



# Ethylene response factors Pp4ERF24 and Pp12ERF96 regulate blue light-induced anthocyanin biosynthesis in ‘Red Zaosu’ pear fruits by interacting with MYB114

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## Abstract

**Key message** Pp4ERF24 and Pp12ERF96 fine tune blue light-induced anthocyanin biosynthesis via interacting with PpMYB114 and promoting the interaction between PpMYB114 and PpbHLH3, which enhances the expression of PpMYB114-induced *PpUFGT*.

**Abstract** The red coloration of pear fruit is attributed to anthocyanin accumulation, which is transcriptionally regulated by the MYB-bHLH-WD40 complex. A number of ethylene response factors (ERF) have been identified to regulate anthocyanin biosynthesis in different plants. In pear, several ERF transcription factor genes were identified to be potentially involved in the light-induced anthocyanin biosynthesis according to transcriptome data. But the molecular mechanism of these ERFs underlying the regulation of anthocyanin accumulation is unknown. In this study, exposure of ‘Red Zaosu’ pear, a mutant of ‘Zaosu’ pear, to blue light significantly induced the anthocyanin accumulation by increasing the expression levels of anthocyanin biosynthetic genes. Gene expression analysis confirmed that the expression of Pp4ERF24 and Pp12ERF96 genes were up-regulated in the process of blue light-induced anthocyanin biosynthesis. Yeast two-hybrid and bimolecular fluorescence complementation assay revealed that Pp4ERF24 and Pp12ERF96 interacted with PpMYB114, but not with PpMYB10. Bimolecular fluorescence complementation assay demonstrated that the interaction between these two ERFs and PpMYB114 enhanced the interaction between PpMYB114 and PpbHLH3. Further analysis by dual luciferase assay verified that these two ERFs increased the up-regulation of PpMYB114-mediated *PpUFGT* expression. Furthermore, co-transformation of Pp12ERF96 with PpMYB114 and PpbHLH3 in tobacco leaves led to enhanced anthocyanin accumulation. Transient overexpression of Pp4ERF24 or Pp12ERF96 alone in ‘Red Zaosu’ pear fruit also induced anthocyanin biosynthesis in pear peel. Our findings provide insights into a mechanism involving the synergistic interaction of ERFs with PpMYB114 to regulate light-dependent coloration and anthocyanin biosynthesis in pear fruits.

**Keywords** Anthocyanin · Pear (*Pyrus*) · ERF · MYB · Blue light

Junbei Ni and Songling Bai contributed equally to this work.

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## Abbreviations

ERF	Ethylene response factor
UFGT	UDP-glucose: flavonoid 3-glucosyltransferase
EBG	Early biosynthetic gene
LBG	Late biosynthetic gene

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PAL	Phenylalanine ammonia lyase
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3'H	Flavanone 3'-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanin synthase
bHLH	Basic helix-loop-helix
DAFB	Days after full bloom

## Introduction

Pear (*Pyrus* spp.) is an economically important fruit in China. The pear fruit peel appears red because of the accumulation of anthocyanins (Huang et al. 2009; Steyn et al. 2005), which are important bioactive compounds responsible for red, blue, and purple color. Thus, anthocyanins affect fruit quality and commercial value. Anthocyanins are biosynthesized via the phenylpropanoid and flavonoid pathway, which involves several enzyme-encoding structural genes. The early biosynthetic genes [i.e., *PAL* (phenylalanine ammonia lyase), *CHS* (chalcone synthase), *CHI* (chalcone isomerase), and *F3'H* (flavanone 3'-hydroxylase)] are involved in the production of common precursors of the flavonoid pathway. In contrast, the late biosynthetic genes [i.e., *DFR* (dihydroflavonol 4-reductase), *ANS* (anthocyanin synthase), and *UFGT* (UDP-glucose: flavonoid 3-glucosyltransferase)] are specific for the anthocyanin biosynthesis pathway (Winkel-Shirley 2001).

The R2R3-MYB proteins interact with a basic helix-loop-helix (bHLH) and a WD-repeat protein to form a MYB-bHLH-WD40 (MBW) complex to transcriptionally regulate anthocyanin biosynthesis (Allan et al. 2008; Ballester et al. 2010; Xie et al. 2012; Xu et al. 2015). Of the MBW components, the MYB transcription factors are the most important regulators of anthocyanin biosynthesis by directly binding to the promoters of anthocyanin structural genes (Allan et al. 2008). In apple, *MdMYB1* cosegregates with the loci for skin color and is the genetic basis for apple skin color, while *MdMYB10-R6* and *MdMYB110* are responsible for the development of red fruit flesh (Tako et al. 2006). In grapevine, *VvMYB1* and *VvMYB2* regulate anthocyanin biosynthesis via the expression of *VvUFGT* (Kobayashi et al. 2002, 2004; Walker et al. 2007). In pear, *PpMYB10* and *PpMYB114* enhance anthocyanin biosynthesis by regulating the expression of structural genes (Feng et al. 2010; Yao et al. 2017).

The ethylene response factor (ERF) family is a large gene family and ERFs are plant-specific transcription factors which play critical roles in various biological processes and responses to environmental stresses (Nakano et al. 2006). For example, *MdERF2* negatively regulates ethylene biosynthesis during apple fruit ripening by suppressing *MdACS1* transcription (Li et al. 2016b). In loquat fruit, *EjAP2-1*,

an *AP2/ERF* factor, directly represses lignin biosynthesis via interaction with *EjMYB1* (Zeng et al. 2015). Several ERFs are also identified to regulate anthocyanin biosynthesis in different plants. In *Arabidopsis*, *aterf4aterf8* double mutant reduces light-responsive anthocyanin accumulation, which indicates that these two ERFs positively regulate light-induced anthocyanin biosynthesis (Koyama and Sato 2018). In apple, *MdERF3* is transcriptionally regulated by *MdMYB1* and involved in anthocyanin biosynthesis (An et al. 2018). In pear, *PpERF3* interacts with *PpMYB114* and *PpbHLH3* to form an *ERF3-MYB114-bHLH3* complex, which co-regulate the coloration of red pear fruits (Yao et al. 2017). In our previous studies, several ERF transcription factors were found to respond to light which might be potentially involved in anthocyanin biosynthesis (Bai et al. 2017). However, it is currently unknown how these ERF genes regulate light-induced anthocyanin biosynthesis in pear.

In this study, four *PpERFs* were selected as candidate genes which might regulate anthocyanin biosynthesis according to transcriptome analysis in pear (Bai et al. 2017). Then we investigated the regulation mechanism of these *PpERFs* on fruit coloration using 'Red Zaosu' pear, a red sport of the green-skinned 'Zaosu' pear which is a hybrid of the Asian pear and *Pyrus communis*. Subsequently, we found that *Pp4ERF24* and *Pp12ERF96* interacted with *PpMYB114* to induce anthocyanin biosynthesis in 'Red Zaosu' pear by enhancing the interaction between *PpMYB114* and *PpbHLH3*. The results presented herein showed a previously uncharacterized anthocyanin biosynthesis regulatory module which provides new insights into the molecular mechanism underlying the red coloration of pear fruits.

## Materials and methods

### Plant materials and treatment

'Red Zaosu' pear fruits were obtained from the orchard at the Research Institute of Horticulture, Henan Academy of Agricultural Sciences in 2016. A total of 150 fruitlets were covered with paper bags (consisting of yellow and black outer and inner layers, respectively) 22 days after full bloom (DAFB). Bagged and uncolored fruits with a similar size and quality were harvested at maturity (155 DAFB) and immediately transported to the laboratory.

