SIBES1 promotes tomato fruit softening through transcriptional inhibition of PMEU1

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Highlights
SIBES1 promotes tomato fruit softening without affecting nutritional quality
SIBES1 inhibits PMEU1-related fruit pectin demethylesterification
SIBES1 represses PMEU1 expression through directly binding to the E-box
Knockout of SIBES1 by CRISPR-Cas9 enhances fruit firmness and extends shelf life
SUMMARY

Fruit softening indicated by firmness determines the texture, transportability, and shelf life of tomato products. However, the regulatory mechanism underlying firmness formation in tomato fruit is poorly understood. Here, we report the regulatory role of SIBES1, an essential component of brassinosteroid hormone signaling, in tomato fruit softening. We found that SIBES1 promotes fruit softening during tomato fruit ripening and postharvest storage. RNA-seq analysis suggested that PMEU1, which encodes a pectin methylesterase, might participate in SIBES1-mediated softening. Biochemical and immunofluorescence assays indicated that SIBES1 inhibited PMEU1-related pectin de-methylesterification. Further molecular and genetic evidence verified that SIBES1 directly binds to the E-box of PMEU1 to repress its expression, leading to fruits softening. Loss-of-function SIBES1 mutant generated by CRISPR-Cas9 showed firmer fruits and longer shelf life during postharvest storage without other quality alteration. Collectively, our results indicated the potential of manipulating SIBES1 to regulate firmness without negative consequence on visual and nutritional quality.
BRs were also found to be actively synthesized and accumulated during tomato fruit ripening (Li et al., 2016), indicating their potential role in tomato fruit ripening. As ethylene is regarded as the master regulator of climacteric tomato fruit ripening, BRs are found responsible for various aspects of fruit ripening, including improvement of carotenoid accumulation and visual quality (Liu et al., 2014), as well as the increase in soluble sugar content during tomato fruit ripening (Li et al., 2016), in either ethylene-dependent or ethylene-independent manner. Genetic and molecular biology studies in Arabidopsis have revealed the core components of the BRs signaling pathway, which constitutes a complete signal pathway from cell surface BR receptor to downstream transcription factors that regulate the expression of BR response genes (Nolan et al., 2020). BRI1-EMS-SUPPRESSOR1 (BES1), a key basic helix-loop-helix TF in the BR signaling pathway, balances plant growth and environment stress tolerance via binding a conserved E-box (CANNTG) and BRRE-box (CGTGC/TG) elements of its target genes (Jiang et al., 2019). Although the mechanism of BES1, acting as a transcription factor in regulating plant growth and development, has been well elucidated in Arabidopsis, its function in fruit softening has remained elusive.

In the present study, the specific role of SIBES1 on softening was investigated in tomatoes. SIBES1-over-expressing lines exhibited enhanced fruit softening while SIBES1-silencing lines showed improved fruit firmness, indicating its positive role in fruit softening. PMEU1, a gene from PME family, which inhibited fruit softening, was downregulated in fruits of SIBES1 RNAi lines. Further genetic analyses along with molecular biology assays (ChIP-qPCR, EMSA, and luciferase [LUC] reporter) reveal that SIBES1 directly binds to the E-box in the promoter of PMEU1 to inhibit its expression, leading to fruit softening. Interestingly, we found knockout or knockdown of SIBES1 in tomato extended shelf life without detrimental effects on visual and nutrition quality, implying SIBES1 will be potential in tomato breeding.

RESULTS AND DISCUSSION
Functional identification of SIBES1 in tomato
To elucidate the function of BES1 in fruit softening, we identified SIBES1, the homologous gene of AtBES1 in tomato (Figures S1A and S1B), and analyzed its expression pattern in BR-deficient and BR-insensitive tomato mutants. The expression level of SIBES1 was decreased in both BR-deficient mutant a and BR-insensitive mutant cu-3 that harbors a mutation in the BR receptor gene BRI1 (Figure S1C). To explore the function of SIBES1 in tomato, we then generated SIBES1-overexpressing transgenic lines, SIBES1-OX-3 and SIBES1-OX-8, as well as SIBES1 RNAi lines, SIBES1-RNAi-8 and SIBES1-RNAi-9 (Figure 1A). In Arabidopsis, the gain-of-function mutant of AtBES1 (bes1-d) has constitutive BR responses including tolerance to BR biosynthesis inhibitors, propiconazole (Pcz) or brassinazole (BRZ), in hypocotyl elongation assays in the dark (Yin et al., 2002; Hartwig et al., 2012). The hypocotyl elongation of SIBES1-OX-3 and SIBES1-OX-8 was not affected by 0.5 μM Pcz in the dark, whereas that of SIBES1-RNAi-8 and SIBES1-RNAi-9 was more susceptible to inhibition (Figures S1D and S1E), which was in agreement with the phenotypes of Arabidopsis BES1 mutants. In addition, BES1/BZR1 repressed the transcription of BR biosynthetic genes, such as DWARF, CPD, and DWARF4, through feedback regulation (He et al., 2005; Yu et al., 2011). In the present study, expression levels of the tomato BR biosynthetic genes (SIDWARF, SICPD, SICYP724B2, and SICYP90B3) were significantly downregulated in SIBES1-OX-3 and SIBES1-OX-8 but upregulated in SIBES1-RNAi-8 and SIBES1-RNAi-9 fruits (Figure S1F). These results indicate that SIBES1 confers conserved function as a transcription factor in BR signaling pathway between tomato and Arabidopsis.

