Brassinosteroids signaling component SlBZR1 promotes fruit ripening in tomato

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SUMMARY

Fruit ripening evolved to be attractive to frugivores that derive energy and nutrition from the fruits in exchange for assisting seed dispersal, which is accompanied by the dramatically change of fruit characteristics, including color, aroma, and texture. The plant hormone ethylene plays a key role in climacteric fruit ripening, while the role of other phytohormones as well as their cross talk with ethylene in modulating fruit ripening remains elusive. Here, we report growth-promoting phytohormone brassinosteroids promote fruit ripening in tomato through regulation of ethylene biosynthesis. Exogenous BR treatment and the increase of endogenous BR content in *SlCYP90B3*-OE promoted ethylene production and fruit ripening. SlBZR1, a central component and positive regulator of BR signaling pathway, promotes ethylene production and carotenoid accumulation through direct transcriptional regulation of *SlACO1, SlACO3* and *SlPSY1*. Furthermore, SlBIN2, a negative regulator of BR signaling upstream of SlBZR1, decreases ethylene production and carotenoid

accumulation. Together, our results demonstrate that BR signaling integrates ethylene and carotenoid biosynthetic pathway to regulate fruit ripening.

Keywords: tomato, brassinosteroid, *SlBZR1*, fruit ripening, ethylene, carotenoids

Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetables as well as a perfect model system to study fruit ripening. Tomato fruit ripening is an intricate process in plant development that also involves overall fruit quality changes, such as color, texture, favor, and aroma [1]. In line with these changes, maturing tomato fruits undergo a series of physiological and metabolic processes, such as respiratory climacteric, cell wall synthesis and degradation as well as carotenoid biosynthesis [2]. Carotenoids are a diverse group of isoprenoid compounds with unique physicochemical properties, which are widely present in ripe tomato fruits, functioning as health-promoting natural pigments that participate in the coloration and quality formation of tomato fruits, as well as assisting seed dispersal by animals [3]. Significant progress has been achieved in recent years on carotenoid biosynthesis and catabolism pathway. *SlPSY1*, encoding phytoene synthase, is a key rate-limiting gene for carotenoid synthesis in tomato. Overexpression of *SlPSY1* accumulates elevated levels of carotenoids in tomato fruits [4]. Carotenoid metabolism in tomato fruits is closely synchronized with the ripening process, which is modulated by the interplay of various factors including developmental cues, environmental signals and phytohormones [3].

As a phytohormone, ethylene is an overall ripening-promoting regulator governing normal ripening and carotenoid accumulation of tomato fruits via a burst of ethylene production followed by an increasing in respiration rate at the onset of fruit ripening [1, 5, 6]. In higher plants, ethylene biosynthesis begins with the supply of S-adenosyl methionine (SAM) via the Yang pathway, which is catalyzed by ACC synthase (ACS) and converted into ACC, subsequent reaction catalyzed by ACC oxidase (ACO) eventually lead to the production of ethylene [7-9]. Previous studies manifest that the expression of *SlACO1* and *SlACO3* is markedly increased when tomato fruit ripening is triggered. Antisense-mediated RNA silencing of *SlACO1* causes delayed ripening and reduced biosynthesis of endogenous ethylene [10-12]. In addition, great progress has been made in identification of transcription factors responsible for tomato fruit ripening

and carotenoid accumulation in an ethylene-dependent manner. Several tomato MADS-box transcription factors and NAC family transcription factors are reported to be involved in ethylene biosynthesis, while also affect the transcription of carotenoid biosynthetic genes. These tomato MADS-box transcription factors, including Ripening Inhibitor (RIN), Agamous-like1 (TAGL1), Fruitfull1 (FUL1), and FUL2 contribute to fruit ripening and carotenoid accumulation with the positive regulations on both ethylene biosynthesis genes, such as *ACS2* and *ACS4*, as well as carotenoid biosynthesis gene *SlPSY1* [13-15]. Moreover, RIN could also interact with TAGL1, FUL1, and FUL2 to form complex which functions as regulator of fruit ripening [13, 16-18]. NAC family transcription factors, including NOR and NOR-like1 positively modulate biosynthetic pathway of ethylene and carotenoid by directly binding to the promoter of *ACS2* and *SlGGPPS2* [19, 20]. Furthermore, other transcription factors such as CNR, AP2a, SlHB1, SlERF6, SGR1 as well as some WRKY transcription factors including SlWRKY35 and SlWRKY32 are also involved in ethylene biosynthesis and carotenoid accumulation during tomato fruit ripening [21-28]. The regulatory mechanism of ethylene in ripening of climacteric fruit, represented by tomato, has been well elucidated. However, fruit ripening is a complex developmental process fine-tuned by various factors, of which the interaction networks of phytohormone signaling play a central role, thus it will be interesting and significant to reveal the function of other phytohormones act either independently or in combination with ethylene in fruit ripening [24]. Moreover, the mechanism of ethylene promoting fruit ripening is primarily applied to extending the shelf life of fruits instead of regulating fruit quality till now. Therefore, elucidating the regulatory mechanism of phytohormone interaction network in fruit ripening will extend our understanding of fruit development as well as provide new strategies for fruit quality improvement.

To date, several phytohormones have been observed to influence ethylene biosynthesis and carotenoid accumulation during fruit ripening. Our previous studies found that Jasmonic acid (JA) and its volatile methyl ester, MeJA, participate in regulation of fruit ripening and carotenoid accumulation partially via an ethylene-independent pathway

[29, 30]. Additionally, JA was also reported to promote ethylene production in apple fruit via direct transcriptional activation of both *MdACS1* and *MdACO1* by MdMYC2 during fruit ripening [31]. Abscisic acid (ABA) concentration gradually increased at the onset of tomato fruit ripening, and application of exogenous ABA promoted ethylene biosynthesis and fruit ripening [32]. Furthermore, treatment of auxin analogue 2,4-D can delay the ripening process of tomato fruit via inhibiting the production of ethylene and enhance the final contents of carotenoids in mature tomato fruits [33].

BRs are a class of steroid phytohormones with broad-spectrum of biological functions, and involved in diverse plant growth and developmental processes, such as seed germination, root growth, stem elongation, leaf morphogenesis, stomatal formation, floral development [34]. To date, the BR signaling pathway has been extensively explored by using multiple approaches. BRs are perceived at the plasma membrane by the extracellular domains of Brassinosteroid-Insensitive1 (BRI1) receptor and its co-receptor BRI1-associated receptor kinase 1 (BAK1). BRI1 and BAK1 trans-phosphorylate each other, which increases the activity of BRI1 resulting in BR signaling kinase 1 (BSK1) phosphorylation and its release from the receptor complex [35, 36]. Activated BSK1 binds to and activates BRI1-Suppressor 1 (BSU1), which leads to dephosphorylation and inhibition of the Brassinosteroid Insensitive 2 (BIN2) [37]. Subsequently, the downstream transcription factors such as Brassinazole Resistant 1 (BZR1) and BRI1-EMS Suppressor 1 (BES1) are dephosphorylated by a cooperative reaction of BIN2 and Protein Phosphatase 2A (PP2A), then accumulate in the nucleus and bind to the promoters of BR-responsive target genes, conferring a specific physiological response [38, 39]. When BRs are absent or present at low levels, BZR1 and BES1 are phosphorylated by active BIN2, which abolishes their DNA-binding activity and leads to their cytoplasmic retention by 14-3-3 proteins. When BRs are present at high levels, BZR1 and BES1 are dephosphorylated by protein phosphatase 2A (PP2A) and transported into the nucleus, then bind to the promoters of their target genes, resulting in gene activation or repression [40].

125 Recent studies have demonstrated that BRs exert effects on fruit development process,
126 especially fruit maturation. The application of exogenous BR promotes the ripening of
127 climacteric fruits, such as tomato, banana, persimmon and mango, as well as non-
128 climacteric fruits, including strawberry and grape via different regulatory mechanisms
129 [41-46]. Moreover, overexpression of the BR biosynthetic gene *DWARF, SlCYP90B3*
130 and *GhDWF4* respectively in tomato, results in elevated level of endogenous BRs,
131 increased ethylene production and carotenoid accumulation as well as earlier ripening
132 in an ethylene-dependent manner [47-49]. Tomato fruits with enhanced BR signaling
133 via over-expression of SlBRI1, the receptor gene, also displayed elevated ethylene
134 production and carotenoid levels during fruit ripening [50]. These results indicate that
135 BRs promotes tomato fruit ripening possibly through activating the expression of
136 ethylene biosynthetic genes and ultimately the ethylene production. Additionally, our
137 former research also showed that BR signaling promoted carotenoid accumulation and
138 quality formation in tomato during fruit ripening period [51]. Several recent studies
139 found that MaBZR1/2, PuBZR1 and DkBZR1 repress ethylene production, while
140 DkBZR2 promotes ethylene production during postharvest fruit ripening [42, 52, 53].
141
142 Our previous reports demonstrated that exogenous application and increase of
143 endogenous BR both promote fruit ripening and carotenoid accumulation, up-regulate
144 the expression of ethylene and carotenoid biosynthetic genes, while the regulatory
145 mechanism of BR signaling in biosynthesis of ethylene and carotenoid during tomato
146 fruit ripening remains unclear. In the present study, with an aim to elucidate the role of
147 BR signaling in tomato fruit ripening, we investigated the function of *SlBZR1* and
148 *SlBES1*, the tomato closest homolog of *AtBZR1* and *AtBES1*. Interestingly, we found
149 that double mutation via *SlBZR1*/*SlBES1*-KO delays tomato fruit ripening and ethylene
150 release as well as reduces carotenoid accumulation. Overexpression of *SlBZR1* and
151 knockout of *SlBIN2* promotes fruit ripening with elevated ethylene production and
152 carotenoid levels. Further tests reveal that *SlBZR1* exerts direct transcriptional
153 activation on ethylene biosynthetic genes *SlACO1* and *SlACO3* as well as carotenoid
154 biosynthetic gene *SlPSY1* to promote ethylene and carotenoid biosynthesis and fruit

155 ripening.

156

157 **Results**

158 **BR promotes fruit ripening**

159

160 Previous studies have shown that the accumulation of endogenous BRs increases during
161 tomato fruit ripening, which indicates that BRs may have a positive effect on tomato
162 fruit ripening [48]. To determine the role of BRs on tomato fruit ripening, we treated
163 tomato fruits collected at mature green (MG) stage with 24-epibrassinolide (eBL) or
164 propiconazole (PCZ) alone as well as the combination of eBL and PCZ (eBL + PCZ)
165 at a concentration of 3 μM, respectively. Compared to the control fruits, eBL-treated
166 fruits showed a significantly higher production of ethylene during storage. Similarly,
167 the peak of climacteric ethylene production occurred about 1 day earlier than the control
168 fruits, while the climacteric ethylene peak was significantly inhibited after PCZ
169 treatment (Supplemental Figure S1A). Moreover, compared with the WT fruits,
170 overexpression of BR biosynthetic gene *SlCYP90B3* increased the level of endogenous
171 bioactive BR in fruits and accelerated fruit ripening, *SlCYP90B3-RNAi* transgenic lines
172 delayed fruit ripening (Supplemental Figure S1B). In line with this, *SlCYP90B3-OE*
173 advanced the onset of the peak of climacteric ethylene production by 1d, and the peak
174 of ethylene production was higher than that in the WT fruits. In contrast, *SlCYP90B3-*
175 *RNAi* caused a lower peak of ethylene production that occurred later about 2 days
176 (Supplemental Figure S1C). The results demonstrate that exogenous eBL treatment and
177 increase in endogenous bioactive BR levels exert a positive role in tomato fruit ripening.

