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CsMYB3 and *CsRuby1* form an 'Activator-and-Repressor' Loop for the Regulation of Anthocyanin Biosynthesis in Citrus

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Regular Paper

Anthocyanins are preferentially accumulated in certain tissues of particular species of citrus. A R2R3-MYB transcription factor (named Ruby1) has been well documented as an activator of citrus anthocyanin biosynthesis. In this study, we characterized *CsMYB3*, a transcriptional repressor that regulates anthocyanin biosynthesis in citrus. *CsMYB3* was expressed in anthocyanin-pigmented tissues, and the expression was closely associated with that of *Ruby1*, which is a key anthocyanin activator. Overexpression of *CsMYB3* in Arabidopsis resulted in a decrease in anthocyanins under nitrogen stress. Overexpression of *CsMYB3* in the background of *CsRuby1*-overexpressing strawberry and *Arabidopsis* reduced the anthocyanin accumulation level. Transient promoter activation assays revealed that *CsMYB3* could repress the activation capacity of the complex formed by CsRuby1/CsbHLH1 for the anthocyanin biosynthetic genes. Moreover, *CsMYB3* could be transcriptionally activated by *CsRuby1* via promoter binding, thus forming an 'activator-and-repressor' loop to regulate anthocyanin biosynthesis in citrus. This study shows that *CsMYB3* plays a repressor role in the regulation of anthocyanin biosynthesis and proposes an 'activator-and-repressor' loop model constituted by *CsRuby1* and *CsMYB3* in the regulation of anthocyanin biosynthesis in citrus.

Keywords: Anthocyanin biosynthesis • Activator • Citrus • Repressor.

Accession numbers: Sequence data of the plant R2R3-MYB repressors used for the phylogenetic tree can be found in the NCBI database under the following accession numbers: AtMYB4 (**NP_195574**), AtMYB3 (**Q9S9K9**), AtMYB32 (**O49608**), PhMYB4 (**ADX33331**), VvMYB4a (**ABL61515**), AmMYB308 (**P81393**), FaMYB1 (**AAK84064**), PtrMYB182 (**AJI76863**), MtMYB2 (**Medtr5g079670**), PpMYB18 (**KT159234**), VvMYBC2-L1 (**ABW34393**), AtMYBL2 (**AEE35154**), PhMYBx (**AHX24371**), AtTRY (**Q8GV05**), VvTRY (**ABW34395**), AtETC2 (**AEC08385**), CPC (**AA21917**), AtETC3 (**AEE81974**) and AtETC1 (**NP_171645**).

Introduction

Anthocyanins, which are natural water-soluble pigments that belong to the flavonoid branch of the phenylpropane pathway, exist in various tissues of plants and are responsible for various colors such as blue, red and purple (Winkel-Shirley 2001). Anthocyanins play important roles, such as protection against ultraviolet and high-light damage and attraction of pollinators and seed distributors in plants (Gould 2004, Mouradov and Spangenberg 2014). In addition anthocyanins are considered as nutrients beneficial for human health owing to their high antioxidant activity, which can protect the human body from damage caused by free radicals (He and Giusti 2010, Li et al. 2017).

The transcriptional regulation of anthocyanin biosynthesis has been widely investigated in various plants. It is well known that the MYB transcription factors (TFs), basic helix-loop-helix (bHLH) and WD40 proteins form MBW complexes to dominate the transcriptional regulation of anthocyanins (Koes et al. 2005, Jaakola 2013, Xu et al. 2015). In addition to the MBW complexes, some important co-TFs such as DELLA and JAZ have been reported to regulate anthocyanin biosynthesis via the MBW complexes under various stress conditions (Jiang et al. 2007, An et al. 2015, Xie et al. 2016, Zhang et al. 2017). MYB repressors of anthocyanin synthesis are also important contributors to anthocyanin regulation (Ma and Constabel 2019). The repressor MYBs in the anthocyanin pathway can be divided into the following two distinct groups: R2R3-MYBs and R3-MYBs. R2R3-MYBs include strawberry FaMYB1, petunia PhMYB27, *Medicago truncatula* MtMYB2, polar PtrMYB182 and PtrMYB57, grapevine VvMYBC2-L1 and peach PpMYB18, which belong to MYB subgroup 4 (Aharoni et al. 2001, Albert et al. 2014, Cavallini et al. 2015, Jun et al. 2015, Yoshida et al. 2015, Wan et al. 2017, Zhou et al. 2019). R2R3 repressor MYBs in subgroup 4 share the C1 motif (LlsrGIDPxT/SHRxI/L) and the C2 motif (pdLNLD/ELxiG/S) with a core consensus sequence of either LxLxL or DLNxxP in their C-terminus, which are required for the repressor activity (Jin et al. 2000, Legay et al. 2007, Dubos et al. 2010, Zhou et al. 2019), and an additional TLLLFR repression motif was also found (Yoshida et al. 2015). R3-MYBs such as CAPRICE (CPC) and TRIPTYCHON (TRY) in *Arabidopsis*, MYBx in petunia and SlMYBATV in tomato, which contain only an intact R3 domain and no repressor motif, are known as competitors of activator MYBs via binding to the same bHLH partner in the MBW complexes (Zhu et al. 2009, Albert et al. 2011, Cao et al. 2017). AtMYBL2 is an R3 MYB containing a unique C-terminal TLLLFR motif and acting as an active repressor of anthocyanin biosynthesis (Dubos

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et al. 2008, Matsui et al. 2008). All the repression motifs mentioned earlier are collectively referred to ERF-associated amphiphilic repression (EAR) motif, and the EAR-motif-containing proteins are known to function as negative regulators in various physiological processes of plants (Kazan 2006, Kagale and Rozwadowski 2011).

Flavonoid-related bHLH TFs can be divided into two subclades, GL3 subclade and TT8 subclade (Montefiori et al. 2015). In *Arabidopsis*, AtMYBL2 might suppress the transcription of *AtDFR* through interacting with AtTT8. In *M. truncatula*, MtTT8 interacts with the proanthocyanidin (PA) and anthocyanin repressor MtMYB2 (Jun et al. 2015). In poplar, PtrMYB57 is an effective repressor of anthocyanin biosynthesis by directly interacting with bHLH131, a homolog of *AtTT8* (Wan et al. 2017).

Opposite or similar expression patterns between activators and repressors suggest that they may provide feedback regulation upon anthocyanin biosynthesis. In *Arabidopsis* and petunia, the expression of passive repressors is upregulated (*AtCPC* and *PhMYBx*) when tissues begin to accumulate anthocyanin pigments (Albert et al. 2014, Nemie-Feyissa et al. 2014), but the active repressors (*AtMYBL2* and *PhMYB27*) are highly expressed during noninductive shade conditions (Dubos et al. 2008, Albert et al. 2011). The feedback regulatory loop formed by activators and repressors is an important feature of flavonoid regulation. As for the feedback regulation of *AtMYBL2* and *AtTT8*, *AtMYBL2* represses the expression of *AtTT8* while *AtTT8* induces the expression of *AtMYBL2*, thus forming a regulatory loop to participate in the feedback regulation of anthocyanin accumulation in *Arabidopsis*. In peach, during the synthesis of anthocyanins and PAs, the relevant MYB activators also activate the expression of *PpMYB18* gene to form a negative feedback regulatory loop, thus preventing the oversynthesis of both anthocyanins and PAs (Zhou et al. 2019). Upregulation of both activators and repressors corroborates that this network includes both positive and negative feedback regulatory loops to maintain metabolic homeostasis in plants.

In the sweet orange (*Citrus sinensis*) genome, 100 R2R3-MYB genes were identified (Liu et al. 2014). However, very few MYBs related to the regulation of anthocyanins have been functionally characterized in citrus. Both the *Ruby1* and *Ruby2* MYB TFs are activators responsible for anthocyanin accumulation in modern and primitive citrus (ButelLi et al. 2017, Huang et al. 2018). In this study, we characterized *CsMYB3* as an R2R3 MYB repressor and demonstrated that it forms an 'activator-and-repressor' regulatory loop in combination with *Ruby1* on anthocyanin biosynthesis in citrus.