SIBES1 promotes tomato fruit softening without affecting nutritional quality
SIBES1 transgenic fruits were then used to investigate the role of BES1 in fruit development. As shown in Figure 1B, no significant difference in fruit appearance at different developmental stages was found between wild-type Ailsa Craig (AC) and transgenic tomato lines. However, SIBES1-OX lines and SIBES1-RNAi lines exhibited reduced and increased fruit firmness compared with AC, respectively (Figure 1C). The varying degrees of firmness correspond to the SIBES1 expression levels in different transgenic lines (Figure 1A), which demonstrated that SIBES1 negatively regulates tomato fruit firmness. Ethylene production in fruits of SIBES1 transgenic lines and wild type (AC) was similar at each development stage (Figure S2A), indicating that SIBES1-mediated fruit softening is via ethylene-independent manner and might be different with the action of formerly reported ripening-related TFs, RIN, and NOR (Osmio et al., 2020). Meanwhile, SIBES1 overexpressing or silencing (RNAi) did not affect nutritional qualities (Table S1), and no significant difference was observed in expression levels of genes related to carotenoid and ascorbic acid biosynthesis between SIBES1-RNAi lines and wild type (Table S2). The fruit weight and fruit number per plant were also not affected in SIBES1-OX or SIBES1-RNAi fruits. Softening due to reduction in firmness is usually essential in determining shelf life, representing postharvest fruit integrity. We found that
Figure 1. Phenotypes of SIBES1-OX and SIBES1-RNAi tomato fruits

(A) Gene expression levels of SIBES1 in leaves and fruits of SIBES1-OX and SIBES1-RNAi plants. L, leaves. MG, mature green stage. B, breaker stage. P, pink stage. R, red ripe stage. Data shown represent the means ± SD of three biological replicates. Different letters indicate significant difference compared to AC (wild type) (one-way ANOVA with Tukey’s test, p < 0.05).

(B) Phenotypes of AC (wild type), SIBES1-OX, and SIBES1-RNAi tomato fruits. Pictures were taken at four developmental stages of fruit. MG, mature green; B, breaker; P, pink; R, red ripe. Scale bar, 1 cm.
The total PME activity was significantly reduced in To test this hypothesis, we measured the change in the pectin metabolic pathway in 
ated pectin metabolic pathway. 
value of fold change of genes in the figure are listed in Table S1. 
contains three biological replicates. B, breaker stage. R, red ripe stage. Accession number, name, description, and the 
discussed above, 
PME activity (Tieman and Handa, 1994). Furthermore, treatment of wild-type fruits with 3 
(EBL), a bioactive BR, significantly promoted fruit softening (Figure S2B). Meanwhile, EBL treatment recovered 
fruit softening in the BR-deficient mutant 
2007; Wen et al., 2013). PMEU1 was reported to negatively regulate tomato fruit softening by promoting cell 
were identified with different gene expression patterns in various tissues by bioinformatics analysis (Wen 
were conspicuously upregulated at both stages of 
softening in the BR-deficient mutant d" (Figure S2C), indicating a positive role of BR in fruit softening. 
SlBES1 inhibited PMEU1-associated pectin metabolic pathway 
Previous studies have established a negative role of PME in de-methylesterification to modulate fruit soft-
ening and maintain fruit integrity (Tieman and Handa, 1994; Phan et al., 2007). Our RNA-seq results indicated 
that PMEU1 and PE1 were upregulated in firmer 
fruits (Figure 1E, Table S3). As discussed above, 
fruits phenocopy the fruit with repressed PME activity. Based on these results, we hypothesize that 
SlBES1-mediated fruit softening might be caused by regulation of the PMEU1-associated pectin metabolic pathway. 

Taken together, all the above results suggested that BR signaling pathway component 
positively modulates fruit softening, possibly through regulation of PMEU1-associated pectin metabolic pathways. 

Figure 1. Continued 
(C) Fruit firmness of AC (wild type), SlBES1-OX, and SlBES1-RNAi fruits at different development stages. MG, mature green. B, breaker. P, pink. R, red ripe. Data shown represent the means ± SD of twenty-four biological replicates from at least six independent fruits. Different letters indicate significant difference compared to AC (wild type) (one-way ANOVA with Tukey’s test, p < 0.05). 
(D) The shelf life of AC (wild type), SlBES1-OX, and SlBES1-RNAi fruits. Fruits were harvested at red ripe stage and stored at room temperature. The progression of fruit deterioration was recorded by time-lapse photography. Time after harvest is specified by days. The storage condition was 25°C and 35% relative humidity. Scale bar, 1 cm. 
(E) RNA-seq results and related metabolic pathway of SlBES1-RNAi fruits at B and R stages. A heatmap showing the expression pattern of firmness-related genes in SlBES1-RNAi fruits and wild type. The values of fold change (log2) with or without significance (p < 0.05) were represented as colored blocks with or without asterisk, respectively. Each group contains three biological replicates. B, breaker stage. R, red ripe stage. Accession number, name, description, and the value of fold change of genes in the figure are listed in Table S1. 

SIBES1-OX fruits have a shorter shelf life, while SlBES1-RNAi fruits have a longer shelf life when compared with the wild type (Figure 1D). The SIBES1-OX lines have a similar phenotype as the loss of integrity in fruits with inhibited PME activity (Tieman and Handa, 1994). Furthermore, treatment of wild-type fruits with 3 μM 24-epibrassinolide (EBL), a bioactive BR, significantly promoted fruit softening (Figure S2B). Meanwhile, EBL treatment recovered fruit softening in the BR-deficient mutant d" (Figure S2C), indicating a positive role of BR in fruit softening.

