

RESEARCH PAPER

Type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening

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Received 3 September 2012; Revised 8 January 2013; Accepted 15 January 2013

Abstract

Although a great deal of progress has been made toward understanding the role of abscisic acid (ABA) in fruit ripening, many components in the ABA signalling pathway remain to be elucidated. Here, a strawberry gene homologous to the *Arabidopsis* gene *ABI1*, named *FaABI1*, was isolated and characterized. The 1641 bp cDNA includes an intact open reading frame that encodes a deduced protein of 546 amino acids, in which putative conserved domains were determined by homology analysis. Transcriptional analysis showed that the levels of *FaABI1* mRNA expression declined rapidly during strawberry fruit development as evidenced by real-time PCR, semi-quantitative reverse transcription-PCR, and northern blotting analyses, suggesting that the Ser/Thr protein phosphatase PP2C1 encoded by *FaABI1* may be involved in fruit ripening as a negative regulator. The results of *Tobacco rattle virus*-induced gene silencing and PBI121 vector-mediated overexpression suggested that the down- and up-regulation of *FaABI1* mRNA expression levels in degreening strawberry fruit could promote and inhibit ripening, respectively. Furthermore, alteration of *FaABI1* expression could differentially regulate the transcripts of a set of both ABA-responsive and ripening-related genes, including *ABI3*, *ABI4*, *ABI5*, *SnRK2*, *ABRE1*, *CHS*, *PG1*, *PL*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*. Taken together, the data provide new evidence for an important role for ABA in regulating strawberry fruit ripening in the processes of which the type 2C protein phosphatase ABI1 serves as a negative regulator. Finally, a possible core mechanism underlying ABA perception and signalling transduction in strawberry fruit ripening is discussed.

Key words: Abscisic acid (ABA), overexpression, strawberry fruit ripening, *Tobacco rattle virus*, type 2C protein phosphatase ABI1, virus-induced gene silencing (VIGS).

Introduction

Abscisic acid (ABA) not only plays a central role in the adaptation of plants to environmental challenges, but also regulates many aspects of plant growth and development (Leung and Giraudat, 1998; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Hirayama and Shinozaki, 2007). In recent years, not only has ABA been shown to play important roles in perception and signal transduction involved in the regulation of *Arabidopsis* seed germination, seedling growth, and stomatal movement (Fujii *et al.*, 2009; Ma *et al.*, 2009; Melcher *et al.*,

2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009; Shang *et al.*, 2010), but much progress has also been made toward a better understanding of the molecular mechanisms that underlie the roles of ABA in the regulation of fleshy fruit ripening (Chai *et al.*, 2011; Jia *et al.*, 2011; Li *et al.*, 2011; Sun *et al.*, 2011, 2012). Nevertheless, many ABA signalling components involved in fruit ripening remain to be elucidated.

To date, two core ABA signalling pathways have been proposed in *Arabidopsis*, namely ABA-PYR/PYL/RCAR-type

2C protein phosphatase (PP2C)–SNF1-related protein kinase 2 (SnRK2) (Fujii *et al.*, 2009) and ABA–ABAR–WRKY40–ABI5 (Shang *et al.*, 2010). Structural biology provides a detailed gate–latch–lock mechanism involved in ABA signal perception and transduction, including ABA–PYR1 perception, PYR1–PP2C interaction, inhibition of PP2C activity, and activation of SnRK2 (Fujii *et al.*, 2009). In this model, the early reported PP2Cs, serving as a central and negatively regulated hub in ABA signalling (Leung *et al.*, 1997; Merlot and Giraudat, 1997; Gosti *et al.*, 1999; Merlot *et al.*, 2001), were first integrated into the canonical ABA signalling network by reversible phosphorylation (Fujii *et al.*, 2009), which is a universal mechanism for regulating diverse biological functions in eukaryotes (Smith, 1996).