Results

Identification and subcellular localization of CsMYB3

Based on previous genome-wide identification of R2R3 MYB genes in sweet orange (*C. sinensis*) (Liu et al. 2014) and further elucidation of the genomic sequences of other citrus species (**http://citrus.hzau.edu.cn/orange/**), we identified four C2 repressor R2R3-MYB TFs. Among them, Cs1g19400, Cs3g22220 and Cs8g02740 had been predicted in previous genome-wide analyses and orange1.1t04785 was newly identified. Multiple sequence alignment revealed that the N-terminal region was highly conserved with a C1 motif (LlsrGIDPxT/SHRxI/L), which is involved in the repression of the genes of anthocyanin biosynthesis, and the C2 motif (pdLNLD/ELxiG/S) was found within the C-terminal domain (Jin et al. 2000, Dubos et al. 2010) (**Supplementary Fig. S1**). The C1 motif and C2 motif are the important characteristics of subgroup 4 MYB proteins (Kranz et al. 1998). In addition to the C2 motif, a number of FaMYB1-like proteins such as PtrMYB182 and VvMYBC2 contain a TLLLFR repression motif (named as C5) originally identified in AtMYBL2 (Matsui et al. 2008, Cavallini et al. 2015, Yoshida et al. 2015), which was not found in the candidate genes of citrus genomes (**Supplementary Fig. S1**).

To better define the relationship between the putative R2R3-MYB repressors from citrus, a phylogenetic analysis of the candidate repressor MYBs and the known flavonoid-related MYB repressors was performed (**Fig. 1A**). Cs1g19400, Cs3g22220 and Cs8g02740 fell into lignin or phenylpropanoid clade consisting of repressor MYBs such as *AtMYB4* (Jin et al. 2000), whereas orange1.1t04785 was clustered into anthocyanin and/or PA biosynthesis clade, which included MYB repressors identified in strawberry, grapevine, *M. truncatula*, poplar and peach (Aharoni et al. 2001, Cavallini et al. 2015, Jun et al. 2015, Yoshida et al. 2015, Zhou et al. 2019). Both the phylogenetic and sequence analyses indicated that orange1.1t04785, which would be referred to as *CsMYB3* hereafter, is a novel R2R3 MYB repressor of anthocyanin and/or PA biosynthesis.

The subcellular distribution of *CsMYB3* was determined by transient expression in a citrus protoplast system. The green fluorescence generated by CsMYB3-GFP was distributed in the same area as the cyan fluorescence generated by OsGhd7-CFP, which is known to be localized in the nucleus (Xue et al. 2008) and was used as a positive control in this study. These results indicated that CsMYB3 is a nuclear-localized TF (**Fig. 1B**).

Expression of *CsMYB3* was associated with *CsRuby1* and anthocyanin accumulation in citrus

The *Ruby1* gene is a critical activator of anthocyanin biosynthesis in citrus (ButelLi et al. 2017). We previously reported a transcriptome analysis of the fruit peel from purple pummelo (*Citrus grandis*) (with anthocyanins) and normal pummelo (without anthocyanins) (Huang et al. 2018). The comparative transcriptome analysis revealed that *CsRuby1* and *CsMYB3* were mainly expressed in the mature fruit peel of purple pummelo, where large amounts of anthocyanins were accumulated. However, these two genes showed low expression levels in the mature fruit peel of normal pummelo, in which no anthocyanins were biosynthesized. Furthermore, we comparatively analyzed the expression patterns of *Ruby1* and *MYB3* in various Citrinae species with anthocyanin accumulation. Chinese box orange (*Atalantia buxifolia*) is recognized as a primitive citrus species, and anthocyanin accumulation was observed in its mature fruit peel (**Fig. 2A**). Trifoliate orange (*Poncirus trifoliata*), a wild species mostly used as citrus rootstock, accumulated anthocyanins in its young leaves (**Fig. 2B**). Lemon (*Citrus limon*) accumulated anthocyanins in both young leaves and flowers (**Fig. 2C**). A special cultivated *Citrus* species, blood

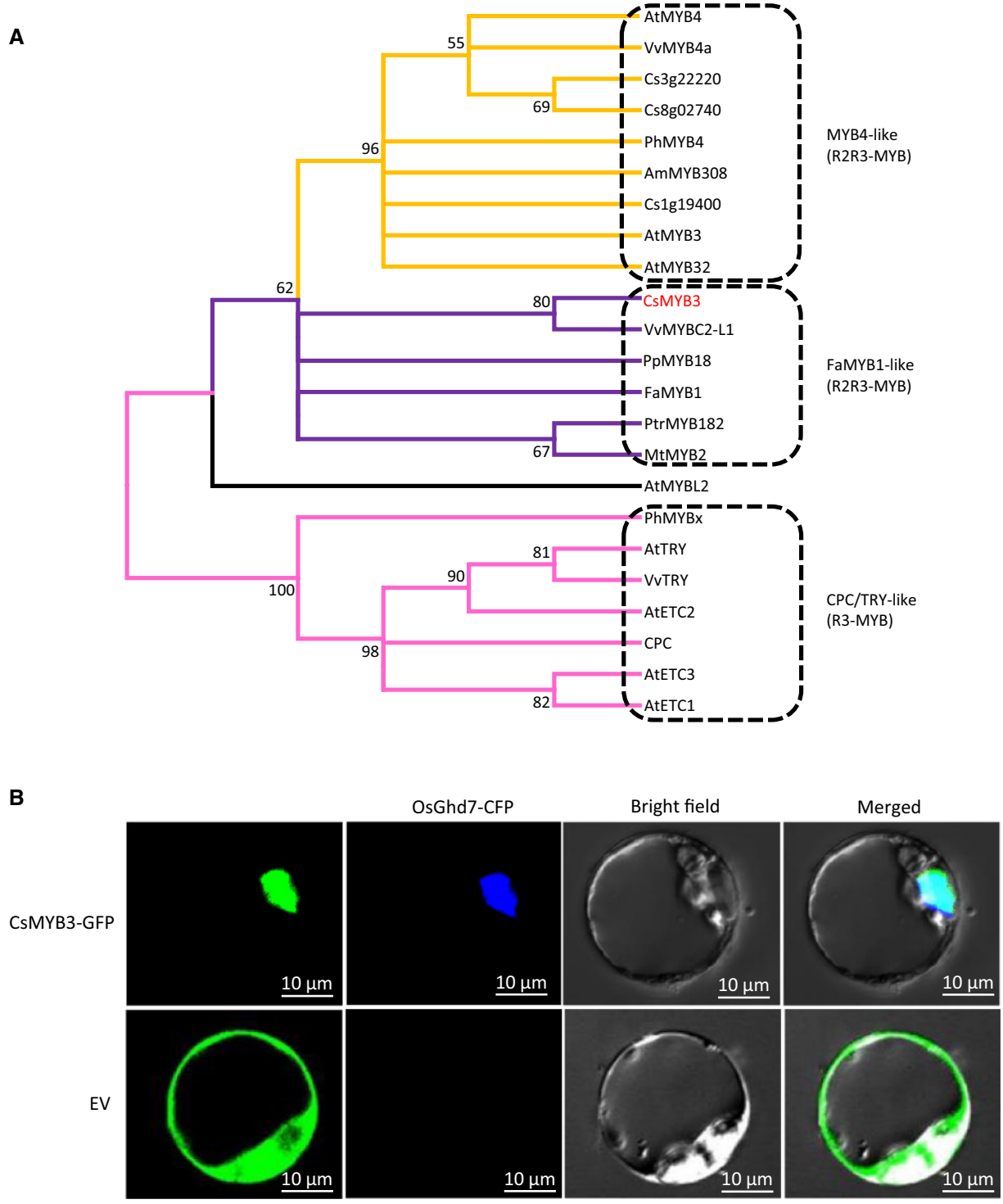

Fig. 1 Phylogenetic analysis and subcellular localization of CsMYB3. (A) Phylogenetic tree of repressor MYB transcription factors based on the N-terminal protein sequences including the R2R3 domain or only the R3 domain for small MYB proteins. The phylogenetic tree was constructed using MEGA 5.05 with 1,000 bootstrap replicates. The numbers indicate the percentage of consensus support. (B) Subcellular localization of CsMYB3 in citrus protoplasts. CsMYB3-GFP and OsGhd7-CFP were cotransformed into citrus callus protoplasts. OsGhd7-CFP was used as a nuclear marker. EV, empty vector, was used as a negative control. Scale bar = 10 μm.

orange (*C. sinensis*), also showed anthocyanin accumulation in its mature fruit flesh (**Fig. 2D**). Quantitative real-time (qRT)-PCR analyses revealed that both *CsRuby1* and *CsMYB3* were highly expressed in anthocyanin-accumulating tissues of all the Citrinae species mentioned earlier, including leaves, flowers and fruits at different developmental stages. Based on these data, we hypothesized that *CsMYB3* may be involved in the regulation of anthocyanin biosynthesis in citrus along with *CsRuby1*.

