MdCOP1 Ubiquitin E3 Ligases Interact with MdMYB1 to Regulate Light-Induced Anthocyanin Biosynthesis and Red Fruit Coloration in Apple

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MdMYB1 is a crucial regulator of light-induced anthocyanin biosynthesis and fruit coloration in apple (Malus domestica). In this study, it was found that MdMYB1 protein accumulated in the light but degraded via a ubiquitin-dependent pathway in the dark. Subsequently, the MdCOP1-1 and MdCOP1-2 genes were isolated from apple fruit peel and were functionally characterized in the Arabidopsis (Arabidopsis thaliana) cop1-4 mutant. Yeast (Saccharomyces cerevisiae) two-hybrid, bimolecular fluorescence complementation, and coimmunoprecipitation assays showed that MdMYB1 interacts with the MdCOP1 proteins. Furthermore, in vitro and in vivo experiments indicated that MdCOP1s are necessary for the ubiquitination and degradation of MdMYB1 protein in the dark and are therefore involved in the light-controlled stability of the MdMYB1 protein. Finally, a viral vector-based transformation approach demonstrated that MdCOP1s negatively regulate the peel coloration of apple fruits by modulating the degradation of the MdMYB1 protein. Our findings provide new insight into the mechanism by which light controls anthocyanin accumulation and red fruit coloration in apple and even other plant species.

Fruit color is an important exterior quality and consideration in consumer choice. Red-skinned apples (Malus domestica) are preferred to apples of other colors because consumers associate redder apples with better taste, ripeness, flavor, and nutrition (King and Cliff, 2002). Anthocyanin and its derivatives, which are synthesized through the flavonoid pathway, are the main pigments that determine fruit coloration in apple, just like in other plant species (Mol et al., 1998; Allan et al., 2008). Light regulates anthocyanin biosynthesis and organ coloration in various plant species, including fruit trees (Mol et al., 1996; Allan et al., 2008), suggesting that these processes are involved in a light-signaling pathway.

Higher plants have an extremely delicate light receiving and signal transduction system. Light information ranging from UV-A to far red is perceived primarily by three classes of photoreceptors. Phytochromes absorb red/far-red light and regulate seed germination, the shade-avoidance response, seedling development, and flowering (Franklin et al., 2005). Phototropins and crytochromes mediate various UV-A/blue-light responses, such as light-controlled phototropism, chloroplast movement, stomatal opening, hypocotyl elongation, cotyledon expansion, anthocyanin accumulation, flowering time, and circadian rhythms (Briggs and Christie, 2002; Liu et al., 2011). UV RESISTANCE LOCUS8 (UVR8) perceives UV-B light and plays important roles in UV-B-mediated photomorphogenesis, induction of flavonoid biosynthesis, and plant defense against UV-B (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012).

Downstream of the light receptors, the ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) acts as a molecular switch of light-induced plant development, such as photomorphogenesis (Osterlund et al., 2000; Holm et al., 2002; Seo et al., 2003; Hong et al., 2008), flowering time (Jang et al., 2008), photoperiodic growth (Yu et al., 2008), and stomatal development (Kang et al., 2009). The subcellular distribution of COP1 is adjusted according to light conditions. In darkness, COP1 is localized to the nucleus, where it presumably targets photomorphogenesis-promoting transcription factors (TFs), such as ELONGATED HYPOCOTYL5 (HY5), LONG AFTER FAR-RED1 (LAF1), LONG HYPOCOTYL IN FAR-RED1 (HFR1), BLUE INSENSITIVE TRAIT1 (BIT1), and CONSTANS (CO), and subsequently mediates their ubiquitination and degradation via the 26S proteasome pathway (Osterlund et al., 2000; Seo et al., 2003; Yang et al., 2005; Hong et al., 2008; Jang et al., 2008). When cells are exposed to light, there is a...
drastic reduction in COP1 abundance in the nucleus, allowing nuclear-localized TFs to reaccumulate. These TFs, in turn, activate the transcription of downstream genes required for plants to undergo photomorphogenesis (Henriques et al., 2009). Among these TFs, HY5 is believed to be one of the central modulators for the coordination of light signals and the regulation of appropriate gene expression (Lee et al., 2007). Furthermore, in vitro and in vivo analyses have shown that it positively regulates anthocyanin accumulation by directly binding to the promoters of anthocyanin biosynthetic genes (Holm et al., 2002; Lee et al., 2007).

MYB TFs belong to one of the largest plant TF families, which play crucial roles in plant growth and development. Some of them are involved in the regulation of anthocyanin biosynthesis. For example, Arabidopsis production of anthocyanin pigment1 (AtPAP1) and AtPAP2 regulate anthocyanin synthesis in Arabidopsis (Arabidopsis thaliana) seed capsules, and transgenic tobacco (Nicotiana tabacum) plants overexpressing AtPAP1 and AtPAP2 exhibit purple plants and flowers, respectively (Borevitz et al., 2000). Anthocyanin biosynthesis is regulated by CI in maize (Zea mays) endosperm (Cone et al., 1993), by PhMYBAN2 in petunia (Petunia hybrida) flowers (Quattrocchio et al., 1999), and by Rose1, Rose2, and Venosa in the flowers of Antirrhinum majus (Schwinn et al., 2006). Among fruit trees, grapevine (Vitis vinifera) is most likely the best-studied species in terms of the regulation of anthocyanin biosynthesis by MYB TFs, such as VvMYBA1, VvMYBA2, VvMYB5a, VvMYB5b, and VvMYBPA1 (Deluc et al., 2008). Similarly, MYB TFs have also been linked to anthocyanin accumulation and fruit coloration in citrus and fruit trees from the family Rosaceae, such as pear (Pyrus communis), plum (Prunus domestica), cherry (Prunus avium), peach (Prunus persica), raspberry (Rubus idaeus), strawberry (Fragaria spp.), and blueberry (Vaccinium corymbosum; Aharoni et al., 2001; Lin-Wang et al., 2010; Butelli et al., 2012; Zifkin et al., 2012).

