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# A novel tomato F-box protein, SlEBF3, is involved in tuning ethylene signaling during plant development and climacteric fruit ripening

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## SUMMARY

**Ethylene is instrumental to climacteric fruit ripening and EIN3 BINDING F-BOX (EBF) proteins have been assigned a central role in mediating ethylene responses by regulating EIN3/EIL degradation in Arabidopsis. However, the role and mode of action of tomato EBFs in ethylene-dependent processes like fruit ripening remains unclear. Two novel EBF genes, *SlEBF3* and *SlEBF4*, were identified in the tomato genome, and *SlEBF3* displayed a ripening-associated expression pattern suggesting its potential involvement in controlling ethylene response during fruit ripening. *SlEBF3* downregulated tomato lines failed to show obvious ripening-related phenotypes likely due to functional redundancy among *SlEBF* family members. By contrast, *SlEBF3* overexpression lines exhibited pleiotropic ethylene-related alterations, including inhibition of fruit ripening, attenuated triple-response and delayed petal abscission. Yeast-two-hybrid system and bimolecular fluorescence complementation approaches indicated that SlEBF3 interacts with all known tomato SlEIL proteins and, consistently, total SlEIL protein levels were decreased in SlEBF3 overexpression fruits, supporting the idea that the reduced ethylene sensitivity and defects in fruit ripening are due to the SlEBF3-mediated degradation of EIL proteins. Moreover, *SlEBF3* expression is regulated by EIL1 via a feedback loop, which supposes its role in tuning ethylene signaling and responses. Overall, the study reveals the role of a novel EBF tomato gene in climacteric ripening, thus providing a new target for modulating fleshy fruit ripening.**

**Keywords: F-box protein, ethylene signaling, fruit ripening, post-transcriptional regulation, feedback loop, tomato (*Solanum lycopersicum*).**

## INTRODUCTION

Ethylene plays an important role in a wide range of developmental processes, including fruit ripening, seed germination, cell elongation, root nodulation, programmed cell death, sex determination, organ senescence and leaf abscission, as well as responses to abiotic stress and pathogen attacks (Lin *et al.*, 2009). Unraveling the ethylene signaling pathway in the model plant *Arabidopsis thaliana* using genetic and molecular approaches uncovered the ethylene transduction pathway is made of a linear cascade of events (Ju and Chang, 2015). According to the established model, in the absence of ethylene, the hormone receptors activate the Raf-like protein kinase CONSTITUTIVE TRIPLE-RESPONSE 1 (CTR1), which in turn inhibits the ER-located membrane protein ETHYLENE INSENSITIVE 2 (EIN2) through phosphorylation of its C-terminal domain (Ju *et al.*, 2012). In the nucleus, the master transcription factors EIN3/EIL1 are targeted for degradation by F-box proteins EBF1/2 (Guo and Ecker, 2003; Potuschak *et al.*, 2003). Upon the presence of ethylene, the receptors are inactivated and therefore the CTR1 kinase is no longer active, resulting in the proteolytic release of the EIN2 C-END, which induces degradation of the F-box proteins EBF1/2 (Ju *et al.*, 2012; Qiao *et al.*, 2012; Wen *et al.*, 2012). The EIN3/EIL1 proteins are consequently stabilized and regulate a transcriptional cascade involving the ERF transcription factors, which regulate the expression of downstream ethylene-responsive genes (Solano *et al.*, 1998; Alonso *et al.*, 2003).

The ubiquitin/26S proteasome system plays an essential role in hormone signaling and response. F-box proteins

are substrate-recruiting subunits of Skp1-cullin1-F-box (SCF)-type E3 ubiquitin ligases, which regulate a wide range of biological processes through ubiquitination and subsequent degradation of substrate proteins (Schulman *et al.*, 2000). It has been well documented that most plant hormone signaling pathways are subjected to F-box protein-dependent regulation (Santner and Estelle, 2010). In Arabidopsis, the F-box protein TIR1 acts as an auxin receptor regulating the stability of Aux/IAA proteins (Gray *et al.*, 2001; Dharmasiri *et al.*, 2005). SLY1 and GID2 are F-box proteins that regulate gibberellin signaling repressors DELLA proteins via interacting with the DELLA-GID1 complex in GA signaling (Dill *et al.*, 2004; Strader *et al.*, 2004; Griffiths *et al.*, 2006). F-box protein COI1 serves as a receptor for JA-Ile/coronatine and targets JAZ proteins for degradation in jasmonate signaling (Yan *et al.*, 2009; Sheard *et al.*, 2010). During ethylene signaling, key components EIN2 and EIN3 are regulated by F-box proteins ETP1/2 and EBF1/2, respectively (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Binder *et al.*, 2007; Qiao *et al.*, 2009).

In the plant model Arabidopsis, two F-box proteins, EIN3-BINDING F-BOX PROTEIN1, EBF1 and EBF2, were shown to play an important role in ethylene signaling via targeting EIN3 and its functional homolog EIN3-like 1 (EIL1) for degradation (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004; Binder *et al.*, 2007). Disruption of either *EBF1* or *EBF2* resulted in increased EIN3 protein levels, and consequently a hypersensitivity to exogenous ethylene at both the phenotypic and molecular levels. Moreover, kinetic studies performed with *ebf1* and *ebf2* mutant indicated that EBF1 primarily degrades EIN3 with no or low ethylene signaling, whereas EBF2 plays a larger role in degrading EIN3 after ethylene responses have been activated (Binder *et al.*, 2007). This suggested that EBF1 and EBF2 have distinct but overlapping roles in regulating ethylene signaling.

Tomato (*Solanum lycopersicum*) is the main plant model for studying ethylene-dependent climacteric fruit ripening. In previous studies, four *EBF* genes (*SlEBF1, SlEBF2*, *SlEBF3* and *SlEBF4*) have been identified based on the presence of conserved F-box domains and Leu-rich repeats through mining the tomato genome sequence (Liu *et al.*, 2015). Silencing of *SlEBF1* and *SlEBF2* genes by TRV-VIGS led to accelerated plant senescence (Yang *et al.*, 2010). However, although *Sl-EBF1* and *Sl-EBF2* were shown to play an important role in ethylene-mediated plant senescence by transient expression system, the function of other EBF family members and the mechanism by which EBF proteins mediate ethylene response and fruit ripening in tomato remain yet to be elucidated.

We show here that a novel tomato F-box protein, SlEBF3, is involved in ethylene-mediated fruit ripening via degradation of EIL proteins in tomato. Transgenic lines with increased expression of *SlEBF3* displayed phenotypes related to ethylene responses, including ethylene sensitivity, petal abscission and fruit ripening. Taken together, our results suggest that SlEBF3 directly interacts with EIL proteins to induce their degradation, which affects the ethylene signaling pathway, resulting in pleiotropic ethylene-related phenotypes.

## RESULTS

### *SlEBF3*, a novel EIN3-binding F-box gene, exhibits a ripening-associated expression pattern in tomato

While the transcriptional regulation associated with fruit ripening has been widely explored (Karlova *et al.*, 2014), studies addressing the role of the post-transcriptional regulation involved in this developmental process remain very scarce. We previously identified two novel EIN3-binding F-box genes based on the presence of conserved F-box domains and Leu-rich repeats (designated as *SlEBF3* and *SlEBF4*; Liu *et al.*, 2015). Phylogenetic analysis showed that SlEBF3 is highly similar to SlEBF2 (Figure S1), displaying 75% identity at the level of amino acid sequence, while SlEBF4 shows higher similarity to SlEBF1.

