MdMADS6 Recruits Histone Deacetylase MdHDA19 to Repress the Expression of the Carotenoid Synthesis-Related Gene MdCCD1 during Fruit Ripening

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Abstract: Fruit ripening is regulated by epigenetic modifications and transcription factors, which may function independently or as protein complexes. Our previous study showed that the apple histone deacetylase19 (MdHDA19) suppresses fruit ripening through the deacetylation of histones in related genes. Here, a MADS-box transcription factor (MdMADS6) was identified using a yeast two-hybrid (Y2H) assay as a candidate protein that interacts with MdHDA19 during apple fruit ripening. Furthermore, Y2H, bimolecular fluorescence complementation (BiFC) and pull-down assays were used to confirm the interaction between MdHDA19 and MdMADS6. Agrobacterium-mediated transient transformation and yeast one-hybrid assays showed that MdMADS6 promoted carotenoid accumulation in apple fruit by acting on the downstream target genes related to carotenoid biosynthesis. In summary, we conclude that, in the early stages of fruit development, the expression of MdMADS6 was maintained at lower levels, where it interacted with MdHDA19 to form a protein complex that inhibited the expression of the downstream genes. At the late stages of fruit development, active expression of MdMADS6 dissociated the protein complex of MdMADS6 and MdHDA19 and consequently promoted the expression of carotenoid biosynthesis genes as well as carotenoid accumulation.

Keywords: MADS transcription factor; protein complex; carotenoid accumulation; fruit ripening

1. Introduction

Numerous physiological and biochemical processes occur during apple fruit ripening and affect its quality, post-harvest life and value [1]. Fruits undergo changes in texture, color and aroma during the ripening process. Previous studies revealed that epigenetic modifications, such as DNA methylation, histone acetylation, phosphorylation and ubiquitination, constitute a major factor in regulating fruit ripening [2]. Histone modification is a dynamic process where acetylation is catalyzed by histone acetyltransferases (HATs) and is related to transcriptional activation, whereas deacetylation is catalyzed by histone deacetylases (HDACs) and is involved in transcriptional inhibition [3].

Many studies have showed that histone deacetylation plays an important role in regulating fruit ripening. For example, the expression of genes involved in carotenoid and ethylene biosynthesis has been reported to decrease after silencing histone deacetylases1 (HDA1) in tomato [4]. The accumulation of carotenoids in fruits of the histone deacetylases3 (SIHDA3)-RNAi lines has been shown to increase simultaneously with up-regulation of

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the expression of ethylene biosynthesis genes, such as 1-aminocyclopropane-1-carboxylate synthase2 (ACS2) and 1-aminocyclopropane-1-carboxylate oxidase1 (ACO1) and other genes related to fruit maturation, such as RIN, CNR and TAGL1 [5].

In addition to epigenetic modifications, transcription factors, such as members of the MADS-box family as well as the ripening inhibitor (RIN), play an important role in regulating fruit ripening. The sepals of the rin mutant were found to be enlarged, and the fruit ripening was inhibited as a result of the activity of the two MADS-box transcription factors, SIMADS-MC and SIMADS-RIN [6]. Knocking out the RIN gene by CRISPR led to synthesis of a kind of RIN-MC protein that inhibited fruit ripening [7]. Another transcription factor, FUL, also plays an important role in fruit development. Overexpression of PpMADS6, a homologue of FUL from peach in Arabidopsis, led to early flowering, apical flowers and multi-fruited pods [8].

In banana fruits, it was found that MaMADS1 showed varying expression levels at different stages of fruit development as well as upon harvesting and treatment with exogenous ethylene and 1-methylcyclopropene (1-MCP). Previous studies demonstrated that MaMADS1 is closely related to fruit ripening [9]. Moreover, it was found that the transcription factor SIMADS1 of MADS-Box has a negative regulatory effect on fruit ripening in tomato [10]. In other fruits, such as grapes, strawberries and bilberries [11–13], MADS-box transcription factors have been shown to be associated with fruit maturation.

Transcription factors function by activating or inhibiting the downstream genes. TAGL1 is highly expressed during fruit ripening and early carpel development and can directly bind to the promoter of SlACS2, a gene of ethylene biosynthesis, to promote tomato fruit ripening [14]. In citrus, CsMADS6 of the MADS-box family functions as a transcriptional activator. It directly binds to the promoters of the carotenoid biosynthesis-related genes phytoene synthase (PSY), phytoene desaturase (PDS) and carotenoid cleavage dioxygenase1 (CCD1) to activate their expression and promote the synthesis of carotenoids. It has also been shown that CsMADS6 has multi-target regulatory effects on carotenoid metabolism [15].

Recent studies have demonstrated that transcription factors can also recruit histone deacetylases to form functional protein complexes. For example, in bananas, the ethylene responsive factor (MaERF11) can initiate MaHDA1 to form an inhibitory protein complex that regulates the expression of maturation-related genes through histone deacetylation, thereby, inhibiting fruit ripening [16].

