The apple bHLH transcription factor MdbHLH3 functions in determining the fruit carbohydrates and malate

Jian-Qiang Yu1, Kai-Di Gu1, Cui-Hui Sun1, Quan-Yan Zhang1, Jia-Hui Wang1, Fang-Fang Ma1, Chun-Xiang You1,2,3, Da-Gang Hu1,2,3,* and Yu-Jin Hao1,2,3,*

1National Key Laboratory of Crop Biology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai’an, Shandong, China
2MOA Key Laboratory of Horticultural Crop Biology and Germplasm Innovation, Tai’an, Shandong, China
3Shandong Collaborative Innovation Center for Fruit and Vegetable Production with High Quality and Efficiency, Tai’an, Shandong, China

Keywords: apple, fruit quality, bHLH, malate accumulation, carbohydrates, photosynthesis.

Introduction

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 majority of apple productions are consumed as fresh fruits, and the rest are mostly processed into concentrates, juices and purées. 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, and colour), defects and decay, hardness and flavour (sugar, acids and aroma volatiles), and the contents of soluble sugars and organic acids that have strong impacts on the consciousness of apple fruit quality (Khan et al., 2013; Lee et al., 2003; Rouphael et al., 2010). Therefore, maintaining a proper balance between sugar and acid content is important for developing a desired apple variety (Visser et al., 1968).

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 (Nour et al., 2010; Wu et al., 2007; Yao et al., 2011; Zhang et al., 2010). Malate has multiple important functions in the life cycle of plants. It is well known that malate is a key intermediate in the tricarboxylic acid (TCA) cycle and is imported into mitochondria as a respiratory substrate (Ferrie et al., 2004; Noguchi and Yoshida, 2008; Sweetman et al., 2009). Malate also has been defined as a pH regulator (Hu et al., 2016a; Mathieu et al., 1986), an essential storage carbon molecule (Martinoia and Rentsch, 1994), and it is also involved in controlling the stomatal function (Lee et al., 2008). Most importantly, malate acts together with other organic acids and soluble sugars to adjust the edible quality of fruits.

Dramatic changes in organic acids and soluble sugars occur during the ripening process of fleshy fruits from the young to fully ripened stage (Chervin et al., 2006; Craker et al., 1971; Defilippi et al., 2004; Fukao et al., 2006; Yao et al., 2011). At the early stage of apple fruit development, most of the imported carbohydrates are rapidly metabolized via glycolysis and the tricarboxylic acid (TCA) cycle (Bahrami et al., 2001; Gibson and McAlister-Henn, 2003; Noguchi and Yoshida, 2008; Sweetman et al., 2009). At this stage, low levels of sugars but higher levels of malate accumulate in apple fruits. As the fruit continues to grow due to cell expansion, carbohydrate metabolism slows down. When more carbon is converted to soluble sugar for storage in apple fruits, the malate content reaches its highest level. At the late stage of apple fruit development, the transport of carbohydrate from leaf to fruit is enhanced, which, along with concomitant, further decline in malate levels and increase in soluble sugar levels, elevates apple fruits’ sweetness to a higher level at maturity. Although the process is well known, the mechanisms involved remain poorly understood in fruit developmental processes, and then elucidating the mechanism of malate and soluble sugar accumulation is thus an important research direction.

The process of malate accumulation is mainly affected by synthesis, transportation and metabolism. Transcription factors involved in the enzymes of malate transporter have been widely reported. For example, the MYB transcription factors MdMYB1/10...
and MdMYB73 modulate malate transportation by directly activating vacuolar transporters, including the tonoplast-localized malate transporter (IPT), aluminium-activated malate transporter 9 (ALMT9/Ma1) and the two primary proton pumps, vacuolar H*-ATPase (V-ATPase) and vacuolar pyrophosphatase (V-PPase), which have been identified play crucial roles in determining fruit acidity (Bai et al., 2012; Hu et al., 2017; Hu et al., 2016a; Sweetman et al., 2009).

Many enzymes involved in malate synthesis and metabolism also have been identified. For example, phosphoenolpyruvate carboxylase (PEPC) is indicated as the key enzyme in fruit malate synthesis, while cytosolic NADP-dependent malic enzyme (cyME) is considered an important enzyme involved in malate degradation (Berüter, 2004; Chollet et al., 1996; Ruffner et al., 1984; Yao et al., 2009). Cytosolic NAD-dependent malate dehydrogenase (cyMDH) is the key enzyme involved in malate synthesis. cyMDH can catalyse the conversion reaction from OAA to malate and affect chloroplast metabolism by regulating the redox (Miller et al., 1998; Wang et al., 2016b; Yao et al., 2011). However, how these malate synthesis and metabolism enzymes are transcriptionally activated or inactivated is still not very clear.

