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Apple RING E3 ligase MdMIEL1 inhibits anthocyanin accumulation by ubiquitinating and degrading MdMYB1 protein

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MdMYB1 is an important regulator for anthocyanin accumulation in apple (*Malus* × *domestica*). Here, an apple RING E3 ligase, MdMIEL1, was screened out as a partner of MdMYB1 with a yeast two-hybrid approach. Pull-down, bimolecular fluorescence complementation and coimmunoprecipitation assays further verified the interaction between MdMIEL1 and MdMYB1 proteins. Subsequently, in vitro and in vivo experiments indicated that MdMIEL1 functioned as a ubiquitin E3 ligase to ubiquitinate MdMYB1 protein, followed by degradation through a 26S proteasome pathway. Furthermore, transgenic studies in apple calli and *Arabidopsis* demonstrated that MdMIEL1 negatively regulated anthocyanin accumulation by modulating the degradation of MdMYB1 protein. Taken together, our findings provide a new insight into the molecular mechanism by which MdMIEL1 negatively regulates anthocyanin biosynthesis by ubiquitinating and degrading MdMYB1 protein.

Keywords: Anthocyanin • posttranslational modification • transcription factor MdMYB1 • ubiquitination • ubiquitin E3 ligase MdMIEL1.

Abbreviations: aa, amino acid; ABA, abscisic acid; AtMIEL1, *Arabidopsis MYB30*-interacting E3 ligase 1; 6-BA, 6-Benzylaminopurine; bHLH, basic helix-loop-helix; BiFC, bimolecular fluorescence complementation; Col-0, *Arabidopsis thaliana* ecotype Columbia; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; GFP, green fluorescent protein; GST, glutathione; HECT, homologous to E6-associated protein C-terminus; HIS, histidine; MBW, MYB-bHLH-WD40/WDR; MS, Murashige and Skoog; NBT, nitroblue tetrazolium; OX, overexpression; RING, Really Interesting New Gene; ROS, reactive oxygen species; TF, transcription factor; WT, wild-type; Y2H, yeast two-hybrid; YFP^C, C-terminal fragment of yellow fluorescent protein; YFP^N, N-terminal fragment of yellow fluorescent protein.

Introduction

Anthocyanins are flavonoid secondary metabolites. They are responsible for not only tolerance to various stresses but also organ color (Mazza and Miniati 1993, Lev-Yadun and Gould 2008). In fruit and floral crops, organ color is an important exterior quality that determines market value. In many plant species, anthocyanins produce the color of red organs such as fruits, flowers and seeds. Anthocyanins are biosynthesized through the phenylpropanoid pathway (Ubi et al. 2006). Generally, their accumulation is accompanied by the ripening or maturation process in organs such as flowers and fruits (Jaakola 2013, Medina-Puche et al. 2014). As a type of compound in the flavonoid metabolic pathway, anthocyanins are biosynthesized through multiple biochemical reactions, and the catalytic enzymes and their encoding structural genes have been identified in various plant species (Jaakola 2013, Xu et al. 2015).

The MYB-bHLH-WD40/WDR (MBW) complex, containing MYB transcription factors (TFs), basic helix-loop-helix (bHLH) and WD-repeat protein, plays a critical role in the regulation of anthocyanin biosynthesis (Allan et al. 2008, Gonzalez et al. 2008). A series of MBW regulators that modulate anthocyanin biosynthesis and fruit coloration have been identified and functionally characterized in a variety of plants (Xu et al. 2015). Among them, R2R3 MYB TFs such as AtPAP1/2 and their homologs are involved in the regulation of anthocyanin biosynthesis by directly binding to the promoters of anthocyanin structural genes (Gonzalez et al. 2008, Zuluaga et al. 2008).

In higher plants, numerous developmental and environmental factors, such as hormones, light, temperature and nutrition, influence the expression of MBW genes, thereby regulating the accumulation of anthocyanins (Jaakola 2013). For example, MYB TF genes *PAP1*, *PAP2*, *AtMYB113* and *AtMYB114* are upregulated by low temperature and high light in *Arabidopsis* (Rowan et al. 2009, Dubos et al. 2010). Similarly, environmental factors also influence the expression of anthocyanin-related *MYB* genes in fruit trees (Steyn et al. 2009, Lin-Wang et al. 2011).

Besides transcriptional regulation, the MBW proteins are also modulated at post-transcriptional levels in response to different stimuli (Jaakola 2013, Maier et al. 2013). The brassinosteroid (BR) signal inhibits MBW activity by phosphorylating WD40 repeat protein TTG1 as well as bHLH TF EGL3 in *Arabidopsis* (Cheng et al. 2014). In apple, MdbHLH3, a homolog of AtGL3, is phosphorylated in response to low temperature and glucose signals (Hu et al. 2016). Besides phosphorylation modification, the ubiquitin/26S proteasome pathway, which contains ubiquitin-activating enzymes (E1s), ubiquitin-

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conjugating enzymes (E2s) and ubiquitin ligases (E3s), plays an important role in regulating plant growth and development by targeting proteins for degradation (Serino and Xie 2013). Among these components, E3 ubiquitin ligase is the key factor that determines the specificity of the protein substrate (Hare et al. 2003, Stone and Callis 2007). E3 proteins are classified into four main enzyme types: HECT (Homologous to E6-associated protein C-Terminus), RING/U-Box (Really Interesting New Gene/U-Box), SCF (a complex of Skp1, CDC53 and F-box protein) and APC (anaphase-promoting complex) ligases (Vierstra 2003, Smalle and Vierstra 2004).

Among these various E3 categories, the RING domain was initially identified in the *Really Interesting New Gene* encoding protein (Freemont 1993) and has subsequently been found in multiple key regulatory proteins (Xie et al. 2002). So far, multiple RING-type proteins have been identified to be involved in various biological processes (Berndsen and Wolberger 2014). For instance, in *Arabidopsis* E3 ligase SDIR1 and KEG confer stress tolerance through regulating abscisic acid (ABA) biosynthesis or signal pathway (Zhang et al. 2007, Liu and Stone, 2010). In rice, OsDSG1 and OsHATS have also been proved to play a key role in plant defense responses (Park et al. 2010, Liu et al. 2016).

Besides stress responses, RING E3 ligases also participate in the regulation of anthocyanin biosynthesis. In both *Arabidopsis* and apple, photoperiod-responsive RING E3 ligase COP1 interacts with and subsequently ubiquitinates and degrades MYB TFs via the 26S ubiquitin–proteasome system, leading to less anthocyanin accumulation (Li et al. 2012, Maier et al. 2013). Furthermore, the E3 ubiquitin ligase NLA has been found to participate in the regulation of anthocyanin biosynthesis in response to limited nutrients (Peng et al. 2008).