In apple, MdMYB1, MdMYB10, and MdMYBA, which are allelic to each other, have been isolated and characterized as key regulatory genes for anthocyanin accumulation and fruit coloration (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). Among them, MdMYB10 constitutively expressed in the whole plant due to its direct binding to its own enhancer promoter in an autoregulatory-loop manner, leading to the production of red-skinned apple fruit (Espley et al., 2009). The transcription of MdMYB1 and MdMYBA is induced by light (Takos et al., 2006; Ban et al., 2007).

Several photomorphogenesis-associated TFs are regulated by light not only at the transcript level but also at the posttranslational level (Hong et al., 2008). However, it is still unknown whether and how anthocyanin-associated MYB TFs are regulated at the posttranslational level in apple and other plant species. In this study, the stability of the MdMYB1 protein was examined under light and dark conditions. Subsequently, MdCOP1-1 and MdCOP1-2 were isolated and characterized. These proteins function in the ubiquitination and degradation of MdMYB1 via the proteasome pathway and regulate fruit coloration in apple. Finally, the potential utilization of MdCOP1 in the genetic improvement of fruit quality is discussed.

RESULTS

MdMYB1 Protein Accumulates under Light But Degrades under Dark in Apple Fruits

Light is an essential environmental factor for fruit coloration in apple (Supplemental Fig. S1, A and B). MdMYB1, which is allelic to MdMYB10 and MdMYBA in apple, is a positive regulator for light-controlled anthocyanin biosynthesis and fruit coloration in apple (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). Its expression is positively induced by light in apple fruits (Takos et al., 2006; Supplemental Fig. S1C). Similarly, the accumulation level of MdMYB1 protein gradually increased with illumination time. Both the transcript and protein levels of MdMYB1 reached a maximum at 48 h after debagging and remained stable after 48 h after debagging (Supplemental Fig. S1C; Fig. 1A). Compared with the immediate increase in MdMYB1 transcripts and proteins (Supplemental Fig. S1C; Fig. 1A), the noticeable increase in anthocyanin content and fruit coloration was delayed (Supplemental Fig. S1, A and B), further indicating that MdMYB1 acts upstream of anthocyanin biosynthesis and fruit coloration in apple.

To examine the stability of the MdMYB1 protein in the dark, shoot tissue cultures of the ‘Gala’ apple cultivar were grown in continuous darkness for 4 d. They were then transferred to the light condition for 1, 6, and 24 h. Finally, they were transferred to darkness again for 1 and 2 d. An immunoblot with anti-MdMYB1 antibody demonstrated that, just like in apple fruit, the MdMYB1 protein increased gradually with illumination time upon exposure to light. After returning to darkness, the abundance of the MdMYB1 protein slightly decreased at 1 d after darkness treatment.
and declined to a very low level at 2 d after darkness (Fig. 1B).

**MdMYB1 Is Degraded through a 26S Proteasome-Dependent Pathway in the Dark**

To test whether MdMYB1 protein degradation in the dark-treated cultures was mediated by the 26S proteasome, the effects of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132, a proteasome inhibitor) and dimethyl sulfoxide (DMSO; control treatment) on MdMYB1 protein degradation in the dark were tested. The result showed that MdMYB1 protein rapidly degraded in dark under dimethyl sulfoxide (DMSO) treatment. However, MG132 treatment noticeably inhibited MdMYB1 degradation compared with DMSO treatment, indicating that the MdMYB1 protein is subject to 26S-proteasome-mediated proteolysis in the dark (Fig. 2A).

To examine if the dark-induced degradation of MdMYB1 protein is mediated by the ubiquitin-dependent proteasomal pathway, the expression vector 35S::MdMYB1-GFP was genetically transformed into apple callus, while wild-type callus was used as a control. Expression and immunoblot analysis showed that transgenic apple callus ectopically expressed MdMYB1 transcripts and produced an obvious level of MdMYB1-GFP proteins (Fig. 2B).

Subsequently, the levels of MdMYB1-GFP ubiquitination were determined using antiubiquitin, anti-GFP, and anti-MdMYB1 antibodies. Almost no immunoblot signal was observed in the control callus under both light and dark conditions. In contrast, there were strong immunoblot signals for transgenic callus under both light and dark conditions. When the anti-GFP and anti-MdMYB1 antibodies were used to detect the ubiquitin level of proteins immunoprecipitated (IP) with anti-GFP antibody, high-molecular-mass forms of MdMYB1, i.e. polyubiquitinated MdMYB1 [Ub(n)-MdMYB1-GFP], were detected under dark conditions but not under light conditions, indicating that ubiquitination of the MdMYB1 protein occurred under dark conditions (Fig. 2C). The modified form of MdMYB1-GFP protein was further confirmed by an antiubiquitin antibody that recognizes only ubiquitinated MdMYB1-GFP. The result showed that the antiubiquitin antibody

**Figure 2.** MdMYB1 proteins are degraded through the ubiquitin-dependent 26S proteasome pathway in the dark. A, Degradation of MdMYB1 protein in the dark and its stabilization by the proteasome inhibitor MG132. After growing for 4 d in the dark, tissue cultures of 'Gala' apple were exposed to light for 24 h and then treated with DMSO or 50 μM MG132, for the indicated times. B, MdMYB1 transcript and protein levels in 35S::MdMYB1-GFP transgenic and nontransgenic wild-type calluses. In A and B, immunoblotting was conducted with anti-MdMYB1 and anti-GFP monoclonal antibodies, respectively. Coomassie Brilliant Blue stained protein served as a loading control. C, Ubiquitination detection of MdMYB1-GFP protein in 35S::MdMYB1-GFP transgenic and wild-type (control) calluses. After pretreatment with 50 μM MG132 to prevent degradation of ubiquitinated proteins, the calluses were treated with light or dark for 12 h. The MdMYB1-GFP protein was IP using anti-GFP antibody. Anti-GFP, anti-MdMYB1, and antiubiquitin antibodies were used to detect MdMYB1-GFP and ubiquitinated MdMYB1-GFP. IB, Immunoblot.
detected a high level of ubiquitinated MdMYB1-GFP proteins in 35S::MdMYB1-GFP transgenic callus under dark conditions but only very-low ubiquitin signal in transgenic callus under light conditions. These results suggest that MdMYB1 is ubiquitinated under dark conditions, providing further evidence for light regulation of MdMYB1 protein abundance via the ubiquitination-mediated 26S proteasome pathway in apple.

