Article

ROS1 promotes low temperature-induced anthocyanin accumulation in apple by demethylating the promoter of anthocyanin-associated genes

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Abstract

Low temperature can affect the growth and development of plants through changes in DNA demethylation patterns. Another known effect of low temperature is the accumulation of anthocyanin pigments. However, it is not known whether the two phenomena are linked, specifically whether DNA demethylation participates in anthocyanin accumulation in response to low-temperature stress. The ROS1 gene is involved in plant DNA demethylation and influences methylation levels in response to low-temperature stress. In this study, using RNA sequencing, we detected the transcription levels of MdROS1, as well as those of anthocyanin biosynthesis-related genes, in apple (Malus domestica) at low temperature. Genomic bisulfite sequencing showed that the methylation levels of the promoters of the anthocyanin-related genes MdCHS, MdCHI, MdF3′H, MdANS, MdUFGT, and MdMYB10 decreased in apple leaves after low-temperature treatment. Similar expression and methylation results were found in apple fruit. Transiently silencing MdROS1 in the leaves and fruit of apple cultivars inhibited the accumulation of anthocyanins and led to decreased expression of anthocyanin biosynthetic genes, and the opposite results were detected in MdROS1-overexpressing leaves and fruit. A promoter binding assay showed that the conserved RRD-DME domains of MdROS1 bind directly to the promoters of MdF3′H and MdUFGT. Taken together, these results suggest that ROS1 affects the anthocyanin biosynthetic pathway by decreasing the methylation level of anthocyanin-related gene promoters, thereby increasing their expression and increasing anthocyanin accumulation.

Introduction

Anthocyanins are phenylpropanoid compounds that are ubiquitous in the tissues and organs of later-diverging land plants. These compounds have been shown to play multiple roles in environmental stress tolerance, in resistance to herbivores and pathogens, and as red, blue, and purple pigments that attract pollinators and seed dispersers [1–3]. Anthocyanins are also helpful in reducing several disease risks for humans as dietary supplements [4–7].

Biotic and abiotic factors such as light and temperature can affect anthocyanin synthesis [8, 9]. In apple, it is known that a lower temperature (12–17°C) promotes anthocyanin accumulation in fruit compared with a higher temperature (24–27°C) [10]. Furthermore, 7°C night temperature can also induce double the amount of anthocyanins compared with a 17°C night temperature [11]. Several studies have shown that low temperature (LT) promotes anthocyanin accumulation by inducing the transcription of anthocyanin biosynthesis-related genes [12–15]. LTs have been shown to facilitate anthocyanin biosynthesis by inducing the expression of several anthocyanin biosynthetic genes [16, 17], as well as members of the R2R3-MYB, basic helix-loop-helix (bHLH) and WD40 transcription factor families, which regulate anthocyanin biosynthesis under LT in crab apple (Malus) leaves [18]. It has also been shown that MdbHLH3, an LT-induced transcription factor, regulates LT-induced anthocyanin biosynthesis by binding to the promoters of the MdDFR and MdUFGT genes and MdMYB1 to promote their expression in apple (Malus domestica) fruit [10]. In contrast, the MdMYB15L repressor binds to the promoter of MdCBF2 and inhibits the expression of MdCBF2, thus decreasing the cold tolerance of apple at low temperature. This repressor also competitively binds with MdbHLH3 and reduces MdbHLH3-induced anthocyanin accumulation [19]. However, the upstream mechanism of the transcriptional regulation of LT-induced anthocyanin accumulation still needs to be examined.

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Results
Identification of the low-temperature-responsive gene ROS1
To investigate the function of genes involved in DNA demethylation during LT-induced anthocyanin accumulation, we searched transcriptome data from leaves of LT-treated apple (M. domestica ‘Gala’) plantlets [36] (Fig. 1a) and identified 18 homologous Arabidopsis DNA demethylation genes. The expression level of four of these genes increased with LT treatment, and expression analysis by quantitative reverse transcription PCR (qRT–PCR) showed that, of these four, MD12G1017600, a ROS1 gene, had increasing expression and the highest transcript accumulation on Day 5 of treatment, so we targeted this gene for further analysis.

