BBX16, a B-box protein, positively regulates light-induced anthocyanin accumulation by activating MYB10 in red pear

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Introduction

Peel colour is an important aesthetic quality of pear (Pyrus pyrifolia) that directly affects consumer appeal (Zhang et al., 2013). Red-coloured European pear has been widely cultivated and accepted worldwide, but green/yellow Asian pears still occupy the majority of the market (Sun et al., 2014). In recent years, along with the introduction of local red pear cultivars into breeding programs, red Asian pears have gradually increased in the market and have gained consumers’ acceptance (Zhang et al., 2013). However, unlike the European pear, which easily colours, the red Asian pear does not easily produce the red-coloured peel, especially in the warmer regions of China (Bai et al., 2017).

The red coloration of Asian pear results from the accumulation of anthocyanin. Anthocyanin is biosynthesized through the flavonoid pathway, in which phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase, dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose: flavonoid 3-glycosyltransferase (UFGT) are the key enzymes (Jin et al., 2016; Qian et al., 2014). The genes encoding these enzymes are transcriptionally regulated by a protein complex formed by an R2R3-MYB, a basic-helix-loop-helix and a WD repeat protein, namely the MBW complex (Xu et al., 2015). This complex binds to the promoters of structural genes and induces their expressions (Dubos et al., 2010). In rosaceous plants, a series of anthocyanin-accumulation-related MYB proteins have been identified, among which PpMYB10 (Feng et al., 2010) and PpMYB114 (Yao et al., 2017) control the red coloration of pear.

The transcriptional activation of structural genes by MYB proteins is the most important step in the regulation of anthocyanin biosynthesis. MYB proteins are transcriptionally and post-translationally regulated by various internal and external factors (Allan and Espley, 2018). Specifically, the transcriptional alteration of MYB genes strongly correlates with anthocyanin accumulation (Xu et al., 2015). Among the various factors, light is indispensable for anthocyanin biosynthesis in pear (Sun et al., 2014). The mechanism of light-induced anthocyanin biosynthesis has been well characterized in the model plant Arabidopsis thaliana as part of photomorphogenesis (Maier et al., 2013). It requires light-responsive elements, including phytochromes and their downstream factors, such as CONSTITUTIVELY
PHOTOMORPHOGENIC 1 (COP1) and LONG HYPOCOTYL 5 (HY5) (Lau and Deng, 2012). In pear, PpHY5 directly binds to the promoter of PpMYB10, but no solid evidence indicates transcriptional activity (Tao et al., 2018). However, the overexpression of apple MdHY5 results in the overaccumulation of anthocyanin in apple calli (An et al., 2017). In addition, ERF, NAC and BBX proteins are involved in the transcriptional regulation of MYB proteins (Allan and Espley, 2018).

B-box (BBX) proteins belong to the zinc finger protein super family and are characterized as containing at least one B-box motif (Gangappa and Botto, 2014). In animals, the B-box motif is always present with RING finger and coiled-coil domains, and these proteins were named as TRIPARTITE MOTIF/RING, B-box and coiled-coil (Borden, 1998). These proteins are involved in transcriptional regulation, cell cycle regulation and ubiquitination (Laubinger et al., 2006). In plants, BBX proteins contain one or two B-box domains near their N termini, and some proteins have a CONSTANS, CO-LIKE and TOC1 motif (CCT domain) in their C termini (Crocco and Botto, 2013). The B-box domains are important for BBX proteins, which affect protein-protein interactions, nuclear bodies and transcriptional regulation (Qi et al., 2012). In Arabidopsis, 32 BBX proteins have been identified, and they are divided into five groups based on their sequences and structures (Gangappa and Botto, 2014). The BBX proteins have also been identified in other plant species, such as rice (Oryza sativa; Huang et al., 2012) and apple (Malus × domestica; Bai et al., 2014).

BBX proteins participate in numerous biological processes in plants, specifically in flower induction and photomorphogenesis. As part of photomorphogenesis, light-induced anthocyanin accumulation involves several BBX proteins. AtBBX21 (Xu et al., 2016), AtBBX22 (Chang et al., 2008) and AtBBX23 (Zhang et al., 2017) are positive regulators of anthocyanin biosynthesis, while AtBBX24 (Job et al., 2018), AtBBX25 (Gangappa et al., 2013a) and AtBBX32 (Holtan et al., 2011) suppress anthocyanin accumulation. In most cases, BBXs directly transcriptionally regulate the anthocyanin biosynthesis-related genes or indirectly regulate these genes through interactions with other proteins, such as HY5 (Gangappa and Botto, 2014). Furthermore, MdCOL11, the homolog of AtBBX22 in apple, may regulate anthocyanin biosynthesis, but solid evidence supporting the regulatory role of MdCOL11 in the anthocyanin biosynthesis is still lacking (Bai et al., 2014). In addition, BBX proteins also mediate hormonal signalling networks. AtBBX20 is involved in the brassinosteroid signalling pathway (Wei et al., 2016), and AtBBX18 promotes hypocotyl growth by increasing gibberellin levels (Wang et al., 2011). BBX21 can bind to the promoters of ABA INSENSITIVE 5, which is a hub of the abscisic acid signalling pathway, and regulate its expression (Xu et al., 2014).

After data mining our previously published transcriptome, a pear BBX gene, namely PpBBX16, was found to be differentially expressed in pear fruit after they were removed from bags, and PpBBX16 may be involved in anthocyanin biosynthesis (Bai et al., 2017). In this work, the expression and function of the PpBBX16 gene were analysed. By applying various approaches, we found that PpBBX16 indirectly activated the expression of PpMYB10, which subsequently activated anthocyanin biosynthesis. In addition, a genomewide analysis of the pear BBX gene family resulted in the further identification of other BBX genes that were differentially expressed during pear coloration. Our work resulted in a regulatory model for PpBBX16 and suggested the potential regulatory functions of other pear BBX proteins in the anthocyanin accumulation process, which helps us to understand the transcriptional regulation that occurs upstream of PpMYB10 during anthocyanin biosynthesis.

