1 **Suggested running title:**

2 MdBT2 modulates malate accumulation in response to nitrate

3

4 **The Title:**

5 BTB-TAZ domain protein MdBT2 modulates malate accumulation by targeting a
6 bHLH transcription factor for degradation in response to nitrate

7

8 **The full names of all the authors:**

9 Quan-Yan Zhang¹, Kai-Di Gu¹, Lailiang Cheng², Jia-Hui Wang¹, Jian-Qiang Yu¹,
10 Xiao-Fei Wang¹, Chun-Xiang You¹, Da-Gang Hu^{1,2*}, Yu-Jin Hao^{1*}

11

12 **The names and address of the institution:**

13 ¹National Key Laboratory of Crop Biology, Shandong Collaborative Innovation
14 Center of Fruit & Vegetable Quality and Efficient Production, College of Horticulture
15 Science and Engineering, Shandong Agricultural University, Tai-An, Shandong
16 271018, China

17 ²Department of Horticulture, Cornell University, 134A Plant Science, Ithaca, NY
18 14853, USA

19

20 **Corresponding authors:**

21 Yu-Jin Hao (haoyujin@sdau.edu.cn), Da-Gang Hu (fap_296566@163.com)

22 Tel: +86-538-824-6692

23 Fax number: +86-538-824-2364

24 Address: College of Horticulture Science and Engineering, Shandong Agricultural
25 University, Tai-An, Shandong 271018, China

26

27 **Keywords:**

28 Apple; Nitrate; *MdBT2*; *MdCibHLH1*; Malate accumulation

29

BTB-TAZ domain protein MdbT2 modulates malate accumulation by targeting a bHLH transcription factor for degradation in response to nitrate

Quan-Yan Zhang¹, Kai-Di Gu¹, Lailiang Cheng², Jia-Hui Wang¹, Jian-Qiang Yu¹, Xiao-Fei Wang¹, Chun-Xiang You¹, Da-Gang Hu^{1,2*}, Yu-Jin Hao^{1*}

¹National Key Laboratory of Crop Biology, Shandong Collaborative Innovation Center of Fruit & Vegetable Quality and Efficient Production, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai-An, Shandong 271018, China

²Department of Horticulture, Cornell University, 134A Plant Science, Ithaca, NY 14853, USA

Corresponding authors: Yu-Jin Hao (haoyujin@sdaa.edu.cn), Da-Gang Hu (fap_296566@163.com)

Abstract

Excessive application of nitrate, an essential macronutrient and a signal regulating diverse physiological processes, decreases malate accumulation in apple fruit, but the underlying mechanism remains poorly understood. Here, we show that an apple BTB/TAZ protein MdbT2 is involved in regulating malate accumulation and vacuolar pH in response to nitrate. *In vitro* and *in vivo* assays indicate that MdbT2 interacts directly with and ubiquitinates a bHLH transcription factor, MdCibHLH1, via the ubiquitin/26S proteasome pathway in response to nitrate. This ubiquitination results in the degradation of MdCibHLH1 protein and reduces the transcription of MdCibHLH1-targeted genes involved in malate accumulation and vacuolar acidification including *MdVHA-A* encoding a vacuolar H⁺-ATPase gene, and *MdVHPI* encoding a vacuolar H⁺-pyrophosphatase gene, as well as *MdALMT9* encoding a aluminum-activated malate transporter gene. A series of transgenic analyses in apple materials including fruits, plantlets and calli demonstrate that MdbT2 controls nitrate-mediated malate accumulation and vacuolar pH at least partially, if not completely, via regulating the MdCibHLH1 protein level. Taken together, these findings reveal that MdbT2 regulates the stability of MdCibHLH1 via

ubiquitination in response to nitrate, which in succession transcriptionally reduces the expression of malate-associated genes, thereby controlling malate accumulation and vacuolar acidification in apples under high nitrate supply.

Introduction

Malate, as a key metabolite, plays a vital role in plant metabolism, pH homeostasis, nutrient uptake, osmotic adjustment and abiotic stress resistance (Fernie and Martinoia, 2009; Finkemeier and Sweetlove, 2009; Bai et al., 2015; Hu et al., 2017). Cellular malate accumulation also largely determines the acidity and perception of sweetness of fleshy fruits and their processed products (Yao et al., 2007; Ye et al., 2017; Butelli et al., 2019; Ma et al., 2019). The majority of malate in the parenchyma cells of fleshy fruits is in the vacuole (Yamaki, 1984). Malate accumulation is a complex process involving synthesis, degradation and transport of malate. Although malate metabolism can alter fruit malate level (Sweetman et al., 2009; Centeno et al., 2011), it appears that transport of malate from the cytosol into the vacuole is the step that largely controls malate accumulation (Etienne et al., 2013; Hu et al., 2016a; Ma et al., 2019).

Vacuolar proton pumps and malate transporters are crucial for malate transport into the vacuole. Vacuolar proton pumps, such as V-ATPase and V-PPase, acidify the vacuoles by pumping protons across the tonoplast. The resulting low pH protonates any malate that crosses the tonoplast from the cytosol, effectively trapping malate in the acid form. This “acid trap” mechanism maintains the malate concentration gradient across the tonoplast for its facilitated diffusion (Martinoia et al., 2007; Etienne et al., 2013; Eisenach et al., 2014; Hu et al., 2016a). Aluminum-activated malate transporter/channel (ALMT) as well as tonoplast dicarboxylate transporter (tDT) enables the transport of malate across the tonoplast (Kovermann et al., 2007; Ma et al., 2015; Ye et al., 2017). *ALMT9* underlies a major malic acid locus, *Ma* in apple (Bai et al., 2012; Khan et al., 2013) and a major QTL for malate level in tomato (Jie et al., 2017).

Upstream of these proton pumps and transporters are various transcription factors (TFs) that regulate their expression. For example, SIWRKY42 directly binds and affects malate transporter SIALMT9 to negatively regulate malate content in tomato fruit (Ye et al., 2017). Furthermore, the MYB TFs together with bHLH TFs and WD40 proteins form MBW complexes to transcriptionally activate the expression

of vacuolar proton pumps and malate transporter genes, thereby promoting malate accumulation in the vacuole (Xie et al., 2012; Hu et al., 2016a, 2017). In apple, MdMYB73 binds directly to the promoters of *MdVHA-A*, *MdVHP1* and *MdALMT9* to regulate malate accumulation (Hu et al., 2017). MdCibHLH1, a bHLH transcription factor homologous to *Arabidopsis* ICE1, interacts with MdMYB73 and enhances its effects on downstream target genes to regulate malate accumulation in apple (Feng et al., 2012; Hu et al., 2017).

Malate accumulation in fleshy fruits is also affected by environmental factors and management practices such as nutrient availability, salinity, temperature, and irrigation (Wu et al., 2002; Burdon et al., 2007; Thakur and Singh, 2012; Etienne et al., 2013). In response to environmental stresses such as low temperature and salinity, plants accumulate malate as a metabolite in coping with these stresses, consequently altering fruit acidity level (Ruffner et al., 1976; Friemert et al., 1988; Hu et al., 2016b). Nitrate is an essential macronutrient for plant growth and development, and also serves as a signal regulating many physiological processes (Liu et al., 2017; Maeda et al., 2018). High nitrate supply has been reported to reduce malate accumulation (Spironello et al., 2004), but how nitrate affects the malate accumulation remains poorly understood.

The BTB-TAZ proteins respond to diverse environmental stimuli such as nutrients and stress and internal signals such as hormones in plants (Mandadi et al., 2009). We recently found that BT2 as a BTB-TAZ protein responds to nitrate in modulating anthocyanin synthesis and accumulation in apple (Wang et al., 2018). In this work, we showed that MdBT2 protein regulates malate accumulation in response to nitrate. A yeast two-hybrid screening library allowed for identification of MdCibHLH1 as a candidate protein for MdBT2 interaction. Subsequently, we uncovered that MdBT2 regulates the stability of MdCibHLH1 via ubiquitination in response to nitrate, which in succession transcriptionally reduces the expression of several malate-associated genes in altering malate accumulation in apple.

127 **Results**

128 **Exogenous application of nitrate reduces malate accumulation**

129 To determine if nitrate has any effect on malate accumulation, we provided apple
130 calli and plantlets with different nitrate concentrations (0 to 5 mM) and measured
131 malate contents in various tissues first. High concentrations of nitrate were found to
132 significantly reduce tissue malate accumulation (Supplemental Fig. S1).