Most phosphorylation events involve transfer of phosphate to serine and threonine residues catalysed by kinases, such as Ca²⁺-dependent protein kinase (CDPK), SnRKs, mitogen-activated protein kinase (MAPK), and a receptor-type kinase (RPK1), while removal of this phosphate is catalysed by Ser/Thr protein phosphatases including type 1 (PP1) and type 2 (PP2), of which PP2 was subsequently divided into three groups based on the metal ion requirements: 2A (not requiring metal ions), 2B (activated by calcium), and 2C (Mg²⁺ dependent) (Cohen and Cohen, 1989; Sopory and Munshi, 1998; Hirayama and Shinozaki, 2007). Note that the *Arabidopsis* strong ABA-insensitive loci *ABII* and *ABII2* have been demonstrated to encode PP2C enzymes, which play a central functional role in the ABA signalling pathway as negative regulators of plant stress, cell differentiation, and growth (Leung *et al.*, 1994; Meyer *et al.*, 1994; Finkelstein *et al.*, 2002; Hirayama and Shinozaki, 2007; Lu and Wang, 2008). Also, plant PP2Cs are reportedly encoded by a large multigene family with 80 and 78 members in *Arabidopsis* and rice, respectively (Xue *et al.*, 2008). In addition to *Arabidopsis* and rice, PP2Cs as negative regulators have been characterized only in several other plants, such as pea, wheat, beech, alfalfa, and maize (MacKintosh *et al.*, 1991, 1992; Meskiene *et al.*, 1998; Sheen, 1998; González-García *et al.*, 2003; Hu *et al.*, 2010).

Following the determination of *Arabidopsis* ABA core signalling function, a great deal of progress has been made toward understanding the role of ABA in fleshy fruit ripening. The down-regulation of the expression of ABA receptor genes Mg-chelatase H subunit (*FaABAR/CHLH*) or pyrabactin resistance 1 gene (*FaPYR1*) can destroy strawberry fruit red colouring, indicating that both *FaABAR/CHLH* and *FaPYR1* proteins are positive regulators of fruit ripening (Chai *et al.*, 2011; Jia *et al.*, 2011; Li *et al.*, 2011). A significant reduction in SINCED1 activity leads to a decline in the transcription of genes encoding major cell wall catabolic enzymes, indicating that ABA affects cell wall catabolism during tomato fruit ripening (Sun *et al.*, 2012). Transcriptional analysis suggested that tomato SIPYL1, SIPYL2, SIPP2C1, SIPP2C5, and SnRK2.3 may be involved in the regulation of fruit ripening (Sun *et al.*, 2011). Although PP2C is an important hub in ABA responses, its defined function in fruit ripening remains unclear. In the present study, silencing and overexpression of the *FaABII* gene in strawberry fruit

were performed. The results showed that PP2C1 encoded by *FaABII* is a negative regulator of strawberry fruit ripening.

Materials and methods

Plant material

Strawberry (*Fragaria×ananassa* cv. Camarosa) was grown in a glass-house (20–25 °C, relative humidity 70–85%, 14h/10h light/dark cycle) during the spring season in 2009–2010. The fruit samples were sampled from seven developmental stages: small green (SG), large green (LG), degreening (DG), white (Wt), initial red (IR), partial red (PR), and full red (FR) for 7, 15, 20, 23, 25, 27, and 31 d after anthesis, respectively. Thirty uniformly sized fruit were sampled at each stage (one replicate). After removing the achenes (seeds), the receptacle (pulp) was cut into cubes measuring 0.5–0.8 cm³, snap-frozen in liquid nitrogen, and quickly stored at –80 °C.

RNA isolation, cloning of the *FaABII* gene, and RT–PCR analysis

Total RNA was extracted from 10 g of fresh or treated strawberry fruit using a modified cetyltrimethylammonium bromide (CTAB) protocol (Jia *et al.*, 2008). Genomic DNA was removed by 15 min incubation at 37 °C with RNase-Free DNase (TaKaRa, Otsu, Japan) followed by an RNA Clean Purification Kit (BioTeke, Beijing, China). The purity and integrity of RNA were analysed both by agarose gel electrophoresis and by the *A*₂₆₀:*A*₂₃₀ and *A*₂₆₀:*A*₂₈₀ ratios. To generate first-strand cDNA, 3 µg aliquots of total RNA were reverse-transcribed using a universal primer (5'-AAGCATGGTA TCAACGCAGAGTAC(T)₃₀VN-3' (where N=A, C, G, or T; V=A, G, or C) supplied with the SMART™ RACE cDNA Synthesis Kit (TaKaRa) according to the manufacturer's protocol.

