The apple bHLH transcription factor MdbHLH3 functions in determining the fruit quality

Jian-Qiang Yu [1], Da-Gang Hu [1*], Yu-Jin Hao [1*]

[1]College of Horticulture Science and Engineering, Shandong Agricultural University, Tai-An, Shandong 271018, China

[*]Corresponding author. Email: haoyujin@sdau.edu.cn; fap_296566@163.com

Background

Apples (*Malus domestica*, 'Borkh'.), one of the most commonly eaten fruits, are planted in temperate regions of the world and are rich in minerals and vitamins. The fruit quality is the most important indices in the production of apples since consumers are most interested in the quality components of the fruits, such as fruit appearance (size, shape, color, and absence of defects and decay), hardness and flavor (sugar, acids, and aroma volatiles), and the contents of soluble sugars and organic acids that has strong impacts on the consciousness of apple fruit quality (Lee et al., 2003; Rouphael et al., 2010; Khan et al., 2013). It is interesting that about 85% of organic acids in apple is malic acid, and malate is the predominant form of malic acid in apple (Wu et al., 2007; Nour et al., 2010; Yao et al., 2011; Zhang et al., 2010).

In apple fruit, malate is synthesized mainly in the cytoplasm through the tricarboxylic acid pathway (Yao et al., 2011). Malate synthesis is influenced by multiple factors (temperature, mineral nutrient deficiencies, drought and salt stress), yet studies on the transcriptional regulation of malate metabolism in apple fruit have been relatively scarce to date (Sweetman et al., 2009; Bai et al., 2012; Hu et al., 2016). Therefore, it is important to identify the transcription factors that regulate apple fruit acidity, to reveal the molecular mechanism of transcription factor regulation of apple fruit acidity, and to expand the theoretical understanding of transcriptional regulation of fruit acidity, as well as to provide theoretical support for molecular breeding and fruit quality improvement of fruit trees.

Methods

Malate and soluble sugar assays

Malate and soluble sugar content were determined using a capillary electrophoresis system (Beckman P/ACE, Palo Alto, CA) and Plant soluble sugar content kit (KeMing, Suzhou, China) as described in a previous study (Wang et al., 2016).

ChIP (Chromatin immunoprecipitation) qPCR, Y1H (yeast one hybrid) and EMSA (Electrophoretic mobility shift assay) assays

The ChIP (chromatin immunoprecipitation) experiment was performed as described by Hu et al. (2019). 35S::Myc and 35S::MdbHLH3-Myc transgenic apple calli were used for the ChIP-qPCR analysis, an anti-Myc antibody (Beyotime) was used for ChIP. All primers used for Chip-PCR are listed in Table S1. The full-length of MdbHLH3 was

ligated into the pGADT7 vector (Clontech). The MdcyMDH promoter 3 and 4 region fragment were ligated into the pHIS2 vector (Clontech). 3-AT (3-Amino-1,2,4-triazole) was used for screening.

The EMSA was conducted as previously described (Hu et al., 2019). The CDSs of MdbHLH3 was cloned into the PET-32a-c vector. The MdbHLH3-His recombinant protein was expressed in *E. coli* BL21 (DE3). The protein was purified using the glutathione sepharose beads (Thermo Scientific, San Jose, CA, USA). The EMSA probe biotin labeling Kit (Beyotime) and the Light Shift Chemiluminescent EMSA Kit (Thermo) were used for the subsequent EMSA. Briefly, the fusion protein MdbHLH3-His and the oligonucleotide probe of the MdcyMDH promoter were incubated in a binding buffer for 20 min at room temperature. The unlabeled probes were used for probe competition.