The blue light irradiation experiments were performed as described previously (Tao et al. 2018), but different samples were used. Briefly, fruits were removed from bags in a darkroom. Fruits lacking mechanical injuries were randomly divided into three groups. The fruits were then transferred to shelves in a phytotron and continuously irradiated with a blue LED (453.2 nm, 21  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 17 °C and 80% relative humidity. Fruit peels collected prior to irradiation

(i.e., 0 h) and after 12, 24, 72, 144, and 240 h were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

### RNA extraction and cDNA synthesis

Total RNA was extracted using a modified CTAB method (Zhang et al. 2012), and the concentration was measured with a BioDrop spectrophotometer (Biochrom, Cambridge, UK). First-strand cDNA was synthesized from 2  $\mu\text{g}$  RNA using the PrimeScript<sup>TM</sup> RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The cDNA was diluted twofold, after which a 1- $\mu\text{l}$  aliquot was used as the template for gene cloning and real-time quantitative PCR (qPCR) analysis.

### Real-time quantitative PCR

Real-time quantitative PCR primers were designed using an online tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Tables S1, S2). Primer efficiency and specificity were verified using melting curves and by sequencing PCR products. The 15- $\mu\text{l}$  qPCR reaction mixture consisted of 7.5  $\mu\text{l}$  SYBR Premix Ex Taq<sup>TM</sup> (Takara, Ohtsu, Japan), 0.5  $\mu\text{l}$  each primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  cDNA, and 5.5  $\mu\text{l}$  RNase-free water. The qPCR was completed using a CFX Connect<sup>TM</sup> real-time PCR system (Bio-Rad, Hercules, CA, USA) with the following program: 95  $^{\circ}\text{C}$  for 3 min; 40 cycles of 95  $^{\circ}\text{C}$  for 10 s and 60  $^{\circ}\text{C}$  for 30 s. A template-free control was included for each run. Relative transcript levels were determined according to the  $2^{-\Delta\Delta\text{Ct}}$  algorithm (Livak and Schmittgen 2001), with the pear *Actin* gene (*PpActin*, JN684184) used for normalizing data.

### Yeast two-hybrid assay

A yeast two-hybrid (Y2H) assay was conducted using the Matchmaker<sup>TM</sup> Gold yeast two-hybrid system (Clontech). The full-length coding sequences (CDSs) encoding the prey and bait proteins were cloned into the AD (pGADT7) and BD (pGBKT7) vectors (Clontech) using the ClonExpress<sup>TM</sup> II One Step Cloning Kit (Vazyme). The primers for constructing vectors are listed in Table S3. The gene-BD plasmid was inserted into Y2HGold cells alone for an auto-activation analysis. The Y2HGold cells were then co-transformed with the AD and BD vectors and added to synthetically defined (SD) medium lacking leucine (Leu) and tryptophan (Trp) (DDO, SD/–Leu/–Trp). The cells were screened for possible physical interactions using SD medium lacking Leu, Trp, adenine (Ade), and histidine (His), but supplemented with 5-bromo-4-chloro-3-indolyl  $\alpha$ -D-galactopyranoside (X- $\alpha$ -gal) and aureobasidin A (AbA) (QDO, SD/–Leu/–Trp/–Ade/–His/X- $\alpha$ -gal/AbA).

### Bimolecular fluorescence complementation assay

The full-length *PpMYB114* and *ERF* gene CDSs were inserted into the p2YN and p2YC vectors using the ClonExpress<sup>TM</sup> II One Step Cloning Kit. *Agrobacterium tumefaciens* GV3101 cells were transformed with the resulting plasmids according to a freeze–thaw method. The cells were incubated overnight and then re-suspended with infiltration buffer (0.2 mM acetosyringone, 10 mM MES, and 10 mM  $\text{MgCl}_2$ ) for an optical density at 600 nm of 1.0. Equal volumes of the different combinations were mixed and used to infiltrate tobacco leaves with a needle-free syringe. The YFP fluorescence was detected using a confocal laser scanning microscope 60 h later.

### Dual luciferase assay

The full-length *trans* gene CDSs were inserted into the pGreenII 0029 62-SK vector, while the promoter sequence of the *cis* gene was cloned into the pGreenII 0800-LUC (firefly luciferase) vector. The resulting plasmids were separately inserted into *A. tumefaciens* GV3101 cells according to a freeze–thaw method. A solution comprising a 10 (SK):1(LUC) ratio was used to infiltrate tobacco leaves with a needle-free syringe. The LUC and Renilla luciferase enzyme activities were analyzed 60 h later using the Dual-Luciferase Reporter Assay System (Promega) with Modulus Luminometers (Promega). Three independent experiments with six biological replicates were conducted for each assay.

### Firefly luciferase complementation imaging assay

A firefly luciferase complementation imaging assay was conducted as previously described (Li et al. 2017). The *PpMYB114* and *PpbHLH3* CDSs were cloned into separate pCAMBIA1300-nLuc and pCAMBIA1300-cLuc vectors. Additionally, *Pp4ERF24* and *Pp12ERF96* were inserted into the pGreenII 0029 62-SK vector. The prepared plasmids were inserted into *A. tumefaciens* GV3101 cells, after which different combinations comprising equal volumes of each vector were used to infiltrate tobacco leaves. After 3 days, luciferase activities were detected using the NightOWL II LB983 low-light cooled CCD imaging apparatus by spraying 0.5 mM luciferin (Promega) on the infiltrated position followed by a 5-min incubation in darkness.

### Transient transformation assay of gene function in tobacco leaves and pear fruits

To verify the gene function of *Pp4ERF24* and *Pp12ERF96* in inducing anthocyanin biosynthesis, the full-length CDSs of *PpMYB114*, *PpbHLH3*, *Pp4ERF24* and *Pp12ERF96* were inserted into the pGreenII 0029 62-SK vector with the

control of the 35S promoter. The prepared plasmids were inserted into *A. tumefaciens* GV3101 cells. Then different combinations comprising equal volumes of each vector were used to infiltrate tobacco leaves as for the dual luciferase assay or fruits of 'Red Zaosu' pear. After infiltration, the pear fruits were put in darkness overnight and moved onto shelves under continuous light ( $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The phenotypes of tobacco leaves were observed 6 days after infiltration, while the phenotypes of pear fruits were observed 3 days after infiltration.

### Statistical analysis

Student's *t*-test and one way ANOVA (Tukey) were completed using the Statistical Product and Service Solutions program (version 19) (SPSS Inc., Chicago, IL, USA).

## Results

### Identification of candidate *ERF* genes according to transcriptome data

Using the previously generated RNA-seq data (Bai et al. 2017), two *PpERF* candidate genes whose expression were correlated with that of *PpMYB10* were identified. These two genes were named as *Pp4ERF24* and *Pp12ERF100* following the nomenclature of an *AP2/ERF* gene family analysis by Li et al. (2018). Further BLAST analysis suggested that another two *ERF* genes, *Pp12ERF96* and *Pp1ERF1*, were paralogs of *Pp4ERF24* and *Pp12ERF100* (Fig. S1A), respectively. The transcriptome data suggested that *Pp4ERF24*, *Pp12ERF100*, and *Pp1ERF1* expression levels increased at 24 h after the removal of bags, while *Pp12ERF96* expression remained low throughout the experiment (Fig. S1B).