To investigate the physiological significance of these two novel *EBF* genes, we first examined their expression pattern in various tomato tissues by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). However, these studies failed to detect any transcript accumulation for *SlEBF4* in various tomato tissues, indicating that this EBF family member might correspond to a pseudogene or that it is expressed in particular tissues or conditions that have not been tested here. By contrast, *SlEBF3* exhibited a constitutive expression in all tissues tested, with the highest levels observed in root, leaf and flower (Figure 1a). In comparison to this expression profile, *SlEBF1* and *SlEBF2* are mainly expressed in fruit and flowers (Figure 1b,c). Remarkably, *SlEBF3* transcript accumulation exhibits a ripening-associated pattern with a dramatic increase at the onset of ripening followed by a sharp decrease at post-breaker (BR) stages (Figure 1a). This expression pattern suggests a potential role for *SlEBF3* in fruit ripening and vegetative growth in tomato.

### SlEBF3 is involved in the feedback regulation of ethylene signaling

To determine whether the ripening-related expression of *SlEBF3* is under ethylene regulation, qRT-PCR was used to monitor its relative mRNA accumulation upon treatment of mature green (MG) fruit with either exogenous ethylene or 1-MCP, the inhibitor of ethylene perception. Two genes, *E4* and *E8*, known to be ethylene responsive (Lincoln *et al.*, 1987), were used as control to validate the efficacy of the treatment. Transcript accumulation assessed by qRT-PCR showed that *SlEBF3* is responsive to exogenous ethylene and that this ethylene-induced expression is significantly

 

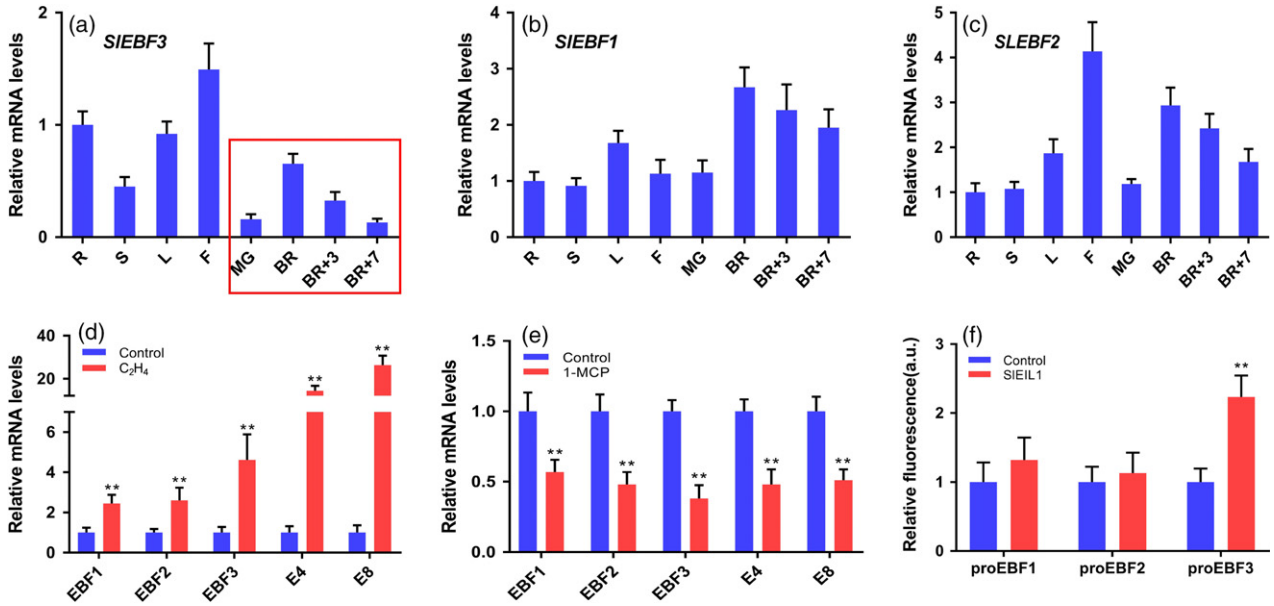


**Figure 1.** Expression pattern and ethylene responsiveness of *SlEBF* family genes.
(a) Relative mRNA levels of *SlEBF3* in different tissues.
(b, c) Relative mRNA levels of *SlEBF1* and *SlEBF2* in different tissues. Accumulation of *SlEBF* transcripts was assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in root (R), stem (S), leaf (L), flower (F), mature green fruit (MG), breaker fruit (BR), 3 days post-BR (BR + 3) and 7 days post-BR (BR + 7). The relative mRNA levels of SlEBF in root were standardized to 1.0, referring to *Sl-Actin* gene as internal control. Values are means ± SD of three biological replicates.
(d) qRT-PCR analysis of *SlEBF* transcripts in wild-type (WT) fruits at MG stage treated with 50 ml $L^{-1}$ ethylene for 6 h.
(e) qRT-PCR analysis of *SlEBF* transcripts in WT fruits at BR stage treated with 1-MCP (1.0 mg $L^{-1}$) for 1 h.
(f) The transcriptional activity of *SlEBF* promoter regulated by EIL1 in a protoplast transactivation assay. Protoplasts were co-transfected with green fluorescent protein (GFP) reporters fused to the EBF1–3 promoters and with an effector plasmid expressing EIL1 protein. Asterisks indicate statistical significance using Student's *t*-test: $^{**}P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

repressed by 1-MCP (Figure 1d,e). *SlEBF3* exhibited stronger response to both ethylene and 1-MCP compared with *SlEBF1* and *SlEBF2* (Figure 1d,e). To further investigate the ethylene regulation of *SlEBF3*, we performed transactivation assays showing that EIL1, a member of the EIN3-like family protein, is able to activate the expression of *SlEBF3* but not that of *SlEBF1* or *SlEBF2* (Figure 1f). Consistent with its ethylene regulation, *in silico* analysis identified conserved ethylene response element (ERE) *cis*-regulatory elements in the promoter region of *SlEBF3* that are not found in *SlEBF1* or *SlEBF2* promoters (Figure S2). These results suggest that *SlEBF3* could be involved in a negative feedback regulation of the ethylene signaling pathway.

### Downregulation of *SlEBF3* resulted in ethylene hypersensitivity

To investigate the functional significance of *SlEBF3* in plant development, transgenic lines underexpressing *SlEBF3* were generated in the MicroTom tomato cultivar through RNA interference (RNAi) approach designed to specifically target *SlEBF3*. More than 10 homozygous transgenic lines corresponding to independent transformation events were obtained. Three representative lines, *SlEBF3-RNAi-5*, *SlEBF3-RNAi-22* and *SlEBF3-RNAi-33* exhibiting significant downregulation of *SlEBF3* expression were selected for further studies (Figure 2a). A strong leaf epinasty phenotype was observed in the three RNAi lines (Figure 2b), suggesting that the downregulation of *SlEBF3* results in ethylene hypersensitivity. Noteworthy, no obvious phenotype related to fruit ripening was observed in these lines (Figure S3), likely due to a compensation mechanism as shown by the upregulation of *SlEBF2* in *SlEBF3*-RNAi fruit at the BR stage (Figure S4).

