The MYB transcription factor PbMYB12b positively regulates flavonol biosynthesis in pear fruit

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Abstract

Background: As a class of natural antioxidants in plants, fruit flavonol metabolites are beneficial to human health. However, the regulatory networks for flavonol biosynthesis in most fruits are largely unknown. Previously, we reported a spontaneous pear bud sport ‘Red Zaosu’ (Pyrus bretschneideri Rehd.) with a high flavonoid content in its fruit. The identification of the flavonol biosynthetic regulatory network in this mutant pear fruit is crucial for elucidating the flavonol biosynthetic mechanism in fruit.

Results: Here, we demonstrated the PbMYB12b positively regulated flavonol biosynthesis in ‘Red Zaosu’ fruit. Initially, we investigated the accumulation patterns of four major quercetin glycosides and two major isorhamnetin glycosides in the fruit of ‘Red Zaosu’ and its wild-type ‘Zaosu’. A PRODUCTION OF FLAVONOL GLYCOSIDES (PFG)-type MYB transcription factor PbMYB12b was also screened for because of its correlation with flavonol accumulation in pear fruit. The biofunction of PbMYB12b was verified by transient overexpression and RNAi assays in pear fruit and young leaves. Overexpression of PbMYB12b enhanced the biosynthesis of quercetin glycosides and isorhamnetin glycosides by positively regulating a general flavonoids biosynthesis gene PbCHSb and a flavonol biosynthesis gene PbFLS. This finding was also supported by dual-luciferase transient expression assay and transient β-glucuronidase (GUS) reporter assay.

Conclusions: Our study indicated that PbMYB12b positively regulated flavonol biosynthesis, including four major quercetin glycosides and two major isorhamnetin glycosides, by promoting the expression of PbCHSb and PbFLS in pear fruit.

Keywords: Flavonol, MYB12, Fruit, Pear

Background

Flavonoids represent a large group of secondary metabolites in plants that generally have a C6-C3-C6 carbon skeleton [1]. Anthocyanins, flavonols, flavonoids and flavanones are common flavonoid compounds found in most fleshy fruits [2–5]. There are various flavonol biofunctions in plants. Antioxidant capacity is a common feature of flavonols and most other flavonoid compounds. In addition, flavonols also have some unique biofunctions. Flavonol derivatives, especially quercetins and kaempferols, have the capacity to inhibit auxin transport [6]. Moreover, flavonols may be involved in the fertilization processes of some higher plants [7, 8].

The biosynthetic pathways of most flavonoid compounds have been verified. The flavonoid biosynthetic pathway is part of the phenylpropanoid pathway. Specifically, phenylalanine ammonia lyase, cinnamate 4-hydroxylase and 4-coumarate: CoA ligase are the biosynthetic enzymes in the general phenylpropanoid pathway. Chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase are the common biosynthetic enzymes in the general phenylpropanoid pathway. Chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase are the common biosynthetic enzymes of most flavonoid compounds. Flavonol synthase (FLS), UDP-glucose: flavonoid 3-glucosyltransferase (UFGT) and quercetin 3-O-methyltransferase (QOMT) are
specifically involved in the biosynthesis of flavonol compounds (Fig. 1) [3, 9].

The flavonoid biosynthetic genes, which encode these enzymes, are transcriptionally regulated by MYB family transcription factors (TFs). Three subgroups among the MYB family were identified as typical activators of the anthocyanin, flavanol and flavonol pathways, respectively [10–12]. To be more specific, PRODUCTION OF ANTHOCYANIN PIGMENT (PAP)-type MYBs, including PAPI(AtMYB75), PAP2(AtMYB90), AtMYB113 and AtMYB114 in Arabidopsis, MYB10 in apple (Malus × domestica), and MYB10 and MYB10b in pear (Pyrus bretscheideri Rehd.), were identified as positive regulators of the flavonol pathway [12, 15]. TRANSPARENT TESTA 2 (TT2)-type genes, including TT2 in Arabidopsis, PbMYB9 in pear and MdMYB9 in apple, were identified as positive regulators of the flavonol pathway [12, 15–17]. PRODUCTION OF FLAVONOL GLYCOSIDES (PFG)-type MYB TFs were first identified as positive regulators of the flavonol pathway in Arabidopsis [18]. These three different types of MYB TFs, PAP, TT2 and PFG, specifically activate the genes encoding the flavonoid biosynthetic enzymes involved in the anthocyanin, flavonol and flavonol pathways, respectively. PAP-type MYB TFs transcriptionally activate the genes encoding leucoanthocyanidin dioxygenase, dihydroflavonol 4-reductase and UFGT [10, 12]. TT2-type MYB TFs transcriptionally activate the genes encoding leucoanthocyanidin reductase and anthocyanidin reductase [16]. PFG-type MYB TFs transcriptionally activate the genes encoding CHS, CHI and FLS [13, 18].

