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ORIGINAL PAPER

Sigma factor FaSigE positively regulates strawberry fruit ripening by ABA

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Abstract Bacterial sigma factor E (SigE) is a positive regulator of sugar catabolism via its interaction with the H subunit of Mg-chelatase (CHLH), which serves as both putative abscisic acid (ABA) receptor (ABAR) in *Arabidopsis thaliana* and positive regulator of strawberry fruit ripening. However, whether SigE influences strawberry fruit ripening has not been determined. We used RNA sequencing and a qPCR to confirm that the expression of the strawberry *SigE* gene (*FaSigE*) is rapidly upregulated in fruits turning red, suggesting FaSigE might mediate strawberry fruit ripening. Silencing *FaSigE* by intron-spliced hairpin RNA-mediated RNA interference significantly inhibited fruit ripening. This observation was confirmed by analyses of fruit firmness, soluble sugar, ABA, and anthocyanin contents, as well as transcript levels of genes related to fruit ripening and ABA signaling. Interestingly, a firefly luciferase complementation assay revealed that FaSigE can interact with FaABAR, while an in vitro fruit disc incubation test indicated ABA induces *FaSigE* expression. Moreover, a surface plasmon resonance assay proved that FaABAR produced in yeast cells can bind to ABA, with a binding dissociation constant of 50 μM. In conclusion, FaSigE can interact with FaABAR and positively regulates strawberry fruit ripening via ABA.

Keywords Strawberry fruit ripening · ABA receptor · CHLH/ABAR · Sigma factor E · Intron-spliced hairpin RNA

Abbreviations

SigE	Sigma factor E
ABA	Abscisic acid
CHLH	The H subunit of Mg-chelatase
ABAR	Putative ABA receptor
RNA-seq	RNA sequence
LUC	Firefly luciferase complementation assay
SPR	Surface plasmon resonance
ihpRNA	Intron splicing hpRNA

Introduction

Over the past few decades, the hormone-regulated molecular mechanisms underlying the ripening of fleshy fruits have been studied extensively, with investigations confirming that ethylene has a key effect on climacteric fruits and abscisic acid (ABA) is important for non-climacteric fruits (Kumar et al. 2014; Shen and Joss 2014). Additionally, FaCHLH/ABAR (H subunit of Mg-chelatase/putative ABA receptor) positively regulates the ripening of strawberry fruits in response to ABA (Jia et al. 2011), while the interaction between CHLH and SigE (sigma factor E) is critical for the catabolism of sugar in *Synechocystis* species (Osanai et al. 2009). However, to the best of our knowledge, whether SigE is involved in the ripening of strawberry fruits has not been determined.

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RNA polymerases play a central role in gene expression and regulation, and are essential for living organisms to adapt to various environmental and physiological conditions. Bacterial RNA polymerase contains four core subunits (RpoA, RpoB, RpoC1, and RpoC2) and nine sigma factors (SigA of group 1, SigB to SigE of group 2, and SigF to SigI of group 3) that bind to the RNA polymerase to initiate transcription in response to an environmental stimulus (Kaneko et al. 1996; Haugen et al. 2008; Imamura et al. 2003; Lemeille et al. 2005). Previous studies revealed that SigE, which is an extra cytoplasmic function sigma factor, is crucial for bacterial stress and inflammatory responses (Missiakas and Raina 1998; Helmann 2002; Staron et al. 2009; Manganelli and Provvedi 2010; Pátek and Nešvera 2011; Buasri and Panbangred 2012; Antal et al. 2016; Gupta et al. 2016). A poorly-conserved TTHB212 protein, which exhibits anti-SigE properties, inhibits transcription by preventing SigE from binding to RNA polymerase (Sakamoto et al. 2008). Interestingly, SigE regulates gene expression specifically during the post-exponential growth phase of *Synechococcus* species (Gruber and Bryant 1998). Thus, as a cell surface stress-related transcription factor, SigE is important for bacterial growth and stress resistance, with diverse effects on cell sporulation (Shuler et al. 1995; Wong et al. 1995; Al-Hinai et al. 2014; Casonato et al. 2014; Kirk et al. 2014), granuloma formation (Giacomini et al. 2006), phagocytosis by macrophages (Ando et al. 2003), cell wall integrity (Paget et al. 1999), fatty acid metabolism (Manganelli et al. 2001), carotenoid biosynthesis (Hakkila et al. 2013), nitrogen starvation and sugar catabolism (Osanai et al. 2005a), and responses to various stresses, including acid (Bansal et al. 2017), oxidative (Bagchi and Ghosh 2006; Pacheco et al. 2012), heat (Pettersson et al. 2015), and salt (Koskinen et al. 2016) stresses.

The PII signaling proteins help coordinate carbon and nitrogen metabolism in bacteria, archaea, and plant chloroplasts. In *Synechocystis* spp. PCC 6803, PamA is a PII-binding transmembrane protein that positively regulates the expression of genes involved in sugar catabolism via SigE (Osanai et al. 2005b, 2006, 2009, 2011; Summerfield et al. 2007; Azuma et al. 2011). Interestingly, the chloroplast H subunit of Mg-chelatase can interact with SigE to inhibit the transcriptional activity of SigE (Osanai et al. 2009). In fact, the complex regulation of SigE activity occurs at the transcriptional, translational, and post-translational levels. Specifically, the transcriptional regulation is mediated by several two-component systems including MprAB (He et al. 2006; Donà et al. 2008), CseB/CseC (Paget et al. 1999; Hong et al. 2002), and PhoP/PhoR (Prágai et al. 2004). Meanwhile, the post-translational regulation is mediated by RseA (Hutchings et al. 2006; Manganelli and Provvedi 2010) and CseE (Park et al. 2008).