### Overexpression of *SlEBF3* resulted in defects in fruit ripening

To gain more insight on the physiological significance of *SlEBF3*, we generated tomato overexpressing lines under the control of the 35S promoter. Sixteen *35S:SlEBF3* independent homozygous lines were obtained that reproduced similar phenotypes. Three representative lines, *SlEBF3-OE-11*, *SlEBF3-OE-12* and *SlEBF3-OE-16*, presenting different expression levels of the transgene (from 20- to 150-fold) and showing a characteristic phenotype were selected for further studies (Figure 3a).

*SlEBF3-OE* lines display a delay of petal abscission (Figure 3b), a typical phenotype associated with ethylene insensitivity. Remarkably, the onset of fruit ripening was

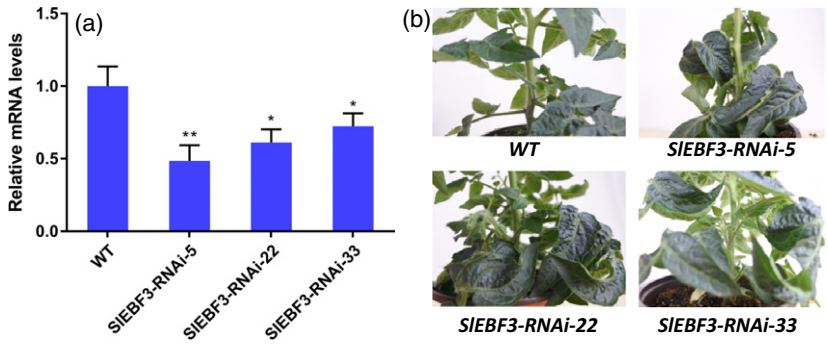


**Figure 2.** Phenotypes of SlEBF3 downregulated tomato lines.
(a) *SlEBF3* transcript accumulation assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) on 4-week-old *SlEBF3-RNAi* plants. Asterisks indicate statistical significance using Student's *t*-test: $^{**}P < 0.01$.
(b) Epinasty phenotype of *SlEBF3-RNAi* lines. *SlEBF3-RNAi-5*, *SlEBF3-RNAi-22* and *SlEBF3-RNAi-33* are three representative independent lines. [Colour figure can be viewed at wileyonlinelibrary.com].

also delayed, with the time from anthesis to BR stage extended by 10 days in *SlEBF3-OE* lines, which is consistent with prior observations that inhibiting ethylene production delays ripening (Hamilton *et al.*, 1990; Oeller *et al.*, 1991). Indeed, the BR stage occurred at 55 days post-anthesis compared with 43 days post-anthesis in wild-type (WT) fruit (Figure 3c). In addition, *SlEBF3* overexpressing lines seem dramatically impaired in their ripening process as the fruit never reach the red-ripe stage. *SlEBF3-OE* fruits remained yellow or orange at the BR +20 days (Figure 3d), while WT fruit reached the red-ripe stage at 7 days post-BR (BR + 7). Therefore, assessing the hue angle value, indicative of color saturation, revealed higher values for *SlEBF3-OE* fruit at BR + 10 comapred with for the WT at BR + 2 (Figure 3e). Furthermore, *SlEBF3-OE* fruits maintained higher firmness compared with WT throughout ripening (Figure 3f), and full ripening could not be recovered upon exogenous ethylene treatment (Figure 3g), suggesting a possible alteration in ethylene perception or response. Overall, the results indicate that not only the attainment of competence to ripen is discriminative between WT and *SlEBF3-OE* lines, but also the subsequent ripening process is impaired in *SlEBF3-OE* fruits. To characterize the ripening inhibition in *SlEBF3-OE* lines at the molecular level, we examined the expression of a set of ripening-related genes. The expression of *PL*, a major fruit pectate lyase gene involved in ripening-related cell wall metabolism (Uluisik *et al.*, 2016), was significantly reduced in *SlEBF3-OE* fruits (Figure 3h), consistent with the enhanced firmness phenotype. In addition, transcript accumulation of key ripening regulatory genes, such as *RIN*, *NOR* and *CNR*, was decreased dramatically at BR and post-BR stages compared with WT (Figure 3i–k). The expression of *E4* and *E8* was also significantly decreased in *SlEBF3-OE* fruits (Figure 3l,m), further supporting the hypothesis of a reduced ethylene response in *SlEBF3-OE* fruits.

### Ethylene production is reduced in *SlEBF3-OE* fruits

The ripening defect phenotype prompted us to investigate climacteric ethylene production in the *SlEBF3-OE* fruits. Monitoring ethylene production from MG stage to 10 days post-BR (BR + 10) clearly showed that *SlEBF3-OE* fruits produced less ethylene than WT at all ripening stages (Figure 4a). In line with this observation, transcripts of *ACS2*, *ACS4* and *ACO1*, the key genes involved in system II climacteric ethylene biosynthesis (Nakatsuka *et al.*, 1998), displayed significantly lower accumulation during ripening in the *SlEBF3-OE* lines (Figure 4b–d). These results suggest that in addition to reduced ethylene sensitivity, lower ethylene production may also account for the ripening defects of *SlEBF3-OE* fruit.

### Carotenoid content was significantly reduced in *SlEBF3-OE* fruits

To investigate the causal factor behind the yellow color phenotype of *SlEBF3-OE* fruits, we examined the dynamic changes of carotenoid and chlorophyll content in WT and two *SlEBF3-OE* lines (*SlEBF3-OE-11* and *SlEBF3-OE-12*) at different ripening stages, including MG, BR, BR + 3, and BR + 10. Both lycopene and β-carotene, the major carotenoids that accumulate during ripening and known to confer red and orange colors, respectively, were dramatically reduced in *SlEBF3-OE* fruits from BR to ripe stages (Figure 4e,f). Noteworthy, the level of lycopene was 10-fold lower in *SlEBF3-OE* lines compared with WT at BR + 10 stage (Figure 4e). In contrast to the decreased carotenoid content, both chlorophyll a and b content were significantly higher in *SlEBF3-OE* lines at BR + 3 and BR + 10 stages (Figure 4g,h).

To further characterize the change in pigment content of *SlEBF3-OE* fruit, we analyzed by qRT-PCR the transcript accumulation of genes involved in the carotenoid biosynthesis pathway at different ripening stages. Transcript levels of *PSY1*, a key regulator of flux through the carotenoid pathway, were dramatically reduced at post-BR stages