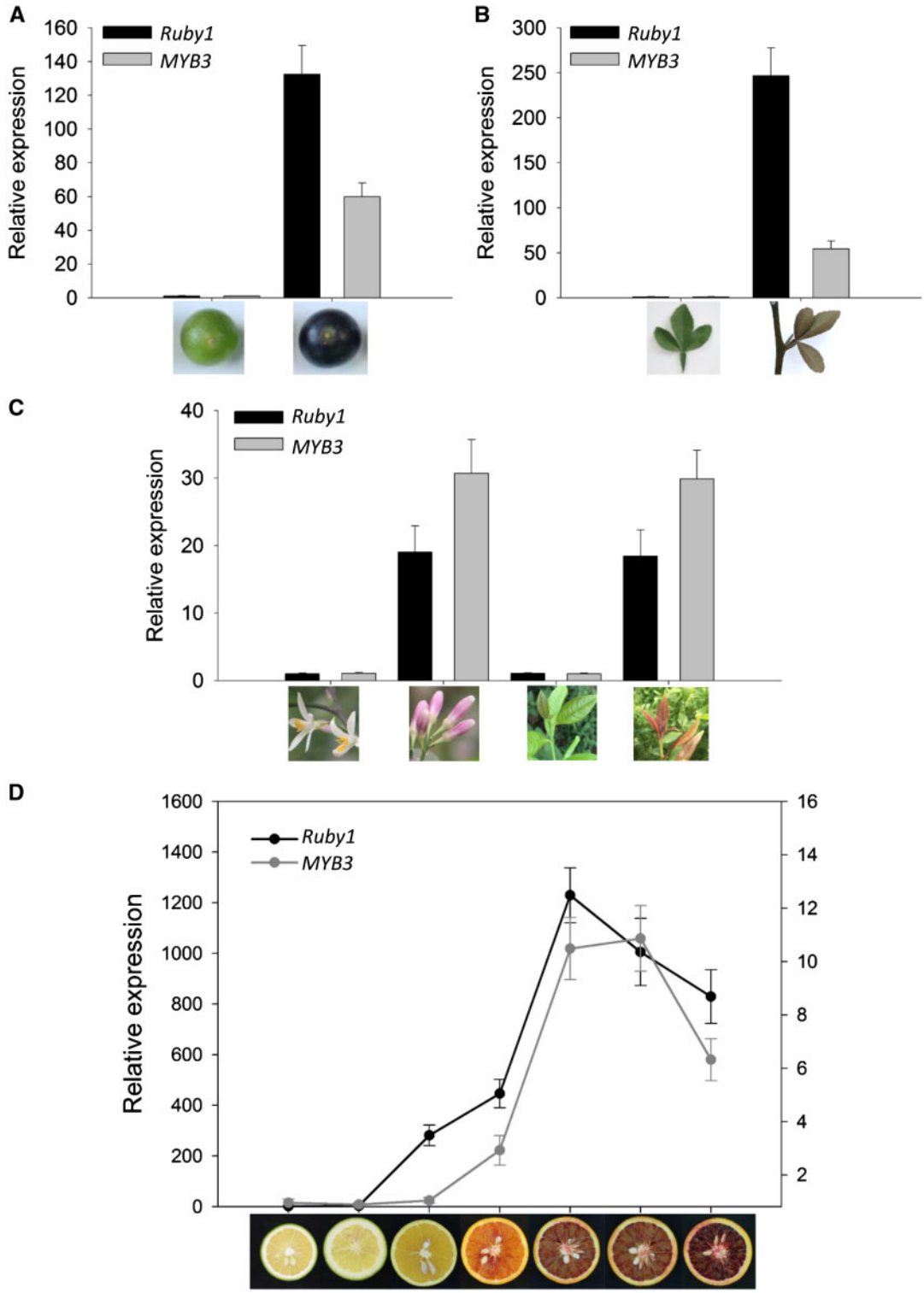

Fig. 2 Expression patterns of *MYB3* and *Ruby1* in *Citrus* and relative species. (A) Analysis of *MYB3* and *Ruby1* expressions in Chinese box orange fruit peel. Values represent the mean ± SE ($n = 3$ biological replicates). (B) Analysis of *MYB3* and *Ruby1* expressions in trifoliate orange leaves. Values represent the mean ± SE ($n = 3$ biological replicates). (C) Analysis of *MYB3* and *Ruby1* expressions in lemon cv. Femminello flowers and leaves. Values represent the mean ± SE ($n = 3$ biological replicates). (D) Analysis of *MYB3* and *Ruby1* expressions in *Citrus sinensis* cv. Tarocco fruit flesh in different fruit development stages. Values represent the mean ± SE ($n = 3$ biological replicates).

Overexpression of *CsMYB3* in *Arabidopsis* led to a decrease in anthocyanins but not in PAs

To investigate the role of *CsMYB3* in the regulation of anthocyanin biosynthesis, heterologous expression experiments were performed on *Arabidopsis*. *CsMYB3* was overexpressed under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Three independent lines with higher expression levels were selected for investigating the function of *CsMYB3*. The transgenic plants overexpressing *CsMYB3* and wild type (WT) showed no remarkable phenotypic differences when grown in Murashige and Skoog (MS) media (**Supplementary Fig. S2**), excluding the possibility that *CsMYB3* functions as a positive regulator of anthocyanin biosynthesis. To further verify its function, low nitrogen media, which can induce anthocyanin accumulation in *Arabidopsis*, were used. WT and 35S:*CsMYB3* *Arabidopsis* seedlings were grown on nitrogen stress (N−) media with no ammonium or nitrate under normal light conditions. Compared with the WT, 35S:*CsMYB3* overexpression plants showed dramatic decreases in the content of anthocyanins under nitrogen stress (**Fig. 3A–C**). The effect of *CsMYB3* on the anthocyanin biosynthetic genes was examined by qRT-PCR analysis. In *CsMYB3*-overexpression lines, the transcript levels of key anthocyanin biosynthetic genes *AtCHS*, *AtF3H*, *AtDFR* and *AtANS* were reduced relative to those in WT under nitrogen stress (**Fig. 3D**), which was consistent with their phenotype of lower anthocyanin content. However, in *CsMYB3*-overexpression lines, the expression of the key regulatory genes *AtPAP1* and *AtPAP2* showed no significant decreases relative to those in WT (**Fig. 3E**), indicating that overexpression of *CsMYB3* led to a decrease in anthocyanins.

Arabidopsis accumulates a large amount of PAs in the seed coat, showing a brown color (Routaboul et al. 2006). Dimethylaminocinnamaldehyde (DMACA) staining could be used for *Arabidopsis* PA assay (Xie et al. 2004). To test whether *CsMYB3* can repress PA accumulation, DMACA staining was performed on the seeds of WT and the same three 35S:*CsMYB3* transgenic *Arabidopsis* lines that were previously subjected to nitrogen stress treatments. Compared with WT, the seeds of 35S:*CsMYB3* transgenic plants showed no significant differences in seed coat color. After DMACA staining, both WT and 35S:*CsMYB3* transgenic *Arabidopsis* seeds changed to dark black (**Supplementary Fig. S3**), indicating that *CsMYB3* cannot reduce PA synthesis in *Arabidopsis*.

