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].

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

151 ripening.

152

153 **Results**

154 **BR promotes fruit ripening**

155

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

174

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

176

177 To better understand BR action and identify the candidate components of BR signaling
178 pathway function in tomato fruit ripening, we manually searched for the *SlBZR1* and
179 *SlBES1*, the homologous gene of *AtBZR1* and *AtBES1* in tomato, encoding proteins of
180 333 and 327 amino acids, with calculated molecular weights of 35.78 and 34.98 kDa,

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

211 of *bzr1bes1-1* and *bzr1bes1-2* lines appeared about 5 days later than in fruits of WT
212 plants (Figure 1B).
213
214 To determine whether fruit quality was altered in *bzr1bes1* mutants, several typical fruit
215 quality parameters were measured at different ripening stages (MG, B, B+3, and B+7).
216 Fruits of both *bzr1bes1-1* and *bzr1bes1-2* lines exhibited increased fruit firmness
217 compared with that of WT at B, B+3 and B+7 stages (Figure 1C), consistent with the
218 delayed ripening of *bzr1bes1* fruits. The contents of lycopene, β-carotene and total
219 carotenoids in fruits of *bzr1bes1-1* and *bzr1bes1-2* lines were much lower than those in
220 fruits of WT at B, B+3 and B+7 stages (Figure 1D). These results suggest that SlBZR1
221 and SlBES1 play a positive role in fruit ripening and carotenoids accumulation.
222
223 ***SlBZR1* promotes tomato fruit ripening**
224
225 Previous studies have shown that *SlBES1* promotes tomato fruit softening without
226 affecting carotenoids contents and ethylene production (Supplemental Figure S3, A and
227 B) [54], which indicates that *SlBZR1* might play a dominant role in tomato fruit ripening
228 and carotenoids accumulation.
229
230 To gain insight into the biological function of SlBZR1 in tomato fruit ripening, we
231 generated *SlBZR1*-overexpression transgenic lines and *SlBZR1* knock-out mutants
232 using CRISPR/Cas9 system in AC. Three transgenic lines (OE-8, OE-9 and OE-14)
233 with elevated *SlBZR1* gene expression levels were selected for further phenotypic and
234 molecular analyses (Supplemental Figure S4, A and B). Likewise, three homozygous
235 *bzr1* mutants (*bzr1-5*, *bzr1-28* and *bzr1-29*) with deletions or insertions that led to
236 translational frame shifts were obtained, which encode truncated SlBZR1 proteins with
237 58, 48 and 49 aa, respectively (Supplemental Figure S4C). These results suggest that
238 *bzr1-5*, *slbzr1-28* and *slbzr1-29* are loss-of-function mutants suitable for functional
239 analysis of *SlBZR1*.
240

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

271 mutant fruits at B stage (Figure 3A). The up-regulated and down-regulated genes in
272 *bzr1* mutant were termed SlBZR1-repressed genes and SlBZR1-induced genes,
273 respectively.
274
275 Moreover, 1986 potential SlBZR1 targets genes were identified by ChIP-seq using a
276 polyclonal anti-GFP antibody and *SlBZR1*-OE fruits at MG stage expressing the BZR1-
277 eGFP fusion protein. We compared the SlBZR1-repressed and SlBZR1-induced
278 differentially expressed genes set obtained by RNA-seq with the SlBZR1 target genes
279 set revealed by ChIP-seq. Cross-referencing the ChIP-seq and RNA-seq data identified
280 276 genes being both *SlBZR1* target and displaying differential expression in *bzr1*
281 mutant fruits (Figure 3B). Interestingly, 73 genes (26.4%) were *SlBZR1*-repressed
282 targets while 203 genes (73.6%) were *SlBZR1*-induced targets, consistent with *SlBZR1*
283 functions as a positive regulator in tomato fruit ripening (Figure 3, B and C).
284
285 The production of ethylene and accumulation of carotenoids as well as fruit softening
286 appears to be a typical feature during tomato fruit ripening. The RNA-seq and ChIP-
287 seq analysis showed that numerous genes involved in fruit ripening including ethylene
288 biosynthesis and carotenoid metabolism as well as transcription factor genes
289 controlling fruit softening and fruit ripening were down-regulated in *bzr1* mutant fruits
290 (Figure 3, D and E).
291
292 The fruit softening-related genes were *SlBZR1*-induced target genes and down-
293 regulated in the *bzr1* mutant fruits, which indicates *SlBZR1* promoted tomato fruit
294 softening by directly regulating fruit softening-related genes. Previous studies have
295 demonstrated that SlBES1 promotes tomato fruit softening through transcriptional
296 inhibition of *PMEU1* without affecting nutritional quality, which indicates that *SlBZR1*
297 and *SlBES1* might perform partially redundant functions in fruit softening and regulate
298 fruit ripening in a reciprocal manner.
299
300 The ethylene biosynthesis genes *SlACO1* and *SlACO3* as well as carotenoid

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

331 biosynthesis in tomato fruit by directly targeting the promoter of *SlACO1* and *SlACO3*.
332
333 **SlBZR1 promotes carotenoid accumulation in a partially ethylene-dependent**
334 **manner**
335
336 To further investigate the influence of the interaction between SlBZR1 and ethylene
337 biosynthesis genes on promoting carotenoid accumulation during tomato fruit ripening,
338 we treated *SlBZR1*-OE (OE-14) and WT fruits collected at mature green stage with 1
339 $\mu L \cdot L^{-1}$ 1-MCP for 12 h. The contents of lycopene and total carotenoids in *SlBZR1*-OE
340 fruits were much higher than in WT fruits after 12 d and 18 d of storage, whereas the
341 accumulation of lycopene, β-carotene and total carotenoids was significantly inhibited
342 by 1-MCP treatment at 6 d, 12 d and 18 d of storage in both *SlBZR1*-OE and WT fruits.
343 Interestingly, the promotion of lycopene and total carotenoid accumulation in the
344 *SlBZR1*-OE fruits were not completely inhibited by 1-MCP treatment. The contents of
345 lycopene and total carotenoids increased in the *SlBZR1*-OE fruits stored for 18 d after
346 1-MCP treatment compared with those in the wild-type fruits (Supplemental Figure S5,
347 A and B). These results implied that *SlBZR1*-OE might promote carotenoid
348 accumulation during tomato fruits ripening in a partially ethylene-dependent manner.
349
350 **SlBZR1 transactivates the expression of *SlPSY1* to regulate carotenoid**
351 **accumulation**
352
353 The expression levels of *SlPSY1* were markedly increased in *SlBZR1*-OE and decreased
354 in *bzr1* mutant fruits compared with WT fruits at MG, B and P stages, which implied
355 SlBZR1 might positively regulate the expression of *SlPSY1* (Figure 5A). To verify the
356 direct regulation of BZR1 on *SlPSY1* expression, we performed promoter analysis of
357 *SlPSY1* gene and found that there was three E-box in the 2-kb upstream regions of the
358 promoters of *SlPSY1*. ChIP-qPCR analysis showed a significantly higher enrichment
359 for the promoters of *SlPSY1*, which indicated that SlBZR1 directly bound to the
360 promoters of *SlPSY1* (Figure 5B).