Transient dual luciferase assays

Transient expression assays were performed in tobacco (*Nicotiana tabacum*) leaves (An et al., 2018). The promoter fragment of MdcyMDH was cloned into the pGreenII 0800-LUC vectors to generate the luciferase reporter gene (MdcyMDHpro-LUC). *MdbHLH3* was cloned into the pGreenII 62-SK vector to generate the effector (35Spro::MdbHLH3). *A. tumefaciens* LBA4404 was used for transforming of the recombinant plasmids. The infiltration was performed as previously described (Xie et al., 2012). A living imaging apparatus was used for luminescence measurement.

Construction of viral vectors and transient expression in apple fruit

The full-length cDNA and antisense partial sequences of MdbHLH3 and MdcyMDH were isolated from 'Gala' apple using RT-PCR. The resulting PCR products of antisense partial sequences of MdbHLH3 and MdcyMDH were inserted into the TRV (tobacco rattle virus) vector in the antisense orientation under the control of the dual 35S promoter. The resulting vectors were named TRV-MdbHLH3 and TRV-MdcyMDH respectively. The resulting PCR products of full-length cDNA of MdbHLH3 and MdcyMDH were cloned into the IL-60 vector under the control of the 35S promoter. The vectors were named IL60-MdbHLH3 and IL60-MdcyMDH, respectively. The resulting vector were used for infiltration of apples by Agrobacterium (GV3101)-mediated transformation as described previously (Xie et al., 2012). The injected apple fruits were placed in the dark at 23°C for two days, then kept in the highlight at 10°C for one week before collecting the injected part for gene expression analysis and malate content determination.

Assay of enzymes involved in sugar metabolism

Enzyme activities were measured according to the instructions from the fructose-1,6-bisphosphatase and sucrose phosphate synthase kits (KeMing, Suzhou, China).

^{13}C pulse labeling

^{13}C pulse labeling was carried out in a gas-tight labeling chamber made with transparent agricultural film at the apple maturity stage (August 10th). First, checked the seal of the labeling chamber, then one gram of $Ba^{13}CO_3$ (^{13}C abundance is 98%, the proportion of ^{13}C in all carbon elements) was put into a beaker and iron powder was reduced into the labeling room. ^{13}C labeling started at 8:00 am (August 10th) with the certain amount of HCl (1 mol/L) injected into the beaker with $Ba^{13}CO_3$ using a syringe. HCl was injected into the beaker every 30 minutes for a duration of 4h in order to maintain the concentration of CO_2. At the same time, another group of unlabeled control plants was used as a blank of ^{13} C labeling (natural abundance of ^{13} C).

The plants were destructively sampled after 72h (at 8:00 am on August 13th). The plant samples were divided into leaves, branches and fruits. Samples were washed by tap water, detergent, tap water and 1% hydrochloric acid in order, and then with deionized water for 3 times.Samples were then dried at 80℃, followed with homogenization by electric grinder and filtration with 0.25 mm mesh screen. The abundance of ^{13}C was measured with DELTAV plus XP advantage isotope ratio mass spectrometer analyzed by National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, Shandong Agricultural University. The calculation method was performed as described by Ding et al. (2017).

Statistical analysis

All experiments were performed in triplicate. Error bars show the standard deviation of three biological replicates. Significant difference was detected by *t*-test using GRAPHPAD PRISM 6.02 software (*, $P < 0.05$; **, $P < 0.01$).

Results (up to 4 figures and tables can be included)

MdbHLH3 promotes malate accumulation in the early stage of apple development by activating malate biosynthetic genes

Three 35S::MdbHLH3-GFP transgenic apple lines overexpressing MdbHLH3 (MdbHLH3-27, MdbHLH3-36, MdbHLH3-44) were generated from our previous study (Xie *et al.,* 2012). To explore the impact of MdbHLH3 overexpression on fruit development, apples were collected from these three lines and the wild-type (WT) control, and tested for the transverse diameter, the vertical diameter, the per fruit weight,

and the malate content at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter and the per fruit weight of the MdbHLH3-overexpressing transgenic apples did not show any significant changes at the various stages of fruit development compared to the WT control (Figure 1b-d). However, both MdbHLH3-27 and MdbHLH3-44 apples accumulated significantly more malate than the WT control ones, especially fruits at 60 days after blooming (DAB) (Figure 1e). The MdbHLH3-36 apples also showed a slightly increase of malate content compared to the wild-type controls (Figure 1e), although not as significant as the other two lines. However, this increase diminished almost completely by day 120 after blooming (Figure 1e), indicating that MdbHLH3-overexpresion increased the malate accumulation mostly in the early stage of apple development.