To identify softening-related genes or cell wall metabolism processes that might participate in SIBES1-induced fruit softening, SIBES1-RNAi fruits were harvested at the breaker (B) and red ripe (R) stages for RNA-seq. Gene ontology (GO) analysis on RNA-seq data (Table S3) in the fruit development period category (p < 0.05) showed that the expression of 24 genes related to the metabolism of cell wall components, including genes involved in pectin metabolism (PMEs and PGs), xyloglucan metabolism (XTHs and CESAs), galactomannan metabolism (CSLAs), and cutin metabolism (CD), as well as other genes encoding cell wall-related proteins, was differentially regulated by SIBES1 (Figure 1E, Table S3). These genes participate in cell wall strengthening through remodeling, degrading or polymerizing cell wall composition as shown in Figure 1E. Among them, pectin degradation gene PG or its homologous genes in different species, such as EXP, CD, and XTHs, have been proved to affect softening-related texture change in fleshy fruit (Seymour et al., 2013; Wang et al., 2019), and the expression levels of XTHs were reported to be regulated by BR during hypocotyl elongation (Nolan et al., 2020). Although these genes have a relationship with softening or might be regulated by BR, the expression levels of most genes were not always influenced by SIBES1 at different stages of fruit development. The expression pattern of PG2 was oppositely regulated at different stages, whereas that of cutin, xyloglucan, and galactomannan metabolism genes, as well as genes encoding other cell wall-related proteins, such as CD, CESAs, XTHs, and EXPB1, was significantly altered at only one stage. However, the expression levels of PME genes, such as PMEU1 and PE1, were conspicuously upregulated at both stages of SIBES1-RNAi fruits. More than fifty PME family genes from tomato were identified with different gene expression patterns in various tissues by bioinformatics analysis (Wen et al., 2020). PMEU1 and PE1 have been initially reported as PME family genes in tomato fruits (Phan et al., 2007; Wen et al., 2013). PMEU1 was reported to negatively regulate tomato fruit softening by promoting cell wall strengthening (Phan et al., 2007), but PE1 did not function in cell wall strengthening of fruits (Wen et al., 2013). It was interesting that PMEU1 was the only one whose expression level was significantly increased at both B and R stages of SIBES1-RNAi fruits. This evidence suggested that PMEU1 and related pectin metabolic pathway might be involved in SIBES1-mediated softening.

Taken together, all the above results suggested that BR signaling pathway component SIBES1 positively modulates fruit softening, possibly through regulation of PMEU1-associated pectin metabolic pathways.
SlBES1-RNAi-8 and SlBES1-RNAi-9 fruits at both mature green (MG) and red ripe (R) stage when compared with the wild type (Figure 2A). PME catalyzes the de-methylesterification of pectin and then attenuates the degree of pectin methylesterification (DM) (Senechal et al., 2014; Figure 2C). With the decrease in total PME activity, the DM of soluble pectin was increased in SlBES1-OX and SlBES1-RNAi fruits at red ripe stage. Monoclonal LM20 antibody probe recognizing highly methylesterified HG, LM19 probe recognizing de-esterified pectin, and 2F4 probe recognizing Ca2+ cross-linked HG were used to label tomato pericarp tissue. Representative sections of fruits from each of AC (wild type), SlBES1-OX-3, and SlBES1-RNAi-8 lines are presented. Scale bar represents 100 µm. The right graphs show the relative fluorescence signal of each antibody. Relative signals are calculated through software ImageJ. Values were normalized with respect to AC (wild type). In (A and B) and (D), each data point represents means ± SD of three determinations. Different letters indicate significant difference between different groups (one-way ANOVA with Tukey’s test, p < 0.05).

Figure 2. SlBES1 represses the pectin de-methylesterification
(A and B) Total pectin methylesterase (PME) activity (A) and degree of methylesterification (DM) of soluble pectin (B) in SlBES1-OX and SlBES1-RNAi fruits at different development stages. MG, mature green stage. B, breaker stage. P, pink stage. R, red ripe stage.
(C) Schematic representation of the relationship between pectin methylesterase (PME) and cell wall strengthening.
(D) Immunolocalization of highly methylesterified HG (homogalacturonan), de-esterified HG, and Ca2+ cross-linked HG in SlBES1-OX and SlBES1-RNAi fruits at red ripe stage. Monoclonal LM20 antibody probe recognizing highly methylesterified HG, LM19 probe recognizing de-esterified pectin, and 2F4 probe recognizing Ca2+ cross-linked HG were used to label tomato pericarp tissue. Representative sections of fruits from each of AC (wild type), SlBES1-OX-3, and SlBES1-RNAi-8 lines are presented. Scale bar represents 100 µm. The right graphs show the relative fluorescence signal of each antibody. Relative signals are calculated through software ImageJ. Values were normalized with respect to AC (wild type). In (A and B) and (D), each data point represents means ± SD of three determinations. Different letters indicate significant difference between different groups (one-way ANOVA with Tukey’s test, p < 0.05).
Figure 3. SIBES1 represses the transcriptional expression of PMEU1
(A) Relative expression levels of PMEU1 in SIBES1-OX and SIBES1-RNAi fruits at different development stages. MG, mature green stage; B, breaker stage; P, pink stage; R, red ripe stage. Each data point represents means ± SD of three determinations. Different letters indicate significant difference between different groups (one-way ANOVA with Tukey’s test, p < 0.05).