We compared the amino acid sequences of the MdROS1 gene in different plants and found that they had high homology (Supplementary Fig. S1). MdROS1 is closely related to PbDME2 (Pyrus breitshneideri), PpROS1 (P. persica), FvROS1 (Pyrus bretschneideri) and FvROS1 (Fragaria vesca subsp. vesca) (Supplementary Fig. S2). To further determine whether this MdROS1 gene responds to an LT signal, we measured its expression in the leaves of ‘Gala’ tissue culture seedlings after 16°C (LT) treatment for 7 days. As shown in Fig. 1b, a bright red color appeared in the leaves after this treatment. High-performance liquid chromatography (HPLC) analysis showed that anthocyanin levels (cyanidin-3-O-glucoside) increased from 0.0 to 22.30 μg/g after 7 days of LT treatment (Fig. 1e). Expression analysis showed that LT treatment significantly promoted the expression of MdROS1, and we also observed that anthocyanin biosynthesis genes (CHS (chalcone synthase), CHI (chalcone isomerase), flavonoid 3′-monooxygenase (F3′H), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UGFT)) and the regulatory gene MdMYB10 showed increased expression (Fig. 1h and k).

We next performed LT treatment on the ever-red crab apple cultivar ‘Royalty’ and the evergreen crab apple cultivar ‘Flame’ (Fig. 1c–d), and we observed similarly increased expression of ROS1 and anthocyanin biosynthesis and regulatory genes and anthocyanin accumulation under LT in these two cultivars (Fig. 1f–g, i–j and l–m), suggesting that ROS1 participates in LT-induced anthocyanin accumulation in Malus plants.

Changes in the expression of anthocyanin-related genes after MdROS1 silencing in Malus leaves
To further confirm the function of MdROS1 in LT-induced anthocyanin accumulation, Agrobacterium tumefaciens cultures containing vectors for the expression of the green fluorescent protein (GFP) reporter alone (TRV-GFP) or GFP together with MdROS1 silencing (TRV-GFP-MdROS1) were individually infiltrated into leaves of the ever-red crab apple cultivar ‘Royalty’ and the green-leaf apple cultivar ‘Gala’. Green fluorescence was observed in all infiltrated leaves. In ‘Royalty’, red
leaves with suppressed MdROS1 expression showed reduced accumulation of anthocyanins, resulting in green coloration in leaves infiltrated with the TRV-GFP-MdROS1 vector after LT treatment (Fig. 2a). In ‘Gala’, there was no obvious variation between wild-type leaves and leaves that were MdROS1-silenced. We observed that LT treatment promoted anthocyanin accumulation and red coloration in control leaves, whereas the red color was weaker in leaves infiltrated with the TRV-GFP-MdROS1 vector (Fig. 2d). Expression analysis showed that MdROS1 was silenced (Fig. 2c and f), and HPLC analysis indicated significantly lower anthocyanin levels (cyanidin-3-O-glucoside) in the MdROS1-silenced leaves than in those of the control (Fig. 2b and e).

Quantitative qRT–PCR analysis suggested a significant decrease in the transcription of the anthocyanin regulatory gene MdMYB10 and the anthocyanin biosynthetic genes MdCHS, MdCHI, MdF3’H, MdANS, and MdUFGT when MdROS1 was silenced in two Malus cultivars under both normal temperature and LT (Fig. 2c and f) conditions. Next, we measured DNA methyltransferase (DNMT) activity in the MdROS1-silenced leaves and observed that activity increased substantially compared with control leaves under both normal and LT conditions (Supplementary Fig. S5).