178

179 **Fruit ripening was inhibited in tomato *bzr1bes1* double mutant**

180

181 To better understand BR action and identify the candidate components of BR signaling
182 pathway function in tomato fruit ripening, we manually searched for the *SlBZR1* and
183 *SlBES1*, the homologous gene of *AtBZR1* and *AtBES1* in tomato, encoding proteins of
184 333 and 327 amino acids, with calculated molecular weights of 35.78 and 34.98 kDa,

185 respectively. Amino acid alignment revealed that SlBZR1 and SlBES1 contain a DNA-
186 binding domain with a nuclear localization signal sequence (NLS), a putative 14-3-3
187 binding site, and a putative PEST sequence, which are typical characteristics of
188 BZR/BES transcription factors. Particularly, 25 serine/threonine residues recognized
189 by the phosphorylation site of GSK-3 kinase BIN2 were also found in SlBZR1 and
190 SlBES1 proteins. In addition, the EAR (ethylene-responsive element binding factor-
191 associated amphiphilic repression) domain motif was found to be localized near the C
192 terminus of SlBZR1 and SlBES1 proteins (Supplemental Figure S2). Phylogenetic tree
193 based on BZR sequences indicated that SlBZR1 and SlBES1 closely clustered with
194 Arabidopsis AtBZR1 and AtBES1 (Supplemental Figure S2).
195
196 To verify whether BRs promotes the ripening of tomato fruits via *SlBZR1* and *SlBES1*,
197 we generated loss-of-function mutants of both *SlBZR1* and *SlBES1* using CRISPR/Cas9
198 system in tomato cultivar *Ailsa Craig* (AC) background. To this end, two specific target
199 sites were designed in the second exon of *SlBZR1* and *SlBES1*. Genomic DNA
200 sequencing revealed two null double mutants *bzr1bes1-1* and *bzr1bes1-2* carried long
201 deletions and single-base deletions causing frameshift and premature termination of
202 translation, likely resulting in complete loss-of-function. Then, two distinct
203 homozygous mutants *bzr1bes1-1* and *bzr1bes1-2* were selected for further analysis, the
204 editing details of the two lines are shown in Figure 1A.
205
206 We observed and recorded the ripening characteristics of *bzr1bes1* mutant fruits.
207 Notably, the fruit ripening progress was markedly delayed in *bzr1bes1-1* and *bzr1bes1-*
208 *2* mutant lines compared with the WT. Fruits of both *bzr1bes1* double mutant lines
209 required an additional 4-5 days from anthesis to the breaker stage compared with the
210 WT fruits. Indeed, fruits of WT plants reached the breaker stage at 35-day post-anthesis
211 (DPA), whereas the average time from anthesis to breaker stage extended to 40 DPA
212 and 39 DPA in fruits of *bzr1bes1-1* and *bzr1bes1-2* lines, respectively. We next
213 investigated climacteric ethylene production in *bzr1bes1* fruits by monitoring ethylene
214 production from 30 to 46 DPA. The peaks of climacteric ethylene production in fruits

215 of *bzr1bes1-1* and *bzr1bes1-2* lines appeared about 5 days later than in fruits of WT
216 plants (Figure 1B).
217
218 To determine whether fruit quality was altered in *bzr1bes1* mutants, several typical fruit
219 quality parameters were measured at different ripening stages (MG, B, B+3, and B+7).
220 Fruits of both *bzr1bes1-1* and *bzr1bes1-2* lines exhibited increased fruit firmness
221 compared with that of WT at B, B+3 and B+7 stages (Figure 1C), consistent with the
222 delayed ripening of *bzr1bes1* fruits. The contents of lycopene, β-carotene and total
223 carotenoids in fruits of *bzr1bes1-1* and *bzr1bes1-2* lines were much lower than those in
224 fruits of WT at B, B+3 and B+7 stages (Figure 1D). These results suggest that SlBZR1
225 and SlBES1 play a positive role in fruit ripening and carotenoids accumulation.
226
227 ***SlBZR1* promotes tomato fruit ripening**
228
229 Previous studies have shown that *SlBES1* promotes tomato fruit softening without
230 affecting carotenoids contents and ethylene production (Supplemental Figure S3, A and
231 B) [54], which indicates that *SlBZR1* might play a dominant role in tomato fruit ripening
232 and carotenoids accumulation.
233
234 To gain insight into the biological function of SlBZR1 in tomato fruit ripening, we
235 generated *SlBZR1*-overexpression transgenic lines and *SlBZR1* knock-out mutants
236 using CRISPR/Cas9 system in AC. Three transgenic lines (OE-8, OE-9 and OE-14)
237 with elevated *SlBZR1* gene expression levels were selected for further phenotypic and
238 molecular analyses (Supplemental Figure S4, A and B). Likewise, three homozygous
239 *bzr1* mutants (*bzr1-5*, *bzr1-28* and *bzr1-29*) with deletions or insertions that led to
240 translational frame shifts were obtained, which encode truncated SlBZR1 proteins with
241 58, 48 and 49 aa, respectively (Supplemental Figure S4C). These results suggest that
242 *bzr1-5*, *slbzr1-28* and *slbzr1-29* are loss-of-function mutants suitable for functional
243 analysis of *SlBZR1*.
244

245 The fruit ripening progress was increasingly promoted in *SlBZR1*-OE lines and
246 inhibited in *bzr1* mutant lines when compared with the WT plants (Figure 2, A and B).
247 Notably, all three *SlBZR1*-OE tomato lines displayed a significantly advanced onset of
248 fruit ripening by 2-3 days, while knockout of *SlBZR1* delayed onset of fruit ripening by
249 1 day (Figure 2, B and C), consistent with the hypothesis that *SlBZR1* positively
250 regulates tomato fruit ripening. The peaks of climacteric ethylene production occurred
251 1-3 days earlier in *SlBZR1*-OE fruits and 1-2 days later in *bzr1* mutant fruits than in the
252 WT (Figure 2D).
253
254 To explore the effects of *SlBZR1* on major physiological characteristics of tomato fruit
255 ripening, we also measured fruit firmness and carotenoid content of *SlBZR1*-OE, *bzr1*
256 mutant and WT fruits. The results showed that the fruit firmness of *SlBZR1*-OE fruits
257 was significantly reduced compared with that of WT fruits at B, B+3 and B+7 stages,
258 while the fruit firmness of *bzr1* mutant fruits was enhanced than that of WT fruit at the
259 same stage (Figure 2, E and F). The contents of lycopene, β-carotene and total
260 carotenoids in *SlBZR1*-OE fruits were significantly higher than those in WT fruits,
261 whereas *bzr1* mutant fruits produced less carotenoids than WT fruits, especially
262 lycopene (Figure 2G). These results indicated that *SlBZR1* was a positive regulator of
263 tomato fruit ripening and carotenoids accumulation.
264
265 ***SlBZR1* is a master regulator of tomato fruit ripening**
266
267 To further elucidate the molecular mechanism of *SlBZR1* in regulating tomato fruit
268 ripening and carotenoids biosynthesis, we performed global transcriptomic profiling by
269 RNA-seq coupled with genome-wide ChIP-seq to identify genes and pathways
270 potentially involved in tomato fruit ripening. *SlBZR1* knockout led to substantial
271 transcriptomic reprogramming with 1,995 and 1,677 genes being differentially
272 expressed (DEGs) in *bzr1* mutant fruits at MG and B stages, respectively. Among these,
273 730 genes were up-regulated and 1265 genes were down-regulated in *bzr1* mutant fruits
274 at MG stage, 603 genes were up-regulated and 1074 genes were down-regulated in *bzr1*

275 mutant fruits at B stage (Figure 3A). The up-regulated and down-regulated genes in
276 *bzr1* mutant were termed SlBZR1-repressed genes and SlBZR1-induced genes,
277 respectively.
278
279 Moreover, 1986 potential SlBZR1 targets genes were identified by ChIP-seq using a
280 polyclonal anti-GFP antibody and *SlBZR1*-OE fruits at MG stage expressing the BZR1-
281 eGFP fusion protein. We compared the SlBZR1-repressed and SlBZR1-induced
282 differentially expressed genes set obtained by RNA-seq with the SlBZR1 target genes
283 set revealed by ChIP-seq. Cross-referencing the ChIP-seq and RNA-seq data identified
284 276 genes being both *SlBZR1* target and displaying differential expression in *bzr1*
285 mutant fruits (Figure 3B). Interestingly, 73 genes (26.4%) were *SlBZR1*-repressed
286 targets while 203 genes (73.6%) were *SlBZR1*-induced targets, consistent with *SlBZR1*
287 functions as a positive regulator in tomato fruit ripening (Figure 3, B and C).
288
289 The production of ethylene and accumulation of carotenoids as well as fruit softening
290 appears to be a typical feature during tomato fruit ripening. The RNA-seq and ChIP-
291 seq analysis showed that numerous genes involved in fruit ripening including ethylene
292 biosynthesis and carotenoid metabolism as well as transcription factor genes
293 controlling fruit softening and fruit ripening were down-regulated in *bzr1* mutant fruits
294 (Figure 3, D and E).
295
296 The fruit softening-related genes were *SlBZR1*-induced target genes and down-
297 regulated in the *bzr1* mutant fruits, which indicates *SlBZR1* promoted tomato fruit
298 softening by directly regulating fruit softening-related genes. Previous studies have
299 demonstrated that SlBES1 promotes tomato fruit softening through transcriptional
300 inhibition of *PMEU1* without affecting nutritional quality, which indicates that *SlBZR1*
301 and *SlBES1* might perform partially redundant functions in fruit softening and regulate
302 fruit ripening in a reciprocal manner.
303
304 The ethylene biosynthesis genes *SlACO1* and *SlACO3* as well as carotenoid

