

# THE ANTHOCYANIN BIOSYNTHETIC REGULATOR MDMYB1 POSITIVELY REGULATES ASCORBIC ACID BIOSYNTHESIS IN APPLE

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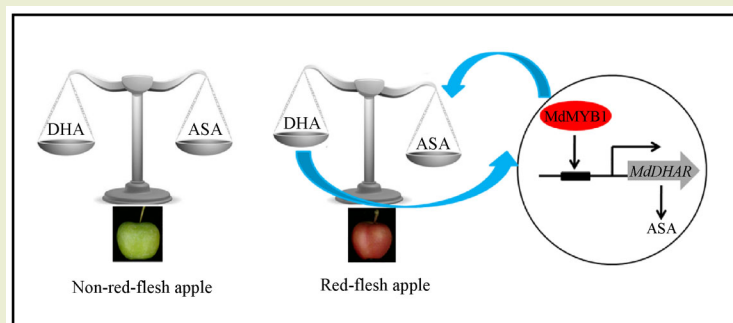
## KEYWORDS

anthocyanin, apples, ascorbic acid, MdMYB1, vitamin C

## HIGHLIGHTS

- The contents of anthocyanin and AsA in red-flesh apples are higher than that in non-red-flesh apples.
- The anthocyanin biosynthetic regulator MdMYB1 directly activates the expression of dehydroascorbate reductase gene *MdDHAR*, thus promoting the activity of the DHAR enzyme and the accumulation of AsA.
- MdMYB1-MdDHAR module may play a key role in AsA-DHA homeostasis.

## GRAPHICAL ABSTRACT



## ABSTRACT

Ascorbic acid (AsA, vitamin C) is involved in the regulation of many aspects of plant growth and development. It is an essential micronutrient for humans and can prevent scurvy, maintain the health of gums and blood vessels, reduce the level of plasma cholesterol and enhance the immune system. Apple cultivars Orin and Guanghui were crossed to obtain a group of hybrid offspring with and without red flesh in the course of assessing apple germplasm resources. Unexpectedly, the red-flesh apples had higher AsA contents than other apples. Further studies showed that the anthocyanin biosynthetic regulator MdMYB1 directly activates the expression of dehydroascorbate reductase gene *MdDHAR*, thus promoting the activity of the DHAR enzyme and the accumulation of AsA. This finding reveals the mechanism leading to high AsA levels in red-flesh apples and suggests a new idea to cultivate red-flesh apples with high AsA contents and produce AsA efficiently and without pollution.

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Ascorbic acid (AsA) is an important antioxidant and has an essential role in plant resistance to oxidative stress<sup>[1]</sup>. In addition to its ability to scavenge reactive oxygen species (ROS), AsA is involved in regulating many aspects of plant growth and development, including seed germination, flower induction, photosynthesis, fruit development, senescence and stress tolerance<sup>[1–7]</sup>. The biosynthesis of AsA occurs mainly through four pathways, namely the D-glucosone, D-galacturonate, myo-inositol and D-mannose/L-galactose pathways<sup>[8–12]</sup>. The D-mannose/L-galactose synthesis pathway is particularly important in AsA biosynthesis. The biosynthesis of AsA is influenced by different stages of plant development (such as germination, fruit development, and senescence) and external light conditions<sup>[2,13]</sup>. However, the molecular mechanism by which the environmental factors regulate the synthesis of AsA remains unclear. AsA is an essential micronutrient for humans and can prevent scurvy, maintain the health of gums and blood vessels, reduce the level of plasma cholesterol and enhance the immune system. However, high cost, waste of resources and environmental pollution in AsA production have restricted the development of industrial AsA production. In the course of assessing apple germplasm resources, we crossed apple cultivars Orin and Guanghui to obtain a group of hybrid offspring with and without red flesh (Fig. 1(a))<sup>[14]</sup>. The red-flesh apples contained large amounts of visible anthocyanin (Fig. 1(a)). In addition, they unexpectedly had higher AsA contents than other apples (Fig. 1(b)). This finding prompted us to explore the mechanism leading to high AsA levels in red-flesh apples.