Overexpressing MdROS1 in Malus leaves alters the expression of anthocyanin-related genes

Deep red coloration was detected in MdROS1-overexpressing ‘Royalty’ leaves (Fig. 3a). The expression level of MdROS1 confirmed that MdROS1 was overexpressed (Fig. 3c and f). HPLC analysis also suggested significantly higher levels of cyanidin-3-O-glucoside in overexpressing leaves than in control ‘Gala’ leaves (Fig. 3b and e). qRT–PCR analysis suggested that the transcription levels of the anthocyanin regulatory gene MdMYB10 and the anthocyanin biosynthetic genes MdCHS, MdCHI, MdF3’H, MdANS, andMdUFGT were significantly higher in the MdROS1-overexpressing leaves than in the control leaves (Fig. 3c and f). DNMT activity was suppressed in the MdROS1-overexpressing leaves compared with the control leaves (Supplementary Fig. S5).
Changes in transcription of anthocyanin-related genes after MdROS1 silencing in apple fruit

To further confirm the function of MdROS1, we also silenced its expression in apple fruit by agroinfiltration. Fruit that was transformed with TRV-GFP-MdROS1 showed less anthocyanin accumulation at the injection sites, resulting in significant yellow coloration (Fig. 5a), while LT treatment led to increased accumulation of anthocyanins in control fruit, with a red coloration at the injection sites. HPLC analysis supported the phenotypic observations since anthocyanin levels (cyanidin-3-O-glucoside) in the MdROS1-silenced fruit were lower than those in the control fruit (Fig. 5b). Expression analysis confirmed that MdROS1 was silenced (Fig. 5c). The expression levels of MdMYB10 and the anthocyanin biosynthetic genes MdCHS, MdCHI, MdF3′H, MdANS, and MdUFGT were lower in MdROS1-silenced fruit. Under LT treatments, the MdROS1-silenced fruit showed lower expression of these genes than the control fruit (Fig. 5c). DNMT activity in the MdROS1-silenced fruit was much higher than that in the control fruit under both normal and LT conditions (Supplementary Fig. S5).

Overexpressing MdROS1 in apple fruit alters expression of anthocyanin-related genes

To further test the function of MdROS1, the pRI-MdROS1 vector was injected into the skin of apple fruit. As shown in Fig. 6a and b, overexpression of MdROS1 promoted anthocyanin accumulation. The expression level of MdROS1 was significantly higher in overexpressed fruit than in the control (Fig. 6c). Furthermore, the MdROS1-overexpressing fruit showed higher MdMYB10, MdCHS, MdCHI, MdF3′H, MdANS, and MdUFGT expression than the control fruit. DNMT activity in the MdROS1-overexpressing fruit was much lower than that in the control fruit (Supplementary Fig. S5). Taken together, these data suggest that MdROS1 promotes LT-induced anthocyanin biosynthesis by regulating the expression of anthocyanin-related genes.

DNA methylation levels in leaves and fruit after low-temperature induction of ROS1

We next measured the methylation levels in the promoters of the anthocyanin regulatory gene MdMYB10 and the anthocyanin biosynthesis genes by bisulfite-sequencing PCR (BSP) of genomic DNA from leaves and fruit. BSP analysis was performed as previously described [37]. Primers were designed by MethPrimer (http://www.urogene.org/methprimer/) and were placed as described in Supplementary Fig. S3. In ‘Royalty’ leaves, BSP analysis indicated that the methylation levels decreased under LT, most notably in MdF3′H and MdUFGT (Fig. 7a). In ‘Flame’ leaves, methylation mainly occurred in the CHH sequence and to a lesser extent in the CHG and CG sequences. Decreased methylation was also detected in ‘Flame’ and ‘Gala’
transient overexpression of ROS1 in apple leaves. a Agroinfiltrated ‘Royalty’ leaves were photographed under UV illumination and normal light at 10 days post-infiltration. Scale bar = 0.5 cm. b Anthocyanin content in inoculated ‘Royalty’ leaves. c Expression of MdROS1 and anthocyanin-related genes was detected by qRT-PCR in infected ‘Royalty’ leaves. d Agroinfiltrated ‘Gala’ leaves were photographed under UV illumination and normal light at 10 days post-infiltration. Scale bar = 1 cm. e Anthocyanin content in inoculated ‘Gala’ leaves. f Expression of MdROS1 and anthocyanin-related genes in inoculated ‘Gala’ leaves was determined using qRT–PCR. qRT–PCR and HPLC analyses were performed with three biological replicates. Error bars indicate the standard error of the mean (SE) of three replicate measurements. Different letters above the bars indicate significantly different values (P < .05), calculated using one-way ANOVA followed by Tukey’s multiple range test.