305 biosynthesis gene *SlPSY1* were also *SlBZR1*-induced target genes and down-regulated
306 in *bzr1* mutant fruits, indicating that *SlBZR1* promoted ethylene production and
307 carotenoids accumulation by directly regulating these candidate ethylene and
308 carotenoid biosynthesis genes (Figure 3, D and E). Moreover, the fruit ripening-related
309 TFs were also *SlBZR1*-induced target genes and down-regulated in *bzr1* mutant fruits.
310 These results were in agreement with the advanced onset of ripening in *SlBZR1*-OE
311 fruits and the delayed ripening initiation in *bzr1* mutant fruits.
312
313 **Ethylene biosynthetic genes *SlACO1* and *SlACO3* as key targets of *SlBZR1* action**
314
315 The expression levels of *SlACO1* and *SlACO3* were markedly increased in *SlBZR1*-OE
316 and decreased in *bzr1* mutant fruits compared with the WT fruits at MG, B and P stages,
317 consistent with the advanced onset of the peak of climacteric ethylene production
318 (Figure 4, A and B). To understand whether SlBZR1 directly regulates transcription of
319 *SlACO1* and *SlACO3* during tomato fruit ripening, we performed promoter analysis of
320 *SlACO1* and *SlACO3* and found that there was one E-box and eight E-box in six regions
321 in the 3-kb upstream regions of the promoters of *SlACO1* and *SlACO3*, respectively.
322 Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis showed a
323 significantly higher enrichment for the promoters of *SlACO1* and *SlACO3*, which
324 indicated that SlBZR1 directly bound to the promoters of both ethylene biosynthesis
325 genes (Figure 4, C and D).
326
327 Next, we tested whether SlBZR1 could activate the transcription of *SlACO1* and
328 *SlACO3* using the dual luciferase reporter assays. The promoter sequences of *SlACO1*
329 and *SlACO3* were fused successfully to the LUC reporter gene, co-transformed with an
330 empty pGreenII 62-SK vector or pGreenII 62-SK-SlBZR1 vector into Nicotiana
331 benthamiana leaf epidermal cells. The results showed that SlBZR1 significantly
332 activated the promoters of the *SlACO1* (1.57-fold) and *SlACO3* (2.33-fold) compared
333 with the activity obtained with the control samples, respectively (Figure 4, E and F).
334 Taken together, the results demonstrated that SlBZR1 positively regulates ethylene

335 biosynthesis in tomato fruit by directly targeting the promoter of *SlACO1* and *SlACO3*.
336
337 **SlBZR1 promotes carotenoid accumulation in a partially ethylene-dependent**
338 **manner**
339
340 To further investigate the influence of the interaction between SlBZR1 and ethylene
341 biosynthesis genes on promoting carotenoid accumulation during tomato fruit ripening,
342 we treated *SlBZR1*-OE (OE-14) and WT fruits collected at mature green stage with 1
343 $\mu L \cdot L^{-1}$ 1-MCP for 12 h. The contents of lycopene and total carotenoids in *SlBZR1*-OE
344 fruits were much higher than in WT fruits after 12 d and 18 d of storage, whereas the
345 accumulation of lycopene, β-carotene and total carotenoids was significantly inhibited
346 by 1-MCP treatment at 6 d, 12 d and 18 d of storage in both *SlBZR1*-OE and WT fruits.
347 Interestingly, the promotion of lycopene and total carotenoid accumulation in the
348 *SlBZR1*-OE fruits were not completely inhibited by 1-MCP treatment. The contents of
349 lycopene and total carotenoids increased in the *SlBZR1*-OE fruits stored for 18 d after
350 1-MCP treatment compared with those in the wild-type fruits (Supplemental Figure S5,
351 A and B). These results implied that *SlBZR1*-OE might promote carotenoid
352 accumulation during tomato fruits ripening in a partially ethylene-dependent manner.
353
354 **SlBZR1 transactivates the expression of *SlPSY1* to regulate carotenoid**
355 **accumulation**
356
357 The expression levels of *SlPSY1* were markedly increased in *SlBZR1*-OE and decreased
358 in *bzr1* mutant fruits compared with WT fruits at MG, B and P stages, which implied
359 SlBZR1 might positively regulate the expression of *SlPSY1* (Figure 5A). To verify the
360 direct regulation of BZR1 on *SlPSY1* expression, we performed promoter analysis of
361 *SlPSY1* gene and found that there was three E-box in the 2-kb upstream regions of the
362 promoters of *SlPSY1*. ChIP-qPCR analysis showed a significantly higher enrichment
363 for the promoters of *SlPSY1*, which indicated that SlBZR1 directly bound to the
364 promoters of *SlPSY1* (Figure 5B).

365

366 Next, a yeast one-hybrid analysis was used to test for interaction between SlBZR1 and
367 the promoter of *SlPSY1*. Yeast cells that were co-transformed with pGADT7- SlBZR1
368 and *SlPSY1pro*-pAbAi were able to grow on the SD/-Leu medium supplemented with
369 AbA (150 ng·ml^{-1}), whereas the negative control co-transformed with pGADT7 and the
370 *SlPSY1pro*-pAbAi did not grow, which confirmed the interaction of SlBZR1 and
371 *SlPSY1* in Yeast (Figure 5C).

372

373 We further performed the dual luciferase reporter assays to test whether SlBZR1 could
374 activate the transcription of *SlPSY1*. The promoter sequences of *SlPSY1* were fused
375 successfully to the LUC reporter gene, co-transformed with an empty pGreenII 62-SK
376 vector or pGreenII 62-SK-SlBZR1 vector into N. benthamiana leaf epidermal cells. The
377 results showed that SlBZR1 significantly activated the promoter of the *SlPSY1* (1.55-
378 fold) compared with the activity obtained with the control sample (Figure 5D). Thus,
379 the results demonstrated that SlBZR1 positively regulates the expression of *SlPSY1* and
380 carotenoid accumulation in ripening tomato fruits.

381

382 **SlBIN2 negatively regulates tomato fruit ripening**

383

384 To further investigate the molecular mechanism of the SlBZR1-mediated regulation of
385 tomato fruit ripening, a Y2H screen of tomato cDNA library was conducted to identify
386 proteins interacting with SlBZR1. We successfully obtained several clones using Y2H
387 screening, and one of them encoding a protein belonging to the Glycogen Synthase
388 Kinase 3 (GSK3)-like kinases caught our attention. A BLAST search of the Phytozome
389 database revealed that the GSK3-like kinase sequence was identical to *SlSK21/BIN2*,
390 encode protein of 437 amino acids, with calculated molecular weights of 49.31 kDa.
391 On the basis of a phylogenetic tree, the homologous genes of GSK3-like kinase family
392 in Arabidopsis and tomato were divided into four subgroups, suggesting that *SlBIN2*
393 was the homologous gene of *AtSK21/BIN2* in tomato, which is a negative regulator of
394 BR signaling (Supplemental Figure S6, A and B). In order to confirm that SlBIN2

395 protein interacts with SlBZR1, we conducted a yeast two-hybrid assays on stringent
396 selective medium (SD/-Trp-Leu-His-Ade) and X-α-gal. The result demonstrated that
397 SlBIN2 interacted with SlBZR1, consistent with the interaction of BIN2 and BZR1 in
398 Arabidopsis (Supplemental Figure S6C).
399
400 To gain insight into the biological function of SlBIN2 in tomato fruit ripening, we
401 generated *SlBIN2*-OE transgenic lines and *SlBIN2* knock-out mutants using
402 CRISPR/Cas9 system in AC. Three transgenic lines (OE-1, OE-2 and OE-4) with
403 elevated *SlBIN2* gene expression were selected for further analysis (Supplemental
404 Figure S6D). At the same time, two homozygous *bin2* mutants (*bin2-5* and *bin2-10*)
405 with 10 bp and 2 bp deletions that led to translational frame shifts were obtained, which
406 encode truncated SlBIN2 proteins with 33 and 10 aa, respectively (Supplemental Figure
407 S6E). These results suggest that *bin2-5* and *bin2-10* are loss-of-function mutants
408 suitable for the functional analysis of *SlBIN2*.
409
410 We observed and recorded the ripening characteristics of *SlBIN2*-OE and *bin2* mutant
411 fruits. Compared with the WT plants, the fruit ripening progress was significantly
412 inhibited in the *SlBIN2*-OE lines and promoted in the *bin2* mutant lines (Figure 6, A
413 and B). *SlBIN2*-OE fruits required an additional 1-2 days, while *bin2* mutant fruits
414 showed 1-2 days earlier from anthesis to the breaker stage when compared with WT
415 fruits (Figure 6C). The peaks of climacteric ethylene production delayed about 1-2 days
416 in *SlBIN2*-OE fruits and occurred 1-2 days earlier in *bin2* mutant fruits than in WT
417 (Figure 6D).
418
419 To study the effects of *SlBIN2* on main physiological characteristics of tomato fruit
420 ripening, we also measured fruit firmness and carotenoid content of *SlBIN2*-OE, *bin2*
421 mutant and WT fruits. The results showed that the firmness of *SlBIN2*-OE fruits was
422 significantly enhanced compared with that of WT fruit at the B, B+3 and B+7 stages,
423 while the firmness of *bin2* mutant fruits was reduced than that of WT fruit at the same
424 stage (Figure 6F). The contents of lycopene, β-carotene and total carotenoids in *SlBIN2*-

425 OE fruits were lower than those in WT fruits, whereas *bin2* mutant fruits produced more
426 carotenoids than WT fruits, especially lycopene (Figure 6G). Consistent with the
427 observed delayed ripening and ethylene production as well as reduced carotenoid levels,
428 the relative expression levels of the ethylene and carotenoid biosynthesis genes *SlACO1*,
429 *SlACO3* and *SlPSY1* were lower in *SlBIN2*-OE fruits compared to WT fruits
430 (Supplemental Figure S7 A, B and C). Taken together, these results indicated that
431 SlBIN2, as a negative regulator of BR signaling, inhibits fruit ripening and carotenoids
432 accumulation in tomato.
433
434 **Discussion**
435
436 Brassinosteroids (BRs) are known as a group of growth-promoting steroid hormones
437 which are required for the regulation of a variety of physiological processes during
438 plant growth and development, including, seed germination, root growth, stem
439 elongation, leaf morphogenesis, stomatal formation, and floral development [34]. Here,
440 our study demonstrates that BR signaling is essential for regulating tomato fruit
441 ripening by interaction with ethylene signaling pathway.
442
443 **Growth-promoting phytohormone BR is essential for tomato fruit ripening**
444
445 Previous reports showed that BRs were involved in the growth and development of
446 tomato fruit. Firstly, exogenous application of BRs to tomato plant enhanced the height
447 of plants as well as the yield and quality of fruits. An increase in fruit number and the
448 levels of lycopene and β-carotene were observed in tomato plants treated with 28-
449 homobrassinolide [55, 56]. Moreover, tomato fruit ripening was significantly inhibited
450 and promoted by Brz and EBR, respectively [41, 57]. Secondly, several BR-deficient
451 and BR-insensitive mutants identified in tomato showed severe dwarfing, delayed fruit
452 ripening and reduced fruit quality [58, 59]. d^x, a tomato BR-deficient mutant with an
453 impaired Dwarf gene encoding a BR C-6 oxidase, resulting in severe dwarfism. d^x fruits
454 showed delayed ripening and reduced fruit yield and dry mass, which could be partially

