Title: FaABI4 is involved in strawberry fruit ripening

Running title: FaABI4 regulates strawberry fruit ripening

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ABSTRACT

Absciscic 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.

Keywords: strawberry fruit; virus-induced gene silencing (VIGS); *FaABI4*; absciscic acid; sugar.

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*, *ADHI*, *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 CO₂ 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 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, *KpnI*); and reverse, 5'-GGGGTACCCAAAAAGAG AGGATCACCGT-3' (the underlined, *EcoRI*). The amplified fragment (from 478 bp to 969 bp) was cloned into the pEASY-T1 cloning vector, digested by *KpnI* and *EcoRI*, and subsequently cloned into the virus vector *KpnI-EcoRI*-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)

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-hydroxylase), *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/cm². 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 a 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 minutes with 200mM mannitol. The equilibration buffer consisted of 50 mM MES (pH 5.5), 10 mM MgCl₂, 10 mM EDTA, 5mM CaCl₂, and 5 mM VC. The discs were divided into four sections, one section was incubated in equilibration buffer with 200 mM mannitol as control, the others were incubated in equilibration buffer with 4% sucrose, 4% glucose, and 100 µM ABA, respectively. The four sections were placed in 250 ml flask and shaken at 25°C for 2 h. After washing with double distilled water, the tissues at each time points were frozen in liquid nitrogen and kept at -80°C until use. The experiment was done with three replications.

3. RESULTS

3.1. Cloning of FaABI4 gene

To clone the *FaABI4* gene, Arabidopsis *ABI4* (AT2G40220) gene was used to BLAST in a strawberry gene library (<https://strawberry.plantandfood.co.nz/index.html>), and a high homologous loc101302022 was found. Based on the nucleotide sequence, the specific primers (named *FaABI4*: forward, 5'-ATGGACGAAGACGACA-3'; reverse, 5'-TCAATCAAATCCTTTAAATCC-3') were designed to amplify a coding sequence of *FaABI4*. The results showed that a 990-bp sequence was obtained and encodes a deduced 330 amino acids. A phylogenetic analysis showed that *FaABI4* and its orthologs were classified largely into three groups, *Prunus persica*, *Prunus mume*, and *Medicago truncatula* were classified into a cluster (Fig. 1A). A protein sequence alignment and protein domain search in NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis indicated that a conserved AP2 domain characteristic to *ABI4* superfamily was founded (Fig. 1B, C), demonstrating that a putative strawberry ABA-insensitive 4 gene *FaABI4*, was isolated successfully.

3.2. Expression patterns of *FaABI4*

In order to investigate the expression patterns of *FaABI4* in developmental strawberry plants, according to fruit colour and size, the developmental strawberry fruits were divided into seven stages: small green (SG), big green (BG), degreening (DG), white (Wt), initial red (IR), partial red (PR), and full red (FR) at 8, 15, 19, 22, 25, 27, and 30d after anthesis, respectively (Fig. 2A). The mRNA expression levels of *FaABI4* were analyzed by SqRT-PCR using 7-stage fruits, and leaves, stems, roots, flowers and white fruits. The results showed that *FaABI4* expressed higher in leaves, followed in flowers and fruits, but less in roots and stems (Fig. 2B). Coupled with fruit red-coloring, the transcripts of the *FaABI4* gene increased rapidly, reached the highest level in ripened fruit (Fig. 2C), suggesting that *FaABI4* might be involved in the fruit ripening.

3.3. Silencing of *FaABI4* by VIGS in strawberry fruit

To identify the function of *FaABI4*, a VIGS system was used to downregulate *FaABI4* expression in strawberry fruit according to the description by Jia et al. (2011). First, a mixture of *Agrobacterium* strain GV3101 cultures containing pTRV1 and pTRV2 carrying a 492-bp fragment of the *FaABI4* gene (pTRV2 - *FaABI4*) in a 1:1 ratio was infiltrated into 2-week-old fruits (Fig. 3A), and control fruits were infiltrated only with TRV alone. Two weeks after infiltration, the control fruits turned full red (Fig.3B). In contrast, RNAi fruits lost red-coloring in the inoculated surface *FaABI4* (Fig. 3C). SqRT-PCR analysis showed that the *FaABI4* transcripts was downregulated in RNAi fruit compared with the control fruits (Fig. 3D). These results showed that *FaABI4* plays a role in the regulation of strawberry fruit ripening at molecular level.