In the past decades, MYB-bHLH-WDR (MBW) complex has been widely studied and is involved in stress responses and anthocyanin regulation, as well as acidity and vacuolar pH determination (Ramsay and Glover, 2005; Wang et al., 2016b). In citrus, Buselli had identified Noemi, which encodes a basic helix-loop-helix (bHLH) transcription factor and which controls the reduction in anthocyanins and fruit acidity (Buselli et al., 2019). In apple, MdbHLH3 was first found by Xie et al. (2012) to interact with MYB TF MdMYB1 in directly activating its downstream anthocyanin biosynthesis genes MdbDFR and MdbUFGT for promoting anthocyanin accumulation and fruit coloration in response to low temperature. In addition to MdMYB1, An et al. (2015) reported that MdMYB9 and MdMYB11 also interacted with MdbHLH3 that are involved in the regulation of the JA-induced biosynthesis of anthocyanin and proanthocyanidin in apples. At present, no other MdbHLH3-interacting MYB TFs were identified except MdMYB1, MdMYB9 and MdMYB11. In addition, we found that apple MdbHLH3 overexpression at the whole-genome level, we conducted an expression profiling experiment of the WT and the three MdbHLH3 transgenic apple fruits at 60 DAB using RNA-seq. About 49.42 M raw reads and 7.41 G raw bases were generated, among which more than 93% were effective bases. Subsequently, expressional abundances of the assembled genes were evaluated using FPKM (fragments per kilobase of transcript per million fragments mapped). Then, we performed pairwise comparisons of transcript abundances to identify differentially expressed genes (P value < 0.05 and |log2 (fold change)| ≥ 1) between the WT and the three MdbHLH3-overexpressing apple samples. Compared to the WT, 616 up-regulated genes and 384 down-regulated genes were found in MdbHLH3-27 fruits, whereas only 296 up-regulated genes and 664 down-regulated genes were found in MdHLH3-36 fruits, but 761 up-regulated genes and 198 down-regulated genes were found in MdbHLH3-44 fruits (Figure 2a; Datasets S1-S3). Among these differentially expressed genes, 27 up-regulated ones and 26 down-regulated ones were common to all three MdbHLH3-overexpressing apple fruits (Figure 2b-c). Subsequently, Gene Ontology (GO) enrichment analysis was performed to further evaluate the global gene expression profiles. We found that most of the fruit acid-related genes were up-regulated (Figure 2d). To validate our RNA-seq expression profiling data, some fruit acid-related genes were selected for qRT-PCR analysis. These genes included the malate-associated genes (MdMDH, MdNADP-ME3, MdALMT9), citrate-associated genes (MdACT3, MdMIDH, MdLike), acetate-associated genes (MdG3H3.1-1, MdG3H3.1-2) and succinate-associated genes (MdchArgL). They were all up-regulated in the three MdbHLH3-overexpressing apple fruits compared with the WT (Figure 2e). Although the exact fold changes in the selected expression levels of MdbHLH3 and its downstream anthocyanin-associated genes (including MdMYB1, MdANS, MdANR and MdbUFGT) at various stages after blooming. The results showed that the anthocyanin contents and the expression levels of MdbHLH3 and its downstream anthocyanin-associated genes (including MdMYB1, MdANS, MdANR and MdbUFGT) in those three MdbHLH3 transgenic lines were higher than the WT control during the whole fruit developmental stages (Figure 1a, Figure S1). These results were consistent with our reports that MdbHLH3 was a key positive regulator in apple (Hu et al., 2020; Xie et al., 2012).

The transverse diameter and vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a). We found that the transverse diameter, vertical diameter, and per fruit weight and malate content in WT and these three were simultaneously tested at various stages after blooming (Figure 1a).
unigenes varied between qRT-PCR analysis and the RNA-seq expression data, a high correlation ($R^2 = 0.3225$) was discovered by a simple linear regression equation ($y = 0.9026x$), indicating a good consistency between the two experimental methods (Figure 2f). In addition, transcript levels of the malate-vacuolar-related transporters including \textit{MdVHA-B1}, \textit{MdVHA-B2}, \textit{MdVHA-E2}, \textit{MdVHP1} and \textit{MdtDT} were found also much higher in the three \textit{MdbHLH3}-overexpressing apple plantlets than in the WT control (Figure S3). Taken together, these results indicated that \textit{MdbHLH3} positively regulates the expressions of acid-related genes, especially malate-associated genes in fruits.

\textbf{MdbHLH3 activates the transcription of \textit{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'-CACGTCG-3') cis-element in the promoters of their target genes (Fisher and Goding, 1992). To determine whether \textit{MdbHLH3} binds to the promoter regions of the malate-associated genes, we analysed the promoter regions of these genes and found four E-box or G-box cis-elements for \textit{MdALMT9}, no E-box or G-box cis-element for \textit{MdNADP-ME3}, and four E-box or G-box cis-elements for \textit{MdcyMDH} (Figure S4). A chromatin immunoprecipitation PCR (ChIP-PCR) assay was performed using \textit{35S::MdbHLH3-Myc} transgenic apple calli. The results showed that approximately 4.5-fold enrichment and 8.5-fold enrichment were detected in the promoter 3 and promoter 4 regions of \textit{MdcyMDH}, respectively, but no enrichment was observed for the promoter of other malate-related genes compared with the control (Figure 3a). These results provided in vivo evidence for the binding of \textit{MdbHLH3} to the promoter region of \textit{MdcyMDH}.

To verify in vitro binding of \textit{MdbHLH3} to the promoters of \textit{MdcyMDH}, yeast one-hybrid (Y1H) assays were conducted. The promoter 3 and promoter 4 regions of \textit{MdcyMDH} were fused to the pHIS2 vector, respectively, and the \textit{MdbHLH3} gene to the activation domain AD. When the fused \textit{MdcyMDH::pHIS} was co-expressed with \textit{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 \textit{MdcyMDH::pHIS} and the AD empty vector, was weaker growth (Figure 3b).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Overexpression of \textit{MdbHLH3} promotes malate accumulation in apple. (a) Fruit phenotype of the three \textit{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 \textit{MdbHLH3} transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 day after blooming (DAB). (c) The measurement of vertical diameter of the three \textit{MdbHLH3} transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 day after blooming (DAB). (d) The measurement of per fruit weight of the three \textit{MdbHLH3} transgenic apple lines and the WT control collected from the different developmental stages of 10, 20, 30, 60, 90 and 120 day 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, $P > 0.05$; *$P < 0.05$; **$P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com]}
\end{figure}
Alternatively, an electrophoresis 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 3c). The formation of these complexes was reduced when increasing amounts of unlabelled E-box competitor probes with the same sequence were added (Figure 3c). This competition was not observed with a mutated version of the probe MdcyMDHm (Figure 3c). 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