To clone the *FaABII* gene, an *Arabidopsis* PP2C protein ABII (At4g26080) was used to BLAST in a strawberry gene library (<https://strawberry.plantandfood.co.nz/index.html>), and a high homology protein with a gene locus 07500 was found. Based on the nucleotide sequence, specific primers (forward, 5'-ATGGAGGA CATGTCTCCAGCAG-3'; reverse, 5'-TCAAGATTTGCTCTTGA ACTTTC-3') were designed to amplify a coding sequence from 'Camarosa' strawberry fruit by reverse transcription–PCR (RT–PCR). PCR was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, with a final extension at 72 °C for an additional 10 min. The PCR products were ligated into a pUC-T vector and subsequently transformed into *Escherichia coli* DH5α. Positive colonies were selected, amplified, and sequenced by Invitrogen China (Shanghai, China).

Primers for semi-quantitative RT–PCR (SqRT–PCR) of *FaABII* were designed based on the determined sequences (forward, 5'-CAGCAGGTAAAGTGGGAGAA-3'; reverse, 5'-AGGG CAAGAGTTGAAAGATAAG-3'). The primers for real-time PCR were designed as showed in Table 1. A Bio-Rad iQ Sequence Detector (Bio-Rad, Hercules, CA, USA) was used for real-time PCR amplification. The quantitative PCR experiment was repeated three times.

Construction of virus vectors, the PBI121 vector, and Agrobacterium-mediated infiltration

The pTRV1 and pTRV2 vectors (Liu *et al.*, 2002) were kind gifts of Dr Liu Yu-le, Qinghua University. A 584 bp cDNA fragment near the 5' end of *FaABII* was amplified using appropriate primers (forward, 5'-GGTAACCATCCCATCAAG-3'; reverse, 5'-GGGTCAAACCTCTGGCTCATTCC-3'), cloned into the pMD19-T vector (TaKaRa), digested with *SacI* and *XbaI*, and subsequently cloned into the virus vector pTRV2 cut with the same restriction enzymes. *Agrobacterium* strain GV3101 containing pTRV1, pTRV2, or the pTRV2 derivative pTRV2-*FaABII*₅₈₄

Table 1. The primers used for real-time PCR.

Genes	Sequences for real-time PCR	Sources of primers
<i>ABI1</i>	Sense: 5'-CAAGAGCCATTCTTTGTCGT-3' Antisense: 5'-TGGAATAATCCAGGGTTTCA-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>SnRK2</i>	Sense: 5'-GCACTTCGCTCCAAGAGTG-3' Antisense: 5'-AGGATATGTAGTGCTGGTAGATT-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>ABI3</i>	Sense: 5'-CGGCGCCTGTATTAGTCCC-3' Antisense: 5'-TGCAGTCTCCAGCGTTTGAT-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>ABI4</i>	Sense: 5'-TCCTCATCACCACCGTCTT-3' Antisense: 5'-ACTCTGGCTCGTTTGCTCT-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>ABI5</i>	Sense: 5'-GGAGCTGGCAATGGTCG-3' Antisense: 5'-AGGCCCGCCTTTCCTT-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>Actin</i>	Sense: 5'-TGGGTTTGTCTGGAGATGAT-3' Antisense: 5'-CAGTAGGAGAACTGGGTGC-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>PYR1</i>	Sense: 5'-GGAGCTGGCAATGGTCG-3' Antisense: 5'-AGGCCCGCCTTTCCTT-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>CHS</i>	Sense: 5'-GCTGTCAAGGCCATTAAGGA-3' Antisense: 5'-GAGCAAACAACGAGAACACG-3'	Chai <i>et al.</i> (2011); Jia <i>et al.</i> (2011)
<i>PG1</i>	Sense: 5'-CGACAGAGTGAAAAATTCCTTAG-3' Antisense: 5'-AGGACTGGGTTAGCAAAATTATTC-3'	GenBank accession no. EF441274
<i>PL1</i>	Sense: 5'-TGACTCCCTTGCTGCTTCTT-3'. Antisense: 5'-TCTACTGCGTGCTCATTCCA-3'	GenBank accession no. EF441273
<i>NCED1</i>	Sense: 5'-CCCAAACGGCACGAAAT-3' Antisense: 5'-GCATCGCTCGCATTCTT-3'	GenBank accession no. HQ290318.1
<i>ABRE1</i>	Sense: 5'-GGTGGTGCGAGAAGATGC-3' Antisense: 5'-TTGGGAAAGATTGGTTGC-3'	Gene 28250 locus in https://strawberry.plantandfood.co.nz/index.html
<i>CHI</i>	Sense: 5'-GTTAAGTGAAGGGCAAGA-3' Antisense: 5'-CCCGTCAGCGGTAGTATCA-3'	GenBank accession number AB201755
<i>F3H</i>	Sense: 5'-TTTTCTGAGCAATGGGAGG-3' Antisense: 5'-CTGGGTTCTGGAATGTCG-3'	GenBank accession no. AB201760
<i>DFR</i>	Sense: 5'-ACGAAGTGATAAAGCCAACA-3' Antisense: 5'-AAACACCAACCTCCGAAC-3'	GenBank accession no. AF029685
<i>ANS</i>	Sense: 5'-CGTGAGACCCAAAGAGGA-3' Antisense: 5'-ATGCCGTGGTTGATAAGG-3'	GenBank accession no. AY695818
<i>UGFT</i>	Sense: 5'-GGTAAGCCACAGGAGGACA-3' Antisense: 5'-TATGAGCACCGAACCAAAA-3'	GenBank accession no. AY575056