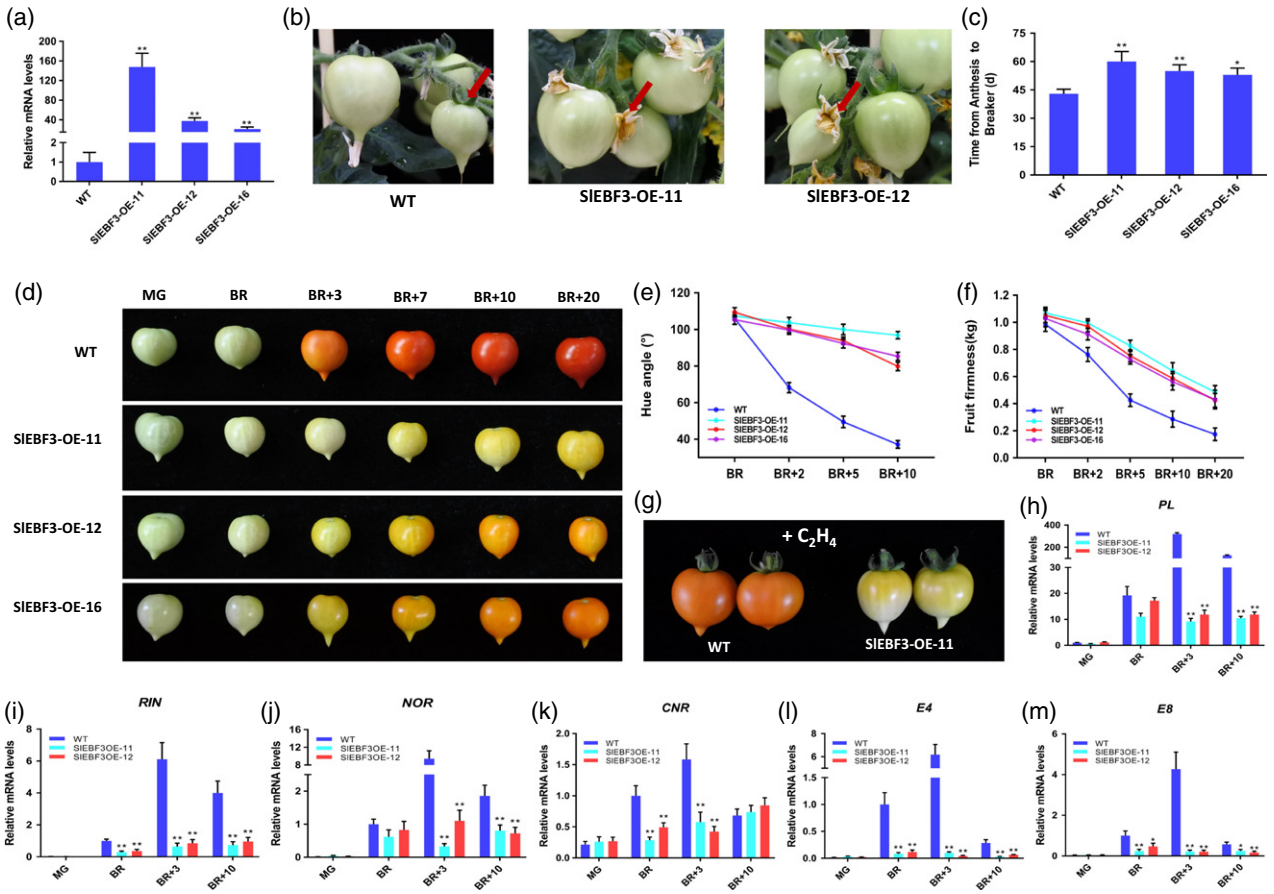


**Figure 3.** Phenotypes of SlEBF3 overexpressing tomato lines.
(a) Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of *SlEBF3* transcripts in *SlEBF3-OE* mature green fruits (MG).
(b) Delay of petal abscission in *SlEBF3-OE* lines.
(c) Time period from anthesis to the breaker stage (BR) in wild-type (WT) and *SlEBF3-OE* lines.
(d) Different ripening stages in WT and *SlEBF3-OE* lines. Fruits from three independent transgenic lines show delayed color development, never reaching a full red color. WT fruits display orange color at BR + 3 stage and reach full red at BR + 7, while *SlEBF3-OE* lines keep yellow (*SlEBF3-OE-11*) or orange color (*SlEBF3-OE-12* and *SlEBF3-OE-16*) even at BR + 20 stage. MG, mature green; BR, breaker stage; BR + 3, 3 days post-BR stage; BR + 7, 7 days post-BR stage; BR + 10, 10 days post-BR stage; BR + 20, 20 days post-BR stage.
(e) Changes in hue angle in WT and *SlEBF3-OE* lines during different ripening stages.
(f) Fruit firmness of WT and *SlEBF3-OE* fruits. Fruits were harvested at the BR stage, kept at room temperate and the firmness was measured at the indicated stages. A total of 20 fruits was used for each measurement, and the values shown are the means ± SD.
(g) Effect of ethylene treatment on WT and *SlEBF3-OE* fruit. MG fruits from WT and *SlEBF3-OE* lines were treated 2 h and three times per day with 10 ppm ethylene. After 7 days, ethylene-treated WT fruit turned full red, while *SlEBF3-OE-11*-treated fruits remained yellow and never got red.
(h–m) Relative expression of ripening-associated genes in WT and *SlEBF3-OE* lines during fruit ripening. Total RNA was extracted from the indicated developmental stages. The relative mRNA levels of each gene in WT at the BR stage were standardized to 1.0, referring to the *SlActin* gene as internal control. Error bar means ± SD of three biological replicates. *SlEBF3-OE-11*, *SlEBF3-OE-12* and *SlEBF3-OE-16* are three representative independent lines. Asterisks indicate statistical significance using Student's *t*-test: $^{**}P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

(Figure 4i). Moreover, a significant decrease in phytoene desaturase expression levels was also observed in *SlEBF3-OE* fruits (Figure 4j). Interestingly, transcript accumulation of *β-LCY1* was increased at post-BR satge, but that of *CYC-β* showed downregulation at BR+3 stage and upregulation at BR+7 stage.

### Dark-grown *SlEBF3-OE* plants showed reduced sensitivity to ethylene

The inability to recover a full ripening behavior upon exogenous ethylene treatment suggests a reduced ethylene perception or response in *SlEBF3-OE* fruit. To determine whether the decreased ethylene sensitivity also affects other tissues, the classical triple-response assay was performed with WT and *SlEBF3-OE* seedlings treated with the ethylene precursor ACC. In the absence of ACC, both the root and hypocotyl elongation exhibited no significant difference between WT and *SlEBF3-OE* lines (Figure 5a). However, upon ACC treatment, *SlEBF3-OE* lines showed marked insensitivity to ethylene with longer hypocotyl and higher root elongation than WT (Figure 5b–d). These results indicate that the