361

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

368

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

377

378 **SlBIN2 negatively regulates tomato fruit ripening**

379

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

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

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

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

481 In maturation process of climacteric fruits promoted by exogenous BR, the endogenous
482 BR content did not change significantly in mango, banana and jujube [42, 44, 64],
483 which increased in tomato and persimmon [43, 48] and decreased in apple and pear
484 [53]. Similarly, the endogenous BR levels were found to be strongly elevated at the
485 onset of fruit ripening in grape berries [46], implying that the mechanisms underlying
486 BRs in controlling fruit ripening might be different depending on the fruit type.
487
488 As one of the primary initiators and regulators of maturation process in climacteric
489 fruits, ethylene plays a key role in promoting ripeness and coloration of climacteric
490 fruits, and its absence or decrease dramatically delays the ripening process [65].
491 Previous studies have demonstrated that BRs can directly regulate the ripening of non-
492 climacteric fruits, but whether BRs regulate the ripening of climacteric fruits directly
493 or via controlling ethylene biosynthesis remains unclear [66]. Interestingly, one study
494 in Arabidopsis proposed that BRs at a low concentration negatively control ethylene
495 production, while BRs at high concentrations positively regulate ethylene production
496 [67, 68]. However, we cannot conclude that BRs either positively or negatively regulate
497 ethylene biosynthesis in a dose-dependent manner, since the effects of of BRs upon
498 different levels on ethylene biosynthesis are still absent in tomato fruits.
499
500 In this work, we found that exogenous application of eBL markedly promotes the
501 production of ethylene, whereas PCZ treatment inhibits the climacteric peak of ethylene
502 production (Supplemental Figure S1A). Furthermore, *SlCYP90B3*-OE accelerated
503 tomato fruit ripening and advanced the onset of the climacteric peak of ethylene
504 production, while *SlCYP90B3*-RNAi retarded tomato fruit ripening and caused a lower
505 peak of ethylene production (Supplemental Figure S1C). Moreover, overexpression of
506 either *SlDWARF*, *GhDWF4* or *SlBRI1* in tomato, results in elevated levels of ethylene
507 production and earlier ripening as well as enhanced carotenoid accumulation [47, 49,
508 50]. In addition, *SlBZR1-OE* or *SlBIN2-KO* accelerated ethylene production and
509 enhanced carotenoid accumulation with up-regulation of ethylene and carotenoid
510 biosynthetic genes (Figure 2 and 6). In contrast, dual *SlBZR1/SlBES1*-KO or *SlBIN2-*

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

accumulation.

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

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

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

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

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AUTHOR CONTRIBUTION

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

Resource availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Qiaomei Wang (qmwang@zju.edu.cn).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The RNA-seq data have been deposited at the NCBI Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/) with the BioProject ID PRJNA635540 and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant Materials and Growth Conditions

We used *Solanum lycopersicum* cv. Ailsa Craig (AC) as the control genotype in the present study. The following tomato genotypes were used in this study: *CYP90B3-OE*, *CYP90B3-RNAi*, *SlBIN2*-OE, *bin2*, *SlBZR1*-OE, *bzr1 and bzr1bes1*. Transgenic plants *CYP90B3-OE* and *CYP90B3-RNAi* were generated during previous studies [48]. Plants were cultivated under a 16 h photoperiod (22/28°C, night/day). *Solanum lycopersicum* flowers were labeled at anthesis and the number of tagged fruits was limited to fewer than four per cluster. The fruits were collected after 30 to 46 DPA stage for the different fruit ripening stages. The fruit ripening stages, including mature green (MG) stage,

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

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

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

677 **Chemical treatment**

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

687 **Measurement of ethylene production**

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

690 2 h, 1 mL of the headspace gas was withdrawn from the container with a 1 mL syringe
691 and immediately injected into a gas chromatograph (Shimadzu GC-17A, Kyoto, Japan),
692 consisting of a flame ionization detector and a hp-5 column (50 m × 0.32 mm × 1.05 μm)
693 with a detection temperature of 200 ℃. The ethylene production was calculated
694 according to commercial standards. Fresh tissues of fruit were mixed frozen in liquid
695 nitrogen and stored at -80 ℃ until use.

696 **Firmness determination**

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

702 **HPLC analysis of carotenoids**

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

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

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

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

727 **Subcellular localization**

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

739 **RNA-seq**

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

744 **ChIP-seq and ChIP-qPCR Assays**

745 Chromatin immunoprecipitation sequencing was performed as previously reported [81].
746 In brief, tomato fruits of *SlBZR1*-OE at MG stage were collected from at least six
747 different plants, sliced into small pieces and fixed in 1x PBS with 1% formaldehyde for
748 15 min under vacuum and ground to fine power under liquid nitrogen. ChIP assay was
749 performed in three independent biological replicates starting with 2 g of each sample

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

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

786 **Yeast one-hybrid assay**

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

800 **Yeast two-hybrid assay**

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

808 Full length coding sequence (CDS) of SlBIN2 was ligated to the binding domain (BD)
809 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.