AsA will be synthesized in large quantities as reactive oxygen scavengers when plants are under stress and excessive ROS are produced<sup>[1]</sup>. AsA is oxidized to dehydroascorbic acid (DHA) by ascorbic acid oxidase in the process of scavenging ROS. Also, the enzyme dehydroascorbic acid reductase (DHAR) catalyzes the formation of AsA from DHA. The AsA-DHA cycle has an important role in maintaining the dynamic balance of AsA and regulating plant growth and stress response<sup>[1,13,15]</sup>. Here, DHA contents and DHAR enzyme activity in red-flesh and other apples were also determined. Red-flesh apples had lower DHA contents and higher DHAR enzyme than other apples (Fig. 1(c,d)). Also, quantitative real-time (qRT)-PCR shows that the expression level of the *MdMYB1* gene (GenBank accession number: MDP0000259614) was positively correlated with the expression level of *MdDHAR* (GenBank accession number: MDP0000175246), that is, the expression levels of *MdMYB1* and *MdDHAR* in red-flesh apples were higher than in other apples (Fig. 1(e)). These results indicate a possible correlation between anthocyanins and AsA.

Anthocyanins are important secondary metabolites in addition to AsA and also have an important role in plant growth and

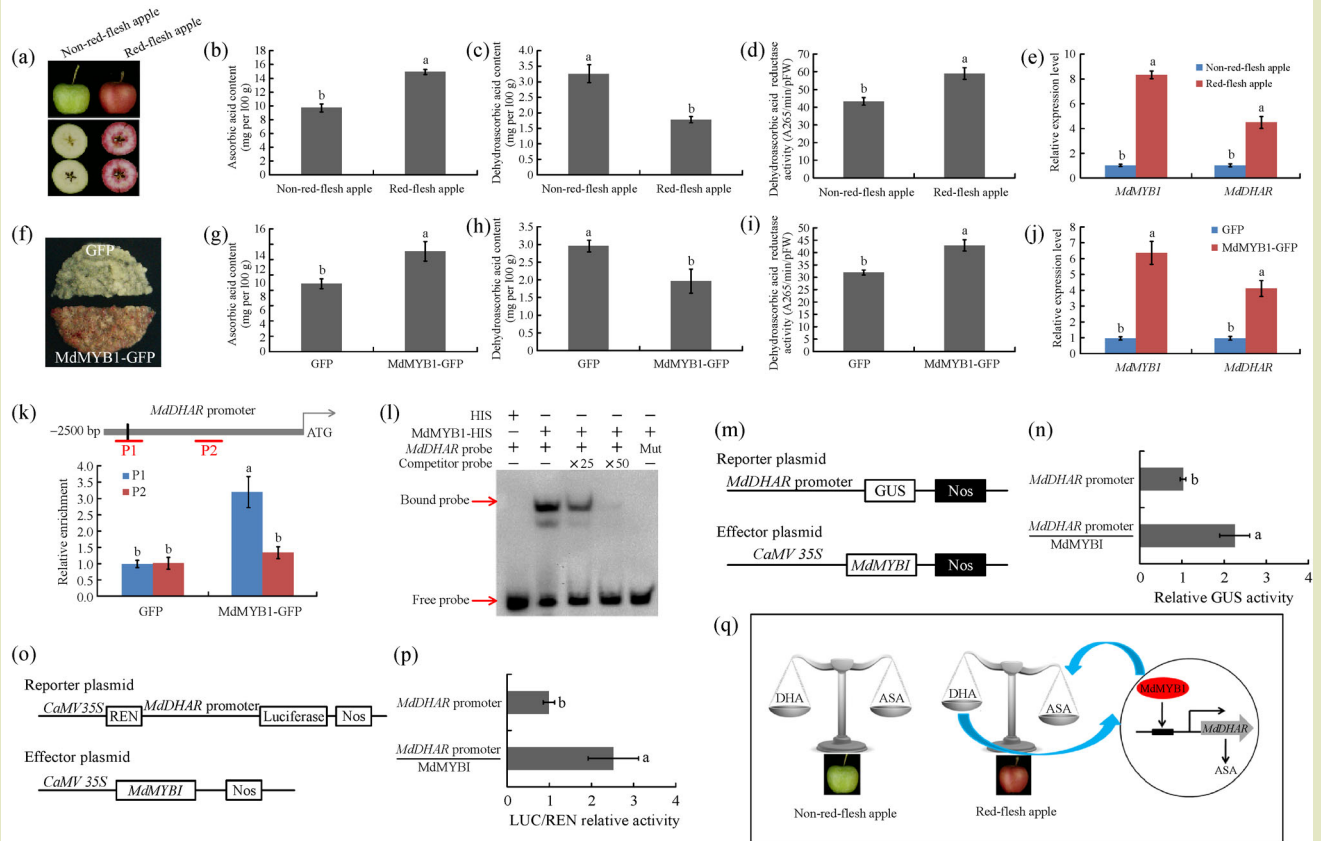
development<sup>[16]</sup>. At the transcriptional level, anthocyanin biosynthesis is regulated by the MYB-bHLH-WD40 protein complex<sup>[17]</sup> and the MYB transcription factor has a central role. *MdMYB1* and its alleles (*MdMYB10* and *MdMYBA*) have been shown to be key positive regulators of anthocyanin biosynthesis in apples<sup>[18–20]</sup>. The red flesh in apples is caused by the overexpression of *MdMYB1* (*MdMYB10*) and we therefore obtained *MdMYB1*-overexpressing apple callus in order to determine the correlation between anthocyanin and AsA (Fig. 1(f)). As expected, the contents of AsA in *MdMYB1*-green fluorescent protein (GFP) transgenic apple callus were higher than in the control (GFP), while the contents of DHA were lower than in the control (GFP) (Fig. 1(g,h)). DHAR enzyme activity and *MdDHAR* gene expression level in *MdMYB1*-GFP transgenic apple callus were higher than in the control (GFP) (Fig. 1(i,j)). These results indicate that overexpression of the *MdMYB1* gene may increase the activity of the DHAR enzyme by increasing the expression of the *MdDHAR* gene, thereby promoting the conversion of DHA into AsA, and finally increasing the contents of AsA. In addition, we found that overexpression of *MdMYB1* in apple callus also promoted the expression of the ascorbate oxidase gene *MdAO* (GenBank accession number: XP\_028958650.1), the ascorbate peroxidase gene *MdAPX1* (GenBank accession number: MDP0000210077) and the monodehydroascorbate reductase gene *MdMDHAR* (GenBank accession number: MDP0000199989) (Fig. 1(j)), suggesting that *MdMYB1* may play a key role in AsA-DHA homeostasis.