Cloning and Functional Identification of MdCOP1-1 and MdCOP1-2

Arabidopsis COP1 is a photomorphogenesis repressor with E3 ubiquitin ligase activity toward light-associated MYB proteins (Seo et al., 2003; Hong et al., 2008). Here, it is hypothesized that MdCOP1 proteins regulate MdMYB1 in apple. To test this hypothesis, MdCOP1 genes were isolated from apple. First, a blast search of the apple genome database found two COP1-like genes, MDP0000245133 and MDP0000259614, which are located on chromosomes 6 and 16, respectively. To test if these two genes are really counterparts of the Arabidopsis COP1 gene, their full-length complementary DNAs (cDNAs) were cloned and named MdCOP1-1 (MDP0000245133) and MdCOP1-2 (MDP0000259614), respectively. The full-length cDNAs of MdCOP1-1 and MdCOP1-2 are 2,087 and 2,213 bp, respectively. The open reading frame of MdCOP1-1 encodes a protein containing 665 amino acid residues with a M, of 74.7 kD and a pI of 6.4, while that of MdCOP1-2 encodes a protein of 659 amino acid residues with a M, of 74.2 kD and a pI of 6.2. The amino acid sequences of the predicted MdCOP1-1 and MdCOP1-2 proteins were highly similar (92.05%) to each other (Supplemental Fig. S2).

Furthermore, both predicted proteins contain a conserved RING domain and a WD-40 motif (Fig. 3A). To analyze the phylogenetic relationship between the MdCOP1s and other COP1s, a neighbor-joining phylogenetic tree was constructed based on the amino acid sequences of MdCOP1-1, MdCOP1-2, and other COP1s from 11 plant species (Fig. 3B). Both MdCOP1s exhibited the highest similarity to the COP1 protein (AAK81856.1) from a Rosa spp. hybrid cultivar, as they were clustered into the same clade. Interestingly, the COP1s from monocots, including maize and rice (Oryza sativa), were clustered into one clade, while the COP1s from dicots clustered into two other clades (Fig. 3B).

To verify the function of MdCOP1s, the Arabidopsis cop1-4 mutant was used to conduct a functional complementation assay. Under dark conditions, wild-type Arabidopsis seedlings showed lengthened hypocotyls, while cop1-4 did not, due to their constitutive photomorphogenic phenotype. Two expression vectors containing the MdCOP1-1 and MdCOP1-2 genes driven by the cauliflower mosaic virus 35S promoter were constructed. These two constructs, 35S::MdCOP1-1 and 35S::MdCOP1-2, were genetically transformed into the Arabidopsis wild type (ecotype ‘Columbia’) and cop1-4 mutant. Expression analysis showed that the MdCOP1-1 and MdCOP1-2 genes were ectopically expressed in the wild-type and cop1-4 backgrounds (Fig. 3C). The transgenic cop1-4 seedlings ectopically expressing MdCOP1s partially recovered the hypocotyl length phenotype under dark conditions, indicating that the two MdCOP1 genes act as counterparts of Arabidopsis COP1s and have COP1 functions (Fig. 3, D and E). In addition, transgenic wild-type seedlings exhibited longer hypocotyls under dark conditions than the wild type themselves (Fig. 3, D and E), further confirming that MdCOP1s function as a negative regulator of light signaling.

MdCOP1s Interacts with MdMYB1 in Vitro and in Vivo

In Arabidopsis, the E3 ubiquitin ligase activity of the COP1 protein is responsible for targeting downstream components for 26S proteasome-mediated degradation in the dark. Generally, COP1 interacts with those proteins in this process. To test if MdCOP1s interact with MdMYB1, a yeast (Saccharomyces cerevisiae) two-hybrid assay was performed. Positive β-gal activity was observed in yeast-containing pGBT9-MdMYB1 plus pGAD424-MdCOP1-1 or pGAD424-MdCOP1-2 grown on -Trp/-Leu/-His/-Ade screening medium but not in those containing pGBT9-MdMYB1 plus the empty pGAD424 vector or pGAD424-MdCOP1s plus the empty pGBT9 vector (Fig. 4A). Therefore, each of the MdCOP1-1 and MdCOP1-2 proteins physically interacted with MdMYB1.

To further characterize the interaction between MdCOP1s and MdMYB1, an in vivo interaction assay using bimolecular fluorescence complementation (BiFC) was carried out. Yellow fluorescent protein (YFP) fluorescence was predominantly detected in the nuclei of the cells cotransformed with two combinations: MdCOP1-1-YN plus MdMYB1-YC and MdCOP1-2-YN plus MdMYB1-YC. In contrast, no fluorescence was observed in the control combinations including the empty pSPYCE-35S vector plus either MdCOP1-1-YN or MdCOP1-2-YN, and the empty pSPYNE-35S plus MdMYB1-YC (Fig. 4B). These results indicate that MdCOP1 and MdMYB1 interact in vivo in plant cells.