It is well known that MdMYB1 and its alleles are positive regulators in anthocyanin biosynthesis in apple (Takos et al. 2006, Ban et al. 2007, Espley et al. 2007). In this study, MdMIEL1, a novel RING-finger protein, was screened as an MdMYB1-interacting protein in apple. Subsequently, it was characterized with the function of regulating anthocyanin accumulation. Finally, the molecular mechanism by which this MdMYB1-interacting RING E3 ligase regulates anthocyanin accumulation is analyzed and discussed.

Results

MdMIEL1 negatively regulates anthocyanin accumulation

Yeast two-hybrid (Y2H) screening through an apple cDNA library was performed using MdMYB1 as a bait. As a result, the gene MDP0000185659 was obtained. Its full-length coding sequence is 804 bp in size and encodes a predicted protein with 267 amino acid (aa) residues. The predicted protein contains a conserved zinc-finger domain (18–99 aa) in the N-terminus and a RING-finger domain (153–195 aa) in the C-terminus (**Fig. 1a**). In addition, its amino acid sequence is highly similar to that of the ubiquitin RING E3 ligase AtMIEL1 (*Arabidopsis MYB30*-Interacting E3 Ligase 1) (**Fig. 1a**), as demonstrated by the phylogenetic analysis (**Fig. 1b**). Hereafter it is referred to as MdMIEL1.

To characterize the function of MdMIEL1, transgenic calli were generated by genetically introducing *35S::MdMIEL1-Flag* or *35S::MdMIEL1-Anti* into the calli of 'Orin' apple. As a result, overexpression calli with a Flag tag (MdMIEL1-OX) and antisense suppression calli (MdMIEL1-Anti) were obtained (Fig. S1a). It was found that, compared with wild-type (WT) controls, MdMIEL1-OX accumulated less anthocyanin but MdMIEL1-Anti accumulated more (**Figs. 1c, d**, S2a–c). Additionally, the expression levels of flavonoid structural genes in the WT, MdMIEL1-OX and MdMIEL1-Anti were analyzed with qRT-PCR assays. The results showed that *MdMIEL1* overexpression slightly downregulated the expression of primary genes such as *MdDFR*, *MdUF3GT*, *MdF3H*, *MdCHI*, *MdCHS* and *MdANR*, which are involved in the anthocyanin biosynthetic pathway in transgenic MdMIEL1-OX calli. In contrast, *MdMIEL1* suppression activated the expression of these genes in transgenic MdMIEL1-Anti calli (**Figs. 1e**, S2d).

To further verify its function, *35S::MdMIEL1-Flag* was genetically transformed into and ectopically expressed in *Arabidopsis*. Three transgenic lines (lines 1, 7 and 8) were obtained and used for further investigation (Fig. S1b). The three transgenic lines produced much less anthocyanin than the WT control (**Fig. 2a, b**), indicating that *MdMIEL1* ectopic expression inhibited the accumulation of anthocyanins. Meanwhile, *MdMIEL1* ectopic expression noticeably suppressed the expression of the anthocyanin biosynthesis-related genes *AtMYB75*, *AtDFR*, *AtUF3GT*, *AtCHI* and *AtCHS* (**Fig. 2c**). These results indicate that MdMIEL1 negatively regulates anthocyanin accumulation in *Arabidopsis*.

Because anthocyanin acts as a reactive oxygen species (ROS) scavenger (Apel and Hirt 2004, Halliwell 2006), ROS content was also measured in transgenic *Arabidopsis* and apple calli. The results showed that MdMIEL1 obviously enhanced ROS accumulation in transgenic *Arabidopsis* and apple calli (Fig. S3).

MdMIEL1 directly interacts with MdMYB1

To verify the interaction between MdMIEL1 and MdMYB1 proteins, an in vitro pull-down assay using the co-purification of recombinant MdMIEL1-GST (GST, glutathione) fusion protein with a histidine (HIS) tag sequence or MdMYB1-HIS was performed. The results showed that MdMYB1-HIS was pulled down by MdMIEL1-GST but HIS failed to be co-purified by MdMIEL1-GST (**Fig. 3a**), indicating that MdMIEL1 interacted with MdMYB1.

In addition, bimolecular fluorescence complementation (BiFC) experiments were performed in agro-infiltrated onion epidermal cells to provide further evidence for molecular interaction between MdMIEL1 and MdMYB1 in plant cells. MdMIEL1 was fused to the N-terminal fragment of yellow fluorescent protein (YFP^N) to generate MdMIEL1-YFP^N, and MdMYB1 was fused to the C-terminal fragment of YFP (YFP^C) generating MdMYB1-YFP^C. As shown in **Fig. 3b**, yellow fluorescence signal was detected in the nuclei when there was pairwise expression of MdMIEL1-YFP^N and MdMYB1-YFP^C. However, no fluorescence was observed in the control combinations of MdMIEL1-YFP^N and YFP^C, or MdMYB1-YFP^c and YFP^N. Therefore, MdMIEL1 protein physically interacts with MdMYB1 in the nucleus.