(B) ChIP-qPCR assays showing that SIBES1 was associated with the locus of candidate genes, LoxA, PMEU1, and PE1. Chromatin of transgenic plants expressing 35Spro:SIBES1-myc (SIBES1-myc) was immunoprecipitated with anti-myc antibody, and the result of ACTIN7 served as control. The relative enrichment for ChIP signal was displayed as the percentage of total input DNA. Values are means ± SD of three biological replicates. Statistical analysis was performed with ANOVA. Bars with asterisks indicate significant difference (**p < 0.01).
Figure 3. Continued
(C) ChIP-qPCR assay to detect the association between SIBES1 and the PMEU1 promoter. The upper graph, schematic diagram of PMEU1, indicating the amplicons used for ChIP-qPCR. Positions of E-box and BRRE-box (BR response element) are indicated. The below graph, ChIP-qPCR results for SIBES1 associated with PMEU1 locus in tomato fruits. The relative enrichment for SIBES1 at two PMEU1 promoter motifs, E-box and BRRE-box, was calculated against the ACTIN2 promoter. AC (wild type) without and with antibody were set as blank control and negative control, respectively. SIBES1-myc without antibody was also set as negative control. The mean value of two technical replicates was recorded for each biological replicate. Values are means ± 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). (D) Transient expression assays showing that SIBES1 represses PMEU1 expression. Representative images of N. benthamiana leaves were taken 48 hr after infiltration. The bottom panel indicates the infiltrated constructs. (E and F) Luminescence intensity (E) and expression level (F) under different treatments as indicated in (D). Values are means ± SD of six biological replicates. Different letters indicate significant difference among groups for each locus (one-way ANOVA with Tukey’s test, p < 0.05). (G) Motif sequence of labeled, unlabeled competitor, and mutant competitor probes. Mutant competitor in which the 5‘-CACCTG-3’ motif was replaced with 5‘-AAAAA-A-3’. (H) DNA electrophoretic mobility shift assay (EMSA) showing that the binding of SIBES1-His to the E-box of PMEU1 promoter in vitro. FAM-labeled probes were incubated with SIBES1-His and the free and bound DNAs were separated on an acrylamide gel.

(Senechal et al., 2014; Silva-Sanzana et al., 2019), the immunolocalization results suggested that the decreased content of the “egg box” structure in SIBES1 fruits was the direct reason for SIBES1-induced softening. Calcofluor-white staining showed no major differences in cell size or patterning between transgenic lines (Figure S3A). The reduced activity of PME and decreased content of its products, as revealed by DM and immunolocalization experiments, support our hypothesis that PME-mediated pectin de-methylesterification was repressed by SIBES1.

The expression pattern of the PME gene during the fruit softening process was further investigated. The expression level of PMEU1 was significantly decreased in SIBES1-OX and increased in SIBES1-RNAi fruits when compared with the wild type at both the B, P, and R stages (Figure 3A), which is consistent with the change of total PME activity and DM (Figures 2A and 2B). Interestingly, the expression level of PMEU1 was also downregulated in EBL-treated fruits, while EBL treatments offset the downregulated PMEU1 expression in BR-deficient mutant d" (Figures S3B and S3C), suggesting a role of BR in repressing PMEU1 expression. These results indicated that both PMEU1-associated pectin de-methylesterification and the expression level of PMEU1 were inhibited by SIBES1. Our data suggest that SIBES1 might repress the expression of PMEU1 to promote fruit softening.

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

To test the hypothesis of possible inhibited expression of PMEU1 by SIBES1, we carried out further tests to prove the transcriptional regulation of SIBES1 on the expression of PMEU1. Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) results showed a significantly higher enrichment for the promoters of PMEU1 than for PE1 (Figure 3B), consistent with a stronger effect of SIBES1 on the transcriptional level of PMEU1 than that of PE1 (Figure 1E, Table S3). The two binding sites, E-box and BRRE-box, were located at −465 bp and −289 bp of the PMEU1 promoter as predicted by the JASPAR database, respectively (http://jaspar.genereg.net/) (Figure 3C). The ChIP-qPCR results further indicated that enrichment of SIBES1 bound to the region containing E-box instead of the BRRE-box (Figure 3C), indicating the amplicons used for ChIP-qPCR. Positions of E-box and BRRE-box (BR response element) are indicated. The below graph, ChIP-qPCR results further indicated that enrichment of SIBES1 bound to the region containing E-box instead of the BRRE-box (Figure 3C), suggesting that SIBES1 might regulate expression by binding to the E-box.

To test if E-box is necessary for regulation of PMEU1 expression by SIBES1, we generated a PMEU1pro:LUC reporter, in which LUC was fused with the PMEU1 promoter. Co-expression of PMEU1pro:LUC with the 35Spro:SIBES1 construct led to a significantly reduced luminescence intensity, suggesting that SIBES1 represses the expression of PMEU1. In addition, when PMEU1pro:LUC was replaced by PMEU1pro:Ebox:LUC, in which the E-box of the PMEU1 promoter was deleted, SIBES1-mediated repression of the PMEU1 expression was abolished, further verifying that E-box rather than BRRE-box was involved in transcriptional regulation of SIBES1 on PMEU1 (Figures 3D–3F).

We then conducted an electrophoretic mobility shift assay with purified SIBES1-His and a 20-bp DNA probe containing the E-box motif. SIBES1-His bound to the DNA probe, and this binding was successfully outcompeted by the unlabeled DNA probe but not by the DNA probe without E-box. These results suggested that SIBES1
Figure 4. Effects of PMEU1 silencing in SIBES1-RNAi on tomato firmness and shelf life

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

(B) Fruit firmness of control, TRV-PMEU1, SIBES1-RNAi, and TRV-PMEU1/SIBES1-RNAi at red ripe stage. Values are means ± SD of twenty-four biological replicates.
directly binds to the PMEU1 promoter through E-box (Figures 3G and 3H). E-box was the binding site of SIBE1 to repress gene expression in tomatoes. BES1/BZR1 usually binds to E-box to activate gene expression and binds to BRRE to repress gene expression (Nolan et al., 2020). There are exceptions; a recent study also showed that OsBZR1 bound to the E-box of PMEU1 promoter and then represses the expression of PMEU1. Less PMEU1 is secreted into the cell wall, and pectin methylesterase activity is attenuated, so the contents of DMHG and Egg box are decreased which causing fruit softening.