In addition, it was found that the three RPD3/HDA1 subfamily histone deacetylases, SIHDA1, SIHDA3 and SIHDA4, interact with the two MADS-box transcription factors, TAG1 and TM29, in tomato, suggesting that these HDACs may participate in regulating the development and maturation of fruits [17]. In longan (Dimocarpus longan Lour.) the histone deacetylase (DIHD2) was reported to interact with the ethylene responsive factor (DIERF1), resulting in enhancing the acetylation level of histone H3 during fruit senescence [18].

Our previous data showed that MdHDA19 inhibits apple fruit ripening. However, the involved regulatory mechanism is still unclear. Therefore, we speculate that MdHDA19 may interact with transcription factors to regulate apple fruit ripening by acting on downstream target genes.

Here, we identified MdMADS6, which can interact with MdHDA19 and activate the expression of the carotenoid biosynthesis-related gene MdCCD1 by binding the CARG element. Furthermore, we demonstrate that MdMADS6 and MdHDA19 may form a protein complex to regulate the ripening of apple fruits.
2. Results
2.1. MdHDA19 Interacts with MdMADS6

To identify the proteins interacting with MdHDA19, we performed a yeast-two-hybrid library screening using MdHDA19 as bait. A total of 420 interacting proteins were screened (Supplementary Table S1), including nine transcription factors. However, according to the protein function and expression levels at various fruit development stages, a MADS-box transcription factor was identified as the candidate protein as related to fruit ripening. The MADS-box transcription factor was identified as MdMADS6 in the Malus domestica through phylogenetic tree analysis and domain analysis (Figure 1A,B).

At the same time, to further investigate the interaction relationship between MdMADS6 and MdHDA19, the vectors pGBK17-MdHDA19 and pGADT7-MdMADS6 were co-transformed into the yeast strain Y2H Gold. The results showed that transformed yeast cells grew on the selective medium lacking Trp, Leu, His and adenine, indicating that MdHDA19 interacted with MdMADS6 (Figure 2B). According to the results of a yeast-two hybrid, the MdMADS6 was verified for further study.

We further tested the direct interaction between MdMADS6 and MdHDA19 in vivo by using the bimolecular fluorescence complementation (BiFC) assay. After the fusion proteins of MdMADS6-pSPYCE and MdHDA19-pSPYNE were co-expressed transiently in tobacco leaf cells, strong YFP fluorescence was observed in the nuclei. As a control, when either of the fusion proteins was co-expressed with the complementary empty vector in tobacco leaf cells, no YFP fluorescence signal was detected, suggesting that MdMADS6 and MdHDA19 interacted in tobacco leaves (Figure 2A).

The interaction of MdMADS6 and MdHDA19 was studied in vitro using a pull-down assay. The purified His-MdMADS6 recombinant protein was incubated with GST-MdHDA19 recombinant protein. The recombinant protein GST-MdHDA19 was pulled down by His-MdMADS6 as shown in Figure 2C, indicating that His-MdMADS6 directly interacted with GST-MdHDA19 in vitro.

Figure 1. Sequence analysis of the MADS transcription factors. (A). Phylogenetic analysis of MdMADS6 and related proteins from other plant species. The scale bar represents 0.05 substitutions per site. The sequence of MADS was the same as that of MADS6 of the Malus domestica. (B). Multiple sequence alignment of MdMADS6 and related proteins. The horizontal lines mark the two conserved domains.
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Figure 2. Interaction between MdMADS6 and MdHDA19 in vitro and in vivo. (A). BiFC assay in tobacco leaf epidermal cells showing the interaction between MdMADS6 and MdHDA19 in the living cells. MdMADS6 fused with pSPYCE and MdHDA19 fused with the pSPYNE were co-transformed into tobacco leaves and visualized using confocal microscopy. Bars = 10 μm. (B). Yeast two-hybrid assay. The coding regions of MdMADS6 and MdHDA19 were cloned into the pGADT7 or pGBK77 vector to create the AD-MdMADS6, BD-MdHDA19, respectively. The ability of yeast cells to grow on synthetic medium lacking Trp, Leu, His and adenine was scored as a positive interaction. (C). Pull-down analysis of the interaction between MdMADS6 and MdHDA19 in vitro.

2.2. MdMADS6 Promotes Apple Fruit Ripening

To investigate the effect of MdMADS6 on fruit ripening, we performed the transient transformation assay using Agrobacterium tumefaciens to overexpress or silence MdMADS6 in apple fruits. The fruits with transiently overexpressed MdMADS6 showed significantly promoted ripening rate (Figure 3A) as well as a higher content of carotenoids (Figure 3C) compared to those transformed with the pRI101 empty vector (Figure 3A). However, the fruits with transiently silenced MdMADS6 had a suppressed ripening phenotype compared with the control (Figure 3A). In addition, we quantified the expression levels of MdMADS6 during stages of fruit development. The results revealed that during the early stages of fruit development, the expression levels of MdMADS6 were significantly lower than those at the late stage (Figure 4A). Therefore, we conclude that MdMADS6 promotes apple fruit ripening.