455 rescued by BR application [58, 60-62]. Thirdly, intense biosynthesis of BRs was
456 detected during tomato fruit development, suggesting BRs was required for tomato fruit
457 development [58, 63]. These studies illuminated that BRs play roles in tomato
458 development, while the effect of endogenous BRs level on tomato fruit ripening is
459 largely unknown.
460
461 Our previous results showed that the transcript levels of BR biosynthetic gene
462 *SlDWARF* and *SlCYP90B3* were up-regulated, followed by an increase in the contents
463 of endogenous BL and CS at the onset of tomato fruit ripening [48]. Additionally,
464 overexpression of *SlCYP90B3* or *SlBRI1* enhanced endogenous BR levels or BR
465 signaling, which promoted tomato fruit ripening [48, 50]. These results are consistent
466 with the identification of several BR-deficient and BR-insensitive mutants showing
467 delayed fruit ripening [58, 59, 62]. Moreover, our study reveals that overexpression of
468 the positive regulator of BR signaling *SlBZR1* or knock-out of the negative regulator of
469 BR signaling *SlBIN2* promote tomato fruit ripening (Figure 2, A and B; Figure 6, A and
470 B). In contrast, *bzr1bes1* and *SlBIN2-OE* lines display delayed fruit ripening (Figure
471 1B; Figure 6, A and B). Our previous results indicate that SlBES1 promotes tomato
472 fruit softening through transcriptional inhibition of *PMEU1* without affecting
473 nutritional quality. The current research demonstrates that SlBZR1 functions as a
474 master regulator of tomato fruit ripening, which directly binds and transactivates
475 ethylene biosynthetic genes *SlACO1* and *SlACO3* and carotenoid biosynthetic genes
476 *SlPSY1* to regulate ethylene production and carotenoid accumulation, thus promoting
477 tomato fruit ripening. Moreover, our results reveal that SlBIN2 which interacts with
478 SlBZR1 as a negative regulator of BR signaling, inhibits SlBZR1-mediated tomato fruit
479 ripening and carotenoid accumulation. Together, these findings indicate that BR
480 signaling promotes fruit ripening.
481
482 **Interplay of the BR and ethylene signaling pathway for regulation of tomato fruit**
483 **ripening**
484

485 In maturation process of climacteric fruits promoted by exogenous BR, the endogenous
486 BR content did not change significantly in mango, banana and jujube [42, 44, 64],
487 which increased in tomato and persimmon [43, 48] and decreased in apple and pear
488 [53]. Similarly, the endogenous BR levels were found to be strongly elevated at the
489 onset of fruit ripening in grape berries [46], implying that the mechanisms underlying
490 BRs in controlling fruit ripening might be different depending on the fruit type.
491
492 As one of the primary initiators and regulators of maturation process in climacteric
493 fruits, ethylene plays a key role in promoting ripeness and coloration of climacteric
494 fruits, and its absence or decrease dramatically delays the ripening process [65].
495 Previous studies have demonstrated that BRs can directly regulate the ripening of non-
496 climacteric fruits, but whether BRs regulate the ripening of climacteric fruits directly
497 or via controlling ethylene biosynthesis remains unclear [66]. Interestingly, one study
498 in Arabidopsis proposed that BRs at a low concentration negatively control ethylene
499 production, while BRs at high concentrations positively regulate ethylene production
500 [67, 68]. However, we cannot conclude that BRs either positively or negatively regulate
501 ethylene biosynthesis in a dose-dependent manner, since the effects of of BRs upon
502 different levels on ethylene biosynthesis are still absent in tomato fruits.
503
504 In this work, we found that exogenous application of eBL markedly promotes the
505 production of ethylene, whereas PCZ treatment inhibits the climacteric peak of ethylene
506 production (Supplemental Figure S1A). Furthermore, *SlCYP90B3*-OE accelerated
507 tomato fruit ripening and advanced the onset of the climacteric peak of ethylene
508 production, while *SlCYP90B3*-RNAi retarded tomato fruit ripening and caused a lower
509 peak of ethylene production (Supplemental Figure S1C). Moreover, overexpression of
510 either *SlDWARF*, *GhDWF4* or *SlBRI1* in tomato, results in elevated levels of ethylene
511 production and earlier ripening as well as enhanced carotenoid accumulation [47, 49,
512 50]. In addition, *SlBZR1-OE* or *SlBIN2-KO* accelerated ethylene production and
513 enhanced carotenoid accumulation with up-regulation of ethylene and carotenoid
514 biosynthetic genes (Figure 2 and 6). In contrast, dual *SlBZR1/SlBES1*-KO or *SlBIN2-*

515 *OE* tomato delayed ethylene production peaks and lower the content of carotenoids
516 (Figure 1 and 6). These results suggest that BRs promote tomato fruit ripening possibly
517 through activating the expression of ethylene biosynthetic genes and ultimately the
518 ethylene production.
519
520 **SlBZR1 and SlBES1 function redundantly in tomato fruit ripening *via* distinct**
521 **regulatory mechanisms**
522
523 BR signaling is dependent on a series of phosphorylation events to modulate the
524 function of BZR1/BES1 transcription factors that regulate the expression of BR-
525 responsive genes [69, 70]. In this study, *SlBZR1* and *SlBES1*, the homologous gene of
526 *AtBZR1* and *AtBES1*, were isolated from tomato. The expression of *SlBZR1* and *SlBES1*
527 increased during the earlier stage of tomato fruit ripening, which suggests that *SlBZR1*
528 and *SlBES1* are crucial regulators in the process of fruit ripening. Dual *SlBZR1/SlBES1*-
529 KO tomato retarded fruit ripening with delayed ethylene production peaks and lower
530 carotenoid content. Considering *SlBES1* promotes tomato fruit softening through
531 transcriptional inhibition of *PMEU1* without affecting carotenoid levels and ethylene
532 production, which indicates that *SlBZR1* might be essential in tomato fruit ripening and
533 carotenoids accumulation [54].
534
535 Our study indicates that *SlBZR1-OE* promoted tomato fruit ripening with elevated
536 ethylene and carotenoid contents, conversely, *SlBZR1-KO* retarded fruit ripening and
537 ethylene production as well as lower carotenoid levels. Unraveling the molecular events
538 underlying *SlBZR1* action by integrating RNA-seq and ChIP-seq data, revealed that
539 knockout of *SlBZR1* results in massive transcriptomic reprogramming involving several
540 families of transcription factors and a large number of fruits ripening related genes that
541 are under the direct regulation of *SlBZR1*. The high number of DEGs involved in
542 ethylene biosynthesis pathway and carotenoids metabolism pathway are influenced in
543 the *SlBZR1*-KO lines support the notion that *SlBZR1* is a master regulator of tomato
544 fruit ripening, in line with the increase in ethylene production and carotenoid

545 accumulation.

546

547 This study showed that SlBZR1 positively regulated the expression of *SlACO1,*
548 *SlACO3* and *SlPSY1* by directly binding to the E-boxes in the promoters of these genes
549 during tomato fruit ripening (Figure 4 and 5). Similarly, in Arabidopsis roots,
550 BES1/BZR2 increase ethylene production for gravitropism by directly binding to the
551 E-boxes in the promoter of *ACO1* [71]. In contrast, BZR1 negatively regulates *ACO4*
552 expression by binding to the BRRE-box of the *ACO4* promoter in arabidopsis seedlings
553 [72]. Furthermore, BZR1 functions as a transcription activator and repressor on
554 different promoters [38]. In this study, *SlBZR1* and *SlBES1*, as a transcriptional
555 activator and repressor, promote tomato fruit ripening by increasing the biosynthesis of
556 ethylene and carotenoid, and inhibiting pectin de-methylesterification, respectively.
557 However, regarding BRs accelerate tomato fruit ripening [41, 48], we suggest that the
558 transcriptional activator *SlBZR1* plays a major role in tomato fruit ripening.

559

560 We observed that SlBZR1 interacts with the SlBIN2 which functions as a negative
561 regulator of BR signaling upstream. Previous studies have demonstrated that the
562 presence of BR results in dephosphorylation of BZR1, and the dephosphorylated BZR1
563 then enters into the nucleus to regulate expression of its target genes [34, 73, 74]. The
564 sub-cellular localization results showed that SlBZR1 are nucleus- and cytoplasm-
565 localized proteins, and EBL treatment induced the nuclear localization of SlBZR1
566 proteins, whereas the interaction between SlBIN2 and SlBZR1 resulted in the
567 phosphorylated SlBZR1 moving into cytoplasm, thus affecting SlBZR1 binding to the
568 promoter of its target genes (Supplemental Figure S6D). These findings are similar to
569 the results that BIN2-induced phosphorylation inactivates BZR1 by promoting their
570 cytoplasmic retention via 14-3-3 proteins [74, 75]. Moreover, *SlBIN2-OE* delayed
571 ethylene production peaks and lower the content of carotenoids, while *SlBIN2-KO*
572 accelerated ethylene production and enhanced carotenoid accumulation (Figure 6). In
573 combination with above observations, it can be concluded that SlBIN2induces the
574 cytoplasmic retention of phosphorylated SlBZR1 via interaction with it, thereby

575 inhibiting transcriptional activation of *SlACO1, SlACO3* and *SlPSY1* by SlBZR1.

576

577 In conclusion, our results support a model that BR signaling regulates tomato fruit
578 ripening (Figure 7). When endogenous BRs are present at low levels, SlBIN2 is
579 constitutively active and interacts with SlBZR1 to promote degradation of cytoplasmic
580 located phosphorylated SlBZR1, thereby inhibit its DNA binding activities, thus retard
581 tomato fruit ripening and decrease the levels of carotenoids. when endogenous BR
582 levels are present at high levels, a series of phosphorylation and dephosphorylation
583 reactions are occurred, leading to the inactivation and degradation of dephosphorylated
584 SlBIN2. SlBZR1 is then rapidly dephosphorylated by BIN2 as substrate and moved
585 into the nucleus to active the transcription of ethylene biosynthetic genes *SlACO1* and
586 *SlACO3* and carotenoid biosynthetic gene *SlPSY1* by direct binding to their promoters,
587 leading to a burst of ethylene production and carotenoids accumulation, thus promoting
588 fruit ripening in tomato.

589

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600

601 **AUTHOR CONTRIBUTION**

602 Q.W., L.L., and F.M. designed the research. F.M., H.L., S.H., C.J., M.Z., S.L., Y.L., J.L.,
603 Y.J., M.W., Z.S., and Y.M. performed the research. F.M. H.L., and S.H. analyzed data.
604 F.M., L.L., and Q.W. wrote the manuscript.

605

606 **DECLARATION OF INTERESTS**

607 The authors declare no competing interests.

608

609 Resource availability

610 *Lead Contact*

611 Further information and requests for resources and reagents should be directed to and

612 will be fulfilled by Qiaomei Wang (qmwang@zju.edu.cn).

613

614 *Materials Availability*

615 This study did not generate new unique reagents.

616

617 *Data and Code Availability*

618 The RNA-seq data have been deposited at the NCBI Sequence Read Archive (SRA)

619 database (http://www.ncbi.nlm.nih.gov/sra/) with the BioProject ID PRJNA635540 and

620 are publicly available as of the date of publication. Accession numbers are listed in the

621 key resources table. This paper does not report original code. Any additional

622 information required to reanalyze the data reported in this paper is available from the

623 lead contact upon request.