133 To confirm the effect of nitrate on malate accumulation in fruits, we treated
134 ‘Royal Gala’ apple fruits with exogenous nitrate from 0 to 5 mM for further analysis
135 (Fig. 1A). Nitrate application led to a significant reduction in fruit malate level (Fig.
136 1C). The expression levels of malate-related genes, including vacuolar proton pumps
137 *MdVHA-A*, *MdVHA-Bs* and *MdVHP1*, and the vacuolar malate channel *MdALMT9* as
138 well as malate-associated MYB TF *MdMYB73*, were significantly decreased when
139 nitrate concentration increased from 0 to 5 mM (Fig. 1B). Subsequently, we measured
140 the hydrolytic and proton-pumping activities of V-ATPase and V-PPase activity.
141 V-ATPase hydrolytic activity and proton-pumping activity and V-PPase activity were
142 decreased to 1/2, 2/3, and 1/3 of the 0 mM N control, respectively, in the 5 mM N
143 treatment (Fig. 1, D-F). Furthermore, measurements of vacuolar pH with the
144 ratiometric fluorescent pH indicator
145 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF) showed that the
146 average vacuolar pH was increased from 3.6 in 0 mM N to 3.7 and 4.1, respectively,
147 in the 0.5 mM and 5 mM N treatments (Fig. 1, G-H). These results suggest that high
148 nitrate level reduces vacuolar acidification by decreasing the proton-pumping
149 activities of both V-ATPase and V-PPase in apple.

151 **BTB/TAZ protein MdBT2 controls malate accumulation in response to nitrate**

152 Considering that BT2 as a BTB-TAZ protein responds to nitrate in plants
153 (Mandadi et al., 2009; Wang et al., 2018), we explored the potential role of BT2 in
154 nitrate-modulated malate accumulation. We first looked at the expression of *MdBT2*
155 in response to nitrate and found that nitrate application increased its transcript level
156 (Supplemental Fig. S2). We then generated 35S::*anti-MdBT2* RNAi transgenic apple
157 calli (35S::*anti-MdBT2*) (Supplemental Fig. S2), and detected higher malate levels in
158 the *anti-MdBT2* calli.

159 To confirm the role of MdBT2 in malate accumulation in response to nitrate,
160 transgenic apple plantlets overexpressing *MdBT2* (MdBT2-OX) were treated with 0

Figure 1. Exogenous application of nitrate reduces malate accumulation. **A**, The ‘Royal Gala’ apple fruits were treated with different concentrations of exogenous nitrate (including 0 mM, 0.5 mM and 5 mM nitrate). **B**, The expression of malate-related genes under different concentrations of exogenous nitrate using qPCR. The genes included about vacuolar proton pumps *MdVHA-A*, *MdVHA-Bs* and *MdVHP1*, and the vacuolar malate channel *MdALMT9* as well as malate-associated MYB TF *MdMYB73*. **C**, Malate contents under different concentrations of exogenous nitrate. **D**, Bafilomycin A1-sensitive ATP hydrolytic activity of V-ATPase under different concentrations of exogenous nitrate. **E**, Proton-pumping activity of V-ATPase under different concentrations of exogenous nitrate was measured by tracking the ATP-dependent quenching of acridine orange using a fluorescence spectrometer with excitation at 493 nm and emission at 545 nm. **F**, V-PPase activity under different concentrations of exogenous nitrate. **G**, Emission intensities of protoplast vacuoles under different concentrations of exogenous nitrate loaded with BCECF at 488 nm (first column) and 458 nm (second column). Scale bar = 10 μ m. **H**, Quantification of the pH in vacuoles under different concentrations of exogenous nitrate. Significant difference was detected by *t*-test. * $P < 0.05$, ** $P < 0.01$.

161 mM N (0 N-MdBT2-OX), while *antiMdBT2* transgenic apple plantlets (*antiMdBT2*)
 162 were treated with 5 mM N (5N-*antiMdBT2*), along with wild-type (WT) control
 163 under 0 mM or 5 mM nitrate. 5 mM nitrate decreased malate accumulation in WT
 164 plantlets; MdBT2-OX reduced the malate level at 0 mM nitrate whereas *antiMdBT2*

Figure 2. BTB/TAZ protein MdbT2 controls malate accumulation in response to nitrate. **A**, Malate contents in WT and transgenic apple plantlets under different concentrations of exogenous nitrate. ‘GL-3’ plantlets were treated with 0 N (0 N-WT). MdbT2 overexpression transgenic apple plantlets were treated with 0 N (0 N-MdbT2-OX). The antiMdbT2 transgenic apple plantlets were treated with 5 N (5 N-antiMdbT2). **B**, Expression of *MdVHA-A*, *MdVHA-Bs*, *MdVHP1* and *MdALMT9* in WT and transgenic apple plantlets under different concentrations of exogenous nitrate using qPCR. **C to E**, The hydrolytic and proton-pumping activities of V-ATPase and V-PPase in WT and transgenic apple plantlets under different concentrations of exogenous nitrate. **F**, Emission intensities of protoplast vacuoles loaded with BCECF at 488 nm and 458nm. Scale bar = 10 μ m. **G**, Quantification of the pH in vacuoles. Significant difference was detected by *t*-test. * $P < 0.05$, ** $P < 0.01$.

165 increased the malate level at 5 mM nitrate (Fig. 2A).

166 Correspondingly, 5 mM nitrate inhibited the expression of malate-associated
 167 genes including *MdVHA-A*, *MdVHA-Bs*, *MdVHP1* and *MdALMT9*, and V-ATPase
 168 hydrolytic and proton-pumping activities as well as V-PPase activity (Fig. 2, B-E);
 169 overexpression of *MdbT2* decreased the expression of these genes and activities of

related enzymes and increased vacuolar pH at 0 mM nitrate whereas antisense repression of *MdBT2* increased the expression of the genes and activities of related enzymes and decreased vacuolar pH at 5 mM nitrate (Fig. 2, B-G).

Taken together, these results demonstrate that BTB/TAZ protein *MdBT2* modulates malate accumulation in response to nitrate in apple.

175

176 ***MdBT2* physically interacts with *MdCibHLH1* via the conserved BTB/BACK** 177 **domain**

To explore the regulatory mechanism by which *MdBT2* is involved in malate accumulation in apple, possible *MdBT2*-interacting proteins were screened using *MdBT2* as a bait through a yeast two hybrid (Y2H) cDNA library. *MdCibHLH1*, a basic helix-loop-helix (bHLH) TF that was previously identified to be involved in malate accumulation and cold resistance, was chosen as a candidate. To determine whether *MdBT2* interacts with *MdCibHLH1* protein, Y2H assays were performed. As *MdBT2* protein contains three conserved domains, BTB, BACK and ZnF_TAZ, we divided the full-length cDNA of *MdBT2* gene into four fragments, i.e., *MdBT2*^{BTB/BACK}, and *MdBT2*^{BTB}, as well as *MdBT2*^{BACK} and *MdBT2*^{BACK/ZnF_TAZ}. Subsequently, the full length cDNA and four truncated mutants of *MdBT2* gene were inserted into the pGBT9 vector, independently, as the bait vectors. Meanwhile, the full-length *MdCibHLH1* cDNA was inserted into the pGAD424 as the prey vector. The different combinations of bait and prey vectors were transformed into yeast for Y2H assays. The full-length *MdBT2* interacted with the full-length *MdCibHLH1* protein (Fig. 3A). Moreover, *MdCibHLH1* interacted with the truncated mutant *MdBT2*^{BTB/BACK} but not with others (Fig. 3A). These results suggest that *MdBT2* *in vitro* interacts with *MdCibHLH1* via the conserved BTB/BACK domain. In addition, a GST pull-down assay showed that a GST-tagged *MdBT2* physically interacted with a His-tagged *MdCibHLH1* *in vitro* (Fig. 3B).

To further confirm the interaction between *MdBT2* and *MdCibHLH1*, an *in vivo* Bimolecular fluorescence complementation (BiFC) assay using tobacco leaf cells was conducted. *MdBT2* and *MdCibHLH1* were ligated to the N-terminal and C-terminal of YFP, respectively, to generate the constructs *MdBT2*-nYFP and *MdCibHLH1*-cYFP. Subsequently, these constructs including empty vectors with pairwise combinations were transiently transformed into tobacco leaf cells by *Agrobacterium*-mediated method. A strong YFP signal was observed in the nucleus of

Figure 3. MdBt2 physically interacts with MdCibHLH1 via the conserved BTB/BACK domain. **A**, MdBt2 interacted with MdCibHLH1 in a Y2H assay. The full-length and the BTB/BACK domain of MdBt2 interacted with the MdCibHLH1 protein. **B**, Pull-down assays of the interaction between MdBt2 and MdCibHLH1. MdBt2-GST was expressed by *E. coli* resulted in the precipitation of MdCibHLH1-HIS using anti-GST antibody and anti-HIS antibody, respectively. GST alone was used as the control. **C**, BiFC assay showed the interaction between MdBt2 and MdCibHLH1 using tobacco leaf cells. MdBt2-nYFP and MdCibHLH1-cYFP interacted in the nucleus of tobacco leaf cells. Scale bar = 10 μm.

tobacco leaf cells in the combination of MdBt2-nYFP and MdCibHLH1-cYFP constructs, with no signal detected in the other two combinations (MdBt2-nYFP+cYFP and nYFP+MdCibHLH1-cYFP) as negative controls (Fig. 3C). These results indicate that MdBt2 physically interacts with the MdCibHLH1 protein.