Although the roles of SigE in bacteria have been relatively well studied, only a few functions of SigE in higher plants have been reported (Fujiwara et al. 2000). The down-regulated expression of *FaCHLH/ABAR* increases the *SigE* transcript level in unripened strawberry fruits (Jia et al. 2011), while an interaction between CHLH and SigE inhibits the transcriptional activity of SigE related to sugar catabolism in *Synechocystis* species (Osanai et al. 2009). These findings compelled us to investigate the role of SigE during strawberry fruit ripening. We cloned a strawberry *SigE* homolog, *FaSigE*, and determined its effects on fruit ripening using intron-spliced hairpin RNA (ihpRNA; Hoffmann et al. 2006). Additionally, we clarified the relationships among FaSigE, FaABAR, and ABA by analyzing the interaction between FaABAR and FaSigE in a firefly luciferase complementation (LUC) assay, determining the effects of ABA on *FaSigE* transcript abundance during an in vitro fruit disc incubation test, and examining the binding of ABA by FaABAR based on surface plasmon resonance (SPR). Our results revealed that FaSigE interacts with FaABAR, and positively regulates strawberry fruit ripening via ABA.

Results

Cloning, bioinformatics, expression and function analysis of *FaSigE* in strawberry fruits

A BLAST search conducted using the *Arabidopsis thaliana SigE* (GenBank: AB021120.1) sequence from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as a query identified a highly homologous *Fragaria vesca* subsp. *vesca* gene (GenBank: XM_004307961) with a 1,981-bp full-length cDNA comprising a 1563-bp coding sequence (172–1,734 bp). Gene-specific primers (forward: 5′-ATGGGAGTTGTGACAGTTTC-3′; reverse: 5′-CAACTA TGTATCTGCGAA G-3′) were designed for a subsequent amplification of the strawberry *SigE* coding sequence in a reverse transcription polymerase chain reaction (RT-PCR) using a cDNA library prepared from strawberry fruits. We then isolated the 1,563-bp amplified product (i.e., *FaSigE*; Fig. 1a), which included an open reading frame encoding a protein with 520 amino acids. The deduced protein contained a conserved domain with the sigma-70 factor, RpoD, which was detected during a homology analysis involving a BLAST search of the NCBI database (Fig. 1b). Thus, the putative strawberry *FaSigE* gene was cloned successfully.

To analyze the *FaSigE* transcript profile during the onset of strawberry fruit ripening, four mixed cDNA libraries derived from ten uniformly-sized fruits at various stages, including large green (LG), white (Wt), initial red (IR), and partial red (PR) stages, underwent RNA-sequencing (RNA-seq) using the Illumina HiSeq 2000