Fleshy fruits are the primary dietary sources of flavonols. Several PFG-type MYB TFs were screened from some fleshy fruits. In grapevine (Vitis vinifera), VvMYBF1 was verified in positively regulating flavonol biosynthesis by activating CHS and FLS [19]. MdMYB22 was proposed as a positive regulator of the flavonol pathway by inducing the expression of MdFLS in apple [20]. The Chinese pear ‘Red Zaosu’ originated as a spontaneous mutation from ‘Zaosu’ (P. bretscheideri Rehd.). The accumulation levels of anthocyanins and flavonols are much greater in ‘Red Zaosu’ fruit than in ‘Zaosu’ fruit [9]. Previously, we verified that PbMYB10b and PbMYB9 are positive regulators of the anthocyanin and flavanol pathways, respectively [15]. However, the flavonol biosynthetic regulatory network in pear fruit is still poorly understood.

In this study, we initially screened for candidate genes that may be involved in flavonol biosynthesis in ‘Red Zaosu’ fruit using a comparative transcriptome analysis. The general flavonoids biosynthesis genes PbCHSa and PbCHSb, the flavonol biosynthesis genes PbFLS and PbUFGT, and one PFG-type MYB TF, PbMYB12b, were used. PbCHSa, PbFLS and PbMYB12b were further investigated because of their high correlations with flavonol glycoside accumulation patterns in ‘Red Zaosu’ fruit. PbMYB12b activated the expressions of PbCHSa and PbFLS, resulting in flavonol glycoside accumulations in ‘Red Zaosu’ fruit. Thus, a PbMYB12b-regulated flavonol biosynthetic pattern in pear fruit was revealed.

**Results**

PbMYB12b is a potential regulator of the flavonol biosynthetic pathway in pear fruit

To investigate the flavonol biosynthetic patterns in the leaves and fruit of ‘Red Zaosu’ and its wild-type ‘Zaosu’, the concentrations of flavonol compounds in their tissue cultured young leaves (three-day-old) and their fruit skin at the fruit developing period (DP) were initially investigated. Quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-rutinoside, quercetin 3-arabinoside, isorhamnetin 3-galactoside and isorhamnetin 3-glucoside were the major flavonol glycosides found in the fruit skins of ‘Red Zaosu’ and ‘Zaosu’ (Fig. 2a). Only four major flavonol compounds including quercetin 3-galactoside, quercetin 3-glucoside, isorhamnetin 3-galactoside and isorhamnetin 3-glucoside were identified in pear leaves (Additional file 1: Figure S2a). We compared the concentrations of these flavonol compounds between ‘Red Zaosu’ and ‘Zaosu’.
The concentrations of most flavonol glycosides were significantly greater in 'Red Zaosu' than in 'Zaosu' (Fig. 2a, Additional file 1: Figure S2a).