2.3. MdMADS6 Binds to the Promoter of the Carotenoid Biosynthesis Gene MdCCD1

In previous research, CsMADS6 was found to promote fruit ripening through increasing carotenoid accumulation by targeting the downstream genes involved in carotenoid biosynthesis [15]. In this study, we measured the expression levels of carotenoid biosynthesis genes at different stages of apple fruit development when MdMADS6 was overexpressed or silenced. The results showed that the expression levels of MdCCD1 and MdPDS were lower at the early than at the late stage of fruit development, especially the expression level of MdCCD1 was significantly increased at the later stage (Figure 4B).
Figure 3. *MdMADS6* promotes apple fruit ripening. (A). Phenotypes of ‘Gala’ fruit in which *MdMADS6* was overexpressed (pRI101 vector) or silenced (pTRV vector) by *Agrobacterium tumefaciens*-mediated transient transformation. Bar = 1 cm. (B). Gene expression levels of *MdMADS6* after overexpression or silencing in ‘Gala’ apple fruits. (C). Carotenoid contents of apple fruits in which *MdMADS6* was overexpressed or silenced by *Agrobacterium tumefaciens*-mediated transient transformation. The transient empty vector was used as a control. Means and SD (*n* = 3) values are shown. * Indicates a statistically significant difference, *p* < 0.05 and ** *p* < 0.01, as determined by a *t*-test. Error bars indicate SD of three biological replicates and three technical replicates.

Figure 4. Correlation analysis between *MdMADS6* and relative expression levels of target genes during fruit developmental stages. (A). Gene expression levels of *MdMADS6*. (B). Gene expression levels of the carotenoid biosynthesis genes *MdCCD1*, *MdPDS* and *MdHYD*. Means and SD (*n* = 3) values are shown. * Indicates a statistically significant difference, *p* < 0.05 and ** *p* < 0.01, as determined by a *t*-test. Error bars indicate SD of three biological replicates and three technical replicates. (C). Correlation analysis of the relative expression levels of *MdMADS6* and *MdCCD1*. (D). Correlation analysis of the relative expression levels of *MdMADS6* and *MdPDS*. (E). Correlation analysis of the relative expression levels of *MdMADS6* and *MdHYD*. $R^2$ means coefficient of determination, *p* value means the significance level, as determined by a *t*-test.
In addition, the expression levels of MdCCD1 showed greater variation in response to modulation of expression of MdMADS6 than did those of MdPDS (Figure 5A). To determine the downstream target genes, we conducted correlation linear analysis between MdMADS6 and each of MdCCD1, MdPDS and MdHYD. The results showed that the expression levels of MdCCD1 during fruit development were closest to those of MdMADS6. An R² value of 0.7428 (Figure 4C) among the genes MdPDS (Figure 4D) and MdHYD (Figure 4E), indicated that MdMADS6 may target MdCCD1 directly.

To confirm the influence of MdMADS6 on MdCCD1 expression, we quantified the expression levels of MdCCD1 and MdPDS in apple fruits in which MdMADS6 was overexpressed or silenced. The results showed that the expression levels of MdCCD1 and MdPDS increased upon overexpression of MdMADS6 as compared with the control. However, silencing of MdMADS6 led to significantly lower expression levels of both genes (Figure 5A), indicating that MdCCD1 is the possible target gene of MdMADS6. We also performed a yeast one-hybrid assay to identify the target gene using pJG4-5-MdMADS6 and pLaZi-MdCCD1 (promoter of the MdCCD1) co-transformed into the EGY48 cells. Upon adding the X-Gal staining fluid to the yeast single spots, they turned blue within 6–8 h (Figure 5B), indicating that MdMADS6 targeting on MdCCD1 directly.

![Figure 5](image-url)  
*Figure 5. MdMADS6 binds to the promoter of MdCCD1 and positively regulates MdCCD1 expression. (A). Gene expression levels of MdCCD1 and MdPDS in the overexpressed or silenced MdMADS6 ‘Gala’ apple fruits. Means and SD (n = 3) values are shown. * Indicates a statistically significant difference, * p < 0.05 and ** p < 0.01, as determined by a t-test. Error bars indicate the SD of three biological replicates. (B). Yeast one-hybrid assay. pJG4-5/MdMADS6 (AD-MdMADS6) constructs were co-transformed with pLaZi/MdCCD1 (BD-cis) separately into the yeast cells EGY48. AD/BD, AD/BD-cis and AD-MdMADS6/BD, were used as negative controls. (C). Gene expression levels of MdCCD1 in overexpressed or silenced MdHDA19 transgenic ‘Orin’ apple calli. Means and SD (n = 3) values are shown. * Indicates a statistically significant difference, * p < 0.05, as determined by a t-test. Error bars indicate SD of three biological replicates and three technical replicates.
Combined with the result of Figure 5A, we concluded that *MdMADS6* targeted *MdCCD1* and positively regulated the expression of *MdCCD1*. To detect the regulating effect of *MdHDA19* on *MdCCD1*, we measured the *MdCCD1* relative expression level in the ‘Orin’ apple calli overexpressed or silenced for *MdHDA19* [19]. The results showed that the expression level of *MdCCD1* decreased compared with the overexpressed pRI101 empty vector but increased significantly compared with overexpressed pRI101-RNAi empty vector, indicating that *MdHDA19* negatively regulated the expression of *MdCCD1* (Figure 5C).