Results
Identification and expression of PpBBX16 under light conditions in ‘Red Zaosu’

In our previous transcriptome analysis, we identified the PpBBX16 gene, which was the homolog of A. thaliana BBX22 and was highly regulated by light exposure. Unexpectedly, when we cloned the PpBBX16 gene, a highly similar gene, PpBBX16-2, which was absent from the predicted pear transcripts, was also isolated from ‘Red Zaosu’ pear. The two proteins encoded by these genes shared a ~90% sequence identity (Figure 1a). A phylogenetic tree showed that PpBBX16 and PpBBX16-2 were closely related to MdBBX22-like genes (MdBBX22-1, MdBBX22-2 and MdBBX22-3) (Figure 1b).

The expression patterns of PpBBX16 and the anthocyanin biosynthesis-related genes during light exposure were further analysed. As shown in Figure 1, anthocyanin continuously accumulated after a 24-h light treatment until the end of the experiment (240 h of light treatment), while a corresponding increase in chlorophyll was not recorded (Figure 1a–c). During the experiment, the expression of PpBBX16 was immediately up-regulated (within 6 h) after exposure to light (Figure 1d), while most of the anthocyanin biosynthetic structural genes were highly up-regulated only after 24 h of treatment (Figure 1e), which was consistent with the anthocyanin accumulation. However, among the regulatory genes, only PpMYB10 was up-regulated by light exposure, while the PpHLH3 and PpWD40 genes were not differentially expressed (Figure 1e).

Ectopic expression of PpBBX16 in Arabidopsis

To verify the function of PpBBX16, a homolog of AtBBX22, it was ectopically expressed in wild-type Arabidopsis. The transgenic lines showed stronger photomorphogenesis phenotypes, including shorter hypocotyls and greater anthocyanin accumulations (Figure 2a–d). However, the PpBBX16-expressing transgenic lines also showed an overaccumulation of anthocyanins at the tops of the floral stems (Figure 2a,d). In the transgenic lines, the expression levels of anthocyanin biosynthesis-related genes were highly regulated, which was consistent with the anthocyanin content (Figure 2e).

Subcellular localization and trans-acting activity of PpBBX16

When the PpBBX16-GFP fusion protein was transiently expressed in the leaves of N. benthamiana with the nuclear expression of mcherry, orange fluorescent signals were observed in the nuclei. The GFP-related fluorescence was distributed throughout the cell. When the empty GFP protein was expressed (Figure 3a). Furthermore, PpBBX16-6D showed a trans-acting ability similar to that of VP16, and the activity was enhanced in BBX16-VP16-6D fusion proteins (Figure 3b). Thus, we confirmed that PpBBX16 is a transcription factor having an intact trans-acting activity.

To determine how PpBBX16 induced anthocyanin biosynthesis, the correlations between PpBBX16 and anthocyanin biosynthesis-related genes were analysed. The direct interactions between PpBBX16 and structural or regulatory genes could not be detected using the yeast one-hybrid assay (Figure 3c). PpBBX16 could not bind to the promoter regions of either PpCHS or
Figure 1  *PpBBX16*’s expression pattern during light treatment in ‘Red Zaosu’ pear. The bagging treatment was performed at 15 DAFB in the orchard. The light treatment was performed using the bagged fruit at 150 DAFB. (a) Colour changes of ‘Red Zaosu’ during the treatment. (b, c) Changes in anthocyanin (b) and chlorophyll (c) contents during the treatment. (d) *PpBBX16*’s expression pattern during the treatment. (e) Anthocyanin biosynthesis-related genes’ expression profiles during the treatment. Error bars represent the standard deviations of three biological replicates.
**Figure 2** Effects of *PpBBX16*’s ectopic expression in Arabidopsis. (a) The phenotypes of wild-type ‘Columbia O’ and transgenic Arabidopsis. Three independent transgenic lines were used in the experiment. The anthocyanins overaccumulated in the seedlings (upper panel) and the tops of flower stalks (bottom panel) in the transgenic lines. (b) The *PpBBX16*’s expression level in transgenic Arabidopsis lines. (c) The ectopic expression of *PpBBX16* resulted in shortened hypocotyls. (d) The anthocyanin contents in the transgenic lines. (e) The expression levels of anthocyanin biosynthesis-related genes (*AtCHS, AtCHI, AtF3H, AtDFR, AtLDOX, and AtPAP1*) in transgenic lines. The error bars represent the standard deviations of three biological replicates. Asterisks indicate significant differences (two-tailed Student’s t-test, *P* < 0.05, **P** < 0.01).

*PpMYB10*, although these regions contained G-box motifs and can be directly bound by HY5, which binds to the same G-box motif as BBX proteins. We further analysed the trans-activation capability of *PpBBX16* on the anthocyanin-related genes in tobacco. When *PpBBX16* and each of the *PpCHI, PpCHS, PpDFR* and *PpMYB10* promoter-driven luciferase were co-infiltrated, significantly greater luciferase activities were observed, which suggested that *PpBBX16* was able to induce the expression levels of these genes (Figure 3d). As HY5 acted as a partner of BBX22 to promote photomorphogenesis in Arabidopsis, we further analysed the physical interaction between *PpBBX16* and PhHY5. The results showed that *PpBBX16* associated PhHY5 in yeast two-hybrid assay and BiFC assay (Figure 3e,f) and such association enhanced the activation in the promoters of *PpMYB10* and *PpCHS* in dual-luciferase assay (Figure 3g).

**Overexpression of *PpBBX16* in pear calli**

To further confirm the function of *PpBBX16* in anthocyanin biosynthesis of pear, we overexpressed the *PpBBX16* gene in pear calli. The pear calli were induced from the flesh of European pear ‘Clapp’s Favorite’ of fruitlets in our laboratory. Empty and *PpBBX16*-containing calli are usually cultured in the dark and are white to light yellow in colour. When calli were moved to continuous white light, an overaccumulation of anthocyanin was observed after 2 days in *PpBBX16* calli but not in wild-type calli (Figure 4a). As expected, the expression levels of *PpBBX16* were high under both dark and light conditions in transgenic calli, but the anthocyanin biosynthesis-related genes were highly expressed only under light conditions (Figure 4b–d). These results indicated that *PpBBX16* is involved in light-induced anthocyanin biosynthesis.