### Expression analysis of genes related to anthocyanin biosynthesis in 'Red Zaosu' pear during red coloration under blue light treatment

The 'Red Zaosu' pear fruits started coloration obviously at 72 h after initiating the blue light treatment, and then continuously turned red until 240 h (Fig. 1a). The transcript abundances of *PpUGT* exhibited a tendency that initially increased and peaked at 72 h, and then subsequently decreased. Additionally, the expression levels of the anthocyanin biosynthetic regulatory genes, *PpMYB10* and *PpMYB114*, exhibited the similar expression trends, and peaked at 24 h and then gradually decreased. The expression levels of *PpbHLH3*, *PpbHLH33* and *PpWD40* slightly increased firstly and then decreased (Fig. 1b).

### Expression analysis of candidate *PpERF* genes in 'Red Zaosu' pear during red coloration

Furthermore, we analyzed the transcript levels of four *ERF* genes in 'Red Zaosu' pear samples treated with blue light. The expression levels of *Pp4ERF24* and its paralog, *Pp12ERF96*, initially increased at 12 h after starting the blue light treatment and then decreased, but increased again at 144 h. In contrast, *Pp12ERF100* and *Pp1ERF1* expression levels exhibited the opposite pattern, with a decrease at 12 h followed by an increase, and then decreased again at 240 h (Fig. 2).

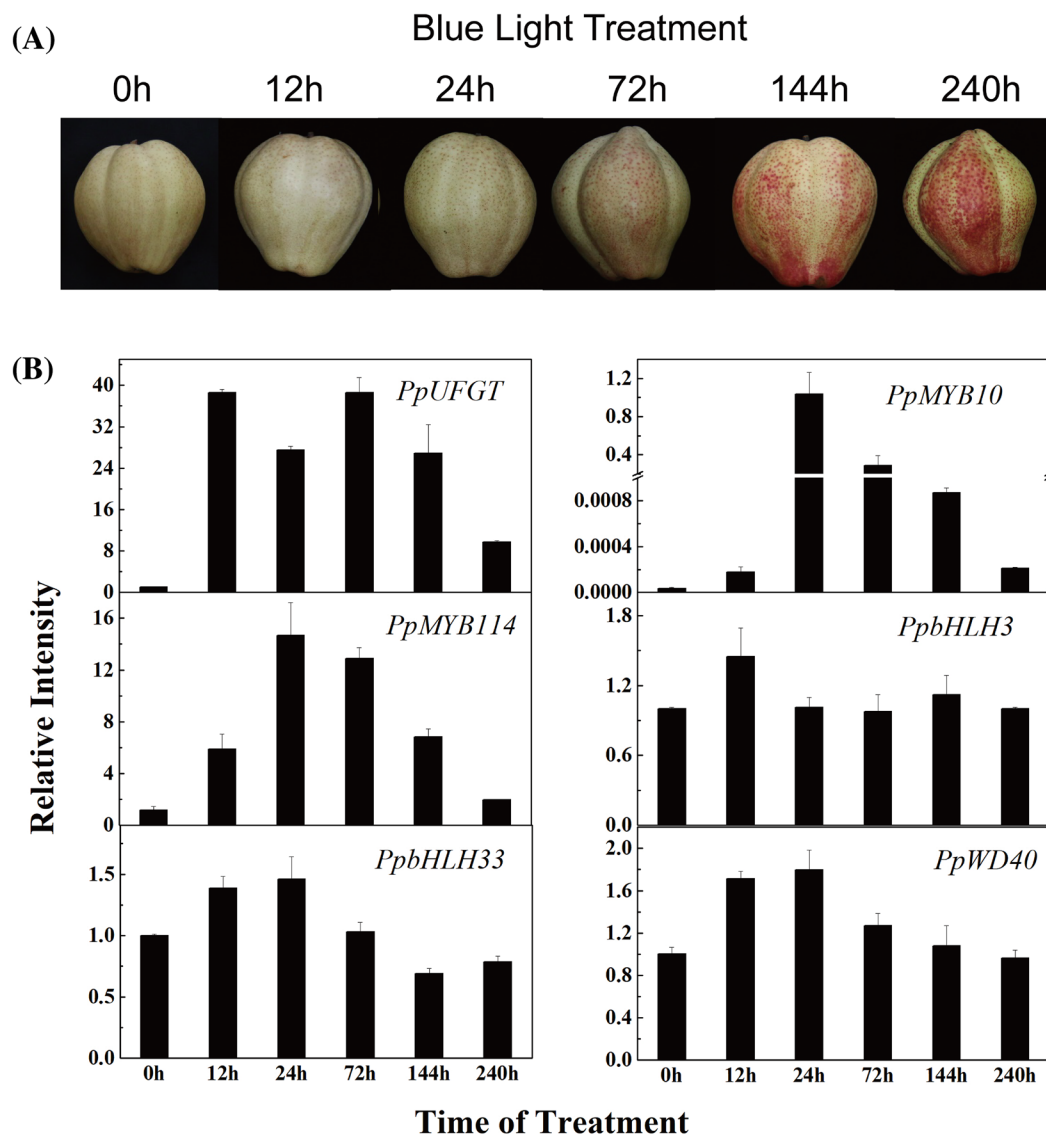
### Interactions between MYB and identified *ERF* transcription factors

According to the expression pattern, we analyzed the interactions between MYB transcription factors and these four *ERF* transcription factors. An Y2H assay indicated that the encoded *ERF* proteins did not interact with PpMYB10 (Fig. S2A), but the physical interaction between Pp4ERF24 and PpMYB114 were detected (Fig. 3a). Unfortunately, because of the auto-activation of PpMYB114, Pp12ERF96, Pp12ERF100, and Pp1ERF1, we were unable to analyze any potential physical interactions among these proteins in an Y2H assay (Fig. S2B). We subsequently investigated the *in vivo* interactions between the *ERF* proteins and PpMYB114 using bimolecular fluorescence complementation (BiFC) assay. As presented in Fig. 3b, green fluorescence was detected in the nuclei when tobacco leaves were co-infiltrated with PpMYB114-YFP<sup>N</sup> and Pp4ERF24-YFP<sup>C</sup> or PpMYB114-YFP<sup>N</sup> and Pp12ERF96-YFP<sup>C</sup>. In contrast, fluorescence was not observed for co-infiltrations with PpMYB114-YFP<sup>N</sup> and Pp12ERF100-YFP<sup>C</sup> or PpMYB114-YFP<sup>N</sup> and Pp1ERF1-YFP<sup>C</sup>. There was also a lack of fluorescence with the control combinations. Therefore, PpMYB114 may interact with Pp4ERF24 and Pp12ERF96.