820 **Reference**

821 1. Klee, H.J., and Giovannoni, J.J. (2011). Genetics and control of tomato fruit
822 ripening and quality attributes. Annu Rev Genet *45*, 41-59.
823 2. Giovannoni, J., Nguyen, C., Ampofo, B., Zhong, S., and Fei, Z. (2017). The
824 Epigenome and Transcriptional Dynamics of Fruit Ripening. Annu Rev Plant Biol
825 *68*, 61-84.
826 3. Liu, L., Shao, Z., Zhang, M., and Wang, Q. (2015). Regulation of carotenoid
827 metabolism in tomato. Mol Plant *8*, 28-39.
828 4. Fraser, P.D., Enfissi, E.M., Halket, J.M., Truesdale, M.R., Yu, D., Gerrish, C., and
829 Bramley, P.M. (2007). Manipulation of phytoene levels in tomato fruit: effects on
830 isoprenoids, plastids, and intermediary metabolism. Plant Cell *19*, 3194-3211.
831 5. Karlova, R., Chapman, N., David, K., Angenent, G.C., Seymour, G.B., and de
832 Maagd, R.A. (2014). Transcriptional control of fleshy fruit development and
833 ripening. J Exp Bot *65*, 4527-4541.
834 6. Cherian, S., Figueroa, C.R., and Nair, H. (2014). 'Movers and shakers' in the
835 regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric
836 fruit. J Exp Bot *65*, 4705-4722.
837 7. Adams, D.O., and Yang, S.F. (1979). Ethylene biosynthesis: identification of 1-
838 aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of
839 methionine to ethylene. Proc Natl Acad Sci U S A *76*, 170-174.
840 8. Alexander, L., and Grierson, D. (2002). Ethylene biosynthesis and action in tomato:
841 a model for climacteric fruit ripening. J Exp Bot *53*, 2039-2055.
842 9. Cara, B., and Giovannoni, J.J. (2008). Molecular biology of ethylene during tomato
843 fruit development and maturation. Plant Science *175*, 106-113.
844 10. Hamilton, A.J., Lycett, G.W., and Grierson, D. (1990). Antisense gene that inhibits
845 synthesis of the hormone ethylene in transgenic plants. Nature *346*, 284-287.
846 11. Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J., and Grierson,
847 D. (1996). Differential expression of the 1-aminocyclopropane-1-
848 carboxylateoxidase gene family of tomato. Plant J *9*, 525-535.
849 12. Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y., and
850 Inaba, A. (1998). Differential expression and internal feedback regulation of 1-
851 aminocyclopropane-1-carboxylate synthase,1-aminocyclopropane-1-carboxylate
852 oxidase, and ethylene receptor genes in tomato fruit during development and
853 ripening. Plant Physiol *118*,1295-1305.
854 13. Martel, C., Vrebalov, J., Tafelmeyer, P., and Giovannoni, J.J. (2011). The tomato
855 MADS-box transcription factor RIPENING INHIBITOR interacts with promoters
856 involved in numerous ripening processes in a COLORLESS NONRIPENING-
857 dependent manner. Plant Physiol *157*, 1568-1579.
858 14. Vrebalov, J., Pan, I.L., Arroyo, A.J., McQuinn, R., Chung, M., Poole, M., Rose, J.,
859 Seymour, G., Grandillo, S., Giovannoni, J., et al. (2009). Fleshy fruit expansion and
860 ripening are regulated by the Tomato SHATTERPROOF gene TAGL1. Plant Cell
861 *21*, 3041-3062.
862 15. Fujisawa, M., Shima, Y., Nakagawa, H., Kitagawa, M., Kimbara, J., Nakano, T.,
863 Kasumi, T., and Ito, Y. (2014). Transcriptional regulation of fruit ripening by tomato

FRUITFULL homologs and associated MADS box proteins. Plant Cell *26*, 89-101.
16. Leseberg, C.H., Eissler, C.L., Wang, X., Johns, M.A., Duvall, M.R., and Mao, L. (2008). Interaction study of MADS-domain proteins in tomato. J Exp Bot *59*, 2253-2265.
17. Bemer, M., Karlova, R., Ballester, A.R., Tikunov, Y.M., Bovy, A.G., Wolters-Arts, M., Rossetto Pde, B., Angenent, G.C., and de Maagd, R.A. (2012). The tomato FRUITFULL homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening. Plant Cell *24*, 4437-4451.
18. Shima, Y., Kitagawa, M., Fujisawa, M., Nakano, T., Kato, H., Kimbara, J., Kasumi, T., and Ito, Y. (2013). Tomato FRUITFULL homologues act in fruit ripening via forming MADS-box transcription factor complexes with RIN. Plant Mol Biol *82*, 427-438.
19. Gao, Y., Wei, W., Fan, Z., Zhao, X., Zhang, Y., Jing, Y., Zhu, B., Zhu, H., Shan, W., Chen, J., et al. (2020). Re-evaluation of the nor mutation and the role of the NAC-NOR transcription factor in tomato fruit ripening. J Exp Bot *71*, 3560-3574.
20. Gao, Y., Wei, W., Zhao, X., Tan, X., Fan, Z., Zhang, Y., Jing, Y., Meng, L., Zhu, B., Zhu, H., et al. (2018). A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening. Hortic Res *5*, 75.
21. Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J., and Seymour, G.B. (2006). A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat Genet *38*, 948-952.
22. Gao, Y., Zhu, N., Zhu, X., Wu, M., Jiang, C.Z., Grierson, D., Luo, Y., Shen, W., Zhong, S., Fu, D.Q., et al. (2019). Diversity and redundancy of the ripening regulatory networks revealed by the fruitENCODE and the new CRISPR/Cas9 CNR and NOR mutants. Hortic Res *6*, 39.
23. Chung, M.Y., Vrebalov, J., Alba, R., Lee, J., McQuinn, R., Chung, J.D., Klein, P., and Giovannoni, J. (2010). A tomato (Solanum lycopersicum) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. Plant J *64*, 936-947.
24. Lee, J.M., Joung, J.G., McQuinn, R., Chung, M.Y., Fei, Z., Tieman, D., Klee, H., and Giovannoni, J. (2012). Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. Plant J *70*, 191-204.
25. Lin, Z., Hong, Y., Yin, M., Li, C., Zhang, K., and Grierson, D. (2008). A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. Plant J *55*, 301-310.
26. Yuan, Y., Ren, S., Liu, X., Su, L., Wu, Y., Zhang, W., Li, Y., Jiang, Y., Wang, H., Fu, R., et al. (2022). SlWRKY35 positively regulates carotenoid biosynthesis by activating the MEP pathway in tomato fruit. New Phytol *234*, 164-178.
27. Zhao, W., Li, Y., Fan, S., Wen, T., Wang, M., Zhang, L., and Zhao, L. (2021). The transcription factor WRKY32 affects tomato fruit colour by regulating YELLOW FRUITED-TOMATO 1, a core component of ethylene signal transduction. J Exp Bot *72*, 4269-4282.

28. Luo, Z., Zhang, J., Li, J., Yang, C., Wang, T., Ouyang, B., Li, H., Giovannoni, J., and Ye, Z. (2013). A STAY-GREEN protein SlSGR1 regulates lycopene and beta-carotene accumulation by interacting directly with SlPSY1 during ripening processes in tomato. New Phytol *198*, 442-452.
29. Liu, H., Meng, F., Miao, H., Chen, S., Yin, T., Hu, S., Shao, Z., Liu, Y., Gao, L., Zhu, C., et al. (2018). Effects of postharvest methyl jasmonate treatment on main health-promoting components and volatile organic compounds in cherry tomato fruits. Food Chem *263*, 194-200.
30. Liu, L., Wei, J., Zhang, M., Zhang, L., Li, C., and Wang, Q. (2012). Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates. J Exp Bot *63*, 5751-5761.
31. Li, T., Xu, Y., Zhang, L., Ji, Y., Tan, D., Yuan, H., and Wang, A. (2017). The Jasmonate-Activated Transcription Factor MdMYC2 Regulates ETHYLENE RESPONSE FACTOR and Ethylene Biosynthetic Genes to Promote Ethylene Biosynthesis during Apple Fruit Ripening. Plant Cell *29*, 1316-1334.
32. Zhang, M., Yuan, B., and Leng, P. (2009). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. Journal of Experimental Botany *60*, 1579-1588.
33. Wu, Q., Tao, X., Ai, X., Luo, Z., Mao, L., Ying, T., and Li, L. (2018). Effect of exogenous auxin on aroma volatiles of cherry tomato (Solanum lycopersicum L.) fruit during postharvest ripening. Postharvest Biology and Technology *146*, 108-116.
34. Nolan, T.M., Vukasinovic, N., Liu, D., Russinova, E., and Yin, Y. (2020). Brassinosteroids: Multidimensional Regulators of Plant Growth, Development, and Stress Responses. Plant Cell *32*, 295-318.
35. Jaillais, Y., Hothorn, M., Belkhadir, Y., Dabi, T., Nimchuk, Z.L., Meyerowitz, E.M., and Chory, J. (2011). Tyrosine phosphorylation controls brassinosteroid receptor activation by triggering membrane release of its kinase inhibitor. Genes Dev *25*, 232-237.
36. Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., and Clouse, S.D. (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. Dev Cell *15*, 220-235.
37. Tang, W., Kim, T.W., Osesprieto, J.A., Yu, S., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L., & Wang, Z. (2008). BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. Science *321*, 557-560.
38. Sun, Y., Fan, X.Y., Cao, D.M., Tang, W., He, K., Zhu, J.Y., He, J.X., Bai, M.Y., Zhu, S., Oh, E., et al. (2010). Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev Cell *19*, 765-777.
39. Yu, X., Li, L., Zola, J., Aluru, M., Ye, H., Foudree, A., Guo, H., Anderson, S., Aluru, S., Liu, P., et al. (2011). A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in Arabidopsis thaliana. Plant J *65*, 634-646.

