoter.

Key words: Flavonoid, MYB transcription factors, pear fruit, plant secondary metabolism, regulatory network, transient.

Introduction

Flavonoid compounds are a large group of secondary metabolites in plants. These metabolites play important roles in various plant biological processes including coloration, resistance to UV-B damage, cell-wall formation, and defence against diseases, and they also possess health beneficial properties (Khan et al., 2010; Winkel-Shirley, 2001; Xu et al., 2014).

Flavonoid biosynthesis has been well documented in various plants (see Supplementary Fig. S1 at *JXB* online). Phenylalanine, as the common precursor, can be catalysed to different types of flavonoid compounds via different biosynthesis pathways. Most enzymes involved in these pathways have been well investigated. To be more precise, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase, and

Abbreviations: VIGS, virus-induced gene silencing; TF, transcription factor; GPP, general phenylpropanoid pathway; PA, proanthocyanidin; PAL, phenylalanine ammonia lyase; CHS, chalone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; UFGT, UDP-glucose:flavonoid 3-glucosyltransferase; ANR, anthocyanin reductase; ANS, anthocyanin synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; TT2, TRANSPARENT TESTA 2; PAP, PRODUCTION OF ANTHOCYANIN PIGMENT; EBGs, early biosynthetic genes; LBGs, late biosynthetic genes; CDS, coding DNA sequence.

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The expression patterns of structural flavonoid pathway genes, which encode these enzymes, are controlled by TFs including MYB-like genes. A number of flavonoid-related MYB TFs have been identified in model plants, such as Arabidopsis thaliana and Zea mays, but few have been identified in woody plants (Dubos et al., 2010; Wang et al., 2013; Li, 2014; Liu et al., 2015). TT2, the first identified PA-related MYB TF, induces the biosynthesis of PAs in the seed coat by activating DFR, ANS, and ANR in A. thaliana (Nesi et al., 2001). In recent studies, several TT2 orthologues have been characterized in woody plants. VvMYBPA2 was identified as the direct regulator of VvMYBPA1 (another PA-related MYB TF) and several structural flavonoid pathway genes in grapevine (Terrier et al., 2009). DkMYB2 in persimmon (Akagi et al., 2010), MdMYB9 in apple (Gesell et al., 2014), and PtMYB134 in poplar (Mellway and Constabel, 2009) were also identified as PA-specific regulators that activate PA-related genes (PAL, DFR, ANR, and LAR). Furthermore, several PA-related MYB TFs, such as DkMYB4 and VvMYBPA1, were phylogenetically placed in a different subclade from the TT2-type MYB TFs. In grapevine, although VvMYBPA1 was regulated by VvMYBPA2, it still acted as a direct regulator of structural flavonoid pathway genes (Bogs et al., 2007). In persimmon, DkMYB4 was characterized as a PA-regulator in fruit flesh and was found to be the specific activator of DkANR but not DkLAR (Akagi et al., 2009). The target gene specificities of PA-related MYBs are mainly dependent on their binding ability with the cis-elements in the promoters of target genes. Although MYB-binding to the cis-elements show various characteristics among different species, conserved motifs were still identified such as MYBCORE and AC-like elements (Solano et al., 1995; Bogs et al., 2007; Akagi et al., 2009).

MYB TFs associated with the anthocyanin pathway have been well studied in various plants, and the PRODUCTION OF ANTHOCYANIN PIGMENT (PAP) family, which specifically induce biosynthesis of anthocyanins in Arabidopsis, share highly homologous similarities in their conserved amino acid sequences (Borevitz et al., 2000; Gonzalez et al., 2008; Heppel et al., 2013). MdMYB10 alleles were the key regulatory factors during the coloration of apple fruits (Takos et al., 2006; Espley et al., 2007; Lin-Wang et al., 2010). In Fragaria × ananassa fruits, FaMYB10 also plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening (Medina-Puche et al., 2014). In grapevine, VvMYBA1 and VvMYBA2 are the specific regulators, by activating UFGT, of the anthocyanin pathway (Kobayashi et al., 2002). Unlike the specific interactions between TT2-type MYB TFs and MYBCORE, PAP-type MYB TFs were found to bind PCE motifs in the promoter regions of their target genes (Dare et al., 2008; Lai et al., 2013). Furthermore, as an atypical anthocyanin regulator, MdMYB3 was identified as a regulator of both the anthocyanin and flavonol pathways (the regulatory mechanism is still unclear) (Vimolmangkang et al., 2013). However, in Arabidopsis, AtMYB4, the orthologue of MdMYB3, acts as a regulator of monogallic and phenolic acid biosynthesis (Jin et al., 2000).