### Effects of Pp4ERF24 and Pp12ERF96 on the interaction between PpMYB114 and PpbHLH3

To examine how the interactions between PpMYB114 and Pp4ERF24 or between PpMYB114 and Pp12ERF96 influence the interaction between PpMYB114 and PpbHLH3, we conducted a firefly luciferase complementation imaging assay. Plasmids were prepared for the production of PpMYB114 and PpbHLH3 fused to the N and C termini of Luciferase, respectively (i.e., PpMYB114-nLuc and PpbHLH3-cLuc). Additionally, Pp4ERF24 and Pp12ERF96 were inserted into the pGreenII 0029 62-SK vector for a subsequent overexpression. Tobacco leaves were co-infiltrated with different combinations of the prepared plasmids





**Fig. 1** Blue light induces anthocyanin biosynthesis in ‘Red Zaosu’ pear fruits. **a** ‘Red Zaosu’ fruit peel color at different time points during a blue light treatment. **b** Anthocyanin biosynthetic structural and

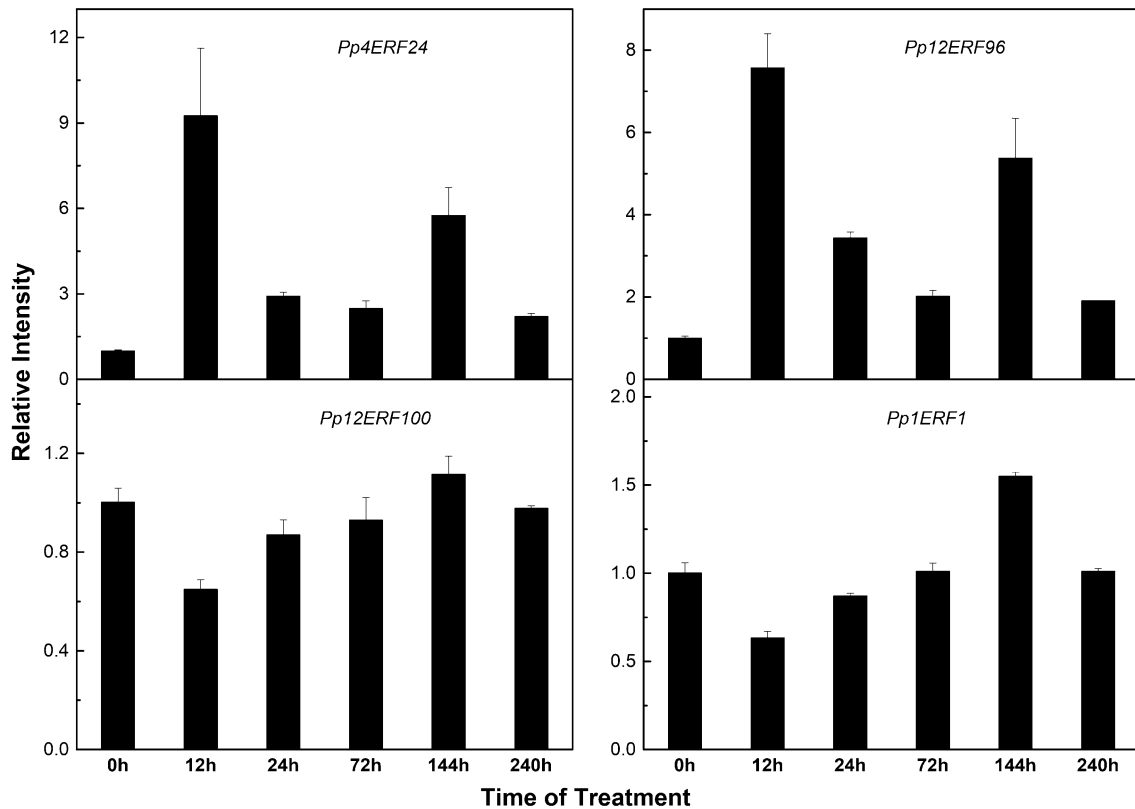
regulatory genes expression levels at different time points during pear coloration. Data are presented as the mean  $\pm$  standard error of three biological replicates

for the production of fusion proteins. A slight luminescence signal was detected in regions containing PpMYB114-nLuc and PpbHLH3-cLuc, while strong luminescence signals were observed when Pp4ERF24-SK or Pp12ERF96-SK were also present (Fig. 4a). These results suggested that Pp4ERF24 and Pp12ERF96 enhanced the interaction between PpMYB114 and PpbHLH3.

#### Effects of Pp4ERF24 and Pp12ERF96 on the transcriptional activation of PpMYB114-mediated *PpUGFT*

Our dual luciferase assay results indicated that PpMYB114 activated the expression of *PpUGFT* (Fig. 4b). Furthermore,

the combined presence of PpMYB114 and bHLH3 increased the activation effect (Fig. 4b). Therefore, the PpMYB114–Pp4ERF24 and PpMYB114–Pp12ERF96 interactions might affect the binding of PpMYB114 to the *PpUGFT* promoter. To verify this possibility, we conducted a dual luciferase assay in which *PpMYB114*, *PpbHLH3*, *Pp4ERF24*, and *Pp12ERF96* were incorporated into the pGreenII 0029 62-SK vector for a subsequent overexpression, while the *PpUGFT* promoter was inserted into the pGreenII 0800-LUC vector. Different plasmid combinations were used to co-transform tobacco leaves (Fig. 4b). Both Pp4ERF24 and Pp12ERF96 were observed to up-regulate *PpUGFT* expression in the presence of PpMYB114 and PpbHLH3. However, *PpUGFT* expression levels in tobacco



**Fig. 2** *Pp4ERF24*, *Pp12ERF96*, *Pp12ERF100* and *Pp1ERF1* expression levels at different time points during pear coloration. Data are presented as the mean  $\pm$  standard error of three biological replicates

leaves with *Pp4ERF24* and *PpMYB114* or *Pp12ERF96* and *PpMYB114* were similar to those in leaves with *PpMYB114* alone (Fig. 4b). These results implied that *Pp4ERF24* and *Pp12ERF96* may indirectly enhance *PpUGFT* expression by enhancing the interaction between *PpMYB114* and *PpbHLH3*.

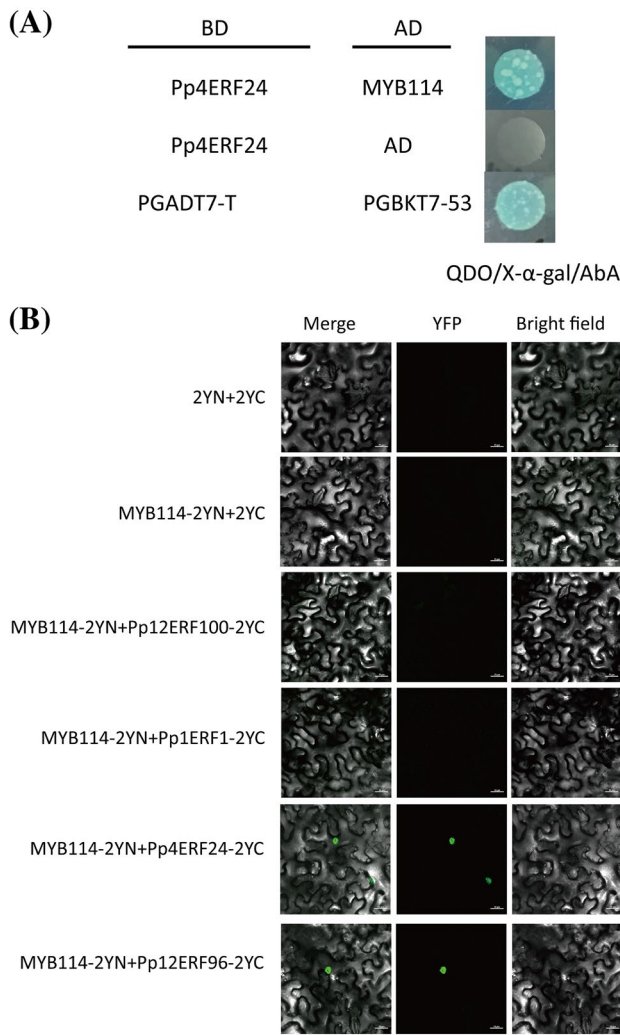
#### Heterologous overexpression of *Pp4ERF24* and *Pp12ERF96* with *PpMYB114* and *PpbHLH3* in tobacco leaves