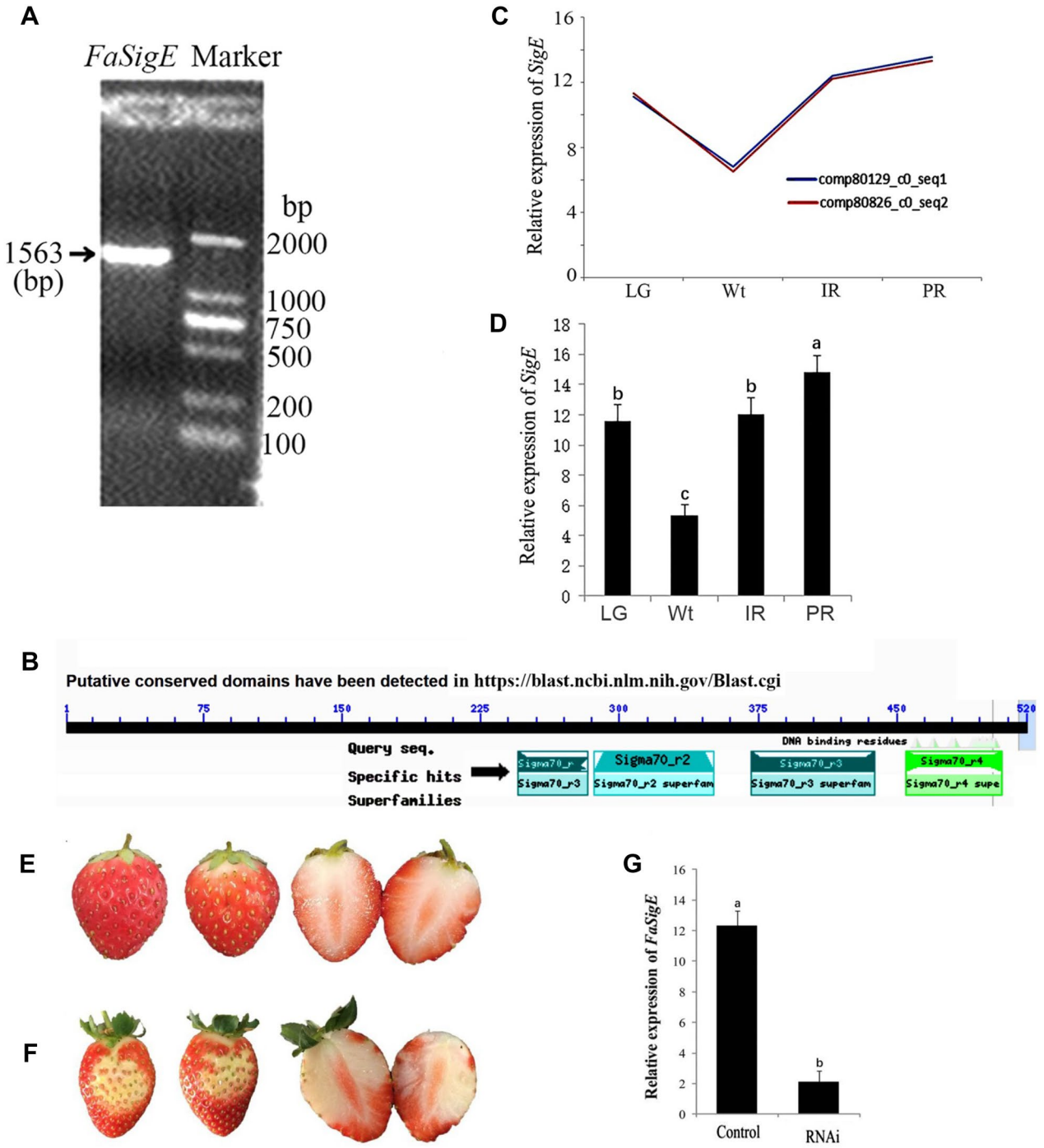


Fig. 1 Cloning, bioinformatics, expression and function analysis of *FaSigE* in strawberry fruits. **a** Agarose gel electrophoresis analysis of a 1563-bp *FaSigE* fragment from strawberry fruits generated by a reverse transcription polymerase chain reaction (PCR). *M* marker, 2000–100 bp. **b** Putative conserved domains (RNA polymerase sigma factor, RpoD-like family, cyanobacteria) were determined based on a 521-amino acid FaSigE sequence in the NCBI database (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The identified protein family was most closely related to the essential sigma-70 factor RpoD in the C-terminal domain model, including the sigma70_r2, sigma70_r3, and sigma70_r4 superfamilies. **c** Two *SigE* gene-like contigs were detected in the RNA sequencing transcriptome data for the fruits collected during the large green (LG), white (Wt), initial red (IR), and partial red (PR) stages. **d** Real-time PCR analysis of *FaSigE* expression in fruits collected during the LG, Wt, IR, and PR. **e** Control fruits infected with empty vector turned red. **f** The fruits in which *FaSigE* was silenced by RNA interference exhibited a chimeric phenotype. **g** Real-time PCR analysis of the *FaSigE* expression levels in transgenic and control fruits. *Actin* expression levels were used as the internal control. *Error bars* represent standard errors ($n=3$). Significant differences determined by variance analysis followed by Duncan's multiple range tests are indicated by *different lowercase letters* ($P<0.05$). (Color figure online)

instrument. Compared with the LG stage, which was associated with 709,101 unigenes, the Wt (537,267 unigenes), IR (534,780 unigenes), and PR (539,813 unigenes) stages had fewer and similar unigenes. The data indicated that the Wt stage represents the transition from green to red fruits, and is a distinct stage during the onset of fruit ripening. We detected two *SigE*-like contigs based on RNA-seq data. The expression levels of contig comp80129_c0_seq1 and its complementary strand, comp80826_c0_seq2, were high in large green fruits, but then decreased and then rapidly increased as fruits turned red (Fig. 1c). Thus, comp80129_c0_seq1 was considered to be *FaSigE*. This result was confirmed by real-time PCR (Fig. 1d). These results suggested that FaSigE might influence strawberry fruit ripening.

To clarify the role of FaSigE in ripening strawberry fruits, *FaSigE* was silenced in fruits using ihpRNA (Intron splicing hpRNA). First, degreening fruits were inoculated with *Agrobacterium tumefaciens* strain GV3101 cells transformed with the pH7FWG vector carrying the *FaSigE*-ihpRNA construct. Fruits treated with bacterial cells containing the empty vectors were used as control. Two-weeks later, the control fruits were completely red (Fig. 1e), unlike the inoculated region of RNAi-treated fruits (Fig. 1f). A qPCR analysis indicated that *FaSigE* expression levels were significantly lower in RNAi-treated fruits (mixed samples) than in control fruits (Fig. 1g). These results implied that FaSigE contributes to the development of red strawberry fruits at the molecular level.

Downregulation of *FaSigE* expression affects a set of ripening-related parameters in RNAi fruits

To further characterize FaSigE functions related to fruit ripening, we analyzed ripening-related physiological parameters, including firmness, soluble sugar concentration, anthocyanin content, and ABA level. Fruits in which *FaSigE* was silenced were firmer than the control fruits (Fig. 2a). Additionally, the anthocyanin (Fig. 2b), soluble sugar (Fig. 2c), and ABA (Fig. 2d) contents were lower in the RNAi-treated fruits. Furthermore, an investigation of the transcript abundance of genes related to ripening and ABA signaling, including *FaCHS, FaUFGT, FaPG1, FaPL1, FaSUT1, FaAI, FaPYR1*, and *FaABI4* (Jia et al. 2011, 2013a), revealed that the expression levels of all genes were downregulated (Fig. 2e). Therefore, the differences between the control and RNAi-treated fruits regarding the expression of genes related to fruit firmness (*FaPG1*and *FaPL1*), sugar content (*FaSUT1*and *FaAI*), anthocyanin content (*FaCHS* and *FaUFGT*), and ABA signaling (*FaPYR1* and *FaABI4*) implied that FaSigE is a positive regulator of strawberry fruit ripening.

