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FaABI4 is involved in strawberry fruit ripening

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ABSTRACT

Abscisic acid (ABA) is regarded as an important regulator in non-climacteric fruit ripening, especially in strawberry fruit. FaABI4, as a critical downstream component of ABA signaling in plants, whether it is involved in strawberry ripening remains unclear. The octoploid strawberry (*Fragaria ananassa*, 'Beinongxiang') fruits were used here to explore this question. Our results showed that *FaABI4* expressed higher in leaves, followed in flowers and fruits, but less in roots and stems, and that coupled with the fruit ripening, its transcripts increased gradually, suggesting that FaABI4 might play a role in the ripening. Using tobacco rattle virus-induced gene silencing (VIGS), downregulation of *FaABI4* transcripts significantly delayed fruit ripening in consistent with changes of firmness, sugar and ABA contents, as well as transcripts of several ripening-related genes, including *CA4H*, *CHI*, *DFR*, *CHS*, *GAL6*, *PE5*, and *XYL2*. Interestingly, ABA, sucrose and glucose all induced *FaABI4* expression through fruit-disc incubation in vitro. In conclusion, our results demonstrate that FaABI4 plays an important role in the regulation of strawberry fruit ripening, and its expression is induced by ABA, sucrose and glucose.

1. Introduction

In recent years, transcript factor ABI4 (ABA-insensitive 4), as one of classical ABA signaling components, is demonstrated to take part in plant diverse processes, including floral transition (Shu et al., 2016), phytohormone crosstalk (Dong et al., 2016; Shu et al., 2015), seed germination (Penfield et al., 2006; Lee et al., 2015), water use efficiency (Easlon et al., 2014), plastid-to-nucleus signaling (Koussevitzky et al., 2007; Giraud et al., 2009; León et al., 2013), sugar responses (Rook et al., 2006; Bossi et al., 2009), lipid mobilization (Penfield et al., 2006), and male sterility (Shu et al., 2014). Although ABA perception and signaling is well documented in regulation of fleshy fruit ripening (Shen and Rose, 2015), whether ABI4 plays a role in strawberry fruit ripening remains unclear.

The *ABI4* was early discovered as an ABA insensitive mutant in Arabidopsis seed development and germination (Finkelstein et al., 1998, 2002), and it promotes seed maturation by inducing LEA, oleosin and dehydrin accumulation (Reeves et al., 2011). Interestingly, *ABI4* alleles were also isolated through screening high sugar insensitive mutants in Arabidopsis germination and seedling, finding that sugar and ABA coordinatedly regulate several photosynthetic and starch biosynthetic genes (Arenas-Huertero et al., 2000; Rook et al., 2001, 2006; Bossi et al., 2009). Indeed, ABI4, as a versatile activator and repressor, alteration of its expression affects a variety of target gene transcripts, including *ABI5*, *SBE2.2*, *AOX1a*, *LHCB*, *RBCS*, *ADH1*, *APL3* (Wind et al., 2013). Notably, recent reports indicated that ABI4, as an evolutionarily conserved protein characteristic to an APETALA 2 (AP2) domain, not only serves as a central and integrative regulator in simultaneous modulation of ABA response, sugar signaling, and CO_2 assimilation (Easlon et al., 2014; Gregorio et al., 2014), but also plays a central role in GA/ABA homeostasis, ethylene production, and floral transition in Arabidopsis (Dong et al., 2016; Shu et al., 2015, 2016).