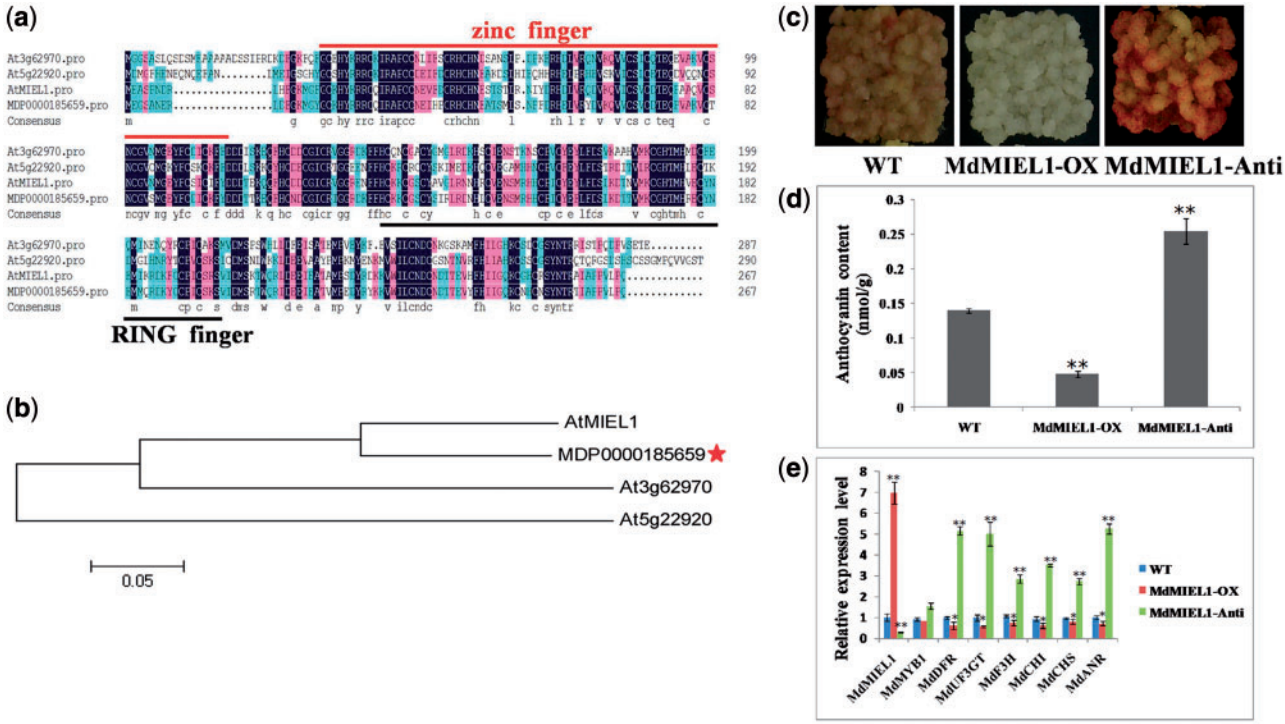

Fig. 1 Functional characterization of MdMIEL1 in apple. (a) Protein alignment for MdMIEL1 and its homologs in *Arabidopsis*. The zinc-finger domain and the RING finger are indicated with red and black bars, respectively. (b) The phylogenetic tree for the MdMIEL1 protein with its homologs. The scale bar indicates branch length. (c, d) Anthocyanin contents in the transgenic calli (MdMIEL1-OX and MdMIEL1-Anti) and the wild-type (WT) control grown on medium. The anthocyanin content of WT is set as control. (e) Relative expression levels of *MdMYB1*, *MdMYB110*, *MdDFR*, *MdUF3GT*, *MdF3H*, *MdCHI*, *MdCHS* and *MdANR* in transgenic calli and WT control. The value for WT was set to 1.

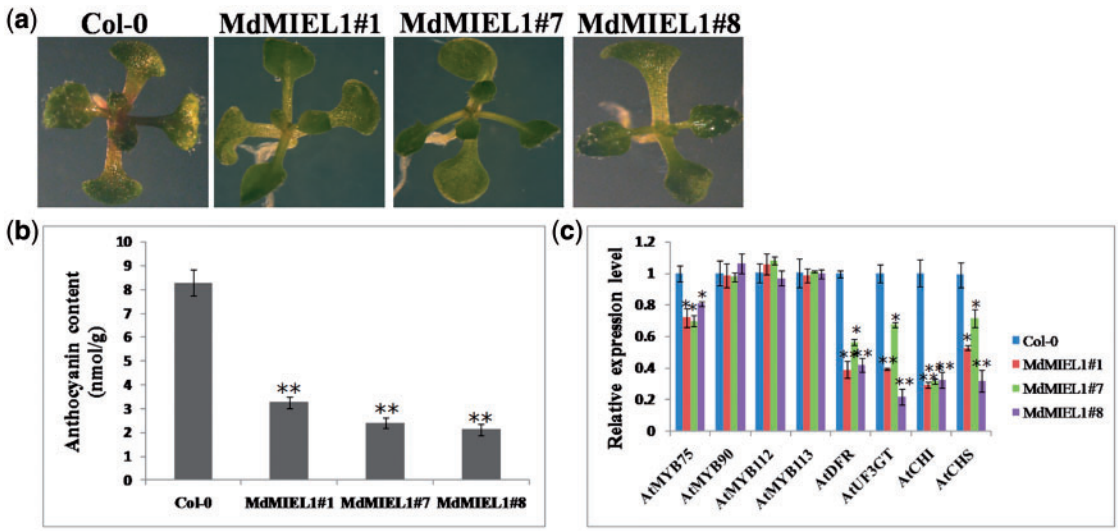

Fig. 2 Ectopic expression of *MdMIEL1* in *Arabidopsis* decreases anthocyanin contents. (a, b) Analysis of anthocyanin contents in 2-week-old wild-type (Col-0) and transgenic plants (#1, #7 and #8) grown in a high-light, low-temperature incubator. The anthocyanin content of Col-0 was set as control. (c) Relative expression levels of genes involved in anthocyanin biosynthesis (*AtMYB75*, *AtMYB90*, *AtMYB112*, *AtMYB113*, *AtDFR*, *AtUF3GT*, *AtCHI*, and *AtCHS*) in transgenic and wild-type *Arabidopsis*. The value for Col-0 was set to 1.

Furthermore, the interaction between MdMIEL1 and MdMYB1 was also confirmed with an in vivo coimmunoprecipitation assay, using MdMYB1-GFP (green fluorescent protein) (single) and MdMYB1-GFP/MdMIEL1-OX (overexpression) (double) transgenic apple calli. Consistent with the results of the pull-down and BiFC assays, MdMIEL1 protein was detected in the presence of the anti-Flag antibody when anti-GFP antibody was used as a negative control (**Fig. 3c**), demonstrating that MdMIEL1 interacts with MdMYB1 in vivo.