Furthermore, the interaction between MdMYB1 and MdCOP1s was confirmed using an in vivo coimmunoprecipitation assay, utilizing MdMYB1-GFP transgenic callus. The result showed that immunoprecipitation of MdCOP1 and MdMYB1 also coprecipitated MdMYB1 and MdCOP1 in MdMYB1-GFP transgenic callus, respectively. Neither MdCOP1 nor MdMYB1 was detected in the absence of the monoclonal antibody or when anti-GST antibody was used as a negative antibody control (Fig. 4C). In addition, GFP transgenic callus was also used as a negative control. The result showed that, in GFP transgenic callus, anti-MdCOP1 antibody IP MdCOP1 protein, but failed to coimmunoprecipitate GFP protein. Similarly, anti-GFP antibody IP GFP
protein, but failed to coimmunoprecipitate MdCOP1 proteins (Fig. 4C). These data indicated that MdMYB1 interacts with MdCOP1 in plant cells.

**MdCOP1s Ubiquitinate MdMYB1 in the Dark**

The fact that MdCOP1s directly interact with MdMYB1 suggests MdMYB1 as a putative substrate for the MdCOP1 proteins. To test whether the MdMYB1 protein is marked for degradation by the 26S proteasome through polyubiquitination by interacting with MdCOP1, an in vitro ubiquitination assay was performed. Considering the high similarity between MdCOP1-1 and MdCOP1-2, MdCOP1-2 alone was chosen for the in vitro ubiquitination assay. The MdMYB1-GST protein was not ubiquitinated in the presence of recombinant MdCOP1-2-GST, detected with anti-ubiquitin or anti-MdMYB1 antibodies (Supplemental Fig. S3). In contrast, when the MdMYB1-GFP proteins IP using anti-GFP antibody from the transgenic apple callus were used instead of the MdMYB1-GST recombinant protein in the reaction, the antiubiquitin and anti-MdMYB1 antibodies both recognized polyubiquitinated MdMYB1-GFP proteins. In addition, the addition of the recombinant MdCOP1-2-GST protein noticeably enhanced the ubiquitination signal (Fig. 5A). This result indicates that MdCOP1-2 has ubiquitin ligase activity when MdMYB1 is used as a substrate in vitro.

To substantiate that the E3 activity of MdCOP1s functions in ubiquitinating the MdMYB1 protein in vivo, the full-length cDNAs of MdCOP1-1 and MdCOP1-2 in
the sense direction as well as a fragment conserved between the two MdCOP1s in the antisense direction were genetically introduced into apple callus, respectively. As a result, MdCOP1-1 overexisted in transgenic callus MdCOP1-1 OE, while MdCOP1-2 transcripts increased in MdCOP1-2 OE. Meanwhile, the transcripts of both MdCOP1-1 and MdCOP1-2 decreased in the transgenic callus harboring the antisense fragment asMdCOP1 (Fig. 5B).

Subsequently, the ubiquitination of MdMYB1 protein were analyzed in the transgenic calluses. In MdCOP1-1 and MdCOP1-2 transgenic calluses, high-molecular-weight polypeptide bands corresponding to the polyubiquitinated forms of MdMYB1 were detected using each of antiubiquitin and anti-MdMYB1 antibodies; these bands were also found in the wild-type callus, but they were much weaker than those in the transgenic calluses (Fig. 5C). However, these bands were almost absent in asMdCOP1 transgenic callus. In addition, MdMYB1 polypeptide bands were detected in all calluses tested in immunoblotting assay using anti-MdMYB1 antibody. The results indicated that MdMYB1 is ubiquitinated in vivo, and the ubiquitination requires MdCOP1s.

The finding that MdCOP1 mediates the in vivo ubiquitination of MdMYB1 prompted us to examine whether MdCOP1s regulate the stability of the MdMYB1 protein. For this purpose, proteins extracted from MdCOP1-1, MdCOP1-2, and asMdCOP1 transgenic calluses as well as the wild-type control were immunoblottedted with anti-MdMYB1 antibody. The wild-type callus produced a high level of MdMYB1 protein under
light conditions. However, after being treated in the dark, the wild-type callus exhibited a substantial reduction in MdMYB1 protein level (Fig. 5D). Compared with the wild-type callus, asMdCOP1 transgenic callus produced a high level of MdMYB1 protein even under dark conditions, while MdCOP1 transgenic calluses accumulated a very low level of MdMYB1 proteins under both light and dark conditions. Furthermore, in the presence of MG132 (50 mM), the MdMYB1 protein level was similar under light and dark conditions in three transgenic calluses and the wild-type control (Fig. 5D, bottom). Therefore, MdCOP1 proteins mediate the proteasome-mediated degradation of the MdMYB1 protein in apple callus.

MdCOP1s Negatively Regulate Fruit Coloration in Apple

To verify the function of MdCOP1s in apple fruit coloration, their expression was enhanced or suppressed with a viral vector-based transformation method. The overexpression of both MdCOP1-1 and MdCOP1-2 inhibited fruit coloration around the injection sites (Fig. 6, B and C), while their suppression promoted the fruit coloration (Fig. 6, A and C). Almost all of the injection sites turned red, likely because wounding enhances anthocyanin accumulation in plants (Mellway et al., 2009).

Subsequently, MdMYB1 transcripts and proteins were detected with semiquantitative reverse transcription