Figure 3. Transient overexpression of ROS1 in apple leaves. a Agroinfiltrated ‘Royalty’ leaves were photographed under UV illumination and normal light at 10 days post-infiltration. Scale bar = 0.5 cm. b Anthocyanin content in inoculated ‘Royalty’ leaves. c Expression of MdROS1 and anthocyanin-related genes was detected by qRT-PCR in infected ‘Royalty’ leaves. d Agroinfiltrated ‘Gala’ leaves were photographed under UV illumination and normal light at 10 days post-infiltration. Scale bar = 1 cm. e Anthocyanin content in inoculated ‘Gala’ leaves. f Expression of MdROS1 and anthocyanin-related genes in inoculated ‘Gala’ leaves was determined using qRT–PCR. qRT–PCR and HPLC analyses were performed with three biological replicates. Error bars indicate the standard error of the mean (SE) of three replicate measurements. Different letters above the bars indicate significantly different values (P < .05), calculated using one-way ANOVA followed by Tukey’s multiple range test.

Figure 4. ROS1 may participate in LT-induced anthocyanin accumulation in apple fruit. a ‘Red Fuji’ fruit under 23°C for 3 days were defined as control (CK). Scale bar = 1 cm. b Anthocyanin content in apple fruit following 3 days of LT treatment. c Expression level of anthocyanin-related genes determined by qRT-PCR. d Expression level of MdROS1 determined by qRT–PCR. Different letters above the bars indicate significantly different values (P < .05) calculated using one-way ANOVA followed by Tukey’s multiple range test.

Figure 4. ROS1 may participate in LT-induced anthocyanin accumulation in apple fruit. a ‘Red Fuji’ fruit under 23°C for 3 days were defined as control (CK). Scale bar = 1 cm. b Anthocyanin content in apple fruit following 3 days of LT treatment. c Expression level of anthocyanin-related genes determined by qRT-PCR. d Expression level of MdROS1 determined by qRT–PCR. Different letters above the bars indicate significantly different values (P < .05) calculated using one-way ANOVA followed by Tukey’s multiple range test.

leaves (Fig. 7b and c). We also observed that methylation also occurred in the fruit peels of ‘Stolav’ under LT, mainly in the CHH sequence and especially in MdUFGT (Fig. 7d).

MdROS1 protein binds to the promoters of anthocyanin biosynthetic genes

The results of the MdROS1 silencing and BSP analyses indicated that the ROS1-mediated DNA demethylation pathway is involved in LT-induced anthocyanin accumulation. To further confirm the function of MdROS1 binding to the promoters of anthocyanin-related genes, a yeast one-hybrid (Y1H) assay was conducted. The main domains, helix-hairpin-helix motif (HHH superfamily, 3769–4285 bp), permuted single zf-CXXC (Perm-CXXC, 4957–5052 bp), and RNA recognition motif-DME (RRD-DME, 5059–5365 bp), were inserted into the pJG4-5 vector. The results showed that MdROS1J and the RRD-DME domain bound to the promoters of MdF3′H and MdUFGT, and that the perm-CXXC domain bound the promoter MdUFGT. This suggested that the anthocyanin biosynthetic genes MdF3′H and MdUFGT might be MdROS1 target genes (Fig. 8a). To provide more evidence for the interaction, we performed a β-glucuronidase (GUS) staining assay using the pGFPGUSPLUS vector containing the MdROS1J or RRD-DME domain in tobacco. The results showed that tobacco expressing both MdROS1J and MdF3′H, RRD-DME and MdF3′H, MdROS1J and MdUFGT, and RRD-DME and MdUFGT were dyed blue (Fig. 8b), indicating that the RRD-DME and MdROS1J sequences had higher affinity for the MdF3′H and MdUFGT promoters. Furthermore, we cut the promoter into three segments, performed Y1H assays (Supplementary Fig. S6) and conducted
Figure 5. Transient silencing of ROS1 in apple fruit. a Infiltrated apple fruits were visualized at 7 days post-infiltration. b Anthocyanin accumulation in inoculated apple fruit. (c) The transcription levels of MdROS1 and anthocyanin biosynthesis genes in inoculated apple fruit were determined using qRT–PCR. qRT–PCR and HPLC analyses were performed with three biological replicates. Error bars indicate the standard error of the mean ± standard error of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way ANOVA followed by Tukey’s multiple range test.