FaSigE interacts with FaABAR that can bind ABA

Previous studies confirmed that FaCHLH/ABAR positively regulates strawberry fruit ripening (Jia et al. 2011) and *Synechocystis* SigE can interact with CHLH (Osanai et al. 2009), suggesting that the regulation of strawberry fruit ripening by FaSigE might be influenced by ABA. To verify this possibility, we first analyzed the interaction between FaSigE and FaABAR in a firefly LUC assay. Because the full-length ABAR/CHLH sequence (i.e., 1,381 amino acids) was too long to be an appropriate bait, we cloned a C-terminal *ABAR* fragment encoding 690 amino acids (ABARc690) that are sufficient for interacting with ABA and transmitting the ABA signal (Wu et al. 2009). The C-terminal *ABAR* fragment and *SigE* were inserted into the pCAMBIA1300-nLUC and pCAMBIA1300-cLUC vectors, which were used to transform *Nicotiana benthamiana* cells. An imaging test indicated that ABARc690 could interact with SigE, unlike the control pairings with ABARc690-nLUC and cLUC or nLUC and SigE-cLUC (Fig. 3a). Second, we analyzed the effects of ABA on *FaSigE* expression levels using an in vitro fruit disc incubation test. The qPCR results indicated that the *FaSigE* transcript abundance was significantly higher in the fruit discs exposed to 100 μM ABA for 2 h than in the mannitol-treated control discs (Fig. 3b). These results implied that ABA induces *FaSigE* expression. Third, another qPCR assay confirmed that the *FaABAR* transcript level was considerably lower in *FaSigE*-silenced fruits (mixed samples) than in control fruits (Fig. 3c), suggesting that FaSigE might positively regulate *FaABAR* expression.

Although the role of FaABAR in ripening strawberry fruits has been determined (Jia et al. 2011), it remains unclear whether FaABAR can bind to ABA. Clarifying this point may help to elucidate the molecular mechanism underlying FaSigE activity. Consequently, we used SPR to assess the interaction between ABA and FaABAR. Because the C-terminal half of *A. thaliana* ABAR interacts with ABA (Wu et al. 2009), a cDNA fragment [i.e., 2104–4143 bp (GenBank No. GQ201451.1)] encoding a 680-amino acid C-terminal region was isolated using a strawberry cDNA library (Fig. 3d). The 2,040-bp *FaABAR* fragment was cloned into pPICZB, which was then linearized and inserted into *Pichia pastoris* X-33 cells. Isolated transformants were treated with 1% methanol to induce the production of a histidine-tagged FaABAR fusion protein. Over the course of a 36-h induction period, production of the 75-kDa recombinant protein initially increased gradually, peaking at 24 h (Fig. 3e). The histidine-tagged FaABAR was purified using the Ni–NTA His Bind Resin and then analyzed in a western blot using an anti-histidine tag antibody (Fig. 3f). The final purified protein solution (0.5 mg/ml) was 93.7% pure. Next, an SPR assay was completed using a Biacore T200 system