MdbHLH3 activates the transcription of MdcyMDH through binding to its promoter

It is known that the bHLH proteins function through the recognition of the E-box (5'-CANNTG-3') or G-box (5'-CACGTG-3') cis-element in the promoters of their target genes. A chromatin immunoprecipitation PCR (ChIP-PCR) assay was performed using 35S::MdbHLH3-Myc transgenic apple calli. The results showed that approximately 4.5-fold, and 8.5-fold enrichment was detected in the promoter 3 and 4 region of MdcyMDH, respectively, but no enrichment was observed for the promoter of other malate-related genes compared to the control (Figure 2a). These results provided in vivo evidence for the binding of MdbHLH3 to the promoter region of MdcyMDH.

To verify in vitro binding of MdbHLH3 to the promoters of MdcyMDH, yeast one-hybrid (Y1H) assays were conducted. The promoter 3 and 4 region of MdcyMDH gene was fused to the pHIS2 vector, respectively, and the MdbHLH3 gene to the activation domain AD. When the fused MdcyMDHpro::pHIS was co-expressed with MdbHLH3-AD, the transformant yeast cells were able to grow well on an SD/-Leu/3-AT 100 plate, but the negative control, which co-expressed the MdcyMDHpro::pHIS and the AD empty vector was weaker growth (Figure 2b).

Alternatively, an electro-phoresis mobility shift assay (EMSA) was developed using purified recombinant His-MdbHLH3 fusion proteins and DNA fragments of the MdcyMDH promoter region containing the E-box sequence as probes. As a result, specific DNA-MdbHLH3 protein complexes were detected when the E-box sequence was used as a labelled probe (Figure 2c). The formation of these complexes was reduced when increasing amounts of un-labelled E-box competitor probes with the same

sequence were added (Figure 2c). This competition was not observed with a mutated version of the probe MdcyMDHm (Figure 2c). This specific competition demonstrated that the interaction between DNA and the MdbHLH3 protein requires the E-box recognition sequence. These results demonstrated that MdbHLH3 binds specifically to the E-box cis-elements within the MdcyMDH promoter *in vitro*.

To verify that MdbHLH3 activates the expression of MdcyMDH, a construct with MdcyMDH fused to the reporter gene luciferase (MdcyMDHpro::Luc) was combined with the 35Spro::MdbHLH3 construct for co-infiltration into tobacco leaves (Figure 3a). The co-expression of these two constructs exhibited significantly higher luminescence signals than the controls did (Figure 3b-c), indicating that MdbHLH3 positively regulates the expression of MdcyMDH in vivo. Secondly, a GUS reporter assay system was set up to transiently express constructs PMdcyMDH::1300 (contains GUS fragments) and 35S::MdbHLH3 in apple calli (Figure 3d). The expression of GUS protein was detected by immunoprecipitation assays. We found that the transgenic-calli-containing PMdcyMDH::1300 plus 35S::MdbHLH3 exhibited a much higher level of GUS staining and GUS proteins than those harboring PMdcyMDH::1300 alone (Figure 3e-f). The addition of 35S alone had little influence on the abundance of GUS protein in PMdcyMDH::1300 transgenic calli (Figure 3e-f). Therefore, overexpression of MdbHLH3 seems to trigger an increase of the MdcyMDH expression by activating MdcyMDH transcription in apple.