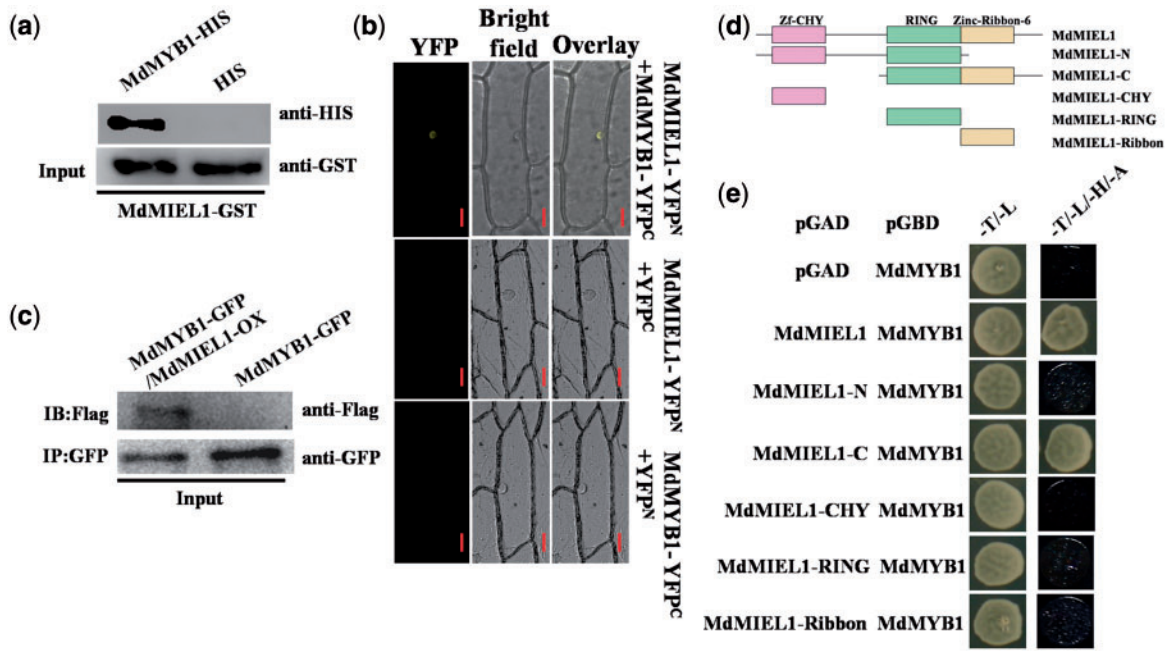

Fig. 3 MdMIEL1 directly interacts with MdMYB1. (a) Pull-down assay analyzing interaction of MdMIEL1 with MdMYB1. *Escherichia coli*-expressed HIS or MdMYB1-HIS proteins were incubated with a cobalt chelate affinity resin containing the immobilized GST-tagged MdMIEL1 protein. The protein mixtures were purified using a GST purification kit. (b) Bimolecular fluorescence complementation assays showing the interaction between MdMIEL1 and MdMYB1 in nuclei of epidermal cells of onion (*Allium cepa*). Bars = 10 μm. (c) Coimmunoprecipitation detection of the interaction. MdMIEL1-Flag and MdMYB1-GFP were immunoprecipitated from protein extracts of MdMYB1-GFP/MdMIEL1-Flag (left) and MdMYB1-GFP (right) transgenic calli using anti-Flag and anti-GFP antibodies, respectively. Input proteins and the immunoprecipitates were analyzed by immunoblotting using anti-Flag and anti-GFP antibodies. (d) Schematic diagram of full-length MdMIEL1 and deletion derivatives used for structural domain analysis and yeast two-hybrid assays. MdMIEL1 (1–267 aa), MdMIEL1-N (1–195 aa), MdMIEL1-C (153–267 aa), MdMIEL1-CHY (18–99 aa), MdMIEL1-RING (153–195 aa) and MdMIEL1-Ribbon (200–258 aa). (e) The C terminus of MdMIEL1 interacts specifically with MdMYB1 in yeast two-hybrid assays. The indicated regions of *MdMIEL1* were cloned into the prey vector pGAD. Empty pGAD plus pGBD-MdMYB1 was used as control. Yeasts grown in SD (−Trp/−Leu) medium and SD (−Trp/−Leu/−His/−Ade) medium are indicated.

To determine the domains of MdMIEL1 involved in the interaction with MdMYB1, Y2H assays using MdMYB1 and truncated regions of the MdMIEL1 protein were performed. The full-length *MdMIEL1* gene and its truncated regions were fused with pGAD (GAL4-DNA activation domain) and named as follows: pGAD-*Ribbon* (200–258 aa), pGAD-*RING* (153–195 aa), pGAD-*CHY* (18–99 aa), pGAD-*MdMIEL1-N* (1–195 aa) and pGAD-*MdMIEL1-C* (153–267 aa) (**Fig. 3d**). The results showed that pGBD-*MdMYB1* and pGAD-*MdMIEL1* yeast co-transformants as well as pGBD-*MdMYB1* and pGAD-*MdMIEL1-C* yeast co-transformants were able to grow on SD/−Trp−Leu−His−Ade selection plates (**Fig. 3e**). These results indicate that the C-terminal region of MdMIEL1, which encompasses the RING-finger domain, is involved in the interaction with MdMYB1. Meanwhile, MdbHLH3 was also selected to detect interaction with MdMIEL1 through Y2H assays because of its role in positively regulating anthocyanin accumulation (Espley et al. 2007, Xie et al. 2012). The results demonstrated that MdMIEL1 did not interact with MdbHLH3 (Fig. S4), suggesting a specific association between MdMIEL1 and MdMYB1.

MdMIEL1 ubiquitinates MdMYB1 protein

Because MdMIEL1 is a putative ubiquitin E3 ligase, it is reasonable to propose that MdMYB1 may be a target protein of MdMIEL1 for ubiquitination and degradation. To verify this hypothesis, an in vitro ubiquitination assay was carried out. MdMYB1 was expressed in *Escherichia coli* as a fusion protein with an HIS tag, while MdMIEL1 was expressed with a GST tag. As shown in **Fig. 4a**, ubiquitination of MdMYB1-HIS protein was found in the presence of ATP, ubiquitin, E1, E2, and MdMIEL1-GST fused protein using an anti-HIS antibody. However, no ubiquitinated MdMYB1 was detected when any one of these factors was absent from the reaction, indicating that MdMIEL1 has ubiquitin E3 ligase activity and that MdMYB1 is its substrate for ubiquitination.

In addition, an in vivo ubiquitination assay was performed to further verify the MdMIEL1-mediated ubiquitination of MdMYB1. MdMYB1 protein was immunoprecipitated with an anti-GFP antibody from MdMYB1-GFP and MdMYB1-GFP/MdMIEL1-OX transgenic calli. The ubiquitinated form of MdMYB1-GFP protein was detected using anti-ubiquitin and anti-GFP antibodies, respectively. The results showed that there were high-molecular-weight polypeptide forms of MdMYB1 in MdMYB1-GFP/MdMIEL1-OX double-transgenic calli, but no corresponding polypeptide bands in the MdMYB1-GFP single-transgenic calli (**Fig. 4b**). The results indicates that MdMYB1 protein is ubiquitinated by MdMIEL1 in vivo.