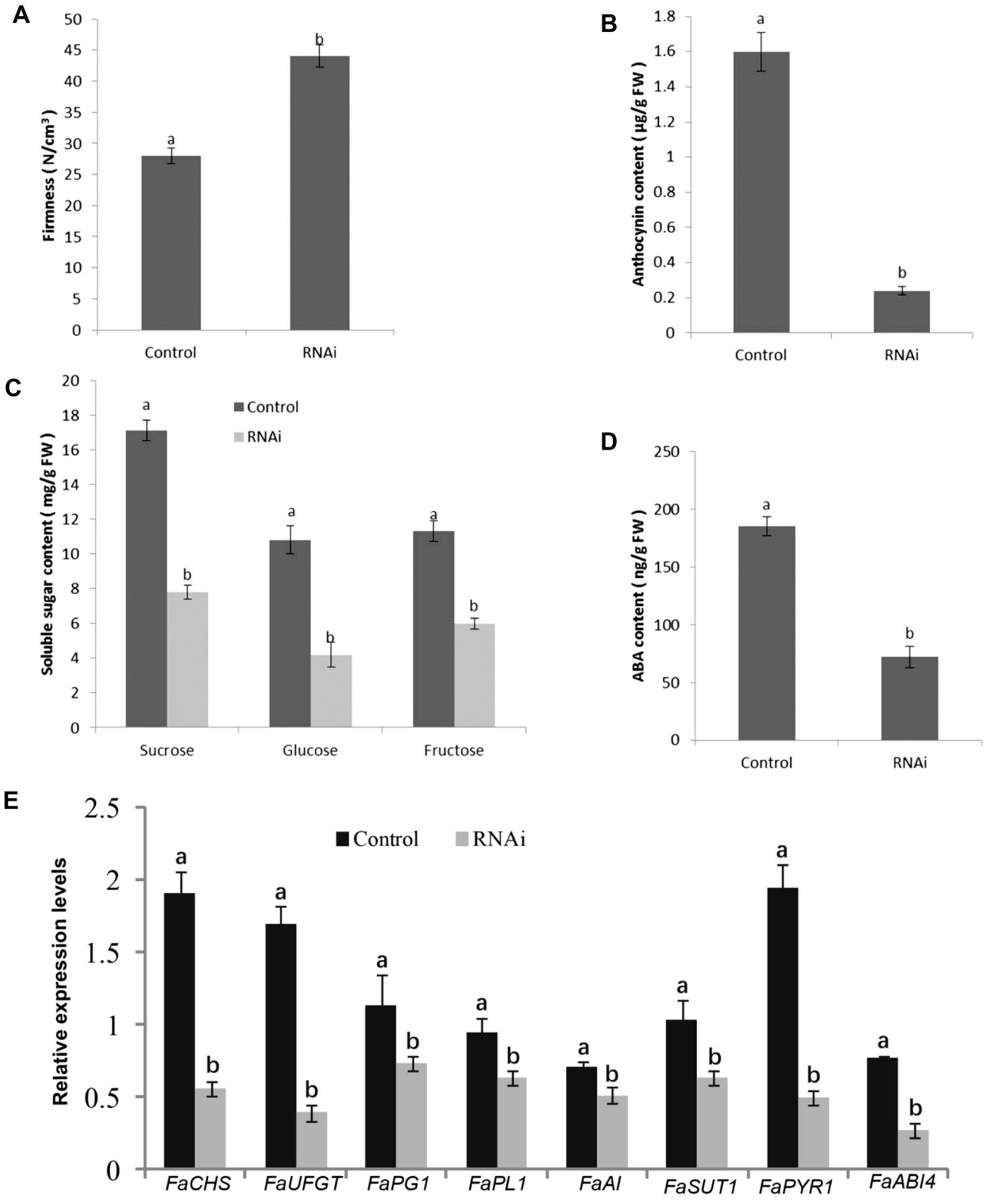
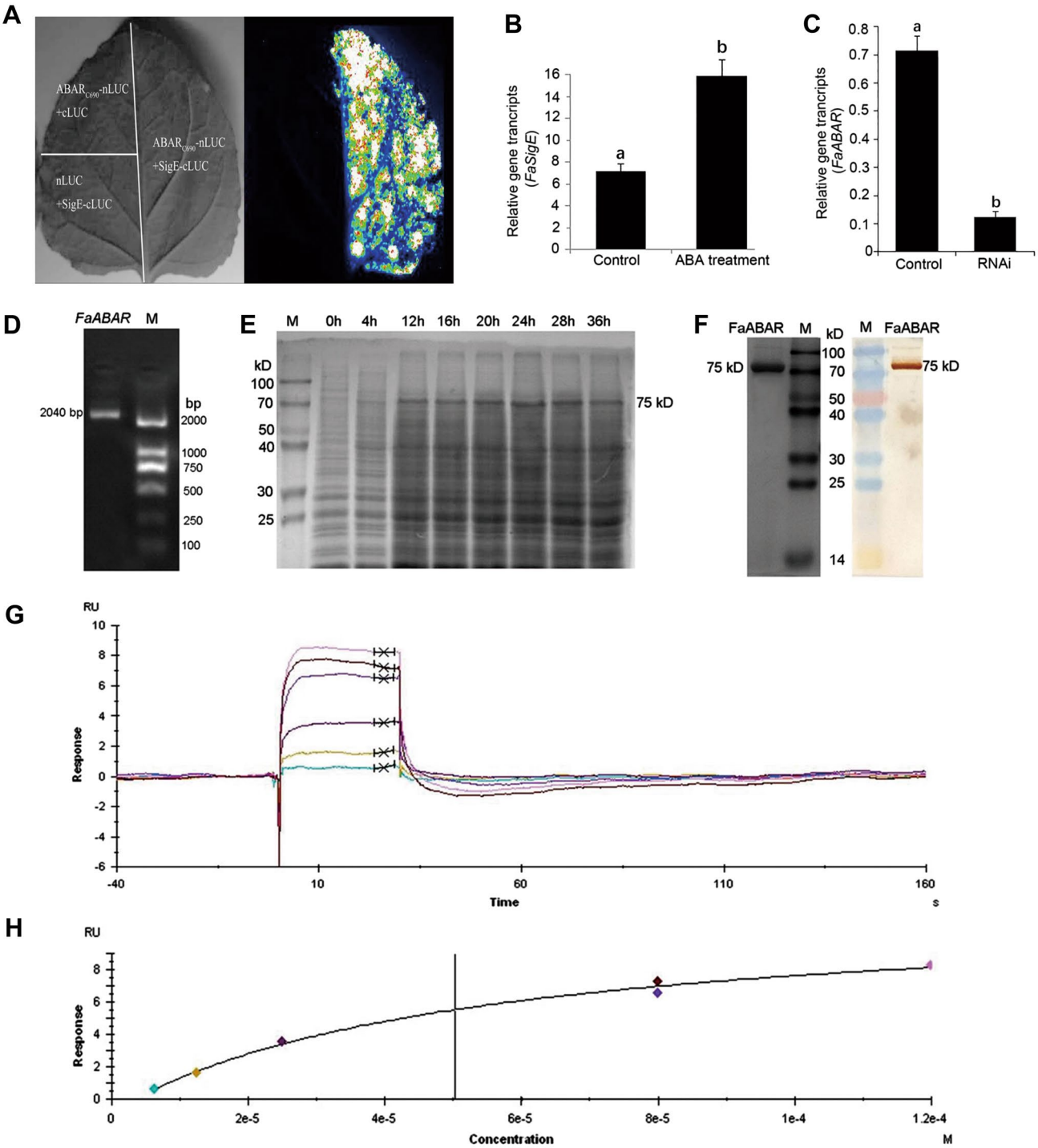


Fig. 2 Changes to several physiological parameters and fruit ripening- and ABA signaling-related gene expression in transgenic and control fruits. **a** Firmness. **b** Anthocyanin contents. **c** Soluble sugar (i.e., sucrose, glucose, and fructose) contents. **d** Abscisic acid (ABA) contents. **e** Relative expression levels of genes related to fruit ripening and ABA signaling. *Error bars* represent standard errors ($n = 3$). Significant differences determined by variance analysis followed by Duncan's multiple range tests are indicated by *different lowercase letters* ($P < 0.05$)



equipped with a certified CM5 sensor chip with carboxyl groups on its surface. We immobilized FaABAR (0.5 mg/ml) to the chip surface at pH 4.0. The analyte with 6–102 μM (+)-ABA in 20 mM phosphate buffer (pH 7.4) was applied at a flow rate of 50 μl/min. Three independent ABA binding measurements confirmed that ABA can bind to strawberry FaABAR, with an average dissociation constant of 50 ± 1.98 μM (Fig. 3g, h).