The Chinese pear ‘Red Zaosu’ originates as a spontaneous mutation from ‘Zaosu’ (Pyrus bretschneideri Rehd.). The flavonoid biosynthesis profiles in both cultivars have previously been investigated: the general phenylpropanoid pathway in both cultivars is similar, but the anthocyanin, the flavonol, and proanthocyanidin pathways are distinct (Zhai et al., 2014). Although we identified UFGT as the key enzyme that directly contributed to the variation in anthocyanin and flavonol biosynthesis, the regulatory mechanism in the proanthocyanidin pathway is still unclear. Furthermore, the flavonoid biosynthesis profile in the fruits of ‘Red Zaosu’ is unique from other pears: the intensity of flavonoid biosynthesis immediately peaks after its flower’s blossom and then continuously decreases during the matura stage. The regulatory mechanism involved in this process is still unclear and understanding this mechanism may provide new perspectives to investigate the regulatory network of flavonoid biosynthesis in vegetative organs.

In this study, we aimed to identify MYB TFs involved in flavonoid biosynthesis in pear fruits. Eighteen MYB genes were grouped into flavonoid-related MYB TFs clades using a phylogenetic analysis. Three candidate MYB TFs, designated as PbMYB10b, PbMYB9, and PbMYB3, were correlated with the flavonoid biosynthesis in pear fruits. As a result, PbMYB10b was identified as the activator of the anthocyanin and PA pathways, and PbMYB9 as the activator of the PA, anthocyanin, and flavonol pathways.

Materials and methods

Plant materials and treatments

The fruits of ‘Zaosu’ (Pyrus bretschneideri Rehd.), ‘Red Zaosu’ (Pyrus bretschneideri Rehd.), ‘Palacer’ (Pyrus communis L.), and ‘E li yihao’ (Pyrus pyrifolia Nakai.) were selected as materials from a commercial orchard in Dali County, Shaanxi Province, China, in 2014 and 2015. The young fruits were harvested 15 days after flower blossom (DAFB). The matured fruits were harvested about 100 DAFB. The fruits that had been bagged were also harvested 100 DAFB. The young bagged fruits of ‘Red Zaosu’ and ‘Palacer’ were selected as the RNAI transient materials. These fruits were kept in bags for about 30 d until the red pigments totally faded. Then, these fruits were harvested and stored at 4 °C for injection. The young naturally coloured fruits of ‘Zaosu’ and ‘Eli yihao’ were selected as the materials for the transient expression analyses. These fruits were harvested at 30 DAFB and stored at 4 °C for injection. The tissues were frozen in liquid nitrogen and stored at ~80 °C for further use.

Flavonoid compounds’ analysis

The extraction and analysis of flavonoid compounds were carried out as described by Zhang et al. (2010). Briefly, the flavonoids were extracted with 70% methanol containing 2% formic acid at 0–4 °C.
The supernatant was filtered through a 0.45 μm syringe filter prior to HPLC analysis. Phenolic compounds were analysed using an HPLC equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA). The Inertsil ODS-3 column (5 μm, 4.0 × 250 mm, GL Sciences Inc., Tokyo, Japan) was used in the separation, preceded by an Inertsil ODS-3 Guard Column (5 μm, 4.0 × 10 mm). Solvent A consisted of 10% formic acid (11.36% of 88% formic acid) dissolved in water and solvent B was 10% formic acid and 1.36% water (11.36% of 88% formic acid) in acetonitrile (HPLC grade, purity: 99.9%). The gradient was 95% A (0 min), 85% A (25 min), 78% A (42 min), 64% A (60 min), and 95% A (65 min). The run-time was 10 min. The flow rate was 1.0 ml min⁻¹ at 30 °C. Simultaneous monitoring was performed at 280 nm for catechin, epicatechin, procyanidin B1, procyanidin B2, arbutin, and gallic acid; 320 nm for chlorogenic acid and caffeic acid; 365 nm for quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-arabinoside, quercetin-3-rutinoside, isorhamnetin-3-galactoside, isorhamnetin-3-glucoside, and isorhamnetin-3-rutinoside; and 520 nm for cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-silane, cyanidin-3-glucoside, and cyanidin-3-rutinoside, respectively. Peaks were identified by comparison of retention time and UV spectra with authentic standards. The concentration of individual phenolic compounds was determined based on peak area and calibration curves were derived from the corresponding authentic phenolic compounds. All of the phenolic standards were obtained from Sigma Aldrich (St Louis, MO, USA), Extrasynthese (Genay Cedex, France), and AApin Chemicals (Abingdon, Oxon, UK).