3. Discussion

Fruit ripening depends on the regulation of the expression of the involved genes by the concerted action of multiple components, such as transcription factors (activator or repressors) and epigenetic modifiers [20]. Previous studies have shown that histone deacetylation is related to transcriptional inhibition [21]. For example, in tomato, down-regulation of the histone deacetylases SIHDA1 and SIHDA2 promoted ethylene biosynthesis and carotenoid accumulation and thereby accelerated fruit ripening [22]. In our previous research, we found that *MdHDA19* inhibited apple fruit ripening.

To perform their functions, proteins may interact with each other to form functional complexes. Transcription factors may be activated or inhibited by epigenetic modifiers. The *AtHDA5* mutants *hda5-1* and *hda5-2* showed a delayed flowering phenotype, and the expression as well as the acetylation levels of the flowering suppressors FLC and MAF1 were increased. In addition, FVE and FLD interacted with HDA5 and HDA6 indicating that they may form complexes to jointly regulate the flowering time [23].

In the molecular regulatory network of fruit ripening, histone deacetylases could be recruited by the relevant transcription factors to form regulatory protein complexes. For example, in bananas, *MaERF11* has been reported to deploy *MaHDA1* to regulate ripening-related genes through histone deacetylation. The expression of transcription factor *MaERF11* and downstream target genes *MaACO1* and *MaEXP2/7/8* revealed the regulatory mechanism of *MaHDA1* in banana fruit ripening [16]. Furthermore, it was found that the three histone deacetylases SIHDA1, SIHDA3 and SIHDA4 interacted with the MADS-box transcription factors *TOMATO AGAMOUS1* (TAG1) and *TOMATO MADS BOX29* (TM29), which are associated with tomato fruit ripening.

This suggested that HDACs may be recruited by MADS-box transcription factors to form protein complexes that regulate fruit ripening [24]. In the present study, we screened out the transcription factor *MdMADS6* as interacting with *MdHDA19* through the Y2H assay. We further identified the interaction relationship between *MdMADS6* and *MdHDA19* in vivo and in vitro through Y2H, bimolecular fluorescence complementation and pull-down experiments.

MADS transcription factors have been proven to be important for the regulation of fruit ripening. Among them, RIN and FUL1 are classical members [6,25]. It was found that the transcript levels of *MdMADS6* were low during the early stages of fruit development, whereas it was expressed at higher levels during later stages. The transient transformation experiment proved that *MdMADS6* promoted fruit ripening. Studies have shown that MADS transcription factors can target a variety of downstream genes related to fruit color to regulate fruit ripening, including genes involved in carotenoid biosynthesis [26].

In citrus, it was also found that *CsMADS6* can activate genes of carotenoid synthesis, leading to carotenoid accumulation, which in turn promoted fruit ripening [15]. In this study, it was found that *MdCCD1*, *MdPDS* and other carotenoid metabolism-related genes were expressed to higher levels at the late stages of fruit development. Moreover, *MdMADS6* interacted with *MdCCD1*, *MdPDS* and other carotenoid metabolism-related genes and positively regulated the expression levels of *MdCCD1* and *MdPDS*. Therefore, we speculated that *MdMADS6* may activate gene expression to promote carotenoid biosynthesis and promote apple fruit ripening.
In conclusion, we propose a two-stage regulatory model of fruit ripening. First, at the early stages of fruit development, the expression of MdMADS6 is low, which interacts with MdHDA19 to form an inhibitory protein complex that depresses the expression of the downstream gene MdCCD1 and, hence, fruit ripening. Second, at the late stages of fruit development, the expression of MdMADS6 increases, causing the protein complex to dissociate and activates the expression of the downstream genes related to carotenoid biosynthesis to promote carotenoid accumulation and apple fruit ripening (Figure 6).

However, whether the promotion of fruit ripening by MdMADS6 is related to the acetylation level of MdMADS6 by MdHDA19 and how the interaction between MdHDA19 and MdMADS6 determines the function of the protein complex have not been addressed. The used assays were not sufficient to specify the stage at which MdHDA19 and MdMADS6 dissociated during fruit development. However, our results do confirm the promotion of apple fruit ripening by MdMADS6. The data also provide novel evidence for transcription factors that deploy histone deacetylases to regulate fruit ripening.