---

Figure 2 MdbHLH3 regulates malate-associated genes in apple fruits. (a) Number of genes showing different expressions in three MdbHLH3 transgenic apple fruits compared with WT (wild-type) by RNA-seq. Venn diagram analysis of common (b) up-regulated and (c) down-regulated genes (DEG; |log2(fold change)| ≥ 1; FDR < 0.01) in three MdbHLH3 transgenic apple fruits compared with WT. (d) Heat map showing different expression of malate-related genes in three MdbHLH3 transgenic apple fruits compared with WT. The complete data are given in Datasets S1-S3. (e) The relative expressions of MdcyMDH (MD13G1214000) and MdcyMDH (MD13G1214000) in WT and three MdbHLH3 transgenic apple fruits determined by qRT-PCR assays. (f) Scatter diagram of log2(fold change) of unigenes in three MdbHLH3 transgenic apple fruits compared with WT. In (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. [Colour figure can be viewed at wileyonlinelibrary.com]
construct for co-infiltration into tobacco leaves (Figure 4a). The co-expression of these two constructs exhibited significantly higher luminescence signals than the controls did (Figure 4b-c), indicating that MdbHLH3 positively regulates the expression of MdcyMDH in vivo. In addition, we performed the Luc assays by injection of tobacco leaves with six construct combinations, 35Spro\(+\)Luc, 35Spro::MdbHLH3\(+\)Luc, 35Spro::MdcyMDHpro-Luc, 35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc, 35Spro::MdMYB1\(+\)35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc and 35Spro::MdMYB73\(+\)35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc. As a result, we found that the relative luminescence intensities in 35Spro::MdMYB1\(+\)35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc and 35Spro::MdMYB73\(+\)35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc were similar to the 35Spro::MdbHLH3\(+\)MdcyMDHpro-Luc-injected sites (Figure S5), suggesting that MdbHLH3-mediated transcriptional activation of MdcyMDH promoter is not dependent on MdMYB1 and MdMYB73.

Subsequently, a GUS reporter assay system was set up to transiently express constructs PMdcyMDH::1300 (contains GUS fragments) and 3SS::MdbHLH3 in apple calli (Figure 4d). The expression of GUS protein was detected by immunoprecipitation assays. We found that the transgenic-calli-containing PMdcyMDH::1300 plus 3SS::MdbHLH3 exhibited a much higher level of GUS staining and GUS proteins than those harbouring PMdcyMDH::1300 alone (Figure 4e-f). The addition of 3SS alone had little influence on the abundance of GUS protein in PMdcyMDH::1300 transgenic calli (Figure 4e-f). Therefore, overexpression of MdbHLH3 seems to trigger an increase in the MdcyMDH expression by activating MdcyMDH transcription in apple.

MdbHLH3 promotes malate accumulation through activating the expression of MdcyMDH

To verify the function of MdbHLH3 in malate accumulation, a viral vector-based transformation method was used to modify its expression in apple fruits. Three viral constructs IL60-MdbHLH3, TRV-MdbHLH3 and TRV-MdcyMDH were created and infiltrated into apple fruits individually or as combination, with the empty vectors as controls (Figure 5a). The expressions of MdbHLH3 and MdcyMDH were confirmed (Figure 5b), and the malate contents were examined in these fruits (Figure 5c). It seemed the overexpression of MdbHLH3 led to a higher level of malate and suppression of MdcyMDH expression decreased the level of malate (Figure 5c). However, when IL60-MdbHLH3 and TRV-MdcyMDH were co-infiltrated to increase the expression of MdbHLH3 and suppress the MdcyMDH expression at the same time, the malate level appeared lower to the empty vector controls (Figure 5b-c), suggesting MdbHLH3 may elevate the malate accumulation through the expression of MdcyMDH in apple fruits.
Similar experiment was also performed in the hairy roots. Agrobacterium rhizogenes MSU440 containing a specific anti-sense MdcyMDH fragment was used to quickly induce the regeneration of MdcyMDH-silenced hairy roots in MdbHLH3 transgenic lines. The successfully transformed roots were identified by their exogenously expressed green fluorescence protein (GFP) and the expressions of the target genes, with the empty vector as a negative control (Figure 5d-e, Figure S6). Similarly, MdcyMDH suppression in the roots reduced the malate content of the MdbHLH3 transgenic lines (Figure 5f). Taken together, MdbHLH3 positively promotes malate accumulation by regulating MdcyMDH at the transcription level in apple.

MdbHLH3 modulates the mitochondrial and chloroplast metabolisms in apple trees

Previous study showed that MdcyMDH overexpression modulated mitochondrial and chloroplast metabolisms by changing the redox activity of MDH during apple development (Wang et al., 2016a; Yao et al., 2011). Therefore, we examined the MDH activities in the cytosol, mitochondria and chloroplast in the MdbHLH3-overexpressing apple trees (Figure 6a-c). For both cyMDH and chMDH, their oxidative activities did not show any difference compared with the WT, but their reductive activities both had significant increase compared with the control (Figure 6a and c). In contrast, neither the oxidative activity nor the reductive activity of mMDH changed in the transgenic plants compared with those in the WT plants (Figure 6b). In addition, the significantly increased malate content showed that the reductive activity of MDH was strengthened against its oxidative activity in the transgenic apples. We further found that MdbHLH3 may have affected the TCA cycle by enhancing the expressions of MdmMDH and MdmSDH (two key genes in TCA cycle) and the ratios of NADH/NAD in the transgenic apple (Figure 6d-e). Thus, in addition to the direct regulation of malate synthesis, MdbHLH3 indirectly modulates TCA cycle and the oxidation–reduction process in mitochondria, affecting the mitochondrial metabolism of transgenic apples.

We also found that the chlorophyll content was elevated in the MdbHLH3 transgenic apple, giving the leaves more green colour (Figure 7a-b). Because MdcyMDH overexpression modulated chloroplast metabolisms by altering the MDH activities in the cytosol and chloroplast (Wang et al., 2016a), we
speculated that the chloroplast metabolisms may have also
been altered in the *MdbHLH3*-overexpressing apple trees. To
test this speculation, we examined the parameters related to
the photosynthesis and chlorophyll fluorescence. As expected,
we observed significant increases in the FV/FM (maximum
quantum yield of PSII photochemistry) and ETR (a quantitative
indicator of the electron transport beyond PSII), and a decrease
in the 1-qP, indicating that the *MdbHLH3* transgenic apple
trees had significantly changed redox levels (Figure 7c-e).

Although the intercellular CO2 concentration in the leaf
was similar between the WT and the transgenic apples, an increase
in the photosynthetic rate and the stomatal conductance was
recorded (Figure 7f-h). Taken together, *MdbHLH3*
overexpression modulated both mitochondria and chloroplast metabolisms compared with WT.