### Isolation of R2R3-MYB genes and a phylogenetic analysis

R2R3-MYB genes were isolated from the coding DNA sequence (CDS) database of the pear genome (Wu et al., 2013; http://peargenome.njau.edu.cn/) as described by Qiao et al. (2015). Briefly, the Myb-like DNA-binding domain Hidden Markov Model profile (HMM profile) (PF00249) was obtained from the Pfam database (Finn et al., 2010) and used as the query to perform the HMM search (Eddy, 2011) against the pear genome database. The corresponding sequences of candidate genes were searched using the BLASTN algorithm against the Pear EST database of the pear genome (http://peargenome.njau.edu.cn/) to confirm that each predicted gene having a high similarity EST sequence (score 300 bp and identity 99%) was expressed in the pear transcriptome.

All pear R2R3-MYBs and all functionally labelled MYB genes from other species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method from other species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method from other species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method from other species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method from other species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method from other species were aligned using Clustal W.

### Expression analysis by quantitative real-time PCR

Total RNA was extracted using the SDS-phenol method as described by Fousca et al. (2004). The RNA concentration and quality were detected by UV spectrophotometry and by running on a 1.2% agar ethidium bromide-stained gel. One mg of total RNA was reverse-transcribed to cDNA using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Every qRT-PCR was performed in three replicates on an iCycler iQ5 (Bio-Rad, Berkeley, CA, USA) with the SYBR Premix Ex Taq II (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Data were analysed by iQ5 2.0 software (Bio-Rad, Berkeley, CA, USA) using the ΔΔCT algorithm. The primers for actin, flavonoid biosynthetic genes, and candidate MYBs are described in Supplementary Table S4.

### Gene/promoter cloning and analysis

The CDS sequences of PhMYB10 (GenBank accession: KT601121), PhMYB10b (GenBank accession: KT601122), PhMYB9 (GenBank accession: KT601123), PhMYB3 (GenBank accession: KT601124), and a 450-bp fragment of the PhUGFT1 promoter (GenBank accession: KT601125) were cloned from ‘Zaosu’ and ‘Red Zaosu’ using primer sets (described in Supplementary Table S4) designed based on their sequence data in the Pear Genome Database (http://peargenome.njau.edu.cn/). cis-elements in the promoters were identified using the FIMO program (http://meme-suite.org/tools/fimo). The characteristic amino sequences of candidate MYBs were identified by several flavonoid-related motifs, including the SG7 ([K/R][D/E][R/K]xGRT[S/L][R/K]), SG7-2 ([W/L]xL[S/L]), [D/E]Lx2(R/K)x3Lx6Lx3R, and [D/E][D/E][E][S][L](D/N)[S/T](D/E) ([D/E]xW) motifs, and the TT2 box (Nesi et al., 2001; Stracke et al., 2001; Zimmermann et al., 2004; Czemmel et al., 2009).

### RNAi transient assay of pear fruit using the virus-induced gene silencing (VIGS) system

The pTRV1 and pTRV2 VIGS vectors (described in Liu et al., 2002) were kindly provided by Dr Dinesh-Kumar, Yale University. The 400–600-bp fragments of the candidate MYB gene were PCR-amplified from pear leaf cDNA sources using primer sets (described in Supplementary Table S4). The resulting products were cloned into pTRV2 to form pTRV2-MYB vectors (described in Supplementary Fig. S2A, B). The Agrobacterium strain GV3101 containing pTRV1 and pTRV2 independently, and their derivatives were used for the VIGS experiments. The Agrobacterium strain GV3101 containing TRV-VIGS vectors was grown at 28 °C in LB medium containing 10 mM MES and 20 mM acetylsyringone with appropriate antibiotics. After 24 h, Agrobacterium cells were harvested and resuspended in the Agrobacterium infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 150 mM acetylsyringone) to a final OD₆₀₀ of 1.2 (for pTRV1 and pTRV2 and their derivatives) and shaken for 4–6 h at room temperature before infiltration. Plant infiltration was performed as described in Ratcliffe et al. (2008) and Spolaore et al. (2001), and the injection volume was modified to about 300 μl in young pear fruit. The negative controls were infiltrated with Agrobacterium containing the empty pTRV1 and pTRV2 vectors, independently.