Silencing PMEU1 caused softer fruits with lower total PME activity and exhibited a complete loss of fruit integrity and shorter shelf life (Figures 4A–D), which was in accordance with previous reports (Tieman and Handa, 1994; Phan et al., 2007). More importantly, knocking down PMEU1 in the SIBE1-RNAi background (TRV-PMEU1/SIBE1-RNAi) suppressed the SIBE1-RNAi phenotypes of higher firmness and the longer shelf life (Figures 4B and 4C). Fruit firmness, shelf life, and total PME activity of TRV-PMEU1/SIBE1-RNAi were restored to almost the same as wild type, suggesting that SIBE1 might modulate fruit softening by inhibition of PMEU1 (Figures 4B–4D). Taken together, these results demonstrated that SIBE1 directly binds to the E-box of PMEU1 to repress its expression, thereby promoting fruit softening.

**Gene editing of SIBE1 by CRISPR-Cas9 enhances fruit firmness without negative effect on nutritional quality**

The above results suggested that SIBE1 might be a potential target to breed tomatoes with longer shelf life and maintaining optimum flavor and nutrients. Then, we generated SIBE1-KO through CRISPR-Cas9-mediated genome editing the 5'-TAGTTGGTGATGAAAGAGGTGG-3' in the second exon of SIBE1 antisense strand with pYLCRISPR/Cas9 (Ma et al., 2013), the independent SIBE1-KO mutant was obtained in AC background, which was the same background with SIBE1-OX and SIBE1-RNAi, with 1 bp deletion (Figure S4A). The expression level of SIBE1 was decreased by 80%, and the expression level of PMEU1 was significantly upregulated as a result of reduced mRNA level of SIBE1 in SIBE1-KO fruits at the red ripe stage (Figure S4B). As expected, the fruit of SIBE1-KO had higher firmness and longer shelf life (Figures S4C and S4D) with negligible effects on other agronomic or quality traits (Table S1), which is in accordance with the phenotypes of SIBE1-OX and SIBE1-RNAi (Figures 1C and 1D; Table S1). Pectin metabolic genes, such as PL or PG, have been used as genome editing targets to obtain fruits with longer shelf life (Uluisik et al., 2016; Wang et al., 2019). Genome editing of upstream TFs that regulate pectin metabolic enzymes might be an alternative way for extending shelf life. However, the reported upstream TFs, RIN or NOR, regulated pectin metabolic genes to provide firmer fruit, while inherited poor quality in the meantime (Osoiro et al., 2020). Our results suggest that SIBE1, as an upstream TF that directly regulates PMEU1 and associated pectin de-methylesterification, could be a powerful target to be explored to extend shelf life without defect in other quality traits to tomato fruits (Figure S4D). A previous study suggested that CRISPR-editing of a hormone biosynthetic gene generated firmer fruit without unfavorable changes in quality (Li et al., 2020). Considering that diverse phytohormone signaling components generally play essential functions via subtle control, such as transcriptional regulation on the target genes (Jiang et al., 2019), it might be potential to achieve improved agronomical traits of crops by genome editing of core signaling components of phytohormones.
In summary, this study provides genetic, biochemical, and molecular biology evidence for SlBES1-mediated fruit softening in an ethylene-independent manner by modulation of the pectin metabolic pathway. At the transcriptional level, SlBES1 directly binds to the E-box of the PMEU1 promoter to repress PMEU1 expression and pectin de-methylesterification, thereby promoting fruit softening (Figure 4E). Fruits with silenced SlBES1 described in this study provide a promising solution to improve fruit firmness and extended shelf life without negative effects on visual and nutritional quality.

Limitations of the study
In this study, we find that SlBES1 represses PMEU1 expression and pectin de-methylesterification to promote fruit softening. Although this study provides a regulation mechanism of SlBES1 on firmness under BR, the relationship between softening and other important homologous TFs, BZR1 or BEHs, in BR signaling remains unknown.

STAR★METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102926.