Transient transformation assay in tobacco leaves was performed to verify the function of the *PpERF* transcription factors. No red pigmentation was observed when *PpMYB114* was overexpressed alone, while the tobacco leaves turned red when *PpMYB114* and *PpbHLH3* were co-overexpressed (Fig. 5a). However, when *PpMYB114* and *Pp12ERF96* were co-overexpressed, the tobacco leaves also remained green (Fig. 5a). When *Pp12ERF96* was co-transformed with *PpMYB114* and *PpbHLH3*, the tobacco leaves turned red (Fig. 5a). Pigment accumulation was quantitatively analyzed, the hue angle decreased (more redness) while total anthocyanin content increased when three TFs were co-expressed compared with both *PpMYB114* and *PpbHLH3*

were over-expressed (Fig. 5b, c). These results indicated that *Pp12ERF96* promoted anthocyanin accumulation through *PpMYB114/PpbHLH3*, probably due to enhancing the interaction between *PpMYB114* and *PpbHLH3*. Unfortunately, no more pigment accumulation was observed when *Pp4ERF24* was co-transformed with *PpMYB114* and *PpbHLH3*, probably because of the instability of heterogeneous overexpression (data not shown).

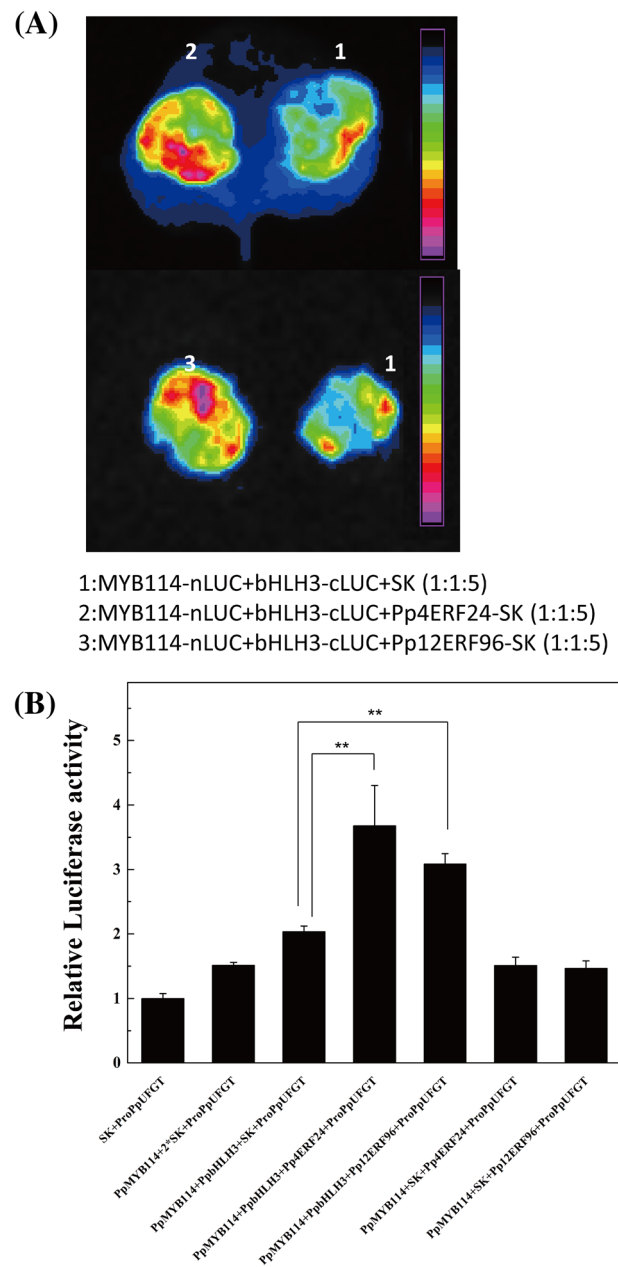
#### Homologous overexpression of *Pp4ERF24* and *Pp12ERF96* in ‘Red Zaosu’ pear fruits

To further confirm that *Pp4ERF24* and *Pp12ERF96* could promote anthocyanin accumulation in pear, the transient transformation of *Pp4ERF24* and *Pp12ERF96* was performed in ‘Red Zaosu’ pear upon maturity. The vectors of *Pp4ERF24*-SK, *Pp12ERF96*-SK were obtained, and the empty vector of pGreenII 62-SK was used as control. As shown in Fig. 6a and b, overexpression of *Pp4ERF24* and *Pp12ERF96* promoted anthocyanin accumulation in pear skin around the injection sites compared with control. RT-qPCR analysis showed that the expression levels of *PpPAL*, *PpCHS*, *PpCHI*, *PpDFR*, *PpANS*, *PpUGFT*, *PpMYB10*, *PpbHLH33* and *PpWD40* were up-regulated