was used for RNA interference (RNAi). For overexpression, the 1641 bp cDNA was amplified using appropriate primers (forward, 5'-TCTAGAATGGAGGACATGTCTCCAGCAG-3'; reverse, 5'-GAGCTCTCAAGATTTGCTCTTGAACCTTC-3'), cloned into the pMD-T simple vector (TaKaRa), digested with *Xba*I and *Sac*I, and subsequently cloned into the binary expression vector PBI121 cut with the same restriction enzymes. *Agrobacterium* strain GV3101 containing PBI121 or the PBI121 derivative PBI121-FaABI1₁₆₄₁ was used for overexpression.

For the virus-induced gene silencing (VIGS) assay, pTRV1 and pTRV2 or recombinant derivatives (pTRV2-FaABI1) were transformed into *Agrobacterium* strain GV3101 by the freeze-thaw method (Fire *et al.*, 1998). A 5 ml culture of each strain was grown overnight at 28 °C in Luria-Bertani (LB) medium (50 mg ml⁻¹ kanamycin and 50 mg ml⁻¹ rifampicin, 10 mM MES, 20 mM acetosyringone). The overnight cultures were inoculated into 50 ml of LB medium and grown at 28 °C overnight. The cells were harvested by centrifugation (5000 rpm, 5 min, 20 °C), resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, 200 mM acetosyringone), adjusted to an optical density (OD) of 1.0–2.0, and left to stand at room temperature for 3 h. About 1 ml of *Agrobacterium* mixture pTRV1, pTRV2, or pTRV2-FaABI1 (1:1 ratio) was infiltrated into every DG fruit with a 50 ml syringe. Ten uniformly sized fruit were used in infiltration experiment, and the experiment was repeated three times.

Determination of fruit firmness and soluble solid, anthocyanin, and ABA contents

The fruit firmness was measured after removal of the skin on three sides using a fruit hardness tester (FHM-5, Takemura Electric Work Ltd, Japan). The solid soluble content of flesh was measured using a hand-held sugar measurement instrument (MASTER-100H, ATAGO Master, Japan), onto which fruit juice was applied to obtain a reading. Anthocyanin measurements were performed as described previously by Fuleki and Francis (1968a, b). ABA measurements were carried out as described by Chai *et al.* (2011). Ten uniformly sized fruit were used for detection of each parameter, and the experiment was repeated three times.

Probe preparation and northern and siRNA hybridization

DIG-labelled probes were synthesized using a PCR-DIG Probe Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. For northern hybridization analysis, aliquots of RNA (15 µg) were separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 M formaldehyde and blotted onto nylon membranes (Hybond N⁺; Amersham Biosciences). RNA blots were hybridized with a 626 bp *FaABI1* probe that was amplified using the appropriate primers (forward, 5'-CAGCAGGTAAAGTGGGAGAA-3'; reverse, 5'-AGGGCAAGAGTTGAAAGATAAG-3'). For the small