MdCibHLH1 is involved in regulating malate accumulation

It is known that MdCibHLH1 interacts with MdMYB73 to modulate malate accumulation by directly activating vacuolar transporter genes including *MdALMT9*, *MdVHA-A* and *MdVHPI* in apple (Hu et al., 2017). To further verify the functions of MdCibHLH1 in the regulation of malate accumulation in apple fruits, the *35S::MdCibHLH1* transgenic apple plantlets (MdCibHLH1-OXs) obtained by Feng et al. (2012) were grafted onto rootstock M9T337, and transgenic apple fruits were harvested at different stages during fruit development in the third growing season. Determination of malate contents revealed that fruits of the three *MdCibHLH1* transgenic lines (MdCibHLH1-OVXs) had much higher malate levels than the wild-type control ('GALA') throughout fruit development, particularly at 40 days after

Figure 4. *MdCibHLH1* is involved in regulating of malate accumulation. **A**, Malate contents in 'GALA' and *MdCibHLH1*-OVXs transgenic apple fruits (*MdCibHLH1*-OVXs) in the whole fruit developmental stages. **B**, The 'GALA' and *MdCibHLH1*-OVXs taken in 40 DAF. **C**, The expression of *MdVHA-A*, *MdVHA-B1*, *MdVHP1*, *MdALMT9* and *MdMYB73* in 'GALA' and *MdCibHLH1*-OVXs. **D** to **F**, The hydrolytic and proton-pumping activities of V-ATPase and V-PPase in 'GALA' and *MdCibHLH1*-OVXs. **G**, Emission intensities of protoplast vacuoles in 'GALA' and *MdCibHLH1*-OVXs with BCECF at 488 nm and 458 nm. Scale bar = 10 μ m. **H**, Quantification of the pH in vacuoles in 'GALA' and *MdCibHLH1*-OVXs. Significant difference was detected by *t*-test. *P < 0.05, **P < 0.01.

221 flowering (DAF) (Fig. 4A). Subsequently, 40 DAF apple fruits were used for further
222 analysis (Fig. 4B).

223 Transcript levels of *MdVHA-A*, *MdVHA-B1* and *MdVHP1*, as well as *MdALMT9*
224 and *MdMYB73*, were significantly higher in the three independent
225 *MdCibHLH1*-OVXs than in 'GALA' fruits control (Fig. 4C). V-ATPase and V-PPase
226 activities were 1.2- to 1.8-fold greater in *MdCibHLH1*-OVXs than in 'GALA' fruits
227 (Fig. 4, D-F). Furthermore, we used BCECF to illustrate the effects of V-ATPase and
228 V-PPase activities on vacuolar pH. As shown in Fig. 4, G-H, the average vacuolar pH
229 in 'GALA' fruits was 4.03 while those of the three *MdCibHLH1*-OVXs were 3.56,
230 3.68 and 3.84, respectively. Hence, higher vacuolar H⁺ concentrations in
231 *MdCibHLH1*-expressing fruits, indicated by the lower pH values, further supports the
232 conclusion that *MdCibHLH1* regulates malate accumulation by altering the tonoplast

233 V-ATPase and V-PPase activities.

234

235 **MdBT2 affects the stability of MdCibHLH1 protein**

236 Earlier work showed that ubiquitination-related MdBT2 scaffold protein targets
237 MYB and bHLH TFs for degradation, thereby regulating plant senescence and
238 anthocyanin biosynthesis (Wang et al., 2018; An et al., 2019). Considering the
239 interaction between MdBT2 and MdCibHLH1, we predicted that MdBT2 probably
240 affects the stability of MdCibHLH1 protein. To verify this, cell-free degradation
241 assays of the prokaryon-expressed and purified MdCibHLH1-HIS fusion proteins
242 were conducted using protein samples extracted from wild-type (WT), 35S::MdBT2
243 transgenic apple calli (35S::MdBT2) and 35S::anti-MdBT2. The MdCibHLH1-HIS
244 protein was more rapidly degraded in the protein extract of the 35S::MdBT2 than in
245 that of the WT (Figure 5A), whereas the protein was more stable in the protein extract
246 of 35S::anti-MdBT2 compared to the WT (Fig. 5A). These results suggest that
247 MdBT2 promotes the degradation of the MdCibHLH1 protein.

248 To further examine whether MdBT2 influences the degradation of the
249 MdCibHLH1 protein *in vivo*, three types of transgenic calli, 35S::MdCibHLH1-GFP,
250 35S::MdBT2-MYC + 35S::MdCibHLH1-GFP, and 35S::anti-MdBT2 +
251 35S::MdCibHLH1-GFP were obtained and used for immunoblotting assays with an
252 anti-GFP antibody. The abundance of MdCibHLH1 was lower in 35S::MdBT2-MYC
253 + 35S::MdCibHLH1-GFP, but higher in 35S::anti-MdBT2 + 35S::MdCibHLH1-GFP
254 than in the WT (Fig. 5B). Meanwhile, cell-free degradation assays were also
255 performed using 35S::MdCibHLH1-GFP and 35S::MdBT2-MYC +
256 35S::MdCibHLH1-GFP. As shown in Fig. 5C, the MdCibHLH1-GFP protein in
257 35S::MdBT2-MYC + 35S::MdCibHLH1-GFP was degraded much greatly than in
258 35S::MdCibHLH1-GFP, just as they did *in vitro*.

259

260 **MdBT2 is involved in the ubiquitination and degradation of MdCibHLH1** 261 **proteins**

262 To examine whether MdCibHLH1 is degraded through a 26S proteasomal
263 pathway, 35S::MdCibHLH1-GFP treated with the translational inhibitor
264 cycloheximide (CHX) and the proteasomal inhibitor MG132 were used for
265 immunoblotting assays with an anti-GFP antibody. The results show that
266 MdCibHLH1 protein accumulation was almost completely inhibited by CHX in the

Figure 5. MdbT2 affects the stability of MdCibHLH1 protein. **A**, The cell-free degradation assays of the prokaryon-expressed and purified MdCibHLH1-HIS fusion proteins were using protein samples extracted from WL, 35S::MdbT2 and 35S::anti-MdbT2. **B**, Three types of transgenic calli, that is, 35S::MdCibHLH1-GFP, 35S::MdbT2-MYC + 35S::MdCibHLH1-GFP and 35S::anti-MdbT2 + 35S::MdCibHLH1-GFP were obtained and used for immunoblotting assays with an anti-GFP antibody. **C**, The cell-free degradation assays were also performed using 35S::MdCibHLH1-GFP and 35S::MdbT2-MYC + 35S::MdCibHLH1-GFP. Total proteins were analyzed by immunoblotting using the anti-GFP antibody. ACTIN was used as the loading control.

267 35S::MdCibHLH1-GFP (Fig. 6A). By contrast, MdCibHLH1 protein highly
 268 accumulated in the transgenic calli treated with MG132 (Fig. 6A), suggesting that the
 269 MdCibHLH1 protein is degraded in a 26S proteasome-dependent manner.

270 26S proteasome-mediated protein degradation is generally associated with
 271 ubiquitination modifications. To examine if MdbT2 participates in the ubiquitination
 272 of the MdCibHLH1 protein, the MdbT2-MYC active protein extracted from
 273 35S::MdbT2-MYC were co-incubated with prokaryon-expressed and purified
 274 MdCibHLH1-HIS protein, E1, E2 and ubi *in vitro* for immunoprecipitation using
 275 anti-HIS and anti-ubi antibodies. A higher amount of high-molecular mass forms of
 276 MdCibHLH1, that is, polyubiquitinated MdCibHLH1 (Ubi(n)-MdCibHLH1-HIS),
 277 was detected when supplemented with MdbT2-MYC active protein (Fig. 6, B and C).
 278 Therefore, it seems that MdbT2 is essential for the ubiquitination of MdCibHLH1
 279 proteins.