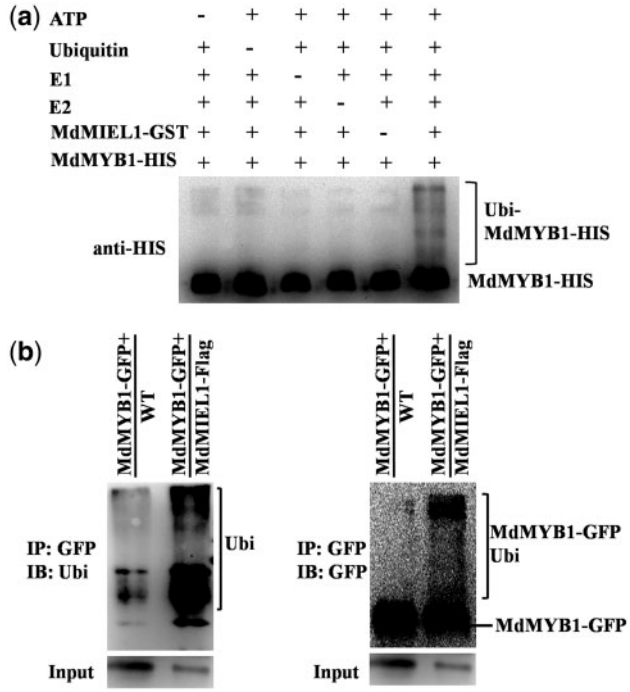

Fig. 4 MdMYB1 is a substrate of MdMIEL1. (a) MdMIEL1 ubiquitinates MdMYB1 in vitro. GST-tagged MdMIEL1 was tested for E3 ubiquitin ligase activity in the presence and absence of ATP, ubiquitin, E1, E2, MdMIEL1-GST and MdMYB1-HIS. The protein gel blot was analyzed using anti-HIS antibody. (b) MdMIEL1 ubiquitinates MdMYB1 in vivo. MdMYB1-GFP was immunoprecipitated using anti-GFP antibody from the two transgenic calli (MdMYB1-GFP, MdMYB1-GFP/MdMIEL1-Flag). The two calli were first treated with the proteasome inhibitor MG132 (50 μM) and then subjected to darkness for 12 h before immunoprecipitation to prevent degradation of ubiquitinated protein. immunoblotting using anti-ubiquitin antibodies is shown on the left and using anti-GFP antibodies on the right.

MdMIEL1 accelerates the degradation of MdMYB1 protein

To examine how MdMIEL1 post-translationally influences the stability of MdMYB1 protein, an in vitro protein degradation assay was performed. WT, MdMIEL1-OX and MdMIEL1-Anti calli extraction solutions were treated with 0.1% DMSO or 100 μM MG132 for 0.5 h, and incubations of extraction solution and MdMYB1-HIS protein were conducted for the indicated times. **Fig. 5a, b** shows that the extraction solution of MdMIEL1-OX increased the decay rate of MdMYB1-HIS protein, while MdMYB1-HIS protein was stabilized in the extraction solution of MdMIEL1-Anti. This result indicates that MdMYB1 is targeted by MdMIEL1 for degradation.

To provide further evidence for the MdMIEL1-mediated degradation of MdMYB1 in vivo, the abundances of MdMYB-GFP proteins were examined in single-transgenic calli (MdMYB1-GFP) and two double-transgenic calli (MdMYB1-GFP/MdMIEL1-OX and MdMYB1-GFP/MdMIEL1-Anti). Proteins extracted from three calli were immunoblotted with anti-GFP antibody. As shown in **Fig. 5c**, the double-transgenic MdMYB1-GFP/MdMIEL1-OX calli accumulated less MdMYB1 protein but MdMYB1-GFP/MdMIEL1-Anti accumulated more of the protein than the MdMYB1-GFP single-transgenic calli. Therefore, MdMIEL1 negatively regulates the protein stability of MdMYB1.

MdMIEL1 negatively regulates MdMYB1-promoted anthocyanin accumulation

To examine how MdMIEL1 influences MdMYB1-promoted anthocyanin accumulation, three kinds of transgenic calli, i.e. MdMYB1-GFP, MdMYB1-GFP/MdMIEL1-OX and MdMYB1-GFP/MdMIEL1-Anti, were used for anthocyanin determination. The results showed that *MdMIEL1* overexpression inhibited, while its suppression promoted, the accumulation of anthocyanin in MdMYB1-GFP transgenic calli (**Figs. 6a, b**, S2a–c).

In addition, transcripts of several anthocyanin biosynthesis structural genes were detected with qRT-PCR. The results showed that *MdMIEL1* overexpression reduced, while its suppression enhanced, the expression levels of these genes, including *MdDFR*, *MdUF3GT*, *MdF3H*, *MdCHI*, *MdCHS* and *MdANR* (**Figs. 6c**, S2d). Therefore, MdMIEL1 negatively regulates MdMYB1-promoted anthocyanin accumulation.

Discussion

The ubiquitin/26S proteasome system is a common post-translational modification that mediates several important processes by removing damaged proteins or destroying the repressor proteins in the plant (Vierstra et al. 2003, Smalle and Vierstra 2004). However, whether and how MBW complexes are regulated at the post-translational level in the anthocyanin biosynthesis pathway is still not well understood, especially in apple and other woody trees, although 688 RING E3 ligases have been identified in apple (Li et al. 2011).

MIEL1 encodes a RING-type E3 ubiquitin ligase, and its functions have been identified in *Arabidopsis* as well as in rice (Marino et al. 2013, Fang et al. 2015). In *Arabidopsis*, the transcription factor MYB30 is regarded as a positive regulator of plant defense and the hypersensitive cell death response. MIEL1 could directly interact with as well as ubiquitinate and degrade MYB30, leading to reduced defense against biotic stresses (Marino et al. 2013). OsSRFP1, a homolog of aMIEL1 in rice, is induced by a variety of abiotic stresses and plays a negative role in cold and oxidative stresses responses (Fang et al. 2015). Recently, MIEL1 has been proved to negatively regulate ABA signaling by promoting protein degradation of MYB96 (Lee and Seo 2016). Additionally, our previous study revealed that ectopic expression of apple *MdMIEL1* promoted lateral root growth by affecting auxin distribution in *Arabidopsis* (An et al. 2017a) and overexpression of *MdMIEL1* negatively regulated salt and oxidative stresses tolerance in apple (An et al. 2017b), indicating that MIEL1 may play critical roles in plant growth and development through targeting the interacting proteins for degradation.