interfering RNA (siRNA) test, siRNA was extracted from 10 g of flesh using the miRcute miRNA isolation kit (Tiangen Biotech, Beijing, China), which was then fractionated in a 15% (w/v) polyacrylamide–urea gel and blotted onto a 0.45 mm nylon membrane (Whatman, Nytran SPC, Sanford, CA, USA) and hybridized with an *ABII*-specific probe corresponding to the *FaABII* region (forward, 5'-GGTAACCATCCATCACAAG-3'; reverse, 5'-GGGTCAAACCTCTGGCTCATT CC-3'). rRNA stained with ethidium bromide was used as a gel loading control. The filters were hybridized overnight with DIG-labelled DNA probes (0.3–1 g ml⁻¹) in high stringency hybridization solution (50% formamide, 2× SSPE buffer, 10 mM dithiothreitol, 1 mg ml⁻¹ herring sperm DNA, 500 µg ml⁻¹ yeast tRNA, and 1 mg ml⁻¹ bovine serum) in a shaking water bath at 50 °C. Following hybridization, the filters were washed twice at 50 °C for 15 min in each of 2× SSC, 1× SSC, and 0.1× SSC. The membranes were then subjected to immunological detection according to the manufacturer's instructions using NBT/BCIP stock solution as a chemiluminescent substrate for alkaline phosphatase (Roche Diagnostics).

Southern hybridization

About 0.5 g of strawberry leaf tissue was used for genomic DNA isolation using a DNA Extraction Kit (BioTeke, Beijing, China) according to the manufacturer's protocol. DIG-labelled probes (*FaABII*, forward, 5'-CAGCAGGTAAAGTGGGAGAA-3', reverse, 5'-AGGGCAAGAGTTGAAAGATAAG-3') were synthesized using a PCR-DIG Probe Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. About 10 µg of DNA was separated by electrophoresis on 0.8% (w/v) agarose gels containing 2.2 M formaldehyde and blotted onto nylon membranes (Hybond N⁺; Amersham Biosciences). The hybridization processes were performed according to the northern analysis above.

Detection of the Tobacco rattle virus (TRV) vector by RT–PCR

Random primers were used to reverse-transcribe RNA for the first strand of infiltrated strawberry fruit to detect the TRV vectors by RT–PCR as described by [Chai *et al.* \(2011\)](#).

Expression and purification of FaPYR1 recombinant protein

The coding sequence of *FaPYR1* ([Chai *et al.*, 2011](#)) was amplified by PCR from a cDNA synthesized above using primers [forward, *CGGATCC ATGGAGAAACCATCATCGGC* (*Bam*HI site); and reverse, *GGCGGCCGC TCAGACCTGGGGAGTTAGCG* (*Not*I site)] and cloned into the expression vector pET28a (Novagen, Germany). The modified fusion protein contains two His-tags at both the N-terminus and the C-terminus. BL21(DE3) cells transformed with the expression plasmid were grown in 1 litre of LB medium containing 50 µg ml⁻¹ kanamycin in an OD₆₀₀ of 0.5–0.6 at 37 °C. Then, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and induction was carried out overnight at 16 °C. Cells were harvested after ~12 h incubation and stored at –80 °C before purification.

Purification was carried out at 4 °C under native conditions. A 1 litre pellet was resuspended in 50 ml of lysis buffer (20 mM TRIS, pH 8.0, 300 mM NaCl, 10 mM imidazole, and Merck's protease cocktail inhibitor) and the cells were sonicated in a Misonix Sonifier in an ice bath. A clear lysate was obtained after centrifugations at 30 000 *g* for 30 min. Proteins were purified on Ni-NTA His Bind Resins (Novagen) in a column procedure. The 8 ml of supernatant filtered on a 0.45 µm filter was added to the Ni-NTA His Bind slurry and mixed gently by shaking at 4 °C for 60 min. The lysate–resin mixture was loaded onto an empty column. The column was washed with lysis buffer supplemented with 20 mM imidazole and 10% glycerol to 20 ml, and eluted with elution buffer (20 mM TRIS, pH 8.0, 300 mM NaCl, 500 mM imidazole, and Merck's protease cocktail inhibitor) for 8 ml. The eluted

fusion protein was stored at –80 °C before isothermal calorimetric analysis.

Isothermal calorimetry analysis

Before the next step of the analysis, the protein was concentrated using an Amicon Ultra-4 centrifugal 10 kDa filter (Millipore) at 3000 *g* for ~10–40 min in a swing bucket rotor (Sigma, USA). Purified FaPYR1 fusion protein was desalted for buffer exchange using a HiTrap Desalting column (GE Healthcare). The HiTrap Desalting column was filled with ITC buffer (20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 20 mM KCl) to remove the ethanol completely in order to equilibrate the column. The sample was applied using a 2–5 ml syringe and the eluted buffer was discarded from the column. The buffer was changed to ITC buffer, and then injection was carried out and the eluted buffer was collected.