280 Subsequently, *in vivo* ubiquitination assays were conducted using
 281 35S::MdCibHLH1-GFP, 35S::MdCibHLH1-GFP + 35S::MdbT2-MYC and
 282 35S::MdCibHLH1-GFP + 35S::anti-MdbT2 (Fig. 6, D and E). The anti-GFP antibody
 283 and anti-ubi antibodies were used to detect the MdCibHLH1-GFP protein
 284 accumulation. The abundance of polyubiquitinated MdCibHLH1-GFP was greater in
 285 35S::MdCibHLH1-GFP + 35S::MdbT2-MYC than in 35S::MdCibHLH1-GFP, but

Figure 6. MdbT2 is involved in the ubiquitination and degradation of MdCibHLH1 proteins. A, 35S::MdCibHLH1-GFP were treated with CHX and MG132 for 6h and then measured the MdCibHLH1-GFP protein level using anti-GFP antibody. B to E, MdbT2 promoted the ubiquitination of MdCibHLH1 protein *in vitro* and *in vivo*. B and C, The MdbT2-MYC active protein extracted from 35S::MdbT2-MYC were co-incubated with prokaryon-expressed and purified MdCibHLH1-HIS protein, E1, E2 and ubi *in vitro* for immunoprecipitation using anti-HIS and anti-ubi antibodies. D and E, Ubiquitination assays *in vivo* were measured using 35S::MdCibHLH1-GFP, 35S::MdCibHLH1-GFP + 35S::MdbT2-MYC and 35S::MdCibHLH1-GFP + 35S::anti-MdbT2. The anti-GFP antibody and anti-ubi antibodies were used to detect the MdCibHLH1-GFP proteins accumulation. IP, Immunoprecipitated. IB, immunoblotted.

286 lower in 35S::MdCibHLH1-GFP + 35S::anti-MdbT2 than in 35S::MdCibHLH1-GFP
 287 (Fig. 6, D and E). These results suggest that the ubiquitination and degradation of the
 288 MdCibHLH1 protein was mediated by MdbT2.
 289
 290 Nitrate participates in the regulation of MdbT2-mediated MdCibHLH1 protein
 291 stability

292 To determine whether nitrate is involved in the regulation of MdBt2-mediated
293 MdCibHLH1 protein stability, the 35S::MdCibHLH1-GFP calli were provided with
294 nitrate (+N) or without nitrate (-N). The total proteins extracted from these calli
295 samples were then used for immunoblotting assays with anti-GFP antibody.
296 Degradation of the MdCibHLH1 protein was accelerated in the presence of nitrate
297 (Fig. 7A). In contrast, the MdCibHLH1 proteins gradually accumulated in the absence
298 of nitrate (Figure 7A). These results suggest that nitrate facilitates the degradation of
299 MdCibHLH1.

300 To examine the effect of nitrate on MdCibHLH1 protein ubiquitination,
301 immunoblotting assays were conducted using 35S::MdCibHLH1-GFP treated with or
302 without nitrate. Exogenous nitrate treatment accelerated the ubiquitination of
303 MdCibHLH1 in 35S::MdCibHLH1-GFP, while the MdCibHLH1 protein
304 ubiquitination was alleviated in the 35S::MdCibHLH1-GFP + 35S::anti-MdBt2 (Fig.
305 7B). These results suggest that nitrate is involved in the regulation of
306 MdBt2-mediated MdCibHLH1 protein ubiquitination.

307 In addition, the level of the ubiquitinated MdCibHLH1 protein was also assessed
308 using an anti-ubi antibody that recognizes only ubiquitinated MdCibHLH1 protein.
309 Similarly, the ubiquitin signal was significantly enhanced in the
310 35S::MdCibHLH1-GFP treated with nitrate compared with absence of nitrate,
311 whereas the ubiquitin signal was reduced in the 35S::MdCibHLH1-GFP +
312 35S::anti-MdBt2 (Fig. 7C)

313 Overall, these results suggest that nitrate acting as a signal regulates
314 MdBt2-mediated MdCibHLH1 protein stability.

315

316 **MdBt2 modulates malate accumulation in an MdCibHLH1-dependent manner**

317 To investigate whether MdBt2 and MdCibHLH1 regulate malate accumulation
318 in apple, a viral vector-based method was applied to alter their expression using
319 vector TRV for suppression. Two viral constructs, including MdBt2-TRV and
320 MdCibHLH1-TRV, were obtained. These constructs including MdBt2-TRV and
321 MdCibHLH1-TRV, as well as two combinations MdBt2-TRV + MdCibHLH1-TRV
322 were used for fruit infiltration, with the empty vectors as controls (Fig. 8A). qPCR
323 assays showed that the transcript levels of *MdBt2* and *MdCibHLH1* genes were
324 significantly decreased after being infiltrated with MdBt2-TRV and
325 MdCibHLH1-TRV (Fig. 8B).

Figure 7. Nitrate participates in the regulation of MdBTL2-mediated MdCibHLH1 protein stability.

A, The protein stability of MdCibHLH1 in response to nitrate. 35S::MdCibHLH1-GFP were incubated without nitrate (-N) and with nitrate (+N) respectively. The total proteins extracted from these calli samples were then used for immunoblotting assays with anti-GFP antibody.

B and C, The immunoblotting assays were conducted using 35S::MdCibHLH1-GFP treated with or without nitrate.

In response to suppression of *MdCibHLH1*, transcript levels of the MdCibHLH1-targeted genes involved in malate accumulation including *MdVHA-A*, *MdVHP1* and *MdALMT9*, were decreased. By contrast, the transcripts of these genes were increased in the MdBTL2-TRV injected apple fruits (Fig. 8B). Subsequently, malate levels, hydrolytic and proton-pumping activities of V-ATPase and V-PPase activity were measured in apple fruit tissues around the infiltration sites. MdCibHLH1-TRV injected apple fruits had lower malate levels and lower proton-pumping activities than the TRV control, whereas MdBTL2-TRV injected apple fruits produced higher malate levels and proton-pumping activities than the control (Fig. 8, C-F). In the MdBTL2-TRV + MdCibHLH1-TRV double-injected apple fruits, *MdCibHLH1* suppression abolished the effect of MdBTL2-TRV infiltration on malate accumulation and proton-pumping activities, indicating that MdBTL2 controls malate accumulation at least partially, if not entirely, via MdCibHLH1 in apple. Similar results were obtained in *MdBTL2* and *MdCibHLH1* transgenic apple plantlets (Supplemental Fig. S3).

In earlier work, we showed that MdCibHLH1 interacts with and activates MdMYB73 to modulate malate accumulation and vacuolar acidification by directly activating *MdALMT9* in apples (Hu et al., 2017). Because MdBTL2 mediates the ubiquitination and degradation of the MdCibHLH1 protein, it is reasonable to predict that MdBTL2 influences the transcription level of MdMYB73-downstream gene *MdALMT9*. To verify this hypothesis, GUS assays were conducted to determine the effect of MdBTL2 on the transcriptional activity of *MdALMT9*. (Supplemental Fig. S4). MdBTL2-MdCibHLH1 regulatory module negatively regulates GUS transcription of MdMYB73-downstream gene *MdALMT9*, which is driven by the *MdALMT9*

Figure 8. MdBT2 modulates malate accumulation in an MdCibHLH1-dependent manner. **A**, Viral vector-based method was applied to apple injection assays. TRV, TRV1 + TRV2; MdBT2-TRV, TRV1 + MdBT2-TRV2; MdCibHLH1-TRV, TRV1 + MdCibHLH1-TRV2; MdBT2-TRV2 + MdCibHLH1-TRV2, TRV1 + MdBT2-TRV2 + MdCibHLH1-TRV2. **B**, The qPCR analysis of the expression of *MdVHA-A*, *MdVHP1* and *MdALMT9*. **C to F**, The malate contents, hydrolytic and proton-pumping activities of V-ATPase and V-PPase were measured in apple fruit tissues around the sites infiltrated with the different viral constructs. Significant difference was detected by *t*-test. **P* < 0.05, ***P* < 0.01.

350 promoter.

351 Taken together, these results further demonstrate that MdBT2 modulates malate
352 accumulation in an MdCibHLH1-dependent manner.