**MdbHLH3 overexpression increased sugar contents in apple**

In the reproductive growth stage, mature leaves are the source of
metabolism and the developing fruits are the main photosynthetic
metabolic sink. 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 with the WT (Figure 8a-b). At the same time, the levels
of sorbitol, sucrose and starch also increased significantly as the
major photosynthetic products in apple leaves (Figure 8c-e).
*MdbHLH3* overexpression produced an increase in the photosynthetic rate in leaves and led to the stronger ability of absorbing

---

**Figure 5** *MdbHLH3* promotes malate accumulation via activating *MdCyMDH* expression. (a) Transient expression of *MdbHLH3* and *MdCyMDH* via the viral
vector-based transformation. The full-length cDNA of *MdbHLH3* gene was cloned into the IL60 vector for overexpression, whereas the antisense cDNA
fragments of *MdbHLH3* and *MdCyMDH* genes were inserted into the TRV vector for suppression. The empty vectors were used as controls. (b) The qRT-PCR
analysis of the relative expression levels of *MdbHLH3* and *MdCyMDH* genes around the injection sites. (c) Malate content of the injected apple fruit peel in
(a). (d) Bright-field images and GFP-green fluorescent images in roots. The full-length cDNA or antisense cDNA fragments of *MdbHLH3* and *MdCyMDH*
genes were cloned into the PRI vector for overexpression or suppression. The empty vectors were used as controls. Bars = 100 μm. (e) The qRT-PCR
analysis of the relative expression levels of *MdbHLH3* and *MdCyMDH* genes in transgenic roots. (f) The content of malate in the transgenic roots. In (b), (c), (e) and
(f), 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. [Colour figure can be viewed at wileyonlinelibrary.com]
from the labelled leaves to the active parts of metabolism and growth (Figure 8f). $^{13}\text{C}$ pulse labelling data showed that $^{13}\text{C}$ was mainly incorporated into fruits at the apple maturity stage, and the relative $^{13}\text{C}$ enrichment of assimilates assigned to the $\text{MdbHLH3}$ transgenic line was significantly higher than WT (Figure S7). The results demonstrated that the transportation and distribution of carbohydrates to fruits increased in the $\text{MdbHLH3}$ transgenic line compared with 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 $\text{MdbHLH3}$ transgenic apples. As expected, the soluble sugar content was significantly increased in the $\text{MdbHLH3}$ transgenic line compared with WT (Figure 8g) which well justified the labelling data presented above.

We also found that two key genes involved in gluconeogenesis, $\text{MdFBP}$ and $\text{MdPEPCK}$, showed higher expression levels in the $\text{MdbHLH3}$ transgenic apple fruits (Figure S8), suggesting that the metabolic pathways might be influenced by the gluconeogenesis. Taken together, $\text{MdbHLH3}$ overexpression seemed to have enhanced transport of carbohydrates into fruits by affecting the source–sink relationship, thereby increasing sugar content in fruits.

### Discussion

Edible quality of fleshy fruits, especially the sugar-acid ratio, has clear impact on overall acceptance by consumers, which is also the main factor affecting its price and market competitiveness (Khan et al., 2013). Fruit quality is controlled by the concerted actions of environmental, developmental and metabolic signalling pathways, and transcription factors play an important role in these processes (Bastías et al., 2011; Hanson et al., 2008). Although the importance of transcription factors for fruit quality has long been suggested, the number of transcription factors identified to be involved in the regulation of fruit quality traits is limited.

### Jack of all trades: $\text{MdbHLH3}$ is a multifunctional regulator in apple

The bHLH transcription factor $\text{MdbHLH3}$ is a multifunctional transcription factor that affects anthocyanin accumulation,
vacuolar acidification and fruit ripening in response to environmental stimuli and developmental events (Hu et al., 2016b; Hu et al., 2019; Xie et al., 2012). Actually, the apple MdMYB1-MdbHLH3-MdTTG1 complex is an important regulatory machinery that modulates anthocyanin synthesis and malate accumulation (Hu et al., 2016). However, this malate regulation was achieved directly by MdMYB1-modulated downstream target genes such as vacuolar proton pumps MdVHAs and malate transporter MdtDT, but not by MdbHLH3. Actually, MdbHLH3 physically interacts with and transcriptionally activates the expression of MdMYB1 (Xie et al., 2012), but does not directly bind to the promoters of MdMYB1 downstream genes (Hu et al., 2016a), suggesting that MdbHLH3 enhances the expression of MdMYB1 downstream genes and malate accumulation in an indirect way. Here, it was found that MdbHLH3 controlled malate accumulation by directly regulating cytosolic malate dehydrogenase MdCyMDH at the early stage of fruit development; this MdbHLH3-modulated malate regulation was specific and did not require a MYB TF. At the fruit ripening period, it is likely that the accumulation of soluble sugar will further modify the taste of fruits from our data. In addition, MdbHLH3 transgenic fruits accumulate higher level of anthocyanin and malate content by regulating the synthesis and transport processes at the stage of fruit development; however, the co-regulation mechanism

Figure 7 MdbHLH3 overexpression alters chloroplast metabolism in the apple trees. (a) Twig and leaf phenotypes of the WT and transgenic apple trees. (b) The content of chlorophyll is measured from the WT and MdbHLH3 transgenic apple leaves. Changes in the (c) maximum quantum yield of PSII (Fv/Fm), (d) photosynthetic electron transport rate (ETR), (e) PSII excitation pressure (1-qP), (f) intercellular CO2 concentration, (g) photosynthetic rate and (h) stomatal conductance in the leaves of the WT and MdbHLH3 transgenic apple trees. In (b–h), 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. [Colour figure can be viewed at wileyonlinelibrary.com]
between the anthocyanin and malate is not clear. MdbHLH3 is a key regulator of photosynthesis and the source–sink relationship that regulates the accumulation and distribution of carbohydrates. Nevertheless, our results demonstrated that MdbHLH3 plays a crucial role in regulating the quality formation during fruit development.