Binding studies were performed using a calorimeter (Microcal ITC200, USA) at 30 °C. The final concentration of FaPYR1 fusion protein was adjusted to 15 mM. Ligand (1 mM S-ABA) in the same buffer was injected into the protein solution. The experiment was repeated three times. Data fitting was performed by using the ORIGIN 7.0 software supplied with the instrument.

Results

Cloning, bioinformatics, and Southern analysis of the *FaABII* gene

To clone the *FaABII* gene, the *Arabidopsis* PP2C protein ABII (At4g26080, GenBank accession no. AY142623) was used for a BLAST search in a strawberry gene library (<https://strawberry.plantandfood.co.nz/index.html>), which yielded a protein showing a high level of identity with the gene locus 07500, on the basis of which specific primers (forward, 5'-ATGGAGGACATGTCTCCAGCAG-3'; reverse, 5'-TCAAGATTTGCTCTTGAACCTTTC-3') were designed to amplify a coding sequence from 'Camarosa' strawberry fruit by RT–PCR. A 1641 bp cDNA homologous to *Arabidopsis* ABII, named *FaABII*, was isolated from strawberry fruit (GenBank accession no. JX989266). The cDNA included an open reading frame encoding a deduced protein of 546 amino acids ([Fig. 1A](#)), in which the putative PP2C conserved domains were detected by homology analysis using BLAST for the *FaABII* protein on the NCBI protein blast website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) ([Fig. 1B](#)), indicating successful isolation of the *FaABII* gene encoding the putative strawberry PP2C.

To investigate further the members of the *FaABII* gene family in the strawberry genome, Southern blot analysis was performed using a probe corresponding to the *FaABII*-coding cDNA sequence. Genomic DNA was digested with *Xho*I, *Xba*I, *Nco*I, and *Bam*HI restriction enzymes, respectively. The probe generated multiple hybridizing bands using every restriction enzyme ([Fig. 1C](#)). The results suggest that the strawberry genome indeed contains a *FaABII* gene family with multiple members.

Expression analysis of the *FaABII* gene during strawberry fruit development

Based on our previous report ([Chai *et al.*, 2011](#)) and changes of fruit size and colour, the fruit development processes of

