Brassinosteroid signaling component SIBES1 promotes tomato fruit softening through transcriptional repression of *PMEU1*

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ABSTRACT

Firmness is one of the most important factors that affect postharvest properties of tomato fruit. However, the regulatory mechanism underlying firmness formation in tomato fruit is poorly understood. Here, we report a novel role of SIBES1, a transcriptional factor (TF) mediating brassinosteroid (BR) signaling, in tomato fruit softening. We first found that SIBES1 promotes fruit softening during tomato fruit ripening and postharvest storage. RNA-seq analysis suggested that *PMEU1*, which encodes a pectin de-methylesterification protein, might participate in SIBES1-mediated fruit softening. Biochemical and immunofluorescence assays in *SIBES1* transgenic fruits indicated that SIBES1 inhibited *PMEU1*-related pectin

de-methylesterification. Further molecular and genetic evidence verified that *SIBES1* directly binds to the E-box in the promoter of *PMEU1* to repress its expression, leading to the softening of the tomato fruits. Loss-of-function *SIBES1* mutant generated by CRISPR/cas9 showed firmer fruits and longer shelf life during postharvest storage without the color, size and nutritional quality alteration. Collectively, our results indicated the potential of manipulating *SIBES1* to regulate fruit firmness via transcriptional inhibition of *PMEU1* without negative consequence on visual and nutrition quality.

Key words: tomato, fruit, *SIBES1*, *PMEU1*, firmness

Introduction

Brassinosteroids (BRs) are well-characterized phytohormone that are critical for plant vegetative growth, development, and response to environmental stimulus, influencing many important agronomic traits (He et al., 2005; Yu et al., 2011; Chen et al., 2019; Cui et al., 2019; Nolan et al., 2020). Recently, BRs were found to be actively synthesized and accumulated during tomato fruit ripening, indicating an important role of BR signaling in fruit (Li et al., 2016; Hu et al., 2020). Thus, the role of BRs in the regulation of fruit ripening remains to be further investigated considering the potential agricultural and research value. Genetic and molecular studies in *Arabidopsis* have revealed many core components of BRs signaling pathway, which constitutes a complete signal pathway from cell surface BR receptor to downstream transcription factors that regulate the expression of BR-regulated genes (Ye et al. 2011; Guo et al. 2013). *BRI1*-*EMS-SUPPRESSOR1* (*BES1*), a key basic helix-loop-helix TF in the BR signaling pathway, balances plant growth and environment stress tolerance (Yu et al., 2011; Nolan et al., 2020) via binding a conserved E-box (CANNTG) and BRRE (CGTGC/TG) elements of its target genes (Cui et al., 2019; Jiang et al., 2019). Although the mechanism of *BES1* transcription factor in regulating plant growth and development has been well elucidated in *Arabidopsis*, its function in fruit softening has remained elusive.

Tomato (*Solanum lycopersicum*) is not only one of the most important vegetables

worldwide, but also a model system for researchers studying fruit ripening. The softening process during ripening indicated by firmness determines overall palatability, transportability, and shelf life of tomato fruit. Fruit firmness is determined by diverse factors, including cell wall structure (Seymour et al., 2013), cell turgor (Saladié et al., 2007), and cuticle properties (Yeats and Rose, 2013). Among them, cell wall and alterations of its structure are considered as the predominant factors, such as changes in the complex of microfibrils and polysaccharides, which are composed of hemicellulose, cellulose, pectin, and other structural proteins. Pectins, known as pectic polysaccharides, are a group of complex polymers containing homogalacturonan (HG), rhamnogalacturonan-I, and rhamnogalacturonan-II and located in the primary cell wall and middle lamella with a high amount (Brummell, 2006). A wide range of enzymes have been investigated to explore the genetic, molecular and biochemical basis of fruit softening. Methyl ester group is removed from HG in a reaction catalyzed by pectin methylesterase (PME/PE, EC 3.1.1.11), and the de-methylesterified pectin forms “egg box” through a cross-link with Ca^{2+} , strengthening the cell wall (Senechal et al., 2014; Silva-Sanzana et al., 2019). In addition, pectin degradation by pectate lyase (PL) and polygalacturonase (PG) were paid attention to targeted control of fruit softening (Ulusik et al., 2016; Wang et al., 2019). Nowadays, tomato cultivars harboring *ripening inhibitor (rin)* and *non-ripening (nor)* mutation produce firm fruits and confer long shelf life. However, they often have a deficiency in coloration and poor nutritional value because of the disturbed ethylene-regulated fruit ripening (Klee and Giovannoni, 2011; Osorio et al., 2011; Osorio et al., 2020). Therefore, targeted control of fruit softening without negative consequences on fruit quality by dissecting the regulatory mechanism becomes an important goal to extend shelf life and improve transportability.