Furthermore, the expression patterns of flavonol biosynthetic genes were screened from a comparative transcriptome analysis between the DP fruit skins of 'Red Zaosu' and 'Zaosu' (Fig. 2b, Additional file 1: Table S1). Several flavonol biosynthetic genes including PbCHSa, PbCHSb, PbFLS and PbUFGT were differentially expressed between 'Red Zaosu' and 'Zaosu'. Consistent with most flavonol glycosides accumulation patterns, the expression levels of PbCHSa, PbCHSb, PbFLS and PbUFGT in 'Red Zaosu' fruit and leaves were greater than those in 'Zaosu' (Fig. 2b, Additional file 1: Figure S2b). Moreover, two PFG-type MYB TFs, designated PbMYB12a and PbMYB12b, were expressed in the transcriptome of the DP fruit skins of 'Red Zaosu' and 'Zaosu' (Additional file 1: Table S1). The expression level of PbMYB12b was significantly greater in 'Red Zaosu' fruit and leaves (Fig. 2b, Additional file 1: Figure S2b). The expression of PbMYB12a in the two cultivars did not show significant difference neither in fruit nor in leaves (Fig. 2b, Additional file 1: Figure S2b). Thus, PbMYB12b was investigated further as a potential regulator of flavonol biosynthesis.

**Identification and molecular characterization of PbMYB12b**

The full-length coding sequences (CDS) of PbMYB12a and PbMYB12b were cloned from 'Red Zaosu' and 'Zaosu'. The lengths of the proteins encoded by PbMYB12a and PbMYB12b were 492 and 485 amino acids, respectively. Moreover, no variations in protein sequences were identified among 'Red Zaosu', 'Zaosu' and the pear reference genome. Typical MYB TFs involved in flavonoid biosynthesis could be classified into three different groups. PAP-type MYB TFs, including PbMYB10 and PbMYB10b in pear, were identified as anthocyanin regulators and placed in group I. TT2-type MYB TFs, including PbMYB9 in pear, were identified as flavanol regulators and placed in group II. Notably, PbMYB12a and PbMYB12b were clustered with PFG-type MYB TFs and placed into group III (Fig. 3a). A protein sequence alignment analysis showed that PbMYB12a and PbMYB12b contain two characteristic motifs, SG7–1 and SG7–2, which belong to PFG-type MYB TFs (Fig. 3b). Thus, PbMYB12a and PbMYB12b were identified as typical PFG-type MYB TFs having the potential capacity to regulate flavonol biosynthesis in pear fruit.

**Sub-cellular localization of PbMYB12b**

The sub-cellular localization of PbMYB12b was inferred from the transient expression of p35S::green fluorescent protein (GFP)-PbMYB12b in onion epidermal cells. The transformed cells expressed GFP strictly in the nuclei (Fig. 4), whereas in control transgenic cells expressing p35S::GFP, the GFP signal was detected throughout the cells (Fig. 4).
The PbMYB12b expression pattern was correlated with flavonol glycosides biosynthesis

The accumulation rates of quercetin 3-galactoside, quercetin 3-arabinoside and isorhamnetin 3-galactoside accelerated after pollination (AP) and slowed down when the fruit in ripening period (RP) in ‘Red Zaosu’ (Fig. 5a). The concentrations of quercetin 3-galactoside and quercetin 3-glucoside remained greater in RP fruit of ‘Red Zaosu’ than in ‘Zaosu’ (Fig. 5a). The expression pattern of PbMYB12b was positively correlated with most...
flavonol glycosides, except for quercetin 3-glucoside (Fig. 5b and c). Moreover, the expression pattern of PbMYB12b was positively correlated with the expression patterns of PbCHSa, PbCHSb and PbFLS, especially the latter two during fruit growth (Fig. 5b and c). Thus, PbMYB12b may positively regulate flavonol glycoside accumulation, and its putative target genes are PbCHSa, PbCHSb and PbFLS.

PbMYB12b could induce flavonol accumulation in pear fruit

The validity of the infection on the leaves and fruit was proved by monitoring the GUS signals (Additional file 1: Figure S3a). Besides, in comparison with the non-infiltrated fruit and leaves, the flavonols concentration or the flavonoid biosynthesis genes did not change too much in those infiltrated fruit and leaves (Additional file 1: Figure S3a).