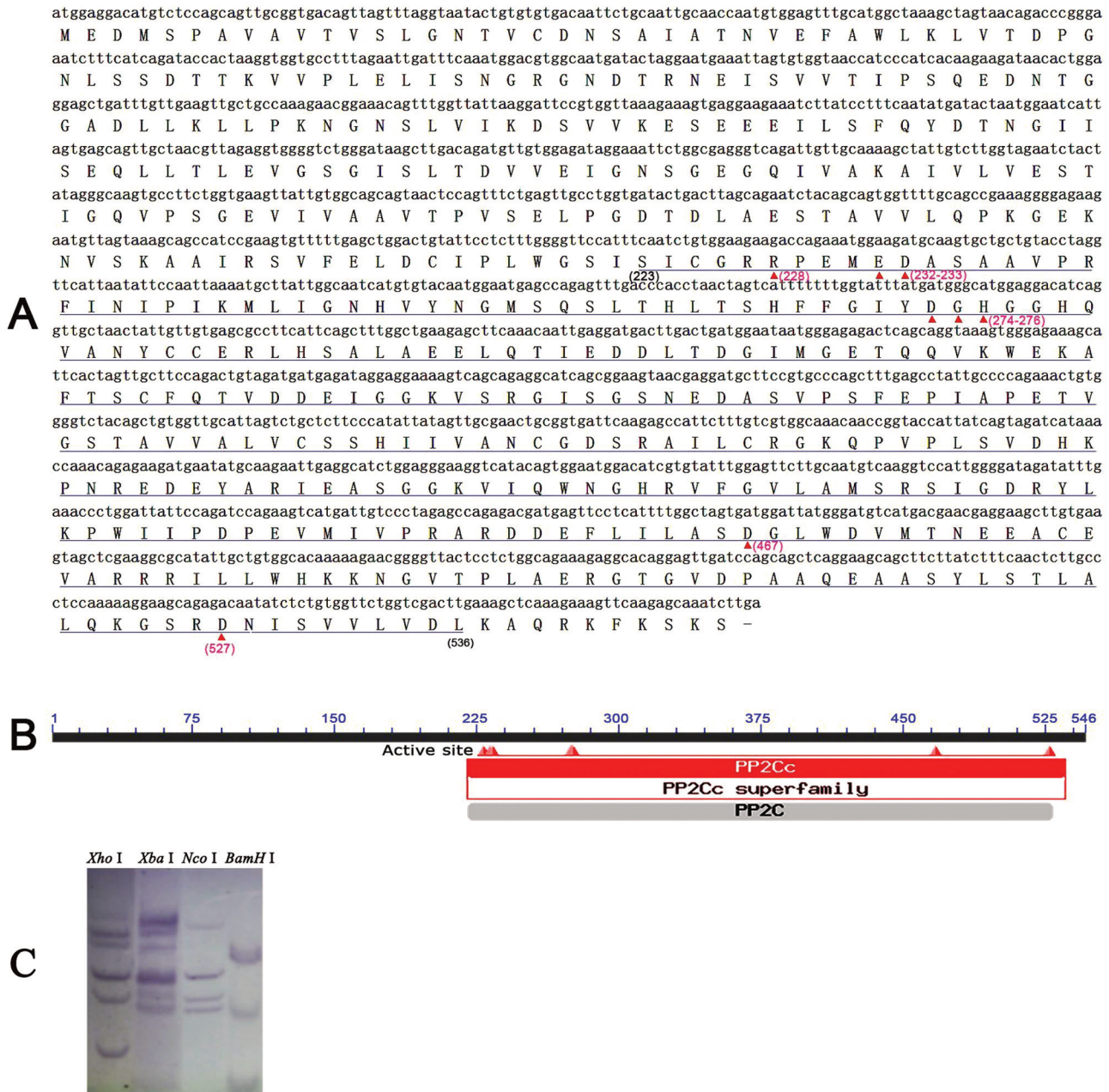


Fig. 1. Characterization of the *FaABI1* gene sequence. (A) The cDNA and deduced amino acid sequences of the *FaABI1* gene. (B) The conserved serine/threonine phosphatase family 2C catalytic domain in the *FaABI1* protein. The amino acids underlined with red triangles represent active sites (228, 232–233, 274–276, 467, and 527). Amino acids 223–536 underlined in blue represent the PP2C superfamily domains. (C) A 10 µg portion of strawberry genomic DNA was digested with *Xho*I, *Xba*I, *Nco*I, and *Bam*HI, electrophoresed on a 0.8% agarose gel, and transferred onto a nylon membrane. The membrane was hybridized with a digoxigenin (DIG)-labelled cDNA fragment of *FaABI1*.

strawberry cultivar ‘Camarosa’ was divided into seven different visual stages: SG, LG, DG, Wt, IR, PR, and FR for 7, 15, 20, 23, 25, 27, and 31 d after anthesis, respectively (Fig. 2A).

To investigate whether the *FaABI1* gene is involved in strawberry fruit ripening, the mRNA expression levels of *FaABI1* were determined by real-time PCR, northern blotting, and SqRT-PCR using the seven-stage fruit (Fig. 2A). The results showed that the mRNA expression levels of *FaABI1* were extremely high in SG fruit, declined rapidly during strawberry fruit development, and finally remained at

extremely low levels at the FR stage (Fig. 2B). The opposite trend between *FaABI1* transcripts and fruit size and colour suggested that *FaABI1* might negatively regulate strawberry fruit ripening.

Silencing of the FaABI1 gene promotes strawberry fruit ripening

To confirm the function of the *FaABI1* gene in strawberry fruit development, a 584bp cDNA fragment of the *FaABI1*