In this study, the specific role of *SIBES1* in softening was investigated in tomato. Firmness phenotypes of *SIBES1*-overexpressing and silencing fruits indicated its positive role in softening. RNA sequencing (RNA-seq) in silencing fruits elucidated the possible target genes, including *pectin methylesterase ubiquitously 1 (PMEU1)*, responsible for softening. More biochemical, molecular and genetic experiments

further reveal that *SIBES1* inhibited PME1-associated pectin metabolic pathway and directly mediated transcriptional regulation of target gene involved in softening. Interestingly, knockout of *SIBES1* in tomato prolonged shelf life without detrimental effects on nutrition quality, implying *SIBES1* could be a potential powerful biotechnological tool in tomato breeding for improvement of shelf life.

Results and discussion

Functional identification of *SIBES1* in tomato

The central role of BES1 in mediating growth and stress response has been widely investigated, but few studies focused on its role in fruit development. To verify the function of BES1 in fruit development, we identified *SIBES1*, the homologous gene of *AtBES1* in tomato (Supplemental Figure S1A and B), and analyzed its expression pattern in BR-deficient and BR-insensitive tomato mutants. The expression level of *SIBES1* was decreased in both BR-deficient mutant *d^x* and BR-insensitive mutant *cu-3* that harbors a mutation in the BR receptor gene *BRI1* (Supplemental Figure S1C). To explore the function of *SIBES1* in tomato, we then generated *SIBES1* overexpressing transgenic lines, *SIBES1-OX-3* and *SIBES1-OX-8*, as well as *SIBES1* RNAi lines, *SIBES1-RNAi-8* and *SIBES1-RNAi-9* (Figure 1A). In *Arabidopsis*, the gain-of-function mutant of *AtBES1* (*bes1-D*) and *AtBZR1* (*bzr1-D*) have constitutive BR responses including tolerance to BR biosynthesis inhibitors, propiconazole (Pcz) or brassinazole (BRZ), in hypocotyl elongation assays in the dark (Yin et al., 2002; Wang et al., 2002; Hartwig et al., 2012). The hypocotyl elongation of *SIBES1-OX-3* and *SIBES1-OX-8* was not affected by 0.5 μ M Pcz in the dark, whereas that of *SIBES1-RNAi-8* and *SIBES1-RNAi-9* was more susceptible to inhibitor (Supplemental Figure S2A and B), which was in agreement with the phenotypes of *Arabidopsis BES1* mutants. In addition, BES1/BZR1 repressed the transcription of BR biosynthetic genes, such as *DWARF*, *CPD*, and *DWARF4*, through feedback regulation (He et al., 2005; Yu et al., 2011). In the present study, expression levels of the BR biosynthetic genes (*SIDWARF*, *SICPD*, *SICYP724B2*, and *SICYP90B3*) were significantly down-regulated in *SIBES1-OX-3* and *SIBES1-OX-8*, but up-regulated in

121 *SIBES1-RNAi-8* and *SIBES1-RNAi-9* fruits (Supplemental Figure S2C). These results
122 indicate that *SIBES1* confers conserved function as a transcription factor in BR
123 signaling pathway between tomato and *Arabidopsis*.

124 ***SIBES1* promotes tomato fruit softening without affecting nutritional quality**

125 Transgenic *SIBES1* fruits were used to elucidate the role of BES1 in fruit
126 development. As shown in Figure 1B, no significant difference in fruit appearance at
127 different developmental stages was found between wild type Ailsa Craig (AC) and
128 transgenic tomato lines. However, *SIBES1-OX* lines and *SIBES1-RNAi* lines exhibited
129 reduced and increased fruit firmness compared with AC, respectively (Figure 1C).
130 These varying degrees of firmness correspond to the *SIBES1* expression levels in
131 different transgenic lines (Figure 1A), which demonstrated that *SIBES1* negatively
132 regulates tomato fruit firmness. The production of ethylene in *SIBES1* transgenic
133 tomato fruits and AC was similar at each development stage (Supplemental Figure
134 S3), indicating *SIBES1*-mediated softening is via ethylene-independent manner and
135 different with firmer but ripening-impaired mutants, *rin* and *nor*. Meanwhile,
136 overexpressing or silencing of *SIBES1* did not affect nutritional qualities
137 (Supplemental Table 3) and the expression levels of most nutrition related genes,
138 including carotenoid and ascorbic acid biosynthetic genes, in *SIBES1-RNAi* kept at the
139 same level with wild type (Supplemental Table 4).