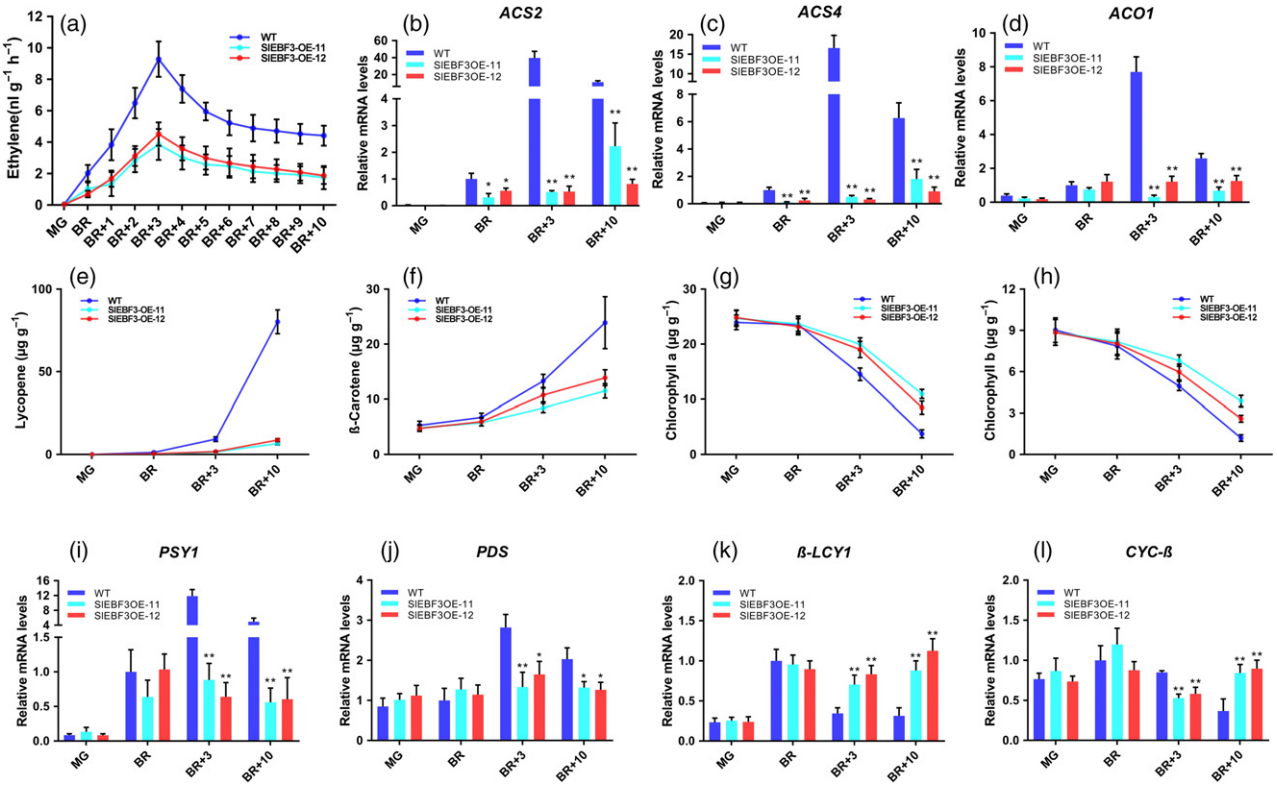


**Figure 4.** Ethylene production and carotenoid accumulation in wild-type (WT) and SlEBF3-OE fruits.
(a) Ethylene production of WT and *SlEBF3-OE* fruits at different ripening stages. Values represent means of at least 15 individual fruits.
(b–d) Expression of ethylene biosynthesis genes in *SlEBF3-OE* fruits at different ripening stages.
(e–h) Lycopene, β-carotene, chlorophyll a and chlorophyll b content in WT and *SlEBF3-OE* lines at different ripening stages.
(i–l) Relative expression of carotenoid biosynthesis genes in WT and *SlEBF3-OE* tomato lines. The relative mRNA levels of each gene in WT at breaker (BR) stage were standardized to 1.0, referring to the *SlActin* gene as internal control. Error bar means ± SD of three biological replicates. *SlEBF3-OE-11* and *SlEBF3-OE-12* are two representative independent lines. Asterisks indicate statistical significance using Student's *t*-test: $^{**}P < 0.01$. MG, mature green; BR, breaker stage; BR < 1, 1 day post-BR stage; BR < 2, 2 days post-BR stage; BR < 3, 3 days post-BR stage; BR < 4, 4 days post-BR stage; BR < 5, 5 days post-BR stage; BR < 6, 6 days post-BR stage; BR < 7, 7 days pos t-BR stage; BR < 8, 8 days post-BR stage; BR < 9, 9 days post-BR stage; BR < 10, 10 days post-BR stage. [Colour figure can be viewed at wileyonlinelibrary.com].

reduced sensitivity to ethylene is not only restricted to fruit.

### EBF3 regulates the accumulation of EIL proteins

In Arabidopsis, EBF proteins were reported to regulate ethylene response via modulating the degradation of EIN3 and EIL1 proteins through the Ub/26S proteasome pathway (Guo and Ecker, 2003; Potuschak *et al.*, 2003). To investigate whether the decreased ethylene sensitivity in *SlEBF3-OE* lines is due to enhanced degradation of EIL proteins in tomato, we first tested the ability of SlEBF3 protein to interact with EIL transcription factors using the yeast-two-hybrid (Y2H) system. The Y2H screening experiment showed that SlEBF3 is able to interact with all four tomato EIL proteins with, however, a lower intensity in the case of SlEIL4 (Figure 6a). The interaction between SlEBF3 and SlEIL proteins was further confirmed by bimolecular fluorescence complementation (BiFC) assays (Figure 6b).

To enable the interaction, SlEBF3 has to accumulate in the same cell compartment compared with SlEIL proteins that are transcription factors targeted to the nuclear compartment. We therefore checked the subcellular localization of SlEBF3 using a transient expression assay in tobacco cell protoplasts with SlEBF3-green fluorescent protein (GFP) fusion construct driven by the 35S CaMV promoter. Fluorescence microscopy analysis clearly showed that SlEBF3-GFP fusion protein strictly localized into the nucleus (Figure 7a). Finally, considering the role of EIL proteins in mediating ethylene responses, we investigated whether the defects in fruit ripening exhibited by *SlEBF3* overexpression lines are associated with a lower accumulation of EIL proteins in the fruit of these lines. Because the existence of functional redundancy among EIL family genes remains a pending issue, we examined the level of EIL proteins at different ripening stages in the *SlEBF3-OE* lines using an antibody that allows the detection of all four EIL proteins, namely EIL1–4. Immunoblot assays showed