### Transient overexpression assay in pear fruit

The complete CDS of the MYB TFs containing introns were fused to the 35S promoter in the pCambia 1301 binary vector (described in Supplementary Fig. S2A, B). The Agrobacterium cultivation and the plant infiltration were the same as described above. The negative controls were infiltrated with Agrobacterium containing the pCambia GUS-intron vector.

### Yeast one hybrid (Y1H) assay

Y1H assays were performed with the Matchmaker Gold Yeast One-Hybrid System Kit (Clontech) according to the manufacturer’s instructions. Based on the distribution of TFs in the PhUGFT1 promoter, we ligated a fragment from -3 to -206 of the PhUGFT1 promoter into pAbAi to construct the pBait-AbAi. At the same time, the complete CDS of the MYB TFs were ligated into pGADT7 vectors to construct the prey-AD vectors. The pBait-AbAi vectors were linearized and transformed into Y1HGold. Then a colony PCR analysis was used to confirm that the plasmids were integrated into the genome. The empty pTRV1 and pTRV2 vectors, independently.

### Statistical analysis

An analysis of variance and significant difference tests were conducted to identify differences among means by one-way ANOVA.
Results
Isolation of flavonoid related MYB TFs

In total, 148 putative R2R3-MYB TFs were isolated from the pear genome, and 18 of them were clustered into four flavonoid-related MYB subclades in the phylogenetic tree (Fig. 1). Pbr016661 (designations are consistent with the accession numbers in the pear genome), Pbr016663, and Pbr042132 belong to the anthocyanin-related MYB clade (S4); Pbr000876, Pbr042766, Pbr20733, Pbr013413, Pbr028725, and Pbr038871 were clustered with AtMYB4 and MdMYB3 in a repressor-type MYB clade (S3); Pbr024977, Pbr045125, Pbr031662, Pbr015230, and Pbr024978 belong to the TT2 clade (S1); and Pbr003370, Pbr036818, Pbr024912, and Pbr018379 belong to another PA-related MYB clade (S2).

Flavonoid biosynthesis patterns and the expression patterns of candidate MYB TFs in pear fruits

The concentrations of general phenylpropanoid pathway products showed no significant differences between the two cultivars. ‘Zaosu’ fruit accumulated more flavanols in its mature fruit flesh and young fruit peel, but no significant differences appeared in its matured fruit peel or bagged fruit peel. More anthocyanins were accumulated in the fruit peel of ‘Red Zaosu’ at both the early stage of fruit development and the mature stage, but no anthocyanins were detected in the flesh or the peel of bagged fruits of either cultivar. The concentrations of flavonols were only higher in ‘Red Zaosu’ compared with ‘Zaosu’ in the young fruits’ peel (Fig. 2A). The specific concentration of each is shown in Supplementary Table S1.

The structural flavonoid pathway genes, except for PbUFGT2, showed relatively higher expression levels in ‘Red Zaosu’ than in ‘Zaosu’ at the early stage of fruit development, but only PbDFR and PbANS retained this higher expression pattern at the mature stage. The EBGs, PbCHI and PbF3H, had higher expression levels in ‘Red Zaosu’ mature flesh and the peel of bagged fruit. By contrast, PbANR had a higher expression level in ‘Zaosu’ in the peel of bagged fruit (Fig. 2B). The relative values of the gene expression levels are listed in Supplementary Table S2.

The expression patterns of candidate MYB TFs were various. The MYB10-like TFs, Pbr016663, Pbr042132, and Pbr020733, had similar expression patterns to PbDFR and PbANS and were thus consistent with their anthocyanin concentrations. Furthermore, the expression patterns of Pbr024978 and Pbr020733 were consistent with the expression patterns of PbANR and PbFLS, respectively (Fig. 2C). The relative values of the gene expression levels are listed in Supplementary Table S3.