Discussion

During the past few decades, much has been made toward understanding the action mechanisms of bacterial SigE (Peters et al. 1991; Goto-Seki et al. 1999; Hong et al. 2002; Paget et al. 1999; Prágai et al. 2004; Osanai et al. 2006, 2009; Donà et al. 2008; Sakamoto et al. 2008; Manganelli and Provvedi 2010). Although FaCHLH/ABAR is a positive

◂**Fig. 3** FaABAR interacts with FaSigE and can bind ABA. **a** Interaction between FaABAR and FaSigE based on a firefly luciferase complementation (LUC) assay. The ABARc690-nLuc and cLuc-SigE constructs were used to assess the interaction between FaABAR and FaSigE in a firefly LUC assay. The fluorescence signals at −110 °C, which were recorded with a 1300B CCD camera (Roper), confirmed that ABARc690 can interact with SigE, while interactions were not detected between ABARc690-nLUC and cLUC or nLUC and SigE-cLUC. **b** Real-time PCR analysis of *FaABAR* expression in *FaSigE*-silenced and control fruits. **c** Effects of abscisic acid on *FaSigE* transcript abundance in an in vitro fruit disc incubation test. *Actin* transcript abundance was used as the internal control. **d** Agarose gel electrophoresis analysis of the 2073-bp *FaABAR*/*CHLH* fragment from strawberry fruits generated by a reverse transcription polymerase chain reaction. *M* marker, 2000–100 bp. **e** Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the recombinant protein (i.e., FaABARc690) produced in yeast cells. *M* marker, 100–25 kDa; 0–36 h, duration of the induction of FaABARc690 production with 1% methanol. **f** Purification of the recombinant protein using Ni–NTA resin and identification of FaABARc690 in a western blot. *M* marker, 100–25 kDa. **g** and **h** Surface plasmon resonance analysis of the binding of FaABARc690 to abscisic acid (ABA). The sample protein was immobilized at the surface of a sensor chip by an amino-coupling process. The binding of (+)-ABA to the immobilized proteins was analyzed based on response data. **g** Data of a representative response record. **h** Corresponding saturation curve of ABA binding to FaABARc690. Error bars represent standard errors ($n=3$). Significant differences determined by variance analysis followed by Duncan's multiple range tests are indicated by different lowercase letters ($P<0.05$)

regulator of strawberry fruit ripening (Jia et al. 2011), whether SigE is involved has not been determined.

In the present study, we prove that FaSigE regulates strawberry fruit ripening via ABA. We revealed that the change in fruit color from green to red coincides with a rapid increase in *FaSigE* transcript abundance, with the highest levels in ripened fruits (Fig. 1). We also observed that the downregulated *FaSigE* expression in de-greening fruits inhibits ripening (Figs. 1, 2). Additionally, FaSigE interacts with FaABAR, which can bind ABA (Fig. 3), and *FaSigE* expression is induced by ABA (Fig. 3). Furthermore, ABA contents and *FaABAR* expression levels are downregulated in fruits in which *FaSigE* is silenced by RNAi (Figs. 2, 3).

It is notable that CHLH negatively regulates *SigE* transcription in *Synechocystis* sp. PCC 6803 (Osanai et al. 2009). Moreover, downregulated *CHLH* expression in unripened strawberry fruits promotes *SigE* transcription, but inhibits the accumulation of soluble sugars (Jia et al. 2011). Considering that *Synechocystis* species SigE positively regulates sugar catabolism (Azuma et al. 2011), it is possible that the inhibited accumulation of soluble sugars is because the increased transcription of *SigE* in unripened fruits results in excessive metabolism of sugars (Jia et al. 2011).

In the present study, the *FaSigE* transcript abundance increased rapidly during the strawberry fruit ripening process (Fig. 1), suggesting that FaSigE might be a positive regulator of ripening. A decrease in *FaSigE* transcripts may inhibit ripening and sugar accumulation in transgenic fruits (Figs. 1, 2), implying FaSigE positively influences sugar catabolism. Additionally, the inhibition of *SigE* expression downregulates *FaABAR* transcription and ABA levels, as a result, inhibits the fruit ripening (Figs. 2, 3). Our data suggest that SigE, FaABAR, sugars, and ABA are associated with a complex regulatory signaling network affecting strawberry fruit ripening. Given important roles of ABA and sugar in ripening (Han et al. 2015; Wang et al. 2015; Razzaq et al. 2016; Zhao et al. 2017), future studies should investigate SigE-related two-component systems and characterize the molecular mechanism that enables SigE to regulate strawberry fruit ripening via ABA signaling pathway.

Materials and methods

Plant materials

Octaploid strawberry (*Fragaria ananassa* cv. 'Hongyan') plants were cultivated on ridges of loam soil with 0.2 m × 0.3 m row spacing in a glasshouse (20–25 °C, 70–85% relative humidity, and a 14-h light/10-h dark cycle) during spring in 2014–2016. One acre (667 m^2) of soil contained 5000 kg organic fertilizer and 30 kg synthetic fertilizer (i.e., nitrogen, phosphorus, and potassium). Plants were irrigated every 5–7 days.

We tagged 200 small green fruits on 50 strawberry plants after flowering. Forty uniformly-sized fruits were collected at 15 (LG), 23 (Wt), 25 (IR), 27 (PR), and 30 (FR) days after anthesis, quickly frozen in liquid nitrogen, and stored at −80 °C until analyzed.