140 Furthermore, treatment of wild type AC fruits with 3 μ M 24-epibrassinolide
141 (EBL) significantly promoted fruit softening (Supplemental Figure S4A). Meanwhile,
142 EBL treatment recovered fruit softening in the BR-deficient mutant *d^{im}* (Supplemental
143 Figure S4B), indicating the role of BR in cell wall homeostasis. Fruit firmness is
144 usually essential in determining fruit shelf life. We also detected a complete loss of
145 fruit integrity and shorter shelf life in *SIBES1-OX* but longer shelf life in
146 *SIBES1-RNAi* when compared with that of the wild type (Figure 1D). The *SIBES1-OX*
147 lines have similar phenotype as the loss of integrity in fruits with inhibited PME
148 activity (Tieman et al., 1992; Tieman and Handa, 1994).

149 To reveal firmness-related genes or metabolism processes that participate in
150 *SIBES1*-induced fruit softening, *SIBES1-RNAi* fruit was harvested at the breaker (B)

151 and red ripe (R) stages for RNA-seq. Gene ontology (GO) analysis on RNA-seq data
 152 in fruit development period category ($p < 0.05$) showed that the expression of 24
 153 genes related to fruit firmness, including the pectin metabolism gene *PME* and
 154 hemicellulose metabolism gene *XTH*, was differentially regulated by *SIBES1* (Figure
 155 1E). The expression pattern of some of those genes, such as *PG2* and *CSLC12*, varied
 156 in different development stages, whereas the others, such as *PE1*, *CD2*, and *XTHs*,
 157 were significantly expressed at only one stage. Only the expression levels of *PMEU1*
 158 were increased at both B and R stages, suggesting that *SIBES1* might affect fruit
 159 softening through regulating *PMEU1*, a gene involved in pectin metabolic pathway.

160 Taken together, these results demonstrated that the BR signaling pathway
 161 component *SIBES1* positively regulates fruit softening through influencing pectin and
 162 other metabolic pathways.

163

164 *SIBES1* inhibited *PMEU1*-associated pectin metabolic pathway

165 Previous studies have established the role of *PME*, especially *PMEU1*, in
 166 modulating fruit softening (Tieman and Handa, 1994; Phan et al., 2007). Fruit with
 167 low total *PME* activity shows a reduced degree of methylesterification, decreased fruit
 168 firmness, and complete loss of fruit integrity (Tieman et al., 1992; Tieman and Handa,
 169 1994; Phan et al., 2007). Three tomato *PME* genes, *PME ubiquitously 1* (*PMEU1*),
 170 *PE1*, and *PE2*, have been identified and their function in fruit firmness has been
 171 proved (Gaffe et al., 1997; Hall et al., 1994). Antisense inhibition of *PMEU1* reduces
 172 the total *PME* activity and accelerates fruit softening (Phan et al., 2007), but *PE1* and
 173 *PE2* have no such effect on firmness of tomato fruit (Wen et al., 2013). Our RNA-seq
 174 results also indicated that *PMEU1* and *PE1* were up-regulated in firmer *SIBES1-RNAi*
 175 fruits (Figure 1E). As discussed above, *SIBES1-OX* fruits phenocopy the fruit with
 176 repressed *PME* activity. Based on these results, we hypothesize that fruit softening in
 177 *SIBES1-OX* lines was caused by reduction of *PME*, mostly *PMEU1*, which is involved
 178 in pectin metabolic pathway

179 To test this hypothesis, we measured the change in pectin metabolic pathway in
 180 *SIBES1* transgenic fruit. The total *PME* activity was reduced in *SIBES1-OX-3* and