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**Figure 5.** MdCOP1s ubiquitinate and degrade MdMYB1. A, COP1 E3 activity was assayed in the presence or absence of rabbit E1, human E2 UbcH5b, His-6-ubiquitin, MdCOP1-GST, and MdMYB1-GFP IP with anti-GFP antibody from MdMYBGFP transgenic callus. Ub(n)-MdMYB1-GFP was detected with protein gel blots using an anti-MdMYB1 monoclonal antibody (right) and an antidualubiquitin antibody (left). B, MdCOP1-1, MdCOP1-2, and MdMYB1 transcripts in transgenic calluses ectopically expressing MdCOP1-1, MdCOP1-2, and asMdCOP1, respectively, and nontransgenic wild-type calluses. C, MdMYB1 was IP using anti-MdMYB1 monoclonal antibody from the three transgenic and wild-type calluses. These calluses were first treated with the proteasome inhibitor MG132 (50 μM) and then subjected to darkness treatment for 16 h before the immunoprecipitation to prevent degradation of ubiquitinated protein. The immunoprecipitate was detected by immunoblotting using anti-ubiquitin (left) and anti-MdMYB1 (right) antibodies. D, MdMYB1 abundance in transgenic and wild-type calluses was detected by immunoblotting using an anti-MdMYB1 monoclonal antibody. The calluses were treated with or without MG132 (50 μM) in light and dark conditions.
(RT)-PCR and immunoblots, respectively. The result showed that the overexpression or suppression of MdCOP1s exerted little influence on MdMYB1 gene expression (Fig. 6D) but great influence on the MdMYB1 protein level (Fig. 6E). Around the injection sites, the accumulation levels of MdCOP1s were negatively correlated with MdMYB1 protein abundance, which is directly positively related to fruit coloration in apple (Fig. 1A). Therefore, MdCOP1s control fruit coloration by negatively modulating the stability and abundance of MdMYB1 proteins in apple. Furthermore, it was also found that the ectopic expression of MdCOP1s inhibited the anthocyanin accumulation and plant coloration in wild-type and cop1-4 mutant Arabidopsis (Supplemental Fig. S4), suggesting that they have E3 ubiquitin ligase activity and the capacity to interact with upstream light receptors. Furthermore, their ectopic expression complemented the photomorphogenetic defects of Arabidopsis cop1-4 mutant, indicating that these proteins are the counterparts of Arabidopsis COP1 in apple. It thus becomes reasonable to speculate that MdCOP1s sense light signals by physically interacting with red, blue, and UV-B light receptors to regulate the downstream proteins in apple.

Light signals regulate the ubiquitination activity of the COP1 protein by changing its interaction with light receptors and modulating its subcellular localization from the cytoplasm to the nucleus. In darkness, nuclear COP1 represses photomorphogenesis by targeting a subset of transcription activators, including the basic helix-loop-helix TF HFRI (Yang et al., 2005), the basic zipper TFs HY5 and HY5 HOMOLOG (Osterlund et al., 2000; Holm et al., 2002; Seo et al., 2003; Hong et al., 2008; Jang et al., 2008; Yu et al., 2008; Kang et al., 2009). In this study, it was found that both MdCOP1-1 and MdCOP1-2 contain highly conserved RING-finger and WD-40 repeat domains at the C and N termini, respectively (Fig. 3A), suggesting that they have E3 ubiquitin ligase activity and the capacity to interact with upstream light receptors. Furthermore, their ectopic expression complemented the photomorphogenetic defects of Arabidopsis cop1-4 mutant, indicating that these proteins are the counterparts of Arabidopsis COP1 in apple. It thus becomes reasonable to speculate that MdCOP1s sense light signals by physically interacting with red, blue, and UV-B light receptors to regulate the downstream proteins in apple.

DISCUSSION

In Arabidopsis, COP1 acts as a central switch in light signal transduction by interacting upstream with light receptors and downstream with target proteins (Ma et al., 2002). There are three types of light receptors: the cryptochromes CRY1 and CRY2, which are responsive to blue light; the phytochromes PhyA and PhyB, which are responsive to red and far-red light; and UVR8, which is responsive to UV-B light. Generally, these receptors physically interact with COP1 through its N-terminal WD-40 repeat domain (Jang et al., 2010; Liu et al., 2011; Christie et al., 2012; Wu et al., 2012). In addition to the N-terminal WD-40 domain, COP1 also contains a RING-finger domain that is commonly conserved in a subclass of ubiquitin protein ligases. The RING-finger domain of the COP1 protein is able to ubiquitinate itself and other TFs downstream, regulating many aspects of plant development (Osterlund et al., 2000; Holm et al., 2002; Seo et al., 2003; Hong et al., 2008; Jang et al., 2008; Yu et al., 2008; Kang et al., 2009).

In this study, it was found that both MdCOP1-1 and MdCOP1-2 contain highly conserved RING-finger and WD-40 repeat domains at the C and N termini, respectively (Fig. 3A), suggesting that they have E3 ubiquitin ligase activity and the capacity to interact with upstream light receptors. Furthermore, their ectopic expression complemented the photomorphogenetic deficits of Arabidopsis cop1-4 mutant, indicating that these proteins are the counterparts of Arabidopsis COP1 in apple. It thus becomes reasonable to speculate that MdCOP1s sense light signals by physically interacting with red, blue, and UV-B light receptors to regulate the downstream proteins in apple.
degradation via a proteasome-mediated ubiquitination pathway. Among these TFs, the abundance of HY5 is directly correlated with the extent of photomorphogenic development, UV-B tolerance, and anthocyanin biosynthesis (Strake et al., 2010). In contrast, a light-controlled nuclear depletion of the COP1 protein permits the accumulation of TFs to activate target genes.

MYB proteins are another type of TF regulated by the COP1 protein. BIT1, a MYB TF, plays an important role in controlling blue-light responses. COP1 interacts with BIT1 and mediates its degradation in darkness, and CRY1 functions to stabilize BIT1 in a blue-light-dependent manner. As a result, BIT1 functions as a positive element in blue-light signaling (Hong et al., 2008). Similarly, another MYB transcription activator, LAF1, that participates in the transmission of PhyA signals to downstream responses also acts as a COP1 substrate for degradation (Seo et al., 2003).

MYB TFs have also been implicated in controlling anthocyanin biosynthesis in several model plants and fruit trees (Aharoni et al., 2001; Allan et al., 2008; Lin-Wang et al., 2010). In Arabidopsis, petunia, maize, and fruit trees, it has been shown that MYB TFs, together with basic helix-loop-helix TFs and WD-40 repeat proteins, regulate anthocyanin biosynthesis and therefore contribute to the red coloration of various organs including the leaves, flowers, and fruits (Mol et al., 1998; Allan et al., 2008). Generally, light induces the expression of anthocyanin-associated MYB genes, and therefore favors red pigment accumulation in plant organs (Mol et al., 1998; Allan et al., 2008; Gonzalez, 2009; Rowan et al., 2009). In apple, the fruit peel does not develop a red color without light, but it rapidly initiates coloration upon exposure to sunlight in a process that is regulated by MdMYB1 and its alleles (Takos et al., 2006; Ban et al., 2007; Allan et al., 2008).

