1 **Short title:** MdPAT16 regulates sugar through palmitoylation.

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9 **The apple palmitoyltransferase MdPAT16 regulates sugar content via an**
10 **MdCBL1-MdCIPK13-MdSUT2.2 pathway**

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20 **One-sentence summary:** Under appropriate salt stress, MdCBL1 was palmitoylation
21 stabilized by MdPAT16 to promote sugar accumulation via an
22 MdCBL1-MdCIPK13-MdSUT2.2 pathway.

23 **Author contributions:** Y.-J.H. and H.J. planned and designed the research. H.J.,
24 Q.-J.M., M.-S.Z., H.-N.G., performed experiments, conducted fieldwork, analyzed
25 data etc. H.J., Y.-Y.L., and Y.-J.H wrote the manuscript.

26 ABSTRACT

27 Protein palmitoylation, a post-translational protein modification, plays an important
 28 role in the regulation of substrate protein stability, protein interactions, and protein
 29 localization. It is generally believed that there are two mechanisms of palmitoylation:
 30 one by acyl-CoA and the other by protein acyltransferase (PAT). In this study, an
 31 MdPAT family member, MdPAT16, was identified and shown to have
 32 palmitoyltransferase activity. We found that this gene responded to salt stress and that
 33 its expression improved plant salt resistance. MdPAT16 was shown to interact with
 34 MdCBL1 and stabilize MdCBL1 protein levels through palmitoylation. MdPAT16
 35 further regulated apple sugar content by stabilizing the MdCIPK13-MdSUT2.2
 36 protein complex. We found that the N-terminal sequence of MdCBL1 contains a
 37 palmitoylation site and that the N-terminal deletion of MdCBL1 leads to changes in
 38 protein stability and subcellular localization. Finally, exogenous salt stress increased
 39 the interaction of MdPAT16 with MdCBL1 and the sugar content in apple. These
 40 findings suggest that MdPAT16 functions as a stable means for the palmitoylation of
 41 downstream protein. It may be a missing link in the plant salt stress response pathway
 42 and have an important impact on fruit quality.

43

44 **Key words:** MdPAT, Palmitoylation, Sugar accumulation, *Malus domestica*

45

46 INTRODUCTION

47 Sugars play multiple important roles in diverse biological processes, such as
 48 physiological metabolism, growth, and developmental stage transitions. Sugars not
 49 only supply energy for plant growth and development, but also act as important
 50 determinants of fruit quality and commodity value. The content, type, and ratio of
 51 sugars directly or indirectly determine fruit flavor, color, and other quality traits.
 52 Sugars also function as signaling molecules to regulate flowering time, nitrogen

53 metabolism, anthocyanin accumulation, and other processes in various plant species
54 (Ohto et al., 2001; Jonassen et al., 2008; Sun et al., 2019; Hu et al., 2016; Liu et al.,
55 2019).

56 Sugars are also used for osmotic adjustment in response to various abiotic
57 stresses. Salinity, drought, and low temperature usually result in the accumulation of
58 sugars (Krasensky et al., 2012). Under long-term salt stress, plants enhance their salt
59 tolerance in response to dehydration and osmotic stress (Chaves et al., 2009). Early
60 salt stress is similar to drought and is mainly affected by ion toxicity and osmotic
61 stress. Therefore, increasing plant tolerance to the early stages of salt stress mainly
62 involves preventing salt from entering the cytoplasm and lowering cell water potential
63 through osmotic adjustment (Sultana et al., 1999; Parida and Das, 2005; Munns and
64 Tester, 2008). The sugars produced by photosynthesis provide necessary energy for
65 the growth and development of tissues in response to environmental stimulation (Lv
66 et al., 2008; Yao et al., 2010). When sugars accumulate to a relatively high level in the
67 vacuole, this produces a high bloating pressure (Gibson, 2005; Moustakas et al., 2011;
68 Rasheed et al., 2011). Therefore, sugars also affect osmotic potential and participate in
69 the response to water and salt stresses.

70 In *Arabidopsis*, sugars participate in abiotic stress responses by affecting osmotic
71 potential, and increased contents of soluble sugar, anthocyanins, and proline occur
72 under stress (Moustakas et al., 2011). In wheat, the contents of glucose, fructose,
73 sucrose, and fructan increase significantly under drought or salt stress (Kerepesi et al.,
74 2000). In apple, the CBL-interacting protein kinase (CIPK) MdCIPK22 interacts with
75 and phosphorylates the sucrose transporter MdSUT2.2 to mediate drought tolerance
76 and sugar accumulation (Ma et al., 2019a). The ABA-related transcription factor
77 MdAREB2 directly binds to the promoter of MdSUT2.2, which plays an important
78 role in ABA-induced sugar accumulation (Ma et al., 2017a). Meanwhile, MdCIPK22
79 also interacts with and phosphorylates MdAREB2, thereby promoting its transcription
80 (Ma et al., 2017b). Therefore, MdCIPK22 both directly and indirectly regulates the
81 downstream protein MdSUT2.2 and sugar accumulation in apple. Another CIPK

82 family gene, MdCIPK13, may also regulate sugar content and salt tolerance (Ma et al.,
83 2019b). However, the upstream regulatory pathways for MdCIPK13 in response to
84 salt stress remain unclear.

85 S-acylation, also called S-palmitoylation, involves the binding of a 16-carbon
86 palmitoyl group to a specific protein cysteine residue through a thioester bond.
87 S-palmitoylation regulates dynamic membrane localization, stability, and transport of
88 proteins between different cellular compartments. It also regulates protein function
89 and protein-protein interactions. Although the mechanism of palmitoylation remains
90 unclear in plants (Hemsley et al., 2013), it is well known that palmitoylation occurs in
91 different membrane structures, such as the endoplasmic reticulum (Batistič et al.,
92 2008), Golgi (Zeng et al., 2007), the plasma membrane (Sorek et al., 2007), and the
93 tonoplast (Batistič, et al., 2012; Zhou et al., 2013). Palmitoylation of proteins also
94 occurs spontaneously without enzyme catalysis. However, in this process, a large
95 number of reaction substrates must be provided (Bizzozero et al., 1987; Duncan and
96 Gilman, 1996). As organisms fail to provide such large amounts of substrate,
97 spontaneous protein palmitoylation is unlikely to occur *in vivo*. The common
98 understanding is that protein palmitoylation is catalyzed by a series of enzymes called
99 protein S-acyl transferases (PATs) (Batistič, et al., 2012; Zhou et al., 2013).

100 Palmitoyl transferase was initially discovered in *Saccharomyces cerevisiae*, and it
101 has been extensively studied in mammals and yeast (Sun et al., 2004; LaGrassa and
102 Ungermann, 2005). The first PAT function genes to be characterized were the
103 Erf2-Erf4 complex and Ark1, which promote palmitoylation of yeast Ras2 protein and
104 Yck2 (yeast casein kinase 2) kinase, respectively (Feng and Davis, 2000; Qi et al.,
105 2014). Both Erf2 and Ark1 have a conserved DHHC (Asp-His-His-Cys) motif in the
106 cysteine residue aggregation domain (CRD), as well as zinc finger structural features
107 (González-Siso et al., 2009; Hou et al., 2005; Ohno et al., 2006; Subramanian et al.,
108 2006). Generally, proteins rich in DHHC motifs have PAT activity. These motifs are
109 important not only for PAT activity, but also for palmitoylation of the DHHC protein
110 itself (Qi et al., 2014).