353

354

Discussion

Malate is a crucial metabolite regulated by various signaling pathways that integrate responses of gene expression to environmental factors such as nutrient availability. Physiological studies have demonstrated that malate and nitrate have intricate regulation mechanisms during plant growth and development (Stitt, 1999; Etienne et al., 2013). Although a connection between nitrate and malate has long been speculated, the exact molecular mechanism has remained unclear. Some reports suggest that nitrogen positively regulates fruit acidity (Reitz and Koo, 1960; Ruhl, 1989; Jia et al., 1999; Radi et al., 2003), while others have shown a negative relationship between nitrate level and fruit acidity (Spironello et al., 2004; Wang et al., 2010). The present study reveals that high nitrate inhibits malate accumulation in apple (Fig. 1; Supplemental Fig. S1). MdBT2, a nitrate-responsive protein, which was shown to be involved in regulating anthocyanin accumulation through interacting with the MdMYB1 in our earlier work (Wang et al., 2018), plays a key role in modulating malate accumulation in response to nitrate. MdBT2 interacts with and ubiquitinates MdCibHLH1 in response to nitrate (Fig. 2-8), providing the molecular link between nitrate availability and malate accumulation.

BT2 is a scaffold protein that recruits CUL3 protein to form the E3 ligase complex or bridges an unknown ubiquitin E3 ligase to ubiquitinate and degrade the target proteins (Robert et al., 2009; Zhao et al., 2016; Wang et al., 2018). MdCUL3 protein is required for the MdBT2-mediated ubiquitination and degradation of MdbHLH104 for iron homeostasis in apple, with the TAZ domain of MdBT2 being essential for the interaction (Zhao et al., 2016). However, MdBT2 promotes the ubiquitination and degradation of MdMYB1 in regulating anthocyanin biosynthesis through an MdCUL3-independent pathway, while the BACK domain of MdBT2 is indispensable for the interaction (Wang et al., 2018). In this study, both BTB domain and BACK domain are required for the interaction between MdBT2 and MdCibHLH1 (Fig. 3). Based on these findings, we speculate that MdBT2 possibly ubiquitinates MdCibHLH1 through an MdCUL3-independent pathway.

In apple, MdCibHLH1, an ICE1-like protein, regulates cold tolerance in a CBF-dependent way (Feng et al., 2012). The ICE1-CBF-COR cold response pathway is one of the dominating cold signaling modules that bring about the cold tolerance response in *Arabidopsis* (Chinnusamy et al., 2003; Shi et al., 2018). It has been known that malate accumulation is affected by temperature. High temperature reduces

malate accumulation in fruit (Buttrose et al., 1971; Lobit et al., 2006; Etienne et al., 2013) whereas low temperature facilitates vacuolar malate accumulation probably by affecting the proton pumps transport activity and membrane fluidity (Murata and Los, 1997; Terrier et al., 2001; Etienne et al., 2013). As malate serves as an osmoticum for plant resistance to low temperature (Ruffner et al., 1976; Friemert et al., 1988; Kovermann et al., 2007) and salinity (Hu et al., 2016b), our previous work (Hu et al., 2017) and current work strongly suggest that MdCibHLH1 regulates plant cold tolerance via more than one pathway. In addition to the CBF-dependent pathway, MdCibHLH1 controls cold tolerance via regulating malate accumulation by the MdCibHLH1-MdMYB73 pathway, which was previously described in our study (Hu et al., 2017).

Malate accumulation involves transcriptional regulation of vacuolar proton pumps and malate transporters (Xie et al., 2012; Li et al., 2017; Hu et al., 2017). It has been reported that MdbHLH3 plays a central role in the accumulation of both malate and anthocyanins by interacting with MdMYB1 and binding to the promoter of MdMYB1 (Xie et al., 2012; Hu et al., 2016a). MdCibHLH1 interacts with MdMYB73 and enhances its transcriptional activity on the downstream target genes in regulating malate accumulation (Fig. 4 and 8; Supplemental Fig. S4; Hu et al., 2017). In both cases, we speculate that the MBW complex is recruited to regulate malate accumulation. However, the target proteins of MdbHLH3 and MdCibHLH1 differ in their functions in regulating malate vs. anthocyanin accumulation. Recent studies have identified *Noemi*, encoding a bHLH transcription factor, controls two co-evolved traits in citrus, fruit acidity and anthocyanin levels (Butelli et al., 2019; Zhu et al., 2019). Our findings here suggest that *Noemi* might regulate fruit acidity and anthocyanin levels via a pathway similar to MdbHLH3-MdMYB1 (Xie et al., 2012; Hu et al., 2016a).

Based on the findings presented in this work and elsewhere (Hu et al., 2017), we propose a working model for the regulation of malate accumulation by nitrate (Fig. 9). In this model, when not ubiquitinated under nitrate deficiency, MdCibHLH1 enhances the activity of MdMYB73, which maintains the transcription of malate-associated genes at a high level, resulting in high malate accumulation. However, MdbT2 ubiquitinates and degrades MdCibHLH1 in response to high nitrate, which reduces the transcription of malate-associated genes by decreasing the activity of MYB73, leading to lower malate accumulation. Our findings provide new insights into the

Figure 9. Work model demonstrating that BTB-TAZ protein MdBT2 ubiquitinates and degrades MdCibHLH1 in response to high nitrate, whereas MdBT2 cannot ubiquitinate MdCibHLH1 in low nitrate to regulate target gene activity of *MdMYB73* to modulate malate accumulation for fruit quality.

423 molecular mechanism by which nitrate affects malate accumulation and fruit quality,
 424 providing the link between two key metabolites in plant metabolism. These findings
 425 not only help us understand the complex regulatory network of malate accumulation
 426 but also are of great significance in guiding molecular breeding programs as well as
 427 traditional apple breeding programs for fruit quality improvement.

428

429 **Materials and methods**

430 **Plant materials and treatments**

431 ‘*GL-3*’ apple (*Malus × domestica*) plantlets, ‘*Royal Gala*’ apple fruits and ‘*Orin*’
 432 calli were used. The ‘*GL3*’ and transgenic apple plantlets were grown on
 433 Murashige-Skoog (MS) medium containing 0.2 mg L⁻¹ NAA, 0.2 mg L⁻¹ GA and 0.6
 434 mg L⁻¹ 6-BA at 25°C under long-day conditions (16 h light / 8 h dark) . ‘*Orin*’ apple
 435 calli were grown on MS medium with 1.5 mg L⁻¹ 2,4-D, and 0.4 mg L⁻¹ 6-BA at 25°C
 436 in the dark. For phenotypic analyses of the nitrate-deficiency response, the ‘*GL-3*’
 437 cultures and ‘*Orin*’ apple calli were transferred from nitrate-sufficient medium (MS
 438 medium with the indicated amount of KNO₃, 5 mM) to nitrate-deficient medium (MS

medium without nitrogen, followed by the indicated amount of KCl, 5 mM, as control), and then grown for 3 days.

441

442 **Measurement of malate contents**

443 Samples were extracted with 95% (v/v) ice-cold methanol first and then 80%
444 (v/v) ice-cold methanol after being frozen and ground in liquid nitrogen. The extracts
445 were evaporated to dryness, dissolved in deionized water, and then centrifuged at
446 4000g. The supernatants were filtered through a 0.45 µm membrane filter and then
447 analyzed with high performance liquid chromatography (HPLC) as described by Hu
448 et al. (2016a).

449

450 **Activity assays of V-ATPase and V-PPase**

451 Isolation of tonoplast membranes was performed as described by Terrier et al.
452 (2001). The bafilomycin A₁-sensitive ATP hydrolytic activity and V-ATPase H⁺
453 transport activity as well as V-PPase activity were measured as described by Hu et al.
454 (2016a, 2017).

455

456 **Measurement of vacuolar pH**

457 Isolation of protoplasts from apple fruit, plantlets and calli as well as vacuolar
458 pH imaging were conducted as previously described by Hu et al. (2016a, 2017). A
459 pH-sensitive fluorescent dye BCECF-AM was used to measure vacuolar pH. The
460 vacuolar pH was quantified by the ratio of 488 nm and 458 nm excitation wavelengths.
461 The ion concentration tool of Zeiss LSM confocal software was used to generate the
462 ratio images.

463

464 **Quantitative RT-PCR (qPCR) assays**

465 Total RNA was extracted from apple flesh, plantlets and calli using RNA Plant
466 Plus Reagent (Tiangen, Beijing, China) and qPCR analysis was performed as
467 described previously (Hu et al., 2019). The primers used for qPCR assays are listed in
468 Supporting Information Table S1. The sequences used in this study were obtained
469 from the GDR databases (<https://www.rosaceae.org/>). The GenBank accession
470 numbers are: *MdClbHLH1* (MDP0000662999), *MdBT2* (MDP0000151000),
471 *MdMYB73* (MDP0000894463), *MdVHA-A* (MDP0000844729), *MdVHPI*
472 (MDP0000688191), *MdALMT9* (MDP0000290997).