Transient overexpression of PbMYB12b in wild-type ‘Zaosu’ fruit revealed its specific function in regulating flavonol accumulation. The concentrations of most flavonol glycosides were up-regulated by the overexpression of PbMYB12b, except for quercetin 3-arabinoside (Fig. 6a). Unexpectedly, the accumulation of the major anthocyanin compound in pear fruit, cyanidin 3-galactoside, was also induced by the overexpression of PbMYB12b (Fig. 6a). For those flavonol biosynthetic genes, the overexpression of PbMYB12b up-regulated the expression of PbCHSb and PbFLS. Moreover, the expression patterns of PbCHSa and PbUFGT were not affected by PbMYB12b (Fig. 6b). The expression levels of PbCHSb and PbFLS were reduced when PbMYB12b expression was transcriptionally interrupted using a virus-induced gene silencing (VIGS) assay in ‘Red Zaosu’ fruit (Fig. 6d). The concentration of quercetin 3-galactoside, a major flavonol compound in pear fruit, was reduced in ‘Red Zaosu’ fruit having a low PbMYB12b expression level (Fig. 6c), but the concentrations of other flavonols were not reduced (Fig. 6d). This phenomenon could be explained by the long period of flavonol glycoside accumulation during DP, while the RNAi effect of VIGS was probably transient.

Therefore, to identify whether PbMYB12b regulates the biosynthesis of most flavonol compounds in pear, we further transient overexpressed and interrupted PbMYB12b expression in the unexpanded young leaves of ‘Zaosu’ and ‘Red Zaosu’ by vacuum infiltration, respectively. Quercetin 3-rubinoside and arabinoside were not identified in the young leaves of ‘Zaosu’ and ‘Red Zaosu’. The young ‘Zaosu’ leaves overexpressing PbMYB12b accumulated more quercetin 3-galactoside and isorhamnetin 3-galactoside than control leaves (Fig. 7a). The expression levels of PbCHSb and PbFLS were also up-regulated by the overexpression of PbMYB12b (Fig. 7b). The biosynthesis of most flavonol
glycosides in young leaves of ‘Red Zaosu’ was inhibited when PbMYB12b expression was interrupted (Fig. 7c). PbCHSb and PbFLS were also lowly expressed in these leaves (Fig. 7d). This result indicated that the regular expression of PbMYB12b was necessary in flavonol glycoside biosynthesis for pears.

Thus, the biofunction of PbMYB12b in flavonol biosynthetic regulation was verified, and two downstream genes of PbMYB12b, PbCHSb and PbFLS, were investigated.

**PbMYB12b activates the expression of PbCHSb and PbFLS**

To determine whether PbMYB12b activates the expression of PbCHSb and PbFLS, we performed dual-luciferase (LUC) assays in tobacco leaves. The promoter regions of PbCHSb and PbFLS were amplified, independently transformed into the pGreenII 0800-LUC vector and co-integrated into tobacco leaves with overexpressing PbMYB12b. When PbMYB12 was independently co-transformed with PbCHSb and PbFLS pro-LUC reporters, the LUC/ Renilla LUC (REN) ratios significantly increased compared with the empty control (Fig. 8a). This was consistent with our observations of GUS activity levels when PbMYB12b was co-expressed with the GUS reporter containing the independent promoter regions of PbCHSb and PbFLS (Fig. 8b). Thus, PbMYB12b positively regulate the expression patterns of PbFLS and PbCHSb by activating their promoters.

**Discussion**

Flavonol glycosides are a group of flavonoid metabolites in plants that have potential health benefits for humans. There are various kinds of flavonol glycosides in different plant species. Quercetin glycosides are common flavonol compounds in fleshy fruit, including apple, pear and grape berry [3, 9, 19, 20]. Nevertheless, as a common fruit, pear has a unique composition of flavonol glycosides owing to the accumulation isorhamnetin glycosides [9, 21, 22]. Although the biosynthetic enzymes of flavonoid compounds have been investigated in pears, neither the biosynthetic pathway of flavonols, nor their regulatory networks, have been specifically studied [3]. Previously, we reported that UFGT was a key biosynthetic enzyme in the accumulation of flavonoid glycosides, including flavonols [9]. In the present study, the expression patterns of the genes encoding CHS and FLS were consistent with the accumulation patterns of quercetin glycosides and isorhamnetin glycosides (Fig. 5). The roles of CHS and FLS in flavonol biosynthesis have
been reported in other plants. The expression of the CHS gene is deficient in the Arabidopsis mutant tt4, and flavonols cannot accumulate in the mutant tt4 [23]. FLS exclusively catalyzes flavonol glycoside biosynthesis, and the regular expression of the FLS gene is necessary for flavonol accumulation in Arabidopsis seedlings [24]. Thus, the biosynthesis of flavonol glycosides may not be determined by a single biosynthetic enzyme but may be influenced by a series of biosynthetic enzymes, including CHS, FLS and UFGT.