111 In plants, the first reported palmitoyl transferase was TIP1 (TIP GROWTH
112 DEFECTIVE 1) in Arabidopsis. It is a member of the palmitoyl transferase family
113 with an ankyrin repeat sequence and is generally expressed in roots, leaves,
114 inflorescence stems, and flowers. TIP1 regulates protein hydrophobicity and affects
115 protein-membrane binding, signal transduction, and intracellular vesicle trafficking
116 (Hemsley et al., 2005). In addition, another Arabidopsis palmitoyltransferase,
117 *AtPAT10*, has palmitoyltransferase activity and participates in the regulation of cell
118 expansion and cell division. It also enhances reproductive capacity (Qi et al., 2014).
119 The *AtPAT10* protein localizes to the Golgi apparatus and the tonoplast (Qi et al.,
120 2013; Zhou et al., 2013). The phenotypes of three *AtPAT10* T-DNA insertion mutants
121 are consistent and include defects in cell expansion and cell division, as well as
122 hypersensitivity to salt stress. *AtCBL2* and *AtCBL3* have been identified as potential
123 substrates of *AtPAT10* and shown to regulate salt tolerance (Zhou et al., 2013).
124 However, it remains unclear how PATs regulate sugar accumulation in response to salt
125 stress.

126 In this study, a palmitoyltransferase family member, *MdPAT16*, was identified in
127 apple. Functional complementation and S-acylation experiments demonstrated that
128 *MdPAT16* has palmitoyltransferase activity, and subsequent experiments
129 characterized its functions in sugar accumulation and salt stress tolerance. Its
130 interacting protein *MdCBL1* was also identified and characterized. Finally, the
131 response of *MdPAT16* to salt stress and its interactions with *MdCBL1* to regulate
132 sugar accumulation and modulate salt tolerance were characterized.

133

134

135 **RESULTS**

136 **MdPAT16 promotes sugar accumulation and enhances salt tolerance**

137 Esculin staining of roots demonstrated that an appropriate concentration of NaCl
138 clearly promoted sucrose transferase activity in apple roots, thereby increasing plant
139 sugar content (Fig. S1). RNA-seq analysis further demonstrated that numerous genes
140 associated with sugar biosynthesis and transportation were markedly upregulated. In
141 addition to sugar-associated genes, the PAT family member *MdPAT16* was also
142 transcriptionally upregulated in response to NaCl treatment (Fig. 1).

143 To further characterize the function of *MdPAT16* in response to salt stress, a
144 pMdPAT16-GUS vector was transiently transformed into apple shoot cultures. The
145 transgenic shoot cultures were then treated with NaCl or H₂O. After GUS staining,
146 NaCl-treated shoot cultures exhibited higher GUS activity than water-treated controls
147 (Fig. S2). To further verify that MdPAT16 functions in salt-induced sugar
148 accumulation, *Agrobacterium rhizogenes*-mediated genetic transformation was
149 performed to obtain MdPAT16 overexpression and suppression transgenic roots. Two
150 weeks after NaCl treatment, MdPAT16-OVX roots grew much better and
151 MdPAT16-Anti roots grew poorly compared with the empty vector (WT) controls, as
152 indicated by lateral root number, root length, and root surface area (Fig. 2A–C).

153 Esculin staining was performed to examine sucrose transport activity in the
154 different transgenic roots. MdPAT16-OVX roots exhibited much stronger blue
155 fluorescence than the WT controls, indicating that MdPAT16 overexpression
156 promoted sucrose transport activity. NaCl treatment further enhanced the fluorescence
157 intensity (Fig. 2 E,F). Sugar contents were also measured, and MdPAT16-OVX roots
158 accumulated much more glucose, sucrose, and soluble sugars than the WT controls,
159 whereas MdPAT-Anti accumulated less (Fig. 2D).

160 To further examine whether MdPAT16 improves sugar accumulation, TRV viral
161 vectors were used for transient overexpression and suppression (Yuval et al., 2007).

Figure 1. RNAseq analysis of GL-3 tissue culture seedlings with and without NaCl treatment.

(A) Gene ontology enrichment of upregulated genes in NaCl-treated and WT seedlings, presented according to $-\log_{10}P$ -value. (B) Venn diagram of the genes upregulated in NaCl-treated and WT seedlings ($\log_2\text{FoldChange} > 2$). (C) KEGG pathway classification of upregulated genes. (D) Normalized heatmap of sugar-related genes, including amino sugar and nucleotide sugar metabolism(MD01G1213100,MD02G1120200), starch and sucrose metabolism(MD03G1098600, MD10G1270400), sucrose transmembrane transporter(MD10G1045900), salt stress response (MD05G1119300, MD06G1147900), and MdPAT16(MD10G1058600).

162 MdPAT16-TRV was transiently transformed into Gala shoot cultures to inhibit the
 163 expression of MdPAT16. The resultant transgenic shoot cultures were used to detect
 164 starch content through iodine staining. MdPAT16 silencing increased starch
 165 accumulation but decreased soluble sugar contents (Fig. 3A,B). Subsequently, a VIGS
 166 experiment was conducted using apple fruits, and the results were the same as those
 167 obtained in apple roots and seedlings (Fig. 3C,D). These results suggested that
 168 MdPAT16 responded to salt stress, improved plant salt resistance, and increased sugar
 169 content.

170 To further characterize its function *in planta*, MdPAT16 was genetically
 171 transformed into Arabidopsis. Esculin staining demonstrated that ectopic expression
 172 of MdPAT16 enhanced fluorescence intensity compared with the WT control. As a

Figure 2. MdPAT16 functions as a positive regulator under salt stress. (A) Root fluorescence identification of MdPAT16 overexpression transgenic roots (Bars = 100 μ m). (B) and (C) Root scan analysis of root shape, root length, surface area, and lateral root number of WT, MdPAT16-OVX, and MdPAT16-anti transgenic roots. Results are given as mean $\pm$ SD. Letters indicate significant differences (*t*-test, $P < 0.01$) (D) Glucose, sucrose, and soluble sugar contents of WT, MdPAT16-OVX, and MdPAT16-anti transgenic roots. (E) and (F) Esculin staining of sucrose transport activity in (E) WT and MdPAT16-OVX transgenic roots, and (F) MdPAT16-OVX roots treated with NaCl and KCl (Bars = 100 μ m).

173 result, ectopic transgenic lines generated more sugars than the WT controls.
 174 MdPAT16 ectopic expression also promoted salt tolerance in transgenic *Arabidopsis*
 175 (Fig. S3).

176 Taken together, these results indicate that *MdPAT16* plays a crucial role in sugar
 177 accumulation in response to salt stress and positively regulates salt tolerance.

179 MdPAT16 is an S-palmitoyltransferase

180 A phylogenetic tree and a sequence alignment analysis demonstrated that
 181 *MdPAT16* is a member of the PAT gene family and that PAT16 sequences are highly
 182 conserved among different plant species (Figs. S4,S5). The yeast mutant *akr1p*, which
 183 is deficient in palmitoyltransferase activity, was used to determine whether MdPAT16
 184 has palmitoylation activity. An MdPAT16-pYES2 expression vector was constructed
 185 and genetically transformed into the *akr1p* mutant and the WT strain BY4741, and the
 186 empty vector was used as a control. MdPAT16 ectopic expression in the *akr1p* mutant
 187 recovered its palmitoyltransferase deficiency phenotype, whereas expression of the
 188 empty vector did not. However, MdPAT16 ectopic expression in the *akr1p* mutant

Figure 3. Overexpression of apple MdPAT16 increases soluble sugar content. (A) Starch staining of MdPAT16-TRV transient suppression Gala tissue culture seedlings and Empty Vector controls. (B) Soluble sugar, glucose, and sucrose contents of MdPAT16-TRV and Empty Vector controls. (C) and (D) visible anthocyanin accumulation conditions (C) and soluble sugar, glucose, and sucrose contents (D) of apple fruits from MdPAT16-IL60 (overexpression), MdPAT16-TRV (suppression) and Empty Vector controls.