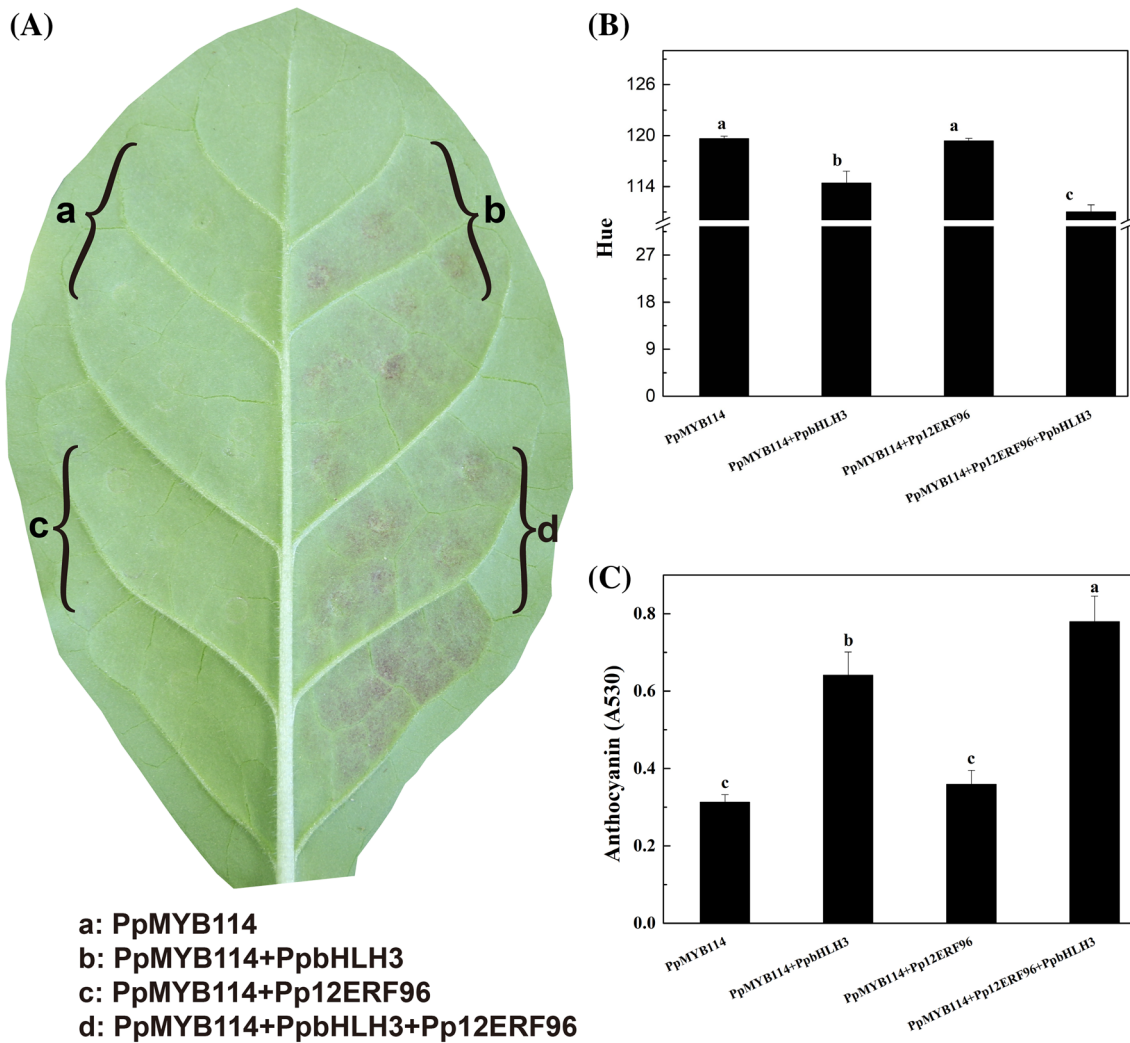


**Fig. 3** Pp4ERF24 and Pp12ERF96 interact with PpMYB114. **a** A yeast two-hybrid (Y2H) assay revealed that Pp4ERF24 interacted with PpMYB114. *Pp4ERF24* gene fragments were inserted into the pGBKT7 vector (BD), while *PpMYB114* was incorporated into the pGADT7 vector (AD). QDO, SD medium lacking Trp, Leu, His, and Ade; QDO/X-α-gal/AbA, QDO medium containing X-α-Gal and AbA. The pGADT7-T and pGBKT7-53 vectors were used as the positive control, while the combination of AD and Pp4ERF24-BD served as the negative control. Blue plaques indicated an interaction between two proteins. The basal AbA concentration was 300 ng ml<sup>-1</sup>. **b** A bimolecular fluorescence complementation (BiFC) assay in *N. benthamiana* leaves revealed that PpMYB114 interacted with Pp4ERF24 and Pp12ERF96, but not with Pp12ERF100 and Pp1ERF1. 2YN and 2YC, PpMYB114-2YN and 2YC (negative control)

in the Pp4ERF24 or Pp12ERF96 injection areas compared with control (Fig. 6c). Furthermore, overexpression of Pp12ERF96 also promoted the expression levels of *PpF3H*, *PpMYB114* and *PpbHLH3* (Fig. 6c). Therefore, these results illustrated that Pp4ERF24 and Pp12ERF96 played positive roles in regulating anthocyanin biosynthesis in ‘Red Zaosu’ pear fruit.



**Fig. 4** Pp4ERF24 and Pp12ERF96 enhance the interaction between PpMYB114 and PpbHLH3. **a** A luciferase complementation imaging assay revealed that Pp4ERF24 and Pp12ERF96 enhanced the interaction between PpMYB114 and PpbHLH3 in tobacco leaves. *Agrobacterium tumefaciens* GV3101 cells harboring different combinations were used to infiltrate different wild tobacco leaf regions. Luciferase activities were recorded in these regions 60 h after infiltration. **b** A dual luciferase assay verified that the co-transformation with different combinations of PpMYB114, PpbHLH3, Pp4ERF24, and Pp12ERF96 affected *PpUFGT* promoter activity. The *PpUFGT* promoter activity is expressed as a ratio of LUC and REN activities. Asterisks indicate significant difference at  $P < 0.01$  by *t* tests. Data are presented as the mean ± standard error of three biological replicates



**Fig. 5** Transient expression assays confirm the function of Pp12ERF96 in regulating anthocyanin in tobacco leaves. **a** The phenotypes of tobacco leaves 6 days after infiltration. **a**, PpMYB114; **b**, PpMYB114+PpbHLH3; **c**, PpMYB114+Pp12ERF96; **d**, PpMYB114+PpbHLH3+Pp12ERF96. **b** The hue angle of tobacco

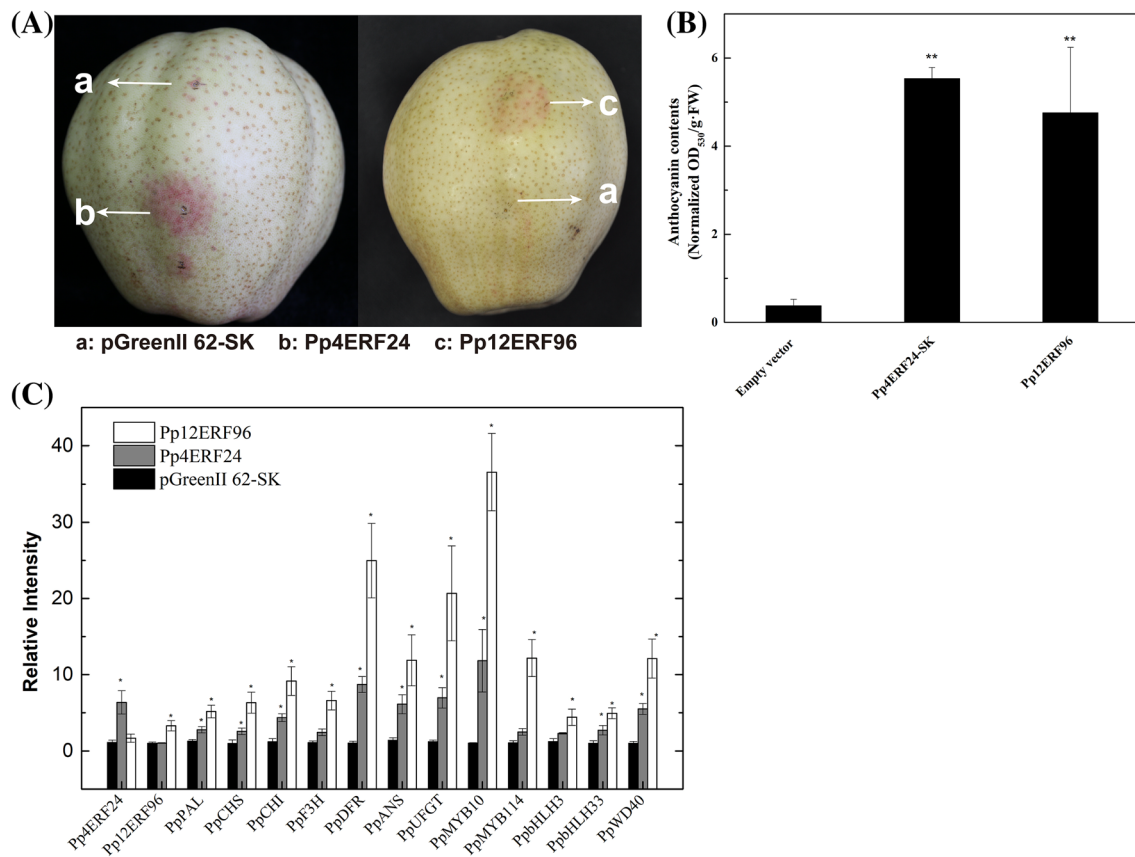
leaves 6 days after infiltration. **c** Measurement of total anthocyanin contents. Lowercase letters indicate significant difference at  $P < 0.05$  by Tukey test. Data are presented as the mean  $\pm$  standard error of three biological replicates