It is interesting to note that in recent years, much progress has been made toward understanding the role of ABA in regulation of strawberry fruit ripening (Jia et al., 2011; Chai et al., 2011; Li et al., 2013; Kadomura-Ishikawa et al., 2015). Downregulation of the transcripts of both ABA biosynthesis (*FaNCED1* or *FaBG3*) or perception (*FaABAR* or *FaPYR1*) genes, all inhibit strawberry fruit ripening, demonstrating that ABA plays an important role in regulation of the fruit ripening, and FaABAR or FaPYR1 is positive regulator for ripening. It is also demonstrated that FaMYB10 is an important component for FaABAR-mediated regulation of anthocyanin biosynthesis during strawberry fruit ripening (Kadomura-Ishikawa et al., 2015). More interestingly, a core ABA signaling pathway 'ABA-PYR/PYL/RCAR-PP2C-SnRK2' in Arabidopsis have been identified by genetics and structural biology, namely, ABA can promote the interaction of PYR1 and PP2C, resulting in PP2C inhibition and SnRK2

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activation, finally phosphorylating the downstream factors such as AREB/ABF, ion channels, and NADPH oxidases (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009), and this core signaling might represent a common mechanism by which ABA regulates strawberry fruit ripening (Jia et al., 2013a). Downregulation of *FaPYR1* expression inhibits the transcript of *ABI4* (Jia et al., 2011), suggesting that ABI4 might play a role in the regulation of strawberry fruit ripening, however, the substantial evidence is lacking.

To investigate a role of ABI4 in fruit development, experiment with strawberry, a model plant for study of nonclimatic fruit ripening, an ABA-insensitive 4 gene, named *FaABI4*, was cloned and silenced by VIGS, as well as its transcriptional regulation by ABA and sugars was also analyzed, finally demonstrating that *FaABI4* is an ABA/sucrose/glucose-induced gene, which plays an important role in the regulation of strawberry fruit ripening.

2. Material and methods

2.1. Plant materials

Strawberry (*Fragaria ananassa* 'Beinongxiang') fruits were planted in a greenhouse environment (20–25 °C, 70–85% relative humidity, and a 14/10 h light/dark regime) in springtime during the period of 2014–2015. During strawberry development, fruits were collected on the following days (d) after anthesis: 8 d (small green), 15 d (big green), 19 d (de-greening), 22 d (white), 25 d (initial red), 28 d (partial red), and 30 d (full red). Three fruits with uniform size were selected at each stage (three replications), then quickly frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from 0.5 g of strawberry fruit using an RNA extraction kit (Easypin rapid plant RNA extraction kit; Biomed, Beijing, China). The purity and integrity of RNA were analyzed both by agarose gel electrophoresis and by the A260: A230 and A260: A280 ratios. To generate first-strand cDNA, 3 μg of total RNA was reverse-transcribed using a first-strand cDNA synthesis super kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol.

2.3. Cloning of FaABI4 coding sequence

The cDNA above was used as a template for amplifying *FaABI4* with specific primers (forward, 5′-ATGGACGAAGACGACA-3′; reverse, 5′-TCAATCAAATCCTTTAAAATCC-3′) designed from a strawberry gene library. PCR was performed under the following conditions: 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with the final reaction being terminated at 72 °C for an additional 10 min. The PCR product was ligated into a pEASY-T1 simple cloning vector and subsequently transformed into Trans1-T1 competent cells. Positive colonies were selected by kanamycin, and sequenced by Huada China (Beijing).

2.4. Construction of virus vectors and agroinfiltration

The pTRV1 and pTRV2 vectors (Liu et al., 2002) were kindly donated by Dr Liu Yu-le, Qinghua University. A 492 bp cDNA fragment of *FaABI4* was amplified using primers forward, 5′-GGAATTCGCCTCTTCGGCTCATCCT TA-3′ (the underlined, *Kpn*I); and reverse, 5′-GGGGTACCCAAAAAGAG AGGATCACCGT-3′ (the underlined, *Eco*RI). The amplified fragment (from 478 bp to 969 bp) was cloned into the pEASY-T1 cloning vector, digested by *Kpn*I and *Eco*RI, and subsequently cloned into the virus vector *Kpn*I-*Eco*RI-cut pTRV2. The *agrobacterium* strain GV3101 containing pTRV1, and pTRV2-*FaABI4* was used for RNAi. *Agrobacterium*-mediated TRV infiltration by syringe injection with a needle into strawberry fruits was performed as described by Fu et al. (2005)

Table 1
The primers used for SqRT-PCR.