189 failed to recover its temperature sensitive phenotype.

190 A mutation from cysteine (Cys) to alanine (Ala) was created in the DHHC-CRD
191 domain of MdPAT16 to produce MdPAT16^{C244A}, and MdPAT16^{C244A} lost the ability to
192 complement the palmitoyltransferase deficiency phenotype (Fig. 4A).

193 The shapes of yeast cells under different treatments were observed with an
194 LSM880 high resolution laser confocal microscope, and quantitative statistics were
195 used to analyze the the percentage of cells that had an altered shape. Observations
196 showed that the expression of MdPAT16 in *akr1p* yeast cells produced a full oval
197 shape similar to that of WT BY4741, whereas *akr1p* yeast cells that expressed
198 MdPAT16^{C244A} exhibited a long rod shape. These observations suggested that
199 MdPAT16 rescued the phenotype of *akr1p* and that this rescue required the cysteine
200 residue of the DHHC catalytic site (Fig. 4B,C). Finally, an ABE (Acyl-Biotin
201 Exchange) assay demonstrated that MdPAT16 had the capacity to palmitoylate itself
202 (Fig. 4D). These observations strongly suggest that MdPAT16 is an
203 S-palmitoyltransferase.

204

Figure 4. MdPAT16 is an S-palmitoyltransferase. (A) Growth test (above) and survival test (below). Under the nonpermissive temperature of 37 °C (right), wild-type (BY4741) yeast cells grew well, but *akr1p* did not. Expression of MdPAT16 in *akr1p* largely restored growth, but MdPAT16^{C244A} failed. (B) and (C) Observations of cell shape using a LSM880 high resolution laser confocal microscope (B). The cell shape of MdPAT16/*akr1p* was indistinguishable from WT, whereas that of MdPAT16^{C244A} differed. Quantitative statistics of all four genotypes grown at 37 °C (C). (D) MdPAT16-V5/*akr1p* and pYES2-V5/*akr1p* by ABE assay were measured by Western blotting with anti-V5 antibody to demonstrate that apple MdPAT16 is auto-acylated.

205 MdPAT16 interacts with the calcineurin B subunit protein MdCBL1

206 To identify MdPAT16-interacting proteins *in planta*, total protein was extracted
 207 from 35S::MdPAT16-GFP and 35S::GFP transgenic apple calli for
 208 co-immunoprecipitation (Co-IP) assays. The IPed proteins were analyzed with mass
 209 spectrometry, and several identified peptides were parts of a calcineurin B subunit
 210 protein homologous to CBL1, hereafter named MdCBL1. Subsequently,
 211 35S::MdPAT16-GFP/MdCBL1-HA and MdPAT16-GFP/HA double transgenic apple
 212 calli were obtained to verify the proteins' interaction *in vivo* using Co-IP. The results
 213 showed that MdPAT16 interacted with MdCBL1 (Fig. 5A). Furthermore, both *in vitro*
 214 pull-down assays and bimolecular fluorescence complementarity (BiFC) assays
 215 confirmed the *in vitro* and *in vivo* interaction between MdCBL1 and MdPAT16 (Fig.
 216 5B,C).

217 Next, a dual-luciferase reporter system was used to determine whether NaCl
 218 treatment influenced the interaction between MdPAT16 and MdCBL1. Full-length
 219 cDNAs of MdPAT16 and MdCBL1 were fused to the N- and C-terminals of the
 220 binary fluorescent vector, respectively. Fluorescence observations demonstrated that

Figure 5. MdCBL1 is a direct substrate of MdPAT16. (A) *in vivo* Co-IP assays between MdPAT16 and MdCBL1 by western blotting with anti-HA and anti-GFP antibodies. (B) *in vitro* GST pull-down assays with MdPAT16-HIS and MdCBL1-GST. Proteins immunoprecipitated with GST-beads were detected using anti-HIS antibody. (C) BiFC was performed to test the interaction between MdPAT16 and MdCBL1, and AHIR1-RFP was co-injected as a plasma membrane marker. Bars = 10 μ m. (D) and (E) The interaction between MdPAT16 and MdCBL1 was visualized with a dual-luciferase reporter system (D) and fluorescence activity was observed with and without NaCl (E).

221 the luciferase activity around the MdPAT16-Nluc and MdCBL1-Cluc co-injection site
222 was much higher than that observed in the empty vector control, further confirming
223 the interaction between MdPAT16 and MdCBL1 (Fig. 5D). Subsequently, 100mM
224 KCl or 100mM NaCl was added to the injection solution and co-injected with
225 MdPAT16-Nluc and MdCBL1-Cluc. Compared with the KCl treatment, the NaCl
226 treatment clearly promoted the interaction between MdPAT16 and MdCBL1 (Fig.
227 5E).

228

229 **MdPAT16 palmitoylates MdCBL1 to determine its subcellular localization and**
230 **protein stability**

231 To determine whether MdPAT16 palmitoylates MdCBL1,
11

Figure 6. MdCBL1 is palmitoylated by MdPAT16 on the 3rd cysteine residue. (A) *in vivo* Co-IP assays between MdPAT16/MdCBL1-HA and MdPAT16-RNAi/MdCBL1-HA double transgenic apple calli. The palmitoylation of MdCBL1 was detected using anti-HA antibodies. (B) Yeast functional complementation assays using MdCBL1^{C3S}/akr1p and MdCBL1^{C138S}/akr1p demonstrated that neither point mutant of MdCBL1 was auto-acylated. (C) ABE assays demonstrated that the 3rd but not 138th cysteine residue determined the palmitoylation of MdCBL1.

232 MdPAT16-OVX/MdCBL1-HA and MdPAT16-RNAi/MdCBL1-HA double transgenic
233 apple calli were obtained and used for Co-IP assays with an anti-HA antibody.
234 MdCBL1 palmitoylation was undetectable in MdPAT16-RNAi transgenic apple calli,
235 whereas MdCBL1 was markedly palmitoylated in MdPAT16-overexpressing apple
236 calli (Fig. 6A), indicating that MdPAT16 is required for MdCBL1 palmitoylation.
237 Furthermore, an online palmitoylation site prediction website, CSS-Palm
238 (<http://csspalm.biocuckoo.org/>), was used to predict the possible palmitoylation sites
239 of MdCBL1. Two Cys residues, the 3rd and 138th cysteines, were scored as possible
240 palmitoylation sites. Functional complementation assays of the yeast *akr1p* mutant
241 demonstrated that MdCBL1 protein that contained mutations at each possible
242 palmitoylation site failed to rescue the deficient phenotype (Fig. 6B), indicating that
243 the two palmitoylation sites are crucial for MdCBL1 function.

244 Acyl-biotin exchange (ABE) assays were performed to further verify the
245 palmitoylation sites. Western blotting indicated that MdCBL1^{C3S}, but not
246 MdCBL1^{C138S}, failed to be palmitoylated by MdPAT16, indicating that the 3rd cysteine
247 residue, but not the 138th cysteine residue, was the palmitoylation site for the
248 MdCBL1 protein (Fig. 6C).