'Activator-and-repressor' relationship between *CsMYB3* and *CsRuby1* in anthocyanin biosynthesis

Considering that the high-level expression of both *CsRuby1* and *CsMYB3* genes was associated with anthocyanin accumulation, we overexpressed *CsMYB3* gene in *CsRuby1*-overexpressing *Arabidopsis* and strawberry lines. The 35S:*CsRuby1* transformants showed a marked accumulation of anthocyanins in *Arabidopsis* seeds, while the 35S:*CsRuby1* + 35S:*CsMYB3* transformants showed remarkable decreases in anthocyanins in *Arabidopsis* seeds (**Fig. 4A, B**). The brownish pigmentation in *Arabidopsis* seeds could only result from the oxidation of PAs but not from the accumulation of anthocyanins (Routaboul et al. 2006). To quantify PA accumulation, soluble and insoluble PAs were extracted and measured. Soluble PA and insoluble PA levels were significantly decreased in the 35S:*CsRuby1* + 35S:*CsMYB3* *Arabidopsis* seeds when compared with those in 35S:*CsRuby1* transformants. The light coloration of *Arabidopsis* seeds was due to the decrease in anthocyanins and PAs. Given that ANR is a key enzyme specific for the conversion of anthocyanidins to PAs (Xie et al. 2003, Xie et al. 2004), we determined its relative transcript levels in the seeds of 35S:*CsRuby1* and 35S:*CsRuby1* + 35S:*CsMYB3* transformants. *AtANR* (*AtBAN*) transcripts in the seeds showed no difference between 35S:*CsRuby1* and 35S:*CsRuby1* + 35S:*CsMYB3* transformants (**Fig. 4C**), excluding the possibility that the light coloration of seeds was due to the conversion of anthocyanidins to PAs catalyzed by ANR. These results suggested that the decrease in PAs may be due to the decrease in its substrate anthocyanidins. To further validate the 'activator-and-repressor' relationship between *CsMYB3* and *CsRuby1*, transient overexpression experiments were performed in yellow strawberry (*Fragaria vesca*) fruits, which are unable to accumulate anthocyanins because of a mutation in the regulatory gene *FaMYB10* (Hawkins et al. 2016). The results showed that overexpression of *CsRuby1* led to an intense red pigmentation after 5 d, whereas overexpression of *CsMYB3* and *CsRuby1* together remarkably reduced the pigmentation (**Fig. 4D**). Taken together, these results demonstrated the 'activator-and-repressor' relationship between *CsMYB3* and *CsRuby1* in anthocyanin regulation.

Repression activity of CsMYB3 did not require physical interaction with CsRuby1 and CsbHLH1 or binding to the promoters of anthocyanin biosynthetic genes

In a previous study, CsRuby1 was shown to interact with CsbHLH1 and enhance the promoter transcriptional activity of anthocyanin biosynthetic genes (Huang et al. 2018). Our yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) results showed that CsMYB3 could not interact with CsbHLH1 (belonging to the GL3 subclade) and CsRuby1 (**Fig. 5A, B**; **Supplementary Fig. S4**). To confirm whether *CsMYB3* directly binds to the promoters of anthocyanin biosynthetic genes, yeast one-hybrid (Y1H) assays were performed. The results revealed that *CsMYB3* could not bind to the promoters of *CsF3'H* or *CsDFR* (**Fig. 5C**). To clarify the regulatory mechanism of *CsMYB3* in repressing the accumulation of anthocyanins, transient expression assays were performed to test whether *CsMYB3* interferes with the transcriptional activation activity of the MBW complexes in *Nicotiana benthamiana*. The results revealed that CsMYB3 reduced the CsRuby1/CsbHLH1 activation capacity for the expression of *CsDFR* and *CsANS* (**Fig. 5D**). Based on the above results, it could be speculated that *CsMYB3* inhibits anthocyanin biosynthesis by repressing the activity of CsRuby1/CsbHLH1 complexes instead of by interacting with CsRuby1 and CsbHLH1 or binding to the promoters of anthocyanin biosynthetic genes.

CsMYB3 was transcriptionally activated by *CsRuby1*

To clarify the mechanism of the similar expression patterns between *CsMYB3* and *CsRuby1*, we analyzed the transcriptional

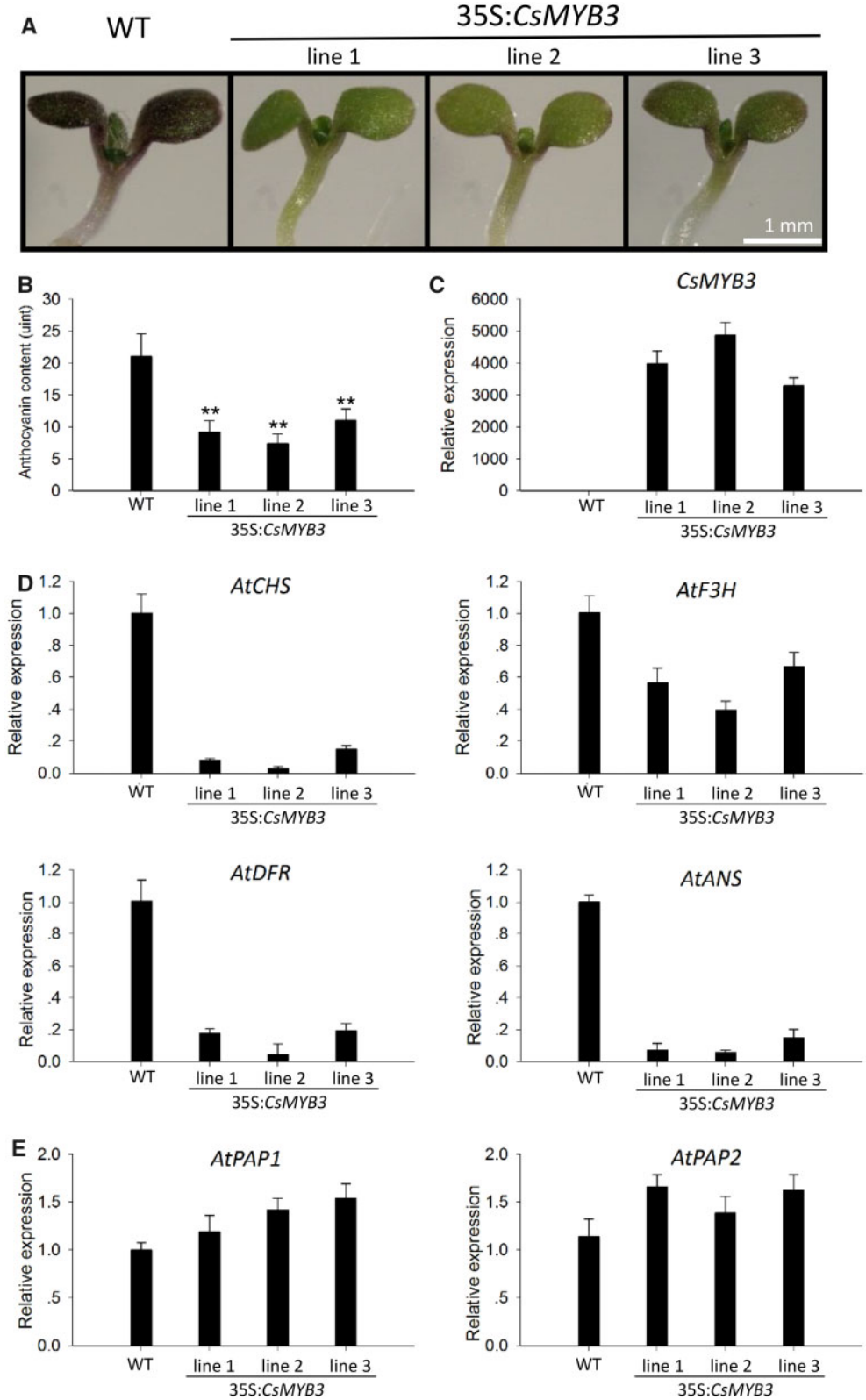

Fig. 3 Effect of *CsMYB3* overexpression on transgenic *Arabidopsis* grown under nitrogen stress. (A) Phenotype of WT and 35S:*CsMYB3* *Arabidopsis* lines under nitrogen stress after 2 weeks of growth. (B) Anthocyanin contents (unit) of WT and 35S:*CsMYB3* *Arabidopsis* lines under nitrogen stress after 2 weeks of growth. (A530 − 0.25 × A657)/fresh weight was considered as anthocyanin content. Values represent the mean ± SD ($n = 3$). $^{**}P < 0.01$ (two-tailed Student's *t*-test). (C) Analysis of *CsMYB3* expression in 35S:*CsMYB3* *Arabidopsis* lines compared with WT under nitrogen stress after 2 weeks of growth. Values represent the mean ± SD ($n = 3$). (D) Analysis of *AtCHS*, *AtF3H*, *AtDFR* and *AtANS* expressions in 35S:*CsMYB3* *Arabidopsis* lines compared with WT under nitrogen stress after 2 weeks of growth. Values represent the mean ± SD ($n = 3$). (E) Analysis of *AtPAP1* and *AtPAP2* expressions in 35S:*CsMYB3* *Arabidopsis* lines compared with WT under nitrogen stress after 2 weeks of growth. Values represent the mean ± SD ($n = 3$).