473

474 **Yeast two-hybrid (Y2H) assay**

475 Y2H assays were performed as described by Wang et al. (2018). The full-length
476 cDNA of MdCibHLH1 was amplified and inserted into pGAD424, while MdBT2
477 cDNAs and truncated sequences (amino acids 1-335, 1-195, 1-128, 129-195, 129-335)
478 were amplified and inserted into pGBT9. The plasmids of pGAD424-MdCibHLH1
479 and the pGBT9-MdBT2s were co-transformed into Y2H Gold (Clontech, Mountain
480 View, CA, USA). The yeasts were grown on -L/-T selection medium for the
481 transformation control and on -L/-T/-H/-A selection medium with or without X-gal
482 for the interaction analysis.

484 **Pull-down assays**

485 The full-length cDNA of MdCibHLH1 was amplified by PCR and inserted into
486 pET-32a to generate HIS-tagged recombinant protein (MdCibHLH1-HIS). The
487 full-length cDNA of MdBT2 was also amplified by RT-PCR and cloned into
488 pGEX-4T to produce GST-tagged fusion protein (MdBT2-GST). The fusion proteins
489 of MdCibHLH1-HIS, MdBT2-GST and empty GST were used for the pull-down
490 assays according to Hu *et al.* (2019).

492 **Bimolecular fluorescence complementation (BiFC) assays**

493 The full-length cDNA of MdBT2 was inserted into the vector
494 35S::pSPYNE-nYFP (MdBT2-nYFP) and MdCibHLH1 was inserted into the vector
495 35S::pSPYCE-cYFP (MdCibHLH1-cYFP). Solutions of *Agrobacterium* harboring
496 recombinant plasmids (MdBT2-nYFP + MdCibHLH1-cYFP) were injected into
497 tobacco leaves. The empty vectors (MdBT2-nYFP + cYFP and nYFP +
498 MdCibHLH1-cYFP) were used as negative controls.

500 **Protein degradation and ubiquitination assays**

501 For the degradation assays of the MdCibHLH1 protein *in vitro*, apple calli
502 (35S::MdBT2, WL and 35S::anti-MdBT2) and the MdCibHLH1-HIS fusion protein
503 were prepared. The extraction buffer contained 25 mM Tris (pH 7.5), 5 mM DTT, 10
504 mM NaCl, 10 mM MgCl₂, 4 mM PMSF and 10 mM ATP. The MdCibHLH1-HIS
505 fusion protein and apple calli were extracted and incubated at 22°C. Samples were
506 collected at the indicated time (0 h, 1 h, 2 h, 4 h and 6 h) and examined using an
507 anti-HIS antibody. For the proteasome inhibitor experiments, 50 µM MG132 was

508 added. For the degradation assays of the MdCibHLH1 protein *in vivo*, two types of
509 apple calli (35S::MdCibHLH1-GFP and 35S::MdCibHLH1-GFP +
510 35S::MdbT2-MYC) were prepared. Total proteins were monitored at the indicated
511 times (0 h, 1 h, 2 h, 4 h and 6 h) when 250 mM CHX was added, and then the
512 anti-GFP antibody was used for immunoblotting assays.

513 For the ubiquitination assays *in vivo*, three types of apple calli
514 (35S::MdCibHLH1-GFP, 35S::MdCibHLH1-GFP + 35S::MdbT2-MYC and
515 35S::MdCibHLH1-GFP + 35S::anti-MdbT2) were prepared and treated with 50 μ M
516 MG132 for 10 h before extraction. The protein extracts were immunoprecipitated
517 using a Pierce Classic Protein A IP Kit (ThermoFisher Scientific, Waltham, MA, USA)
518 with anti-GFP and anti-ubi antibodies. For the ubiquitination assays *in vitro*,
519 35S::MdbT2-MYC apple calli were treated with 50 μ M MG132 for 10 h to obtain the
520 MdbT2-MYC active protein using Pierce Classic Protein A IP Kit. The incubation
521 buffer contained 50 mM Tris (pH 7.5), 2 mM DTT, 50 mM MgCl₂, 2 mM ATP, 100 ng
522 rabbit E1, 100 ng human E2 and 1 μ g ubi. The buffer and MdCibHLH1-HIS protein
523 with or without MdbT2-MYC active protein *in vivo* were co-incubated at 30°C for 24
524 h. Protein ubiquitination was detected using anti-HIS and anti-ubi antibodies.

525

526 **Apple injection assays**

527 Fruit injection assays were carried out as described previously (Hu et al., 2016).
528 The CDSs of MdbT2 and MdCibHLH1 were amplified and inserted into viral vector
529 to obtain MdbT2-TRV (TRV1 + MdbT2-TRV2), MdCibHLH1-TRV (TRV1 +
530 MdCibHLH1-TRV2) and MdbT2-TRV + MdCibHLH1-TRV (TRV1 + MdbT2-TRV2
531 + MdCibHLH1-TRV2), with the TRV vector (TRV1 + TRV2) used as control. The
532 mixture of vectors and the *A. tumefaciens* solutions were injected into apple fruit peel
533 and flesh and tissues around the injection hole.

534

535 **Statistical analysis**

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

539

540 **Supplemental Data**

541 **Supplemental Fig. S1.** Malate contents under a series of nitrate concentrations in

542 apple.

543 **Supplemental Fig. S2.** BTB/TAZ protein MdBT2 controls malate accumulation in
544 response to nitrate.

545 **Supplemental Fig. S3.** MdBT2 modulates malate accumulation in an
546 MdCibHLH1-dependent manner in response to nitrate.

547 **Supplemental Fig. S4.** The MdBT2-MdCibHLH1 regulatory module negatively
548 regulates MdMYB73-downstream gene *MdALMT9*.

549 **Supplemental Table S1.** The primers used in this study.

550

551 **Acknowledgements**

552 We would like to thank Prof. Takaya Moriguchi of National Institute of Fruit Tree
553 Science, Japan, for ‘*Orin*’ apple calli. This work was supported by grants from the
554 National Key Research and Development Program of China (2018YFD1000200), the
555 National Natural Science Foundation of China (31972375, 31772288), the Ministry of
556 Agriculture of China (CARS-28), Shandong Province (SDAIT-06-03), and Nanjing
557 Agricultural University (ZW201805).

558

559 **Author Contributions**

560 Y.-J.H. and D.-G.H. planned and designed the research. Q.-Y.Z., K.-D.G., J.-H.W.,
561 J.-Q.Y., X.-F.W. and C.-X.Y. performed experiments, conducted fieldwork, analysed
562 data etc. Q.-Y.Z., D.-G.H., Y.-J.H. and L.C wrote the manuscript.

563

564 **Figure legends**

565 **Figure 1. Exogenous application of nitrate reduces malate accumulation.**

566 **A,** The ‘*Royal Gala*’ apple fruits were treated with different concentrations of
567 exogenous nitrate (including 0 mM, 0.5 mM and 5 mM nitrate).

568 **B,** The expression of malate-related genes under different concentrations of
569 exogenous nitrate using qPCR. The genes included about vacuolar proton pumps
570 *MdVHA-A*, *MdVHA-Bs* and *MdVHP1*, and the vacuolar malate channel *MdALMT9* as
571 well as malate-associated MYB TF *MdMYB73*.

572 **C,** Malate contents under different concentrations of exogenous nitrate.

573 **D,** Bafilomycin A1-sensitive ATP hydrolytic activity of V-ATPase under different
574 concentrations of exogenous nitrate.

575 **E,** Proton-pumping activity of V-ATPase under different concentrations of exogenous

576 nitrate was measured by tracking the ATP-dependent quenching of acridine orange
 577 using a fluorescence spectrometer with excitation at 493 nm and emission at 545 nm.
 578 **F**, V-PPase activity under different concentrations of exogenous nitrate.
 579 **G**, Emission intensities of protoplast vacuoles under different concentrations of
 580 exogenous nitrate loaded with BCECF at 488 nm (first column) and 458 nm (second
 581 column). Scale bar = 10 μ m.
 582 **H**, Quantification of the pH in vacuoles under different concentrations of exogenous
 583 nitrate. Significant difference was detected by *t*-test. **P* < 0.05, ***P* < 0.01.

584

585 **Figure 2. BTB/TAZ protein MdbT2 controls malate accumulation in response to**
 586 **nitrate.**

587 **A**, Malate contents in WT and transgenic apple plantlets under different
 588 concentrations of exogenous nitrate. ‘GL-3’ plantlets were treated with 0 N (0 N-WT).
 589 MdbT2 overexpression transgenic apple plantlets were treated with 0 N (0
 590 N-MdbT2-OX). The antiMdbT2 transgenic apple plantlets were treated with 5 N (5
 591 N-antiMdbT2).