Figure 6. Transient overexpression of ROS1 in apple fruit. a Infiltrated apple fruits were visualized at 3 days post-infiltration. b Anthocyanin accumulation in inoculated apple fruit. c Transcription levels of MdROS1 and anthocyanin biosynthesis genes in inoculated apple fruit were determined using qRT–PCR. qRT–PCR and HPLC analyses were performed with three biological replicates. Error bars indicate the standard error of the mean ± standard error of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way ANOVA followed by Tukey’s multiple range test.

a) a biolayer interferometry (BLI) assay to quantify the binding affinities of the RRD-DME and MdROS1J protein sequences to the promoters of $MdF3'H$ and $MdUFGT$. The results showed that RRD-DME and MdROS1J had a significant interaction with the $MdUFGT$ promoter. The kinetic values indicated that RRD-DME had a higher affinity for the $MdUFGT$ promoter than did MdROS1J (Fig. 8c).

**Discussion**

LT promotes anthocyanin accumulation in *Malus* plants [38], and anthocyanins play an important role in plant adaptation to changes in environmental conditions [1–3]. Here, we demonstrate that ROS1, a DNA demethylation protein, is a positive regulator of cold tolerance and anthocyanin biosynthesis in apple leaves and fruit. In addition, ROS1 was confirmed to bind directly to downstream gene promoters to regulate their methylation levels.

**Under low-temperature stress, ROS1 participates in the formation of anthocyanins**

ROS1-mediated DNA demethylation has been studied extensively in plants. For example, the DNA demethylase ROS1 can regulate seed dormancy by controlling imprinted gene transcription in the endosperm in *Arabidopsis* [31]. The silencing of EPIDERMAL PATTERNING FACTOR 2 (EPF2) in *Arabidopsis* with mutated ROS1 resulted in the overproduction of stomatal lineage cells [32]. In rice, defects in male and female gametogenesis were observed on knocking out the OsROS1 mutant [39]. Moreover, OsROS1 can promote aleurone formation by reducing the expression levels of two putative transcription factor genes, RISBZ1 (rice seed b-Zipper 1) and RPBF (rice prolamin box binding factor) [40]. ROS1 also plays an important role during plant defense against pathogens. The expression of several immunity-related genes was enhanced by demethylating the promoters of these genes by ROS1 to restrict attack by the bacterium *Pseudomonas syringae* [30]. These studies suggested that ROS1 participates in various physiological activities during plant growth and development.

We also noticed that several studies showed that DNA demethylation and methylation can affect flavonoid biosynthesis during cold treatment [34, 35]. For example, demethylation of the *Artemisia annua* AaPAL1 promoter strongly correlated with anthocyanin accumulation [46], and the expression level of the DNA demethylase ROS1 gene peaked at the pink stage of strawberry ripening [50]. In our present study, we found that LT treatment induced the expression of ROS1, accompanied by anthocyanin accumulation, as well as anthocyanin-related gene
expression. Functional assays also suggested that the expression variation in MdROS1 can alter the coloration of Malus leaves and fruit, suggesting that ROS1 may be an anthocyanin regulatory gene at the transcription level, promoting anthocyanin accumulation during LT stress.

**ROS1-mediated DNA demethylation is a supplementary mechanism for LT-induced anthocyanin accumulation**

Anthocyanin synthesis is induced by various environmental factors, such as light, LT, and salinity [41–43]. Several studies have shown that LT leads to an increase
in anthocyanin biosynthesis mainly by promoting the transcription of relevant transcription factors [10, 12, 15, 44]. In Arabidopsis thaliana, MYB75, MYB90, MYB113, and MYB114 contribute to LT-induced anthocyanin accumulation [45–47]. BoPAP1, a MYB transcription factor, has been shown to increase the expression level of anthocyanin biosynthesis genes and promote coloration in purple kale (Brassica oleracea) during LT exposure [44]. An apple MYB transcription factor, MdMYB15L, was found to inhibit anthocyanin accumulation by competitively interacting with MdbHLH33 under cold treatment [19]. Finally, MdSIZ1 was identified to regulate anthocyanin accumulation by sumoylating MdMYB1 in response to LT in apple [48].