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AUTHOR CONTRIBUTIONS
Q.W., C.L., and H.L. designed the research. H.L., M.Z., C.J., M.Q., F.M., S.H., Z.S., and D.L. performed the research. H.L. D.L., and Y.L. analyzed data. H.L., L.L., Q.W., Y.Y., and C.J. wrote the manuscript.
DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| LM19                | Plantprobes | Cat# LM19, RRID: AB_2734788 |
| LM20                | Plantprobes | Cat# LM20, RRID: AB_2734789 |
| 2F4                 | Plantprobes | Cat# 2F4 |
| Alexa Fluor 488 goat anti-rabbit | Jackson ImmunoResearch | Cat# 111-545-003, RRID: AB_2338046 |
| **Bacterial and virus strains** |        |            |
| E.coli strain DH5α  | N/A | N/A |
| A. tumefaciens GV3101 | N/A | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| 24-epibrassinolide   | Sigma-Aldrich | CAS# 78821-43-9 |
| Propiconazole        | Sigma-Aldrich | CAS# 60207-90-1 |
| Ni-His Resin         | Thermo Fisher | 88221 |
| pET28a-SlBES1-His    | This paper | N/A |
| **Critical commercial assays** |        |            |
| QIAquick PCR Purification Kit | QIAGEN | 28104 |
| Gateway LR Clonase II Enzyme mix | Thermo Fisher | Cat. 11791020 |
| **Deposited data**  |        |            |
| RNA-seq data         | This paper | PRJNA635540 |
| **Experimental models: Organisms/strains** |        |            |
| Tomato: SlBES1-OX 3  | This paper | N/A |
| Tomato: SlBES1-OX 8  | This paper | N/A |
| Tomato: SlBES1-RNAi 8 | This paper | N/A |
| Tomato: SlBES1-RNAi 9 | This paper | N/A |
| Tomato: SlBES1-KO    | This paper | N/A |
| **Oligonucleotides** |        |            |
| More than 10, see Table S4 |        |            |
| **Recombinant DNA**  |        |            |
| Plasmid: Pro35S:SlBES1-myc | This paper | N/A |
| Plasmid: pHANNIBAL-SlBES1 | This paper | N/A |
| Plasmid: pYLCRISPR/Cas9-SlBES1 | This paper | N/A |
| Plasmid: pGWB35-PMEU1pro-LUC | This paper | N/A |
| Plasmid: pGWB35-PMEU1proE-box: LUC | This paper | N/A |
| Plasmid: SlBES1-1300  | This paper | N/A |
| Plasmid: pTRV2-PMEU1  | This paper | N/A |
| **Software and algorithms** |        |            |
| ImageJ               | National Institutes of Health, USA | https://imagej.nih.gov/ij/ |
| MEGA X               | N/A | https://www.megas |
| SPSS 19              | N/A | https://www.ibm.com/cn-zh/analytics/spss-statistics-software |
| Indigo software      | Berthold Technologies | Combined with equipment |
RESOURCES 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
All transgenic lines were constructed in tomato cultivars Ailsa Craig (AC). Cultivars Lycopersicon pimpinellifolium (Spim), Condine Red (CR), and Craigella are the parental line of cu-3, dim, and dx (Bishop et al., 1996; Scheer et al., 2003; Li et al., 2015), respectively. Plants were cultivated under a 16 h photoperiod (22/28°C, night/day). The number of tagged flowers at anthesis was limited to fewer than five per cluster. The fruit ripening stages, including mature green stage (MG), breaker stage (B), pink stage (P), and red ripe stage (R), were defined based on fruit color as described previously (Giovannoni, 2004). After firmness determination and immunofluorescence, three biological replicates for transgenic fruit pieces (each biological replicate consisting of at least three fruits) or fully expanded leaves from 4-week-old BR mutants were frozen with liquid nitrogen as samples, and then stored at -80°C for further tests.

Accession numbers
The accession numbers for the genes described in this report are as follows: SIBES1 (Solyc04g079980), PMEU1 (Solyc03g123630), PE1 (Solyc07g064170), SIDWARF (Solyc02g089160), SICPD (Solyc06g051750), SICYP72AB2 (Solyc07g056160), SICYP90B3 (Solyc02g085360), LOXA (Solyc08g014000), ACTIN7 (Solyc03g078400), ACTIN2 (Solyc11g005330).

METHOD DETAILS

Vector constructs and plant transformation
Vector constructs for transgenic were generated following standard molecular biology protocols. For SIBES1-OX plants, the full-length coding sequence of SIBES1 without termination codon was amplified via PCR and inserted into the pGWB17 vector using Gateway (Invitrogen) technology (Nakagawa et al., 2007) to get the Pro35S:SIBES1-myc construct. For SIBES1-RNAi construct, a fragment of SIBES1 with length of 305 bp was amplified and then inserted into intermediate vector pHANNIBAL in the positive orientation, thereby generating vector pHANNIBAL-SIBES1. The same fragment of SIBES1 was inserted into pHANNIBAL-SIBES1 in the reverse orientation, generating the vector pHANNIBAL-SIBES1i. The target inverted repeat sequences were obtained by SacI and SpeI digestion of pHANNIBAL-SIBES1i. Eventually, these sequences were inserted into pBIN19 under the control of CaMV 35S promoter to generate pBIN19-SIBES1-RNAi.

The above constructs were introduced into tomato cultivars AC via Agrobacterium tumefaciens LBA4404-mediated transformation (Liu et al., 2019). For SIBES1-OX lines, thirteen T1 lines were obtained according to their resistance to Kanamycin on the screening for regenerated shoots. Among them, six lines showed an increased expression level of SIBES1 in leaves. Two lines, SIBES1-OX 3 and SIBES1-OX 8, showed the significantly enhanced expression level of SIBES1 in leaves and fruits. Homozygous T2 or T3 transgenic plants from these two lines were used for further researches. For SIBES1-RNAi lines, six T1 lines were selected according to their resistance to Kanamycin. Two of them, SIBES1-RNAi 8 and SIBES1-RNAi 9, have a decreased expression level of SIBES1 in fruits and homozygous T2 or T3 transgenic plants from these two lines were used for further researches.
For fruits of overexpressing lines, the expression level of SIBE1 in SIBE1-OX 3 was increased by 32%, 208%, 250%, and 35% at MG, B, P, and R stage, respectively. The expression level of SIBE1 in SIBE1-ox 8 was increased by 6%, 79%, 50%, and 25% at MG, B, P, and R stage, respectively (Figure 1A). Meanwhile, for fruits of silencing lines, the expression level of SIBE1 in SIBE1-RNAi 8 was down-regulated by 81%, 95%, 94%, and 94% at MG, B, P, and R stage, respectively. The expression level of SIBE1 in SIBE1-RNAi 9 was reduced by 60%, 95%, 93%, and 93%, at MG, B, P, and R stage, respectively (Figure 1A).

Generation of the CRISPR/Cas9 mutant

The guide RNA sequence CCACCTCTTTCATCACCAACTA for SIBE1 was cloned into pYLCRISPR/Cas9 (Ma et al., 2015), and the base C at 556bp was deleted. After inducing construct into AC by Agrobacterium-mediated transformation, sequences containing guide RNA target sites were amplified and sequenced to confirm mutations in targeted regions. Homozygous T2 transgenic plants were selected for further experiments.