3.4. Understanding the role of *FaABI4* in the regulation of fruit ripening

To further understand the role of *FaABI4* in the regulation of fruit ripening, we analyzed the ripening-related events and genes, including a set of pigment and softening-associated genes, such as *CA4H*, *CHI*, *DFR*, *CHS*, *GAL6*, *PE5*, and *XYL2*. The results showed that firmness was increased in RNAi fruit (Fig. 4A), in which soluble solid concentrations (representing sugars, Fig.4B) and ABA contents (Fig. 4C), and the mRNA expression levels of *CA4H*, *CHI*, *DFR*, *CHS*, *GAL6*, *PE5*, and *XYL2* were all down-regulated (Fig. 4D). The changes of the ripening-related events and gene transcripts in RNAi fruits are consistent with a role of *FaABI4* in the regulation of strawberry fruit ripening.

To explore the regulation of *FaABI4* expression, effects of ABA, sucrose (Suc), and glucose (Glu) on the mRNA expression levels of *FaABI4* were analyzed using a fruit-disc-incubation test *in vitro*. The SqRT-PCR analysis showed that compared with the control incubated with mannitol after 2h incubation, 100 μ M ABA, 4% sucrose or 4% glucose all significantly promoted the mRNA expression levels of *FaABI4*, respectively (Fig. 4E). The results demonstrated that *FaABI4* expression could be induced by ABA, sucrose and glucose.

4. Discussion

Over the past years, much progress has been made toward understanding the role of ABI4 in plant growth and development, as well as environmental stress by integration of multiple plant cell signals, such as ABA, sugar, osmotic stress, and plastid signals (Rook et al., 2006; Fernández and Strand, 2008; León et al., 2013; Wind et al., 2013). However, whether ABI4 is involved in nonclimatic fruit ripening is yet unknown.

In the present study, we have provided several physiological and molecular evidences to confirm that FaABI4 plays an important role in the regulation of strawberry fruit ripening as follows: (1) the expression levels of *FaABI4* transcription factor increased gradually during strawberry fruit development, and reached the highest expression level in ripened fruit (Fig. 2); (2) downregulation of *FaABI4* gene expression levels led to unripening fruit via phenotypic, physiological, and molecular analysis (Fig. 3), namely, compared with the control fruits, the RNAi fruits lost red color and had less sugar contents, but fruit firmness increased; meantime, the expression levels of both anthocyanin- and softening-associated genes were downregulated; (3) ABA contents were downregulated in *FaABI4*-RNAi fruits.

Notably, it is previously reported that a rapid increase in both ABA and sugar contents is coupled with strawberry fruit ripening (Jia et al., 2011), to a large extent, sucrose, as a signal, promotes strawberry fruit ripening by stimulation of ABA biosynthesis (Jia et al., 2013b, c). In fact, the previous and our results indicate that synergetic accumulation of ABA, sugar, and *ABI4* transcripts occurred coupled with the red-coloring, maybe, certain link might be present among them.

In another nonclimatic fruit, grape berries, Gambetta et al. (2010) found that sugar and ABA signaling orthologs, including AP2 transcription factors (TF), are activated at the onset of grape berry ripening (Gambetta et al., 2010). In the present study, downregulation of the mRNA expression levels of an AP2-type protein gene *FaABI4* inhibited both ABA and soluble sugar contents (Fig. 4), as a result, led to unripening fruits, manifesting that ABI4 is a positive regulator for the ripening. Although ABA, sucrose and glucose all could promote the transcripts of *FaABI4* (Fig. 4), less ABA and sugar contents in *FaABI4*-RNAi fruits might disrupt a synergetic-accumulation relationship of these three components,

which maintain a certain level to be required for strawberry fruit ripening.

Conclusions

From the present study, we provide a set of phenotypic, physiological and molecular evidences clarified that FaABI4 is an APETALA 2 (AP2)-type protein, and it expressed ubiquitously in strawberry plants and could be induced by ABA, sucrose and glucose. In consistent with higher transcripts of *FaABI4* in red-coloring fruits, downregulation of its transcripts in developmental fruit inhibited both ABA and sugar accumulation, as a result, retarded ripening, demonstrating that ABI4 is a positive regulator for the ripening. In the future, experiment with the non-climatic model plant, strawberry, integration of FaABI4 into the crosstalk of ABA and sugar signaling is charming research work.

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Figure legends

Fig. 1 A phylogenetic tree and protein sequence alignment of FaABI4 analogues with a putative conserved domains.