952 40. Mao, J., and Li, J. (2020). Regulation of Three Key Kinases of Brassinosteroid
953 Signaling Pathway. Int J Mol Sci *21*.
954 41. Zhu, T., Tan, W.-R., Deng, X.-G., Zheng, T., Zhang, D.-W., and Lin, H.-H. (2015).
955 Effects of brassinosteroids on quality attributes and ethylene synthesis in
956 postharvest tomato fruit. Postharvest Biology and Technology *100*, 196-204.
957 42.Guo, Y.F., Shan, W., Liang, S.M., Wu, C.J., Wei, W., Chen, J.Y., Lu, W.J., and Kuang,
958 J.F. (2019). MaBZR1/2 act as transcriptional repressors of ethylene biosynthetic
959 genes in banana fruit. Physiol Plant *165*, 555-568.
960 43. He, Y., Li, J., Ban, Q., Han, S., and Rao, J. (2018). Role of Brassinosteroids in
961 Persimmon (Diospyros kaki L.) Fruit Ripening. J Agric Food Chem *66*, 2637-2644.
962 44. Zaharah, S.S., Singh, Z., Symons, G.M., and Reid, J.B. (2011). Role of
963 Brassinosteroids, Ethylene, Abscisic Acid, and Indole-3-Acetic Acid in Mango
964 Fruit Ripening. Journal of Plant Growth Regulation *31*, 363-372.
965 45. Chai, Y.-m., Zhang, Q., Tian, L., Li, C.-L., Xing, Y., Qin, L., and Shen, Y.-Y. (2012).
966 Brassinosteroid is involved in strawberry fruit ripening. Plant Growth Regulation
967 *69*, 63-69.
968 46. Symons, G.M., Davies, C., Shavrukov, Y., Dry, I.B., Reid, J.B., and Thomas, M.R.
969 (2006). Grapes on steroids. Brassinosteroids are involved in grape berry ripening.
970 Plant Physiol *140*, 150-158.
971 47. Li, X., Chen, X., Guo, X., Yin, L., Ahammed, G. J., Xu, C., Chen, K., Liu, C., Xia,
972 X., Shi, K., Zhou, J., Zhou, Y., and Yu, J. (2016) DWARF overexpression induces
973 alteration in phytohormone homeostasis, development, architecture and carotenoid
974 accumulation in tomato. Plant Biotechnol J *14*, 1021-1033.
975 48. Hu, S., Liu, L., Li, S., Shao, Z., Meng, F., Liu, H., Duan, W., Liang, D., Zhu, C.,
976 Xu, T., et al. (2020). Regulation of fruit ripening by the brassinosteroid biosynthetic
977 gene SlCYP90B3 via an ethylene-dependent pathway in tomato. Hortic Res *7*, 163.
978 49. Ye, S.-e., Li, F., Li, X.-b., Hong, Q.-b., Zhai, Y.-l., Hu, M.-y., Wei, T., Deng, S.-s.,
979 Pei, Y., and Luo, M. (2015). Over-expression of GhDWF4 gene improved tomato
980 fruit quality and accelerated fruit ripening. Journal of Integrative Agriculture *14*,
981 1980-1991.
982 50. Nie, S., Huang, S., Wang, S., Cheng, D., Liu, J., Lv, S., Li, Q., and Wang, X. (2017).
983 Enhancing Brassinosteroid Signaling via Overexpression of Tomato (Solanum
984 lycopersicum) SlBRI1 Improves Major Agronomic Traits. Front Plant Sci *8*, 1386.
985 51. Liu, L., Jia, C., Zhang, M., Chen, D., Chen, S., Guo, R., Guo, D., and Wang, Q.
986 (2014). Ectopic expression of a BZR1-1D transcription factor in brassinosteroid
987 signalling enhances carotenoid accumulation and fruit quality attributes in tomato.
988 Plant Biotechnol J *12*, 105-115.
989 52. He, Y., Liu, H., Li, H., Jin, M., Wang, X., Yin, X., Zhu, Q., and Rao, J. (2021).
990 Transcription factors DkBZR1/2 regulate cell wall degradation genes and ethylene
991 biosynthesis genes during persimmon fruit ripening. J Exp Bot *72*, 6437-6446.
992 53. Ji, Y., Qu, Y., Jiang, Z., Yan, J., Chu, J., Xu, M., Su, X., Yuan, H., and Wang, A.
993 (2021). The mechanism for brassinosteroids suppressing climacteric fruit ripening.
994 Plant Physiol *185*, 1875-1893.
995 54. Liu, H., Liu, L., Liang, D., Zhang, M., Jia, C., Qi, M., Liu, Y., Shao, Z., Meng, F.,

Hu, S., et al. (2021). SlBES1 promotes tomato fruit softening through transcriptional inhibition of PMEU1. iScience *24*, 102926.