Furthermore, the Empty and *PpBBX16*-overexpression calli were subjected to RNA-Seq analysis (Figure 5). Light treatment induced the transcription changes of more than 6000 genes, among which 1283 genes overlapped with *PpBBX16*-overexpression calli samples. On the other hand, 2353 genes were identified as light-induced differentially expressed genes specifically in *PpBBX16*-overexpression calli (Figure 5a). Furthermore, the KEGG enrichment analysis was carried out in the differential expression genes between Empty and *PpBBX16*-overexpressive calli under light treatment. The results showed that besides flavonoid biosynthesis pathway, other biological pathways, such as carotenoid biosynthesis, biosynthesis of amino acids, etc., also showed relatively high P-value in this analysis (Figure 5b). These results further confirmed that *PpBBX16* was involved in the flavonoid biosynthesis and other pathways as well.

**Transient expression of *PpBBX16* in pear fruit**

Because the calli were induced from the flesh of European pear, to further confirm the involvement of *PpBBX16* in anthocyanin biosynthesis in the pear peel, we transiently overexpressed *PpBBX16* in ‘Korla’ pear and silenced it in ‘Red Zaoxu’ pear. The transient overexpression of *PpBBX16* in ‘Korla’ pear induced anthocyanin biosynthesis surrounding the injection site after 5 days of light treatment, while no red colour was observed after the injection of *Agrobacteria* containing the empty vector. In contrast, after silencing *PpBBX16* in ‘Red Zaoxu’ fruitlets, anthocyanin biosynthesis was suppressed around the injection site (Figure 6a–d). A further analysis showed that the expression of *PpBBX16* and anthocyanin biosynthetic-related genes were in accordance with the anthocyanin accumulation (Figure 6e).
Because many members of the BBX family have been characterized as light-responsive genes in model plants, we further analysed the BBX family in pear and monitored their expression levels during light-induced anthocyanin biosynthesis. In addition to the 37 BBX proteins identified from published pear genome data, we isolated two additional BBX proteins, PpBBX16-2 and PpBBX21-2, by homologous cloning. In total, 39 BBX family members were identified in pear and were named based on their chromosomal distribution (Figure S2). On the basis of the numbers and distributions of B-box and CCT domains, these BBX proteins were divided into five groups (Figures S2 and S3). Group I contained 11 members that contained two B-box domains in their N termini and one CCT domain near their C termini. Groups II and IV had 11 and 6 members, respectively, with a reverse B-box domain. Groups III (four members) and V (six members) were identified as lacking the CCT domain (Figure S3).

qPCR was performed to determine the expression patterns of BBX gene family members during light exposure at 0, 6, 24, 72, 120 and 240 h. PpBBX1, PpBBX5, PpBBX16, PpBBX16-2, PpBBX35 and PpBBX36 showed sharp increases in expression during the first 24 h, and PpBBX9 and PpBBX17 reached their maximum expression levels by 6 h of light treatment. On the contrary, several genes had greater expression levels during the latter stage of the treatment. The expression levels of PpBBX2, PpBBX4, PpBBX10, PpBBX22, PpBBX23, PpBBX24, PpBBX26, PpBBX28 and PpBBX32 peaked at 120 h. However, the expressions of some BBX genes, such as PpBBX5, showed little change during the entire experiment (Figure S4). The primers are listed in Table S1.

**Discussion**

Multiple lines of evidences showed that although apple and pear are genetically related, their regulation of anthocyanin biosynthesis seems different. Firstly, UV-B irradiation is less effective on pear than that on apple (Zhu et al., 2018). Secondly, apple and pear showed opposing effects of some hormones, such as jasmonic acid and ethylene. Therefore, we speculated that the regulation pathway of pear anthocyanin biosynthesis might be different from that in apple. To uncover such differences, we have clarified the protein-protein interactions and protein-DNA interactions among

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**Figure 3**  PpBBX16 and PpHY5 jointly activated the anthocyanin biosynthesis. (a) Subcellular localization of PpBBX16 expressed in tobacco leaf cells. The β-galactosidase activities reflected the trans-acting activities. (c) Yeast one-hybrid assays of PpBBX16 and the promoter of PpCHS or PpMYB10. PpBBX16 could not interact with either promoter region, even though they harboured G-boxes and can be directly bound by PpHY5 (Tao et al., 2018). (d) PpBBX16 transcriptionally induced the activity of anthocyanin biosynthesis-related genes (PpCH5, PpChit, PpDFR and PpMYB10) in dual-luciferase assay. (e, f) PpBBX16 interacting with PpHY5. The physical interaction of PpBBX16 and PpHY5 was tested by yeast two-hybrid assays (e) and BiFC assays (f). (g) PpBBX16 and PpHY5 jointly promoted the expression of PpMYB10 and PpCHS. Error bars for dual-luciferase assays represent the standard deviation of three independent experiments each with six technical replicates. Lower case letters above bars indicate a significant difference determined by two-way ANOVA followed by multiple comparisons with Tukey’s test (P < 0.05). Asterisks indicate significant differences (two-tailed Student’s t-test, * P < 0.05, ** P < 0.01).
PpCRYs, PpCOP1, PpHY5 and PpMYB10 within the conserved light-responsive pathway in red pear (Tao et al., 2018), but could not find any differences. In the present work, we further analysed light-responsive B-Box proteins in pear. PpBBX16 responds to light and induces anthocyanin biosynthesis

Light is required for pear fruit coloration (Sun et al., 2014). In our previous reports, we confirmed the presence of the conserved light-induced pathway, including the COP1-HY5-MYB10 regulatory module that is responsible for anthocyanin biosynthesis, in the red pear cultivar ‘Red Zaosu’, but this model could still not fully explain its red coloration. The corresponding anthocyanin extracts are shown above each bar. (d) The expression levels of anthocyanin biosynthesis-related genes (PcCHS, PcCHI, PcDFR, PcANS, PcUFGT and PcMYB10) in pear calli. The error bars represent the standard deviations of three biological replicates. Asterisks indicate significant differences (two-tailed Student’s t-test, * P < 0.05, ** P < 0.01).