249 To determine whether MdPAT16-mediated palmitoylation of MdCBL1 influenced
250 its subcellular localization, MdPAT16-GFP and MdCBL1-RFP were transiently
251 expressed in *Nicotiana benthamiana* leaves. AtCBL1-GFP and AtHIR1-RFP
252 fluorescent proteins were used as plasma membrane markers to co-localize with
253 MdPAT16 and MdCBL1. Both MdPAT16 and MdCBL1 showed a significant
254 co-localization with the plasma membrane marker (Fig. 7A). MdCBL1^{C3S}-RFP was
255 then used to check whether MdPAT16-mediated MdCBL1 palmitoylation influenced
256 its subcellular localization. MdCBL1-RFP was localized to the plasma membrane in
257 the presence of MdPAT16-GFP, whereas MdCBL1^{C3S}-RFP was not (Fig. 7B). In
258 addition, an uptake assay was performed to further confirm the subcellular migration
259 of MdCBL1. Cellular compartments such as nuclei, cytoplasm, and membrane
260 structures were isolated from tobacco leaves that transiently expressed MdCBL1-GFP
261 and MdCBL1^{C3S}-GFP. Western blotting assays were performed to measure the
262 expression levels of MdCBL1-GFP and MdCBL1^{C3S}-GFP in nuclei, cytoplasm, and
263 membrane structures. Anti-Histone3(H3) and anti-actin were used as loading controls
264 for nuclei and cytoplasm, respectively, while AtHIR1-RFP was co-injected as a
265 loading control for membrane structures. The results indicated that the subcellular
266 localization of MdCBL1 changes when palmitoylation is absent (Fig. 7C).

267 In addition, 35S::MdPAT16-OVE/MdCBL1-RFP,
268 35S::MdPAT16-RNAi/MdCBL1-RFP, 35S::Empty Vector/MdCBL1-RFP, and
269 35S::MdPAT16-OVE/MdCBL1^{C3S}-RFP were genetically transformed into apple roots
270 using an *Agrobacterium rhizogenes*-mediated transformation system. The resultant
271 transgenic roots were used to determine whether the MdPAT16-mediated
272 palmitoylation of MdCBL1 influences its subcellular localization. MdPAT16 was

Figure 7. Localization of MdCBL1 to the plasma membrane depends on the function of MdPAT16. (A) Subcellular localization of MdCBL1-RFP and MdPAT16-GFP in *N. benthamiana* leaves. AtCBL1-GFP and AtHIR1-RFP were used as plasma membrane markers. (B) Subcellular localization of MdCBL1^{C3S}-RFP. (C) Qualitative detection of MdCBL1 and MdCBL1^{C3S} in different cellular compartments by western blotting. Anti-Histone3 and anti-actin were used as loading controls for nuclei and cytoplasm, respectively. AtHIR1-RFP was co-injected as a loading control for plasma membrane. (D) Subcellular localization of MdCBL1/WT, MdCBL1/MdPAT16-OVE, MdCBL1/MdPAT16-RNAi, and MdCBL1^{C3S}/MdPAT16-OVE in transgenic roots of GL-3.

273 required for the plasma membrane localization of MdCBL1-RFP protein, and
 274 MdCBL1^{C3S}-RFP failed to localize to plasma membrane even together with MdPAT16
 275 in 35S::MdPAT16-OVE/MdCBL1^{C3S}-RFP transgenic apple roots (Fig. 7D). Therefore,
 276 MdPAT16-mediated palmitoylation of MdCBL1 promotes its subcellular localization
 277 to the plasma membrane.

278 Cell-free degradation assays were performed to examine whether
 279 MdPAT16-mediated palmitoylation affects the protein stability of MdCBL1.
 280 Prokaryotic-expressed MdCBL1-GST and eukaryotic-expressed MdCBL1^{C3S}-V5
 281 were incubated with total proteins extracted from the WT control and from
 282 MdPAT16-GFP and MdPAT16-RNAi transgenic apple calli. MdCBL1-GST was more
 283 stable in MdPAT16-GFP transgenic apple calli, but its stability was markedly
 284 decreased in MdPAT16 RNAi transgenic calli. Meanwhile, MdCBL1^{C3S}-V5 degraded
 285 much more quickly than MdCBL1-GST. Therefore, MdPAT16-mediated

286 palmitoylation of MdCBL1 influences its stability.

287

288 **MdCIPK13 is required for MdPAT16-mediated sugar accumulation**

289 To determine how MdPAT16 and MdCBL1 are involved in the regulation of
290 sugar accumulation, viral vectors were used to perform transient expression analyses
291 in apple fruits. MdCBL1-IL60 overexpression and MdCBL1-TRV suppression vectors
292 were constructed and injected into fruits. Like MdPAT16, MdCBL1 overexpression
293 clearly increased sugar content, whereas its suppression decreased sugar content. As
294 mentioned above, MdPAT16 overexpression increased sugar content (Figure 3C,D).
295 When MdPAT16-IL60 and MdCBL1-TRV were co-injected into apple fruit, MdCBL1
296 suppression almost completely abolished the MdPAT16-mediated increase in fruit
297 sugar content (Fig. 9A-C). In addition, *Agrobacterium rhizogenes*-mediated genetic
298 transformation was performed to obtain 35S::MdCBL1-OVX, 35S::MdCBL1-RNAi,
299 and 35S::MdCBL1^{C3S}-OVX transgenic apple roots. Increasing activity of sucrose
300 transferase was detected in the resultant transgenic roots, respectively. MdCBL1
301 overexpression enhanced sucrose transferase activity in transgenic apple roots, and
302 MdCBL1 suppression inhibited it (Fig. 9D). Furthermore, mutation of the MdCBL1
303 palmitoylation site abolished its role in enhancing sucrose transferase activity (Fig.
304 9D), indicating that the palmitoylation site plays a crucial role in MdCBL1 function.

305 In our previous report, salt stress induced sugar accumulation in the vacuole
306 through a MdCBL1/MdCIPK13-MdSUT2.2 pathway (Ma et al., 2019). Considering
307 that MdCBL1 interacts with MdCIPK13, it is reasonable to propose that MdCIPK13
308 is involved in MdPAT16-mediated sugar accumulation in response to salt stress. To
309 verify this hypothesis, viral vectors were used to perform transient expression
310 experiments in apple fruits. MdCIPK13-IL60 overexpression and MdCIPK13-TRV
311 suppression vectors were constructed and used for injection. MdCIPK13-IL60
312 promoted sugar accumulation in apple fruit, whereas MdCIPK13-TRV inhibited it
313 (Fig. 10A). In addition, MdCIPK13 suppression abolished the MdPAT16-mediated

Figure 9. MdCBL1 is required for sugar accumulation. (A) and (B) Anthocyanin accumulation of MdCBL1-IL60, MdCBL1-TRV and Empty Vector apple fruits (A), and MdPAT16-IL60/MdCBL1-TRV, IL60/MdCBL1-TRV, and Empty Vector apple fruits (B). (C) Soluble sugar, glucose, and sucrose contents of different fruits. (D) Esculin uptake assay for sucrose transport activity in transgenic roots of MdCBL1, MdCBL1^{CS}, and MdCBL1-RNAi.