MdbHLH3 participates in a wider spectrum of metabolic processes by interfacing with TCA cycle

Malic acid, in the form of its anion malate, is present in all metabolizing cells. In addition to being a fruit quality component, malate can also be imported into tricarboxylic acid (TCA) cycle as

Figure 8 MdbHLH3 overexpression increases sugar content in apple fruits. (a) The FBP (fructose-1,6-bisphosphatase) activity is measured in the WT and MdbHLH3 transgenic apple leaves. (b) The SPS (sucrose phosphate synthase) activity is measured in the WT and MdbHLH3 transgenic apple leaves. (c) The content of sucrose is measured in the WT and MdbHLH3 transgenic apple leaves. (d) The content of starch is measured in the WT and MdbHLH3 transgenic apple leaves. (e) The content of sorbitol is measured in the WT and MdbHLH3 transgenic apple leaves. (f) The content of absorbing 13C is measured in the WT and MdbHLH3 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 day after blooming (DAB). (a–e) The mature leaves are measured after 120 DAB from the WT and MdbHLH3 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. [Colour figure can be viewed at wileyonlinelibrary.com]
a respiratory substrate (Noguchi and Yoshida, 2008; Sweetman et al., 2009), it functions as a key intermediate in TCA cycle in mitochondria, and it is also a component of the cytosolic pyruvate metabolism. The equilibrium position favours malate production (Figure 1e), and more malate may underlie the enhanced TCA cycle-related gene expression in MdbHLH3 transgenic apple (Figure 6d). On the other hand, in the light, MdcmYDH is thought to be to facilitate the synthesis of malate from oxaloacetic acid (OAA) (Hanning and Heldt, 1993; Yao et al., 2011).

MdbHLH3 may participate in the metabolic processes of the TCA cycle as well as its interactions with photosynthesis (Nunes-Nesi et al., 2011; Nunes-Nesi et al., 2007), photosynthesis (Bauwe et al., 2010) and nitrate assimilation (Foyer et al., 2011) via regulating MdcyMDH. Hence, it may also be related to a redox perturbation. At the same time, the TCA cycle is clearly involved in a wider metabolic network by regulating the level of accumulation of metabolic intermediates, and allows TCA cycle activity to contribute to other metabolic processes, such as glycolysis, gluconeogenesis, flavonoids and fatty acid metabolism (Araujo et al., 2012). MdbHLH3 participates in a wider spectrum of metabolic processes by interfacing with TCA cycle.

MdbHLH3 plays a key role in the crosstalk process between malate and soluble sugar metabolisms

On the other hand, during the fruit development of apples, both sorbitol and sucrose are synthesized in leaves and utilized in sink organs (mainly apple fruits). MdbHLH3 overexpression enhances photosynthesis of apple fruit trees to increase the content of carbohydrates such as sorbitol, sucrose and starch (Figure 8c-e). Sorbitol accounts for about 70% of all photosynthates produced in apple leaves (Bielecki, 1969; Cheng et al., 2005). In fruits, sorbitol is mainly converted to fructose (Yamauchi et al., 1994), which feeds into the sucrose–sucrose cycle (Li et al., 2012) or the ‘futile sucrose recyclers’ (Nguyen-Quoc and Foyer, 2001). Almost all sorbitol is converted to fructose in this manner. Interestingly, the content of sucrose in MdbHLH3 transgenic apple leaves is abnormally high and is positively correlated with the expression level of MdbHLH3 (Figure 8c). Therefore, there may be some connections between the sucrose content and the MdbHLH3 expression level. It is estimated that at least 80% of the total carbon flux goes to soluble sugars in apple fruit (Li et al., 2012). However, the mechanism by which the carbon flux is directed towards sugar accumulation or glycolysis/TCA cycle during fruit development is still not completely understood.

In MdbHLH3 overexpression apple fruits of 60 DAB, malate content was dramatically boosted, accompanied by the enhanced content of soluble sugars (Figures 1e and 8f), suggesting that MdbHLH3 is involved in malate synthesis and has a possible role in the interconversion of malate to sugars. In addition, altered expression levels of MdFBP and MdPEPC (two key genes involved in gluconeogenesis) in MdbHLH3 transgenic apple trees suggested that MdbHLH3 was involved in the regulation of gluconeogenesis (Figure S8). MdbHLH3 overexpression up-regulated the transcription of MdcyMDH, the content of malate and the MDH activities (Figures 5 and 6). Yao et al. (2011) reported that overexpression of cyMDH (cytosolic NAD-dependent malate dehydrogenase) increased the activities of cytosolic malic enzyme and other enzymes involved in the primary carbon metabolism in the transgenic apple calluses. In addition, transcript levels of malate-vacuolar-related transporters including MdVHA-B1, MdVHA-B2, MdVHA-E2, MdVHP1 and MdtDT were significantly increased in the MdbHLH3-overexpressing apple plantlets (Figure S3). Taken together, these results suggested the existence of crosstalk between malate and soluble sugar metabolisms during the growth and development of apples, and MdbHLH3 plays a key role in this process.

The content of organic acids and soluble sugars directly affects the taste of the fleshy fruits (Khan et al., 2013). In addition, obtaining a moderate sugar-to-acid ratio is the main goal of breeding programmes for these edible fruits. Elucidating the mechanisms involved in the accumulation and conversion of the organic acids and the soluble sugars is a key step to understanding the fruit developmental process. Our study found that an apple bHLH transcription factor MdbHLH3 activates the transcription of MdcyMDH and promotes the synthesis and metabolism of malate, which enhances the accumulation and allocation of carbohydrates from source to sink, and the accumulation of soluble sugars. These results provided new insights into the roles MdbHLH3 play during the regulation of malate and soluble sugar accumulation and in apple fruit development. As a key regulator, MdbHLH3 linked the fruit developmental process to the metabolism of malate and soluble sugars. This mechanism also provides a potential biotechnological strategy in creating new apple varieties with improved taste and flavour. We summarized our findings and hypothesis on the function of MdbHLH3 in the regulation of fruit quality into a working model (Figure 9). We found that an apple bHLH transcription factor MdbHLH3 increases the content of malate by activating the transcription of MdcyMDH, and enhances the conversion and transportation of carbohydrates from leaves to fruits, which promotes the accumulation of soluble sugars in the fruits.