RNA sequencing and data analysis

Total RNA was extracted from the receptacles of six frozen fruits per analyzed stage (i.e., LG, Wt, IR, and PR) using the RNeasy Plant Mini kit (Qiagen, Dusseldorf, Germany). The extracted RNA was treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA and then used as the template to generate cDNA libraries with the RNA Library Prep kit (New England BioLabs, Ipswich, MA, USA). The prepared libraries were sequenced using the Illumina HiSeq 2000 platform (Illumina, USA). The data analysis was done according to the description (Benjamini and Yekutieli 2001; Mortazavi et al. 2008; Wang et al. 2010, 2017; Langmead and Salzberg 2012; Ming et al. 2012).

RNA isolation and cDNA synthesis

Total RNA was extracted from 0.5 g strawberry receptacles from three frozen fruits of each stage using the Easyspin Rapid Plant RNA Extraction kit (Biomed, Beijing, China).

The purity and integrity of the extracted RNA were analyzed by agarose gel electrophoresis and absorbance ratios (i.e., 260:230 nm and 260:280 nm), after which 3 μg RNA was reverse transcribed using the TransScript First-strand cDNA Synthesis SuperMix kit (Biomed).

Cloning of the *FaSigE* coding sequence

The cDNA obtained as described in the previous section was used as the template for amplifying *FaSigE* with gene-specific primers (forward, 5′-ATGGGAGTTGTGACAGTTTC-3′; reverse, 5′-TCAACTATGTATCTGCGAAG-3′) designed based on a sequence in a strawberry gene library database (https://strawberry.plantandfood.co.nz/index.html). The PCR program used to amplify the *FaSigE* sequence was as follows: 94 °C for 5 min; 34 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s; 72 °C for 10 min. The PCR products were ligated into a pEASY-T1 simple vector for a subsequent transformation of *Escherichia coli* DH5α cells.

Construction of the *FaSigE* RNA interference vector

For the ihpRNA-mediated silencing of *FaSigE*, a 461-bp *FaSigE* cDNA fragment was amplified using the following primers: forward, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCCTCCTGTTCTTAGTTCCTCTG-3′ (attB1, underlined); reverse, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGATTTCTGCCCTGACC-3′ (attB2, underlined). The 461-bp amplified fragment was cloned into the pDONR™ 221 vector (Invitrogen) using the Gateway BP Clonase II enzyme mix, and into the pK7GWIWG2(II)-RR vector using the Gateway LR Clonase II enzyme mix. The *FaSigE*-RNAi recombinant plasmid was inserted into *A. tumefaciens* strain GV3101 cells, which were grown at 28 °C in Luria–Bertani liquid medium containing 10 mM MES, 20 μM acetosyringone. When the culture reached an optical density at 600 nm (OD_{600}) of 0.8, the cells were harvested and resuspended in infection buffer [10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 200 μM acetosyringone]. The cell suspension was mixed for 2 h at room temperature before 200-μl aliquots were injected into whole fruits using a sterile 1 ml hypodermic syringe.

Determination of anthocyanin and soluble sugar contents

The anthocyanin content of three fruits of each stage was measured by reversed-phase high-performance liquid chromatography (HPLC) using a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent) with the following linear gradient: Solution A (acetonitrile) 0 to 20% for 13 min, 20–40% for 20 min, and 0% for 25 min and Solution B (10% formic acid) at a flow rate of 1 ml/min. The detection wavelength was 520 nm, the column temperature was 25 °C, and the injection volume was 20 μl. Pelargonidin-3-*O*-glucoside was used as the standard. The HPLC-analysis of soluble sugar content was done according to the description (Jia et al. 2011). The analysis was repeated three times.

Quantitative real-time polymerase chain reaction

The qRT-PCR analyses were completed in a CFX Sequence Detector (Bio-Rad, Hercules, CA, USA) using 20-μl samples consisting of 10 μl SYBR Premix Ex Taq (Takara), 0.4 μl forward and reverse primers, and 2 μl cDNA template. The PCR program was as follows: 95 °C for 2 min; 40 cycles of 94 °C for 20 s and 54 °C for 30 s. *Actin* was used as a reference gene. Relative gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The following qRT-PCR primers were used in Table 1.

Firefly luciferase complementation assay

To analyze the interaction between ABAR and SigE, we generated the ABARc690-nLuc and cLuc-SigE constructs. The pCAMBIA1300-nLUC and pCAMBIA1300-cLUC plasmids were kindly provided by Dr. Gong Zhi-Zhong of China Agricultural University. All constructs were inserted into *A. tumefaciens* strain GV3101 cells, which were subsequently used to transform *Nicotiana benthamiana* plants as follows. A single clone of each strain was cultured in 5 ml YEB supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin at 28 °C for 24 h. The bacteria were then collected and resuspended with 3 ml injection buffer 10 mM MES (pH 5.7), 10 mM $MgCl_2$, and 250 mM acetosyringone for an OD_{600} of 0.5. The bacterial solutions were kept at room temperature for 2 h before being injected into *N. benthamiana* leaves. Three days later, 1 mM luciferin was sprayed onto the lower epidermis, after which the leaves were maintained in darkness for 5 min. The fluorescence signals were observed and recorded at −110 °C using a 1300B CCD camera (Roper). The assay was completed with three replicates.

Determination of firmness and abscisic acid content

Three uniform strawberry fruits of each stage were used to assess flesh firmness. Two sides of each fruit were analyzed using a GY-4 fruit penetrometer (Digital Force Gauge, Shanghai, China) and flesh firmness was recorded as N/cm^2. The experiment was completed with three replicates.