181 *SIBES1-OX-8* from mature green (MG) to R stage, and it was increased in
 182 *SIBES1-RNAi-8* and *SIBES1-RNAi-9* from B to R stage (Figure 2A). PME catalyzes
 183 the demethylesterification of pectin, and then attenuates the degree of pectin
 184 methylesterification (DM) (Senechal et al., 2014; Figure 2C). As the total PME
 185 activity decreased, the DM of soluble pectin was increased in *SIBES1-OX-3* and
 186 *SIBES1-OX-8* from MG to R stage and decreased in *SIBES1-RNAi-8* and
 187 *SIBES1-RNAi-9* from B to R stage (Figure 2B). Additionally, the content and location
 188 of highly methylesterified HG, de-esterified HG, and “egg box” were identified
 189 through the immunofluorescence of LM20, LM19, and 2F4, respectively (Figure 2D).
 190 Compared with the wild type, the signal of methylesterified HG was higher in
 191 *SIBES1-OX* and lower in *SIBES1-RNAi* (Figure 2D), whereas the signal of
 192 de-esterified HG was lower in *SIBES1-OX* and higher in *SIBES1-RNAi* fruit (Figure
 193 2D), consistent with the total PME activity and DM in *SIBES1* transgenic fruit. The
 194 “egg box” signal was significantly elevated in *SIBES1-RNAi* and lowered in
 195 *SIBES1-OX* when compared with that in the wild type (Figure 2D). Given the positive
 196 role of the “egg box” in cell wall strengthening (Senechal et al., 2014; Silva-Sanzana
 197 et al., 2019), the immunolocalization results suggested that decreased content of the
 198 “egg box” structure in *SIBES1* fruit was the direct reason for *SIBES1*-induced fruit
 199 softening. Calcofluor-white staining showed no major differences in cell size or
 200 patterning between transgenic lines (Supplemental Figure S5). The reduced activity of
 201 PME and decreased content of its products, as revealed by DM and
 202 immunolocalization experiments, support our hypothesis that PME, which is involved
 203 in the pectin metabolic pathway, was repressed in *SIBES1-OX* fruit.

204 Then, the PME gene required for the fruit softening process was investigated. To
 205 date, *PMEU1*, has been proved to be the only PME gene that affects both total PME
 206 activity and fruit firmness in tomato (Phan et al., 2007). The expression level of
 207 *PMEU1* was decreased in *SIBES1-OX* from the MG to the R stage and increased in
 208 *SIBES1-RNAi* from the B to the R stage (Figure 3A); this corresponds to the total
 209 PME activity and DM changes (Figure 2A and B). Interestingly, the expression level
 210 of *PMEU1* was also down-regulated in EBL-treated fruits, while EBL treatment offset

the down-regulated *PMEU1* expression in d^{im} (Supplemental Figure S6A and B), suggesting a role of BR in repressing *PMEU1* expression. These results indicated that both *PMEU1* mediated pectin metabolic pathway and the expression level of *PMEU1* was repressed by SIBES1. All genetic and molecular results suggest that SIBES1 represses the expression of *PMEU1* to promote fruit softening.

SIBES1 mediated direct transcriptional inhibition of *PMEU1* to confers fruit softening

To test the hypothesis of possible inhibited expression of *PMEU1* by SIBES1, we carried out further experiments to prove the transcriptional regulation of SIBES1 in the expression of *PMEU1*. We first selected several candidate genes including *PMEU1* on the basis of our results and previous proteomics data (Liu et al., 2016). ChIP-qPCR results showed a significantly higher enrichment for the promoters of *PMEU1* than for *PEI* (Figure 3B). Transcriptional factor BES1 preferentially binds to E-box (CANNTG) or BRRE-box (CGTG(T/C)G) to regulate the expression of downstream genes in *Arabidopsis* (Yu et al., 2011). The two binding sites, E-box and BRRE-box, were located at -465 bp and -289 bp of the *PMEU1* promoter as predicted by Jaspar database (<http://jaspar.genereg.net/>) (Figure 3C). The ChIP-qPCR results further indicated enrichment of SIBES1 binding to the region containing E-box but not to that of the BRRE-box (Figure 3C), suggesting that SIBES1 might regulate *PMEU1* expression by binding to the E-box.

To test if E-box is necessary for SIBES1 regulation of the *PMEU1* gene, we generated a *PMEU1pro:LUC* reporter, in which luciferase (LUC) was fused with the *PMEU1* promoter. Co-expression of *PMEU1pro:LUC* with the *35Spro:SIBES1* construct led to a significantly reduced luminescence intensity, suggesting that SIBES1 represses the expression of *PMEU1*. Importantly, when *PMEU1pro:LUC* was replaced by the *PMEU1proΔEbox:LUC*, in which the E-box of the *PMEU1* promoter involved in the transcriptional regulation of SIBES1 on *PMEU1* was deleted, SIBES1-mediated repression of the *PMEU1* expression was abolished (Figure 3D to F).