624

625 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

626 **Plant Materials and Growth Conditions**

627 We used *Solanum lycopersicum* cv. Ailsa Craig (AC) as the control genotype in the

628 present study. The following tomato genotypes were used in this study: *CYP90B3-OE*,

629 *CYP90B3-RNAi*, *SlBIN2*-OE, *bin2*, *SlBZR1*-OE, *bzr1 and bzr1bes1*. Transgenic plants

630 *CYP90B3-OE* and *CYP90B3-RNAi* were generated during previous studies [48]. Plants

631 were cultivated under a 16 h photoperiod (22/28°C, night/day). *Solanum lycopersicum*

632 flowers were labeled at anthesis and the number of tagged fruits was limited to fewer

633 than four per cluster. The fruits were collected after 30 to 46 DPA stage for the different

634 fruit ripening stages. The fruit ripening stages, including mature green (MG) stage,

635 breaker (B) stage, pink (P) stage, and red ripe (R) stage, which were defined based on
636 fruit color as described previously [76]. At the MG stage, fruits reach full size and
637 display jelly placental tissues. The fruits displaying their first signs of color change
638 were identified as B stage, 3 and 7 days after B stage were classified as P and R stage,
639 respectively. Three biological replicates for pericarp tissues of the fruits (each
640 biological replicate consisting of at least three fruits) were collected after harvesting,
641 frozen in liquid nitrogen immediately, and then stored at -80°C for further tests.
642
643 **Accession Numbers**
644 The accession numbers for the genes described in this report are as follows: *SlBZR1*
645 (Solyc12g089040), *SlBES1* (Solyc04g079980), *SlPSY1* (Solyc03g031860), *SlACO1*
646 (Solyc07g049530), *SlACO3* (Solyc07g049550), *SlBIN2* (Solyc07g055200),
647 *SlCYP90B3* (Solyc02g085360), *ACTIN7* (Solyc03g078400), *ACTIN2*
648 (Solyc11g005330).
649
650 **METHOD DETAILS**
651 **Vector Construction and Plant Transformation**
652 To generate the $35S_{pro}$:*SlBZR1*-eGFP construct, the full-length coding sequence (CDS)
653 of *SlBZR1* without termination codon was amplified via PCR, cloned into pQB-V3, and
654 recombined with the binary vector pK7FWG2.0 (*35S* promoter, N-eGFP). To generate
655 the $35S_{pro}$:*SlBIN2*-myc construct, *SlBIN2* CDS was PCR amplified, cloned into pQB-
656 V3, and recombined with the binary vector PGWB17 (*35S* promoter, c-4myc). All
657 vector constructs were generated following standard molecular biology protocols and
658 using Gateway (Invitrogen) technology. Primers used for vector construction are listed
659 in Supplemental Table. All above constructs were introduced into tomato cultivars AC
660 via *Agrobacterium tumefaciens* LBA4404-mediated transformation. Transformants
661 were selected based on their resistance to kanamycin [77]. Homozygous T2 or T3
662 transgenic plants were used for phenotypic and molecular characterization. T2 progeny
663 of independent transgenic lines *SlBZR1*-OE (OE-8, OE-9 and OE-14) as well as
664 *SlBIN2*-OE (OE-1, OE-2 and OE-4) were used in this study.

665 **Generation of *slbzr1, slbin2 and slbzr1 slbes1* Using CRISPR/Cas9 Technology**

666 CRISPR-P (http://cbi.hzau.edu.cn/crispr/) was used to design the single guide RNA
667 (sgRNA) sequence that target either *SlBZR1* or *SlBIN2* and two guide RNA sequences
668 which targeted both *SlBZR1* and *SlBES1* genes. Sequences of the sgRNAs are listed in
669 Supplemental Table. The CRISPR/Cas9 system was used to generate the *slbzr1* and
670 *slbin2* mutant as previously described. The sgRNA expression cassette with target
671 sequence were assembled to *pYAO*:hSpCas9 binary plasmid [78]. The CRISPR/Cas9
672 mutagenesis of *slbzr1 slbes1* was performed by expressing two guide RNA sequences
673 which targeted both *SlBZR1* and *SlBES1* genes in pYLCRISPR/Cas9 vector [79]. After
674 confirmation by sequencing, three final binary vectors containing sgRNA and Cas9 was
675 transferred into *Agrobacterium tumefaciens* LBA4404, then introduced into AC as
676 described previously. CRISPR/Cas9-induced mutations were genotyped by PCR
677 amplification and DNA sequencing. The induced genomic mutations and the primers
678 used for confirming mutation are given in Fig. 1 and Supplemental Table, respectively.
679 Homozygous T2 transgenic plants *bzr1-5*, *bzr1-28*, *bzr1-29*, *bin2-5* and *bin2-10* as well
680 as *bzr1bes1-1* and *bzr1bes1-2* were selected for further experiments.

681 **Chemical treatment**

682 The fruits of AC and *SlBZR1-OE* (OE-14) were harvested at mature green stage (the
683 MG stage). The fruits of AC were treated with 3 μM 24-epibrassinolide (EBL, Sigma,
684 St. Louis, MO) or 5 μM Pcz as well as the combination of 3 μM eBL and 5 μM PCZ,
685 respectively [51]. Fruits treated with solvent were used as a control. After the treatments,
686 the fruits were stored at at 24 ℃ and 80% relative humidity and sampled at 1st, 3rd,
687 6th, and 9th day for further tests. For 1-MCP treatment, the fruits of AC and *SlBZR1-*
688 *OE* was placed in 25 L containers and treated with 1 μL L^{-1} 1-MCP for 16 h. After
689 treatment with 1-MCP, the fruits were removed from the containers and stored at 24 ℃
690 and 80% relative humidity and collected at 0, 1st, 4th, 7th, and 10th day for further tests.

691 **Measurement of ethylene production**

692 Ethylene production of tomato fruit was detected according to former reports with some
693 minor modifications. Five fruits were enclosed in 1.0 L airtight container at 20 ℃ for

694 2 h, 1 mL of the headspace gas was withdrawn from the container with a 1 mL syringe
695 and immediately injected into a gas chromatograph (Shimadzu GC-17A, Kyoto, Japan),
696 consisting of a flame ionization detector and a hp-5 column (50 m × 0.32 mm × 1.05 μm)
697 with a detection temperature of 200 ℃. The ethylene production was calculated
698 according to commercial standards. Fresh tissues of fruit were mixed frozen in liquid
699 nitrogen and stored at -80 ℃ until use.

700 **Firmness determination**

701 The fruits of wild type and transgenic lines were harvested at each development stage.
702 Firmness was measured at the fruit equatorial region with a TA-XT2i texture analyzer
703 (Stable Micro Systems Ltd., Godalming, UK). The penetration depth was set at 10 mm
704 and the penetration speed of a 7.5mm probe was set at 1 mm s^{-1}. The unit of force for
705 firmness is Newton.

706 **HPLC analysis of carotenoids**

707 The extract and analysis of carotenoids was conducted as early described using high
708 performance liquid chromatography (Shimadzu, Kyoto, Japan) with minor
709 modification [30]. About 0.5 g of tomato fruit powder was extracted with 30 mL of
710 hexane, acetone and ethanol (1:1:1, v/v/v). The mixture was shaken and then 15 mL of
711 double distilled water added. The extracts were centrifuged at 12000 g for 15 min at
712 4 ℃. The supernatant was eluted through a 0.22 μm filter membrane then evaporated
713 to dryness with nitrogen blowing instrument. The condensed extract was dissolved in
714 tetrahydrofuran: acetonitrile: methanol (15:30:55, v/v/v) with 1.5 mL dissolution. Then
715 the solutions were loaded into liquid phase bottle for HPLC analysis.

716 The analysis of carotenoids was determined using HPLC instrument (Shimadzu, Kyoto,
717 Japan), consisting of a SPD-M20A diode array detector and chromatographic column
718 C18 (5 μm particle size, 4.6 mm × 250 mm, Elite analytical instruments Co., Ltd.,
719 Dalian, China), The mobile phase was methanol: acetonitrile (90:10, v/v) containing
720 0.05 % TEA. The current velocity and the wavelength were set as 1.2 mL/min and
721 475 nm, respectively. Peak area of carotenoids was selected based on the spectral
722 characteristics and then calculated according to standard compounds (lutein, lycopene,
723 and β-carotene; Sigma, USA).

724 **RNA extraction and qRT-PCR analysis**

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

731 **Subcellular localization**

732 The *35S*$_{pro}$:*SlBZR1*-eGFP and *35S*$_{pro}$:*SlBIN2*-myc constructs was co-infiltrated with a
733 mCherry-labeled nuclear marker NF-YA4-mCherry into *N. benthamiana* leaves using
734 *A. tumefaciens*-mediated infiltration [80]. The N. benthamiana plants were kept in the
735 dark for 48 h after infiltration. Then EBR (10 μM) was injected into the infiltrated *N.*
736 *benthamiana* leaves and imaging was performed 12 h after EBR treatment. GFP
737 fluorescence was observed under a confocal microscope (TCS SP8; Leica Wetzlar,
738 Germany). For green fluorescence observation, the excitation wavelength was 488 nm
739 and the emission wavelengths were 520~540 nm; for red fluorescence observation, the
740 excitation wavelength was 561 nm and the emission wavelengths were 610~630 nm.
741 *35S*$_{pro}$:eGFP was used as a control. All transient expression assays were repeated at least
742 3 times, and the representative results were shown.

743 **RNA-seq**

744 Total RNA was extracted from tomato fruits of WT and *bzr1* at MG and B stages and
745 Illumina MiSeq library was constructed as manufacturer's instructions (Illumina, San
746 Diego, CA, USA) described. Then each sample library was sequenced with the Illumina
747 Miseq platform (Shanghai Personal Biotechnology Cp., Ltd., Shanghai, China).