**Figure 4** The effects of PpBBX16 overexpression in pear calli. (a) PpBBX16’s overexpression resulted in an anthocyanin accumulation after 2 days of the light treatment. (b) PpBBX16’s expression level in transgenic pear calli. (c) Anthocyanin contents in transgenic pear calli after the light treatment. The corresponding anthocyanin extracts are shown above each bar. (d) The expression levels of anthocyanin biosynthesis-related genes (PcCHS, PcCHI, PcDFR, PcANS, PcUFGT and PcMYB10) in pear calli. The error bars represent the standard deviations of three biological replicates. Asterisks indicate significant differences (two-tailed Student’s t-test, * P < 0.05, ** P < 0.01).

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protein (Figure 3a), which is important for its function as a transcription factor. Indeed, PpBBX16 showed a strong transactivation activity in yeast, which indicated that PpBBX16 positively regulated downstream genes (Figure 3b). Thus, PpBBX16 activated the expression of several structural and regulatory genes, such as PpCHI, PpCHS, PpDFR and PpMYB10 (Figure 3e), which is consistent with some reports that BBX genes in Arabidopsis can increase anthocyanin biosynthesis by regulating anthocyanin biosynthesis-related genes’ expression levels (Datta et al., 2008). In transgenic Arabidopsis, these anthocyanin biosynthesis genes were also activated by exogenous PpBBX16 (Figure 2e). To confirm that PpBBX16 contributed to anthocyanin biosynthesis, it was overexpressed in pear flesh-originated calli, which showed that a high level of PpBBX16 expression mediated

![Figure 5](image)

Figure 5  RNA-Seq analysis of PpBBX16 overexpression calli under light/dark condition. (a) The Venn graph of the numbers of differential expression genes between Empty and PpBBX16 overexpression calli under dark/light conditions. (b) The enrichment analysis of KEGG pathways in the differential expression genes between Empty and PpBBX16 overexpression calli under light condition.
the light-induced red coloration of calli under light conditions (Figure 4). RNA-Seq analysis using the calli indicated the enrichment of flavonoid pathway in differential expression genes between light treated Empty and PpBBX16 calli (Figure 5). Further transient assays involving pear peel also confirmed that PpBBX16 was a positive regulator of anthocyanin biosynthesis (Figure 6). These results, together with the results from heterogeneous system, indicated that PpBBX16 is a crucial player in the process of light-induced anthocyanin biosynthesis in pear.

PpBBX16 interacts with PpHY5 and induces the expression of anthocyanin structural genes and transcription factors in light

PpBBX16 could not directly bind the promoter of PpCHS or PpMYB10 in the yeast one-hybrid assay (Figure 3e) even such fragments used in this assay contained G-box motifs but can be bound by PpHY5 (Tao et al., 2018). BBX family members in Arabidopsis, such as AtBBX21, have been identified to bind to the G-box motif and are involved in the photomorphogenesis (Xu et al., 2016), while the apple BBX22 homolog, MdCOL11, potentially binds an unknown motif because a deletion assay identified a region without a G-box that corresponds to the trans-acting activity of MdCOL11 (Bai et al., 2014). The present work, however, failed to observe the physical interaction between PpBBX16 and PpMYB10 promoter, but PpBBX16 was able to induce the promoter activities of PpCHS, PpMYB10 and other anthocyanin-related genes in the dual-luciferase assay. The further analysis showed that PpBBX16 and PpHY5 physically interacted with each other in vivo and in vitro and the PpBBX16/PpHY5 complex strongly induced the promoter activity of PpMYB10 (Figure 3e–g). Therefore, we proposed that full function of PpBBX16 might require the assistance of other transcription factor(s), such as HY5 (Figure 3e–g and Datta et al., 2008) to provide the DNA binding activity. However, PpBBX16 itself had the trans-acting activity, at least in yeast (Figure 3b), suggesting it might function by itself in a PpHY5-independent manner. RNA-Seq analysis identified many other KEGG pathways modulated by the overexpression of PpBBX16 (Figure 5), suggesting that PpBBX16 also functioned in other biological pathways.

Potential functions of other BBX family members in anthocyanin accumulation

BBX genes have been identified in Arabidopsis (Crocco and Botto, 2013), rice (O. sativa L. ssp. Japonica) (Huang et al., 2012) and other plants (Almada et al., 2009). In this work, we identified 39 BBX family members in pear (Figures S2 and S3), which was more than in Arabidopsis (32) or rice (30) (Wu et al., 2012). However, because the pear genome is 3.9 times larger than that of Arabidopsis, the number of BBX family members is not consistent with the greater genome size, indicating that the BBX family expanded to various degrees among different species, probably owing to the different genome duplication events. Additionally, several BBX proteins were not correctly predicted in the published pear genome, such as PpBBX16-2 and PpBBX1-2, which suggested that the pear BBX family might have more members. Because tandem and large-scale segmental duplications affect gene family expansion during plant evolution (Cannon et al., 2004), to detect the origins of duplicated BBX family genes in pear, we analysed the collinearity of the pear BBX family. However, no collinearity was found within the pear BBX family, probably as a result of the limited number of BBX members in the pear genome (Figure S5).
Several BBX family members regulate photomorphogenesis, including anthocyanin accumulation. In Arabidopsis, AtBBX4, AtBBX20, AtBBX21 and AtBBX22 have contradictory functions (Gangappa and Botto, 2014). The B-box domains play crucial roles in the interactions with other proteins. In Arabidopsis, AtBBX21 (Xu et al., 2016), AtBBX22 (Datta et al., 2008), AtBBX24 (Job et al., 2018) and AtBBX25 (Gangappa et al., 2013a) physically interact with HY5, and three BBX proteins interact with HOMOLOG OF HY5 through B-box domains (Gangappa et al., 2013b). A point mutation in the B-box domain impedes the interaction with HY5 (Datta et al., 2007). Additionally, BBX proteins interact with other BBX family members. AtBBX32 interacts with AtBBX21 and suppresses its binding to HY5 (Holtan et al., 2011). Therefore, pear BBX proteins may interact with HY5 or other BBX proteins to induce anthocyanin biosynthesis.