55. Ali, B., Hayat, S., Hasan, S.A., and Ahmad, A. (2006). Effect of root applied 28-homobrassinolide on the performance of Lycopersicon esculentum. Scientia Horticulturae *110*, 267-273.
56. (2014). Response of Tomato Cultivars on Yield and Quality Attributes Applied With Two Different Modes of BR Analogues: A Comparative Study. In International Conference on Advances in Agricultural, Biological & Environmental Sciences (AABES-2014) Oct 15-16, 2014 Dubai (UAE).
57. Vardhini, B.V., and Rao, S.S.R. (2002). Acceleration of ripening of tomato pericarp discs by brassinosteroids. Phytochemistry *61*, 843-847.
58. Lisso, J., Altmann, T., and Mussig, C. (2006). Metabolic changes in fruits of the tomato dx mutant. Phytochemistry *67*, 2232-2238.
59. Koka, C.V., Cerny, R.E., Gardner, R.G., Noguchi, T., Fujioka, S., Takatsuto, S., Yoshida, S., and Clouse, S.D. (2000). A putative role for the tomato genes DUMPY and CURL-3 in brassinosteroid biosynthesis and response. Plant Physiol *122*, 85-98.
60. Bishop, G.J., Harrison, K., & Jones, J.D. (1996). The tomato Dwarf gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. Plant Cell *8*, 959-969.
61. Bishop, G.J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takatsuto, S., Jones, J.D., and Kamiya, Y. (1999). The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proc Natl Acad Sci U S A *96*, 1761-1766.
62. Nomura, T., Kushiro, T., Yokota, T., Kamiya, Y., Bishop, G.J., and Yamaguchi, S. (2005). The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in Arabidopsis. J Biol Chem *280*, 17873-17879.
63. Montoya, T., Nomura, T., Yokota, T., Farrar, K., Harrison, K., Jones, J.D., Kaneta, T., Kamiya, Y., Szekeres, M., and Bishop, G.J. (2005). Patterns of Dwarf expression and brassinosteroid accumulation in tomato reveal the importance of brassinosteroid synthesis during fruit development. Plant J *42*, 262-269.
64. Zhu, Z., Zhang, Z., Qin, G., and Tian, S. (2010). Effects of brassinosteroids on postharvest disease and senescence of jujube fruit in storage. Postharvest Biology and Technology *56*, 50-55.
65. Yue, P., Lu, Q., Liu, Z., Lv, T., Li, X., Bu, H., Liu, W., Xu, Y., Yuan, H., and Wang, A. (2020). Auxin-activated MdARF5 induces the expression of ethylene biosynthetic genes to initiate apple fruit ripening. New Phytol *226*, 1781-1795.
66. Yang, N., Zhou, Y., Wang, Z., Zhang, Z., Xi, Z., and Wang, X. (2021). Emerging roles of brassinosteroids and light in anthocyanin biosynthesis and ripeness of climacteric and non-climacteric fruits. Crit Rev Food Sci Nutr, 1-13.
67. Hansen, M., Chae, H.S., and Kieber, J.J. (2009). Regulation of ACS protein stability by cytokinin and brassinosteroid. Plant J *57*, 606-614.
68. Lv, B., Tian, H., Zhang, F., Liu, J., Lu, S., Bai, M., Li, C., and Ding, Z. (2018). Brassinosteroids regulate root growth by controlling reactive oxygen species

1040 homeostasis and dual effect on ethylene synthesis in Arabidopsis. PLoS Genet *14*,
1041 e1007144.
1042 69. He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (2002). The GSK3-like
1043 kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the
1044 brassinosteroid signaling pathway in arabidopsis. Proc Natl Acad Sci U S A *99*,
1045 10185-10185.
1046 70. Yin, Y., Wang, Z., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J.
1047 (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate
1048 gene expression and promote stem elongation. Cell *109*, 181-191.
1049 71. Park, C.H., Seo, C., Park, Y.J., Youn, J.H., Roh, J., Moon, J., and Kim, S.K. (2020).
1050 BES1 directly binds to the promoter of the ACC oxidase 1 gene to regulate
1051 gravitropic response in the roots of Arabidopsis thaliana. Plant Signal Behav *15*,
1052 1690724.
1053 72. Moon, J., Park, Y.J., Son, S.H., Roh, J., Youn, J.H., Kim, S.Y., and Kim, S.K. (2020).
1054 Brassinosteroids signaling via BZR1 down-regulates expression of ACC oxidase 4
1055 to control growth of Arabidopsis thaliana seedlings. Plant Signal Behav *15*,
1056 1734333.
1057 73. Bishop, G.J., and Koncz, C. (2002). Brassinosteroids and plant steroid hormone
1058 signaling. Plant Cell *14 Suppl*, S97-110.
1059 74. Ryu, H., Kim, K., Cho, H., Park, J., Choe, S., and Hwang, I. (2007).
1060 Nucleocytoplasmic shuttling of BZR1 mediated by phosphorylation is essential in
1061 Arabidopsis brassinosteroid signaling. Plant Cell *19*, 2749-2762.
1062 75. Gampala, S.S., Kim, T.W., He, J.X., Tang, W., Deng, Z., Bai, M.Y., Guan, S.,
1063 Lalonde, S., Sun, Y., Gendron, J.M., et al. (2007). An essential role for 14-3-3
1064 proteins in brassinosteroid signal transduction in Arabidopsis. Dev Cell *13*, 177-
1065 189.
1066 76. Giovannoni, J.J. (2004). Genetic regulation of fruit development and ripening. Plant
1067 Cell *16 Suppl*, S170-180.
1068 77. Shao, Z., Zhao, Y., Liu, L., Chen, S., Li, C., Meng, F., Liu, H., Hu, S., Wang, J., and
1069 Wang, Q. (2020). Overexpression of FBR41 enhances resistance to sphinganine
1070 analog mycotoxin-induced cell death and Alternaria stem canker in tomato. Plant
1071 Biotechnol J *18*, 141-154.
1072 78. Yan, L., Wei, S., Wu, Y., Hu, R., Li, H., Yang, W., and Xie, Q. (2015). High-
1073 efficiency genome editing in Arabidopsis using YAO Promoter-Driven
1074 CRISPR/Cas9 system. Mol Plant *8*, 1820-1823.
1075 79. Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H.,
1076 Lin, Y., et al. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency
1077 multiplex genome editing in monocot and dicot plants. Mol Plant *8*, 1274-1284.
1078 80. Zhang, S., Feng, M., Chen, W., Zhou, X., Lu, J., Wang, Y., Li, Y., Jiang, C.Z., Gan,
1079 S.S., Ma, N., et al. (2019). In rose, transcription factor PTM balances growth and
1080 drought survival via PIP2;1 aquaporin. Nat Plants *5*, 290-299.
1081 81. Lu, P., Yu, S., Zhu, N., Chen, Y.R., Zhou, B., Pan, Y., Tzeng, D., Fabi, J.P., Argyris,
1082 J., Garcia-Mas, J., et al. (2018). Genome encode analyses reveal the basis of
1083 convergent evolution of fleshy fruit ripening. Nat Plants *4*, 784-791.

82. Liu, Y., Du, M., Deng, L., Shen, J., Fang, M., Chen, Q., Lu, Y., Wang, Q., Li, C., and Zhai, Q. (2019). MYC2 Regulates the Termination of Jasmonate Signaling via an Autoregulatory Negative Feedback Loop. Plant Cell *31*, 106-127.