However, it remains unknown whether and how anthocyanin-associated MYB TFs are regulated at the posttranslational level in apple and other plant species. In this study, it was found that MdCOP1s physically interact with MdMYB1 and subsequently mediate its ubiquitination and degradation in apple (Figs. 4 and 5). In the in vitro ubiquitination assay, only the MdMYB1-GFP protein IP with anti-GFP antibody, but not MdMYB1-GST purified from prokaryotic expression, could be ubiquitinated (Fig. 5A; Supplemental Fig. S3). This result may have occurred because the GST-tagged recombinant protein is not functional and/or needs posttranslational modification or cofactors for protein interaction and ubiquitination. In the unicellular red alga Cyanidioschyzon merolae, the purified recombinant CmMYB1 protein binds to the promoter regions of nitrogen assimilation genes only in an Electrophoretic Mobility Shift Assay analysis using a C. merolae crude cell extract (Imamura et al., 2009). Our findings support that MdCOP1s posttranslationally ubiquitinate the MdMYB1 protein to control its degradation via the 26S proteasome pathway in darkness. Considering the reports on the MYB TFs LAF1 and BIT1 (Seo et al., 2003; Hong et al., 2008), it seems that the mechanism by which COP1s modulate the light-controlled stability of MYB TFs may be conserved in higher plants.

Because MdCOP1s negatively regulate the abundance of the MdMYB1 protein, which is a positive regulator for anthocyanin biosynthesis and fruit coloration (Takos et al., 2006), the levels of MdCOP1s transcripts are negatively related with the extent of red fruit coloration in apple (Fig. 6A), indicating that MdCOP1s are negative regulators of apple fruit coloration. In Arabidopsis, mutant cop1-4 plants produced more anthocyanin and appeared much redder in color than the wild type, and the ectopic expression of MdCOP1s inhibited anthocyanin accumulation and red organ coloration in both the cop1-4 and wild-type backgrounds (Supplemental Fig. S4), indicating that COP1 proteins function beyond species boundary. Therefore, it can be speculated that COP1 proteins inhibit anthocyanin accumulation and red organ coloration in a way that is highly conserved between herbaceous and woody plants.

We provide here new insight into the mechanism underlying light-controlled anthocyanin accumulation and red fruit coloration in apple and even other plant species. The light receptors such as MdPHYs, MdCRYs, and MdUVR8, perceive light signals with different wavelengths. In Arabidopsis these light receptors interact with AtCOP1 that is a negative regulator for photomorphogenesis. MdCOP1s are the counterparts of Arabidopsis COP1 in apple; therefore, it is reasonable to propose that MdCOP1s should sense light signals by interacting with photoreceptors in a similar way as found in Arabidopsis. In darkness, the nuclear MdCOP1 proteins interact with MdMYB1 and mediate its ubiquitination and degradation via a 26S proteasome pathway. In light, nuclear depletion of the COP1 protein prevents the MdMYB1 protein from being ubiquitinated and degraded. As a result, MdMYB1 accumulates and directly binds to the promoters of anthocyanin biosynthetic genes, which are another type of TF regulated by COP1 proteins inhibit anthocyanin accumulation and red organ coloration in a way that is highly conserved between herbaceous and woody plants.

Figure 7. A model for light-induced apple fruit coloration.
genes such as MdUFGT and MdDFR to activate their expression (Takos et al., 2006; Han et al., 2010). Finally, anthocyanin accumulation leads to red coloration in apple fruit. A model summarizing our findings is presented in Figure 7.

The peel color is one of the most important agronomic traits for fruit crops. Together with fruit size and shape, it contributes to the overall appearance of fresh fruit and largely determines the diet and market values in fruit industries (King and Cliff, 2002). In addition, anthocyanins also contribute to or even determine additional features that are of special relevance in fruit crops. For example, anthocyanins are of interest in human nutrition as they have been reported to possess a wide range of biological properties imparting benefits to health (Butelli et al., 2008). There is considerable interest in the control of their biosynthesis, and therefore fruit color is a major target in fruit tree breeding programs. Our findings regarding the regulatory mechanism by which MdCOP1 regulates the MdMYB1 protein and resuultantly inhibit fruit coloration in a light-dependent manner may be used in a breeding program with a novel biotechnological strategy to create new apple cultivars with enhanced anthocyanin content and pleasing colors.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis (Arabidopsis thaliana)* ecotype ‘Columbia’ and *cop1-4* mutant were used. After being treated for 3 d at 4°C, seeds were sown on Murashige and Skoog medium. Seedlings and plants were grown at 21°C for a 16-h light period (200 μmol m⁻² s⁻¹), unless stated otherwise.

Tissue cultures of apple (*Malus domestica* ‘Gala’) were subcultured at 1-month intervals on Murashige and Skoog medium supplemented with 0.3 mg l⁻¹ 6-benzylaminopurine and 0.2 mg l⁻¹ naphthalacetic acid at 25°C for a 16-h light period (100 μmol m⁻² s⁻¹), unless stated otherwise. Wild-type and transgenic calluses of apple ‘Gala’ were maintained at 3-week intervals on Murashige and Skoog medium containing 0.5 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 6-benzylaminopurine in the dark at 25°C, unless stated otherwise. Bagged fruits of apple ‘Red Delicious’ were harvested at 140 d after blooming.