592 **B**, Expression of *MdVHA-A*, *MdVHPI* and *MdALMT9* in WT and transgenic apple
 593 plantlets under different concentrations of exogenous nitrate using qPCR.

594 **C to E**, The hydrolytic and proton-pumping activities of V-ATPase and V-PPase in
 595 WT and transgenic apple plantlets under different concentrations of exogenous nitrate.

596 **F**, Emission intensities of protoplast vacuoles loaded with BCECF at 488 nm and
 597 458nm. Scale bar = 10 μ m.

598 **G**, Quantification of the pH in vacuoles. Significant difference was detected by *t*-test.
 599 **P* < 0.05, ***P* < 0.01.

600

601 **Figure 3. MdbT2 physically interacts with MdCibHLH1 via the conserved**
 602 **BTB/BACK domain.**

603 **A**, MdbT2 interacted with MdCibHLH1 in a Y2H assay. The full-length and the
 604 BTB/BACK domain of MdbT2 interacted with the MdCibHLH1 protein.

605 **B**, Pull-down assays of the interaction between MdbT2 and MdCibHLH1.
 606 MdbT2-GST was expressed by *E. coli* resulted in the precipitation of
 607 MdCibHLH1-HIS using anti-GST antibody and anti-HIS antibody, respectively. GST
 608 alone was used as the control.

609 **C**, BiFC assay showed the interaction between MdbT2 and MdCibHLH1 using

610 tobacco leaf cells. MdBT2-nYFP and MdCibHLH1-cYFP interacted in the nucleus of
611 tobacco leaf cells. Scale bar = 10 μ m.

612

613 **Figure 4. MdCibHLH1 is involved in regulating of malate accumulation.**

614 **A**, Malate contents in ‘*GALA*’ and *MdCibHLH1-OVXs* transgenic apple fruits
615 (*MdCibHLH1-OVXs*) in the whole fruit developmental stages.

616 **B**, The ‘*GALA*’ and *MdCibHLH1-OVXs* taken in 40 DAF.

617 **C**, The expression of *MdVHA-A*, *MdVHA-B1*, *MdVHP1*, *MdALMT9* and *MdMYB73* in
618 ‘*GALA*’ and *MdCibHLH1-OVXs*.

619 **D to F**, The hydrolytic and proton-pumping activities of V-ATPase and V-PPase in
620 ‘*GALA*’ and *MdCibHLH1-OVXs*.

621 **G**, Emission intensities of protoplast vacuoles in ‘*GALA*’ and *MdCibHLH1-OVXs*
622 with BCECF at 488 nm and 458 nm. Scale bar = 10 μ m.

623 **H**, Quantification of the pH in vacuoles in ‘*GALA*’ and *MdCibHLH1-OVXs*.
624 Significant difference was detected by *t*-test. **P* < 0.05, ***P* < 0.01.

625

626 **Figure 5. MdBT2 affects the stability of MdCibHLH1 protein.**

627 **A**, The cell-free degradation assays of the prokaryon-expressed and purified
628 MdCibHLH1-HIS fusion proteins were using protein samples extracted from WL,
629 35S::MdBT2 and 35S::anti-MdBT2.

630 **B**, Three types of transgenic calli, that is, 35S::MdCibHLH1-GFP,
631 35S::MdBT2-MYC + 35S::MdCibHLH1-GFP and 35S::anti-MdBT2 +
632 35S::MdCibHLH1-GFP were obtained and used for immunoblotting assays with an
633 anti-GFP antibody.

634 **C**, The cell-free degradation assays were also performed using
635 35S::MdCibHLH1-GFP and 35S::MdBT2-MYC + 35S::MdCibHLH1-GFP. Total
636 proteins were analyzed by immunoblotting using the anti-GFP antibody. ACTIN was
637 used as the loading control.

638

639 **Figure 6. MdBT2 is involved in the ubiquitination and degradation of**
640 **MdCibHLH1 proteins.**

641 **A**, 35S::MdCibHLH1-GFP were treated with CHX and MG132 for 6h and then
642 measured the MdCibHLH1-GFP protein level using anti-GFP antibody.

643 **B to E**, MdBT2 promoted the ubiquitination of MdCibHLH1 protein *in vitro* and *vivo*.

644 **B and C**, The MdBt2-MYC active protein extracted from 35S::MdBt2-MYC were
 645 co-incubated with prokaryon-expressed and purified MdCibHLH1-HIS protein, E1,
 646 E2 and ubi *in vitro* for immunoprecipitation using anti-HIS and anti-ubi antibodies.
 647 **D and E**, Ubiquitination assays *in vivo* were measured using 35S::MdCibHLH1-GFP,
 648 35S::MdCibHLH1-GFP + 35S::MdBt2-MYC and 35S::MdCibHLH1-GFP +
 649 35S::anti-MdBt2. The anti-GFP antibody and anti-ubi antibodies were used to detect
 650 the MdCibHLH1-GFP proteins accumulation. IP, Immunoprecipitated. IB,
 651 immunoblotted.

652

653 **Figure 7. Nitrate participates in the regulation of MdBt2-mediated**
 654 **MdCibHLH1 protein stability.**

655 **A**, The protein stability of MdCibHLH1 in response to nitrate. 35S::
 656 MdCibHLH1-GFP were incubated without nitrate (-N) and with nitrate (+N)
 657 respectively. The total proteins extracted from these calli samples were then used for
 658 immunoblotting assays with anti-GFP antibody.

659 **B and C**, The immunoblotting assays were conducted using 35S::MdCibHLH1-GFP
 660 treated with or without nitrate.

661

662 **Figure 8. MdBt2 modulates malate accumulation in an MdCibHLH1-dependent**
 663 **manner.**

664 **A**, Viral vector-based method was applied to apple injection assays. TRV, TRV1 +
 665 TRV2; MdBt2-TRV, TRV1 + MdBt2-TRV2; MdCibHLH1-TRV, TRV1 +
 666 MdCibHLH1-TRV2; MdBt2-TRV2 + MdCibHLH1-TRV2, TRV1 + MdBt2-TRV2 +
 667 MdCibHLH1-TRV2.

668 **B**, The qPCR analysis of the expression of *MdVHA-A*, *MdVHPI* and *MdALMT9*.

669 **C to F**, The malate contents, hydrolytic and proton-pumping activities of V-ATPase
 670 and V-PPase were measured in apple fruit tissues around the sites infiltrated with the
 671 different viral constructs. Significant difference was detected by *t*-test. **P* < 0.05, ***P*
 672 < 0.01.

673

674 **Figure 9. Work model demonstrating that BTB-TAZ protein MdBt2**
 675 **ubiquitinates and degrades MdCibHLH1 in response to high nitrate, whereas**
 676 **MdBt2 cannot ubiquitinate MdCibHLH1 in low nitrate to regulate target gene**
 677 **activity of MdMYB73 to modulate malate accumulation for fruit quality.**

678

679

Parsed Citations

- An JP, Zhang XW, Bi SQ, You CX, Wang XF, Hao YJ (2019) MdbHLH93, an apple activator regulating leaf senescence, is regulated by ABA and MdbT2 in antagonistic ways. New Phytol 222:735-751**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Bai Y, Dougherty L, Cheng LL, Xu KN (2015). A co-expression gene network associated with developmental regulation of apple fruit acidity. Mol Genet Genomics 290: 1247-1263**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Burdon J, Lallu N, Yearsley C, Osman S, Billing D, Bolding H (2007) Postharvest conditioning of Satsuma mandarins for reduction of acidity and skin puffiness. Postharvest Biol and Tec 43: 102-114**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Butelli E, Licciardello C, Ramadugu C, Durand-Hulak M, Celant A, Recupero GR, Martin C (2019) Noemi controls production of flavonoid pigments and fruit acidity and illustrates the domestication routes of modern citrus varieties. Curr Biol 29: 158-164**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Buttrose MS, Hale CR, Kliewer WM (1971) Effect of temperature on the composition of Cabernet Sauvignon berries. Am J Enol Viticult 22: 71-75**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Centeno DC, Osorio S, Nunes-Nesi A, Bertolo AL, Carneiro RT, Araújo WL, Oliver SN (2011) Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. Plant Cell 23: 162-184**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Gene Dev 17: 1043-1054**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Etienne A, Génard M, Lobit P, Mbéguié-A-Mbéguié D, Bugaud C (2013) What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. J. Exp. Bot 64: 1451-1469**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Eisenach C, Baetz U, Martinoia E (2014) Vacuolar proton pumping: more than the sum of its parts? Trends Plant Sci. 19: 344-346**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Feng XM, Zhao Q, Zhao LL, Qiao Y, Xie XB, Li HF, Hao YJ (2012) The cold-induced basic helix-loop-helix transcription factor gene MdCIBHLH1 encodes an ICE-like protein in apple. BMC Plant Biol. 12: 22**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Fernie AR, Martinoia E (2009) Malate. Jack of all trades or master of a few? Phytochemistry 70: 828-832**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Finkemeier I and Sweetlove LJ (2009) The role of malate in plant homeostasis. F1000 Biol. Rep 1: 47**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Friemert V, Heininger D, Kluge M, Ziegler H (1988) Temperature effects on malic-acid efflux from the vacuoles and on the carboxylation pathways in Crassulacean-acid-metabolism plants. Planta 174:453-461**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Hu DG, Sun CH, Ma QJ, You CX, Cheng LL, Hao YJ (2016a) MdMYB1 regulates anthocyanin and malate accumulation by directly facilitating their transport into vacuoles in apples. Plant Physiol 170: 1315-1330**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Hu DG, Sun CH, Sun MH, Hao YJ (2016b) MdSOS2L1 phosphorylates MdVHA-B1 to modulate malate accumulation in response to salinity in apple. Plant Cell Rep 35: 705-718**

- Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Hu DG, Li YY, Zhang QY, Li M, Sun CH, Yu JQ, Hao YJ (2017) The R2R3-MYB transcription factor MdMYB73 is involved in malate accumulation and vacuolar acidification in apple. Plant J 91: 443-454**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Hu DG, Yu JQ, Han PL, Xie XB, Sun CH, Zhang QY, Hao YJ (2019) The regulatory module MdPUB29-MdbHLH3 connects ethylene biosynthesis with fruit quality in apple. New Phytol 221: 1966-1982**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Jia D, Shen F, Wang Y, Wu T, Xu X, Zhang X, Han ZH (2018) Apple fruit acidity is genetically diversified by natural variations in three hierarchical epistatic genes: MdSAUR37, MdPP2CH and MdALMTII. Plant J 95: 427-443**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Jia H, Hirano K, Okamoto G (1999) Effects of fertilizer levels on tree growth and fruit quality of 'Hakuho' peaches (*Prunus persica*). J Jpn Soc Hortic Sci 68: 487-493**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Kovermann P, Meyer S, Hortensteiner S, Picco C, Scholz-Starke J, Ravera S, Lee Y, Martinoia E (2007) The Arabidopsis vacuolar malate channel is a member of the ALMT family. Plant J 52: 1169-1180**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Li SJ, Yin XR, Wang WL, Liu XF, Zhang B, Chen KS (2017) Citrus CitNAC62 cooperates with CitWRKY1 to participate in citric acid degradation via up-regulation of CitAco3. J. Exp. Bot 68: 3419-3426**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Liu KH, Niu Y, Konishi M, Wu Y, Du H, Chung HS, Ishida T (2017) Discovery of nitrate–CPK–NLP signalling in central nutrient-growth networks. Nature 545: 311**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Lobit P, Genard M, Soing P, Habib R (2006) Modelling malic acid accumulation in fruits: relationships with organic acids, potassium, and temperature. J Exp Bot 57: 1471-1483**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Ma BQ, Liao L, Zheng HY, Chen J, Wu BH, Ogutu C, Li SH, Korban SS, Han YP (2015) Genes encoding aluminum-activated malate trans-porter II and their association with fruit acidity in apple. Plant Genome-US 8: 3**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Ma B, Liao L, Fang T, Peng Q, Ogutu C, Zhou H, Han YP (2019) A Ma10 gene encoding P-type ATPase is involved in fruit organic acid accumulation in apple. Plant Biotechnol J 17: 674-686**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Maeda Y, Konishi M, Kiba T, Sakuraba Y, Sawaki N, Kurai T, Yanagisawa S (2018) ANIGT1-centred transcriptional cascade regulates nitrate signalling and incorporates phosphorus starvation signals in Arabidopsis. Nat commun 9: 1376**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Mandadi KK, Misra A, Ren S, McKnight TD (2009) BT2, a BTB protein, mediates multiple responses to nutrients, stresses, and hormones in Arabidopsis. Plant Physiol 150: 1930-1939**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J Exp Bot 58: 83-102**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Murata N, Los DA (1997) Membrane fluidity and temperature perception. Plant Physiol 115: 875**
 Pubmed: [Author and Title](#)
 Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Radi M, Mahrouz M, Jaouad A, Amiot MJ (2003) Influence of mineral fertilization (NPK) on the quality of apricot fruit (cv. Canino). The effect of the mode of nitrogen supply. Agronomie 23: 737-745

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Reitz HJ, Koo RC (1960) Effect of nitrogen and potassium fertilisation OB yield, fruit quality, and leaf analysis of Valencia orange. In Proceedings. Am Soc HorticSci 75: 244-252

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Robert HS, Quint A, Brand D, Vivian-Smith A, Offringa R (2009) BTB and TAZ domain scaffold proteins perform a crucial function in Arabidopsis development. Plant J 58: 109-121

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ruffner HP, Hawker JS, Hale CR (1976) Temperature and enzymic control of malate metabolism in berries of Vitis vinifera. Phytochemistry 15: 1877-1880

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ruhl EH (1989) Effect of potassium and nitrogen supply on the distribution of minerals and organic acids and the composition of grape juice of Sultana vines. Austn J Exp Agr 29: 133-137

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shiratake K and Martinoia E (2007) Transporters in fruit vacuoles. Plant Bio 24: 127-133

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shi Y, Ding Y, Yang SH (2018) Molecular regulation of CBF signaling in cold acclimation. Trends Plant Sci 23: 623-637

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Spironello A, Quaggio JA, Teixeira LJ, Furlani PR, Sigrist JM (2004) Pineapple yield and fruit quality effected by NPK fertilization in a tropical soil. Rev brasa de frutic 26: 155-159

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Stitt M (1999) Nitrate regulation of metabolism and growth. Curr Opin Plant Biol 2: 178-186

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sweetman C, Deluc LG, Cramer GR, Ford CM, Soole KL (2009) Regulation of malate metabolism in grape berry and other developing fruits. Phytochemistry 70: 1329-1344

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Terrier N, Sauvage FX, Romieu C (2001) Changes in acidity and in proton transport at the tonoplast of grape berries during development. Planta 213: 20-28

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Thakur A, Singh Z (2012) Responses of 'Spring Bright' and 'Summer Bright' nectarines to deficit irrigation: fruit growth and concentration of sugars and organic acids. Sci Hortic 135: 112-119

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Wu BH, Génard M, Lescourret F, Gomez L, Li SH (2002) Influence of assimilate and water supply on seasonal variation of acids in peach (cv Suncrest). J Sci Food Agr 82:1829-1836

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Wang HC, Ma FF, Cheng LL (2010) Metabolism of organic acids, nitrogen and amino acids in chlorotic leaves of 'Honeycrisp' apple (Malus domestica Borkh) with excessive accumulation of carbohydrates. Planta 232: 511-522

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Wang XF, An JP, Liu X, Su L, You CX, Hao YJ (2018) The nitrate-responsive protein MdBT2 regulates anthocyanin biosynthesis by interacting with the MdMYB1 transcription factor. Plant Physiol 178: 890-906

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Xie XB, Li S, Zhang RF, Zhao J, Chen YC, Zhao Q, Hao YJ (2012) The bHLH transcription factor MdbHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. Plant Cell Environ 35: 1884-1897

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yao YX, Li M, Liu Z, Hao YJ, Zhai H (2007) A novel gene, screened by cDNA-AFLP approach, contributes to lowering the acidity of fruit in apple. Plant Physiol Bioch 45: 139-145

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ye J, Wang X, Hu T, Zhang F, Wang B, Li CX, Ye ZB (2017) An InDel in the promoter of Al-ACTIVATED MALATE TRANSPORTER9 selected during tomato domestication determines fruit malate contents and aluminum tolerance. Plant Cell 29: 2249-2268

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zhao Q, Ren YR, Wang QJ, Wang XF, You CX, Hao YJ (2016) Ubiquitination-related MdbT scaffold proteins target a bHLH transcription factor for iron homeostasis. Plant Physiol 172:1973-1988

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zhu F, Wen W, Fernie AR (2019) Finding noemi: The transcription factor mutations underlying trait differentiation amongst Citrus. Trends Plant Sci 24:384-386

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)