The expression patterns of PbMYB10, PbMYB10b, PbMYB9, and PbMYB3 are correlated with flavonoid biosynthesis in pear fruits

The expression patterns of Pbr016663, Pbr042132, Pbr020733, and Pbr024978 showed correlations with the expression patterns of most structural flavonoid pathway genes (Table 1). This result was consistent with the previous observation in Fig. 2. Thus, these four MYB TFs were predicted to be the potential regulators of flavonoid biosynthesis and were designated as PbMYB10, PbMYB10b, PbMYB9, and PbMYB3, respectively.

Cloning of PbMYB10, PbMYB10b, PbMYB9, and PbMYB3 and an analysis of sequence characteristics

The primer pairs used to amplify the CDS of PbMYB10, PbMYB10b, PbMYB9, and PbMYB3 in ‘Zaosu’ and ‘Red Zaosu’ were designed based on their sequences in the pear genome (see Supplementary Table S4). No sequence differences were found between the two cultivars. The sequences of the PbMYB10b R3 region in both cultivars were different, containing a 222 bp insert, from those in the pear genome.

The alignment of the amino acid sequences of PbMYB10, PbMYB10b, PbMYB9, and PbMYB3 with other labelled MYB TFs showed that all of these MYB TFs were conserved in their R2 region and the bHLH-acting region in R3, but more diversity was found in the C-terminal region. Six conserved motifs,
MYB transcription factors regulate flavonoid biosynthesis in pear fruit

PbMYB10b, PbMYB9, and PbMYB3 are regulators of different flavonoid pathways.

PbMYB10b promoted the expression of PbDFR and induced the biosynthesis of PAs and anthocyanins in its overexpression fruits. However, no changes were detected in the expression level of structural genes or the concentrations of flavonoid compounds in its RNAi fruits. This indicated that PbMYB10b was a specific regulator of the anthocyanin and PA pathways, but that its function could be complemented by other MYB TFs, such as PbMYB10.

The expression levels of PbANR and PbUFGT1 were up-regulated in the PbMYB9 overexpression fruits. The biosynthesis patterns of flavonols, flavanols, and anthocyanins were in accordance with the expression patterns of PbANR and PbUFGT1. PbMYB9 RNAi fruits, the PA, flavonol, and anthocyanin pathways were suppressed, and the expression of PbANR and PbUFGT1 was also suppressed. This indicated that PbMYB9 was a regulator of the PA, flavonol, and anthocyanin pathways, and it has essential roles in these pathways.

PbMYB3 activated the expression of PbUFGT1, PbFLS, and PbMYB10 but did not induce the accumulation of any flavonoid compounds in the overexpression fruits. However, in its RNAi fruits, PbMYB10 and PbUFGT1 were slightly suppressed, and the anthocyanin and flavonol pathways were suppressed. That indicated that PbMYB3 was a potential activator of PbFLS and PbMYB10.

The PbUFGT1 promoter could be specifically bound by PbMYB10, PbMYB10b, and PbMYB9

The MYBCORE-like element was identified in ANR promoters in each species, but in UFGT promoters, the...
**Discussion**

**PbMYB10, PbMYB10b, PbMYB9, and PbMYB3 are related to flavonoid biosynthesis in pear fruits**

The expression patterns of *PbMYB10* and *PbMYB10b*, which belong to PAP-type MYB TFs, have positive correlations with the expression patterns of most structural flavonoid pathway genes and were in accordance with the expression patterns of *PbDFR* and *PbANS* in pear fruits. These observations are consistent with previous studies focused on anthocyanin-related MYB TFs. In strawberry, *FaMYB10*, a PAP-type MYB TF, regulates the expression of most EBGs and LBGs involved in anthocyanin production in ripened fruit receptacles (Medina-Puche et al., 2014). The expression level of *MdMYB1* is co-ordinated with the expression levels of genes in the anthocyanin pathway in apple fruits (Takos et al., 2006). *PbMYB9* is consistent with *PbANR* in its expression level, which implied that this TT2-type MYB TF may contribute to regulating PA biosynthesis. *PbMYB3* only showed a slight difference in its expression level between the two cultivars at the young stage of fruit development, but its expression pattern was correlated with the expression levels of the most structural genes. It was also consistent with flavonol synthesis at each stage of fruit development and in fruit flesh. Consistent with our observations, *MdMYB3*, the orthologue of *PbMYB3*, activates several flavonoid pathway genes, including CHS, CHI, UFGT, and FLS in apple fruits (Vimolmangkang et al., 2013). Thus we hypothesize that these four MYB TFs are potential regulators of flavonoid biosynthesis in pear fruits.