For MG132 and DMSO treatments, cv Gala tissue cultures and callus were grown in light for 4 d, and then transferred to liquid Murashige and Skoog medium and incubated overnight with DMSO or with 50 μM MG132 in the dark. After the incubation, the materials were washed three times (5 min each) with liquid Murashige and Skoog medium to remove residual DMSO or MG132 before protein extraction.

**Isolation of Full-Length *MdCOP1*-1 and *MdCOP1*-2 cDNAs, Sequence Comparison, and Semiquantitative RT-PCR Analysis**

Plant materials were harvested, frozen in liquid nitrogen, and then ground under RNase-free conditions. The RNA was extracted using the TRIzol reagent (Invitrogen), following the manufacturer’s instructions, and then treated with DNase I at 37°C for 30 min. The RNA was then reverse transcribed using the PrimeScript first-strand cDNA synthesis kit (Takara), following the manufacturer’s instructions. A 10-μl aliquot of the diluted cDNA was used for PCR.

Full-length cDNAs of *MdCOP1*-1 and *MdCOP1*-2 were obtained by RT-PCR using the primer pairs MdCOP1-1F/R and MdCOP1-2F/R (Supplemental Table S1). The full-length cDNA sequence was used to search homologous sequences via the National Center for Biotechnology Information BLASTX.

Multiple alignments of amino acid sequences were performed between apple and other plants using ClustalW. A phylogenetic tree was then constructed by the neighbor-joining method.

Semiquantitative RT-PCR was carried out in 25-μl reactions with 2 μl of diluted cDNA template. The PCR profile was 94°C for 3 min, 20 to 30 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 30 s, followed by a 5-min extension at 72°C. The primer pairs used were SQMYB-F and SQMYB-R for *MdMYB1*, SQPI-F and SQPI-R for *MdCOP1*-1, and SQP2-F and SQP2-R for *MdCOP1*-2 (Supplemental Table S1). *MdACTIN* was used as the external control. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel and viewed under UV light after standard staining with ethidium bromide.

**Protein Extraction and Western Blotting**

Approximately 500 mg of tissue cultures, fruit skin, or callus was ground in a buffer containing 100 mM Tris (pH 8.0), 1 mM EDTA, 0.1% (v/v) polyvinylpyrrolidone, 10 mM β-mercaptoethanol, 200 mM Suc, and 0.5% (w/v) protease inhibitor mixture (Sigma-Aldrich). After homogenization, the mixture was clarified by centrifugation, and the protein concentration was determined using the Bradford reagent (Sigma-Aldrich) with bovine serum albumin as a standard.

Anti-MdMYB1 and anti-MdCOP1 monoclonal antibodies were commissioned from GenScript Company. Protein extracts (10 μl) were separated on an 8% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Millipore) using an electrotransfer apparatus (Bio-Rad). The membranes were incubated with anti-MdMYB1, anti-GFP (Sigma-Aldrich), or antishiubiquitin (Sigma-Aldrich) primary antibodies and peroxidase-conjugated secondary antibodies (Abcam) before visualization of immunoreactive proteins using ECL kits (Millipore). Coomassie Brilliant Blue-stained protein served as a protein loading control.

**Vector Construction and Genetic Transformation**

The full-length cDNA of *MdMYB1* was amplified using the primer pair MdMYB-GFPF and MdMYB-GFPR and then cloned into the pBIN expression vector, which has a GFP tag, under the control of the 35S promoter (Supplemental Table S1). Meanwhile, the full-length cDNAs of *MdCOP1*-1 and *MdCOP1*-2 were cloned into the expression vector pBI121 under the control of the 35S promoter. A 426-bp fragment of *MdCOP1*-2 (591-1,017 bp) is highly conserved between *MdCOP1*-1 and *MdCOP1*-2, was amplified using the primer pair asMdCOP1F and asMdCOP1R and cloned into pBI121 to construct an *MdCOP1* antisense expressing vector (Supplemental Table S1). Subsequently, the resultant vectors were genetically transformed into *Arabidopsis* by the floral-dip method as described by Clough and Bent (1998) and into apple callus as reported by Li et al. (2002).

**Construction of the Viral Vectors and Agroinfiltration in Apple Fruit**

To observe the effects of *MdCOP1*-1 and *MdCOP1*-2 overexpression or suppression in apple fruits, viral vectors were used as described by Ratcliff et al. (2001) and Perez et al. (2007). The full-length cDNAs of *MdCOP1*-1 and *MdCOP1*-2 were amplified using the primer pairs BDP1-F/-R and BDP2-F/-R and then cloned into the pRI vector under the control of the 35S promoter (Supplemental Table S1). The overexpression constructs were named pRI-MdCOP1-1 and pRI-MdCOP1-2. To generate an antisense expression vector for *MdCOP1*, the same 426-bp fragment of *MdCOP1*-2 (591-1,017 bp) was used in the above-mentioned genetic transformation was cloned into the tobacco rattle virus (TRV) vector in an antisense orientation under the control of the dual 35S promoter. The resultant vector was named TRV-MdCOP1.

Approximately 200 ng of plasmid DNA (in 100 μl) of the empty vector pLR, pLR-MdCOP1-1, and pLR-MdCOP1-2 was injected to fresh-bagged apple fruit skin using a needleless syringe. The helper plasmid IL-60-BS was used in all sense infiltrations. The injected apples were kept overnight in the dark at room temperature and were subsequently treated with 24 h continuous white light (200 μmol m⁻² s⁻¹) with supplemental UV-B (280-320 nm) at 17°C in a growth chamber for 2 to 4 d before observation.