Several BBX family members are regulated by light. The expression levels of PpBBX1, PpBBX8 and PpBBX35 peaked 24 h after the light treatment and then decreased (Figure 5A), which was the same as some anthocyanin biosynthesis-related genes, such as PpPAL, PpLFTP, PpCHS and PpMYB10 (Figure 1e). However, PpBBX4 and PpBBX26 peaked 120 h after the light treatment (Figure 5A). The expression of PpBBX16 increased at 6 h after the light treatment (Figure 1d), which was prior to the increased expression of anthocyanin biosynthesis-related genes and might control those genes’ expression. In addition, the sequence of PpBBX16-2 was very similar to that of PpBBX16. However, they had totally different expression patterns, with PpBBX16 peaking at 6 h, while PpBBX16-2 peaked at 72 h. This might be attributed to the promoter differences that resulted in different expression patterns (Figure 5A). PpBBX16-2 was not found in the genome data, even though it highly similar to MdBXX22, which indicated that BBX family might be larger than predicted by the genome data (Figure S1).

The usage of pear calli in the gene function studies of pear

Since apple calli were firstly used in the studies of anthocyanin-related genes (Xie et al., 2012), they have been widely adopted in gene function studies in the apple, including flavonoid biosynthesis (Hu et al., 2016), the fruit acidity (Zhao et al., 2016), fruit ripening (Li et al., 2017) and abiotic stress (Sun et al., 2018). The use of apple calli enabled the researchers to easily observe effects of genes in the homologous system, thus greatly accelerating the study of gene functions in apples. Recently, the application of citrus calli has also been reported (Lu et al., 2018). The present work firstly reported the application of pear calli for the study of gene function and PpBBX16 was verified to successfully increase the anthocyanin biosynthesis in the transgenic pear calli (Figure 4). We believe that pear calli reported herein will advance the study of gene function in Pyrus species.

Conclusions

Here, we identified a BBX family member, PpBBX16, which, along with the well-characterized CP01–HY5–MYB10 regulatory module, is involved in light-induced anthocyanin biosynthesis. Moreover, other BBX proteins were shown to be involved in light responses and anthocyanin accumulation (Figure 7). Our results provide a series of new target genes involved in the determinants of red coloration in pear that are located upstream of PpMYB10.

**Experimental procedures**

**Plant materials and light treatment**

Six-year-old trees of red pear cultivar ‘Red Zaosu’ in the orchard of the Institute of Horticulture, Henan Academy of Agricultural Sciences (E 113.71°, N 34.71°), were used as materials. The fruitlets were covered with double-layered light-proof paper bags 15 days after full bloom (DAFB), and mature fruits were harvested with bags at 150 DAFB. Approximately 300 uniform, defect-free fruits were collected for postharvest light treatments. Half of these fruits were bag-removed while the other half remained with the bag. All of these fruits were then treated in the phytotron at 17 °C with continuously white light (699 lux). The fruit peels were collected after 0, 6, 24, 72, 144 and 240 h of light treatment, immediately frozen in liquid nitrogen and stored at -80 °C until use.

**Colour and pigment measurements**

Measuring the fruits’ colour was carried out with a chroma meter (CR-400, Konica Minolta, Tokyo, Japan) at four evenly distributed equatorial sites.

The anthocyanins of fruit and pear calli were extracted according to Bai’s protocol with minor modifications (Bai et al., 2014). In brief, 0.1 g of frozen peels was placed in 1 mL methanol: acetic acid (99 : 1, v : v) overnight in the dark at 4 °C. The absorbance of each 100-μL sample extracted was assessed using a spectrophotometer (DU800, Beckman Coulter, Brea, CA, USA) at 530, 620 and 650 nm. The anthocyanin content was calculated using the formula: Absorbance = (A530 - A650, 0.2 × (A650 - A620))/0.2.

To measure the anthocyanin pigments in Arabidopsis, 15 plants were frozen in liquid nitrogen and ground. The powders were immersed in 1 mL methanol: acetic acid (99 : 1, v : v) overnight in the dark at 4 °C. After centrifugation (13 400 g), the liquid phase was used to test the absorption at 530 nm using a spectrophotometer.

Total chlorophyll was extracted and measured using the method described by Huang et al. (2009). A total of 0.5 g fruit peel was homogenized in 3 mL 80% cold acetone. Then, the mixture was centrifuged at 4 °C and 9400 g for 20 min. The absorbance of the extract was measured using a DU800 spectrophotometer at 645 and 663 nm. The total chlorophyll content was calculated using the formula: chlorophyll = (20.2 × A663) + (8.02 × A645).

**Pear fruit RNA isolation and reverse transcription quantitative PCR (qPCR)**

Total RNA of fruit peel was extracted using a modified CTAB method according to Zhang et al.’s protocol (2013). First-strand cDNA was synthesized from 4 μg DNA-free RNA using the oligo(dT) PrimerScriptTM RT reagent Kit with gDNA Erase (RR047Q, Takara, Otsu, Japan) following the manufacturer’s instructions. The cDNA was diluted threefold and used as the template for gene cloning and qPCR analysis. qPCR was performed using a CFX96 real-time PCR detection system (CFX96, Bio-Rad, Hercules, CA, USA). A template-negative control for each primer pair was included for each run. All relative expression levels were calculated using the 2^−ΔΔCt method against the pear Actin or Arabidopsis PP2A (JN684184 or AT1G13320, respectively) gene. The analysis was performed with three biological replicates. The primers were designed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
Subcellular localization analysis

The subcellular localization of PpBBX16 was determined using the method of Yang et al. (2018). In brief, the full-length CDSs (without termination codons) of the target genes were independently cloned into the pCambia1300 vector, which contained the CaMV 35S promoter and GFP gene, resulting in fusion genes driven by the 35S promoter (primer listed in Table S1). Agrobacterium tumefaciens lines harbouring the vectors were independently infiltrated into the leaves of Nicotiana benthamiana transgenic lines containing red fluorescent protein expression in the nuclei. The fluorescence was observed by confocal laser scanning microscopy (A1, Nikon, Tokyo, Japan).