314 sugar increase in MdPAT16-IL60+MdCIPK13-TRV co-injected apple fruits (Fig.
 315 10A,B), indicating that MdCIPK13 is required for MdPAT16-mediated sugar
 316 accumulation under salt stress.

317

Figure 10. MdPAT16 mediates sugar accumulation through the MdCIPK13 pathway. Anthocyanin accumulation (A) and soluble sugar, glucose, and sucrose contents (B) of MdCIPK13-IL60, MdCIPK13-TRV, MdPAT16-IL60/ MdCIPK13-TRV, and Empty Vector apple fruits.

318 Discussion

319 Salt stress impedes plant growth and development via osmotic adjustment
 320 (Munns, 2002). It also alters stomatal conductance and transpiration rate and affects
 321 plant ion balance (Chaves et al., 2009). Soluble sugars, as compatible osmolytes, are
 322 thought to function mainly in the stabilization of proteins and cellular structures under
 323 stress (Sanchez et al., 2008). In addition, sugars reduce the stress-induced
 324 accumulation of reactive oxygen species (ROS) (Hu et al., 2012), thereby increasing

stress resistance. Although attention has been paid to the function of sugars under salt stress, the specific mechanisms and upstream regulation of this process are still unclear. In this study, a palmitoylation transferase family member, MdPAT16, was identified in apple. In transgenic materials and VIGS fruits, MdPAT16 overexpression significantly increased soluble sugar content and root sucrose transferase activity under salt stress (Fig. 3), thereby positively modulating sugar accumulation and salt tolerance. A calcineurin B-like (CBL) protein, MdCBL1, was shown to be a substrate of MdPAT16. Further experiments demonstrated that MdPAT16 participated in the MdCBL1-MdCIPK13-MdSUT2.2 regulatory pathway through palmitoylation of MdCBL1, thereby regulating sugar accumulation (Figs. 9,10; Ma et al., 2019a).

PATs respond to abiotic stress. The loss-of-function mutant *pat10* exhibits hypersensitivity to salt stress in *Arabidopsis thaliana* (Cheng et al., 2005; Krebs et al., 2010; Bassil et al., 2011b; Barragán et al., 2012; Zhou et al., 2013). In maize, the S-palmitoylation family member ZmTIP1 interacts with the calcium-dependent protein kinase ZmCPK9 to regulate root hair length and drought tolerance (Zhang et al., 2019). In apple, MdPAT16 was identified as a DHHC-type PAT (Figure S5). It played roles not only in the modulation of salt tolerance (Figure 2), but also in the regulation of sugar accumulation (Figure 3).

The critical functions of PATs do not depend exclusively on the DHHC functional group, and not all DHHC-type PATs show palmitoyltransferase activity. Some PAT activity deficient proteins also function to the resistance to external stress, and PATs can act as cation transporters to withstand adverse circumstances (Hines et al., 2010; Ohno et al., 2012). Indeed, we could not initially exclude the possibility that the functions of MdPAT16 in salt stress resistance and sugar accumulation derived not from its palmitoylation of substrates but from its potential role as a cation transporter. However, functional complementation assays demonstrated that MdPAT16 rescued the palmitoyltransferase deficient phenotype of the *akr1p* yeast mutant, whereas MdPAT16^{C244A} failed, indicating that MdPAT16 is a typical palmitoyltransferase. These results suggested that MdPAT16 functions through its PAT activity.

354 The functions of PATs are usually achieved through palmitoylation of substrate
355 proteins. Palmitoylation regulates protein activity, protein sorting, and protein-protein
356 interactions (Hemsley et al., 2013; Zhou et al., 2013). Palmitoylation also influences
357 protein stabilization, promoting the function of substrate proteins. The human
358 palmitoyltransferase ZDHHC14 has been shown to exert a tumor suppressor function
359 through palmitoylation of its substrate proteins (Marc et al., 2014). A series of
360 calmodulin proteins in plants that respond to calcium signals have been identified as
361 PAT substrates, including CPKs (Leclercq et al., 2005; Martín and Busconi, 2000;
362 Zhang et al., 2019), CaMs (Wang et al., 2005), and CBLs (Batistič et al., 2010; Zhou
363 et al., 2013). Here, the CBL family protein MdCBL1 was identified as a direct
364 substrate of MdPAT16 using mass spectrometry. MdCBL1 directly interacted with
365 MdPAT16 *in vivo* and *in vitro*. Experiments demonstrated that the palmitoylation of
366 MdCBL1 was dependent on MdPAT16, and MdCBL1 created a phenotype
367 indistinguishable from that of MdPAT16 (Fig. 5). Therefore, MdCBL1 was a direct
368 substrate of MdPAT16.

369 Substrate proteins lose membrane localization and biological function in the
370 absence of corresponding PAT modifications. CBL2, 3, and 6 in *Arabidopsis thaliana*
371 are substrates of PAT10, and palmitoylation site deletion mutants of CBL2, 3, and 6
372 fail to localize to the tonoplast (Batistič, 2012; Zhou et al., 2013). The *cbl2* and *cbl3*
373 double mutant exhibits developmental abnormalities, including leaf tip necrosis and
374 defects in the reproductive process, that are similar to the phenotype of the *pat10*
375 single mutant (Tang et al., 2012; Zhou et al., 2013). In apple, the membrane
376 localization and function of MdCBL1 also depended on its palmitoylation. The
377 palmitoylation site mutant MdCBL1^{C3S} no longer localized to the plasma membrane,
378 and 35S-driven MdCBL1^{C3S} transgenic roots showed a functional absence of sucrose
379 transferase activity without corresponding PAT modifications (Figs. 7,9D). Thus,
380 palmitoylation modification played an important role in the function of substrate
381 proteins. Taken together, these results suggest that the function of MdCBL1 in the
382 regulation of sugar content is dependent on palmitoylation by MdPAT16.

Transgenic analyses suggested that MdCBL1^{C3S} was mislocalized from the plasma membrane to the cytoplasm and nucleus (Fig. 7) and lost its regulatory function in sugar accumulation. Ubiquitination in plants modulates the nuclear entry and degradation of substrate proteins. Therefore, a ubiquitination enzyme may facilitate the nuclear entry and degradation of MdCBL1. Studies in humans indicate that a competitive inhibition exists between palmitoylation and ubiquitination of substrate proteins, in which palmitoylation inhibits the activity of ubiquitination enzymes (Rebecca et al., 2019). The palmitoylation modifications of PD-L1 by DHHC3 significantly inhibit the ubiquitination modifications of PD-L1 (Han et al., 2019). Therefore, MdCBL1 may be modified by ubiquitination in the absence of appropriate palmitoylation. This ubiquitination modification may be directly or indirectly affected by palmitoylation.

The biological functions of CBLs are performed through interaction with CIPKs (Cheong, 2003; Li et al., 2006; Pandey et al., 2004). In *Arabidopsis*, AtCBL1 shows a conserved interaction with AtCIPK24/SOS2 that mediates sodium ion homeostasis under salt stress (Albrecht et al., 2003). In *M. domestica*, the overexpression of MdCIPK6L causes significant improvements in salt tolerance, and the heterologous expression of MdCIPK6L rescues the salt-sensitive phenotype of *sos2* (Wang et al., 2012). MdSOS2 exhibits high similarity with AtCIPK24/SOS2, which also shows high salt tolerance (Hu et al., 2012). Apple MdSUT2.2 enhances sugar accumulation and stress resistance by the phosphorylation of MdCIPK13 (Ma et al., 2019a). Our results suggest that MdPAT16 is involved in the MdCIPK13 regulatory pathway through its interactions with MdCBL1. The palmitoylation stabilization of MdCBL1 probably stabilizes the MdCBL1-MdCIPK13 protein-protein interaction and promotes the phosphorylation of MdSUT2.2, thereby causing sugar accumulation. Previous studies have also demonstrated that another *M. domestica* CIPK family member, MdCIPK22, functions similarly to MdCIPK13, interacting with MdSUT2.2 in response to drought stress to promote sugar accumulation (Ma et al., 2019b). Whether PAT is also involved in the upstream regulation of this process requires further

412 investigation.