Figure 6. A model showing the influence of a protein complex involving MdMADS6 and MdHDA19 on fruit ripening. At the early stages of fruit development, the expression levels of MdMADS6 are at low levels where it interacts with MdHDA19 to form a protein complex to inhibit downstream gene expression. At the late stages of fruit development, the active expression of MdMADS6 dissociates the protein complex of MdMADS6 and MdHDA19 activates the expression of carotenoid biosynthesis genes MdCCD1 and promotes carotenoid accumulation and fruit ripening.

4. Materials and Methods

4.1. Plant Materials

Malus domestica ‘Gala’ and MdHDA19-transgenic ‘Orin’ apple calli were used in this study [19]. Fruits were harvested at 90–110 d after flower blossoming. Fruit peels were sampled and frozen in liquid nitrogen for subsequent analysis. Each sample comprised three biological replicates, and each replicate included three fruits.

4.2. DNA Extraction, Total RNA Isolation and cDNA Synthesis

The DNA extraction, total RNA isolation and cDNA synthesis were conducted as previously described [26].

4.3. Gene Cloning

The cDNA sequences of MdHDA19 and MdMADS6 were downloaded from the Apple Genome Database (https://www.rosaceae.org/, accessed on 15 January 2022). Using gene-specific primers, the coding sequences were cloned from cDNA, and the gene fragments were cloned into TOPO-Blunt vector (Aidlab) and then sequenced (Beijing Shenggong, Beijing, China). The total volume of each PCR reaction was 50 μL, including 25 μL of 2 × Phanta Max Buffer, 1 μL of dNTP Mix, 1 μL of each of the upstream and downstream primers, 1 μL of Phanta Max Super-Fidelity DNA Polymerase, 1 μL of template and ddH2O up to 50 μL. The thermal cycler procedure was: 95 °C pre-denaturation for 3 min, 95 °C denaturation for 15 s, 58 °C annealing for 30 s, 72 °C extension for 90 s, and 72 °C final extension for 5 min.
4.4. Protein Purification

The pET-32a and pGEX6P-1 vectors were digested with EcoRI, and the target gene fragments cloned from ‘Gala’ cDNA were inserted into pET-32a and pGEX6P-1 vectors separately using a seamless cloning method. After confirming the correct sequences, the integrated vectors were transformed into E. coli BL21 (DE3) cells. The density of E. coli was adjusted to an OD of 0.5. The strong inducer IPTG at a concentration of 0.3 mM was added to induce protein synthesis overnight at 4 °C. Next day, the bacterial cells were broken via sonication and centrifuged, and the supernatant and precipitate were collected. Finally, the supernatant and precipitate were subjected to western blotting.

After inducing the GST-MdHDA19 and His-MdMADS6 protein, the His-tagged protein purification kit (CWBIIO, catalog number: CW0894S) was used to pass the purification column to allow GST-MdHDA19 binding to His-MdMADS6 and finally perform western blot detection [27]. His-MdMADS6 was immobilized on the chromatographic column, the solution was passed through the chromatographic column, the flow-through was collected, and the flow-through was incubated with GST antibody. The pull-down assays were performed as previously described [19]. The primers used are listed in Supplementary Table S2.

4.5. Yeast Two-Hybrid Assay

The yeast library was constructed by Clontech, the yeast two-hybrid vectors used were pGADT7 and pGBKT7, and the used yeast strain was Y2HGold (Shanghai Weidi biotechnology CAT#: YC1002). The vectors pGADT7 and pGBKT7 were digested with EcoRI. The MdMADS6 cloned fragment was inserted into pGADT7, and the MdHDA19 cloned fragment was inserted in pGBKT7. The MdHDA19 gene had no self-activation activity. The constructed baits and prey vectors were introduced into the Y2HGold yeast strain. The yeast cells were spread onto SD/-Leu/-Trp deficient medium and then transferred to the SD/-Leu/-Trp/-His/-Ade medium. The Yeast Two-Hybrid assay was performed as previously described [28].

4.6. BiFC Assay

The pSPYCE155 and pSPYNE173 vectors were digested by BamHI. The MdHDA19 cloned gene fragment was inserted into the pSPYNE173 linear vector, and the MdMADS6 cloned fragment was inserted into the pSPYCE155. Then, the constructed vectors were transformed into GV3101 Agrobacterium separately, and the positive clones were picked into 2 mL LB Medium (50 µg/mL Rif, 100 µg/mL Kan). The clones were shaken overnight at 28 °C, transferred into 50 mL of LB medium (50 µg/mL Rif, 100 µg/mL Kan, 10 mM MES and 20 mM acetosyringone) and incubated overnight at 28 °C. Finally, the bacterial cells were collected by centrifugation. The infection buffer (10 mM MgCl2, 10 mM MES and 200 mM acetosyringone) was used to re-suspend the solution, the OD600 was adjusted to 1.0, and the cultures were incubated in the dark for 3 h.