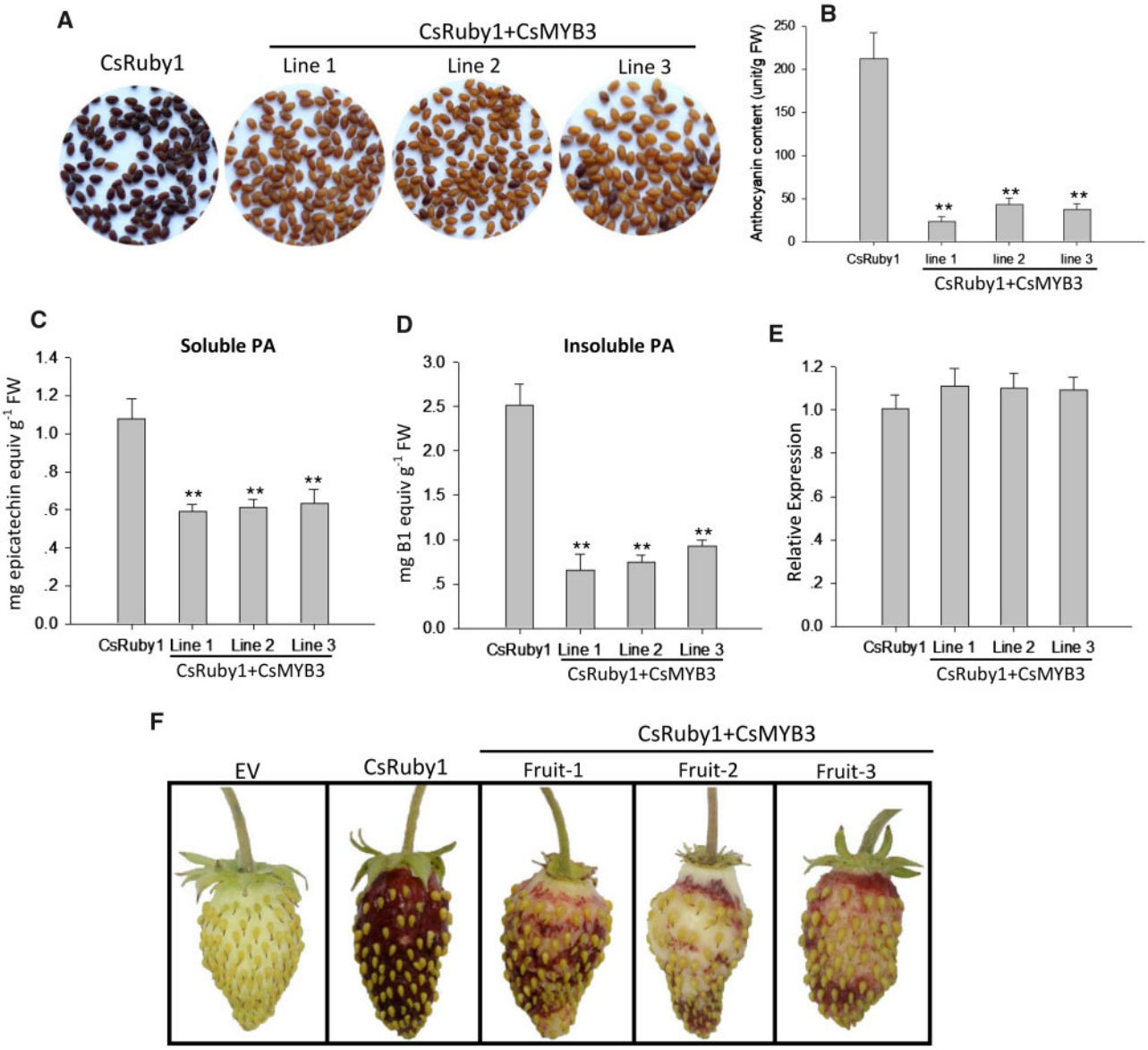

Fig. 4 Effect of overexpression *CsMYB3* on anthocyanin-accumulating *Arabidopsis* and strawberry lines overexpressing the *CsRuby1* gene. (A and B) Pigmentation phenotype (A) and anthocyanin content (unit) (B) of the *Arabidopsis* seeds of 35S:*CsRuby1* and 35S:*CsMYB3* + 35S:*CsRuby1* T_2 lines. (A530 − 0.25 × A657)/fresh weight was considered as the anthocyanin content. Values represent the mean ± SD ($n = 3$). $^{**}P < 0.01$ (two-tailed Student's *t*-test). (C) Soluble PA levels quantified with DMACA reagent and expressed as epicatechin equivalents. Data are mean ± SD ($n = 3$). $^{**}P < 0.01$ (two-tailed Student's *t*-test). (D) Insoluble PA levels quantified by the butanol–HCl method and expressed as procyanidin B1 equivalents. Data are mean ± SD ($n = 3$). $^{**}P < 0.01$ (two-tailed Student's *t*-test). (E) Relative expression levels of *ANR* in the seeds of 35S:*CsRuby1* and 35S:*CsRuby1* + 35S:*CsMYB3* transformants. The *actin* gene was used as internal control. (F) Transient overexpression of *CsRuby1* and *CsMYB3* + *CsRuby1* in the yellow *F. vesca* variety Pineapple Crush that is unable to synthesize anthocyanins. EV, empty vector.

cis-elements of *CsMYB3*. The *MYB3* promoter sequences were cloned from sweet orange (*C. sinensis*) and pummelo (*C. grandis*), and those of other citrus species were downloaded from the citrus genome database (**http://citrus.hzau.edu.cn/orange/**), including the sequences of citron (*Citrus medica*). Multiple sequence alignments indicated that the 1-kb upstream region of the promoter was highly conserved and contained two conserved MYB-recognition elements (MREs) (**Supplementary Fig. S5**), suggesting that Ruby1 can bind to the promoter of *MYB3* in citrus. To confirm this hypothesis, electrophoretic mobility shift assay (EMSA) was performed using the HIS-Ruby1 fusion protein. The results revealed that CsRuby1 could specifically bind to the two conserved MREs from the promoters of *CsMYB3* (**Fig 6A**). The promoter activity assays in the transient expression study revealed that CsRuby1 and the CsRuby1/CsbHLH1 complexes enhanced *CsMYB3* promoter activity and CsMYB3 could repress the activity of CsRuby1/CsbHLH1 complexes (**Fig. 6B**). These results implied that *CsMYB3* and *CsRuby1* probably form a common regulatory loop to regulate anthocyanin accumulation in citrus.