Materials and Methods

Plant materials, growth conditions and treatments

‘Gala’ apple in vitro shoot cultures were grown on MS (Murashige and Skoog) solid medium containing 0.2 mg/l IAA (indole-3-acetic acid) and 0.8 mg/l 6-BA (6-benzylaminopurine) agar at 25 °C under a 16-h photoperiod.

‘Orin’ apple (Malus domestica Borkh.) calli were induced from young embryos. They were subcultured three times at 3-week intervals on MS medium plus 1.5 mg/l IAA, 2.4-D (2,4-dichlorophenoxyacetic acid) and 0.4 mg/l 6-BA at 25°C in the dark before being used for further studies.

To obtain MdbHLH3 transgenic and WT apple fruits, the MdbHLH3-27 (3 trees), MdbHLH3-36 (2 trees), MdbHLH3-44 (4 trees) transgenic and WT (5 trees) apple trees were grown in the field at an experimental farm at April 2011, until they bore fruits at the end of August 2016. These trees were covered with 10-μm nylon fabric to prevent the spread of pollen, which could avoid genetic contamination for other trees. Fruits collected 10, 20, 30, 60, 90 and 120 days after blooming were used for further investigations. At least 10 apple fruits were collected from each tree to eliminate genetic variations. 90 to 120 days after blooming, WT and MdbHLH3 transgenic apple trees were used for the MDH activity, photosynthesis, chlorophyll fluorescence and enzyme activity assays.

RNA extraction, RT-PCR and qRT-PCR

The extraction of total RNA was performed using the RNeasy Plant Miniprep Kit (TIANGEN, Beijing, China). Then, RNA was converted into ss cDNA using digestion by DNase I and M-MLV
reverse transcriptase (TaKaRa, Dalian, China). RT-PCR and qRT-PCR were performed as previously described by Hu et al. (2019).

Construction of the plasmid and genetic transformation
To construct MdbHLH3- and MdcyMDH-overexpressing and antisense suppressing vectors, the full-length cDNA and antisense partial 5'-UTR sequences of MdbHLH3 and MdcyMDH were isolated from ‘Gala’ apple using RT-PCR. The resulting PCR products were inserted into the pCXSN, pCXSN::GFP or pCXSN::Myc vectors under the control of the 35S promoter. The genetic transformation was performed with methods described by Xie et al. (2012). All primers used in this study are listed in Table S1.

Malate and soluble sugar assays
Malate and soluble sugar contents 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., 2016a).

RNA-Seq analysis
Total RNAs were extracted from apple fruits of WT or MdbHLH3 transgenic fruit trees at 60 DAB. RNA-Seq assays were performed with the methods as described by Hu et al. (2019).

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, and 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 promoter 4 region fragments were ligated into the pHIS2 vector (Clontech). 3-AT (3-amino-1,2,4-triazole) was used for screening. The yeast one-hybrid assay was conducted as previously described (Li et al., ).

The EMSA was conducted as previously described (Hu et al., 2019). The CDSs of MdbHLH3 were 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 LightShift 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 unlabelled probes were used for probe competition (Hu et al., 2019).

Transient dual-luciferase assays
Transient expression assays were performed in tobacco (Nicotiana tabacum) leaves (An et al., 2018a). The promoter fragment of MdcyMDH was cloned into the pGreenII 0800-LUC vector 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 the recombinant plasmids. The infiltration was performed as previously described (Xie et al., 2012). A living imaging apparatus was used for luminescence measurement.

GUS detection
Reporter construct containing the promoter sequences of MdcyMDH was prepared as previously described (An et al., 2018b; Hu et al., 2017).
Construction of viral vectors and transient expression in apple fruit

The full-length cDNA and antisense partial sequences of MdbhHLH3 and MdcyMDH were isolated from ‘Gala’ apple using RT-PCR. The resulting PCR products of antisense partial sequences of MdbhHLH3 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-MdbhHLH3 and TRV-MdcyMDH, respectively. The resulting PCR products of full-length cDNA of MdbhHLH3 and MdcyMDH were cloned into the IL-60 vector under the control of the 35S promoter. The vectors were named IL60-MdbhHLH3 and IL60-MdcyMDH, respectively. The resulting vector was 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 2 days and then kept in the highlight at 10°C for 1 week before collecting the injected part for gene expression analysis and malate content determination.

Agrobacterium rhizogenes MSU440 transformation

The antisense MdbhHLH3 (anti-MdbhHLH3) or antisense MdcyMDH (anti-MdcyMDH) vector was transformed into Agrobacterium rhizogenes (MSU440) and used for root infection as described previously (Sun et al., 2018). The green fluorescent protein expressed in the resulting MSU440 was used as a marker for transformation screen.

MDH activity and NADH/NAD+ determination

MDH activity assays were performed with the methods as described by Wang et al. (2016a). The MDH reductive activity was measured in 1 mL of a reaction mixture containing 50 mM Tris-HCl (pH 7.8), 2 mM MgCl2, 0.5 mM EDTA, 0.2 mM NADH and 2 mM OAA. The MDH oxidative activity was measured in 1 mL of a reaction mixture containing 50 mM Tris-HCl (pH 8.9), 2 mM MgCl2, 0.5 mM EDTA, 0.2 mM NAD and 25 mM malate. The NADH/NAD+ was measured according to the method of Coenzyrne I NAD(H) Content Kit (KeMing, Suzhou, China).

Measurement of chlorophyll content, photosynthesis and chlorophyll fluorescence

Chlorophyll content was measured with the methods as described by Zhu et al. (1990). Photosynthesis and chlorophyll fluorescence were measured using a portable photosynthesis modulated fluorometer (Hansatech, Norfolk, UK), respectively. Photosynthesis and chlorophyll fluorescence that were measured at least three times for and at least nine leaves for each apple tree were tested. Measurement of photosynthesis was made between 8:30 and 11:00 AM on a sunny day. CO2 concentration was maintained at 396 ± 21 µL/L, the air temperature was approximately 25 °C and the relative humidity was 85 ± 0.9%. For chlorophyll fluorescence measurements, the steady-state fluorescence (Fs) was recorded after 2 min of continuous illumination by using light at 100 µW/m²/s from the FMS-2 pulse light source, the maximum fluorescence in the light-adapted state (Fm) was obtained by imposing 0.8 s of saturating light of 8000 µW/m²/s, and the minimum fluorescence in the light-adapted state (F0) was determined by illuminating 3 s with far-red light after turning off the actinic light. The related parameters were calculated with the methods as described by Wang et al. (2016a).