413 MdCIPK13 and MdSUT2.2 are co-localized on the tonoplast, unlike MdCBL1
414 and MdPAT16 that co-localize to the plasma membrane. Studies on AtCBL1
415 demonstrate that when AtCIPK1 interacts with plasma-membrane-localized AtCBL1
416 or tonoplast-localized AtCBL2, the subcellular localization of AtCIPK1 changes
417 correspondently, resulting in different membrane localization (Batistič et al., 2008). A
418 similar situation may occur for MdCIPK13, which exhibits tonoplast localization
419 when interacting with MdSUT2.2 and plasma membrane localization when interacting
420 with MdCBL1. Therefore, MdCIPK13 probably migrates during the interaction with
421 different proteins, resulting in different localizations. Further experiments should be
422 performed to assess this hypothesis.

423 Taken together, previous reports and our current study suggest a working model
424 that summarizes our findings. Under salt stress in apple, MdPAT16 stabilizes the
425 expression of MdCBL1 through palmitoylation and promotes sugar accumulation
426 through the regulatory pathway of MdCBL1-MdCIPK13-MdSUT2.2 (Fig. S6).
427 Meanwhile, there exists a ubiquitin ligase that mediates the nuclear entry and
428 degradation of MdCBL1 in the absence of palmitoylation. This process may be
429 directly or indirectly regulated by palmitoylation.

430 Sugar content in apple is a decisive factor for fruit quality. Sugars accumulate to
431 maintain the intracellular ion balance in response to salt stress and also contribute to
432 fruit quality and commodity values (Hu et al., 2016). Our work reveals a new
433 mechanism for the regulation of sugar content and fruit quality and contributes to a
434 better understanding the pathway by which apple responds to salt stress. Increases in
435 carbohydrate concentration following moderate salt stress raise the question of the
436 role of carbohydrate availability in plant growth under stress. In total, our data support
437 the proposal that moderate upregulation of MdPAT16 has a potential role in the
438 promotion of fruit quality during salt stress. Therefore, our research provides new
439 ideas for the simultaneous improvement of fruit quality and stress resistance through

440 breeding methods.

441

442 **Materials and methods**

443 **Plant material, growth conditions, and salt treatments**

444 GL-3 apple (*Malus x domestica* Borkh.) tissue cultures were used for
445 transformation and stress treatments (Chen et al., 2019). GL-3 and transgenic apple
446 cultures were cultured and subcultured in Murashige & Skoog (MS) medium
447 supplemented with 0.3 mg/L 6-benzylaminopurine (6-BA), 0.2 mg/L indoleacetic acid
448 (IAA), and 0.1 mg/L gibberellin (GA). The cultures were maintained at a constant
449 temperature of 25°C under long-day conditions (16 h light/8 h dark) and were
450 subcultured every 30 days.

451 Seeds of *Malus hupehensis* were harvested and stratified at low temperature and
452 humidity for more than 30 days. When the seeds began to germinate, they were
453 transplanted into substrate under long-day greenhouse conditions (16 h light/8 h dark).
454 Four weeks after germination, seedlings of consistent size were selected for further
455 experiments.

456 Apple calli used in this article were induced from embryos of ‘Orin’ apples
457 (*Malus x domestica* Borkh.). Calli were grown and subcultured on MS medium with
458 1.5 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L 6-BA at a constant
459 temperature of 25°C in the dark and subcultured every 15 days.

460 For short-term salt treatments, 150 mM NaCl was used in hydroponic
461 experiments. For long-term salt treatments, apple plantlets were treated with 150 mM
462 NaCl under the greenhouse conditions described above.

463 Different concentrations of NaCl(0mM; 1mM; 10mM; and 100mM) were applied
464 to *Malus hupehensis* apple seedlings after germination and transplanting into
465 vermiculite. The pictures were obtained after two weeks under the same greenhouse

466 conditions described above.

467

468 **Ca²⁺-ATPase activity, Rhizosphere pH staining, and Solution pH.**

469 The activities of Ca²⁺-ATPase in *Malus hupehensis* apple seedlings were detected
470 by colorimetry method. The method was followed by Ca²⁺/Mg²⁺-ATPase activity
471 detection kit(BC0965, Solarbio).

472 The treated roots of *Malus hupehensis* apple seedlings were placed in the
473 solution mixed of 0.01% bromocresol violet, 0.2mm CaSO₄, and 0.7% Agar (pH =
474 6.5), and photographs were taken after 45 minutes in dark.

475 Solution pH was detected by PHS-3C pH-meter.

476

477 **Genetic transformation**

478 The full-length coding sequences (CDS) of *MdPAT16* (MD10G1058600) and
479 *MdCBL1* (MD00G1132600) were identified from the apple genome (GDDH13 v1.1)
480 and amplified. The CDS of *MdPAT16* and *MdCBL1* were inserted into the pRI101-An
481 plasmid with a GFP tag to build overexpression vectors. The forward and reverse
482 fragments were also inserted into the pRNAi-E vector (Song et al., 2017) to construct
483 RNAi vectors. Both overexpression vectors and RNAi vectors were introduced into
484 *Agrobacterium Tumafaciens* EHA105 competent cells (Transgen). The genetic
485 transformation procedure was performed as described in Ma et al. (2017b).

486 The full length *MdPAT16* sequence was then ligated with a GFP tag to build
487 MdPAT16-GFP, and the full length *MdCBL1* sequence was ligated with an HA tag to
488 build MdCBL1-HA. These vectors were introduced into ‘Orin’ apple calli using the
489 *Agrobacterium* method as described in Ma et al. (2017a).

490 Transgenic root systems of MdPAT16 and MdCBL1 were induced by

491 *Agrobacterium Rhizogenesis* K599 competent cells (Transgen) under *Malus*
492 *hupehensis* and GL-3 tissue culture backgrounds according to the procedure of Meng
493 et al. (2019).

494 For Arabidopsis transformation, the MdPAT16 overexpression vector was
495 introduced into *A. Tumaficiens* strain LBA4404. MdPAT16 was then transformed into
496 Col-0 using the floral dip method, and the seeds were screened on 1/2 MS medium
497 with 25 mg/L kanamycin. Positive seedlings were detected using qRT-PCR, and the
498 screened T3 generation seedlings were used for further analysis.

499

500 **RNA extraction and qRT-PCR assays**

501 Total RNA was extracted from rooted tissue cultures using the RNA Extraction
502 Kit (Tiangen). The extracted RNA was purified using the PrimeScript First Strand
503 cDNA Synthesis Kit (TaKaRa, Dalian, China), and the oligo DT Kit was used to
504 generate cDNA.