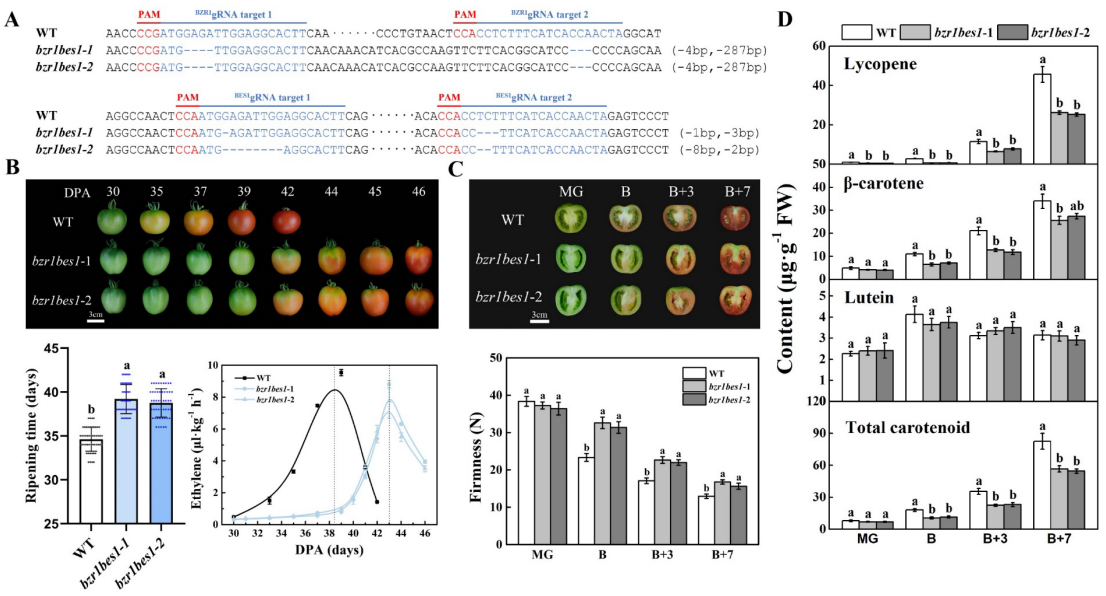

Figure 1. SlBZR1/SlBES1 double gene knockout abolish BR signaling delays tomato fruit ripening and ethylene production, reduces carotenoid accumulation.

(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 ($P < 0.05$).

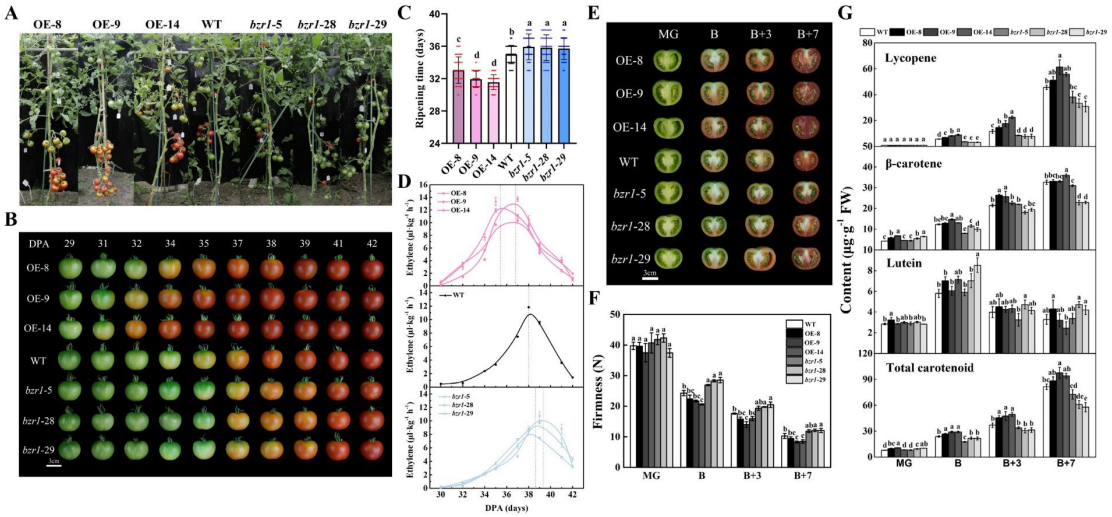

Figure 2. *SlBZR1* promotes the fruit ripening schedule and carotenoids accumulation.

(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).

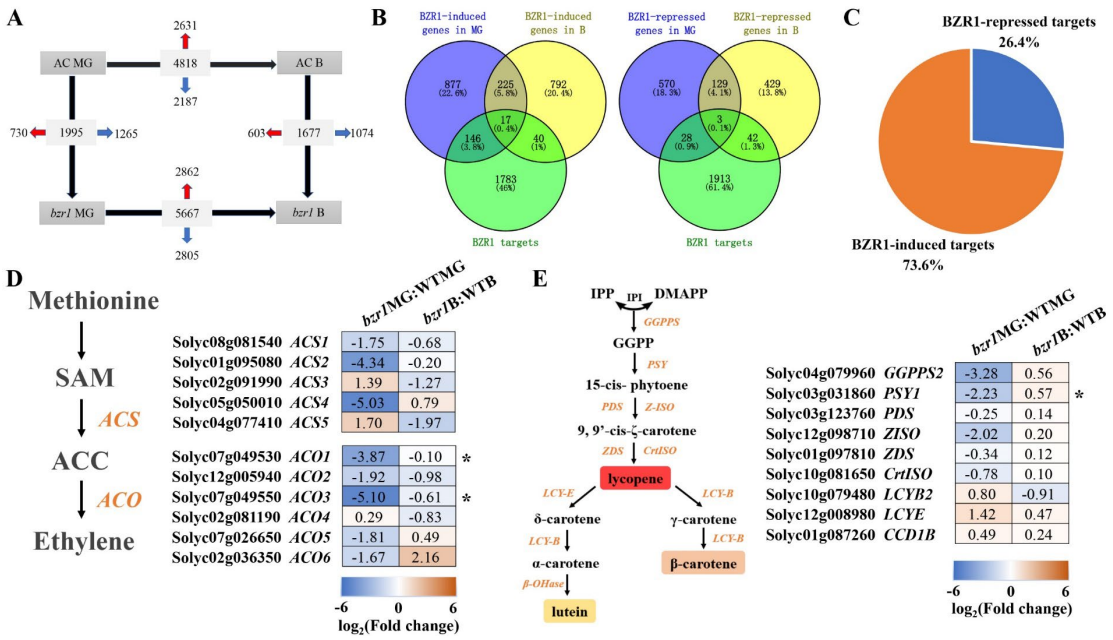

Figure 3. *SlBZR1* target genes identified by combined genome-wide RNA-seq and ChIP-seq approaches.

(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.