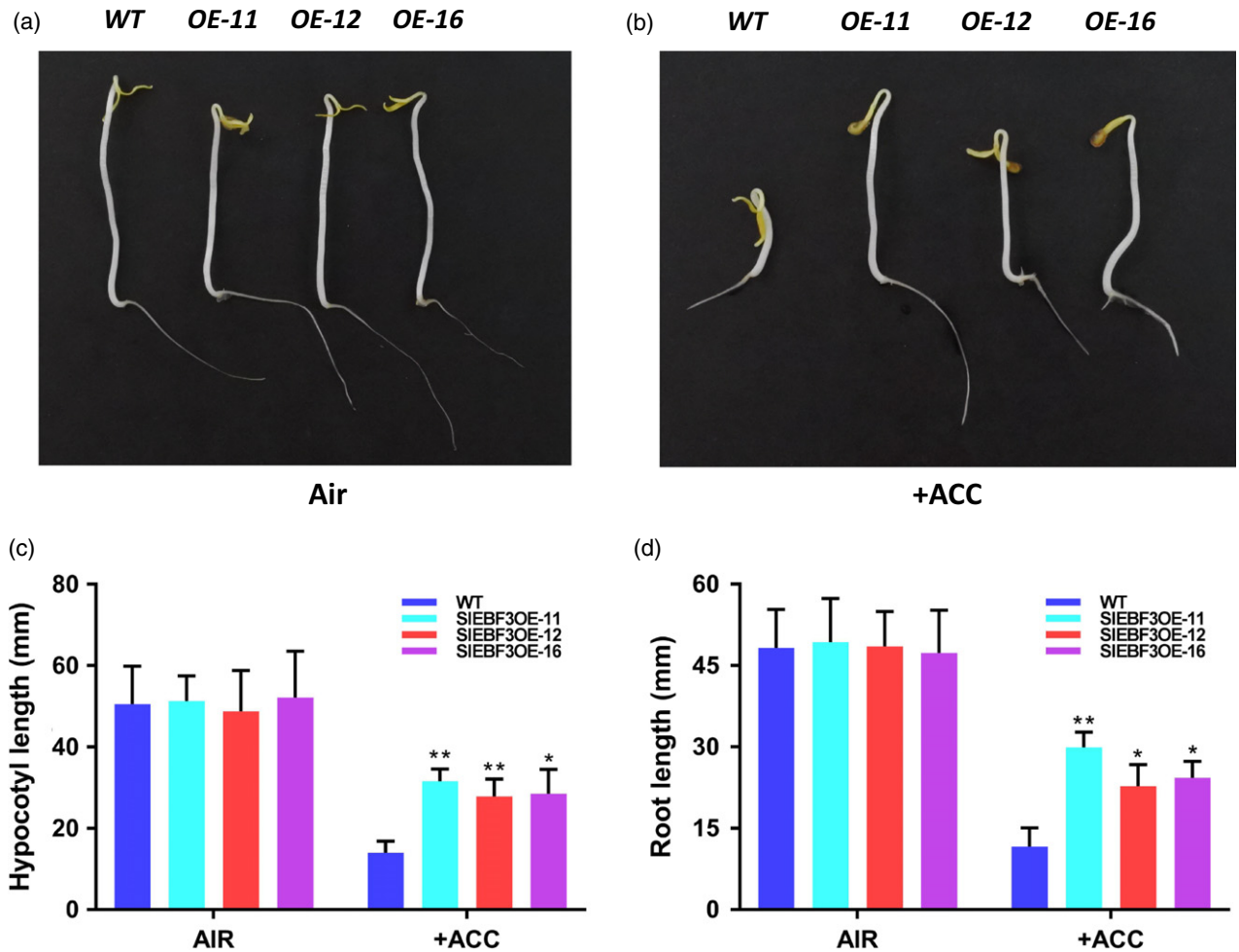


**Figure 5.** Ethylene triple-response in seedlings of wild-type (WT) and *SlEBF3-OE* lines.
(a) Etiolated WT and *SlEBF3-OE* seedlings in the absence of ACC treatment.
(b) Etiolated *SlEBF3-OE* seedlings displayed reduced ethylene response under ACC treatment. Seeds were surface sterilized and germinated on MS/2 with 5 μM ACC and incubated at 25°C in the dark for 7 days. At least 30 seedlings were measured.
(c) Hypocotyl elongation of WT and *SlEBF3-OE* lines with or without ACC treatment.
(d) Root length of WT and *SlEBF3-OE* lines with or without ACC treatment.
Values are means ± standard deviation (SD; $n \geq 30$) of three replicates. $^{**}P < 0.01$ (Student's *t*-test). [Colour figure can be viewed at wileyonlinelibrary.com].

that total EIL protein amounts were significantly reduced at BR and BR + 3 stages in *EBF3-OE* transgenic fruits compared with WT (Figure 7b). These results strongly suggest that the reduced ethylene sensitivity and inhibition of fruit ripening are likely due to the degradation of EIL proteins in *EBF3-OE* lines. Taken together, the data support the hypothesis that SlEBF3 is able to interact with EIL transcription factors leading to their degradation and thus impairing ethylene-dependent fruit ripening in tomato.

## DISCUSSION

Though post-transcriptional regulation is known to play an important role in controlling plant responses to a variety of endogenous and exogenous cues, its role in fruit ripening has been only addressed in very rare cases. EIN3-binding F-box proteins are central regulators of ethylene signal transduction pathway in Arabidopsis through targeting EIN3 and its homolog EIL1 for degradation (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004). However, it is still unknown whether EBFs operate in the tomato according to the same mechanism in controlling ethylene signaling and ethylene-dependent developmental processes such as climacteric fruit ripening. So far, data on EBF and their role in fruit ripening remain poorly documented (Yang *et al.*, 2010), and the present study brings evidence that *SlEBF3*, a novel *EBF* gene, is involved in fruit ripening via interacting with EIL proteins in tomato and mediating their degradation.

In contrast to *SlEBF1* and *SlEBF2*, expression of *SlEBF3* is highly induced by ethylene and repressed by 1-MCP, thus supporting the hypothesis that SlEBF3, but not SlEBF1 or SlEBF2, is involved in the feedback regulation of

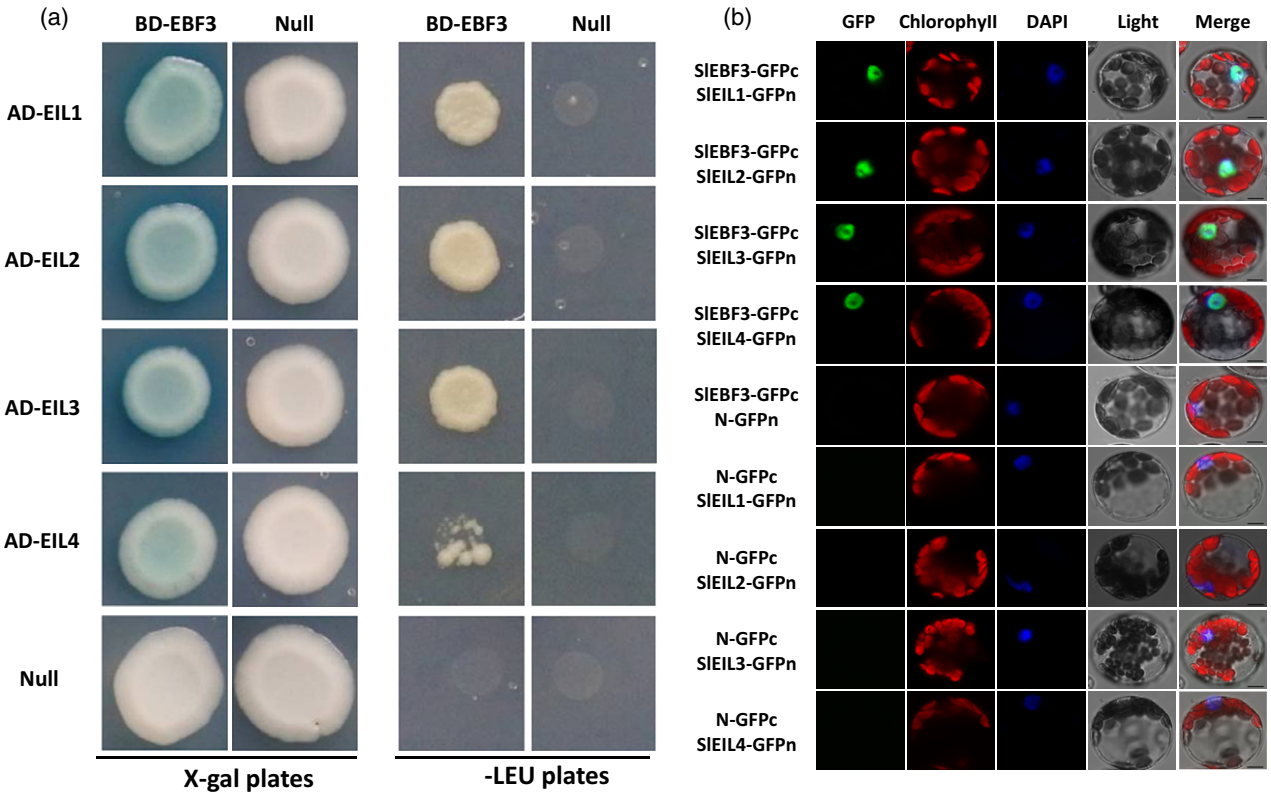


**Figure 6.** Interaction between SlEBF3 and EIL proteins.
(a) Analysis of the interaction between SlEBF3 and EILs in yeast system. Null represents empty vector. Pictures of plates were taken after 3 days at 28°C.
(b) Bimolecular fluorescence complementation (BiFC) analysis showed the interaction between SlEBF3 and EILs in Arabidopsis protoplasts. Green indicates green fluorescence protein (GFP) fluorescence, and blue indicates nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). [Colour figure can be viewed at wile yonlinelibrary.com].