The re-suspended bacterial solutions were mixed in equal proportions and co-injected into tobacco leaves [26]. The leaves were kept for 1 d in the dark and then for 2 d in the light. The leaves were placed around the injection spot on a glass slide and covered with a cover glass. The material was examined under a FV3000 confocal microscope (Olympus) to observe the fluorescence. The excitation and emission wavelengths were 407–457 nm for 4,6-diamidino-2-phenylindole and 514 and 527 nm for enhanced YFP, respectively. The BiFC assay was performed as previously described [27].

4.7. Agrobacterium-Mediated Transient Transformation and Treatments

The pTRV (tobacco rattle virus) and the pRI101 vectors were digested with BamH1 (New England Biolabs, code NO.R3142), and the MdMADS6 gene cloned fragment was inserted into the overexpression and silencing vectors. The constructed vector was introduced into GV3101 Agrobacterium, and positive clones were picked and grown on 2 mL of LB medium (50 µg/mL Rif, 100 µg/mL Kan) with shaking at 28 °C overnight. The cells
were then incubated in 50 mL of LB medium (50 µg/mL Kan, 100 µg/mL Kan, 10 mM MES and 20 mM acetosyringone) at 28 °C overnight and collected by centrifugation. The infection buffer (10 mM MgCl₂, 10 mM MES and 200 mM acetosyringone) was used to re-suspend the bacterial suspension to an OD600 value of 1.0 and then allowed to stand for 3 hr.

The apple fruits were infected under vacuum (0.08 MPa), eight apples per gene and control were used for each treatment. After 24 hr of co-cultivation in the dark, they were cultured under light in a 25 °C constant temperature incubator for about one week to observe the phenotype. After the fruit appeared with a phenotype, pictures were taken, and samples were taken one week later for fluorescence quantitative PCR experiment [26]. In order to facilitate the observation of fruit phenotype, the transient overexpression assay of MdMADS6 was conducted before the transient silencing MdMADS6 assay.

To assess whether increased expression could hasten ripening (color change), fruit were harvested at 90–100 d after flower blossoming for transient overexpression. To assess whether reduced expression would inhibit ripening, fruit were harvested at 100–110 d after the flower blossom stage for transient silencing. The pTRV and pRI101 vectors were preserved in the lab.

4.8. Yeast One-Hybrid Assay

The pLacZi and pJG4-5 vectors were digested with SalI and EcoR1, respectively. Then, the MdCCD1 promoter cloned fragment was inserted into the pLacZi vector, and the cloned fragment of MdMADS6 was inserted into the pJG4-5 vector. The constructs were co-transformed into EGY48 yeast cells, which were subsequently spread on the -Trp/-Ura two-deficiency medium and incubated for 48 hr. After the yeast spots appeared, single spots were transferred to a medium containing the BU component. After the spot grew, 2 µL of X-gal was added [29].

4.9. Real-Time Fluorescence Quantification

A reaction mixture consisting of 10 µL of 2 × Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Q712), 0.5 µL of upstream and downstream primers, 1 µL of Template cDNA and finally made up to 20 µL with ddH₂O was used. Actin (Malus domestica) was used as an internal control. The procedure was conducted using ABI QuantStudio™ 6 Flex system (Applied Biosystems Inc., Foster City, CA, USA) with MightyAmp (SYBR Plus) (Takara; code no. R075A). The procedure was as follows: 95 °C pre-denaturation 30 s, 95 °C denaturation 10 s and 60 °C annealing 30 s for 40 cycles, and the melting curve was: 95 °C: 15 s, 60 °C: 60 s and 95 °C: 15 s. Actin was used as an internal reference, and the calculation method of relative expression was 2⁻∆∆Ct, which contained three biological replicates and three technical replicates [30].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11050668/s1, The sequence data of this article can be found in the Apple Genome Database (http://www.rosaceae.org, accessed on 15 January 2022) and the National Center for Biotechnology Information (NCBI, https://www.Ncbi.nlm.nih.gov, accessed on 15 January 2022). The accession numbers are as follows: MdHDA19 (MD10G1145400), MdCCD1 (MD07G1011500), MdMADS6 (MD06G1204300), MdPDS (MD04G1023800), MdHYD (MD01G1208300), Malus domestica MADS6 (NP_001280849.1), Prunus ussuriensis x Pyrus communis MADS6 (KAB2633630.1), Prunus pseudocerasus MADS (AIU94287.1), Solanum lycopersicum MADS1 (NP_001234380.1), Citrus sinensis MADS (XP_006477713.1), Musa acuminate MADS2.3 (AIIU94432.1), Diospyros kaki MADS1 (ABD65406.1), Vitis vinifera MADS2 (NP_001268109.1), Carica papaya MADS1 (ACD39982.1), Fragaria x ananassa MADS1 (AEFS9025.1). Table S1: MdHDA19 interacting proteins. Table S2: Primier sequences.