Dual-luciferase assay

Dual-luciferase assays were performed with tobacco (N. benthamiana) in accordance with a previous publication (Niu et al., 2016). In brief, the full-length CDS of the BBX gene was cloned into pGreenII 0029 without the termination codon. The promoter sequence of the gene was inserted into pGreenII 0800-LUC. Both constructs were individually transformed into A. tumefaciens GV3101 (containing the pSoup vector) using the freeze–thaw method. Agrobacterium infiltration was carried out with a needle-free syringe. The Firefly luciferase and Renilla luciferase activities were analysed 72 h after infiltration using the Dual-Luciferase Reporter Assay System (E710, Promega, Madison, WI, USA) with Modulus Luminometers (GloMax96, Promega). Both luciferase activities were analysed in three independent experiments with at least six biological replications for each assay.

Yeast one-hybrid assays

Yeast one-hybrid assays were performed using the Matchmaker Gold Yeast One-Hybrid System Kit (TaKaRa) according to the manufacturer’s protocol. Briefly, promoter fragments were ligated into the pAbAi vector, and PpBBX16 was cloned into the pGADT7 vector. The pAbAi vector was linearized and transformed into the yeast strain Y1HGold. Transformsants were selected on plates containing a selective synthetic dextrose medium lacking uracil. The prey vectors were then transformed into Y1HGold cells harbouring the pAbAi-bait and tested on SD/-Leu/AbA plates.

Yeast two-hybrid assays

The yeast two-hybrid assays were performed using the Matchmaker Gold Yeast Two-Hybrid System Kit (TaKaRa, Dalian, China) in accordance with the manufacturer’s instructions with a minor modification. Briefly, 500 ng pGADT7 (AD) and 500 ng pGBK7 (BD) vectors were simultaneously transformed into Y2HGold yeast cells using the polyethylene glycol/lithium acetate method. The transformsants were selected on QDO/X plates (SD/-Trp/-Leu/-His/Ade/X-α-gal). The pGADT7-T and pGBK7-T3 vectors provided with the kit were used as positive controls.

Bimolecular fluorescence complementation (BiFC) assays

For bimolecular fluorescence complementation (BiFC) assays, the coding sequences (CDSs, without the termination codon) were cloned into the YFP (p2YN) or YFP* (p2YC). Then, A. tumefaciens strain GV3101 harbouring the constructs was transiently co-expressed in all possible combinations of p2YN and p2YC fusion proteins in Nicotiana benthamiana leaves. Fluorescence was observed according to subcellular localization assays.

Transient transformation analysis in pear fruit

For the transient overexpression assay, the mature fruit of ‘Korla’ pear (Pyrus sinkiangensis, the hybrid species of Pyrus communis and East Asian pear cultivars) was infected. ‘Korla’ pear trees bear...

Figure 7 Proposed model of the mechanism of regulating anthocyanin accumulation through PpBBX16 in pear. (a) Red pear under dark conditions cannot accumulate anthocyanin. The dashed lines represent pathways already reported in Arabidopsis: Line 1, BBX proteins could be degraded through the 26S proteasome pathway by interactions with E3 ubiquitin ligases, such as COP1 (Datta et al., 2008). Line 2, The functions of BBX members can be suppressed by other BBX proteins (Holtan et al., 2011). (b) Red pear under light conditions accumulate anthocyanin through the PpBBX16-involved pathway: PpBBX16 and PpHY5 jointly activate the expression of structural genes (Arrow 3) and PpMYB10 (Arrow 4), which further induces the expression levels of structural genes.
fruit that are blushed on the side exposed to sun, which indicates the presence of an intact anthocyanin biosynthesis pathway in this cultivar, which makes it a suitable material for observing the induction of coloration. The fruits were infiltrated with the pCambia1301-PpBBX16 vector in A. tumefaciens “GV3101” with injection syringes according to Li et al.’s protocol with slight modifications (Li et al., 2012). Briefly, A. tumefaciens was grown to saturation in Luria-Bertani medium. After centrifugation (13,400 g), the pellet was resuspended in the infection solution (10 mM MgCl₂, 10 mM MES and 150 mM acetoxyrroline), and the mixture was kept at room temperature for 1 h. After infiltration, the fruits were kept under dark conditions for 1 day and then treated with continuous white light for 5 days. The empty pCambia1301 vector was used as the negative control (Empty). After images were taken, the fruit peel surrounding the injection sites was collected, immediately frozen in liquid nitrogen and stored at −80 °C until use.

For the virus-induced gene silencing (VIGS) assay, a PpBBX16 fragment (417-772 bp) was amplified using the primer pair BBX16VIGS-F/BBX16VIGS-R and cloned into the pTRV2 vector (pTRV2-PpBBX16). The resulting vector was introduced into A. tumefaciens strain EHA105 and then used for pear fruit infiltration. The ‘Red Zaosu’ pear fruitlets (50 DAFB) were used for the VIGS assays because they can achieve a full dark red colour, making them ideal for the analysis of coloration inhibition. The injection solution harbouring pTRV2-PpBBX16 was co-infiltrated with pTRV1 in fruitlets of ‘Red Zaosu’ pear on the tree. Then, 10 days after infiltration, the peels were collected, immediately frozen in liquid nitrogen and stored at −80 °C until use. Empty pTRV1 and pTRV2 were co-infiltrated as the negative control (pTRV).