748 **ChIP-seq and ChIP-qPCR Assays**

749 Chromatin immunoprecipitation sequencing was performed as previously reported [81].
750 In brief, tomato fruits of *SlBZR1*-OE at MG stage were collected from at least six
751 different plants, sliced into small pieces and fixed in 1x PBS with 1% formaldehyde for
752 15 min under vacuum and ground to fine power under liquid nitrogen. ChIP assay was
753 performed in three independent biological replicates starting with 2 g of each sample

754 tissue. Nuclei were purified using a one-step Percoll gradient centrifugation as
755 previously described. ChIP-Seq was performed with the anti-GFP antibody (Millipore)
756 using transgenic plants expressing their cDNA fused to GFP under the 35S promoter.
757 For conventional TruSeq libraries, the reverse crosslinked DNA was end-repaired, dA-
758 tailed, ligated to Y-adapters, and PCR amplified prior to sequencing. For the more
759 recent Tn5-based ChIP-Seq libraries, 1-2 uL of purified and assembled transposome (1
760 OD) was added to the washed magnetic beads in the presence of 30 uL 1x Tn5 buffer
761 (10 mM $MgCl_2$, 25 mM Tris pH 8.0, 10% DMF). The tagmentation reaction was
762 performed at 37 °C for 30 min. The beads were then washed with low salt, high salt,
763 and TE washing buffers and reverse crosslinked overnight. The purified DNA was then
764 PCR amplified using N70x and N50x index primers. Two to three biological replicates
765 were sequenced for each experiment.
766 For ChIP-qPCR, 3 g of 0.5 cm^2 fruit pieces of *SlBZR1*-OE were collected at mature
767 green stage and cross-linked using 1%(v/v) formaldehyde under vacuum for 10 min
768 and ground to powder in liquid nitrogen. Then, the chromatin complexes were isolated
769 following former research, sonicated with Biorupter plus (Diagenode, Belgium,
770 Antwerp), and immunoprecipitated with 5 μg Mouse monoclonal anti-GFP antibody
771 (clone 9E10, IgG, Roche, Switzerland). About 200~300 bp of ChIP DNA and input
772 DNA were recovered and dissolved in water for further qPCR analysis [82]. The
773 primers used for ChIP-qPCR were designed around E-box in the enriched regions and
774 are listed in Supplemental Data. Each ChIP value was normalized to its respective input
775 DNA value. The fold enrichment on each candidate genes promoter was calculated
776 against the ACTIN7 or ACTIN2, respectively. Mean value of two technical replicates
777 was recorded for each biological replicate. The values of three independent biological
778 replicates were collected and error bars represented the standard error from these
779 experiments.
780 **Dual luciferase transactivation assay**
781 The whole length of SlBZR1 CDS was fused with pGreen II-SK vector to generate an
782 effector construct. The promoter was cloned into the vector pGreen II-LUC to generate
783 a reporter construct using gene-specific primers. These constructs were introduced into

784 Agrobacterium cell GV3101 together with the pSoup helper plasmid. Tobacco leaves
785 were agroinfiltrated with the indicated plasmids, and harvested 3 d later. NightOWL II
786 LB983 Ultrasens backlit (Berthold Technologies, Bad Wildbad, Germany) with Indigo
787 software was used to capture LUC expression image and to quantify LUC luminescence
788 intensity. 100 mM luciferin was sprayed on leaves and incubated in dark for 5 min
789 before detection. Six independent determinations were performed.

790 **Yeast one-hybrid assay**

791 The SlBZR1 CDS was amplified and inserted into pGADT7 to generate a prey construct.
792 The promoter fragments of *SlPSY1* (1,835-bp upstream of the predicted translation start
793 site), *SlACO1* (546-bp upstream of the predicted translation start site) or *SlACO3* (2828-
794 bp upstream of the predicted translation start site) were cloned separately into the
795 pAbAi vector to create bait constructs. After digesting with BstB1, these bait constructs
796 were used to transform yeast strain Y1HGold and integrated into the yeast genome to
797 create the bait reporter strains. The minimal inhibitory concentration of aureobasidin A
798 (AbA) was screened to avoid self-activation. The prey vector and the empty vector
799 (AD), serving as the negative control, were transformed separately into the bait reporter
800 strains and grown on SD medium lacking Leu (SD/-Leu) plates with or without AbA at
801 30℃ for 72 h. The positive clones were picked and diluted in double-distilled water to
802 a final concentration of OD600 = 1.0. The suspension was spotted on SD/-Leu medium
803 with or without AbA to assess protein-DNA interactions.

804 **Yeast two-hybrid assay**

805 The TAD deletion constructs of SlBZR1 were used for the Y2H screening of the
806 pGADT7-based tomato cDNA library, which was generated with mRNAs isolated from
807 toamto leaves, roots, stems, flowers and fruits. The SlBZR1 CDS was introduced into
808 the pGBKT7 vector and then used as bait to screen the cDNA library based on the
809 Matchmaker Gold Yeast Two-Hybrid System (Clontech). Yeast transformants were
810 exhaustively selected on SD/-Ade/-His/-Leu/-Trp/X-α-Gal medium. Putative SlBZR1
811 interacting clones were characterized and sequenced.

812 Full length coding sequence (CDS) of SlBIN2 was ligated to the binding domain (BD)
813 in the pGBKT7 vector, and full-length CDS of SlBZR1 was introduced into the

activation domain (AD) vector (pGADT7). The BD and AD vectors were co-transformed into the Y2H Gold yeast strain (AH109). Transformed yeast cells were grown on SD/-Leu/-Trp medium and SD/-Ade/-His/-Leu/-Trp medium containing x-a-gal to determine protein-protein interactions.

Statistical analysis

The experimental data were analyzed with SPSS 19.0 software. Multiple comparisons were subjected to ANOVA using Duncan's test， while pairwise comparisons were computed using Student's *t*-test ($P < 0.05$). Statistically signifificant differences ($P < 0.05$) are represented by different lowercase letters.

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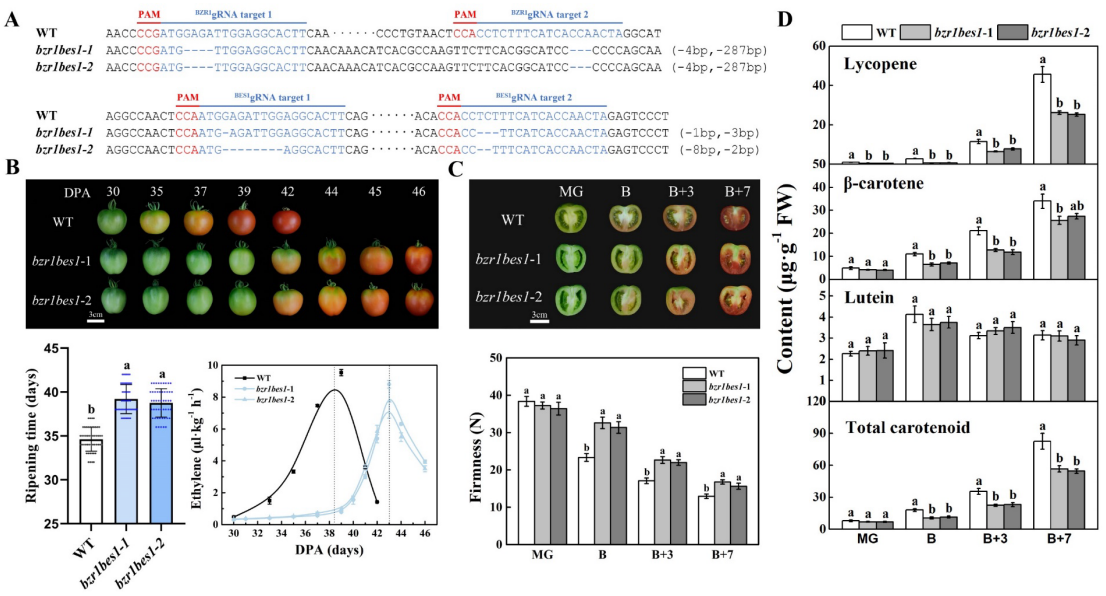

Figure 1. SlBZR1/SlBES1 double gene knockout abolish BR signaling delays tomato fruit ripening and ethylene production, reduces carotenoid accumulation.

(A) Genotype of mutations in the SlBZR1 and SlBES1 locus generated by the CRISPR/Cas9 genome editing system. Two target sequences were designed to specifically target the second exons with a 221-bp interval. The red and blue letters indicate the sequences of protospacer adjacent motif (PAM) and target, respectively. The mutations in the transgenic plants were confirmed by sequencing the genomic regions flanking the target sites. The sequences of the wild-type (WT) plants and two homozygous mutant lines (*bzr1bes1-1* and *bzr1bes1-2*) are shown. (B) Ripening phenotype of *bzr1bes1* null mutant fruits. WT and *bzr1bes1* mutant fruits at 30, 35, 37, 39, 42, 44, 45, and 46 days post anthesis (DPA) are shown. Ripening time (days from anthesis to breaker stage) of WT and *bzr1bes1* mutant fruits. Changes in ethylene production of WT and *bzr1bes1* mutant fruits. (C) Longitudinal section phenotypes of WT and *bzr1bes1* mutant fruits at four different developmental stages. Fruit firmness and carotenoids content (D) were measured. MG, mature green stage; B, breaker; B + 3, 3 d after breaker stage; B + 7, 7 d after breaker stage. Biological replicates (3-4 fruits per fruit ripening stage) were performed in triplicate, and the data are presented as means ± SE. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$).

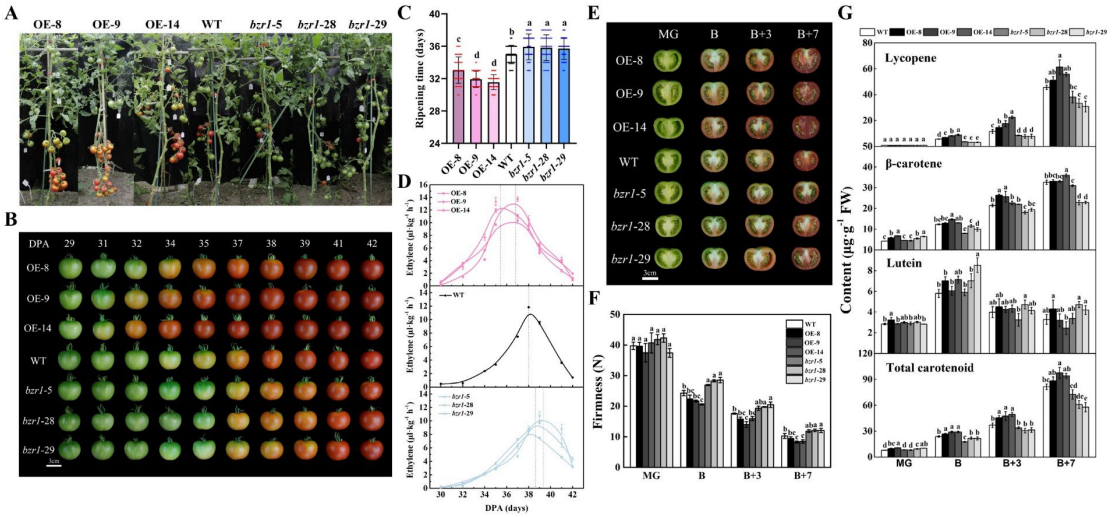

Figure 2. *SlBZR1* promotes the fruit ripening schedule and carotenoids accumulation.

(A) Phenotypes of *SlBZR1*-OE and *bzr1* mutant lines. (B) Ripening phenotype of *SlBZR1*-OE and *bzr1* mutant fruits. Fruits from *SlBZR1*-OE lines ripen earlier while *bzr1* mutant lines show a delayed ripening phenotype. (C) Ripening time (days from anthesis to breaker) of WT and *SlBZR1*-OE and *bzr1* mutant fruits. (D) Ethylene production in WT, *SlBZR1*-OE and *slbzr1* mutant fruits at different developmental stages. (E) Cross-sectioned WT, *SlBZR1*-OE and *slbzr1* mutant fruits at four different developmental stages. (F) Fruit firmness and carotenoids content (G) in WT, *SlBZR1*-OE and *slbzr1* mutant fruits at four different developmental stages. MG, mature green stage; B, breaker; B + 3, 3 d after breaker stage; B + 7, 7 d after breaker stage. Biological replicates (3–4 fruits per fruit ripening stage) were performed in triplicate, and the data are presented as means ± SE. The asterisks indicate statistically significant differences between the WT and transgenic fruits (*P < 0.05).