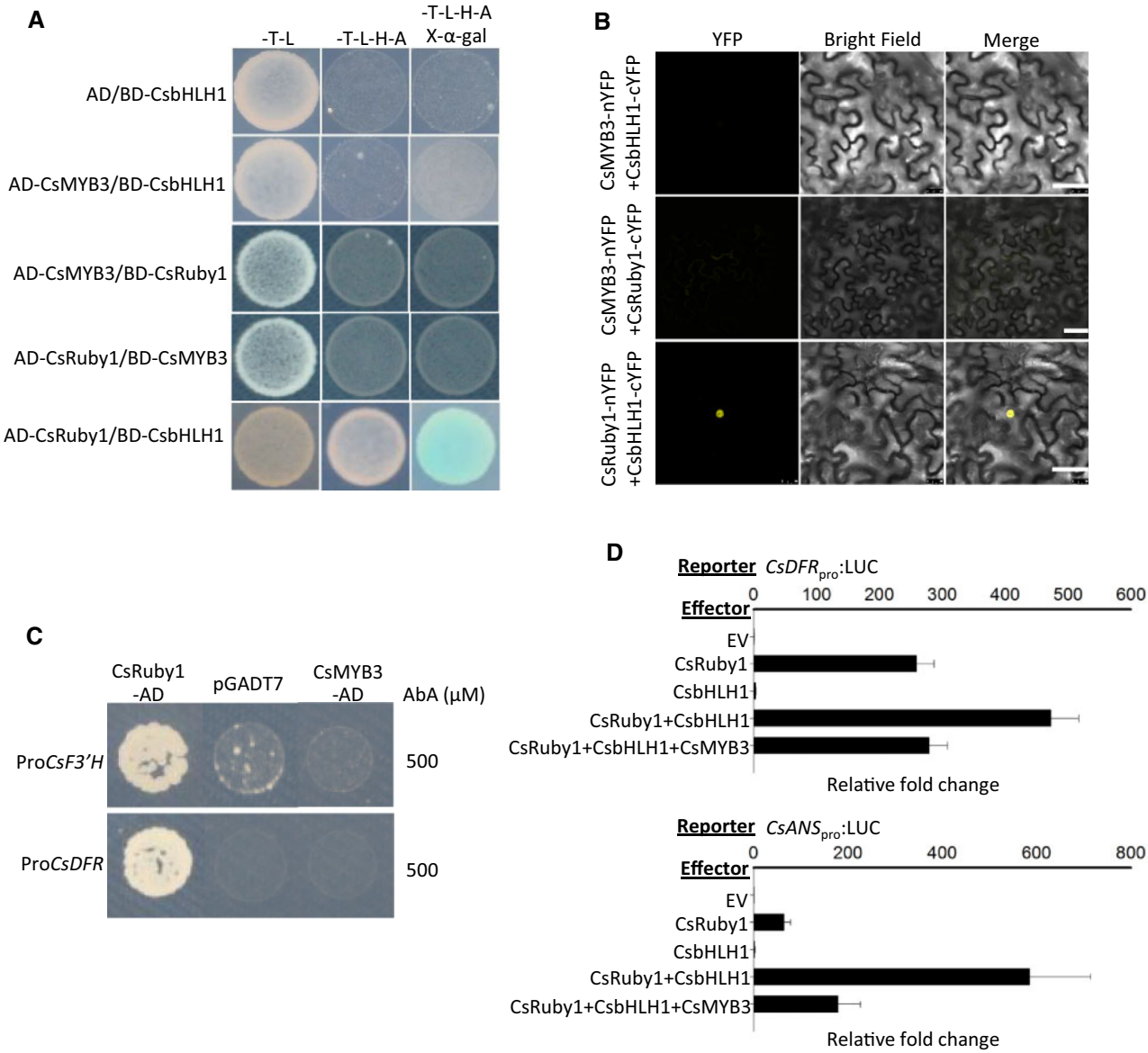

Fig. 5 Interaction analysis of CsMYB3 with CsRuby1, CsbHLH1 and anthocyanin biosynthetic genes. CsMYB3 cannot interact with CsRuby1 and CsbHLH1 in yeast two-hybrid assays (A) and in vivo by BiFC assays (B). eYFP signals are localized in the nucleus and are shown in yellow, indicating a positive interaction signal. Scale bar = 50 μm. The interaction between CsRuby1 and CsbHLH1 is a positive control. (C) CsMYB3 cannot bind to the promoters of *CsF3'H* and *CsDFR* in yeast one-hybrid assays. (D) Transient promoter activity assays using *CsDFR* promoter and *CsANS* promoter driving *LUC* as a reporter along with effectors (CsRuby1, CsbHLH1, CsRuby1 + CsbHLH1, CsRuby1 + CsbHLH1 + CsMYB3); the empty vector (EV) serves as an internal control. Values represent the mean ± SD ($n = 8$ replicate reactions).

Discussion

CsMYB3 plays a repressor role in regulation of anthocyanin biosynthesis in citrus

The regulation of anthocyanin biosynthesis is complex, and MYBs may play either activating or repressing roles in the developmental, environmental and stress-related anthocyanin biosynthesis (Albert et al. 2011, Albert et al. 2014, Xie, et al. 2016). The *CsRuby1* gene is a critical R2R3 MYB transcriptional activator that controls anthocyanin biosynthesis in citrus (ButelLi et al. 2017). However, the MYB repressors involved in anthocyanin biosynthesis in citrus have not been characterized. In this study, we characterized a novel citrus R2R3-MYB repressor *CsMYB3* and demonstrated its role together with *CsRuby1* in regulating anthocyanin biosynthesis. *CsMYB3* belongs to anthocyanin/PA group, which includes *M. truncatula MtMYB2* (Jun et al. 2015), grapevine *VvMYBC2-L1* (Cavallini et al. 2015), polar *PtrMYB182* and *PtrMYB57* (Yoshida et al. 2015, Wan et al. 2017) and peach *PpMYB18* (Zhou et al. 2019). Our results showed that *CsMYB3*-overexpressing *Arabidopsis* seedlings had lower anthocyanin accumulations than the WT, which is consistent with the reduced transcripts of anthocyanin biosynthetic genes in transgenic *Arabidopsis* seedlings under nitrogen or high-light stress conditions, confirming that CsMYB3 represses

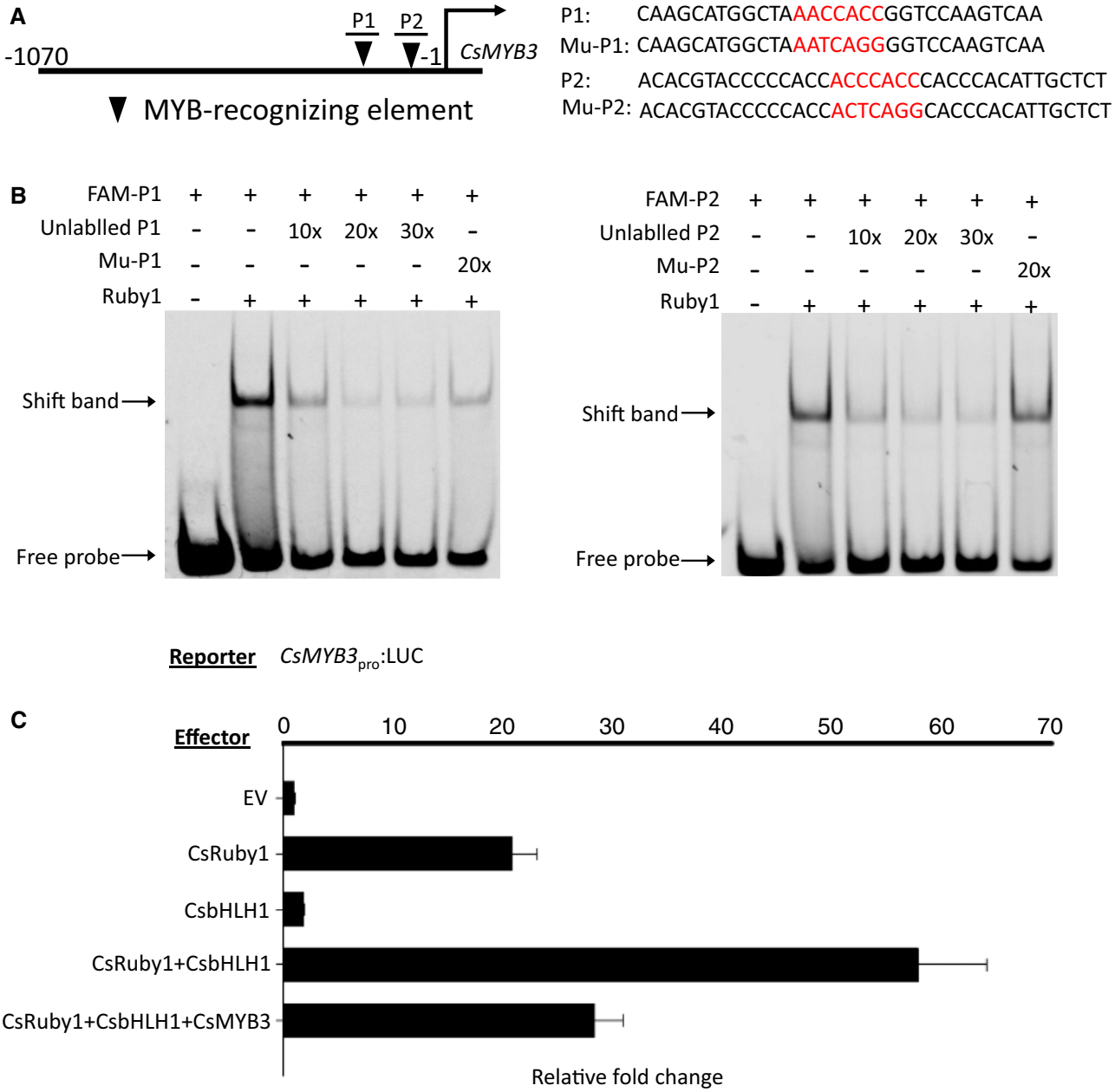

Fig. 6 Binding of CsRuby1 to the *CsMYB3* promoter and activation of its expression. (A) Schematic representation of the *CsMYB3* promoter (left) and sequences used in the EMSA (right). (B) EMSA showing the binding of CsRuby1 to the promoters of *CsMYB3*. The 10-, 20- and 30-fold excess of nonlabelled probes (unlabeled P1 or P2) and the 20-fold excess of mutant probes (Mu-P1 or P2) were used for competition. (C) Transient promoter activity assays were carried out using the conserved *CsMYB3* promoter driving *LUC* as a reporter along with effectors (CsRuby1, CsbHLH1, CsRuby1 + CsbHLH1, CsRuby1 + CsbHLH1 + CsMYB3); the empty vector (EV) serves as an internal control. Values represent the mean ± SD ($n = 8$ replicate reactions).

anthocyanin biosynthesis. Unlike other reported MYBs in the FaMYB1-like group such as *MtMYB2* (Jun et al. 2015), *PpMYB18* (Zhou et al. 2019), *PtrMYB182* (Yoshida et al. 2015) and *PtrMYB57* (Wan et al. 2017), *CsMYB3* possibly does not repress PA accumulation.