505 A 20 µl reaction system with SYBR Green Supermix (Takara) was used for
506 qRT-PCR analysis. The total reaction system included 10 µl SYBR Green mixture, 7
507 µl double distilled water, 1 µl cDNA template, and 1 µl each of upstream and
508 downstream primers. Relative expression was quantified using the (Ct) 2^{-DDCt} method,
509 and *MdActin* (GenBank accession number CN938024) was selected as the internal
510 control gene. Prism software was used to generate the chart, and the significance of
511 treatment differences was assessed by the Data processing system software using
512 One-way ANOVA method.

513

514 **Yeast functional complementation assay**

515 The yeast palmitoylation mutant *akr1p* and its original strain, BY4741, were
516 obtained from Thermo Scientific. The pYES2-DEST52 empty vector,

517 MdPAT16-pYES2, and MdPAT16^{C244A}-pYES2 vectors were transformed into akr1p
518 and BY4741. For the growth assay, yeast cells were grown to stationary phase on
519 glucose minimal liquid media. 5 µl per yeast strain with an OD₆₀₀ of approximately
520 0.5 was placed onto two individual galactose minimal agar medium plates. These
521 plates were incubated at 28 °C and 37 °C, respectively. Cells were observed under a
522 LSM880 laser scanning confocal microscope to obtain cell shape data, and the mutant
523 rate was counted using ImageJ (ver. 1.41).

524

525 **Acyl-Biotinyl Exchange (ABE) assay**

526 Auto-acylation of MdPAT16 was measured using an ABE assay.
527 MdPAT16-pYES2 and MdPAT16^{C244A}-pYES2 vectors were transformed into BY4741.
528 Total proteins were then extracted and measured by western blotting using β-actin
529 antibody and anti-V5 antibody. The detected proteins were selected for the ABE assay
530 following the methods of Wan et al. (2007) .

531

532 **Root shape, root length, surface area, and lateral root number**

533 The shape of roots were photographed by LA-S root scanner, and root length,
534 surface area, and lateral root number were detected by root analyse system software
535 by LA-S root scanner.

536

537 **Glucose; Sucrose; and Soluble sugar contents detection**

538 The sugar contents were detected of same size (about 500mg) plant samples with
539 three individual duplications. Sucrose, glucose, and soluble sugar assay kits(Keming
540 biotechnology co. LTD) were used to detected sugar contents in samples.

541

542 **Esculin uptake**

543 The roots of transgenic root systems of MdPAT16 and MdCBL1 were rinsed and
544 mounted on glass slides in 1/2 MS liquid media with 10 mM esculin under normal or
545 150mM saline conditions. Fluorescence was scanned using a 367 nm excitation
546 wavelength and a 454 nm emission wavelength as described in Ma et al. (2018).

547

548 **Virus-induced gene silencing assays**

549 Virus-induced gene silencing (VIGS) assays were performed to verify the
550 expression patterns of MdPAT16 and MdCBL1 in apple fruit. The full length CDS of
551 MdPAT16 and MdCBL1 were inserted into IL60-2 vectors, to construct
552 MdPAT16-IL60-2 and MdCBL1-IL60-2, and the IL60-1 vector was used as an
553 auxiliary plasmid. Antisense gene fragments were also used to construct
554 MdPAT16-TRV-2 and MdCBL1-TRV-2. The TRV1 vector was used as an auxiliary
555 plasmid. The TRV vectors were introduced into *A. Tumefaciens* strain LBA4404. The
556 mixed vectors and *A. Tumefaciens* solutions were injected into the peels of apple fruits,
557 and stored under 24°C with constant light. VIGS assays were performed according to
558 the method of An et al. (2018).

559

560 **Co-IP assay**

561 Proteins were extracted from apple calli that were transformed with
562 MdPAT16-GFP/HA and MdPAT16-GFP/MdCBL1-HA. The target proteins were
563 absorbed by Protein A/G agarose beads (Thermo Fisher). The absorbed proteins were
564 measured by western blotting using anti-MYC and anti-HA antibodies.

565

566 **Pull-down assay**

567 The full length of CDS of MdPAT16 and MdCBL1 were ligated to His and GST
568 targets to build MdPAT16-His and MdCBL1-GST. The resulting plasmids were
569 transformed into *Escherichia coli* BL21 (DE3; Transgene), and 10 μ M isopropyl β -D-
570 1-thiogalactopyranoside (IPTG) was used to induce for 6 hours. Proteins were then
571 separated and purified. The MdPAT16-His target protein was co-incubated separately
572 with GST and MdCBL1-GST. The mixtures were eluted from glutathione-agarose
573 beads and measured with Anti-His and Anti-GST antibodies via western blotting.

574

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

576 Bimolecular fluorescence complementation (BiFC) assays were performed to
577 verify the protein-protein interaction *in vivo*. The full-length CDS of MdPAT16 and
578 MdCBL1 were inserted into separate fluorescence plasmids to build
579 MdPAT16-pSPYNE and MdCBL1-pSPYCE. The two vectors and the empty vector
580 control were transformed into tobacco (*Nicotiana benthamiana*) leaves. LSM880 laser
581 scanning confocal microscopy (488–534 nm wavelength) was used to visualize green
582 fluorescence, and AtCBL1, a known membrane-localized protein, was used as a
583 plasma membrane marker.

584

585 **Subcellular localization**

586 MdPAT16 was ligated with a GFP target, MdCBL1 was ligated with an RFP tag,
587 and MdCBL1^{C3S} was ligated with an RFP tag. The resulting plasmids were
588 transformed into *Agrobacterium* LBA4404. Membrane-localized GFP and RFP
589 proteins were used as markers. All strains were transiently transformed into *N.*
590 *benthamiana* leaves to observe the single localization and co-localization conditions.
591 The proteins were extracted from leaves to perform a separation assay, and western
592 blotting was used to verify.

593 *Agrobacterium rhizogenes*-induced transgenic roots were scanned using an

594 LSM880 laser scanning confocal microscope (488–543 nm) to measure GFP
595 fluorescence, and FM4-64 was used as membrane-associated marker.

596

597 **Cell-free degradation**

598 The MdCBL1-GST protein was induced and mixed with total proteins extracted
599 from MdPAT16-OVE and MdPAT16-RNAi transgenic apple calli, with WT apple calli
600 proteins used as a control. The reaction mixes were incubated at 22 °C and
601 periodically sampled. The samples were boiled and measured by western blotting
602 using anti-Actin and anti-GST antibodies.

603

604 **SUPPLEMENTAL DATA**

605 **Supplementary Figure S1. Salt stress in apple promotes sugar accumulation.**

606 **Supplementary Figure S2. The MdPAT16 promoter responds to salt stress.**

607 **Supplementary Figure S3. Ectopic expression of MdPAT16 in Arabidopsis**
608 **increases sugar content and salt resistance.**

609 **Supplementary Figure S4. Phylogenetic tree analysis of MdPATs and AtPATs.**

610 **Supplementary Figure S5. Sequence analysis of MdPAT16 and PAT16 in other**
611 **species.**

612 **Supplementary Figure S6. Proposed model of the mechanism regulating sugar**
613 **content.**

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

615

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621

622 **Figure legends**

623 **Figure 1. RNAseq analysis of GL-3 tissue culture seedlings with and without**
624 **NaCl treatment.**

625 **(A)** Gene ontology enrichment of upregulated genes in NaCl-treated and WT
626 seedlings, presented according to $-\log_{10}P$ -value. **(B)** Venn diagram of the genes
627 upregulated in NaCl-treated and WT seedlings ($\log_2\text{FoldChange} > 2$). **(C)** KEGG
628 pathway classification of upregulated genes. **(D)** Normalized heatmap of sugar-related
629 genes, including amino sugar and nucleotide sugar
630 metabolism(MD01G1213100,MD02G1120200), starch and sucrose
631 metabolism(MD03G1098600, MD10G1270400), sucrose transmembrane
632 transporter(MD10G1045900), salt stress response (MD05G1119300,
633 MD06G1147900), and MdPAT16(MD10G1058600).