Chemical treatment

AC and dim at mature green stage were treated with 24-epibrassinolide (EBL, Sigma, St. Louis, MO). Lanolin with or without 3 μM EBL were used to cover fruits totally and treated fruits were put in a phytotron (Qiushi environment company, Hangzhou, China) with 16 h photoperiod at 24 °C and 80% relative humidity (Liu et al., 2014) and then collected at 1st, 3rd, 6th, and 9th day for further tests. Tomato seeds were sown in half-strength MS containing 0.5 μM propiconazole (Picz, Sigma, St. Louis, MO). Plants were grown at 28 °C with total darkness for 6 d, then the lengths of seedlings hypocotyls and roots were measured through software ImageJ after photographed.

Firmness determination

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

RNA extraction and relative quantitative PCR

For RNA extraction, 0.1 g of leaves or fruits was mixed with 1 mL RNAiso plus according to manufacturer’s instruction (Takara, Kusatsu, Japan), then RNA was reverse-transcribed into cDNA using PrimeScript RT reagent with gDNA Eraser (Takara, Kusatsu, Japan). TB Green (Takara, Kusatsu, Japan) was then used in Step One Real-Time PCR System (Applied biosystem, CA, USA) for relative quantitative PCR (qPCR). The gene-specific primers used were listed in Table S4.

RNA-Seq

AC and SIBE1-RNAi-8 fruits at breaker and red ripe stages were collected for total RNA extraction and Illumina MiSeq library was constructed as described by manufacturer’s instructions (Illumina, San Diego, CA, USA) and then sequenced with the Illumina Miseq platform.

ChIP-qPCR

Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) for fruits was performed following previous reports (Liu et al., 2019). In brief, 3 g of 0.5 cm² fruit pieces of SIBE1-OE-3 at mature green stage were collected and cross-linked using 1% (v/v) formaldehyde under vacuum for 10 min and ground to powder in liquid nitrogen. Then, the chromatin complexes were isolated, sonicated with Biorupter plus (Diagenode, Belgium, Antwerp), and immunoprecipitated with 5μg Mouse monoclonal anti-c-myc antibody (clone 9E10, IgG, Roche, Basel, Switzerland). About 200~300 bp of ChIP DNA and input DNA was recovered and dissolved in water for further ChIP-qPCR analysis. For ChIP-qPCR, primer pairs were used to analyze the ChIP DNA (Table S4). Each ChIP value was normalized to its respective input DNA value. The fold enrichment on each candidate gene or PMEU1 promoter was calculated against the ACTIN7 or ACTIN2, respectively. The mean value of two technical replicates was recorded for each biological replicate. The values of three independent biological replicates were collected and error bars represented the standard error from these tests.

Transient expression assay in tobacco leaves

Transient expression assay was performed in tobacco (Nicotiana benthamiana) (Liu et al., 2019). The PMEU1 promoter was cloned with KOD FX (Toyobo, Japan, Osaka) and inserted into PQB vector using DNA Ligation Kit (Takara, Kusatsu, Japan). Then the promoter was fused with the LUC reporter gene.
into the plant binary vector pGWB35 using Gateway cloning kit (Thermo, Boston, USA) to generate the PMEU1pro:LUC reporter construct. SIBES1-1300 was used as an effector construct. These constructs were transformed into Agrobacterium cell GV3101 with P19 protein and pSoup vector, and then the cells were incubated, harvested, and re-suspended in infiltration buffer (10 mM MES, 40 mM AS, and 10 mM MgCl₂) to a final concentration of OD₆₀₀= 1.0. Equal volumes of transformed cells with different combinations were mixed and then co-infiltrated into tobacco leaves with a needleless syringe. Infiltrated plants were placed at 28 °C for 48 h before imaging. NightOWL II LB983 Ultrasens backlit (Berthold Technologies, Bad Wildbad, Germany) with Indigo software was used to capture LUC expression image and to quantify LUC luminescence intensity. 100 mM luciferin was sprayed on leaves and incubated in dark for 5 min before detection. Six independent determinations were performed.

EMSAs
The full-length coding region of SIBES1 was amplified and then inserted into pET28a vector. The recombinant protein, SIBES1-His were expressed in E. coli Rosetta cells at 28°C and purified to homogeneity with Ni-His Resin (Thermo, Boston, USA) after identified by SDS-PAGE. Oligonucleotide probes were synthesized and labeled with FAM at 3’ ends. EMSAs (electrophoretic mobility shift assay) was performed as previously described (Liu et al., 2019) with minor modification. In brief, the FAM-labeled binding box probe, probe without any label (competitor), and mutant probe (mutant competitor) were incubated with SIBES1-His proteins at room temperature for 20 min, respectively. Bound and free probes were separated via PAGE. Typhon FLA7000 (GE, Fairfield, USA) was used to capture the final image. Probes were listed in Table S4.