A correlation analysis showed that the expression levels of *PbMYB10, PbMYB10b, PbMYB9*, and *PbMYB3* correlate with the expression pattern of most structural genes in a similar way, and thus formed a collinearity-system. The existence of the collinearity-system implied that these TFs may be regulated by a single unknown regulator, or even by one of themselves. Furthermore, the concentrations of PA were not correlated with the expression levels of *PbANR* and *PbMYB9* in the young fruits or flesh, which could be explained by the PAs accumulation in the fruits at an early stage. However, the mechanism still needs to be studied in future research.

**PbMYB10b is an activator of PA biosynthesis and anthocyanin biosynthesis**

*PbMYB10b* induced the expression of *PbDFR* in its overexpression fruits and slightly reduced the expression of *PbDFR*.
in its RNAi fruits. However, with contrast with the significantly increased concentration of PAs and anthocyanins in its overexpression fruits, the concentration of all these flavonoid compounds barely changed in its RNAi fruits. In \(PbMYB10b\) RNAi fruits, \(PbMYB10\) still stayed at a relatively high level, and we speculated that this MYB TF could be functionally complementary with \(PbMYB10\) and \(FaMYB10\), an orthologue of the MYB10 family in strawberry, plays a major role in the regulation of flavonoid/phenylpropanoid metabolism and the anthocyanin pathway was completely suppressed in its RNAi fruits (Medina-Puche et al., 2014). However, in European pear (\(Pyrus communis\) L.), although the expression data indicate that \(PcMYB10\) is very important in anthocyanin production, the mapping data indicate that \(PcMYB10\) is not directly responsible for the red skin variation (Pierantoni et al., 2010). This result implies that the anthocyanin pathway in pear fruits may not be controlled by a single MYB TF. This redundancy could be further explained by the fact that, although \(PbMYB10b\) has the anthocyanin-related motifs, the ANDV motif, and \([K/R]Pxxx[K/T][F/Y]\) in its amino acid sequence, the last two residues in each motif is not conserved. This change may lead to partially lost functions, such as lacking the ability to regulate the expression of \(PbUFGT1\). This finding was also supported by our observation in the young fruits of ‘Eli yihao’ (\(Pyrus pyrifolia\) Nakai; see Supplementary Fig. S3A).

\(PbMYB9\) is an essential activator of the PA, anthocyanin, and flavonol pathways

Significant suppression of the PA, flavonol, and anthocyanin pathways was observed in \(PbMYB9\)-RNAi fruits, consistent with the increased concentrations of PA, flavonols, and anthocyanins in \(PbMYB9\)-overexpression fruits. \(PbMYB9\), as a TT2-type MYB TF, induced or reduced the expression of \(PbUFGT1\) and \(PbANR\) in its overexpression or RNAi fruits, respectively. In previous studies, TT2-type MYBs have been identified as the PA pathway regulators that activate ANR or LAR expression, or even as activators of DFR and ANS which are involved in both the PA and anthocyanin pathways (Baudry et al., 2004; Gesell et al., 2014). Interestingly, TT2-type MYBs have never been reported as activators of the anthocyanin pathway or as direct activators of UFGT. \(PbMYB9\) is a typical TT2-type regulator containing all of the TT2 characteristic motifs in its C-terminal region. These characteristics increased the likelihood of binding the promoters of target genes containing cis-elements, like MYBCORE, and thus the existence of the MYBCORE-like motif in \(PbUFGT1\).
Zhai et al. makes the interaction possible. Furthermore, RNAi assays indicated that *PbMYB9* is essential for the biosynthesis of PA products and anthocyanins in pear fruits. These findings were also supported by our observations in the young fruits of ‘Palacer’ (*Pyrus communis*; see Supplementary Fig. S3B).

However, in the PA pathway, procyanidin B1 and procyanidin B2 (the polymers of catechin and epicatechin) accumulated to a different extent from catechin and epicatechin. MYBs did not directly induce the biosynthesis of procyanidin B1 or procyanidin B2 but, in the long term, the accumulation of...
catechin and epicatechin caused by MYBs may induce the biosynthesis of procyanidin B1 and procyanidin B2. More attention should be taken on this point in future work (see Supplementary Fig. S4).