In this study, a MYB TF was identified as being involved in regulating flavonol glycoside biosynthesis in pear fruit. PbMYB12b is a typical PFG-type MYB TF, and its expression pattern is consistent with the flavonol accumulation pattern in pear fruit (Figs. 3 and 5). PbMYB12b largely expressed in young ‘Red Zaosu’ fruit

![Fig. 7 PbMYB12b-regulated flavonol biosynthesis in young pear leaves.](image)

![Fig. 8 PbMYB12b activates PbFLS and PbCHSb promoters.](image)
(AP) and kept decreasing during the fruit development (DP and RP), this result is also consistent with the expression patterns of PbMYB12 in ‘Dangshansu’ pear fruit during its development [25]. Overexpressed PbMYB12b induced the biosynthesis of most quercetin glycosides and isorhamnetin glycosides in pear fruit (Fig. 6). In apple calli, the overexpression of MdMYB22, a PFG-type MYB TF, also induces flavonol accumulation but reduces anthocyanin biosynthesis [20]. However, in our study, the anthocyanin concentration increased in ‘Zaosu’ fruit overexpressing PbMYB12b but was not decreased in ‘Red Zaosu’ fruit with interrupted PbMYB12b (Fig. 6).

The result indicated that PbMYB12b could be a positive regulator of anthocyanin biosynthesis but not the dominant or necessary TF of anthocyanin biosynthesis.

Transient silenced PbMYB12b by VIGS only reduced the concentration of quercetin 3-galactoside in pear fruit skin (Fig. 6c and d). However more flavonol glycosides were significantly affected by PbMYB12b silencing in leaves (Fig. 7c and d). This result indicated that PbMYB12b is more functional in leaves and probably not fruit specific. Besides, levels of flavonol glycosides in leaves are in fact lower in comparison with fruits (Fig. 2, Additional file 1: Figure S2). The leaves with less flavonol accumulation may provide a more sensitive environment to detect the effects of transient silenced PbMYB12b. Although the flavonol biosynthetic related genes were suppressed by silenced PbMYB12b, the high level flavonol background in DP fruit maybe still overshadowed the effect of silenced PbMYB12b on the flavonol concentration level. The low flavonols background in leaves also could explain why more flavonol glycosides were significantly affected by PbMYB12b silencing in leaves.

MdMYB22 specifically up-regulates the expression of MdFLS but down-regulates that of MdCHS in apple. VvMYBF1, a PFG-type MYB TF in grapevine, activates the expression of CHS and FLS by binding their promoters [19]. In the present study, we verified that PbMYB12b positively regulated the expression of PbCHSb and PbFLS by activating their promoters (Fig. 8a and b). Interestingly, PbMYB12b and VvMYBF1 both have a conserved SG7–2 motif with four residues, WLLS, that is not found in MdMYB22 (Fig. 3). This observation indicated that there were two types of PFG MYB TFs that both induced flavonol accumulation but one could inhibit anthocyanin biosynthesis at the same time. The correlations between the sequence variations in the two types of PFG MYB TFs and their biofunctions in different regulatory networks for flavonol biosynthesis will be studied in the future.