Mechanism underlying the repression activity of CsMYB3

Our dual-luciferase assay data showed that the CsRuby1/CsbHLH1 complexes could activate the *CsDFR* and *CsANS* promoters directly in *N. benthamiana*, whereas when *CsMYB3* was co-expressed with *CsRuby1* and *CsbHLH1* constructs, it could reduce the activation capacity of CsRuby1/CsbHLH1 complexes by >50%. In previous studies, R2R3-MYB repressors were shown to inhibit flavonoid biosynthesis through the following two ways: acting directly to repress the activity of MBW complexes via their EAR motif (Jun et al. 2015, Yoshida et al. 2015) and binding directly to the promoters of flavonoid biosynthetic genes to repress their expression via the EAR motif (Wan et al. 2017). Besides, the activity of some R2R3-MYB repressors

requires physical interaction with their bHLH partners, such as polar *PtrMYB57* (Wan et al. 2017). In this study, the Y1H assay results excluded the possibility that *CsMYB3* directly binds to the promoters of anthocyanin biosynthetic genes, suggesting that *CsMYB3* does not directly repress the expression of anthocyanin biosynthetic genes but through decreasing the activity of CsRuby1–CsbHLH1 complexes, which would lead to the suppression of anthocyanin biosynthesis. In addition, the repression activity of CsMYB3 does not require physical interaction with CsbHLH1, or with CsRuby1, which is a positive regulator in the MBW complexes for anthocyanin biosynthesis.

An 'activator-and-repressor' loop model constituted by *CsMYB3* and *CsRuby1* regulates anthocyanin biosynthesis in citrus

The expression data indicated that *CsRuby1* and *CsMYB3* are highly expressed in most citrus species with anthocyanin accumulation, including primitive species, wild species and cultivated species, and in different tissues such as leaves, flowers and fruits, suggesting that *CsMYB3* and *CsRuby1* form a regulatory loop in citrus anthocyanin biosynthesis. Between these two MYB regulators, the role of *Ruby1* as a positive regulator of anthocyanin biosynthesis has been clearly elucidated in citrus (Butelli et al. 2012, ButelLi et al. 2017, Huang et al. 2018). *CsMYB3* is an active repressor and can be activated by *CsRuby1*. The *CsMYB3* gene counterbalances the activation role of *CsRuby1*, which could prevent excess accumulation of anthocyanins in citrus. Hence, we propose that there may be an 'activator-and-repressor' loop model to maintain the homeostasis of anthocyanin accumulation in citrus and its wild relatives. In this model, *CsRuby1* acts as an activator, while *CsMYB3* acts as a repressor of anthocyanin biosynthesis. In anthocyanin-accumulating tissues of citrus, the expression of the *CsRuby1* gene is highly induced and the CsRuby1/CsbHLH1 complexes activate the expression of anthocyanin biosynthetic genes and induce anthocyanin accumulation. Moreover, the *CsRuby1* gene binds to the *CsMYB3* promoter to activate its expression. In turn, *CsMYB3* represses the activity of CsRuby1/bHLH1 complexes to feedback regulate anthocyanin biosynthesis (**Fig. 7**). In summary, *CsRuby1* and *CsMYB3* form an 'activator-and-repressor' regulatory loop to regulate anthocyanin accumulation in citrus.

Conclusions

In this study, a novel R2R3 MYB repressor *CsMYB3*, which is involved in the anthocyanin biosynthesis pathway, was identified in citrus. Given the long juvenile period (5–8 years) of citrus, we used the stable transformation of *Arabidopsis* and transient transformation of strawberry to elucidate the 'activator-and-repressor' relationship between *CsMYB3* and *CsRuby1* in anthocyanin biosynthesis. The expression levels of both *CsMYB3* and *CsRuby1* genes were consistent with anthocyanin accumulation in the leaves, flowers and fruits of different citrus species. Interaction assays indicated that CsMYB3 can repress the activation capacity of MBW complexes and can be activated by CsRuby1. Finally, we proposed an 'activator-and-repressor' loop model for the regulation of anthocyanin biosynthesis in citrus.

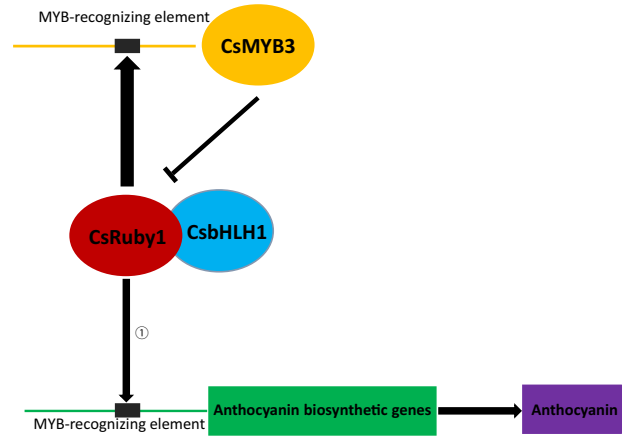

Fig. 7 Proposed 'activator-and-repressor' loop model for the regulation of anthocyanin accumulation in citrus. *CsRuby1* acts as the activator by directly and positively regulating the expression of anthocyanin biosynthetic genes and *CsMYB3*, whereas *CsMYB3* acts as the repressor by inhibiting the activity of the CsRuby1–CsbHLH1 complexes. The arrows and blunt-ended lines indicate activation and repression, respectively. ① indicates the pathway that has been previously reported (Butelli et al. 2012, Huang et al. 2018).

Materials and Methods

Plant materials

Chinese box orange (*A. buxifolia*), trifoliate orange (*P. trifoliata*), lemon (*C. limon*) and blood orange (*C. sinensis* cv. Tarocco) were grown in an experimental orchard at Huazhong Agricultural University (Wuhan, China). The trees used for sample collection were cultivated with standard horticultural practices and methods for disease and insect control. Leaves, flowers and fruits were obtained from different species as indicated in **Fig. 2**. Blood orange fruit flesh was collected at six different developmental stages based on fruit coloration: 100 d after flowering (DAF), 130 DAF, 200 DAF, 250 DAF, 270 DAF, 300 DAF and 320 DAF (**Fig. 2**). Three fruits from the same plant and stage were used. All the materials were collected and immediately frozen in liquid nitrogen and stored at −80°C until use.

Growth and stress conditions

For all experiments in this study, seeds were sterilized as described by Engineer and Kranz (2007) and germinated on 0.8% agar plates. After 2 d at 4°C, the plates were transferred to growth chambers with a 16-h light/8-h dark light cycle at an intensity of 6,000 lx at 22°C. An MS-modified basal salt mixture (Phyto-Technology Laboratories, USA) was used for the growth media. For nitrogen stress, an MS-modified basal salt mixture without nitrogen (Phyto-Technology Laboratories, USA) was used for the growth media. For high-light stress, the light intensity was set to 50,000 lx.

Sequence alignments and phylogenetic analysis

The full-length amino acid sequences of CsMYB3 from citrus and R2R3-MYB repressors from other plants were aligned using ClustalW. A phylogenetic tree was constructed using the MEGA 5.05 software (Tamura et al. 2011). Node support was estimated using a neighbor-joining bootstrap analysis (1,000 bootstrap replicates).

Real-time quantitative PCR analysis

One microgram of total RNA was used for cDNA synthesis with a HiScriptII Q RT SuperMix (with gDNA wiper) Kit (Vazyme Biotech). Quantitative reverse

transcription-PCR was performed with an SYBR FAST qPCR Kit (KAPA), and amplification was performed using 384-well plates and an ABI 7900 Fast Real-Time System (PE; Applied Biosystems). For each biological replicate, quantifications were conducted in triplicate. Primer information is presented in **Supplementary Table S1**. The *Actin* gene was used as the internal control. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ analysis method. Primer sequences of *AtCHS* (AT5G13930), *AtF3H* (AT3G51240), *AtDFR* (AT5G42800) and *AtANS* (At4g22880) were used according to a previous study (Jun et al. 2015). Primers for *AtPAP1* (AT1G56650) and *AtPAP2* (AT1G66390) were designed in this study.