The PbUFGT1 promoter has both MYBCORE-like and PCE-like motifs in its sequence

MYBCORE is a TT2 specific target element usually identified in ANR or LAR promoters (Bogs et al., 2007; Akagi et al., 2009). PCE is identified as the target specifically bound by PAP-type TFs and usually identified in the promoters of anthocyanin related genes, such as DFR, ANS or UFGT (Dare et al., 2008). Unlike the sequence in the pear genome, the PbUFGT1 promoter in both cultivars contained an inserted fragment which contributed to the existence of the PCE-like motif, G-box, and MYBCORE-like motif in a region of less than 100 bp. The G-box was adjacent to the bHLH and played an important role in the interaction of MYB TFs with their target genes. When lacking the G-box that was adjacent to the ACC-motif, MdMYB9 cannot perform its normal function (Gesell et al., 2014). Furthermore, the [DE]Lx2[RR]x3Lx6Lx3R motif, identified in the R3 regions of PbMYB10, PbMYB10b, PbMYB9, and PbMYB3, potentially contributes to the specificity of the bHLH partner in the stable MYB-bHLH complex (Zhao et al., 2013). This implied that, not only is the existence of the G-box important, but that the relative location between these two motifs is also quite important. The PCE motif was also adjacent to the bHLH motif. The cis architecture has been verified to play a more important role than the TF complex in determining the variation in promoter activity (Zhu et al., 2015). Thus, the special MYBCORE-G-box-PCE structure is essential for flavonoid biosynthesis in pear fruits.

PbMYB3, a potential regulator of PbMYB10?

The PbMYB3-overexpression fruits showed no significant differences in the level of flavonoid compounds. In its RNAi fruits, flavonols and anthocyanins were reduced. Among the structural flavonoid pathway genes, only PbFLS and PbUFGT1 were induced or reduced by PbMYB3. However, we found that the expression level of PbMYB10 was also correlated with PbMYB3. Thus the expression pattern of PbUFGT1 could be explained, and the result is consistent with the regulation pattern of MdMYB3 in apple (Vimolmangkang et al., 2013). Thus we speculated that PbMYB3 is a putative regulator of PbMYB10. More attention should be focused on this MYB TF in future studies.

In this study, the correlation of flavonoid biosynthesis with expression patterns of MYB TFs were clarified in pear fruits. Three candidate MYB TFs, designated as PbMYB10b, PbMYB9, and PbMYB3 were cloned and functionally verified by overexpression and RNAi transient assays. The results suggest that PbMYB10b acts as an activator of the anthocyanin and PA pathways but could be functionally complemented by other MYB TFs. PbMYB9 is an activator of the PA, anthocyanin, and flavonol pathways, and its function was essential for flavonoid biosynthesis in pear fruits. PbMYB3 is a potential regulator of PbMYB10 (Fig. 6).

Supplementary data

Supplementary data can be found at JXB online.

Table S1. The concentrations of flavonoid compounds in the two cultivars.

Table S2. The expression pattern of flavonoid structural genes in the two cultivars.

Table S3. The expression pattern of putative MYB TFs in the two cultivars.

Table S4. Primer list.

Figure. S1. Flavonoid biosynthesis pattern in pear fruit.

Figure. S2. Construction of the recombinant plasmids.

Figure. S3. The MYB overexpression transient fruits of ‘Eli yihao’ (A) and the MYB RNAi transient fruits of ‘Palacer’ (B).

Figure. S4. The concentrations of flavonoid compounds in MYB-overexpression ‘Zaosu’ fruits and MYB-RNAi ‘Red Zaosu’ fruits.

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References


Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L. 2004. TT2, TT8, and TTG1 synergistically specify the regulation pattern of MdMYB3 in apple (Vimolmangkang et al., 2013). Thus we speculated that PbMYB3 is a putative regulator of PbMYB10. More attention should be focused on this MYB TF in future studies.

In this study, the correlation of flavonoid biosynthesis with expression patterns of MYB TFs were clarified in pear fruits. Three candidate MYB TFs, designated as PbMYB10b, PbMYB9, and PbMYB3 were cloned and functionally verified by overexpression and RNAi transient assays. The results suggest that PbMYB10b acts as an activator of the anthocyanin and PA pathways but could be functionally complemented by other MYB TFs. PbMYB9 is an activator of the PA, anthocyanin, and flavonol pathways, and its function was essential for flavonoid biosynthesis in pear fruits. PbMYB3 is a potential regulator of PbMYB10 (Fig. 6).

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References