MdbHLH3 overexpression increased sugar contents in apples

In the reproductive growth stage, mature leaves are the source of metabolism and the developing fruits are the main metabolic pool. The carbohydrate metabolic enzyme activities in the source leaves are usually used to measure the source intensity. We found that the activities of the fructose-1,6-bisphosphatase (FBP) and the sucrose phosphate synthase (SPS) were significantly increased in the leaves of MdbHLH3 transgenic apple trees compared to the WT (Figure 4a-b). At the same time, the levels of sorbitol, sucrose and starch also increased significantly as the major photosynthetic products in apple leaves (Figure 4c-e). MdbHLH3 overexpression produced an increase in the photosynthetic rate in leaves, and led to the stronger ability of absorbing ^{13}C from the labeled leaves to the active parts of metabolism and growth (Figure 4f). ^{13}C pulse labeling data showed that ^{13}C in WT and MdbHLH3 transgenic line was mainly incorporated into fruits at the apple maturity stage, and the relative ^{13}C enrichment of assimilates assigned to MdbHLH3 transgenic line was significantly higher than WT.

The results demonstrated that the transportation and distribution of carbohydrates to fruits increased in MdbHLH3 transgenic line compared to WT. Since sucrose in the source leaf is the main substrate for metabolism in sink tissues and sorbitol is the main translocated form of carbon in apple trees, we measured the soluble sugar content in the MdbHLH3 transgenic apples. As expected, the soluble sugar content was significantly increased in MdbHLH3 transgenic apples compared to WT (Figure 4g) which well justified the labeling data presented above

Taken together, MdbHLH3 overexpression seemed to have enhanced the transport of carbohydrates into fruits by affecting the source-sink relationship, thereby increasing sugar content in fruits.