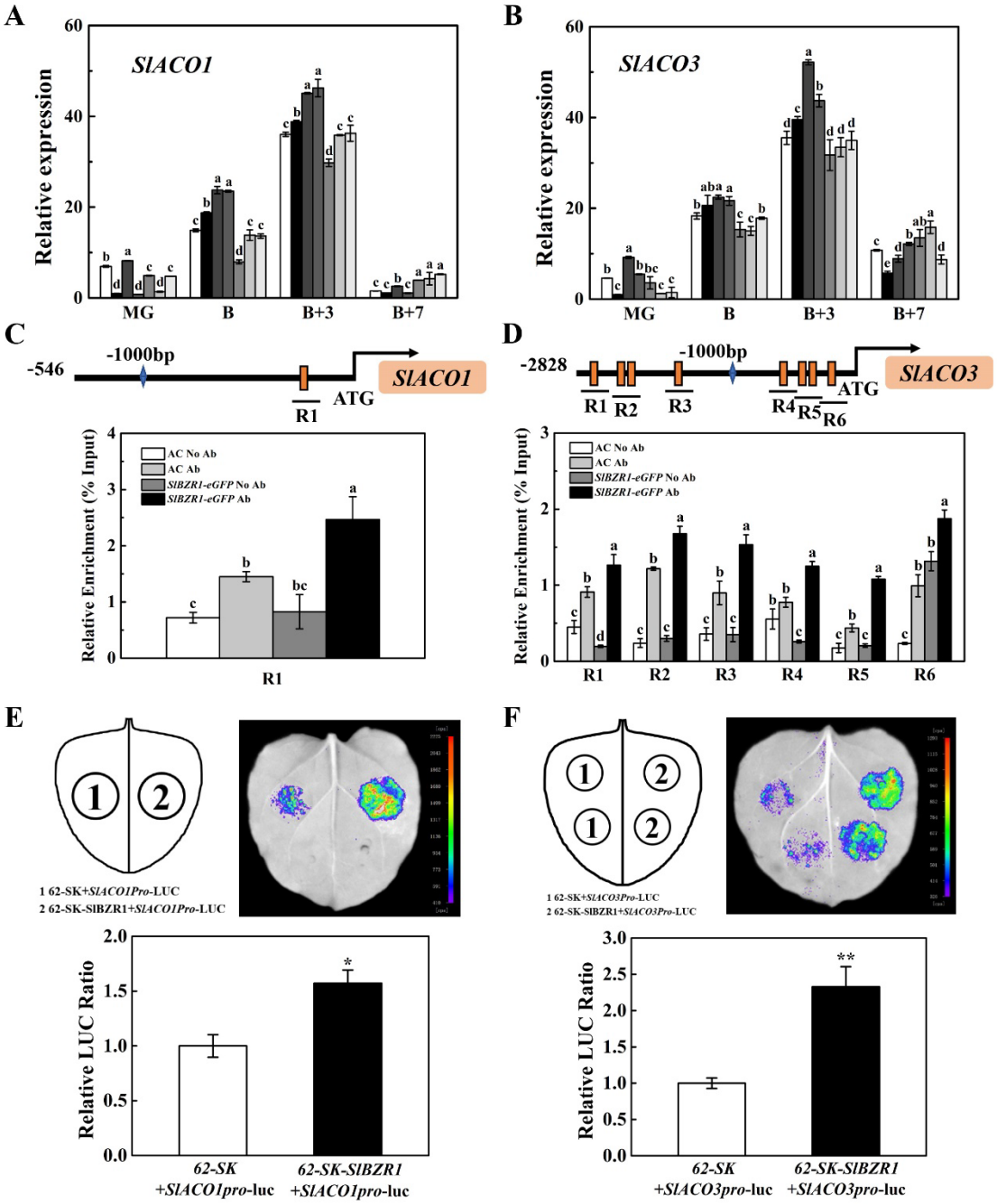

Figure 4. SlBZR1 directly binds and activates the expression of *SlACO1* and *SlACO3*

(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

1144 DNAs. AC (wild type) without and with antibody were set as blank control and negative control,
1145 respectively. SlBZR1-GFP without antibody was also set as negative control. The mean value of two
1146 technical replicates was recorded for each biological replicate. Values are means ± SD of three biological
1147 replicates. Different letters indicate significant difference among groups for each locus (one-way
1148 ANOVA with Tukey's test, $P < 0.05$). (E, F) Transient expression assay for SlBZR1 activation of the
1149 promoters of *SlACO1* and *SlACO3*. The reporter and effector vectors in each experiment were co-
1150 introduced into tobacco (*N. benthamiana*) leaves by Agrobacterium GV3101. SK represents empty
1151 vector control. Representative images of tobacco leaves were taken 3d after infiltration. Luminescence
1152 intensity values are means ± SD of six biological replicates. Different letters indicate significant
1153 difference among groups for each locus (one-way ANOVA with Tukey's test, $P < 0.05$).

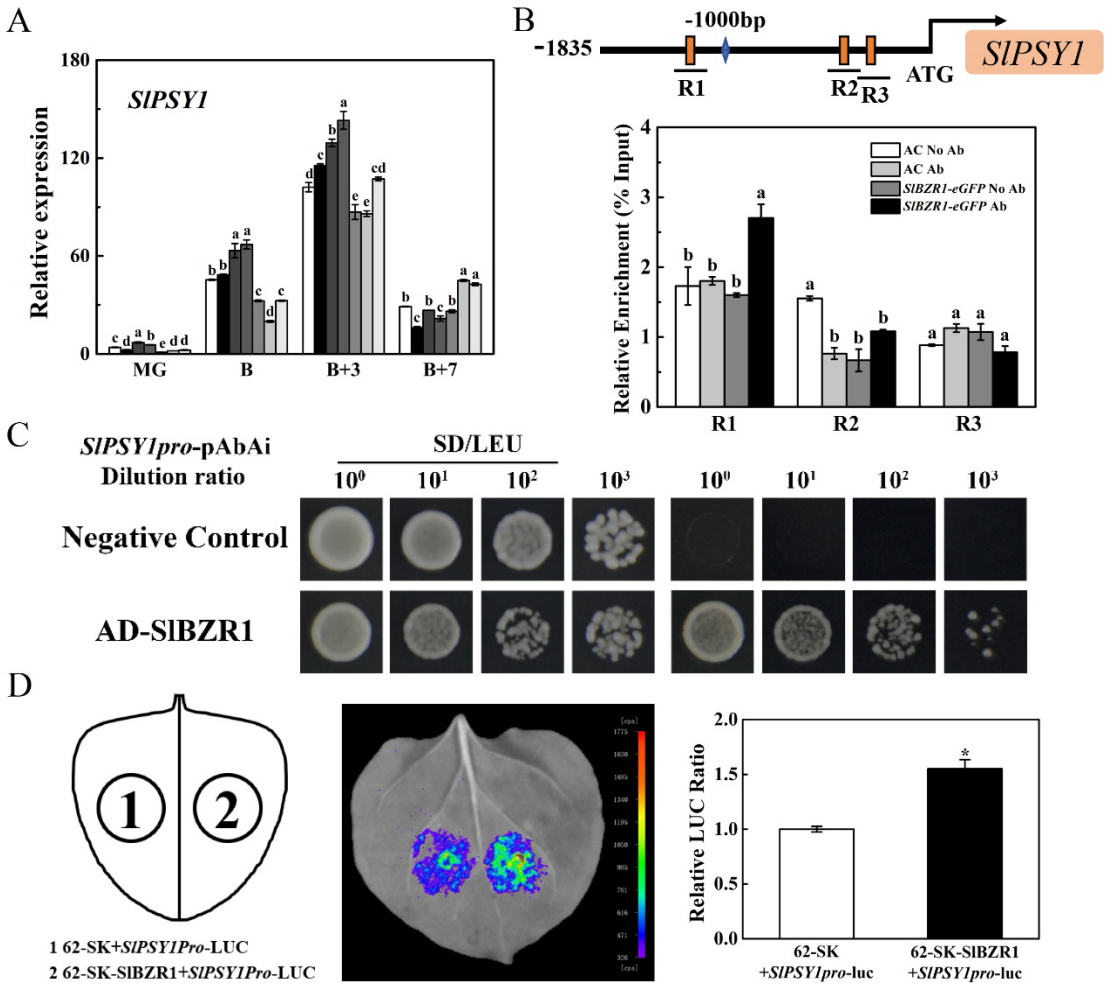

Figure 5. SlBZR1 positively regulates the expression of *SlPSY1* involved in carotenoid accumulation in ripening tomato fruits

(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.