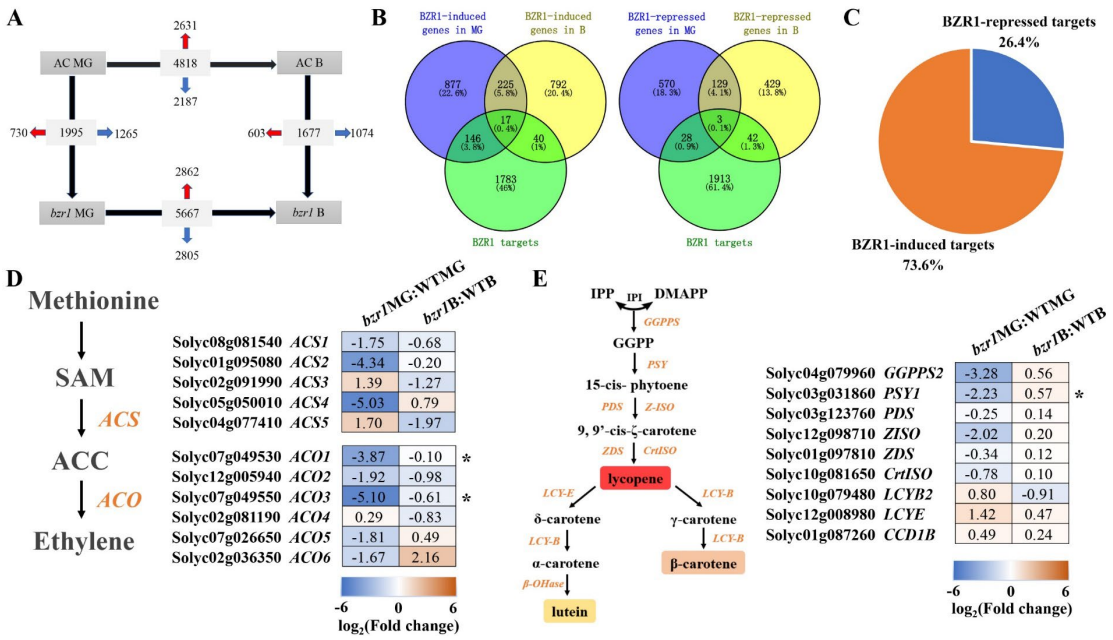

Figure 3. *SlBZR1* target genes identified by combined genome-wide RNA-seq and ChIP-seq approaches.

(A) Number of differentially expressed genes (|$\log_2$(fold change)|>1 and p-value<0.05) between WT and *bzr1* at MG and B stage identified by RNA-seq. Indicated are total numbers (boxed) and numbers of up-regulated (➡) and down-regulated genes (➡) between stages and genotypes. (B) Venn diagram showing the numbers of genes common to *SlBZR1*-regulated genes and SlBZR1 target genes. (C) Percentage of SlBZR1-repressed and SlBZR1-induced target genes (in total 276). (D) Heatmap showing the differentially expressed genes involved in ethylene biosynthesis identified from the comparison of the *bzr1* mutant versus WT fruits at MG and B stage based on RNA-seq analysis. (E) Heatmap showing the differentially expressed genes involved in carotenoid biosynthesis identified from the comparison of the *bzr1* mutant versus WT fruits at MG and B stage based on RNA-seq analysis.

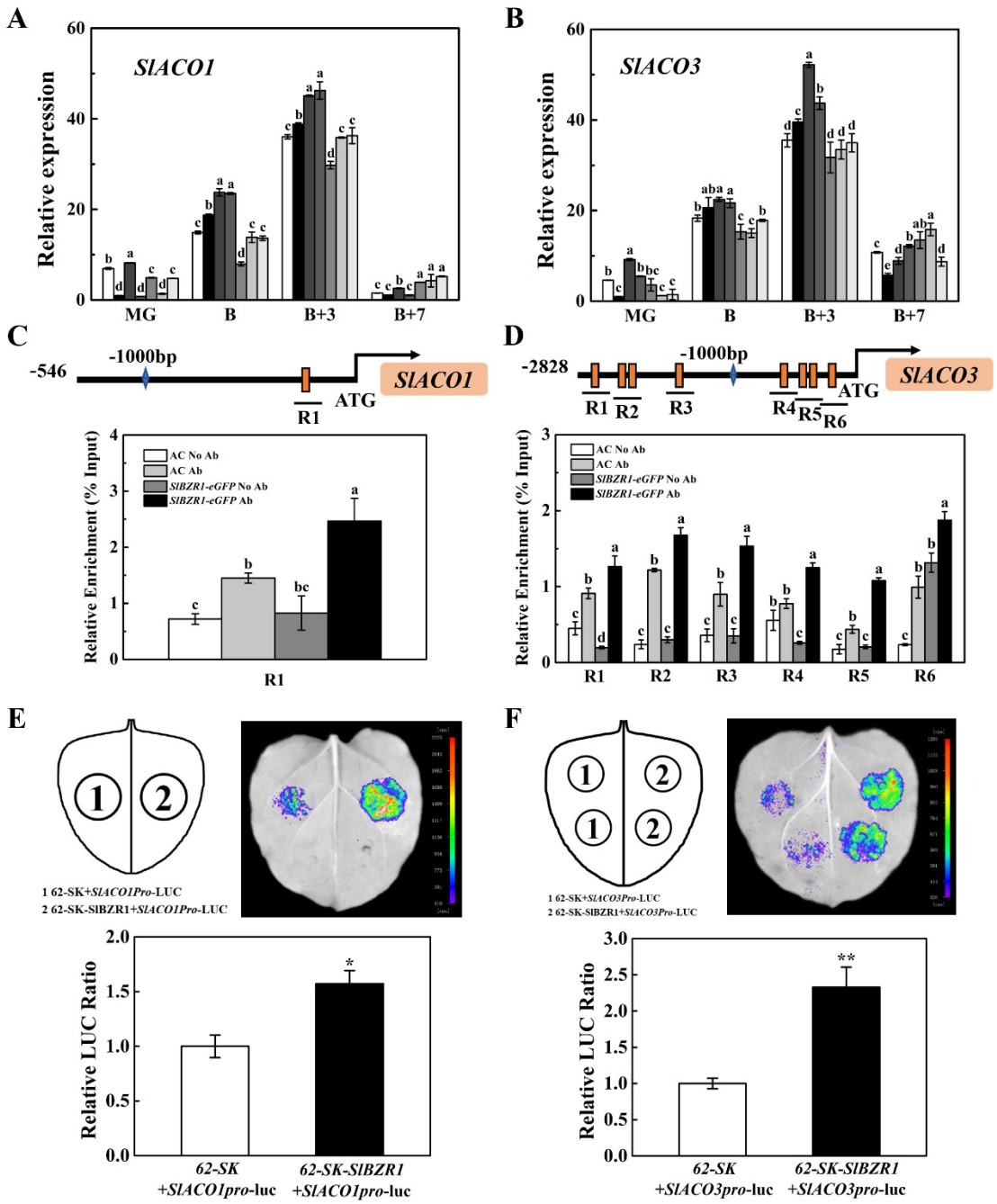

Figure 4. SlBZR1 directly binds and activates the expression of *SlACO1* and *SlACO3*

(A) Relative expression levels of *SlACO1* and *SlACO3* (B) in SlBZR1-OE and *bzr1* mutant fruits at different development stages. MG, mature green stage; B, breaker; B + 3, 3 d after breaker stage; B + 7, 7 d after breaker stage. Biological replicates (3-4 fruits per fruit ripening stage) were performed in triplicate, and the data are presented as means ± SE. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$). (C) ChIP-qPCR assays showing that SlBZR1 binds to the promoters of *SlACO1* and *SlACO3* (D). The promoter structures of the SlBZR1 target genes *SlACO1* and *SlACO3* are shown. The orange boxes represent E-box elements, and the numbers indicate the positions of these motifs relative to the translational start site. The black lines with uppercase letters represent the regions used for ChIP-qPCR. The relative enrichment values show the percentages of DNA fragments that coimmunoprecipitated with anti-GFP antibodies relative to the input

DNAs. AC (wild type) without and with antibody were set as blank control and negative control, respectively. SlBZR1-GFP without antibody was also set as negative control. The mean value of two technical replicates was recorded for each biological replicate. Values are means ± SD of three biological replicates. Different letters indicate significant difference among groups for each locus (one-way ANOVA with Tukey's test, $P < 0.05$). (E, F) Transient expression assay for SlBZR1 activation of the promoters of *SlACO1* and *SlACO3*. The reporter and effector vectors in each experiment were co-introduced into tobacco (*N. benthamiana*) leaves by Agrobacterium GV3101. SK represents empty vector control. Representative images of tobacco leaves were taken 3d after infiltration. Luminescence intensity values are means ± SD of six biological replicates. Different letters indicate significant difference among groups for each locus (one-way ANOVA with Tukey's test, $P < 0.05$).

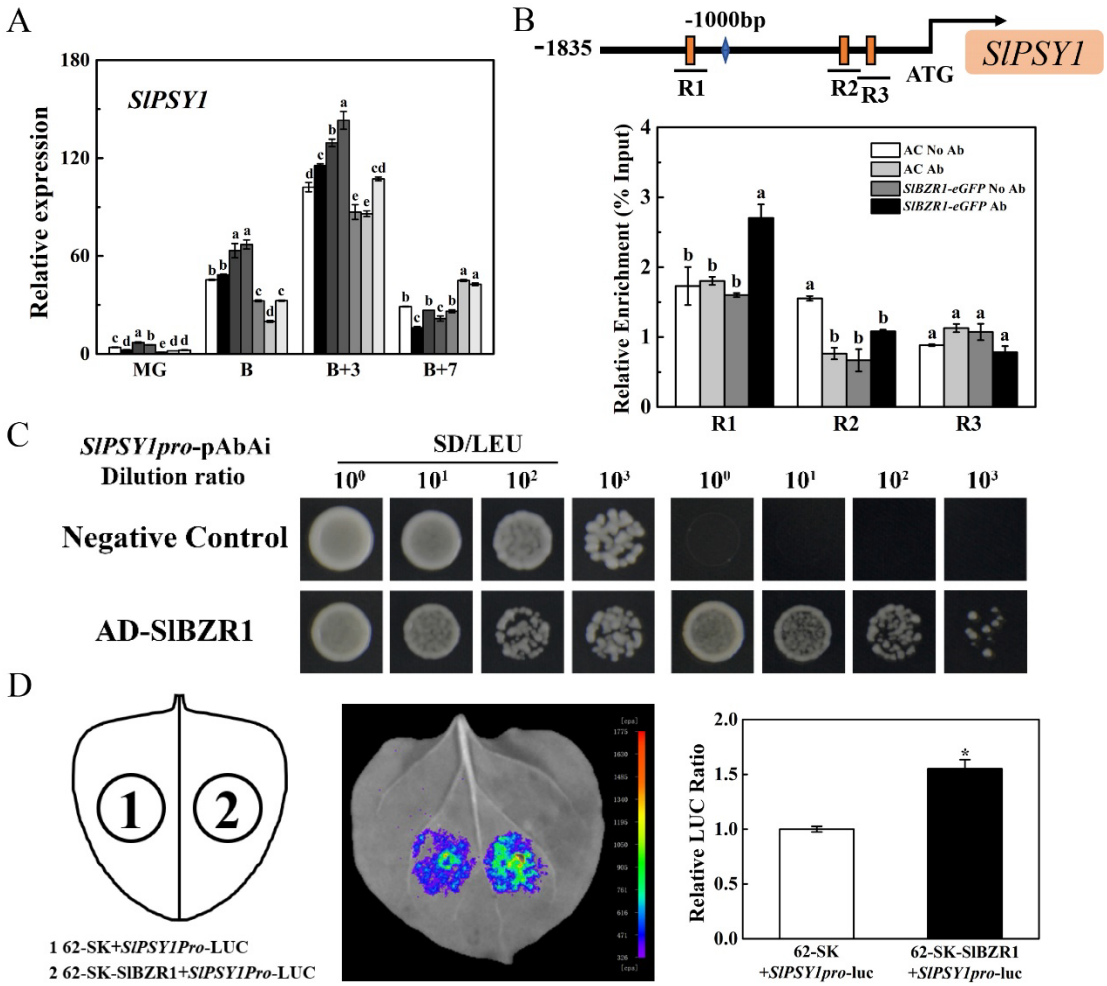

Figure 5. SlBZR1 positively regulates the expression of *SlPSY1* involved in carotenoid accumulation in ripening tomato fruits