Another recent study showed that massive expression of AtROS1 in tobacco leads to a reduction in the methylation of the CHS, CHI, F3H, FLS, DFR, and ANS promoters and induces their expression, resulting in an elevated flavonoid content [33]. Moreover, lower methylation levels in the promoters of anthocyanin biosynthesis genes and regulatory genes were positively related to the higher expression levels of these genes in LT-treated peach fruit flesh [34]. The methylation levels of both the DFR and Ruby promoter regions were strongly decreased in the area of high anthocyanin accumulation during cold storage. This report suggested that anthocyanin accumulation in blood orange fruit is related to epigenetic control mechanisms such as promoter methylation [35]. Therefore, we deduced that DNA demethylation in anthocyanin biosynthesis gene promoters may play an important role in regulating LT-induced anthocyanin accumulation in Malus plants. Our results showed that the methylation level of anthocyanin biosynthesis gene promoters was decreased in LT-treated Malus leaves or fruit, and the expression level of anthocyanin biosynthesis genes was significantly changed with ROS1 overexpression or silencing, which suggested that LT can induce the demethylation of anthocyanin biosynthesis genes by ROS1 and promote anthocyanin accumulation by increasing the expression of related genes. Furthermore, we hypothesized that ROS1-mediated demethylation of anthocyanin biosynthesis gene promoters may be a coordinated regulatory mechanism, with transcription factors controlling anthocyanin-related gene expression and regulating anthocyanin accumulation under LT.

The RRD-DME domain of MdROS1 interacts directly with the promoter of MdUFGT

High levels of AtROS1 expression in tobacco plants affect the methylation status of flavonoid biosynthesis genes and antioxidant-related gene promoters. A. thaliana ros1 mutants have been reported to have hypermethylated genes/transgenes, resulting in transcriptional gene silencing [22]. NtGPDL-like genes were shown to be demethylated under abiotic stress conditions [33]. However, these studies were limited to epigenetic analysis and did not show how ROS1 interacts with the promoters of target genes.

A previous study showed that Arabidopsis antisilencing factor SUVH1 (Su(var)3-9 homolog) is required for the transcription of promoter-methylated genes without altering DNA methylation [51]. In a Chip-seq (chromatin immunoprecipitation combined with sequencing) assay SUVH1 was significantly enriched in the promoters of methylated genes, which suggested that SUVH1 can directly bind to these promoters [51]. These results suggest that MdROS1 may have the same mechanism when regulating DNA demethylation.

Our results showed that MdROS1 can bind to the promoters of anthocyanin biosynthetic genes to participate in anthocyanin accumulation. MdROS1 has three structures in the conserved domain area: an HHH superfamily domain, a perm-CXXC domain, and an RRD-DME domain. The RRD-DME domain is an important structural domain in plant DNA glycosylases and participates in demethylation [52, 53]. The perm-CXXC domain has been shown to bind to non-methyl sites [53], and the HHH superfamily domain has DNA binding properties [54, 55]. Y1H assays showed that MdROS1J (the combined HHH motif, perm-CXXC domain, and RRD-DME domain) and the RRD-DME domain interacted with the promoters of MdF3′H and MdUFGT, and GUS staining assays and BLI further confirmed that the MdROS1J and RRD-DME domains interacted with the promoters of MdF3′H and MdUFGT.

We deduce that MdROS1-mediated DNA demethylation promotes anthocyanin biosynthesis in Malus plants by binding to the promoters of MdF3′H and MdUFGT under LT conditions and that the RRD-DME domain of MdROS1 is necessary for interaction with the target gene. Furthermore, ROS1, DME, DML1, DML2, and DML3 belong to the 5-mC DNA glycosylase family of proteins and are responsible for initiating active DNA demethylation in Arabidopsis [22, 52, 56]. Moreover, the ROS1 loss-of-function mutant showed no obvious developmental defects, and we predict that these five 5-mC DNA glycosylase family proteins are functional complements in plants. In our results, we noticed that the methylation levels of anthocyanin-related genes, including MdMYB10, were reduced. Furthermore, Y1H, electrophoretic mobility shift assay (EMSA), and BLI assays suggested that MdF3′H and MdUFGT are direct targets of MdROS1. Therefore, we deduced that the DME, DML1, DML2, and DML3 proteins may also participate in the LT-induced anthocyanin biosynthesis process, and we will elucidate this hypothesis in our future study.