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).

13C pulse labelling

13C pulse labelling was carried out in a gas-tight labelling chamber made with transparent agricultural film at the apple maturity stage (August 10). First, the seal of the labelling chamber was checked, and then one gram of Ba13CO3 (13C abundance is 98%, the proportion of 13C in all carbon elements) was put into a beaker and iron powder was reduced into the labelling room. 13C labelling started at 8:00 am (August 10) with the certain amount of HCl (1 mol/L) injected into the beaker with Ba13CO3 using a syringe. HCl was injected into the beaker every 30 min for a duration of 4h in order to maintain the concentration of CO2. At the same time, another group of unlabelled control plants was used as a blank of 13C labelling (natural abundance of 13C).

The plants were destructively sampled after 72 h (at 8:00 am on August 13). 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 °C, followed with homogenization by electric grinder and filtration with 0.25-mm mesh screen. The abundance of 13C was measured with DELTA V plus XP advantage isotope ratio mass spectrometer analysed by the 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 G_RAPH_PAO Prism 6.02 software (**, P < 0.05; ***, P < 0.01).

Acknowledgements

This work was supported by grants from the National Key Research and Development Program of China (2018YFD1000200), National Natural Science Foundation of China (31972375, 31772288 and 31902049), the Ministry of Agriculture of China (CARS-28), Ministry of Education of China (IRT15R42), Shandong Province (SDAIT-06-03 and ZR2019QC006) and Nanjing Agricultural University (ZW201805).

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

H.Y.J. and H.D.G. conceived and designed the experiments. Y.J.Q., H.D.G., G.K.D., S.C.H., Z.Q.Y., W.J.H., M.F.F. and Y.C.X. performed the experiments. H.D.G. and Y.J.Q. wrote the article.

References

An, X.H., Tian, Y., Chen, K.Q., Liu, X.J., Liu, D.D., Xie, X.B. and Hao, Y.J. (2015) MdMYB9 and MdMYB11 are involved in the regulation of the JA-induced...
biosynthesis of anthocyanin and proanthocyanidin in apples. Plant Cell Physiol. 56, 650–662.

An, J.P., Yao, J.F., Xu, R.R., You, C.X., Wang, X.F. and Hao, Y.J. (2018a) An apple NAC transcription factor enhances salt stress tolerance by modulating the ethylene response. Physiol. Plantarum. 164, 279–289.

An, J.P., Wang, X.F., Li, Y.Y., Song, L.Q., Zhao, L.L., You, C.X. and Hao, Y.J. (2018b) EIN3-LIKE1, MYB1, and ETHYLENE RESPONSE FACTOR3 act in a regulatory loop that synergistically modulates ethylene biosynthesis and anthocyanin accumulation. Plant Physiol. 178, 808–823.

Araujo, W.L., Nunes-nesi, A., Nikolosi, Z., Sweetlove, L.J. and Fernie, A.R. (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant Cell Environ. 35, 1–21.

Bahrami, A.R., Chen, Z.H., Walker, R.P., Leegood, R.C. and Gray, J.E. (2001) Ripening-related occurrence of phosphoenolpyruvate carboxykinase in tomato fruit. Plant Mol. Biol. 47, 499–506.

Bai, Y., Dougherty, L., Li, M.J., Fazio, G., Cheng, L.L. and Xu, K.N. (2012) A natural mutation-led truncation in one of the two aluminum-activated malate transporter-like genes at the Ma locus is associated with low fruit acidity in apple. Mol. Genet. Genom. 287, 663–678.

Baslas, A., Lopez-Climent, M., Valcarcel, M., Rosello, S., Gomez-Cadenas, A. and Casariego, J.A. (2011) Modulation of organic acids and sugar content in tomato fruits by an abscisic acid-regulated transcription factor. Physiol. Plantarum. 141, 215–226.

Bauwe, H., Hagemann, M. and Fernie, A.R. (2010) Photospiration: players, partners and origin. Trends Plant Sci. 15, 330–336.

Berütter, J. (2004) Carbohydrate metabolism in two apple genotypes that differ in malate accumulation. J. Plant Physiol. 161, 1011–1029.

Bielecki, R.L. (1969) Accumulation and translocation of sorbitol in apple phloem. Aust. J. Bot. 22, 611–620.

Butelli, E., Licciodello, C., Ramadugu, C., Durand-Hulak, M., Celant, A., Recupero, G.R., Froelicher, Y. et al. (2019) Noemi controls production of flavonoid pigments and fruit acidity and illustrates the domestication routes of modern citrus varieties. Plant Physiol. 177, 158–164.

Cheng, L., Zhou, R., Reidel, E.J., Xiao, X.B., Starbuck, M.J. and O'Leary, M.H. (1996) Phospho enol pyruvate carboxykinase in spinach (Spinacia oleracea L.) leaves partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. Plant Physiol. 103, 1147–1154.

Hanson, J., Hansen, M., Wiese, A., Hendriks, M.M. and Smeekens, S. (2008) The sucrose regulated translation factor bZIP11 affects amino acid metabolism by regulating the expression of ASPARAGINE SYNTHETASE1 and PROLINE DEHYDROGENASE2. Plant J. 53, 935–949.

Hu, D.G., Sun, C.H., Ma, Q.J., You, C.X., Cheng, L.L. and Hao, Y.J. (2016a) MdMYB1 regulates anthocyanin and malate accumulation by directly facilitating their transport into vacuoles in apples. Plant Physiol. 170, 1315–1330.

Hu, D.G., Sun, C.H., Zhang, Q.Y., An, J.P., You, C.X. and Hao, Y.J. (2016b) Glucose sensor MdhxK1 phosphorylates and stabilizes MdHLEH3 to promote anthocyanin biosynthesis in apple. PLoS Genet. 12, e1006273.