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References
1. Tucker, G.; Yin, X.; Zhang, A. Ethylene and fruit softening. *Food Qual. Saf.* 2017, 1, 253–267. [CrossRef]
2. Kouzarides, T. Chromatin modifications and their function. *Cell* 2007, 128, 693–705. [CrossRef] [PubMed]
3. Liu, X.; Yang, S.; Zhao, M.; Luo, M.; Yu, C.-W.; Chen, C.-Y.; Tai, R.; Wu, K. Transcriptional repression by histone deacetylases in plants. *Mol. Plant* 2014, 7, 764–772. [CrossRef] [PubMed]
4. Guo, J.E.; Hu, Z.; Zhu, M.; Li, F.; Zhu, Z.; Lu, Y.; Chen, G. The tomato histone deacetylase SIHDA1 contributes to the repression of fruit ripening and carotenoid accumulation. *Sci. Rep.* 2017, 7, 7930. [CrossRef] [PubMed]
5. Guo, J.E.; Hu, Z.; Yu, X.; Li, A.; Li, F.; Wang, Y.; Tian, S.; Chen, G. A histone deacetylase gene, SIHDA3, acts as a negative regulator of fruit ripening and carotenoid accumulation. *Plant Cell Rep.* 2018, 37, 125–135. [CrossRef]
6. Vrebakov, J.; Ruzewsky, D.; Padmanabhan, V.; White, R.; Medrano, D.; Drake, R.; Schuch, W.; Giovannoni, J. A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. *Science* 2002, 296, 343–346. [CrossRef]
7. Li, S.; Xu, H.; Ju, Z.; Cao, D.; Zhu, H.; Fu, D.; Grierson, D.; Qin, G.; Luo, Y.; Zhu, B. The RIN-MC fusion of MADS-box transcription factors has transcriptional activity and modulates expression of many ripening genes. *Plant Physiol.* 2018, 176, 891–909. [CrossRef]
8. Xu, Y.; Zhang, L.; Ma, R. Functional characterization and mapping of two MADS box genes from peach (*Prunus persica*). *Chin. Sci. Bull.* 2008, 53, 537–543. [CrossRef]
9. Liu, C.; Xi, W.; Shen, L.; Tan, C.; Yu, H. Regulation of floral patterning by flowering time genes. *Dev. Cell* 2009, 16, 711–722. [CrossRef]
10. Dong, T.; Hu, Z.; Deng, L.; Wang, Y.; Zhu, M.; Zhang, J.; Chen, G. A tomato MADS-box transcription factor, SLMADS1, acts as a negative regulator of fruit ripening. *Plant Biol.* 2013, 163, 1026–1036. [CrossRef]
11. Boss, P.K.; Vivier, M.; Matsumoto, S.; Dry, I.B.; Thomas, M.R. A cDNA from grapevine (*Vitis vinifera* L.), which shows homology to AGAMOUS and SHATTERPROOF, is not only expressed in flowers but also throughout berry development. *Plant Mol. Biol.* 2001, 45, 541–553. [CrossRef] [PubMed]
12. Seymour, G.B.; Ryder, C.D.; Cevik, V.; Hammond, J.P.; Popovich, A.; King, G.J.; Vrebalov, J.; Giovannoni, J.J.; Manning, K. A SEPALLATA gene is involved in the development and ripening of strawberry (*Fragaria × ananassa* Duch.) fruit, a non-climacteric tissue. *J. Exp. Bot.* 2011, 62, 1179–1188. [CrossRef] [PubMed]
13. Jaakola, L.; Poole, M.; Jones, M.O.; Kämäräinen-Karppinen, T.; Koskimäki, J.J.; Häggman, H.; Fraser, P.D.; Manning, K.; King, G.J.; et al. A Squamosa MADS box gene involved in the regulation of anthocyanin accumulation in bilberry fruits. *Plant Physiol.* 2010, 153, 1619–1629. [CrossRef]
14. Itkin, M.; Seybold, H.; Breitel, D.; Rogachev, I.; Meir, S.; Aharoni, A. Tomato Agamous-like 1 is a component of the fruit ripening regulatory network. *Plant J.* 2009, 60, 1081–1095. [CrossRef] [PubMed]
15. Lu, S.; Zhang, Y.; Zhu, K.; Yang, W.; Ye, J.; Chai, L.; Xu, Q.; Deng, X. The citrus transcription factor CsMADS6 modulates carotenoid metabolism by directly regulating carotenogenic genes. *Plant Physiol.* 2018, 176, 2657–2676. [CrossRef] [PubMed]
16. Han, Y.C.; Kuang, J.F.; Chen, J.Y.; Liu, X.C.; Xiao, Y.Y.; Fu, C.C.; Wang, J.-N.; Wu, K.-Q.; Lu, W.J. Banana transcription factor MaERF11 recruits histone deacetylase MaHDA1 and represses the expression of MaMAC1 and expansins during fruit ripening. *Plant Physiol.* 2016, 171, 1070–1084. [CrossRef] [PubMed]
17. Zhao, L.; Lu, J.; Zhang, J.; Wu, P.-Y.; Yang, S.; Wu, K. Identification and characterization of histone deacetylases in tomato (*Solanum lycopersicum*). *Front. Plant Sci.* 2015, 5, 760. [CrossRef] [PubMed]
18. Kuang, J.; Chen, J.; Luo, M.; Wu, K.-Q.; Sun, W.; Jiang, Y.-M.; Lu, W.-J. Histone deacetylase HD2 interacts with ERF1 and is involved in longan fruit senescence. *J. Exp. Bot.* 2012, 63, 441–454. [CrossRef]
19. Hu, Y.N.; Han, Z.Y.; Wang, T.; Li, H.; Li, Q.; Wang, S.; Tian, J.; Wang, Y.; Zhang, X.; Xu, X.; et al. Ethylene response factor MdERF4 and histone deacetylase MdHDA19 suppress apple fruit ripening through histone deacetylation of ripening-related genes. *Plant Physiol.* 2022, 202, kia016. [CrossRef]
20. Berger, S.L. The complex language of chromatin regulation during transcription. *Nature* 2007, 447, 407–412. [CrossRef]
21. Yinglin, J.; Mingyang, X.U.; Wang, A. Recent advances in the regulation of climacteric fruit ripening. *Front. Agric. Sci. Eng.* 2021, 8, 314–334.
22. Guo, J.E.; Hu, Z.; Li, F.; Zhang, L.; Yu, X.; Tang, B.; Chen, G. Silencing of histone deacetylase SIHDT3 delays fruit ripening and suppresses carotenoid accumulation in tomato. *Plant Sci.* 2017, 265, 29–38. [CrossRef] [PubMed]
23. Luo, M. Regulation of flowering time by the histone deacetylase HDA5 in Arabidopsis. *Plant J.* 2015, 82, 925–936. [CrossRef] [PubMed]