DNA methylation and demethylation are dynamic processes

The biological processes of phosphorylation and acetylation are reversible, and the methylation of histones at lysine residues is also reversible [57]. During ROS1-mediated DNA demethylation, METHYL-CpG-BINDING DOMAIN 7 (MBD7) interacts with Increased DNA Methyltransferase 2 (IDM2) and Increased DNA Methyltransferase 3 (IDM3) to recruit Increased DNA Methyltransferase 1
(IDM1) for histone acetylation and provides the proper chromatin environment for ROS1 function [58]. ROS1 antagonizes RNA-directed DNA methylation (RdDM) to avoid DNA hypermethylation at specific loci, and the expression of ROS1 decreases in all known RdDM mutants, indicating that ROS1 expression is influenced by DNA methylation and demethylation pathways [49, 59, 60]. Studies have also shown that ROS1 expression is promoted by DNA methylation and antagonized by DNA demethylation [61], and ROS1 expression is also inhibited in RdDM silencing and in met1 mutants [61]. Moreover, there is a cyclically reversible dynamism between DNA methylation and DNA demethylation during the temperature seasonality response in perennial American ginseng [62], which suggests that DNA methylation and demethylation are dynamic processes in plants.

The methylation levels of the MdMYB10 promoter were related to the different apple peel color patterns [63]. Moreover, the expression of MdMYB1 is mainly regulated by the CHH methylation levels in the MR3 region (~1246 bp to ~780 bp) of the MdMYB1 promoter. These results suggested that the expression of MYB1 is mainly controlled by DNA methylation [64]. In our study, BSP analysis suggested that the methylation level of the anthocyanin regulatory gene MdMYB10, as well as anthocyanin biosynthetic genes, decreased under LT. Therefore, we deduced that LT promotes the expression of ROS1, enhances the demethylation of anthocyanin-related genes, and induced anthocyanin accumulation. This suggests that methylation and demethylation synergistically control the transcription of MdMYB10 and anthocyanin biosynthetic genes in response to temperature variation.

In conclusion, this study indicates that ROS1 promotes anthocyanin accumulation under LT conditions by binding directly to the promoters of anthocyanin-related genes, resulting in dynamic methylation and demethylation. Moreover, ROS1-mediated DNA demethylation is a supplementary mechanism for LT-induced anthocyanin accumulation.

**Materials and methods**

**Plant materials**

The experimental materials used in this study were *M. domestica* ‘Gala’, 'Red Fuji’, 'Stolav', *Malus* cv. 'Royalty', and *Malus* cv. 'Flame'. The explants were taken from the annual branches in the apple germplasm resource garden of Beijing Agricultural University before germination in the spring, and were cultured in Murashige and Skoog (MS) medium. The culture temperature was 23–26°C, the relative humidity was controlled at 60–70%, the light duration was 16 hours/8 hours, and the light intensity was 10,000 lux. Bagged 'Stolav' fruit were collected 140 days after blooming. We used three trees with similar growth conditions and collected fruit samples from annual branches growing in the southeast direction.

Fruit skins were collected by peeling to generate samples with <1 mm of cortical tissue.

**RNA extraction and qRT–PCR analysis**

RNA samples were extracted from apple peels and crab apple leaves using an RNA Extraction Kit (Biomed, Beijing, China). SYBR Green qPCR Mix (TaKaRa, Ohtsu, Japan) and a Bio-Rad CFX96 Real-Time PCR System (BIO-RAD, USA) were used to analyze the expression of related genes as described before [18]. The $2^{-ΔΔCt}$ method [65] was used to calculate transcription levels.