We analyzed the promoter sequence of *MdDHAR* in order to further reveal the transcriptional regulation mechanism of *MdMYB1* on the *MdDHAR* gene. The *MdDHAR* promoter sequence was found to contain a binding site (P1) for the MYB transcription factor. Chromatin immunoprecipitation (ChIP)-PCR assays were conducted to determine the binding of *MdMYB1* to the promoter of *MdDHAR*. *MdMYB1* protein precipitated from *MdMYB1*-GFP transgenic callus and enrichment of *MdDHAR* promoter sequence detected by qRT-PCR. This indicates that the enrichment of the P1 region was higher than the control region (P2, Fig. 1(k)), and that *MdMYB1* may directly bind to the P1 region of the *MdDHAR* promoter. We also conducted electrophoretic mobility shift assays to verify the interaction between *MdMYB1* and the *MdDHAR* promoter. As shown in Fig. 1(l), *MdMYB1* directly bound to the 5'-CTGTTG-3' site of the *MdDHAR* promoter, while *MdMYB1* did not bind when the 5'-CTGTTG-3' site was mutated to 5'-CGGTGG-3'. These data indicate that *MdMYB1* binds to the *MdDHAR* promoter.

To study the transcriptional regulation function of *MdMYB1* on *MdDHAR*, the promoter sequence of *MdDHAR* was cloned to

the pCambia1391-GUS vector and transformed into the apple callus (Fig. 1(m)). GUS activity detection results show that compared with MdDHAR-GUS callus alone, MdMYB1 overexpressed on MdDHAR-GUS basis significantly increased its

GUS activity (Fig. 1(n)). In addition, the promoter sequence of MdDHAR was cloned to the pGreen0800-LUC vector and full length MdMYB1 was inserted into the pGreen62-SK vector (Fig. 1(o)). The recombinant plasmids were transformed into