Subcellular protein localization

Subcellular localization of the CsMYB3 protein was assessed using citrus callus protoplasts (HB pummelo, *C. grandis* L. Osbeck) in accordance with the previously described method (Liu et al. 2016). A CsMYB3-GFP fusion construct was produced by inserting the full length of *CsMYB3* into a pM999-GFP vector. Plasmids were extracted and purified using a Plasmid MidiKit (Qiagen) following the manufacturer's protocol. The OsGhd7 in rice was used as a nuclear maker. The CsMYB3-GFP and OsGhd7-CFP plasmids were then cotransformed into citrus protoplasts. Florescence images were subsequently captured using a confocal laser scanning microscope (TCS SP2; Leica). Primer information is presented in **Supplementary Table S1**.

Transient expression assays

A dual-luciferase reporter assay was conducted in *N. benthamiana* leaves according to a previous report (Hellens et al. 2005). For DNA-promoter interaction assays, the putative promoters (~1.5-kb upstream of the ATG start codon) of the *CsDFR* and *CsANS* genes and 1-kb upstream of the ATG start codon of *CsMYB3* were amplified from sweet orange cv. Tarocco genomic DNA. These fragments were then inserted into the cloning site of pGreen0800-LUC. The full-length *CsRuby1*, *CsMYB3* and *CsbHLH1* were amplified and cloned into an effector vector (a modified pK2GW7) driven by the 35S promoter. A pK2GW7 empty vector was used as a negative control. Primer information is provided in **Supplementary Table S1**.

Arabidopsis transformation

Arabidopsis thaliana ecotype Columbia was used for genetic transformation. The coding sequence of *CsMYB3* was amplified and cloned into a pK2GW7 vector. The construct was then electroporated into *Agrobacterium tumefaciens* strain GV3101 and transformed into WT *Arabidopsis* using the floral dip method to obtain 35S:*CsRuby1* + 35S:*CsMYB3* *Arabidopsis* plants. The coding sequence of *CsMYB3* was amplified and cloned into a pB2GW7 vector. The pB2GW7-*CsMYB3* construct was then electroporated into *A. tumefaciens* strain GV3101, which was subsequently transformed into *CsRuby1*-overexpressing homozygous transgenic *Arabidopsis* plants using the floral dip method.

Anthocyanin extractions and measurements

Anthocyanin contents were determined based on a previously reported protocol (Mita et al. 1997) with modifications. Seedlings or seeds of *Arabidopsis* were collected and weighed, after which anthocyanins were extracted in 1 ml of 100% methanol and 0.1% (v/v) HCl for 30 min at 4°C with ultrasonic vibration. The extracts were then centrifuged for 10 min at 13,000 rpm. The resulting supernatant (200 µl) was used to measure the A530 and A657 values, and (A530 − 0.25 × A657)/fresh weight was considered as the anthocyanin content.

DMACA staining

Arabidopsis T_2 seeds were stained for the presence of PAs using 1% (w/v) 4-DMACA in methanol: 6 N HCl (Pang et al. 2008). After DMACA staining for 2 d at room temperature, seeds were washed three times with 70% ethanol, visualized and photographed with a stereomicroscope (Leica MZFL III).

Quantification of PAs

For PA analysis, 0.01 g (seeds of *A. thaliana*) samples were extracted with 5 ml of 70% acetone/0.5% acetic acid (extraction solution) by vortex and then sonicated at room temperature for 1 h, followed by centrifugation at 2,500 × *g* for 10 min. The pooled supernatants were then extracted three times with chloroform and three times with hexane, and the supernatants (containing soluble PAs) and residues (containing insoluble PAs) were collected separately.

Total soluble PA content was calculated spectrophotometrically after reaction with DMACA reagent for 30 min (0.2%, w/v, DMACA in methanol, 3 N HCl) at 640 nm, with (+)-catechin (MedChemExpress, USA) as standard.

For the quantification of insoluble PAs, 2 ml of butanol–HCl reagent was added to the residues and the mixture was sonicated at room temperature for 1 h, followed by centrifugation at 2,500 × *g* for 10 min. A total of 6 ml of butanol–HCl reagent and 200 µl of 0.2% ammonium sulfate solution were added to 1 ml of supernatant. The mixture was boiled for 40 min and cooled on ice, and the absorption of the supernatants was measured at 550 nm; the absorbance values were converted into PA equivalents using a standard curve of procyanidin B1 (MedChemExpress, USA).

Y2H assays

For the transactivation activity assays, the coding sequence of *CsbHLH1* was amplified and inserted into a pGBKT7 vector. The fusion constructs were then introduced into yeast strain Y2H GOLD following the manufacturer's instructions (Yeastmaker; Clontech), and the transformed yeast cells were grown on SD–Trp and SD–Trp–His–Ade plates to check for transactivation activity. For Y2H assays, the full-length coding sequences of *CsMYB3* and *CsRuby1* were individually cloned into pGADT7 (Clontech) to produce fusion proteins that contained a GAL4 activation domain. Various combinations of BD and AD vectors were cotransformed into yeast strain AH109. After the yeasts were mated, they were plated onto SD–Trp–Leu plates. Positive colonies were validated by PCR amplification and then streaked on new SD–Trp–Leu and SD–Trp–Leu–His–Ade plates. Finally, the positive colonies were transferred onto SD–Trp–Leu–His–Ade + X-a-Gal plates to further validate positive interactions between the two tested genes. For the transactivation test, photographs were taken 3–5 d after inoculation on media and 3–4 d for the Y2H test. All the primers used are listed in **Supplementary Table S1**.

Bimolecular fluorescence complementation

The coding sequences of *CsMYB3*, *CsRuby1* and *CsbHLH1* were amplified using the primer pairs (**Supplementary Table S1**) and then cloned into PCL112-35S and PCL113-35S vectors, which contained DNA encoding the N- or C-terminal regions of YFP (yellow fluorescent protein) (YFPN or YFPC), respectively. BiFC experiments were performed by cotransfecting different combinations of the constructs into 5-week-old *N. benthamiana* leaves via *A. tumefaciens* strain GV3101 transformation using 150 µM acetosyringone and setting the final optical density of each *Agrobacterium* suspension to 0.8. YFP-dependent fluorescence was detected 3 d after transfection using a confocal laser scanning microscope (Zeiss LSM 510 Meta).

Y1H assays

Y1H assays were performed using a Matchmaker Gold Yeast One-Hybrid System Kit (Clontech) in accordance with the manufacturer's protocol. The *CsRuby1* and *CsMYB3* genes were ligated to pGADT7 to generate AD-*CsRuby1* and AD-*CsMYB3* constructs. The fragments of the promoters of *CsF3'H*and *CsDFR* were ligated to a pAbAi vector to generate pAbAi-bait plasmids, which were then linearized and transformed into yeast strain Y1H Gold and then selected with a plate of selective synthetic dextrose medium that lacked uracil. The constructs of AD-MYB were transformed into strain Y1H Gold holding pAbAi-bait and screened on an SD/-Ura/AbA plate. Primer information is given in **Supplementary Table S1**.

Electrophoretic mobility shift assay

The EMSA was conducted as described previously (Zhu et al. 2017) with some modifications. Briefly, the His-fused Ruby1 was expressed and purified as in our previous study (Huang et al. 2018). The 5′FAM-labeled oligonucleotide probes were directly synthesized and labeled by the Shanghai Sangon Company (Shanghai, China). Unlabeled probes with the same or mutated probes were used as cold competitors. To perform binding reactions, the binding solution [0.1% Nonidet P-40, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 100 ng μl^{-1} of poly(dI–dC), 1,500 ng of purified protein and 1 µl of the FAM-labeled probe (10 μmol^{-1})] was mixed together and incubated at 4°C for 45 min. For the competition assays, the unlabeled probe or

mutated probe was incubated with protein and binding buffer at 4°C for 45 min. Next, 1 µl of the FAM-labeled probe (10 µmol^{-1}) was added and incubated at 4°C for 45 min. The samples were loaded onto a prerun 6% polyacrylamide gel. Electrophoresis was performed at 4°C using 0.59 Tris–Borate–EDTA as the electrophoresis buffer in the dark for 1 h. Gel images were acquired using the Amersham Imager 600 (GE Healthcare, Tokyo, Japan). Probe information is presented in **Supplementary Table S1**.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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