Determination of PME enzyme activity and degree of pectin methylesterification
Total PME activity and degree of pectin methylesterification (DM) were determined as previously reported (Kyomugasho et al., 2015). Alcohol-insoluble substances (AIS) from cell wall was first obtained and then soluble and insoluble pectin were extracted from AIS. 4 g of fresh tomato pericarp at different fruit stage was boiled in 25 mL 95% ethanol for 20 min and then mixed for 60 s. The precipitate was left after centrifuged at 1500 g for 10 min. Then the precipitate was mixed with 25 mL 80% ethanol and the mixture was centrifuged at 1500 g for 20 min to obtain precipitates. This step was repeated three times until the supernatant showed colorless. The crude cell wall pellet was dried under air steam and then suspended in DMSO: water (9:1, v/v, 20 mL/g). After stirring in room temperature for 24 h, the slurry was centrifuged at 1500 g for 20 min to remove DMSO. The pellet was washed repeatedly in 95% ethanol and then was dried under air. The dried material was washed once with acetone. Then this cell wall extraction was freeze-dried and weighted. It was centrifuged at 10500 g for 25 min after suspending in sterilized distilled water for 1 h and this step was repeated for once. Supernatant and precipitate in these two steps were collected, respectively. The supernatant was soluble pectin while the precipitate was insoluble pectin. The pH was adjusted to 6 with 0.1M NaOH to ensure total ion amount of carboxyl group.

For pectin methylesterase (PME, EC3.1.1.11) activity determination, titration with an automatic titration was used. Weighted crude protein from cell wall was assayed in determination solution, 2 % (w/v) citrus pectin (pH=7.0). The volume of 5 mM NaOH consumed for titration was recorded over 5 min. Total PME activity was calculated as the volume of consumed NaOH per min (U·min⁻¹·g⁻¹).

Immunofluorescence
Fresh tomato pericarp materials were fixed with FAA for at least 24h. Fixed tissue was dehydrated by serial incubations at 4°C in solution with an increasing concentration of ethanol from 10 % to 100 % and then submersed with LR White resin (Sigma-Aldrich, St. Louis, MO) to place into capsules. 1 μm sections were obtained from the embedding block and then placed on glass slides for immunofluorescence assays. LM19 and LM20 mouse monoclonal antibodies bind demethylated and hypermethylated HGs, respectively. Additionally, 2F4 mouse monoclonal antibodies are used to bind “egg box” epitopes formed of chain dimer of de-esterified HG linked with calcium ions (Silva-Sanzana et al., 2019). The non-specific binding sites were blocked by incubating the slide with the sample portion at room temperature for 30 min with 5% fat-free milk powder dissolved in 1× PBS and washed once with 1×PBS. The primary antibody was diluted with blocking solution (5%, 1×PBS) at a ratio of 1: 5, and the solution was incubated for 90 min at room temperature. Then samples were washed three times with 1×PBS before secondary antibody incubation. The secondary antibody Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch, Pennsylvania, USA) was diluted with blocking solution (5%, 1×PBS) at a ratio of 1: 100 and incubated for 20 min at room temperature.
60 min at room temperature. Then 1\times PBS solution was used to wash for three times. 0.25 mg/mL Calcofluor White (Sigma-Aldrich, St. Louis, MO) dissolved in 1\times PBS was added for 5 min to fix the cell wall. This sample was washed with 1\times PBS twice and anti-fluorescence decay quencher Citifluor (Agar Scientific, Stansted, UK) was added before placing the coverslip. The images were observed with a NIKON ECLIPSE Ci-L upright microscope and an objective lens CFI 10\times/22. The immunolabels of different treatments were done at least three times, and the most representative batch of processed images was selected for display. The relative signals of images were calculated through software ImageJ.

**Virus-inducing gene silencing**

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

Agrobacterium GV3101 containing pTRV1 or pTRV2-PMEU1 were harvested and resuspended in the Agrobacterium infiltration buffer (10 mM MgCl2, 10 mM MES, pH 5.6, 150 mM acetosyringone) to a final OD 600 of 1.0. After shaking for 4-6 h at 28°C, the mixture of Agrobacterium GV3101 cultures containing pTRV1 and pTRV2-PMEU1 in a 1:1 ratio was syringe-infiltrated into the carpopodium of fruit at MG stage. Tomato fruit infiltrated with empty TRV alone was used as the control.

**Carotenoid and ascorbic acid analysis**

For carotenoid analysis (Liu et al., 2018), 0.4 g fruit powder was added with 30 ml extracting solution (hexane:acetone:ethanol=1:1:1 by volume) and mixed at 150 r min\(^{-1}\) for 30 min. After adding with 15 ml double distilled water, the mixture was centrifuged at 1500 g for 10 min. The supernatant was concentrated by nitrogen and dissolved with 1.5 ml dissolution (tetrahydrofuran: acetonitrile: methanol=15:30:55 by volume) as a sample for analysis in HPLC (Shimadzu, Kyoto, Japan). 20 \(\mu\)l sample was injected into a C18 column (5 \(\mu\)m particle size, 4.6mm\(\times\)250 mm, Elite analytical instruments Co., Ltd., Dalian, China). Mobile phase (methanol:acetonitrile=9:1, with 0.05% triethylamine) was set at a flow rate of 1.2 ml min\(^{-1}\). The absorbance of 475 nm was detected via an SPD-M20A diode array detector. Authentic carotenoids (lycopene, \(\beta\)-carotene, and lutein; Sigma, St Louis, MO, USA) were chosen to calculate the amounts of carotenoids.

For ascorbic acid analysis (Liu et al., 2018), 0.4 g powder was mixed with 2.5 ml of 1% oxalate and centrifuged at 7000rpm for 10 min at 4°C. 20 \(\mu\)l sample from filtered supernatant was injected onto the same specification C18 column as carotenoid analysis described. 0.1% oxalic acid solution was chosen as the mobile phase with a flow rate of 1 ml min\(^{-1}\). The absorbance of 243 nm was detected via an SPD-M20A diode array detector. Authentic ascorbic acid was chosen as a standard chosen to calculate the amount of ascorbic acid.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses were performed using SPSS 19. Details of the statistical tests applied, including the statistical methods, number of replicates, mean and error bar details and significances, are indicated in the relevant figure legends. All replicates are biological, unless otherwise noted in the figure legend.