Conclusions
Based on our results, PbMYB12b’s regulation of flavonol biosynthesis was investigated. PbMYB12b could up-regulate the expression levels of PbCHSb and PbFLS, and their high-level expression resulted in the accumulation of flavonol compounds, including quercetin glycosides and isorhamnetin glycosides, in pear fruit. PbMYB12b also contributes slightly to the accumulation of anthocyanin in pear fruit. An increased understanding of regulatory networks for flavonol biosynthesis in pear fruit could help to breed flavonoid-enriched cultivars, providing nutritious fruit.

Materials and methods
Plant materials and growing conditions
In the present study, ‘Red Zaosu’ (P. bretschneideri Rehd.) and ‘Zaosu’ (P. bretschneideri Rehd.) were collected from the Horticultural Research Base of Northwest A&F University in Yangling Distinct, Shaanxi Province, China. The pear fruit were harvested and sampled at four representative points during the whole-fruit growth period, including 5 d before pollination (BP), 15 d after pollination (AP), 50 d after pollination (DP) and 110 d after pollination (fruit ripen period, RP). Moreover, the pear fruit in DP and three-day-old leaves of tissue cultured pear plants were selected to transiently verify the bio-function of PbMYB12b. The detailed experimental design was illustrated in Additional file 1: Figure S1.

Fresh plant tissues for later use were immediately frozen, ground to a powder in liquid nitrogen and stored at –80 °C.

DNA and RNA extraction and purification
Total DNA and RNA were extracted and purified by SDS solubilization and phenol extraction [26].

RNA-seq and analysis
During the DP, 3 μg total RNA were extracted from receptacles and fruit skins of ‘Red Zaosu’ and ‘Zaosu’, with three biological replicates. These were used for sequencing. Briefly, total RNA was randomly fragmented, reversed-transcribed, amplified and purified to form cDNA libraries. After the assessment of the cDNA libraries using an Agilent Bioanalyzer 2100 system, the library preparations were paired-end sequenced (100 bp) on an Illumina HiSeq2500 platform. Clean reads were screened by removing reads containing adapters and poly-Ns, and low-quality reads, from the raw data. Paired-end clean reads were aligned to the pear genome using TopHat [27]. Genes with q-values < 0.05, as assessed by the DESeq R package, and fold changes > 2 were assigned as differentially expressed genes between ‘Red Zaosu’ and ‘Zaosu’ [28].
RT-qPCR
In total, 2 μg purified RNA was reverse-transcribed to cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). The primer pairs of selected genes and PbActin (internal control) are listed in Additional file 1: Table S2. PCR reactions were performed on a StepOne-Plus PCR system (Applied Biosystems) with SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer’s instructions. Expression data of three biological replicates were analyzed according to the cycle threshold 2−ΔΔCt method.

Transient assay
The transient expression assays were performed as previously described, with a modified infiltration method [15]. For the overexpression of PbMYB12b, the full-length PbMYB12b CDS was PCR-amplified from ‘Red Zaosu’ cDNA sources and then replaced the GFP-coding sequence in the pCambia 1301 binary vector using ClonExpress One Step Cloning Kit (Vazyme) based on homologous recombination technology. For the RNAi of PbMYB12b using VIGS, a 479-bp fragment of the PbMYB12b homologous recombination technology. The construct was transiently introduced into onion epidermal cells by injection, and the un-infiltrated DP fruit and leaves were also sampled as controls. For transient RNAi, the DP fruit and young leaves of ‘Red Zaosu’ co-infiltrated by pTRV2 and pTRV1 and the un-infiltrated DP fruit and leaves were also sampled as controls. For GUS staining, the plant materials were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 12 as described [29]. The primer pairs are listed in Additional file 1: Table S2.