Meanwhile, the antisense expression vector TRV-MdCOP1 and empty vector TRV were transformed into *Agrobacterium tumefaciens* GV3101. After the *A. tumefaciens* grew to saturation in Luria-Bertani medium, the culture was centrifuged. Subsequently, the pellet was resuspended in 10 mM MgCl₂, 10 mM MES, 10 mM KCl, and 0.1 mM ABT to form a 100 μg ml⁻¹ solution containing 10 μg ml⁻¹ ABT and 0.05% (v/v) acetone.
Measurement of the Total Anthocyanin Concentration

Total anthocyanin was extracted with a methanol-HCl method (Lee and Wicker, 1991). Samples (0.1 g) were soaked and incubated overnight in 5 mL methanol and 0.1% (v/v) HCl in the dark at room temperature. The absorbance of each extract was measured at 530, 620, and 650 nm with a spectrophotometer (UV-1600, Shimadzu). The relative anthocyanin content was determined by the formula: optical density (OD) = (OD650 - OD620) / 0.1 - (OD530 - OD620). One unit of anthocyanin content was expressed as a change of 0.1 optical density (unit × 10^3 g^-1 fresh weight).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Apple fruit coloration, anthocyanin accumulation and MdMYB1 transcript levels in light in apple fruit.

Supplemental Figure S2. Schematic representing the alignment of MdCOPI-1 and McCOPI-2 cDNAs with the corresponding genes.

Supplemental Figure S3. In vitro ubiquitination of MdMYB1-GST.

Supplemental Figure S4. Function of MdCOPIs in anthocyanin accumulation in the Arabidopsis cop1-4 mutant.

Supplemental Table S1. Primers used in this study.

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and 150 ms acetosyringone and kept at room temperature for 2 h. For TRV infections, separate cultures containing pTRV1 and TRV-MdCOP1 were mixed in a 1:1 ratio, and then infiltrated into fresh-bagged apple fruit skin using a needleless syringe. The injected apples were treated in darkness at 4°C for 7 d and then transferred to 24 h continuous white light (200 μmol m^-2 s^-1) with supplemental UV-B (280–320 nm) at 17°C in a growth chamber for 4 to 6 d before observation. The apples infiltrated with either pLR or TRV were used as controls.

**BiFC**

The full-length cDNAs of MdCOPI-1, MdCOPI-2, and MdMYB1 were amplified using the primer pairs BiFCP1-F/-R, BiFCP2-F/-R, and BiFCMYB-F/-R (Supplemental Table S1), respectively, and then cloned into pSPYNE-35S and pSPYCE-35S vectors, which contain DNA encoding the N- or C-terminal regions of YFP (YFP-N or YFP-C), respectively. Onion (*Allium cepa*) epidermal cells were transiently co-expressed using an *A. tumefaciens* infection method with different combinations of these constructs as described by Walter et al. (2004). YFP-dependent fluorescence was detected 24 h after transfection using a confocal laser-scanning microscope (Zeiss LSM 510 Meta).

**Yeast Two-Hybrid Assays**

Yeast (*Saccharomyces cerevisiae*) two-hybrid assays were performed using the Matchmaker GAL4-based two-hybrid system as recommended by the manufacturer (Clontech). Full-length cDNAs of *MdCOPI-1* and *MdCOPI-2*, as well as an *MdMYB1* fragment as described by Xie et al. (2012), were inserted into pGAD424 and pGBT9 (Clontech). All of the constructs were transformed into yeast strain AH109 using a lithium acetate method. Yeast cells were cultured on minimal medium/-Leu/-Trp according to the manufacturer’s instructions (Clontech). Transformed colonies were plated onto minimal medium/-Leu/-Trp/His/-Ade with or without 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside to test for possible interactions.

**In Vitro Ubiquitination Assays and in Vivo Coimmunoprecipitation**

The open reading frames of *MdMYB1* and *MdCOPI1* were amplified using the primer pairs PEN/F/-R and PENB/F/-R (Supplemental Table S1), respectively, and then inserted into pGEX-4T-1 (Amersham Biosciences) to generate the *MdMYB1-GST* and *MdCOPI1-GST* fusion constructs, respectively. All of the constructs were transformed into *Escherichia coli* strain BL-21 cells. After growing to saturation, the transformed cells were treated with isopropyl-β-D-thiogalactoside to induce fusion protein expression and then purified using a Pierce GST spin purification kit (Thermo) following the instructions.

In vitro ubiquitination assay was performed according to Seo et al. (2003). Each 30-μL reaction mixture contained 100 ng protein substrate (MdMYB1-GST or *MdCOPI1-GST*), 20 ng rabbit E1 ubiquitin-activating enzyme (Boston Biochem), 20 ng human E2 ubiquitin-conjugating enzymes UbcH13 (Boston Biochem), 10 μg His-6 ubiquitin (Sigma-Aldrich), and 200 ng E3 (recombinant *MdCOPI2* protein, *MdCOPI1*-GST). The reactions were carried out at 30°C for 4 h. A 10-μL portion of the reaction mixture was separated on an 8% SDS-PAGE gel, and ubiquitinated *MdMYB1* was detected by western blot with anti-MdMYB monoclonal antibody and antiubiquitin (Sigma-Aldrich) antibody.

For in vivo detection of ubiquitinated *MdMYB1*, *MdMYB1-GFP* transgenic and wild-type apple calli were pretreated with 50 μM proteasome inhibitor MG132 for 16 h in the light or dark. A Pierce classic IP kit (Thermo) was used to immunoprecipitate *MdMYB1-GFP* with anti-GFP antibody (Sigma-Aldrich), and the precipitate was analyzed by immunoblot with antiubiquitin antibody (Sigma-Aldrich).

For in vivo coimmunoprecipitation, *MdMYB1-GFP* and GFP transgenic calli treated with MG132 (50 μM) for 16 h was ground in liquid nitrogen. A Pierce classic IP kit (Thermo) was applied to immunoprecipitate the *MdMYB1-GFP* (or GFP) and *MdCOPI1* proteins with anti-GFP and anti-MdCOPI1 monoclonal antibodies. The eluted proteins were analyzed by western blot with the opposite antibodies.

**Hypocotyl Length Measurements**

The hypocotyl lengths of at least 30 Arabidopsis seedlings grown in the dark were measured. The experiments were performed with at least three independent biological replications.
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