To extract ABA, 0.5 g receptacles were ground using a mortar and pestle and homogenized in extraction solution [80% (v/v) methanol]. Extracts were centrifuged at 10,000×*g* for 20 min and the supernatants were added to Sep-Pak C18 cartridges (Waters, Milford, MA, USA) to

Table 1 The primers used for real-time PCR

Genes	Sequences for SqRT-PCR
FaPYR1	Sense, 5′-GGAGCTGGCAATGGTCG-3′; antisense, 5′-AGGCCCGCCTTTCCTT-3′
FaABI4	Sense, 5′-TCCTCATCACCACCGTCTT-3′; antisense, 5′-ACTCTGGCTCGTTTGCTCT-3′
FaSUT1	Sense, 5′-TTCAAGCGACAGAAATACCC-3′; antisense, 5′-ACCCAATCCAGTTTAGACCAG-3′
FaSS	Sense, 5′-TTATCCCTCGCATTCTTATT-3′; antisense, 5′-CAATTCCCTTCTCGGTTCTA-3′
FaCHS	Sense, 5′-GAGCAAACAACGAGAACACG-3′; antisense, 5′-GCTGTCAAGGCCATTAAGGA-3′
FaUFGT	Sense, 5′-GGTAAGCCACAGGAGGACA-3′; antisense, 5′-TATGAGCACCGAACCAAAA-3′
FaPG1	Sense, 5′-CGACAGAGTGAAAAATTC-3′; antisense, 5′-AGGACTGGGTTAGCAAAATTA-3′
FaPL1	Sense, 5′-TGACTCCCTTGCTGCTTCTT-3′; antisense, 5′-TCTACTGCG TGCTCATTCCA-3′
Actin	Sense, 5′-TGCATATATCAAGCAACTTTACAC-3′; antisense: 5′-ATAGCTGAGATGGATCTTCC-3′

remove polar compounds. Samples were stored at −20 °C prior to being used in an enzyme-linked immunosorbent assay. The experiment was completed with three replicates.

In vitro fruit disc incubation test

White strawberry fruit disc tissues were treated with ABA in an in vitro incubation test as described by Jia et al. (2013b). The discs were divided into two sections, with one section incubated in equilibration buffer with 200 mM mannitol (i.e., control) and the other section incubated in equilibration buffer with 100 μM ABA. The two sections were kept in a 250-ml flask at 25 °C for 2 h with shaking. The sections were washed with double-distilled water and then frozen in liquid nitrogen and kept at −80 °C. The experiment was completed with three replicates.

Cloning of the *FaABAR* abscisic acid-binding region

The cDNA generated as described above was used as the template for amplifying the C-terminal half of *FaABAR* with the following primers: forward, 5′-GAATTCAAAAATGGC TGCCAACAATCCATCTG-3′ (*Eco*RI, underlined); reverse, 5′-GCGGCCGCCCGATCGATTCCCTCAATTTTG-3′ (*Not* I, underlined). The PCR program was as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 130 s; 72 °C for 10 min. The amplification products were ligated into a pEASY-T1 simple vector and inserted into *E. coli* DH5α cells (TransGen Biotech). The plasmid DNA from positive colonies were amplified and sequenced by Invitrogen (Shanghai, China).

Purification of recombinant FaABAR produced in yeast cells

Recombinant FaABAR was expressed and purified using the EasySelect™ *Pichia* Expression Kit and the ProBond™ Purification System (Invitrogen, USA), respectively. A partial *FaABAR* sequence of N terminal was removed from a pEASY-T1 simple vector carrying $FaABAR_{2073}$ by digesting with *Eco*RI and *Not*I. The fragment was then cloned into the expression vector, pPICZB, digested with the same enzymes. The recombinant pPICZB plasmid was inserted into *E. coli* cells and transformants were selected in Petri dishes containing agar-solidified low-salt Luria–Bertani medium supplemented with 25 μg/ml Zeocin™. Twenty transformants were selected for sequencing to confirm the target fragment was inserted correctly (i.e., in frame with the C-terminal tag). The purified and linearized recombinant plasmids were inserted into *P. pastoris* cells and Zeocin™-resistant yeast transformants were selected in Petri dishes consisting of agar-solidified YPDS medium supplemented with an appropriate Zeocin™ concentration. The FaABAR fusion protein was expressed and purified using the Ni–NTA His Bind Resin and analyzed in a western blot involving an anti-histidine tag antibody according to manufacturer-recommended protocols (Novagen, USA). The eluted fusion protein was stored at −80 °C until analyzed further.

Surface plasmon resonance assay

The SPR assay was completed using a Biacore T200 system (GE Healthcare) equipped with a certified CM5 sensor chip with carboxyl groups on its surface. Sample proteins (90% pure based on size exclusion chromatography) were covalently immobilized to saturate the sensor chip surface using the Amine Coupling kit (Biacore). The surface of flow cell 2 was activated for 7 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at a flow rate of 10 μl/min. The FaABAR (0.5 mg/ml in 10 mM sodium acetate, pH 4.0) was immobilized on the sensor chip surface at a density that saturated the surface. Meanwhile, flow cell 1 was left blank to serve as a control. The chip surface was then blocked with a 7 min injection of 1 M ethanolamine, pH 8.0. To collect kinetic and affinity binding data at 25 °C, the analyte consisting of (+)-ABA in 20 mM phosphate buffer (150 mM NaCl and 30 mM KCl, pH 7.4) was injected over

flow cells 1 and 2 at 5–120 μM and a flow rate of 50 μl/min. The complex was allowed to associate and dissociate for 60 s, and data were collected and globally fitted to a steady-state model in the Biacore S200 Evaluation Software v1.0. The experiment was completed with three replicates.

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