Analysis of flavonoid compounds
The extraction and analysis of flavonoid compounds were carried out as previously described [30]. 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 analyzed using a HP1200 Liquid Chromatograph equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA). An Inertsil ODS-3 column (5.0 μm, 4.0 × 250 mm, GL Sciences Inc., Tokyo, Japan) was used in the separation, preceded by an Inertsil ODS-3 Guard Column (5.0 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 (11.36% of 88% formic acid) and 1.36% water in acetonitrile (HPLC grade, purity: 99.9%). The gradient was 95% solvent A (0 min), 85% solvent A (25 min), 78% solvent A (42 min), 64% solvent A (60 min) and 95% solvent A (65 min). The post-run-time was 10 min. The flow rate was 1.0 mL min−1 at 30°C. Simultaneous monitoring was performed at 365 nm for quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-arabinoside, quercetin-3-rutinoside, isorhamnetin-3-ga-lactoside and isorhamnetin-3-glucoside, and at 520 nm for cyanidin-3-galactoside. Peaks were identified by a comparison of retention times and UV spectra with those of authentic standards. The concentrations of individual phenolic compounds were determined based on peak areas and calibration curves that 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 AAp Chemicals (Abingdon, Oxon, UK).

Sub-cellular localization of the PbMYB12b protein in the onion epidermis
The full-length PbMYB12b CDS was amplified and fused into pCambia 2300 to generate the transgene construct p35S::GFP-PbMYB12b using a ClonExpress One Step Cloning Kit (Vazyme) based on homologous recombination technology. The construct was transiently introduced into onion epidermal cells by injection, and control cells were transformed in the same way with p35S::GFP. After their transformation, the onion epidermal samples were held for 16 h at 25°C in the dark, after
which GFP fluorescence was imaged using a BX51 + PD72 + IX71 microscopic imaging system (OLYMPUS).

**Dual-LUC reporter assays in tobacco leaves**

To assay the effects of PbMYB12b on PbCHSb and PbFLS, their promoters were amplified and inserted into pGreenII 0800-LUC double-reporter plasmid as reporters (primer pairs are listed in Additional file 1: Table S2) [31]. The effector plasmid was constructed by inserting PbMYB12b into the p62-SK vector. The constructed effector and reporter plasmids, in different combinations, were co-transformed into tobacco leaves.

The activities of LUC and REN were quantified 48 h after infiltration with a dual LUC assay kit (Promega) using a Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific). The transcriptional capability of PbMYB12 was assessed by the LUC/REN ratio. Five biological repeats were included for each pair.

**GUS reporter assays in tobacco leaves**

To assay the effects of PbMYB12b on PbCHSb and PbFLS, their promoters were individually inserted into the pBI121-GUS plasmid as reporters. An effector plasmid was constructed by inserting PbMYB12b into the p62-SK vector. The constructed effector and reporter plasmids, in different combinations, were co-transformed into tobacco leaves. The GUS activity was measured as described previously [29].

**Statistical analysis**

To determine significant differences among the data, Student’s t test was conducted using SPSS 16.0.

**Additional file**

Additional file 1: Figure S1. The illustration of experimental design. Figure S2. Flavonol glycoside concentrations and the expression patterns of related genes in tissue cultured pear leaves. Figure S3. The transient efficiency in pear fruit and leaves. Table S1. FPKM values of flavonol biosynthesis related genes. (DOCX 1624 kb)

**Abbreviations**

AP: After pollination; AP: After pollination; BP: Before pollination; Chl: Chalcone isomerase; CHS: Chalcone synthase; DP: Developing period; F3H: Flavanone 3-hydroxylase; FLS: Flavonol synthase; PAP: PRODUCTION OF ANTHOCYANIN PIGMENT; PFG: PRODUCTION OF FLAVONOL GLYCOSIDES; QOMT: Quercetin 3-O-methyltransferase; RP: Ripen period; TF: Transcription factor; TT2: TRANSPARENT TESTA 2; UFGT: UDP-glucose: flavonoid 3-glucosyltransferase; VIGS: Virus induced gene silencing

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**Availability of data and materials**

All data generated and analyzed during this study are included in this published article.

**Authors’ contributions**

RZ, YZ, JY, XL and TW prepared the plant samples, flavonoid extraction and quantification. RZ, MW, HL and CY performed bioinformatics analyses. RZ, YZ, FL and MW quantified genes expression. RZ, ZW, TW, FL and XL performed transient assay. RZ, HL, CY, ZW, FM, LX and MW developed the overall strategy, designed experiments and coordinated the project. RZ, ZW, CY, LX and MW prepared the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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