We then conducted electrophoretic mobility shift assay (EMSA) with purified

241 SIBES1-His and a 20-bp DNA probe containing this E-box motif. SIBES1-His bound
242 to the DNA probe, and this binding was successfully outcompeted by unlabeled DNA
243 probe but not by the DNA probe without E-box. These results suggested that SIBES1
244 directly binds to the *PMEUI* promoter through E-box (Figure 3G and H). E-box was
245 the binding site of SIBES1 to repress gene expression in tomato. Although
246 BES1/BZR1 mostly bind to E-box to activate gene expression and to BRRE to repress
247 genes, there are exceptions (Nolan et al., 2020). A recent study also showed that
248 OsBZR1 bind to E-box to inhibit downstream gene expression in rice (Fang et al.,
249 2020).

250 Silencing *PMEUI* caused softer fruits with lower total PME activity and exhibited
251 a complete loss of fruit integrity and shorter shelf life (Figure 4A to D), which was in
252 accordance with previous studies (Tieman and Handa, 1994; Phan *et al.*, 2007). More
253 importantly, knocking down *PMEUI* in the *SIBES1-RNAi* background
254 (TRV-*PMEUI*/*SIBES1-RNAi*) suppressed the *SIBES1-RNAi* phenotypes of higher
255 firmness and the longer shelf life. Fruit firmness, shelf life, and total PME activity of
256 TRV-*PMEUI*/*SIBES1-RNAi* were almost the same as wild type, indicating that
257 SIBES1-mediated fruit softening is mainly mediated by the inhibition of *PMEUI*
258 (Figure 4 A to D).

259 Taken together, these results demonstrated that SIBES1 directly binds to the
260 E-box of *PMEUI* to repress its expression, thereby promoting fruit softening.

261

262 **Gene editing of *SIBES1* by CRISPR/Cas9 enhances fruit firmness without** 263 **negative effect on nutritional quality**

264 Above results suggested *SIBES1* may be a potential gene that can be used for
265 breeding tomatoes with longer shelf life and maintaining optimum flavor. Then we
266 generated *SIBES1-KO* by using CRISPR/Cas9 through targeting the
267 5'-TAGTTGGTGATGAAAGAGG TGG-3' in the second extrinsic of SIBES1
268 antisense strand with *pYLCRISPR/Cas9* (Ma et al., 2015), and the result showed that
269 the fruit of *SIBES1-KO*, a *SIBES1* knockout mutant line, had higher firmness and
270 longer shelf life without loss of nutritional quality (Supplemental Figure S7 and Table

271 3).

272

273 In summary, this study provides genetic, biochemical, and molecular evidence for
274 SIBES1-mediated repression of the pectin metabolic pathway and fruit softening. At
275 the transcriptional level, SIBES1 directly binds to the E-box of the *PMEU1* promoter
276 to repress *PMEU1* expression, thereby promoting fruit softening (Figure 4 E). BR also
277 plays a positive role in this process. Fruits with silenced *SIBES1* described in this
278 study provide a promising solution to improve fruit firmness and extended shelf life
279 without negative effect on visual and nutritional quality.

280

281 **Methods**

282 **Plant Materials and Growth Conditions**

283 All transgenic lines were constructed in tomato cultivars Ailsa Craig (AC). Cultivars
284 *Lycopersicon pimpinellifolium* (Spim), Condine Red (CR), and Craigella are the
285 parental line of *cu-3* (Scheer et al., 2003), *d^{im}*, and *d^x* (Li et al., 2015), respectively.
286 Plants were cultivated under a 16 h photoperiod (22/28 □, night/day). Fruits ripening
287 stages were defined as described previously (Giovannoni, 2004). More details are
288 described in Supplemental Methods.

289

290 **Vector Constructs and Plant Transformation**

291 Recombinant plasmid *35S pro:SIBES1-myc* and *pBIN19-SIBES1-RNAi* were used for
292 *SIBES1-OX* and *SIBES1-RNAi* construct, respectively. Constructs were introduced into
293 AC via *Agrobacterium*-mediated transformation (Shao et al., 2019). Homozygous T2
294 transgenic plants were selected based on resistance to kanamycin and then used for
295 further tests. More details are described in Supplemental Methods.

296

297 **Generation of the CRISPR/Cas9 Mutant**

298 The guide RNA sequence CCACCTCTTTCATCACCAACTA for *SIBES1* was cloned
299 into *pYLCRISPR/Cas9* as described previously (Ma et al., 2015). After inducing
300 construct into AC by *Agrobacterium*-mediated transformation, sequences containing

301 guide RNA target sites were amplified and sequenced to confirm mutations in targeted
302 regions. Homozygous T2 transgenic plants were selected for further experiments.