Induction of pear calli and their transformation

The pear calli were induced from the flesh of young ‘Clapp’s Favorite’ (P. communis) fruit on the NN69 (NITSCH and NITSCH 1969) solid medium with addition of sucrose (30 g/L), 6-benzylaminopurine (0.5 mg/L) and 2,4-dichlorophenoxyacetic acid (1.0 mg/L). The first-generation calli were subcultured several times, and the rapidly growing soft calli were screened and maintained under dark conditions on the MS (Murashige and Skoog) solid medium supplemented with sucrose (30 g/L), 6-benzylaminopurine (0.5 mg/L) and 2,4-dichlorophenoxyacetic acid (1.0 mg/L). The transformation of pear calli was performed as follows: pear calli were soaked in A. tumefaciens strain EHA105 (0.5 OD₆₀₀) containing either the pCambia1301-PpBBX16 vector or the empty pCambia1301 vector for 10 min. After 3 days coculture, the calli were then screened on MS solid medium mentioned above by 10 mg/L hygromycin B under continuous dark conditions at 24 °C. For the light treatment, the newly subcultured empty or PpBBX16-containing calli were moved to the light conditions (16-h light/8-h dark) for 2 day and then used for observation.

RNA-Seq analysis

We used the pear calli harbouring Empty vector and pCambia1301-PpBBX16 vector for RNA-Seq analysis. The calli were incubated under dark or light for 2 days for RNA-Seq. The calli of three different lines were simultaneously treated and used as biological replicates. The total RNAs were extracted as described above. Ten micrograms of total RNA each was used for next-generation sequencing. The library construction and sequencing were performed by Novogene (Beijing, China) using the HiSeq X (Illumina, San Diego, CA) platform with a 150-bp pair-end strategy. The clean reads were mapped to the European pear genome sequence (Chagné et al., 2014; http://www.rosaceae.org) using HISAT2 (Pertea et al., 2016) with default parameters. The reads were then assembled into transcripts and compared with reference gene models using StringTie (Pertea et al., 2016). The differentially expressed gene analysis was performed using DESeq2 (Love et al., 2014).

Arabidopsis’ hypocotyl measurement

Seeds of wild-type (Col-0) A. thaliana and overexpression lines (35S:PpBBX16) in the Col-0 background were grown on 1/2 MS medium. Seeds were subjected to a chilling treatment at 4 °C for 72 h and then transferred into white light at 24 °C under long-day conditions (16-h light/8-h dark). Five-day-old Arabidopsis seedlings were used for hypocotyl measurements. At least 10 seedlings were imaged, and hypocotyl lengths were measured using ImageJ software (https://imagej.nih.gov/ij/).

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Conflict of interest

The authors declare that they have no competing interests.

Authors’ contributions

SB and YTeng conceived and planned the study; RT performed most of the experiments; JN collected the samples and extracted total RNAs for qPCR; YTang and YM produced the pear calli from pear fruit; YL and ZY helped with the qPCR assays and the transformation of Arabidopsis and pear calli; QY and XY were involved in the bioinformatics analysis; ZW managed the pear trees and collected the fruits from the orchard; and SB, RT and YTeng wrote the manuscript. All authors read and approved the final manuscript.

References

Allan, A.C. and Espley, R.V. (2018) MYBs drive novel consumer traits in fruits and vegetables. Trends Plant Sci. 23, 693–705.
Almada, R., Cabrera, N., Casaretto, J.A., RuizLara, S. and González, V.E. (2009) WCO and WCOL1, two CONSTANS homologous genes, are regulated during flower induction and dormancy in grapevine buds. Plant Cell Rep. 28, 1193–1203.
An, J.P., Qu, F.J., Yao, J.F., Wang, X.N., You, C.X., Wang, X.F. and Hao, Y.J. (2017) The bZIP transcription factor MdHY5 regulates anthocyanin accumulation and nitrate assimilation in apple. Hortic. Res. 4, 17023.
Bai, S.L., Saito, T., Honda, C., Hatsuyma, Y., ito, A. and Moriguchi, T. (2014) An apple B-box protein, MdCOL11, is involved in UV-B- and temperature-induced anthocyanin biosynthesis. Planta. 240, 1051–1062.
Bai, S.L., Sun, Y.W., Qian, M.J., Yang, F.X., Ni, J.B., Tao, R.Y., Lin, L. et al. (2017) Transcriptome analysis of bagging-treated red Chinese sand pear peels reveals light-responsive pathway functions in anthocyanin accumulation. Sci. Rep. 7, 63.
Borden, K.L. (1998) RING fingers and B-boxes: zinc-binding protein-protein interaction domains. Biochem. Cell Biol. 76, 351–358.
Cannon, S.B., Mitra, A., Baumgarten, A., Young, N.D. and May, G. (2004) The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol.* 4, 1–21.

Chagné, D., Crowhurst, R.N., Pindo, M., Thrimawithana, A., Deng, C., Ireland, H., Fiers, M. et al. (2014) The draft genome sequence of European pear (*Pyrus communis L. ‘Bartlett’*). *PloS ONE* 9, e92644.

Chang, C.S.J., Li, Y.H., Chen, L.T., Chen, W.C., Hsieh, W.P., Shin, J., Jane, W.N. et al. (2008) LFZ1, a HYS-regulated transcriptional factor, functions in Arabidopsis de-etiolation. *Plant J.* 54, 205–219.

Crocco, C.D. and Botto, J.F. (2013) BX3 proteins in green plants: insights into their evolution, structure feature and functional diversification. Gene 531, 44–52.

Datta, S., Hettiarachchi, C., Johansson, H. and Holm, M. (2007) SALT TOLERANCE HOMOLOG2, a B-box protein in Arabidopsis that activates transcription and positively regulates light-mediated development. *Plant Cell* 19, 3242–3255.

Datta, S., Johansson, H., Hettiarachchi, C., Irigoyen, M.L., Desai, M., Rubio, V. and Holm, M. (2008) LFZ1/SALT TOLERANCE HOMOLOG3, an Arabidopsis B-box protein involved in light-dependent development and gene expression, undergoes COP1-mediated ubiquitination. *Plant Cell* 20, 2324–2338.

Duan, H., Cvracke, R., Grotewold, E., Weisshaar, B., Martin, C. and Lepiniec, L. (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci. 15, 573–581.

Feng, S.Q., Wang, Y.L., Yang, S., Xu, Y.T. and Chen, X.S. (2010) Anthocyanin biosynthesis in pears is regulated by a R2R3-MYB transcription factor PmMYB10. *Planta* 232, 245–255.

Gangappa, S.N. and Botto, J.F. (2014) The BX3 family of plant transcription factors. *Trends Plant Sci.* 19, 460–470.