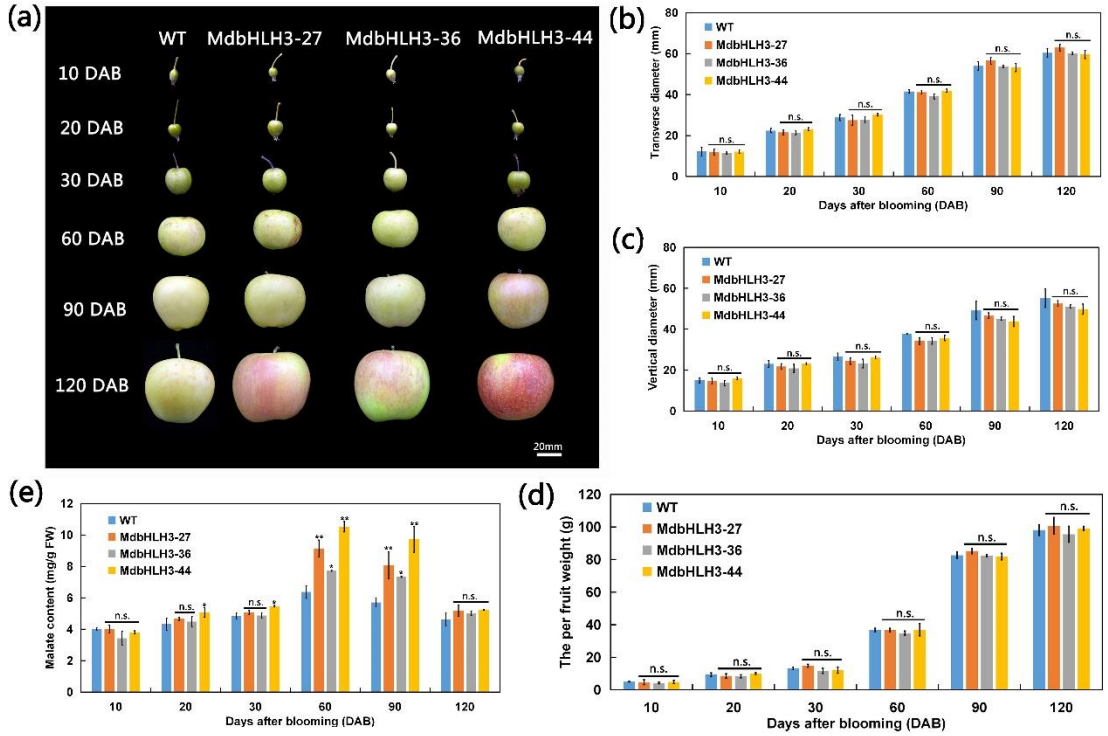

Figure 1 Overexpression of MdbHLH3 promotes malate accumulation in apple. (a) Fruit phenotype of the three MdbHLH3 transgenic apple lines and the WT (wild-type) control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 d after blooming (DAB). (b) The measurement of transverse diameter of the three MdbHLH3 transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 d after blooming (DAB). (c) The measurement of vertical diameter of the three MdbHLH3 transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 d after blooming (DAB). (d) The measurement of per fruit weight of the three MdbHLH3 transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 d after blooming (DAB). (e) The measurement of malate content of the three MdbHLH3 transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and

120 d after blooming (DAB). In (b-e), the data are shown as the mean ± SE, based on more than nine replicates. Statistical significance was determined using Student's t-test. ns, P > 0.05; *P < 0.05; **P < 0.01.