303

304 **Chemical Treatment**

305 AC and d^{im} at mature green stage were treated with 24-epibrassinolide (EBL, Sigma,
306 St. Louis, MO) as described previously (Liu et al., 2014) and then collected at 1st, 3rd,
307 6th, and 9th day for further tests. Tomato seeds were sown in half-strength MS
308 containing 0.5 μ M propiconazole (Pcz, Sigma, St. Louis, MO). Plants were grown at
309 28 °C with total darkness for 6 d, then the lengths of seedlings hypocotyls and roots
310 were measured through software ImageJ after photographed.

311

312 **Firmness Determination**

313 Firmness was tested at the fruit equatorial region with a texture analyzer (TA-XT2i,
314 Godalming, UK) and a 7.5 mm probe with 1 mm s⁻¹ penetration speed as described
315 previously (Liu et al., 2018).

316

317 **RNA Extraction and Relative Quantitative PCR**

318 For RNA extraction, 0.1g of leaves or fruits was mixed with 1 mL RNA iso plus
319 according to manufacturer's instruction (Takara, Kusatsu, Japan), then RNA was
320 reverse-transcribed into cDNA using PrimeScript RT reagent with gDNA Eraser
321 (Takara, Kusatsu, Japan). TB Green (Takara, Kusatsu, Japan) was then used in Step
322 One Real-Time PCR System (Applied biosystem, CA, USA) for relative quantitative
323 PCR (qPCR). The gene-specific primers used were listed in Supplemental Table 2.

324

325 **RNA-Seq**

326 AC and *SIBES1-RNAi-8* fruits at breaker and red ripe stages were collected for total
327 RNA extraction and Illumina MiSeq library was constructed as described by
328 manufacturer's instructions (Illumina, San Diego, CA, USA) and then sequenced with
329 the Illumina Miseq platform.

330

331 **ChIP-qPCR, Transient Expression Assay in Tobacco and EMSA**

332 Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) for fruits was
333 performed following previous reports (Liu et al., 2019). Used primer pairs were listed
334 in Supplemental Table 2. Transient expression assay in tobacco (*Nicotiana*
335 *benthamiana*) and EMSA was performed as described previously (Shao et al., 2019).
336 More details are described in Supplemental Methods.

337

338 **Determination of PME Enzyme Activity and Degree of Pectin** 339 **Methylesterification**

340 Total PME activity and degree of pectin methylesterification (DM) were determined as
341 previously reported (Tucker et al., 1992; Freitas et al., 2012; Kyomugasho et al., 2015;
342 Chylinska et al., 2016). More details are described in Supplemental Methods.

343

344 **Immunofluorescence**

345 Immunofluorescence was performed as described previously (Silva-Sanzana et al.,
346 2019). Fresh pericarps were fixed with FAA and submerged with LR White resin
347 (Sigma, St. Louis, MO) to obtain sections for treating with LM20, LM19, and 2F4
348 antibodies (Plantprobes, Leeds, UK). More details are described in Supplemental
349 Methods.

350

351 **Virus-Inducing Gene Silencing**

352 A coding region fragment of *PMEU1* (1-300 bp) was inserted into pTRV2 to generate
353 pTRV2-*PMEU1*. pTRV1 and pTRV2-*PMEU1* were induced into AC fruits following
354 the previous protocol (Fu et al., 2005).

355

356 **Carotenoid and Ascorbic Acid Analysis**

357 The concentrations of carotenoid and ascorbic acid were determined as described
358 previously (Liu et al., 2018).

359

360 **Data Availability**

361 The RNA-seq data of this study were submitted to the NCBI Sequence Read Archive
362 (SRA) database (<http://www.ncbi.nlm.nih.gov/sra/>) with the BioProject ID
363 PRJNA635540.

364

365 **ACCESSION NUMBERS**

366 The accession numbers for the genes described in this report are as follows: *SIBES1*
367 (Solyc04g079980), *PMEU1* (Solyc03g123630), *PE1* (Solyc07g064170), *SIDWARF*
368 (Solyc02g089160), *SICPD* (Solyc06g051750), *SICYP724B2* (Solyc07g056160),
369 *SICYP90B3* (Solyc02g085360), *LOXA* (Solyc08g014000), *ACTIN2*
370 (Solyc11g005330).

371

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377

378 **AUTHOR CONTRIBUTION**

379 Q.W., C.L., and H.L. designed the research. H.L., M.Z., C.J., M.Q., S.H., F.M., Z.S.,
380 and D.L. performed the research. H.L. D.L., and Y.L. analyzed data. H.L., L.L, C.J.,
381 and Q.W. wrote the manuscript.