Hu, D.G., Li, Y.Y., Zhang, Q.Y., Li, M., Sun, C.H., Yu, J.Q. and Hao, Y.J. (2017) R2R3-MYB transcription factor MdMYB73 is involved in malate accumulation and vacuolar acidification in apple. Plant J. 91, 443–454.

Hu, D.G., Yu, J.Q., Han, P.L., Xie, X.B., Sun, C.H., Zhang, Q.Y. and Hao, Y.J. (2019) The regulatory module MdIPU829-MdhbHLH3 connects ethylene biosynthesis with fruit quality in apple. New Phytol. 221, 1566–1982.

Hu, D.G., Sun, C.H., Zhang, Q.Y., Gu, K.D. and Hao, Y.J. (2020) The basic helix-loop-helix transcription factor MdHLEH3 modulates leaf senescence in apple via the regulation of dehydratase-enolase-phosphate complex 1. J. Exp. Bot. 7, 1–6.

Khan, S.A., Beekwilder, J., Schaart, J.G., Mumm, R., Soriano, J.M., Jacobsen, E. and Schouten, H.J. (2013) Differences in acidity of apples are probably mainly caused by a malic acid transporter gene on LG16. Tree Genomes. 9, 475–487.

Lee, K.W., Kim, Y.J., Kim, D.O., Lee, H.J. and Lee, C.Y. (2003) Major phenolics in apple and their contribution to the total antioxidant capacity. J. Agr. Food Chem. 51, 6516–6520.

Lee, M., Choi, Y., Birla, B., Kim, Y.Y., Jeon, B., Maeshiba, M., Yoo, J.-Y. et al. (2008) The ABC transporter AtABC14 is a maltose importer and modulates stomatal response to CO2. Nat. Cell Biol. 10, 1217–1223.

Li, F., Lei, H., Zhao, X., Tian, R. and Li, T. (2012) Characterization of three sorbitol transporter genes in micropropagated apple plants grown under drought stress. Plant Mol. Biol. Rep. 30, 123–130.

Li, S., Wang, X., He, S., Li, J., Huang, Q., Imaizumi, T. and Gu, H. (2016) CFLAP1 and CFLAP2 are two bHLH transcription factors participating in synergistic regulation of ACF1L-mediated cuticle development in Arabidopsis. PLoS Genet. 12, e1005744.

Martinoa, E. and Rentsch, D. (1994) Malate compartmentation-responses to a complex metabolism. Annu. Rev. Plant Biol. 45, 447–467.

Mathieu, Y., Guern, J., Pean, M., Pasquier, C., Beloeil, J.C. and Lallamand, J.Y. (1986) Cytoplasmic pH regulation in Acer pseudoplantus cells. II. Possible mechanisms involved in pH regulation during acid load. Plant Physiol. 82, 846–852.

Miller, S.S., Driscoll, B.T., Gregerson, R.G., Ganitt, J.S. and Vance, C.P. (1998) Alfaifa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH. Plant J. 15, 173–184.

Nguyen-Quoc, B. and Foyer, C.H. (2001) A role for ‘futile cycles’ involving inverte and sucrose synthesize in sucrose metabolism of tomato fruit. J. Exp. Bot. 52, 881–889.

Noguchi, K. and Yoshida, K. (2008) Interaction between photosynthesis and respiration in illuminated leaves. Mitochondrion. 8, 87–99.

Nour, V., Trandafir, I. and Ionica, M.E. (2010) Compositional characteristics of fruits of several apple (Malus domestica Borkh.) cultivars. Not. Bot. Horti. Agrobot. 38, 228–233.

Nunes-Nesi, A., Sweetlove, L.J. and Fernie, A.R. (2007) Operation and function of the tricarboxylic acid cycle in the illuminated leaf. Physiol. Plantarum. 129, 45–56.

Nunes-Nesi, A., Araujo, W.L. and Fernie, A.R. (2011) Targeting mitochondrial metabolism and machinery as a means to enhance photosynthesis. Plant Physiol. 155, 101–107.

Ramsay, N.A. and Glover, B.J. (2005) MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci. 10, 63–70.

Rouphael, Y., Schwartz, D., Krumbein, A. and Colla, G. (2010) Impact of grafting on product quality of fruit vegetables. Sci. Hortic. 127, 172–179.
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The anthocyanin contents and the expression levels of MdbHLH3 and its downstream anthocyanin-associated genes (including MdbMYB1, MdbANS, MdbANR, MdbUFGT, and etc.) in WT and those three MdbHLH3 transgenic lines.

Figure S2 The ratio of TSS and TA in WT and MdbHLH3 transgenic apple fruits.

Figure S3 Relative expressions of malate-vacuolar related transporters in WT and those three MdbHLH3-over-expression apple plantlets.

Figure S4 Promoter sequence analysis for MdbHLH3 binding motifs in the malate-associated genes.

Figure S5 The luciferase assays showing that MdbHLH3 activates the expression of MdcyMDpro::Luc that is not dependent on MdbMYB1 and MdbMYB73 in the representative images of Nicotiana benthamiana leaves by transient infiltration.

Figure S6 Induced regeneration of the hairy roots by Agrobacterium rhizogenes MSU440 in WT line, which was used as a negative control.

Figure S7 The relative content of 13C in WT and MdbHLH3 transgenic apple fruits. MdbHLH3-27, MdbHLH3-36 and MdbHLH3-44 are three transgenic apple lines.

Figure S8 The relative expression levels of MdbFBP and MdbPEPCK (two key genes involved in gluconeogenesis) genes in three MdbHLH3 transgenic lines compared to WT.

Table S1 The primers used for RT-PCR and qRT-PCR in this study.

DataSet S1 Annotation of all unigenes in WT and MdbHLH3-27 transgenic apple fruits.

DataSet S2 Annotation of all unigenes in WT and MdbHLH3-36 transgenic apple fruits.

DataSet S3 Annotation of all unigenes in WT and MdbHLH3-44 transgenic apple fruits.