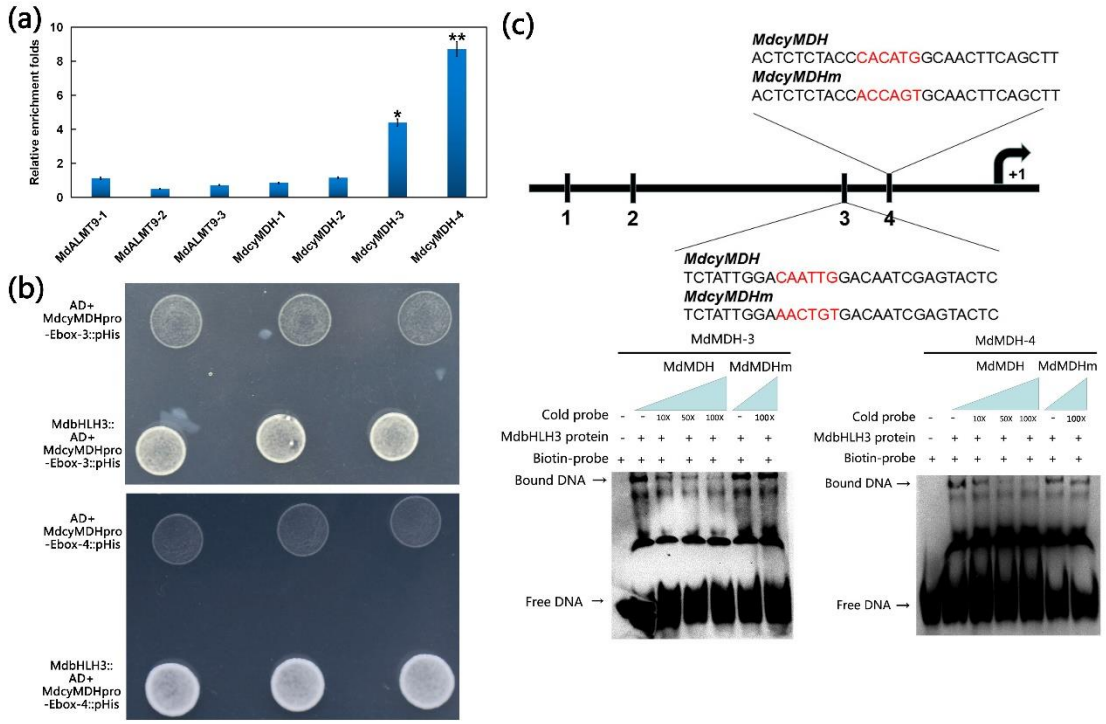

Figure 2 MdbHLH3 binds to the promoter of MdcyMDH. (a) The relative enrichment levels of the malate-related gene promoter fragments by CHIP (Chromatin immunoprecipitation)-PCR assays. (b) Yeast one-hybrid assays showing that MdbHLH3 binds to the promoter 3 and 4 fragments of MdcyMDH, containing the E-box or G-box motifs. The screening concentration of 3-AT was 100 mmol. The empty vector and the MdcyMDH promoter (AD-Empty+MdcyMDH-Promoter) were used as negative controls. (c) The determination of the interaction between MdbHLH3 and labeled DNA probes in the promoters of MdcyMDH by EMSA (Electrophoretic mobility shift assay). In (a), data are shown as the mean ± SE, based on more than nine replicates. Statistical significance was determined using Student's t test. *, P < 0.05; **, P < 0.01.