382

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389

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Figure 1. Phenotypes of *SIBESI-OX* and *SIBESI-RNAi* Tomato Fruits.

(A) Gene expression levels of *SIBESI* in leaves and fruits of *SIBESI-OX* and *SIBESI-RNAi* plants. L, leaves. MG, mature green stage. B, breaker stage. P, pink stage. R, red ripe stage. Data shown represent the means $\pm$ SD of three biological replicates. Different letters indicate significant difference compared to AC (wild type) (one-way ANOVA with Tukey's test, $p<0.05$).

(B) Phenotypes of AC (wild type), *SIBESI-OX* and *SIBESI-RNAi* tomato fruits. Pictures were taken at four developmental stages of fruit. MG, mature green; B, breaker; P, pink; R, red ripe. Scale bar = 1 cm.

(C) Fruit firmness of AC (wild type), *SIBESI-OX* and *SIBESI-RNAi* fruits at different development stages. MG, mature green. B, breaker. P, pink. R, red ripe. Data shown represent the means $\pm$ SD of twenty-four biological replicates from at least six independent fruits. Different letters indicate significant difference compared to AC (wild type) (one-way ANOVA with Tukey's test, $p<0.05$).

(D) Tomato shelf life of AC (wild type), *SIBESI-OX* and *SIBESI-RNAi* fruits. Fruits were harvested at red ripe stage and stored at room temperature. The progression of fruit deterioration was recorded by time-lapse photography. Time after harvest is specified by days. The storage condition was 25°C and 35% relative humidity. Scale bar = 1 cm.

(E) RNA-seq results and related metabolic pathway of *SIBESI-RNAi* fruits at B and R stages. A heat map showing the expression pattern of firmness related genes in *SIBESI-RNAi* fruits and wild type. The values of fold change ($\log_2$) with or without significance ($p<0.05$) were represented as coloured block with or without asterisk, respectively. Each group contains three biological replicates. B, breaker stage. R, red ripe stage. Accession number, name, description and the value of fold change of genes in figure were listed in Supplemental Table 1.

Figure 2. SIBES1 Represses the Pectin Metabolic Pathway.

(A-B) Total pectin methylesterase (PME) activity (A) and degree of methylesterification (DM) of soluble pectin (B) in *SIBES1-OX* and *SIBES1-RNAi* fruits at different development stages. MG, mature green stage. B, breaker stage. P, pink stage. R, red ripe stage.

(C) Schematic representation of the relationship between pectin methylesterase (PME) and cell wall strengthening.

(D) Immunolocalization of highly methylesterified HG (homogalacturonan), de-esterified HG and Ca²⁺ cross-linked HG in *SIBES1-OX* and *SIBES1-RNAi* fruits at red ripe stage. Monoclonal LM20 antibody probe recognizing highly methylesterified HG, LM19 probe recognizing de-esterified pectin, and 2F4 probe recognizing Ca²⁺ cross-linked HG were used to label tomato pericarp tissue. Representative sections of fruits from each of AC (wild type), *SIBES1-OX-3* and *SIBES1-RNAi-8* lines are presented. Scale bar represents 100 μ m. The right graphs show the relative fluorescence signal of each antibody. Relative signals are calculated through software ImageJ. Values were normalized with respect to AC (wild type).

In (A-B) and (D) Each data point represents means $\pm$ SD of three determinations. Different letters

576 indicate significant difference between different groups (one-way ANOVA with Tukey's test,
577 $p<0.05$).
578

Figure 3. SIBES1 Represses the Transcriptional Expression of *PMEU1*.

(A) Relative expression levels of *PMEU1* in *SIBES1-OX* and *SIBES1-RNAi* fruits at different development stages. MG, mature green stage. B, breaker stage. P, pink stage. R, red ripe stage.

Each data point represents means $\pm$ SD of three determinations. Different letters indicate significant difference between different groups (one-way ANOVA with Tukey's test, $p<0.05$).

(B) ChIP-qPCR assays showing that SIBES1 associated with the locus of candidate genes, *LoxA*, *PMEU1* and *PE1*. Chromatin of transgenic plants expressing *35Spro:SIBES1-myc* (*SIBES1-myc*) was immunoprecipitated with anti-myc antibody, and results of gene *ACTIN7* served as control. The relative enrichment for ChIP signal was displayed as the percentage of total input DNA. *PMEU1* locus contains both E-box and BRRE-box. Values are means $\pm$ SD of three biological replicates. Statistical analysis was performed with ANOVA. Bars with asterisks indicates significant difference ($*p<0.05$).