634

635 **Figure 2. MdPAT16 functions as a positive regulator under salt stress. (A)** Root
636 fluorescence identification of MdPAT16 overexpression transgenic roots (Bars = 100
637 μm). **(B) and (C)** Root scan analysis of root shape, root length, surface area, and
638 lateral root number of WT, MdPAT16-OVX, and MdPAT16-anti transgenic roots.
639 Results are given as mean $\pm$ SD. Letters indicate significant differences (t -test, $P < 0.01$)
640 **(D)** Glucose, sucrose, and soluble sugar contents of WT, MdPAT16-OVX, and
641 MdPAT16-anti transgenic roots. **(E) and (F)** Esculin staining of sucrose transport
642 activity in (E) WT and MdPAT16-OVX transgenic roots, and (F) MdPAT16-OVX
643 roots treated with NaCl and KCl (Bars = 100 μm).

644

645 **Figure 3. Overexpression of apple MdPAT16 increases soluble sugar content. (A)**
646 Starch staining of MdPAT16-TRV transient suppression Gala tissue culture seedlings
647 and Empty Vector controls. **(B)** Soluble sugar, glucose, and sucrose contents of
648 MdPAT16-TRV and Empty Vector controls. **(C) and (D)** visible anthocyanin
649 accumulation conditions(C) and soluble sugar, glucose, and sucrose contents (D) of
650 apple fruits from MdPAT16-IL60 (overexpression), MdPAT16-TRV (suppression) and
651 Empty Vector controls.

652

653 **Figure 4. MdPAT16 is an S-palmitoyltransferase. (A)** Growth test (above) and
654 survival test (below). Under the nonpermissive temperature of 37°C (right), wild-type
655 (BY4741) yeast cells grew well, but *akr1p* did not. Expression of MdPAT16 in *akr1p*
656 largely restored growth, but MdPAT16^{C244A} failed. **(B) and (C)** Observations of cell
657 shape using a LSM880 high resolution laser confocal microscope (B). The cell shape
658 of MdPAT16/*akr1p* was indistinguishable from WT, whereas that of MdPAT16^{C244A}
659 differed. Quantitative statistics of all four genotypes grown at 37°C (C). **(D)**
660 MdPAT16-V5/*akr1p* and pYES2-V5/*akr1p* by ABE assay were measured by Western
661 blotting with anti-V5 antibody to demonstrate that apple MdPAT16 is auto-acylated.

662

663 **Figure 5. MdCBL1 is a direct substrate of MdPAT16. (A)** in vivo Co-IP assays
664 between MdPAT16 and MdCBL1 by western blotting with anti-HA and anti-GFP
665 antibodies. **(B)** in vitro GST pull-down assays with MdPAT16-HIS and
666 MdCBL1-GST. Proteins immunoprecipitated with GST-beads were detected using
667 anti-HIS antibody. **(C)** BiFC was performed to test the interaction between MdPAT16
668 and MdCBL1, and AtHIR1-RFP was co-injected as a plasma membrane marker. Bars
669 = 10 μm. **(D) and (E)** The interaction between MdPAT16 and MdCBL1 was
670 visualized with a dual-luciferase reporter system (D) and fluorescence activity was
671 observed with and without NaCl (E).

672

673 **Figure 6. MdCBL1 is palmitoylated by MdPAT16 on the 3rd cysteine residue. (A)**
 674 in vivo Co-IP assays between MdPAT16/MdCBL1-HA and
 675 MdPAT16-RNAi/MdCBL1-HA double transgenic apple calli. The palmitoylation of
 676 MdCBL1 was detected using anti-HA antibodies. **(B)** Yeast functional
 677 complementation assays using MdCBL1^{C3S}/akr1p and MdCBL1^{C138S}/akr1p
 678 demonstrated that neither point mutant of MdCBL1 was auto-acylated. **(C)** ABE
 679 assays demonstrated that the 3rd but not 138th cysteine residue determined the
 680 palmitoylation of MdCBL1.

681

682 **Figure 7. Localization of MdCBL1 to the plasma membrane depends on the**
 683 **function of MdPAT16. (A)** Subcellular localization of MdCBL1-RFP and
 684 MdPAT16-GFP in *N. benthamiana* leaves. AtCBL1-GFP and AtHIR1-RFP were used
 685 as plasma membrane markers. **(B)** Subcellular localization of MdCBL1^{C3S}-RFP. **(C)**
 686 Qualitative detection of MdCBL1 and MdCBL1^{C3S} in different cellular compartments
 687 by western blotting. Anti-Histone3 and anti-actin were used as loading controls for
 688 nuclei and cytoplasm, respectively. AtHIR1-RFP was co-injected as a loading control
 689 for plasma membrane. **(D)** Subcellular localization of MdCBL1/WT,
 690 MdCBL1/MdPAT16-OVE, MdCBL1/MdPAT16-RNAi, and MdCBL1^{C3S}/
 691 MdPAT16-OVE in transgenic roots of GL-3.

692

693 **Figure 8. MdPAT16 stabilizes MdCBL1 through palmitoylation. (A), (B) and (C)**
 694 Cell-free degradation assay in which MdCBL1-GST was recombined with total
 695 proteins extracted from WT (A), MdPAT16-OVE (B), and MdPAT16-RNAi (C) apple
 696 calli under a water bath for 1, 2, and 3 h. The expression of MdCBL1 was measured
 697 by western blotting. **(D)** Cell-free degradation assay in which MdCBL1^{C3S}-V5 was
 698 recombined with total proteins extracted from MdPAT16-OVE apple calli under a

Figure 8. MdPAT16 stabilizes MdCBL1 through palmitoylation. (A), (B) and (C) Cell-free degradation assay in which MdCBL1-GST was recombined with total proteins extracted from WT (A), MdPAT16-OVE (B), and MdPAT16-RNAi (C) apple calli under a water bath for 1, 2, and 3 h. The expression of MdCBL1 was measured by western blotting. (D) Cell-free degradation assay in which MdCBL1^{C3S}-V5 was recombined with total proteins extracted from MdPAT16-OVE apple calli under a water bath for 1, 2, and 3 h.

699 water bath for 1, 2, and 3 h.

700

701 **Figure 9. MdCBL1 is required for sugar accumulation.** (A) and (B) Anthocyanin
702 accumulation of MdCBL1-IL60, MdCBL1-TRV and Empty Vector apple fruits (A),
703 and MdPAT16-IL60/MdCBL1-TRV, IL60/MdCBL1-TRV, and Empty Vector apple
704 fruits (B). (C) Soluble sugar, glucose, and sucrose contents of different fruits. (D)
705 Esculin uptake assay for sucrose transport activity in transgenic roots of MdCBL1,
706 MdCBL1^{C3S}, and MdCBL1-RNAi.

707

708 **Figure 10. MdPAT16 mediates sugar accumulation through the MdCIPK13**
709 **pathway.** Anthocyanin accumulation (A) and soluble sugar, glucose, and sucrose
710 contents (B) of MdCIPK13-IL60, MdCIPK13-TRV, MdPAT16-IL60/
711 MdCIPK13-TRV, and Empty Vector apple fruits.

712

713

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