ethylene signaling. This is in line with the presence of conserved ERE in *SlEBF3* promoter and the ability of SlEIL1 to regulate its promoter activity as shown by the transactivation assays. In Arabidopsis, both AtEBF1 and AtEBF2 were shown to function as repressors of EIN3-dependent transcription, but it was reported that AtEBF2, but not AtEBF1, is involved in the feedback regulation of ethylene signaling (Konishi and Yanagisawa, 2008). It is well established that feedback regulation of ethylene signaling participates to the fine tuning of ethylene response (Zhao and Guo, 2011). Therefore, our data are consistent with the hypothesis that SlEBF3 is a component of the feedback loop regulating autocatalytic ethylene production that is a typical trait of climacteric fruit ripening (Liu *et al.*, 2015).

Downregulation of *SlEBF3* resulted in the upregulation of *SlEBF2* in tomato fruit, suggesting the presence of an active compensation mechanism involving EBF family members that might be essential to maintaining homeostasis of ethylene signaling. Functional compensation involving components of ethylene signaling has been already described (Tieman *et al.*, 2000), and it was reported that a compensation mechanism allows maintaining a threshold level of EBF transcripts in both Arabidopsis and tomato (Potuschak *et al.*, 2003; Yang *et al.*, 2010). Overexpression of AtEBF1 in Arabidopsis resulted in the downregulation of endogenous AtEBF1 and AtEBF2 (Potuschak *et al.*, 2003). Likewise, *SlEBF1* transcript accumulation is enhanced in *SlEBF2* silenced tomato plants and, conversely, the level of SlEBF2 transcripts in SlEBF1-silenced lines is higher than in control plants (Yang *et al.*, 2010). Functional compensation among EBF members suggests that appropriate levels of EBF are essential for ethylene signaling in both Arabidopsis and tomato, and further supports the central role of EBF in tuning ethylene responses.

Single gene-silenced plants for either *SlEBF1* or *SlEBF2* were indistinguishable from control tomato plants (Yang *et al.*, 2010), suggesting that *SlEBF1* and *SlEBF2* are functionally redundant. Interestingly, we show here that downregulation of *SlEBF3* results in leaf epinasty phenotype likely due to ethylene hypersensitivity. These data suggest that SlEBF3 may play a more prominent role in ethylene signaling than other EBF members in tomato. *AtEBF1* and *AtEBF2* were shown to be functionally redundant and both *ebf1* and *ebf2* mutants were hypersensitive to ethylene, with *ebf1* showing also dwarfism and reduced fertility in Arabidopsis (Guo and Ecker, 2003). This suggests that

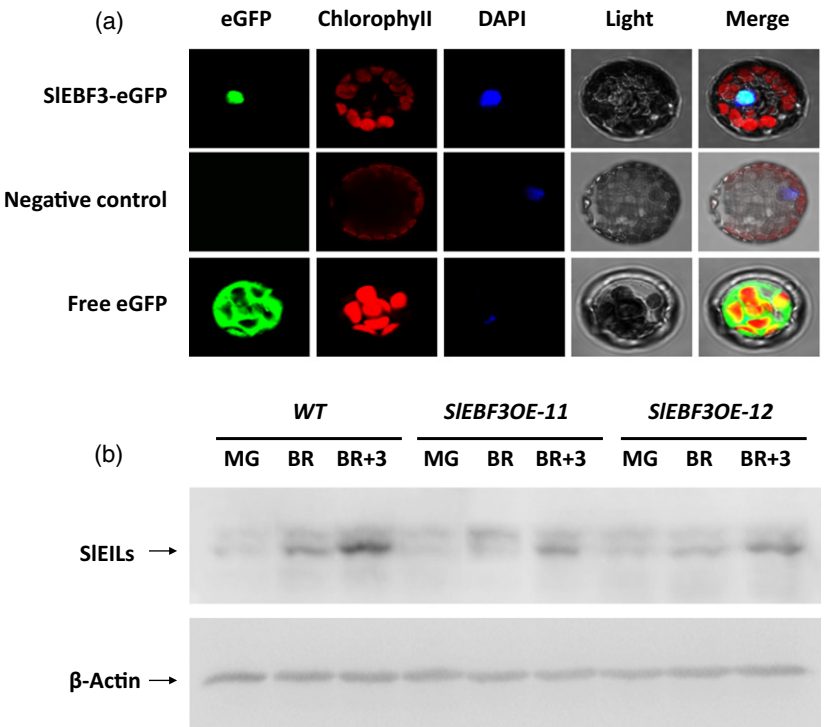


**Figure 7.** Reduced abundance of EIL proteins in *SlEBF3* overexpression fruits.
(a) Green fluorescent protein (GFP)-tagged fusion proteins were expressed in Tobacco cell protoplast after transfection under the control of the 35S promoter.
(b) Total protein extracts from wild-type (WT) and *SlEBF3-OE* fruits at different ripening stages were subjected to immunoblot with anti-EIL1-4 antibody. MG, mature green; BR, breaker stage; BR + 3, 3 days post-BR stage. *SlEBF3-OE-11* and *SlEBF3-OE-12* are two independent SlEBF3 overexpression lines. [Colour figure can be viewed at wileyonlinelibrary.com].

despite the known functional redundancy among EBF members, disruption of a single EBF is sufficient to alter ethylene sensitivity, sustaining the idea that different EBF members may possess their own functional specificities.

*SlEBF3* overexpression lines exhibited reduced ethylene sensitivity with defects in fruit ripening and delay in petal abscission. These phenotypes resemble those of tomato lines repressed in multiple *SlEIL* genes that display ethylene insensitivity, impaired fruit ripening, lack of the triple-response and delay in petal abscission (Tieman *et al.*, 2001; Yokotani *et al.*, 2009). Whether or not EIL family genes are functionally redundant remains an unresolved issue. Indeed, while tomato EILs were reported to be functionally redundant (Tieman *et al.*, 2001), evidence against this idea has been also provided showing that constitutive expression of EIL1 failed to fully restore ripening in the ethylene-insensitive *Nr* tomato mutant (Chen *et al.*, 2004). In tomato, there is no evidence, so far, showing that EBFs are able to regulate the levels of EIL proteins. The present study revealed a direct interaction between SlEBF3 and all tomato SlEIL proteins, suggesting that SlEBF3 can potentially regulate these transcription factors at the post-translational level. In support of this idea, the levels of total SlEIL proteins observed in *SlEBF3* overexpression fruits are significantly reduced. Therefore, it is conceivable that SlEBF3 may control ethylene signaling and fruit ripening through targeting EIL proteins for degradation via ubiquitin/26S proteasome system.