(C) The upper graph, schematic diagram of *PMEU1*, indicating the amplicons used for ChIP-qPCR. Position of E-box and BRRE-box (BR Response Element) are indicated. The below graph, ChIP-qPCR assays, showing that SIBES1 associated with *PMEU1* locus in tomato fruits. The relative enrichment for SIBES1 at two *PMEU1* promoter motifs, E-box and BRRE-box, was calculated against the *ACTIN2* promoter. AC (wild type) without and with antibody were set as blank control and negative control, respectively. *SIBES1-myc* without antibody was also set as negative control. The mean value of two technical replicates was recorded for each biological replicate. Values are means $\pm$ SD of three biological replicates. Different letter indicates a significant difference among groups for each locus (one-way ANOVA with Tukey's test, $p<0.05$).

(D) Transient expression assays showing that SIBES1 represses *PMEU1* expression. Representative images of *N. benthamiana* leaves are taken 48 h after infiltration. The bottom panel indicates the infiltrated constructs.

(E-F) Luminescence intensity (E) and *SIBES1* expression level (F) in different treatment as indicated in (D). Values are means $\pm$ SD of six biological replicates. Different letter indicates a significant difference among groups for each locus (one-way ANOVA with Tukey's test, $p<0.05$).

(G) Motif sequence of labeled, unlabeled competitor and mutant competitor probes. Mutant competitor in which the 5'-CACTTG-3' motif was replaced with 5'-AAAAAA-3'.

(H) DNA electrophoretic mobility shift assay (EMSA) showing that the SIBES1-His binds to E-box of *PMEU1* in *vitro*. FAM-labeled probes were incubated with SIBES1-His and the free and bound DNAs were separated on an acrylamide gel.

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Figure 4. Effects of Silencing *PMEU1* in AC and *SIBES1-RNAi* on Tomato Firmness and Shelf Life.

(A) Relative expression levels of *SIBES1* and *PMEU1* in fruits of AC (Control), virus-induced *PMEU1* silencing lines (TRV-*PMEU1*), *SIBES1-RNAi*, and virus-induced *PMEU1* silencing in *SIBES1-RNAi* (TRV-*PMEU1*/*SIBES1-RNAi*) at red ripe stage.

(B) Fruit firmness of Control, TRV-*PMEU1*, *SIBES1-RNAi*, and TRV-*PMEU1*/*SIBES1-RNAi* at red ripe stage. Values are means $\pm$ SD of twenty-four biological replicates.

(C) The tomato shelf life of Control, TRV-*PMEU1*, *SIBES1-RNAi*, and TRV-*PMEU1*/*SIBES1-RNAi*. Fruits were harvested at red ripe stage and stored at room temperature. The progression of fruit deterioration was recorded by time-lapse photography. Time after harvest is specified by days. The storage condition was 25°C and 35% relative humidity.

(D) Total PME activity of Control, TRV-*PMEU1*, *SIBES1-RNAi*, and TRV-*PMEU1*/*SIBES1-RNAi* fruits at red ripe stage.

(A) to (D) Expected for fruit firmness, values are means $\pm$ SD of three biological replicates. Different letter indicates a significant difference among groups (one-way ANOVA, $p < 0.05$, Tukey's test).

(E) Schematic representation of relationship between transcriptional regulation of *slbes1* on *pmeu1* and fruit firmness.

When BRs are absent as left showed, receptor BRI and co-receptor BKI1 are separated. Transcriptional factor SIBES1 with phosphate group couldn't inhibit pectin methylesterase gene *PMEU1* expression. Enzyme protein PMEU1 is formed and secreted into the cell wall. Then PMEU1 de-esterified HMHG and produces DMHG, DMHG formed "Egg box" through cross-link with Ca^{2+} . Egg box provides strengthening support to fruit firmness. When BRs are present, BRs bind to BRI and BKI1. A series of phosphorylation and dephosphorylation are occurred, which lead to the dephosphorylation of SIBES1. Dephosphorylated SIBES1 binds to the E-box of *PMEU1* promoter and then represses the expression of *PMEU1*. Fewer PMEU1 are secreted into the cell wall and pectin methylesterase activity is attenuated, so the contents of DMHG and Egg box are decreased which causing fruit softening.

BRI1, BRASSINOSTEROID INSENSITIVE 1; BAK1, BRI1-ASSOCIATED KINASE1; BES1, BRI1-EMS-SUPPRESSOR 1; PMEU1, PECTIN METHYLESTERASE UBIQUITOUSLY 1; HMHG, highly methylesterified HG; DMHG, demethylesterified HG.