**Fig. 1** MdMYB1 positively regulates AsA biosynthesis by activating *MdDHAR*. (a) Representative non-red-flesh and red-flesh apples. Apple crossbreeding groups (non-red-flesh and red-flesh apples) were harvested 140 days after full bloom. Measurement of (b) ascorbic acid (AsA) and (c) dehydroascorbic acid (DHA) contents. Error bars denote standard deviation. Different letters above the bars indicate significant differences ( $P < 0.05$ ) obtained by one-way analysis of variance. (d) Measurement of dehydroascorbic acid reductase activity. The experiments were repeated three times and each experiment contained 3–5 apple fruits per variety. A representative picture is shown here. (e) Detection of the expression levels of *MdMYB1* and *MdDHAR* in non-red-flesh and red-flesh apples. The value for non-red-flesh apple was set to 1. qRT-PCR was conducted in three biological replicates and three technical replicates, and each sample contained 3–5 apple fruits. (f) Representative wild-type (GFP) and *MdMYB1*-overexpressing (*MdMYB1*-GFP) apple callus. Measurement of (g) AsA and (h) DHA contents. (i) Measurement of dehydroascorbic acid reductase activity. A representative picture is shown here. (j) Detection of the expression levels of *MdMYB1*, *MdDHAR*, *MdMDHAR*, *MdAO* and *MdAPX1* in transgenic apple callus. The value for GFP was set to 1. (k) Chromatin immunoprecipitation (ChIP)-PCR assays of *MdMYB1* binding to the promoter of the *MdDHAR* gene. The predicted 5'-CTGTTG-3' sequences are indicated by the black line. Chromatin from the empty vector control (GFP) and 35S: *MdMYB1*-GFP apple callus (*MdMYB1*-GFP) were immunoprecipitated with and without anti-GFP antibodies. Two regions (P1 and P2) were examined by qRT-PCR. The enrichment of GFP was set to 1. (l) Electrophoretic mobility shift assay results showing that the *MdMYB1*-HIS fusion protein bound directly to the *MdDHAR* promoter. Unlabeled probes were used as competitors. In the mutated probe (Mut), the 5'-CTGTTG-3' motif was replaced by 5'-CGGTGG-3'. (m) Schematic representation of the GUS reporter vector containing the *MdDHAR* promoter and the effector vector containing *MdMYB1*. (n) GUS activity detection of *MdDHAR*-promoter and *MdDHAR*-promoter/*MdMYB1* transgenic apple callus. *MdDHAR*-promoter, *MdDHAR*-promoter transgenic apple callus; *MdDHAR*-promoter/*MdMYB1*, *MdDHAR*-promoter and 35S:*MdMYB1*-GFP co-transformed apple callus. The value for *MdDHAR*-promoter was set to 1. (o) Schematic representations of the effector vectors containing *MdMYB1*, and the firefly luciferase reporter vectors containing the *MdDHAR* promoter. *MdDHAR*-promoter, *MdDHAR*-promoter-pGreen0800-LUC + pGreen62-SK; *MdDHAR*-promoter/*MdMYB1*, *MdDHAR*-promoter-pGreen0800-LUC + *MdMYB1*-pGreen62-SK. (p) Relative LUC/REN activity of effector plasmids and reporter plasmids. The value for *MdDHAR*-promoter was set to 1. (q) A working model of *MdMYB1* functioning in AsA biosynthesis.

*Agrobacterium tumefaciens* and injected into tobacco leaves. The fluorescence detection results show that *MdMYB1* overexpression significantly increased the fluorescence activity of *MdDHAR* (Fig. 1(p)). These results suggest that *MdMYB1* activates the expression of *MdDHAR* by directly binding to its promoter.

Anthocyanin and AsA play similar biological roles in plant growth and development<sup>[1,16]</sup>. The crosstalk between them has never been revealed. Here, we found that apples with high anthocyanin content contained more AsA, suggesting that there may be a positive correlation between anthocyanin and AsA. We hypothesized that anthocyanin biosynthesis might disrupt AsA-DHA homeostasis. The biological function of anthocyanin may

also require the coordination of AsA. Of course, the relationship between anthocyanin and AsA needs further study.

In summary, our studies reveal why the AsA contents of red-flesh apples are higher than those of other apples. This is because excessive *MdMYB1* breaks the homeostasis of AsA and DHA in apple fruits, and *MdMYB1* directly activates the expression of the dehydroascorbate reductase gene *MdDHAR*, thus promoting the activity of the DHAR enzyme and the accumulation of AsA (Fig. 1(q)). This finding reveals the mechanism leading to high AsA levels in red-flesh apples and provides new information that is useful in the cultivation of red-flesh apples with high AsA contents to produce AsA efficiently and without pollution.

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### Compliance with ethics guidelines

Jianping An, Xiaofei Wang, Chunxiang You, and Yujin Hao declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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