Gangappa, S.N., Crocco, C.D., Johansson, H., Datta, S., Hettiarachchi, C., Holm, M. and Botto, J.F. (2013a) The Arabidopsis B-BOX protein BBX25 interacts with HYS, negatively regulating BBX22 expression to suppress seedling photomorphogenesis. *Plant Cell* 25, 1243–1257.

Gangappa, S.N., Holm, M. and Botto, J.F. (2013b) Molecular interactions of BBX24 and BBX25 with HYH, HYS HOMOLOG, to modulate Arabidopsis seedling development. *Plant Signal. Behav.* 8, e25208.

He, Y., Li, W., Lv, J., Jia, Y.B., Wang, M.C. and Xia, G.M. (2012) Ecotypic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 1511–1522.

Holm, H.E., Bandong, S., Marion, C.M., Adam, L., Tiwari, S., Shen, Y., Maloof, J.N. et al. (2011) LZF1, a HY5-regulated transcriptional factor, functions in *Arabidopsis de-etiolation*. *J. Exp. Bot.* 62, 4603–4615.

Holtan, H.E., Bandong, S., Marion, C.M., Adam, L., Tiwari, S., Shen, Y., Maloof, J.N. et al. (2011) LZF1, a HY5-regulated transcriptional factor, functions in *Arabidopsis de-etiolation*. *J. Exp. Bot.* 62, 4603–4615.

Jiang, Y. (2017) *Genome Res.* 27, 864–876.

Jin, W., Wang, Z.W., Shi, Z.B., Zhang, S., Ming, R., Zhu, S.L., Khan, M.A. et al. (2012) The genome of pear (*Pyrus bretschneideri Rehd.*). *Genome Res.* 23, 396–408.

Xie, X., Li, S., Zhang, R., Zhao, J., Chen, Y., Zhao, Q., Yao, et al. (2012) The bHLH transcription factor MdhHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. *Plant Cell Environ.* 35, 1884–1897.

Xu, D.Q., Li, J.G., Ganganna, S.N., Hettiarachchi, C., Lin, F., Andersson, M.X., Wang, Y., Xu, D.Q., Li, J.G., Gangappa, S.N., Lin, F., Andersson, M.X., Wang, Y. et al. (2014) The BBX family of plant transcription factors. *J. Exp. Bot.* 65, 4288–437.

Yang, Q.S., Li, W., Lv, J., Jia, Y.B., Wang, M.C. and Xia, G.M. (2012) Ecotypic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 1511–1522.

Jiang, Y. (2017) *Genome Res.* 27, 864–876.

Xie, X., Li, S., Zhang, R., Zhao, J., Chen, Y., Zhao, Q., Yao, et al. (2012) The bHLH transcription factor MdhHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. *Plant Cell Environ.* 35, 1884–1897.

Xu, D.Q., Li, J.G., Ganganna, S.N., Hettiarachchi, C., Lin, F., Andersson, M.X., Wang, Y. et al. (2014) The BBX family of plant transcription factors. *J. Exp. Bot.* 65, 4288–437.

Yang, Q.S., Li, W., Lv, J., Jia, Y.B., Wang, M.C. and Xia, G.M. (2012) Ecotypic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 1511–1522.

Jiang, Y. (2017) *Genome Res.* 27, 864–876.

Xie, X., Li, S., Zhang, R., Zhao, J., Chen, Y., Zhao, Q., Yao, et al. (2012) The bHLH transcription factor MdhHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. *Plant Cell Environ.* 35, 1884–1897.

Xu, D.Q., Li, J.G., Ganganna, S.N., Hettiarachchi, C., Lin, F., Andersson, M.X., Wang, Y. et al. (2014) The BBX family of plant transcription factors. *J. Exp. Bot.* 65, 4288–437.

Yang, Q.S., Li, W., Lv, J., Jia, Y.B., Wang, M.C. and Xia, G.M. (2012) Ecotypic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 1511–1522.

Jiang, Y. (2017) *Genome Res.* 27, 864–876.
bud dormancy transition in ‘Suli’ pear (Pyrus pyrifolia white pear group). Plant Physiol. Biochem. 127, 355–365.

Yao, G.F., Ming, M.L., Allan, A.C., Gu, C., Li, L.T., Wu, X., Wang, R.Z. et al. (2017) Map-based cloning of the pear gene MYB114 identifies an interaction with other transcription factors to coordinately regulate fruit anthocyanin biosynthesis. Plant J. 92, 437–451.

Zhang, D., Qian, M.J., Yu, B. and Teng, Y.W. (2013) Effect of fruit maturity on UV-B-induced post-harvest anthocyanin accumulation in red Chinese sand pear. Acta Physiol. Plant. 35, 2857–2866.

Zhang, X.Y., Huai, J.L., Shang, F.F., Xu, G., Tang, W.J., Jing, Y.J. and Lin, R.C. (2017) A PIF1/PIF3-HY5-BBX23 transcription factor cascade affects photomorphogenesis. Plant Physiol. 174, 2487–2580.

Zhao, Q., Ren, Y.R., Wang, Q.J., Wang, X.F., You, C.X. and Hao, Y.J. (2016) Ubiquitination-related MdBT scaffold proteins target a bHLH transcription factor for iron homeostasis. Plant Physiol. 172, 1973–1988.

Zhu, Y.F., Su, J., Yao, G.F., Liu, H.N., Gu, C., Qin, M.F., Bai, B. et al. (2018) Different light-response patterns of coloration and related gene expression in red pears (Pyrus L.). Sci. Hortic. 229, 240–251.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Alignment and phylogenetic tree of PpBBX16.b

Phylogenetic tree analysis of PpBBX16 and PpBBX16-2.

Figure S2 The phylogenetic analysis of BBX proteins according to their protein sequences.

Figure S3 Structures of the PpBBX proteins.

Figure S4 qRT-PCR analysis of the expression patterns of members of BBX gene family.

Figure S5 Collinearity analysis detected the genome-wide collinear gene pairs but no BBX genes were detected.

Table S1 Primer list used in the present work.