1175 benthamiana) leaves by Agrobacterium GV3101. SK represents empty vector control. Representative
1176 images of N. benthamiana leaves were taken 3d after infiltration. Luminescence intensity values are
1177 means ± SD of six biological replicates. Different letters indicate significant difference among groups
1178 for each locus (one-way ANOVA with Tukey's test, $p < 0.05$).

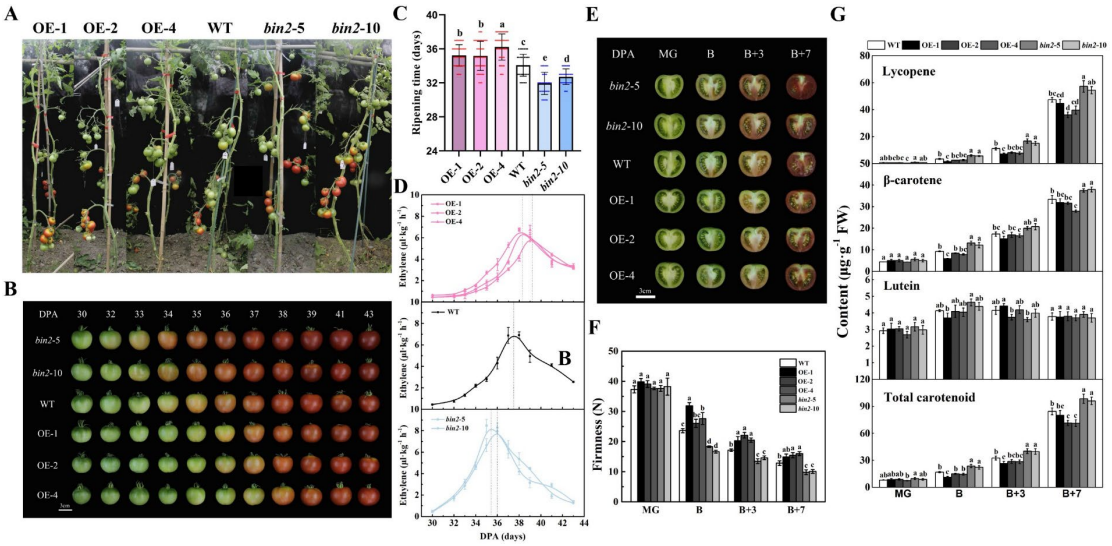

Figure 6. SlBIN2 delays tomato fruit ripening and reduces carotenoid accumulation.

(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 ($P < 0.05$).

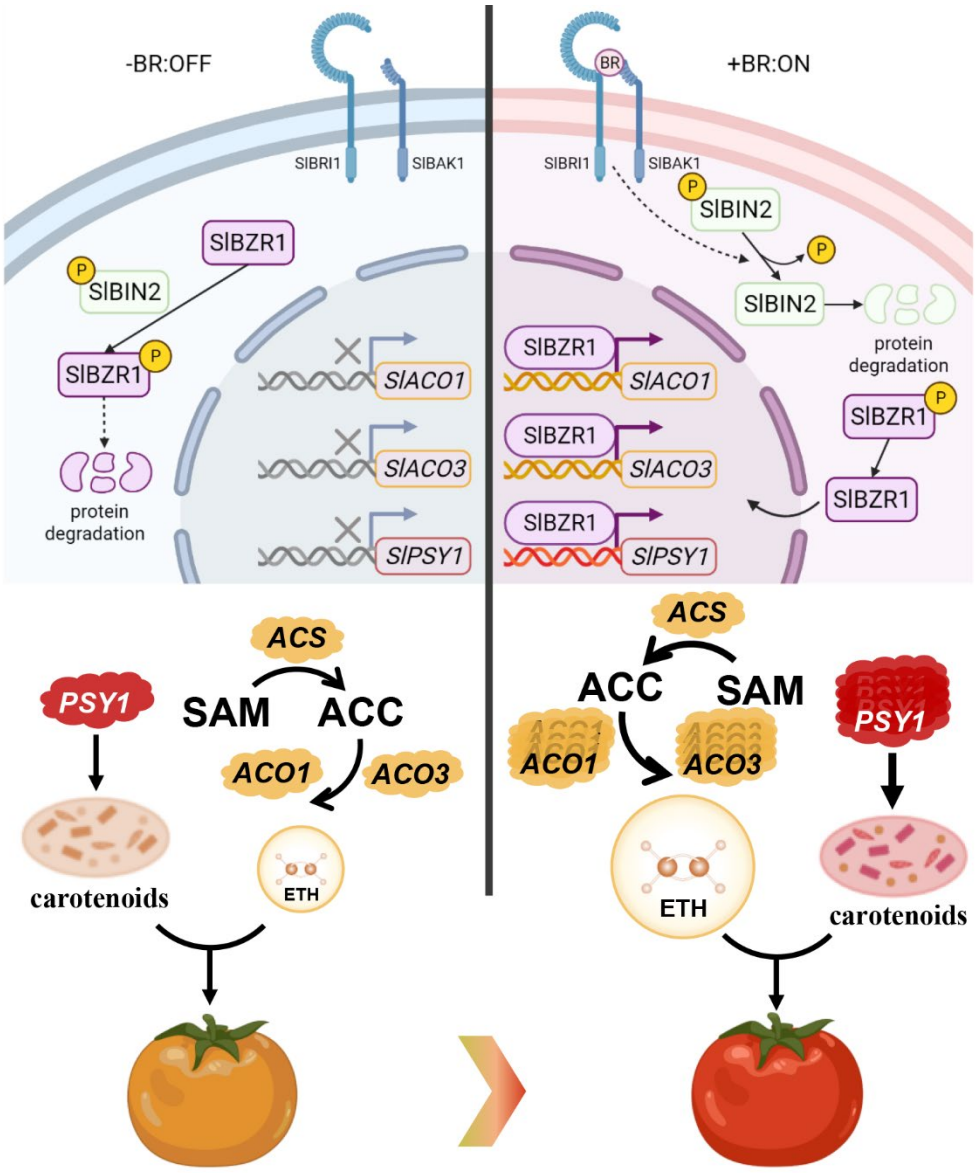

Figure 7. A proposed regulatory mechanism of BR on tomato fruit ripening and carotenoid accumulation.

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.