24. Bemer, M.; Karlova, R.; Ballester, A.R.; Tikunov, Y.M.; Boyv, A.G.; Wolters-Arts, M.; de Barros Rossetto, P.; Angenent, G.C.; de Maagd, R.A. The Tomato FRUITFULL Homologs TDR4/FUL1 and MBP7/FUL2 Regulate Ethylene-Independent Aspects of Fruit Ripening. *Plant Cell.* 2012, 24, 4437–4451. [CrossRef]

25. Fujisawa, M.; Shima, Y.; Nakagawa, H.; Kitagawa, M.; Kimbara, J.; Nakano, T.; Kasumi, T.; Ito, Y. Transcriptional Regulation of Fruit Ripening by Tomato FRUITFULL Homologs and Associated MADS Box Proteins. *Plant Cell.* 2014, 26, 89–101. [CrossRef]

26. Han, Z.; Hu, Y.; Lv, Y.; Rose, J.K.; Sun, Y.; Shen, F.; Wang, Y.; Zhang, X.; Xu, X.; Wu, T.; et al. Natural variation underlies differences in Ethylene Response Factor17 activity in fruit peel degreening. *Plant Physiol.* 2018, 176, 2292–2304. [CrossRef]

27. Hu, Y.; Han, Z.; Sun, Y.; Wang, S.; Wang, T.; Wang, Y.; Xu, K.; Zhang, X.; Xu, X.; Han, Z.; et al. ERF4 affects fruit firmness through TPL4 by reducing ethylene production. *Plant J.* 2020, 103, 937–950. [CrossRef]

28. Li, X.; Shen, F.; Xu, X.; Zheng, Q.; Wang, Y.; Wu, T.; Li, W.; Qiu, C.; Xu, X.; Han, Z.; et al. An HD-ZIP transcription factor, MxHB13, integrates auxin—regulated and juvenility—Determined control of adventitious rooting in Malus xiaojinensis. *Plant J.* 2021, 107, 1663–1680. [CrossRef]

29. Mao, Y.B.; Liu, Y.Q.; Chen, D.Y.; Chen, F.Y.; Fang, X.; Hong, G.J.; Wang, L.J.; Wang, J.W.; Chen, X.Y. Jasmonate response decay and defense metabolite accumulation contributes to age-regulated dynamics of plant insect resistance. *Nat. Commun.* 2017, 8, 13925. [CrossRef]

30. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the \( 2^{-\Delta \Delta CT} \) method. *Methods* 2001, 25, 402–408. [CrossRef]