Overall, the outcome of the study supports a model in which tomato SlEBF3 is involved in regulating autocatalytic ethylene production associated with climacteric ripening via mediating the degradation of EIL proteins. Interestingly, it was recently shown that the expression of *SlEBF3* is downregulated in cold-treated fruit (Zhang *et al.*, 2016) and in auxin-treated samples (Li *et al.*, 2016). These results suggest that SlEBF3 may play a central role in regulating several ethylene-dependent developmental processes in plants.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

Tomato (*S. lycopersicum* MicroTom) plants were grown under standard glasshouse conditions, and the conditions in the culture chamber room were set as follows: 14-h day/10-h night cycle, 25°C/20°C day/night temperature, 80% relative humidity, 250 μmol $m^{-2}$ $sec^{-1}$ light intensity.

### Plant transformation

*Agrobacterium tumefaciens*-mediated tomato plants transformation was performed as described (Wang *et al.*, 2005). The transformed lines were then selected on a kanamycin-containing medium. Homozygous lines from F3 or later generations were used in all experiments.

### RNA extraction and qRT-PCR

Total RNA from different tissues were isolated using a Plant RNA Purification Reagent (Invitrogen, cat. no. 12322-012) according to the manufacturer's instructions. Genomic DNA contamination treatment, first-strand complementary DNA generation and qRT-PCR analyses were carried out in accordance with the described protocols (Liu *et al.*, 2013).

### Color measurement

Minolta chromameter (CR-200, 78903131; Ramsey, NJ, USA) was used to measure the *L*, *a* and *b* values of fruit at different ripening stages (BR, BR + 2, BR + 5 and BR + 10). Hue angle values were calculated according to the following equation: hue angle = $\tan^{-1}(b/a)$ if $a > 0$ or $180 + \tan^{-1}(b/a)$ if $a < 0$.

### Fruit firmness

Twenty fruits from each line were harvested at the BR stage, and the firmness was assessed with a TA.XT plus texture analyzer (Stable Microsystems, Godalming, UK) with a P/2 columnar probe (2 mm diameter). The test parameters were set as follows: pre-test speed 1 mm $sec^{-1}$; test speed 1 mm $sec^{-1}$; post-test speed 5 mm $sec^{-1}$; penetration depth 2 mm; trigger force 10 g. The value of firmness was calculated by Texture Exponent 32.

### Triple-response assay

Seeds of WT and transgenic lines were sterilized and put on MS/2 medium with 5.0 μM ACC or without ACC, and then transferred to 25°C for germination in the dark for 7 days. The seedling triple-response was scored by assessing hypocotyl and root length. For each line, at least 50 seedlings were measured.

### Ethylene measurement

To minimize the effect of wound ethylene caused by picking, fruits at different ripening stages were harvested and placed in open 120-ml jars for 2 h. Jars were then sealed and incubated for 2 h at room temperature, and 1 ml of headspace gas was then injected into an GC1120 gas chromatography equipped with a flame ionization detector (Sunny Hengping, Shanghai, China). Ethylene production of fruits was compared with ethylene standards of known concentration and normalized for fruit weight.

### Carotenoid and chlorophyll determination

Carotenoid and chlorophyll were determined according to the method described by Nagata and Yamashita (1992). Briefly, 1.0 g ground tomato fruit powder was extracted with acetone and hexane (2:3 by volume), then optical densities of the supernatant at 663 nm, 645 nm, 505 nm and 453 nm were measured by spectrophotometer at the same time. The content of chlorophyll a, b, lycopene and β-carotene could be calculated from the equations given as follows: chlorophyll a (mg 100 $ml^{-1}$) = $0.999A663 - 0.0989A645$; chlorophyll b (mg 100 $ml^{-1}$) = $-0.328A663 + 1.77A645$; lycopene (mg 100 $ml^{-1}$) = $-0.0458A663 + 0.204A645 + 0.372A505 - 0.0806A453$; β-carotene (mg 100 $ml^{-1}$) = $0.216A663 - 1.22A645 - 0.304A505 + 0.452A453$.

### Subcellular localization of SlEBF3 protein

The coding sequences (CDS) of EBF3 were cloned into the pART2a to generate the *35S::EBF3-GFP* transient expression vector. The released protoplast cells from leaves of 20-day-old tobacco were isolated and transformed via the PEG-mediated protoplast transfection method. After 1 night grown, 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleic acids of the protoplasts. Signals from GFP and DAPI were visualized with laser-scanning confocal microscopy.

### Transient transactivation assay

The transient transactivation assay was performed as described previously (Liu *et al.*, 2016). Briefly, protoplasts used for transfection were isolated from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells. The reporter construct was generated with the EBF1-3 promoters fused to GFP. Protoplast co-transfection assays were performed using the reporter plasmids and effector vectors carrying 35S:EIL1. GFP expression was analyzed and quantified by flow cytometry 16 h following protoplast transfection.

### Y2H assays

Yeast two-hybrid assays were performed according to the procedure described (Golemis *et al.*, 2009). The CDS of *EBF3* were introduced into pEG202 vector to create bait constructs, while the CDS of EIL1, EIL2, EIL3 and EIL4 were cloned into pJG4-5 to create prey construct vectors. Different combinations of bait and prey constructs were then co-transformed into yeast strain EGY48, and 2–4 days later these yeast strains were tested on selective plate medium.

### BiFC analysis

The entire open reading frames of EBF3, EIL1, EIL2, EIL3 and EIL4 were cloned into both the vector pE3228 containing N-terminal GFP fragments ($GFP^N$) and pE3242 containing C-terminal GFP fragments ($GFP^c$). Mesophyll protoplasts were isolated from 2-week-old Arabidopsis leaves, and were used for PEG-mediated protoplast transfection of different combinations of $GFP^N$ and $GFP^c$ constructs. The transformed protoplasts were grown at 25°C overnight. GFP fluorescence was visualized with laser-scanning confocal microscopy. Meanwhile, DAPI was used to mark the nucleic acids that were then observed at an excitation wavelength of 405 nm.

### Protein isolation and immunoblot analysis

Fruit tissues were harvested at different stages, including MG, BR and BR + 3. Protein isolation was performed as described previously (Huang *et al.*, 2013). Isolated protein extracts were mixed with 2× or 5× sodium dodecyl sulfate (SDS) loading buffer. The blended extracts were boiled for 5 min. The samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes, then blocked in blocking buffer (1 × TTBS with 5% dried non-fat milk) for 1 h at room temperature. The blocked membranes were then incubated with anti-EIL1-4 antibody for another 2 h, and additionally incubated for 1 h with secondary antibody of goat anti-rabbit IgG. EIL-specific antibody was produced by ABclonal Biotechnology (Wuhan, China).

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Phylogenetic analysis of tomato SlEBF family genes.
**Figure S2.** The presence of EREs in the promoter of SlEBF genes.
**Figure S3.** Different ripening stages of WT and SlEBF3-RNAi lines.
**Figure S4.** Expression of SlEBF1 and SlEBF2 in SlEBF3-OE fruits at BR stage.

 

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