## Discussion

The anthocyanin biosynthesis pathway is controlled by various regulatory factors in addition to the MBW complex. For example, several transcriptional regulators, such as MdJAZ (An et al. 2015), MdHXK1 (Hu et al. 2016), MdCOL11 (Bai et al. 2014), MdEIL1 (An et al. 2017a), MdHY5 (An et al. 2017b), and MdSIZ1 (Zhou et al. 2017), function as either activators or repressors in fine tuning anthocyanin biosynthesis in apple, indicating the essential roles of transcription factors in the coloration of fruits. Several ERF transcription factors have also been shown to regulate anthocyanin biosynthesis in different plants. In apple, MdERF3 is involved in ethylene-induced anthocyanin accumulation via the following pathway: MdEIL1 directly binds to the promoter

of *MdMYB1* and transcriptionally activated its expression, while MdMYB1 interacts with the promoter of *MdERF3*, thereby promoting ethylene biosynthesis, which finally forming a positive feedback (An et al. 2018). In *Arabidopsis*, the class II repressors, AtERF4 and AtERF8, positively regulate anthocyanin biosynthesis in light responses, probably *via* repressing the expression of a negative regulator of the anthocyanin biosynthesis (Koyama and Sato 2018). In pear, Yao et al. (2017) found that PpMYB114 interacts with PpERF3 to form a new complex (ERF3-MYB114-bHLH3) and promotes anthocyanin biosynthesis by binding to the promoters of the anthocyanin biosynthetic structural genes (Yao et al. 2017). In our study, Pp4ERF24 and Pp12ERF96 also participated in fine tuning anthocyanin biosynthesis (Figs. 3, 4, 5, 6). Based on the phylogenetic analysis, we





**Fig. 6** Transient expression assays confirm the function of Pp4ERF24 and Pp12ERF96 in regulating anthocyanin in 'Red Zaosu' pear skins. **a** The phenotypes of 'Red Zaosu' pear 3 days after infiltration. **a**, pGreenII 62-SK; **b**, Pp4ERF24; **c**, Pp12ERF96. **b** Measurement of total anthocyanin contents. Two asterisks indicated significant difference at  $P < 0.01$  by t tests. Data are presented as the mean  $\pm$  standard

error of three biological replicates. **c** Analysis of expression levels of anthocyanin biosynthesis genes in 'Red Zaosu' pear skins using RT-qPCR. Asterisk indicates significant difference at  $P < 0.05$  by t tests. Data are presented as the mean  $\pm$  standard error of three biological replicates

confirmed that the sequences of these two ERF proteins differed from PpERF3 (Fig. S1A). Furthermore, in the pear genome database, *Pp4ERF24* and *Pp12ERF96* locate on chromosomes 4 and 12, separately, while *PpERF3* locates on chromosome 5. Pp4ERF24 and Pp12ERF96 were not indispensable for pear red coloration, but they acted as accessory factors by interacting with PpMYB114 to enhance the interaction between PpMYB114 and PpbHLH3 (Figs. 3, 4a), which led to the enhanced expression of MYB114-induced anthocyanin biosynthetic structural genes (Fig. 4b), and finally promoted anthocyanin accumulation. Heterologous transient assay in tobacco leaves also confirmed that Pp12ERF96 significantly induced anthocyanin accumulation when co-expressed with PpMYB114 and PpbHLH3 (Fig. 5). However, overexpression of Pp4ERF24 with PpMYB114 and PpbHLH3 failed to induce redder coloration in tobacco leaves, which might be attribute to the specificity of this ERF in pear, and the instability of heterogeneous overexpression. Furthermore, when Pp4ERF24 or Pp12ERF96 was

overexpressed in 'Red Zaosu' pear fruits, anthocyanin accumulation was promoted, respectively (Fig. 6). These results implied that Pp4ERF24 and Pp12ERF96 are positive regulators of anthocyanin biosynthesis and confirmed the existence of a previously uncharacterized regulatory module.

It has been reported that several factors posttranscriptionally regulate MBW complex by interacting with MYB protein and then indirectly regulate anthocyanin biosynthesis. In *A. thaliana*, light-induced mitogen-activated protein kinase 4 positively regulates anthocyanin biosynthesis by interacting with AtMYB75 and increasing its stability (Li et al. 2016a). In *Arabidopsis*, the bHLH transcription factor TCP3 interacts with several flavonoid-regulating R2R3-MYBs (AtMYB75/AtMYB90/AtMYB113/AtMYB114/AtMYB12/AtMYB111) to promote flavonoid biosynthesis (Li and Zachgo 2013). In this study, two *ERF* genes (*Pp4ERF24* and *Pp12ERF100*) that were highly co-expressed with *PpMYB10* (correlation coefficient of 0.95) were identified according to the light-induced anthocyanin biosynthesis transcriptome

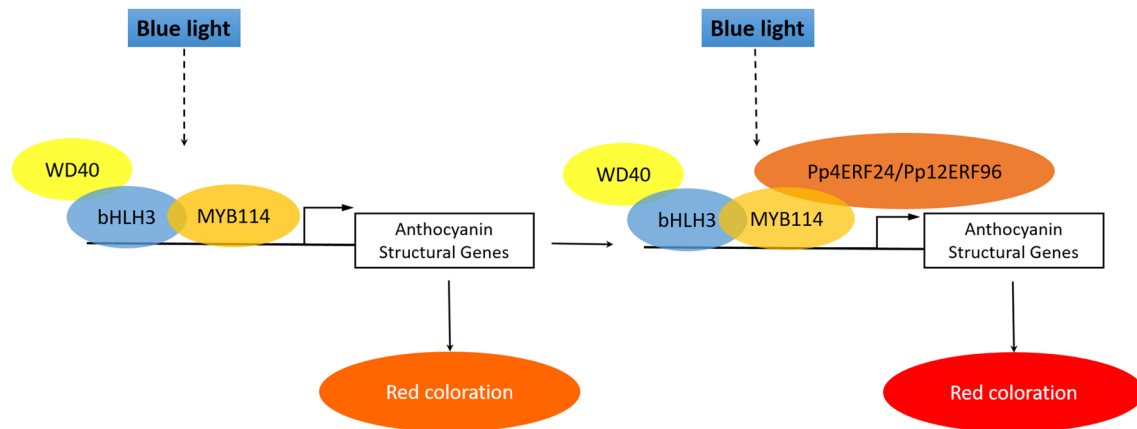
data (Bai et al. 2017). Additionally, we identified another two *ERF* genes, *Pp12ERF96* and *Pp1ERF1*, which are paralogs of *Pp4ERF24* and *Pp12ERF100* (Fig. S1A), respectively. Due to the high correlation coefficient between these *ERFs* and *PpMYB10*, we hypothesized that these ERF proteins might interact with PpMYB10 and co-regulate anthocyanin biosynthesis. However, an Y2H assay revealed that none of these proteins interacted with PpMYB10 (Fig. S2A). Additionally, *PpMYB114*, which was co-expressed with *PpMYB10*, was not identified as a differentially expressed gene because of inaccuracies in its predicted transcript sequence (Pbr042132.1), which lacked an important 222-bp R3 domain, resulting in neglect of this gene. Thus, we analyzed the interaction between PpMYB114 and these four ERF proteins, and found that Pp4ERF24 and Pp12ERF96 interacted with PpMYB114 (Fig. 3a, b). In pear, *PpMYB10* is an ortholog of *MdMYB1* gene (Feng et al. 2010), while *PpMYB114* is probably unique, which may lead to the different interaction between these two MYBs and ERFs (Figs. S2, 3).