(A) Relative expression levels of *SlPSY1* in SlBZR1-OE and *bzr1* mutant fruits at different development stages. MG, mature green stage; B, breaker; B + 3, 3 d after breaker stage; B + 7, 7 d after breaker stage. Biological replicates (3-4 fruits per fruit ripening stage) were performed in triplicate, and the data are presented as means ± SE. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$). (B) ChIP-qPCR assays showing that SlBZR1 binds to the promoters of *SlPSY1*. The promoter structures of the SlBZR1 target genes *SlPSY1* are shown. The orange boxes represent E-box elements, and the numbers indicate the positions of these motifs relative to the translational start site. The black lines with uppercase letters represent the regions used for ChIP-qPCR. The relative enrichment values show the percentages of DNA fragments that coimmunoprecipitated with anti-GFP antibodies relative to the input DNAs. AC (wild type) without and with antibody were set as blank control and negative control, respectively. SlBZR1-GFP without antibody was also set as negative control. The mean value of two technical replicates was recorded for each biological replicate. Values are means ± SD of three biological replicates. Different letters indicate significant difference among groups for each locus (one-way ANOVA with Tukey's test, $p < 0.05$). (C) Y1H analysis showing that SlBZR1 binds to the promoter of *SlPSY1*. AbA, a yeast cell growth inhibitor, was used as a screening marker. The basal concentration of AbA was 150 $ng \cdot mL^{-1}$. The empty vector and the *SlPSY1* promoter were used as negative controls. (D) Transient expression assay for SlBZR1 activation of the promoters of *SlPSY1*. The reporter and effector vectors in each experiment were co-introduced into tobacco (N.

benthamiana) leaves by Agrobacterium GV3101. SK represents empty vector control. Representative images of N. benthamiana leaves were taken 3d after infiltration. Luminescence intensity values are means ± SD of six biological replicates. Different letters indicate significant difference among groups for each locus (one-way ANOVA with Tukey's test, $p < 0.05$).

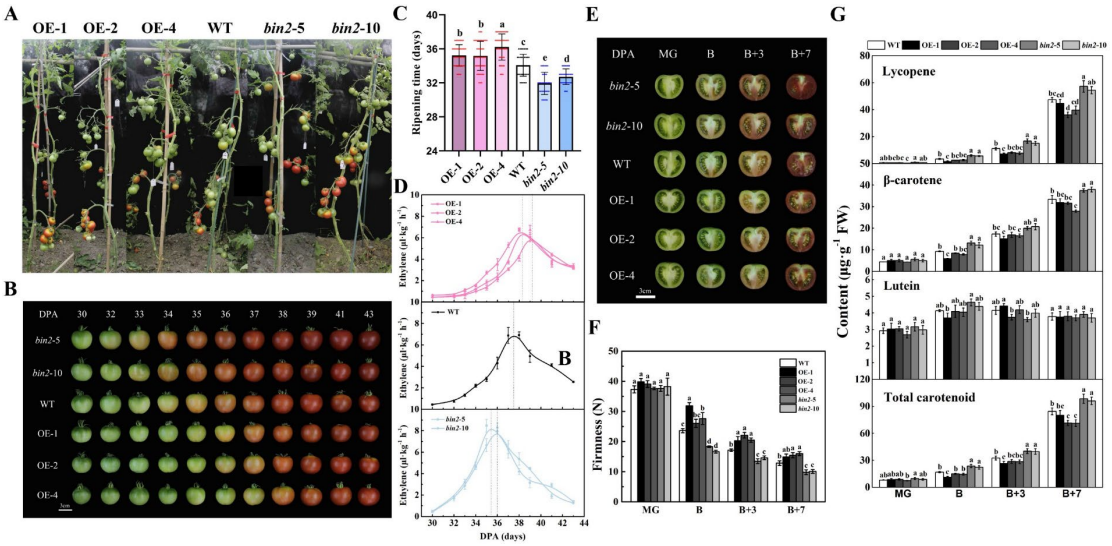

Figure 6. SlBIN2 delays tomato fruit ripening and reduces carotenoid accumulation.

(A) Phenotypes of *SlBIN2*-OE and *bin2* mutant lines. (B) Ripening phenotype of *SlBIN2*-OE and *bin2* mutant fruits. Fruits from *SlBIN2*-OE lines show a delayed ripening phenotype while *bzr1* mutant lines ripen earlier. (C) Ripening time (days from anthesis to breaker) of WT, *SlBIN2*-OE and *bin2* mutant fruits. (D) Ethylene production of WT, *SlBIN2*-OE and *bin2* mutant fruits at different developmental stages. (E) Cross-sectioned WT, *SlBIN2*-OE and *bin2* mutant fruits at four different developmental stages. (F) Fruit firmness and carotenoids content (G) in WT, *SlBIN2*-OE and *bin2* mutant fruits at four different developmental stages. MG, mature green stage; B, breaker; B + 3, 3 d after breaker stage; B + 7, 7 d after breaker stage. Biological replicates (3-4 fruits per fruit ripening stage) were performed in triplicate, and the data are presented as means ± SE. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$).

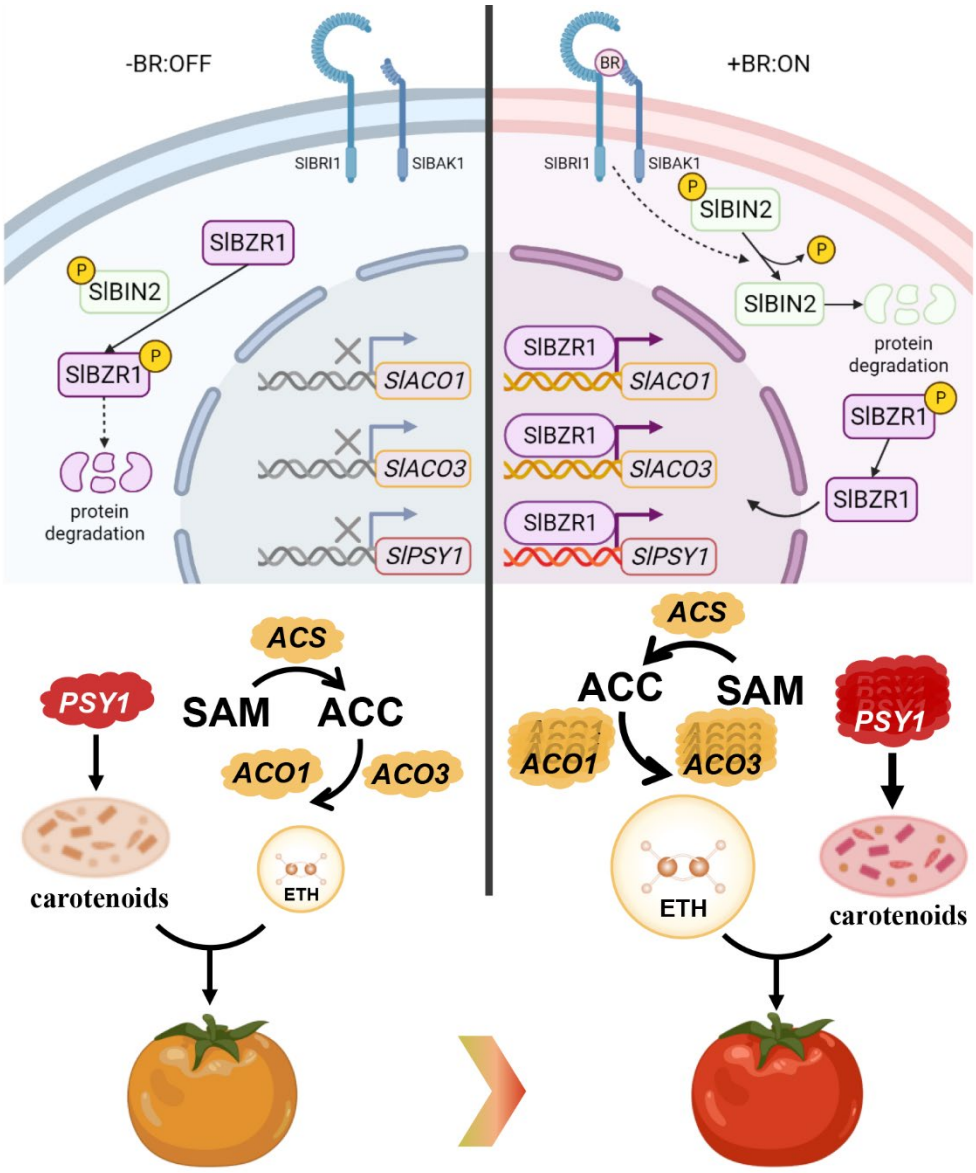

Figure 7. A proposed regulatory mechanism of BR on tomato fruit ripening and carotenoid accumulation.

When BRs (brassinosteroids) are absent (left), BRI1 (Brassinosteroid-Insensitive1) and its co-receptor BAK1 (BRI1 Associated receptor Kinase 1) are kept inactive. BIN2 (Brassinosteroid-Insensitive 2) is constitutively active and phosphorylates BZR1 (Brassinazole-resistant1) transcription factor to promote its degradation and 14-3-3-mediated cytosolic retention, and to directly inhibit their DNA binding activities. When BRs are present (right) and bind to the extracellular domains of BRI1 and its co-receptor BAK1 to activate the two receptor kinases, a series of phosphorylation and dephosphorylation reactions are occurred, leading to the degradation of dephosphorylated SlBIN2. Phosphorylated SlBZR1 is dephosphorylated and moved into the nucleus to active the transcription of ethylene biosynthetic genes (*SlACO1* and *SlACO3*) and carotenoid biosynthetic gene *SlPSY1* by direct promoter binding, leading to a burst of ethylene production and carotenoids accumulation.