Genes	Sequences for SqRT-PCR
FaABI4	Sense: 5′- ACAACGAGAGCGGTGCTGAAA −3′ Antisense: 5′- CGTAAGGATGAGCCGAAGAGG −3′
GAL6	Sense: 5′-GACGGTCAGCATAAGATTC-3′ Antisense: 5′-GGCATACCATACTCATCAAG-3′
PE5	Sense: 5′-GCTCAACACAACCGTAAAC-3′ Antisense: 5′-GAACCTCCTCCAACACTTT-3′
XYL2	Sense: 5′-GCCTCTATCTACTGTGCGTC-3′ Antisense: 5′-TGAATCCCAAACTGGTCTAC-3′
CA4H	Sense: 5′-GTGACTTTATTCCCGTGCT-3′ Antisense: 5′-CTGGAGGAGGTAACATCTCA-3′
CHS	Sense: 5′-AGAATCCCAGTATGTGCG-3′ Antisense: 5′-TGAGGAGGTGAAATGTGAG-3′
CHI	Sense: 5′-CCAGCAATACTCCGAGAA-3′ Antisense: 5′-GTCCACTTTCCCATTTCC-3′
DFR	Sense: 5′-CGGAAAGTAAAGATGACTGG-3′ Antisense: 5′-GTTCTCCTCAATGCCCTT-3′
Actin	Sense: 5′-TGCATATATCAAGCAACTTTACACTGA-3′ Antisense: 5′-ATAGCTGAGATGGATCTTCCTGT-3′

2.5. Semiquantitative PCR (SqRT-PCR)

For SqRT-PCR analysis of *FaABI4* gene expression, the first-strand cDNA from the RNAi and control fruits was used as a template for PCR amplification. *Actin* was used as an internal control. The ripening-related genes include *CA4H* (cinnamate 4-hydroxylae), *CHI* (chalcone isomerase), *DFR* (dihydroflavonol 4-reductase), *CHS* (chalcone synthase), *GAL6* (beta-galactosidase 8-like), *PE5* (pectin methyl esterase) and *XYL2* (D-xylulose reductase). *Actin* mRNA (accession number: XM 011470684) was used as an internal control. The primers for SqRT-PCR were designed as showed in Table 1. The experiment was done with three replications.

2.6. Determination of firmness, soluble solid concentration and ABA content

Three uniform strawberry fruits were used for the detection of firmness. Flesh firmness was analyzed on two sides of each fruit using a GY-4 fruit penetrometer (Digital Force Gauge, Shanghai, China). The strength of flesh firmness was recorded as N/cm^2. Soluble solid concentrations (representing sugars) were measured by Digital refractometer (TD-45, China). The experiment was done with three replications.

For ABA extraction, 0.5 g receptacle was ground in a mortar and homogenized in extraction solution (80% methanol, v/v). Extracts were centrifuged at 10,000*g* for 20 min. The supernatant liquid was eluted through an Sep-Pak C18 cartridge (Waters, Milford, MA, USA) to remove polar compounds, and then stored at −20 °C for enzyme-linked immune sorbent assay (ELISA) as described by Zhang et al. (2009). The experiment was done with three replications.

2.7. Incubation of fruit disc in vitro

Treatment on fruit disc tissues of strawberry fruit with sucrose, glucose, and ABA by an in vitro incubation was done as described by Jia et al. (2013b). After washing samples with distilled water, the freshly harvested berries were pre-cooled to 4 °C. Discs of berry mesocarp, 10 mm in diameter and 1 mm in thickness, were prepared with a cork borer. The discs were immediately immersed in the equilibration buffer (Archbold, 1999) for 30 min with 200 mM mannitol. The equilibration buffer consisted of 50 mM MES (pH 5.5), 10 mM $MgCl_2$, 10 mM EDTA, 5 mM $CaCl_2$, and 5 mM VC. The discs were divided into four sections, one section was incubated in equi-