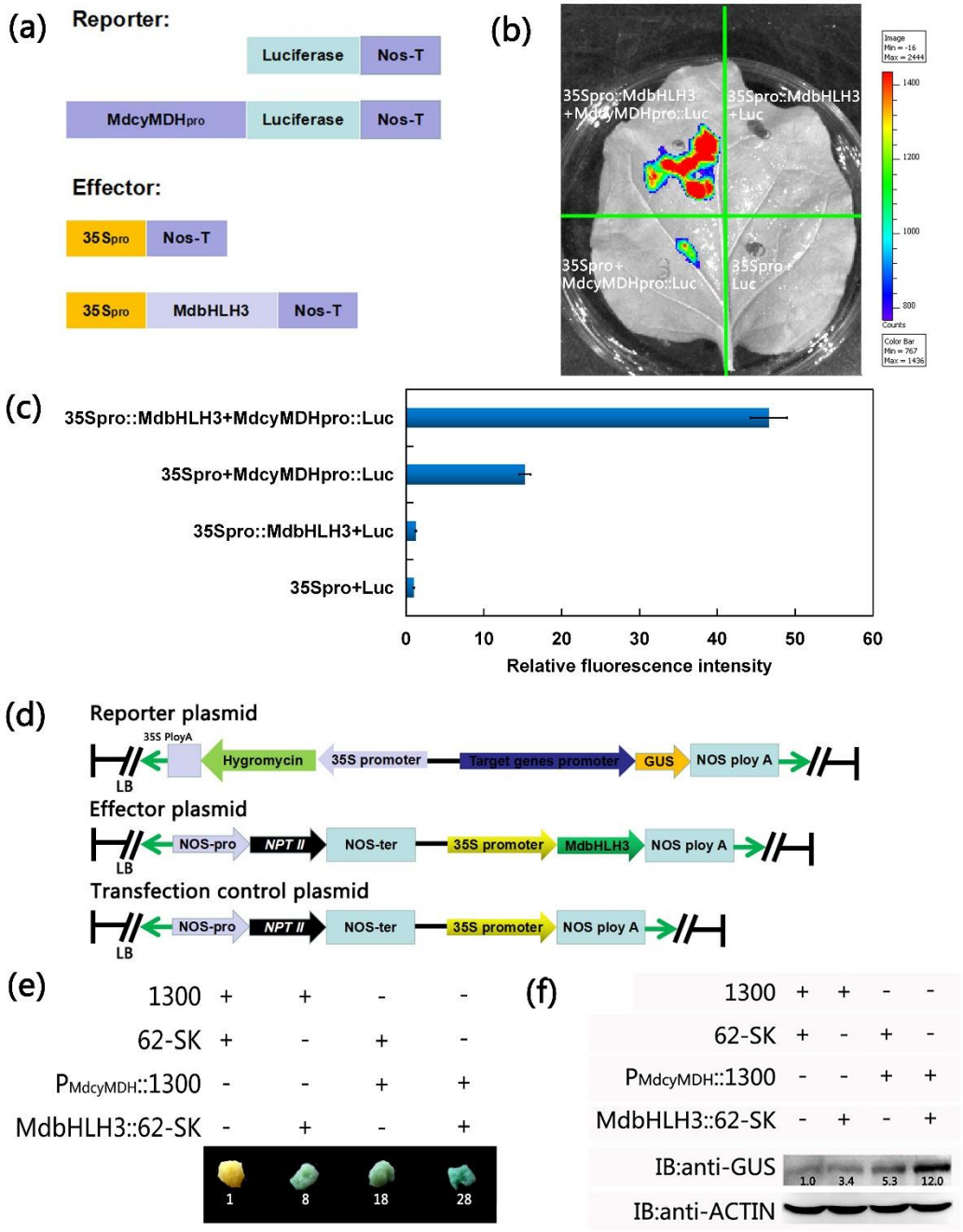

Figure 3 MdbHLH3 enhances its expression by activating the transcription of MdcyMDH. (a) Schematic representation of the Luc reporter vector (MdcyMDHpro::Luc) and the effector vector (35Spro::MdbHLH3). (b) The expression assays showing that MdbHLH3 activates the expression of MdcyMDHpro::Luc in the representative images of Nicotiana benthamiana leaves by transient infiltration. (c) The relative quantitative analysis of luminescence intensity by setting the value for luminescence intensity in 35S pro +Luc samples to 1. (d) Schematic representation of the GUS reporter plasmid (PMdcyMDH::1300) and the effector plasmid (MdbHLH3::62-SK). (e) The effector (MdbHLH3::62-SK) and reporter (PMdcyMDH::1300) constructs in the binary vectors were introduced into apple calli for staining to visualize GUS activity. (f) The abundance of recombinant protein was determined in transgenic-calli-containing PMdcyMDH::1300 plus MdbHLH3::62-SK

by using an anti-GUS antibody.