In this study, it was found that a RING E3 ligase, MdMIEL1, interacted with MdMYB1 (**Fig. 3**). It ubiquitinated and degraded MdMYB1 proteins, leading to a decrease in anthocyanin abundance (**Figs. 4–6**), suggesting that MdMIEL1 regulates anthocyanin accumulation primarily at a post-translational

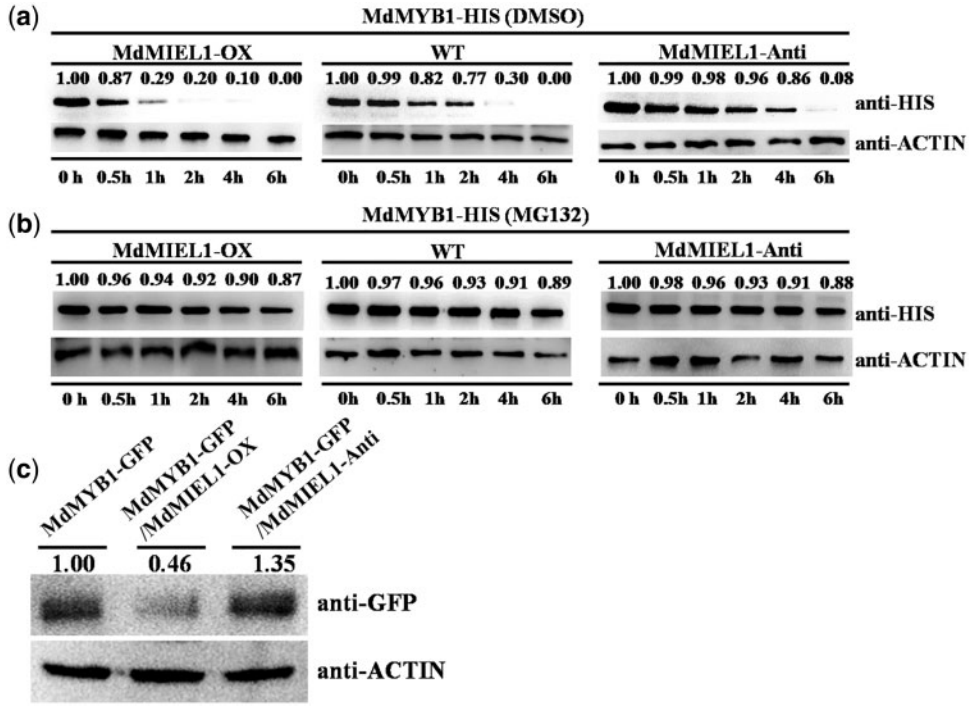

Fig. 5 MdMIEL1 accelerates MdMYB1 degradation via ubiquitination. (a, b) MdMIEL1 degrades MdMYB1 in vitro. MdMIEL1 promotes the degradation of MdMYB1-HIS protein and its stabilization by the proteasome inhibitor MG132. For MG132 and DMSO treatments, extracts of wild-type (WT) and transgenic (MdMIEL1-OX and MdMIEL1-Anti) calli were treated with 0.1% DMSO or 100 μM MG132 for 0.5 h and then incubated with MdMYB1-HIS protein for the indicated times. SDS-PAGE shows the residual levels of the MdMYB1-HIS protein. Actin levels as detected by anti-actin were used as loading controls. Protein levels at 0 h were set to 1.00. (c) MdMYB1-GFP abundance in transgenic calli was assessed by immunoblotting using anti-GFP antibody from the three transgenic calli. The protein abundance of MdMYB1-GFP was set to 1.00.

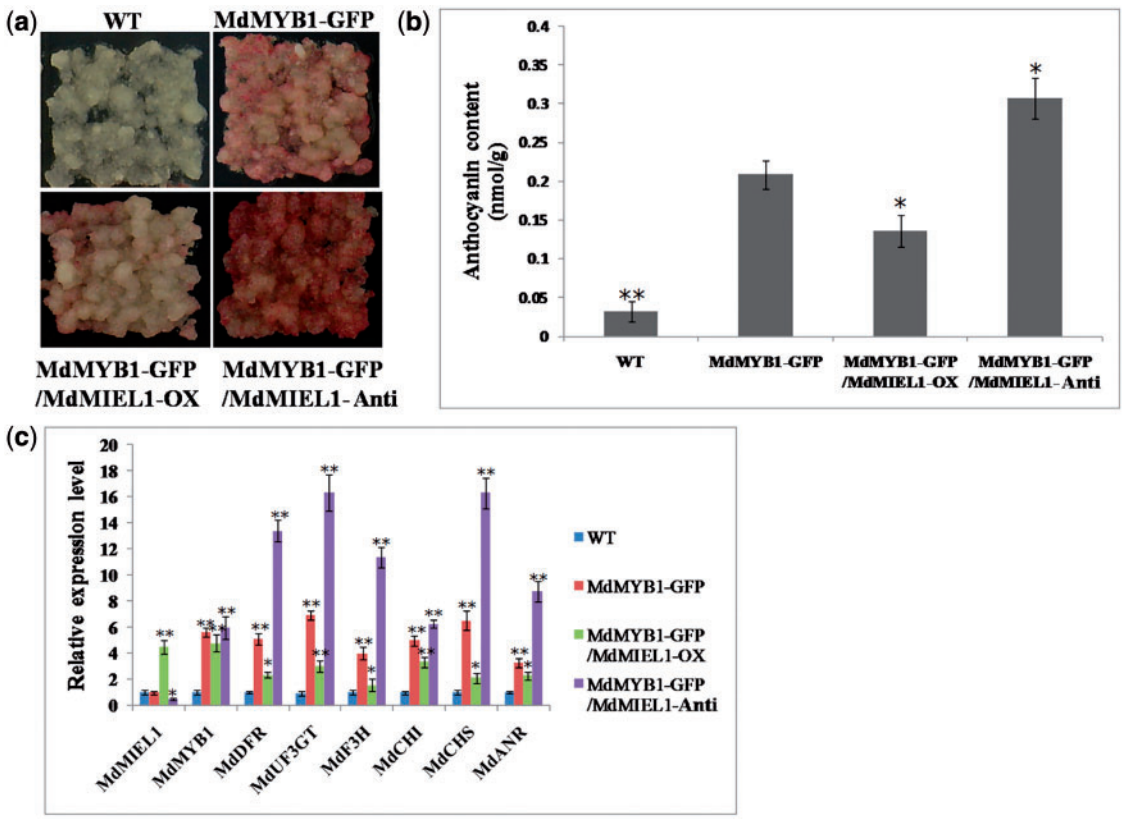

Fig. 6 MdMIEL1 decreases anthocyanin accumulation in *MdMYB1* transgenic apple calli. (a, b) Anthocyanin contents in wild-type (WT) and three transgenic calli grown on medium. The anthocyanin content of MdMYB1-GFP was set as control. (c) qRT-PCR analysis of *MdMIEL1*, *MdMYB1*, *MdMYB110*, *MdDFR*, *MdUF3GT*, *MdF3H*, *MdCHI*, *MdCHS* and *MdANR* in apple calli. The value for WT was set to 1.