**Measurement of methylation levels**

Genomic DNA (1.0 μg) was obtained using the DNA Bisulfite Extraction Kit (Aidlab, Beijing, China) to conduct bisulfite treatment. The Methylation-Specific PCR Kit (Tiangen Biotech, Beijing, China) was used for PCR analysis. The methylation level was calculated after sequencing 8–12 times.

**Yeast one-hybrid assays**

As the effector constructs, the open reading frame of MdROS1 and the HHH superfamily, perm-CXXC, and RRM-DME structural domains were separately cloned into pG4-5 vector (Clontech, Palo Alto, CA, USA) under the galactokinase 1 (GAL1) promoter. The MdCHS, MdCHI, MdF3H, MdANS, MdUFGT, and MdMYB10 promoter sequences were inserted in the pLaCZ vector. The EGY48 yeast (Saccharomyces cerevisiae) strain was used to conduct the Y1H assay. All transformation and screenings were performed three times.

**Transient expression in tobacco and β-glucuronidase staining assays**

Transient overexpression of MdROS1J, RRD-DME and Perm-CXXC in tobacco (*Nicotiana benthamiana*) leaves was performed using the pBI121 vector containing the MdROS1J, RRD-DME and Perm-CXXC sequence, using a previously described protocol [66]. The MdUFGT and MdF3′H promoter was cloned into the pBI101 vector. Transient expression assays in *N. benthamiana* plants were carried out as previously described [67]. GUS staining was performed as previously described [68]. All experiments were carried out with three biological replicates.

**Biolayer interferometry assays**

GST-MdROS1J and GST-RRD-DME solution (20 μg/ml) diluted with 10 mM sodium acetate pH 4.5 was detected with sensors for 10 min to analyse the binding ability of MdROS1 with target promoters. Promoter sequences were diluted to five different concentrations in BLI buffer [0.01 M PBS, pH 7.4, 0.005 % (v/v) Tween 20] to serve as analyte samples [69].

**HPLC analysis**

Apple leaf and fruit peel samples (0.8–1.0 g fresh weight) were ground in 10 ml extraction solution (methanol:water:formic acid:trifluoroacetic acid, 70:27:23–26 and Skoog (MS) medium. The culture temperature was 8–16°C, the relative humidity was controlled at 60–70%, the light duration was 16 hours/8 hours, and the light intensity was 10,000 lux. Bagged ‘Stolav’ fruit were collected 140 days after blooming. We used three trees with similar growth conditions and collected fruit samples from annual branches growing in the southeast direction.
and incubated at 4°C in the dark for 72 hours [70]. The supernatant was passed through filter paper and then through a 0.22-μm Millipore™ filter (Billerica, MA, USA). An HPLC instrument (Agilent 1100, Agilent Technologies Inc., USA) with a 150-mm column was used for determination of anthocyanin content. When the retention time was ~5.9 minutes, the peak represented cyanidin-3-O-glucoside [3, 70, 71]. The peak area (x) was used to calculate anthocyanin content by the following formula:

Anthocyanin content = \( (R \times x + a) \times \frac{v}{m} \)

where \( R \) and \( a \) are the coefficients of the standard curve as measured with cyanidin-3-O-glucoside standard samples, \( x \) is the peak area, \( v \) is the volume of extraction solution, and \( m \) is the weight of samples.

**Transient expression assays in apple plantlets and apple fruit**

The MdROS1 (434 bp) fragment was cloned into pTRV2 vector at the XbaI and KpnI sites for a silencing assay [72]. The full length of MdROS1 was cloned into a modified pRI101-eGFP vector at the NdeI and BamHI sites for over-expression analysis. The infiltration protocol and culture methods for transient expression assays in crab apple plantlets and apple fruit were as previously described [18, 73]. All samples were analyzed in at least three biological replicates.

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**Author contributions**

Experiments were designed by J.T. and Y.Y. and performed by L.Y., Y.S., X.Z. and M.C. Data were analyzed by T.W., J.T., Y.X., J.Z. and Y.Y. Contributed reagents/materials were contributed by J.T. and Y.Y. The paper was written by L.Y., J.T. and Y.Y.

**Data availability**

RNA-sequencing data in this study have been deposited in the NCBI Bioproject database under accession number (PRJNA732234).

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Supplementary data**

Supplementary data is available at Horticulture Research online.

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