(A) A phylogenetic tree of FaABI4 analogues was performed by MEGA6 software through Neighbor-joining method

(1000 bootstrap replications); (B) A protein sequence alignment of FaABI4 analogues; (C) An AP2 putative

conserved domain was founded in FaABI4 protein via a BLAST in the NCBI database

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Fa: *Fragaria vesca* subsp. Vesca; Pp: *Prunus persica*; Pm: *Prunus mume*,

Md: *Malus domestica*; Mt: *Medicago truncatula*; Gm: *Glycine max*; Zj: *Ziziphus jujube*; Cs: *Citrus sinensis*; Cc:

Citrus clementina; Vv: *Vitis vinifera*; Me: *Manihot esculenta*; Pt: *Populus trichocarpa*; Tc: *Theobroma cacao*; Gr:

Gossypium raimondii; Ca: *Capsicum annuum*; Nt: *Nicotiana tabacum*; Si: *Sesamum indicum*; Bn: *Brassica napus*; Br:

Brassica rapa; At: *Arabidopsis thaliana*.

Fig. 2 SqRT-PCR analysis of expression pattern of *FaABI4* in strawberry plants.

(A) Changes in fruit size and color. (B) Transcripts of *FaABI4* in roots, stems, leaves, flowers and fruits. (C)

Transcripts of *FaABI4* in developmental fruits. The rRNA indicates the loading control of the RNA samples stained with

ethidium bromide. *Actin* mRNA was used as an internal control. SG: small green, BG: big green, DG: de-greening, Wt:

white, IR: initial red, PR: partial red, FR: full red.

Fig. 3 Silencing of *FaABI4* by VIGS in strawberry fruit.

(A) Two-week-old BG fruits still attached to the plants were used for inoculation. (B) The control fruits inoculated with

Agrobacterium containing the TRV only, could turn full red. (C) The RNAi fruit inoculated with *Agrobacterium*

containing TRV carrying a *FaABI4* fragment, lost red-coloring in the inoculated surface. (D) SqRT-PCR analysis of the

FaABI4 transcripts in receptacles of the control and RNAi fruits. The rRNA indicates the loading control of the RNA samples stained with ethidium bromide. *Actin* mRNA was used as an internal control.

Fig. 4 Changes of the ripening-related parameters in RNAi fruits and effects of sugar and ABA on *FaABI4* transcripts *in vitro*.

(A) Firmness. (B) Sugar contents. (C) ABA contents. (D) SqRT-PCR analysis of transcripts of *CA4H*, *CHI*, *DFR*, *CHS*, *GAL6*, *PE5*, and *XYL2*. (E) Effects of sucrose (Suc), glucose (Glu), and ABA on *FaABI4* transcripts in fruit-disc incubation *in vitro*. The rRNA indicates the loading control of the RNA samples stained with ethidium bromide. *Actin* mRNA was used as an internal control. Error bars represent the SE (n = 3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

Tables

Table 1. The primers used for SqRT-PCR.

Genes	Sequences for SqRT-PCR
<i>FaABI4</i>	Sense: 5'- ACAACGAGAGCGGTGCTGAAA -3' Antisense: 5'- CGTAAGGATGAGCCGAAGAGG -3'
<i>GAL6</i>	Sense: 5'-GACGGTCAGCATAAGATTC-3' Antisense: 5'-GGCATACCATACTCATCAAG-3'
<i>PE5</i>	Sense: 5'-GCTCAACACAACCGTAAAC-3' Antisense: 5'-GAACCTCCTCCAACACTTT-3'
<i>XYL2</i>	Sense: 5'-GCCTCTATCTACTGTGCGTC-3' Antisense: 5'-TGAATCCCCAACTGGTCTAC-3'
<i>CA4H</i>	Sense: 5'-GTGACTTTATTCCCGTGCT-3' Antisense: 5'-CTGGAGGAGGTAACATCTCA-3'
<i>CHS</i>	Sense: 5'-AGAATCCCAGTATGTGCG-3' Antisense: 5'-TGAGGAGGTGAAATGTGAG-3'
<i>CHI</i>	Sense: 5'-CCAGCAATACTCCGAGAA-3' Antisense: 5'-GTCCACTTTCCCATTTC-3'
<i>DFR</i>	Sense: 5'-CGGAAAGTAAAGATGACTGG-3' Antisense: 5'-GTTCTCCTCAATGCCCTT-3'
<i>Actin</i>	Sense: 5'-TGCATATATCAAGCAACTTTACACTGA-3' Antisense: 5'-ATAGCTGAGATGGATCTTCCTGT-3'

Figures

Fig. 1

Fig. 2

Fig. 3

Fig .4