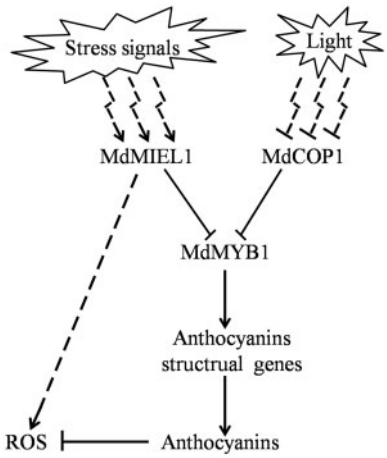

Fig. 7 A model of MdMIEL1-regulated anthocyanin accumulation in apple.

level. As is well known, MdMYB1 works together with its bHLH partners such as MdbHLH3 and MdbHLH33 to regulate anthocyanin accumulation in response to light, temperature and other environmental factors (Li et al. 2012, Xie et al. 2012). However, MdMIEL1 did not interact with MdbHLH3 protein (Fig. S4). Therefore, MdMYB1 may be a specific substrate of MdMIEL1 in the regulation of anthocyanin biosynthesis. Interestingly, the expression of *AtMYB75*, but not other MYB transcription factors, was significantly reduced in *MdMIEL1*-overexpressing lines, indicating that, besides post-translational regulation, MdMIEL1 may also regulate *AtMYB75* at the transcriptional level, which needs further investigation.

As important signaling molecules, ROS play an indispensable role in stress responses (Apel and Hirt 2004, Møller et al. 2007). Under normal growth conditions, ROS homeostasis is maintained at a low level. When the homeostasis is disrupted by environmental stimuli, excess ROS lead to severe cellular damage (Vranova et al. 2002).

Based on the fact that MdMIEL1 inhibits anthocyanin biosynthesis and simultaneously promotes ROS accumulation, it is interesting to speculate whether MdMIEL1 is probably involved in the delicate balance between anthocyanin and ROS accumulation. When plants grow in the absence of stresses, ROS and anthocyanins are all maintained at low levels (Vranova et al. 2002, Møller et al. 2007). However, under stresses, anthocyanins accumulate as a consequence of the triggering of expression of anthocyanin biosynthetic genes, and then influence ROS content, leading to tolerance of these stresses (Apel and Hirt 2004, Halliwell 2006). MdMIEL1 may participate in this process.

A model summarizing our findings regarding this regulatory pathway is presented in **Fig. 7**. In this model, ubiquitin E3 ligase MdMIEL1 responds to environmental stimuli and interacts with MdMYB1 to ubiquitinate it, finally negatively regulating anthocyanin biosynthesis in the plant. This provides a new insight into the ubiquitination-mediated post-translational regulation of MdMYB1 protein for anthocyanin biosynthesis and ROS accumulation in response to environmental factors, while MdCOP1 may play a critical role in light-mediated protein stabilization of MdMYB1 and anthocyanin accumulation (Li et al. 2012).

These results suggest that MdMIEL1 regulates anthocyanin accumulation by modulating the stability and abundance of MdMYB1. This may be a novel mechanism underlying the MdMIEL1-mediated regulation of anthocyanin biosynthesis independent of light.

Materials and Methods

Plant materials and growth conditions

Calli of the 'Orin' cultivar (An et al. 2016) and *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were used in experiments. *Arabidopsis* seeds were germinated on Murashige and Skoog (MS) agar medium after 3 d at 4°C. Seedlings were grown at 22°C under normal light conditions with a 16 h light/8 h dark photoperiod. 'Orin' calli were grown on MS medium containing 1.5 mg·l^{-1} of 2,4-D and 0.5 mg·l^{-1} of 6-Benzylaminopurine (6-BA) at 24°C in the dark, and subcultured at 15-d intervals.

For high-light and low-temperature treatments, calli and seedlings were transferred to a phytotron at 15°C with constant high light (photon flux density about 70 μmol s^{-1} m^{-2}). For the MG132 and DMSO treatments, apple callus extraction solutions were treated with 0.1% DMSO or 100 mM MG132 for 0.5 h.

Molecular cloning of MdMIEL1 and plasmid construction

To isolate the full-length cDNA sequence of *MdMIEL1* in the apple genome, the known *Arabidopsis MIEL1* gene was used as a query to search similar sequences in apple by mining the apple Gene Family Database (GFDB) (http://www.applegene.org/) and the Genome Database for Rosaceae (GDR) (http://www.rosaceae.org/). RT-PCR was conducted using cDNA templates from in vitro 'Gala' apple tissue cultures. *MdMIEL1* (MDP0000185659), a gene containing an 804-bp open reading frame (ORF), was obtained (An et al. 2017a, An et al. 2017b).

The overexpression vector *MdMIEL1-Flag* was constructed by inserting the DNA fragment of the *MdMIEL1* ORF into the modified vector pCAMBIA1300-Flag. The suppression vector MdMIEL1-Anti was constructed by inserting the DNA fragment of the *MdMIEL1* reverse complement into the transformed vector pCAMBIA1300. All of the primers used are shown in Supplementary Data Table S1.

Generation of transgenic calli and transgenic Arabidopsis

Agrobacterium tumefaciens LBA4404 strain was grown in Lysogeny broth (LB) medium supplemented with 50 mg·ml^{-1} rifampicin. The MdMIEL1-Flag construct was the *MdMIEL1* coding sequence under the control of the *Cauliflower Mosaic Virus* 35S promoter. For transformation of 'Orin' calli, 10-d-old calli were co-cultured with LBA4404 carrying an MdMIEL1-Flag. The calli were co-cultured on MS medium containing 1.5 mg·l^{-1} 2,4-D and 0.5 mg·l^{-1} 6-BA for 2 d at 24°C. Subsequently, the calli were washed three times with sterile water and transferred to MS medium supplemented with 250 mg·l^{-1} carbenicillin and 30 mg·l^{-1} hygromycin for transgene selection. The transgenic apple calli were co-cultured in selective medium containing appropriate concentrations of antibiotic.

Transgenic *Arabidopsis* plants were generated through the floral dip transformation method (Clough and Bent 1998).