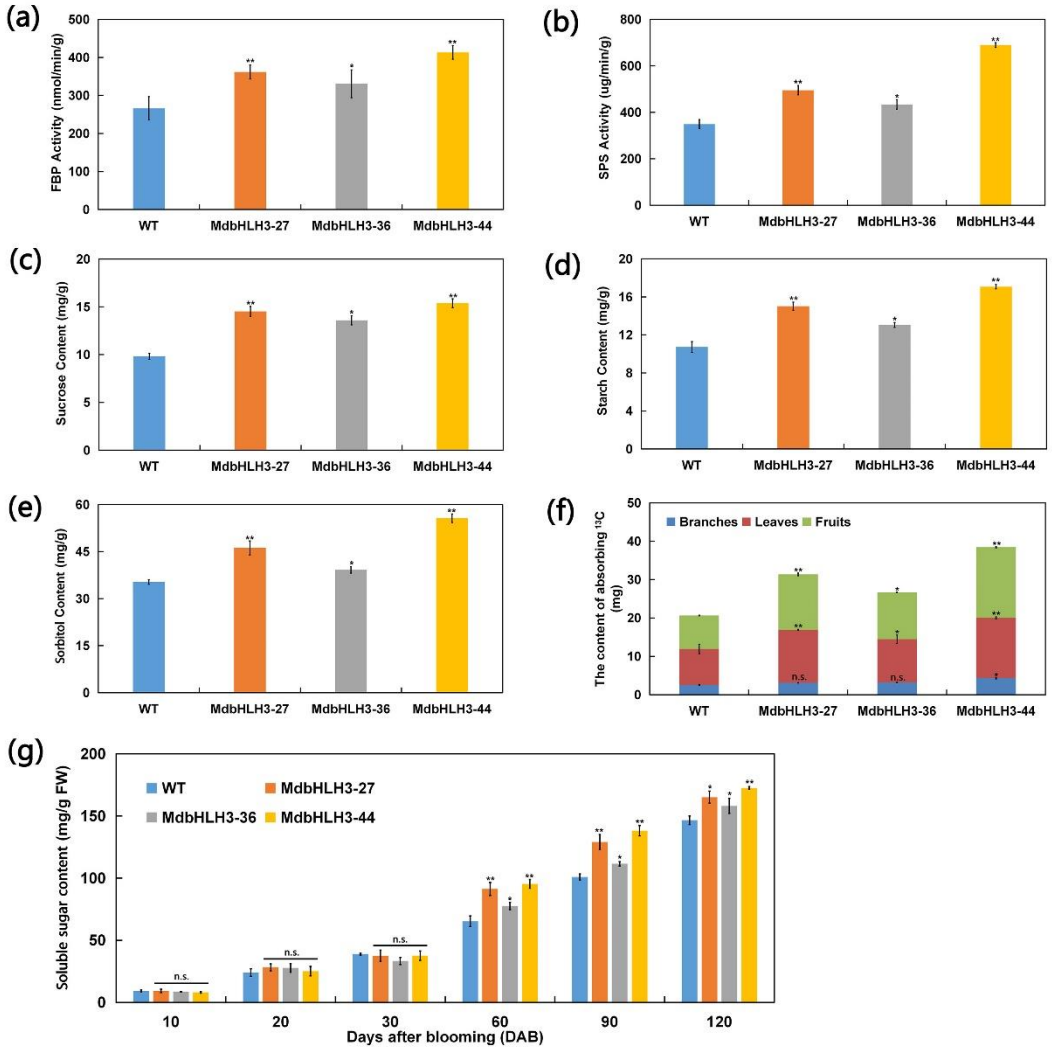

Figure 4 MdbHLH3-overexpression increases sugar content in apple fruits. (a) The FBP (fructose-1,6-bisphosphatase) activity is measured in the WT and transgenic apple leaves. (b) The SPS (sucrose phosphate synthase) activity is measured in the WT and transgenic apple leaves. (c) The content of sucrose is measured in the WT and transgenic apple leaves. (d) The content of starch is measured in the WT and transgenic apple leaves. (e) The content of sorbitol is measured in the WT and transgenic apple leaves. (f) The content of absorbing 13C is measured in the WT and transgenic apple trees. (g) The measurement of soluble sugar content of the WT and MdbHLH3 transgenic apple fruits collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 d after blooming (DAB). (a-e) The mature leaves are measured after 120 DAB from the WT and transgenic apple trees. Data are shown as the mean ± SE, based on more than nine replicates. Statistical significance was determined using Student's t test. n.s., $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

Conclusion

Changes in carbohydrates and organic acids largely determine the palatability of edible tissues of horticulture crops. Elucidating the potential molecular mechanisms involved

in the change of carbohydrates and organic acids, as well as their temporal and spatial crosstalk are key steps in understanding fruit developmental processes. Here, we used apple (*Malus domestica*, 'Borkh') as research materials, and found that MdbHLH3, a basic helix-loop-helix transcription factor (bHLH TF), modulates the accumulation of malate and carbohydrates. Biochemical analyses demonstrated that MdbHLH3 directly binds to the promoter of MdcyMDH that encodes an apple cytosolic NAD-dependent malate dehydrogenase, activating its transcriptional expression, thereby promoting malate accumulation in apple fruits. Additionally, MdbHLH3 overexpression increased the photosynthetic capacity and carbohydrate levels in apple leaves, and also enhanced the carbohydrate accumulation in fruits by adjusting carbohydrate allocation from sources to sinks. Overall, our findings provide new insights into the mechanism of how the bHLH TF MdbHLH3 modulates the fruit quality. It directly regulates cytosolic malate dehydrogenase MdcyMDH to coordinate carbohydrate allocation and malate accumulation in apple.

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