The anthocyanin biosynthesis pathway is controlled by various factors including environmental factors and developmental factors (Jaakola 2013). Light is one of the most important environmental factors which regulates anthocyanin biosynthesis. The light-responsive factors, which contain the photoreceptors and their downstream regulators [e.g., CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and ELONGATED HYPOCOTYL 5 (HY5)], negatively or positively influence light-induced anthocyanin biosynthesis (Li et al. 2012; Maier et al. 2013; Shin et al. 2013; Zhang et al. 2011). In apple, MdCOP1 inhibits anthocyanin biosynthesis by interacting with MdMYB1, resulting in ubiquitination and degradation (Li et al. 2012). MdHY5 induces anthocyanin biosynthesis by binding to the promoter of *MdMYB1* (An et al. 2017b). According to these findings, light regulates anthocyanin biosynthesis via transcriptionally or post-transcriptionally regulating MdMYB1. In this study, the expression patterns of four *ERF* genes in transcriptome data of pear fruit and ‘Red Zaosu’ pear suggested their possible involvement in the light-response (Figs. S1B, 2). We analyzed the promoters of *Pp4ERF24* and *Pp12ERF96*, which consisted of many light-responsive elements, including G-box, Sp1, and L-box (Fig. S3). The G-box is a well-known light-responsive element that is regulated by HY5, which positively mediates photomorphogenesis (Chattopadhyay et al. 1998). Thus, we speculated that *Pp4ERF24* and *Pp12ERF96* expression levels might be regulated by blue light via HY5 or other transcription factors. Our findings may be associated with light-induced anthocyanin biosynthesis, which remains to be explored.

Previous studies confirmed that PpMYB10 and PpMYB114 induce the expression of *PpUFGT* by binding to its promoter, with *PpUFGT* expression levels

correlated with those of *PpMYB10* and *PpMYB114* (Qian et al. 2014; Yao et al. 2017). In grape, *VvUFGT* expression is regulated by VvMYBA1, and its expression level is correlated with that of *VvMYBA1* (Zheng et al. 2013). In apple, *MdUFGT* and *MdMYB1* exhibit similar expression patterns (Takos et al. 2006). However, in our study, the *PpUFGT* expression level rapidly increased at 12 h after starting the blue light treatment, while *PpMYB10* and *PpMYB114* expression levels exhibited a slow increase over the first 24 h. Obviously, *PpUFGT* expression was not closely correlated with *PpMYB10* or *PpMYB114* expression (Fig. 1b), implying other factors may directly or indirectly regulate *PpUFGT* transcription. We also observed that the *Pp4ERF24* and *Pp12ERF96* expression levels quickly increased at 12 h after initiating the light treatment (Fig. 2). In addition, luciferase assay confirmed the indirectly positive regulation of *PpUFGT* by Pp4ERF24 and Pp12ERF96 (Fig. 4b). Therefore, we speculated that the synergistic regulation by these two ERF transcription factors and two MYB proteins led to the inconsistent expression patterns of *PpUFGT* and *PpMYB* genes.

In addition, the *Pp4ERF24* and *Pp12ERF96* expression patterns in ‘Red Zaosu’ pear fruits were different from those in ‘Meirensu’ fruits according to an analysis of transcriptome data (Fig. 2, S1B). These differences were probably due to the genetic background of red coloration in different pear cultivars. ‘Meirensu’ pear is a hybrid of ‘Kosui’ pear and ‘Huoba’ pear, of which the red color is inherited from ‘Huoba’ pear. While ‘Red Zaosu’ pear is a sport from ‘Zaosu’ pear, of which the bud mutation mechanism remains unknown. As to the coloration profiles, ‘Meirensu’ pear only accumulates anthocyanin in mature fruits, but ‘Red Zaosu’ pear accumulates anthocyanin in the young leaves, flowers, fruitlets and mature fruits (Qian et al. 2014). Therefore, the regulatory patterns of red coloration in ‘Meirensu’ and ‘Red Zaosu’ pears differ from each other. Furthermore, it has been well characterized that the rearrangement of multiple repeats in the promoter of *MdMYB10* produces a red foliage and fruit core phenotypes in apple (Espley et al. 2009). However, no difference of the promoter of *PpMYB10* was found between ‘Red Zaosu’ pear and ‘Zaosu’ pear (Qian et al. 2014) and there was no coloration in the fruit core of ‘Red Zaosu’, indicating that the red coloration of ‘Red Zaosu’ was also different from MYB10-R6 apple and may specifically be regulated by some other patterns. On the other hand, the different expression patterns of *Pp4ERF24* and *Pp12ERF96* in ‘Red Zaosu’ and ‘Meirensu’ were potentially due to the different treatments in these two experiments, as we exposed ‘Red Zaosu’ pear fruits to continuous blue light, while the ‘Meirensu’ pear fruits examined in the previous study were treated with natural sunlight (i.e., circadian rhythm) (Bai et al. 2017).



**Fig. 7** A model showing Ethylene Response Factors Pp4ERF24 and Pp12ERF96 regulate blue light-induced anthocyanin biosynthesis in ‘Red Zaosu’ pear fruits by interacting with PpMYB114. Pp4ERF24 and Pp12ERF96 interact with PpMYB114 and promote the interac-

tion between PpMYB114 and PpbHLH3, thus, enhancing the transactivation of PpMYB114 to *PpUGFT* and inducing more anthocyanin biosynthesis. Overlap of PpMYB114 and PpbHLH3 means enhanced interacting effect

Overall, the data presented herein indicate that Pp4ERF24 and Pp12ERF96 fine tune blue light-induced anthocyanin biosynthesis partly through the following pathway: Pp4ERF24 and Pp12ERF96 interact with PpMYB114 and promote the interaction between PpMYB114 and PpbHLH3, which enhances the transcriptional activation of *PpUGFT* expression by PpMYB114, ultimately inducing anthocyanin biosynthesis (Fig. 7). These findings suggest that there is a previously uncharacterized regulatory module of light-induced anthocyanin biosynthesis which is mediated by ERF transcription factors, and provide new evidences that the red coloration of pear fruits is regulated by a complex network.

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**Author contributions** JN, SB, and YT conceived and planned the study. RT and LY helped collect samples and extracted the total RNA. JN and YZ completed the qPCR, vector construction, and bioinformatics analysis. JN and LG conducted the Y2H, BiFC, dual luciferase, firefly luciferase complementation imaging assays and the transient transformation assay of gene function in tobacco leaves and ‘Red Zaosu’ fruits. JN, SB, MQ and YT wrote the manuscript. All of the authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interest.

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