Real-time quantitative PCR (qRT-PCR)

Transcription levels of *MdMIEL1* and anthocyanin-biosynthesis related genes were examined using specific primers. *MdACTIN* was used as the control. All of the primers used are shown in Supplementary Data Table S1. Each experiment was repeated at least three times. The results were based on the average of three parallel experiments.

In vitro pull-down assay

To determine the MdMIEL1 interaction with MdMYB1, a pull-down assay was performed using a Profound™ Pull-Down PolyGST Protein:Protein interaction

Kit (Thermo, USA) following the manufacturer's instructions. The ORF of *MdMYB1* as prey was cloned into the PET-32a(+) vector, which contained an HIS tag sequence for detection and purification, and subsequently expressed in *E. coli* BL21 (DE3) (Transgene, Beijing, China). MdMIEL1 as bait was cloned into the PGEX-4T-1 vector, and the recombinant proteins were expressed in *E. coli* BL21 (DE3), forming fusion proteins with GST. The bait proteins were immobilized to a cobalt chelate affinity resin. Then, the prey protein was added to the cobalt chelate affinity resin containing the immobilized GST-tagged bait protein, and incubated at 4°C for 1 h with rotation. After washing, samples were desaturated, and assessed through Western blot analysis with anti-HIS or anti-GST antibodies (Abcam, UK).

BiFC assay

The full-length cDNAs of *MdMIEL1* and *MdMYB1* were cloned into pSPYNE-35S and pSPYCE-35S, which contained DNA encoding the N- or C-terminal regions of YFP (YFP^N or YFP^C), respectively, to generate the plasmids MdMIEL1-YFP^N and MdMYB1-YFP^C according to previous protocols (Walter et al. 2004).The constructs were transformed into *A. tumefaciens* LBA4404. The combination of MdMIEL1-YFP^N and MdMYB1-YFP^C was used for experiments, and the combinations of MdMIEL1-YFP^N and YFP^C or MdMYB1-YFP^C and YFP^N were used as controls. These combinations of *Agrobacterium* strains were mixed and co-infected into onion (*Allium cepa*) epidermal cells. YFP fluorescence was analyzed with a confocal laser-scanning microscope (Zeiss LSM 510 Meta, Jena, Germany).

In vivo coimmunoprecipitation assay

Two-week-old calli of MdMYB1-GFP/ MdMIEL1-Flag and MdMYB1-GFP were treated with 50 μM MG132 for 16 h and ground in liquid nitrogen. A Pierce Classic IP (immunoprecipitation) Kit (Thermo Fisher Scientific, San Jose, CA, USA) was applied to immunoprecipitate the MdMYB1-GFP protein with anti-GFP antibodies. And the eluted proteins were detected by Western blotting using an anti-Flag antibody.

Yeast two-hybrid assay (Y2H)

The Y2H experiments were performed according to the manufacturer's instructions (Clontech, USA). cDNAs (full-length, N-terminus, C-terminus, RING-domain, CHY domain, Ribbon domain) of *MdMIEL1* and *MdMYB1* were inserted into pGAD and pGBD vectors. The plasmids were co-transformed into the yeast strain Y2H Gold by using the lithium acetate method. The cells were plated on medium lacking Trp and Leu (SD/−Trp−Leu) and cultured at 28°C. For interaction screening, the colonies were transferred to medium lacking Trp, Leu, His and Ade (SD/−Trp−Leu−His−Ade) (Xie et al. 2012).

In vitro protein degradation assay

Protein degradation assays were performed to study the post-translational regulation of MdMYB1 in vitro. WT apple calli and transgenic callus extraction solutions were extracted with a buffer (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl, 5 mM dithiothreitol (DTT), 10 mM ATP, 4 mM Phenylmethanesulfonyl fluoride (PMSF)). The incubations of the extraction solution and MdMYB1-HIS protein were conducted up to the indicated times. The relative protein level of MdMYB1 was detected by Western blotting with anti-HIS monoclonal antibody (Wang et al. 2009)

In vitro ubiquitination assay

In vitro ubiquitination assays were performed according to Seo et al. (2003). The full-length *MdMIEL1* coding region was amplified using the primers listed in Supplementary Data Table S1 and cloned into the *BamH*I/*Sma*I restriction enzyme sites of the PET-32a(+) vector (GE Healthcare, Fairfield, CT, USA). The MdMIEL1-HIS fusion protein was expressed in the Rosetta *E. coli* strain and purified using HIS Sepharose beads (GE Healthcare, Fairfield, CT, USA). Human E1 (UBA1, GI: 23510338), yeast ubiquitin (GI: 209599) and human E2 (UBCh5b, GI: 4507773) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The reaction mixture (30 μl) contained 100 ng of protein substrate, 20 ng of rabbit E1 ubiquitin-activating enzyme (Boston Biochem), 20 ng human of E2 ubiquitin-conjugating enzymes UbcH5b (Boston Biochem), 10 μg of HIS-6-ubiquitin (Sigma-Aldrich) and 200 ng of E3 (MdMIEL1-GST). Reactions were incubated at 30°C for 4 h. Ubiquitination was detected by Western blotting with anti-HIS monoclonal antibody (Xie et al. 2002).

Measurement of total anthocyanin content

Anthocyanin was extracted with a methanol–HCl method (Lee and Wicker 1991). Approximately 2 g of the sample was soaked and incubated in 5 ml of 1% (v/v) methanol-HCl overnight in the dark at 24°C. The absorbance of each extract was measured at 530, 620 and 650 nm with a spectrophotometer (UV-1600, Shimadzu). The relative anthocyanin content was quantified using the following formula: optical density (OD)=(OD530−OD620)−0.1(OD650−OD620). One unit of anthocyanin content was expressed as a change of 0.1 OD (unit × $10^3 g^{-1}$ fresh weight).

Measurement of ROS content

In situ O_2^- accumulation was detected by histochemical staining with nitro-blue tetrazolium (NBT) (Shi et al. 2010). For H_2DCF-DA staining, samples were infiltrated with 5 μM H_2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) staining solution for 5 min and then examined with an Olympus BZX16 microscope using the same parameters without reaching signal saturation. Quantification of fluorescence intensity of samples was performed using ImageJ software (http://rsbweb.nih.gov/ij/) (Xie et al. 2014).

Statistical analysis

Statistical analysis was performed with appropriate methods using R (3.0.2) with the R Commander package. Differences were considered statistically significant when $*P < 0.05$ and $**P < 0.01$. All the results were based on the average of three parallel experiments.

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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