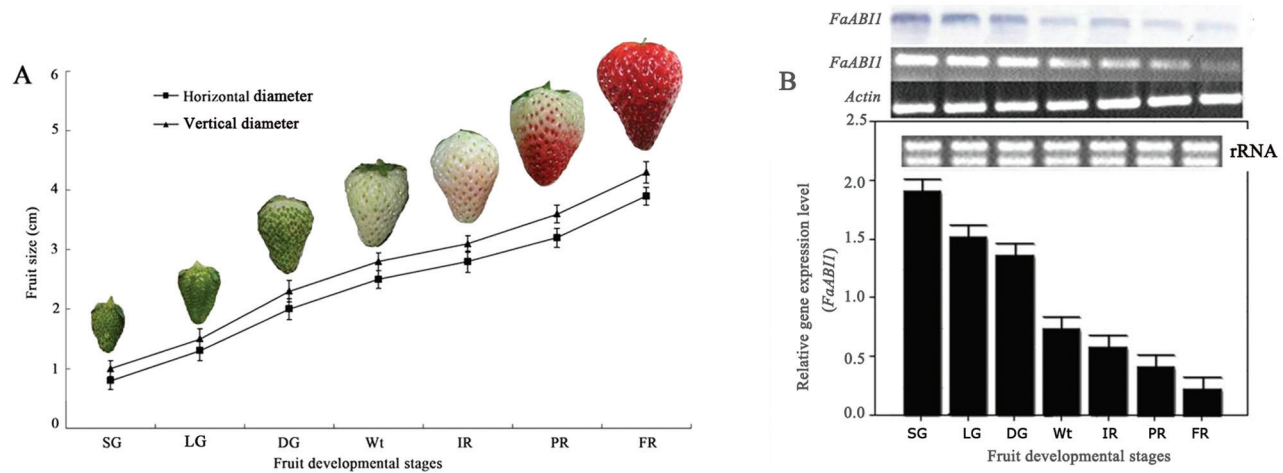


Fig. 2. Variations in transcripts of the *FaABI1* gene in strawberry fruit during the seven developmental stages. (A) Development was divided into seven stages based on changes in fruit size and colour: small green (SG), large green (LG), degreening (DG), white (Wt), initial red (IR), partial red (PR), and full red (FR). (B) Changes in *FaABI1* transcript levels during the seven developmental stages were determined by northern blotting, semi-quantitative RT-PCR, and real-time PCR analyses (from top to bottom). rRNA indicates the loading control of the RNA samples stained with ethidium bromide. *Actin* mRNA was used as an internal control. The error bars represent the standard error ($n=3$).

gene (from 207 bp to 790 bp) was cloned based on the coding sequence isolated above and inserted into the TRV2 virus vector using the restriction enzymes *Xba*I and *Sac*I. A mixture of *Agrobacterium tumefaciens* strain GV3101 cultures containing pTRV1 and pTRV2 or pTRV2 carrying the 584 bp fragment of the *FaABI1* gene (pTRV2-*FaABI1*₅₈₄) in a 1:1 ratio was inoculated into DG fruit using a syringe (Fig. 3A). Control fruit were inoculated with TRV alone (empty vector). Unexpectedly, the surface of the inoculated RNAi fruit developed a red colour 5 d after injection (Fig. 3C), while the surface of the control fruit remained at the Wt stage (Fig. 3B).

To validate the suppression of *FaABI1* at the molecular level, a series of analyses were performed by SqRT-PCR, real-time PCR, northern blotting, and siRNA analyses. The results showed that the *FaABI1* transcripts were markedly down-regulated in RNAi fruit compared with the control fruit (Fig. 4A); 300 bp TRV-RNA1 and 900 bp TRV-RNA2 were both detected in *Agrobacterium*-mediated TRV-inoculated fruit (Fig. 4B, C, lanes 2–4), but not in fruit inoculated with *Agrobacterium* alone (Fig. 4B, C, lane 1); *FaABI1*-related siRNA for specific RNA silencing was also detected in RNAi fruit but not in control fruit (Fig. 4D). Taken together, the *FaABI1* gene was silenced successfully in strawberry fruit and thus led to the promotion of red colour development.

Overexpression of the *FaABI1* gene inhibits strawberry fruit ripening

To confirm further the negative regulatory role of *FaABI1* in strawberry fruit ripening, the 1641 bp coding sequence of *FaABI1* was cloned into the plant binary expression construct PBI121 using *Xba*I and a *Sac*I restriction enzyme named PBI121-*FaABI1*₁₆₄₁. A mixture of *A. tumefaciens* strain GV3101 cultures containing PBI121 (control) or

PBI121-*FaABI1*₁₆₄₁ was inoculated into DG fruit using a syringe (Fig. 3A). Twelve days after inoculation, the surface of control fruit turned full red (Fig. 3D), whereas the inoculated sector on the surface of the overexpressing (OE) fruit remained white (Fig. 3E). SqRT-PCR and real-time PCR analyses indicated that the *FaABI1* gene mRNA level was up-regulated by 60% in OE fruit compared with control FR fruit (Fig. 4E). These results indicated that overexpression of the *FaABI1* gene inhibited strawberry fruit ripening.

Alteration of *FaABI1* expression affects a set of ABA-responsive and ripening-related gene transcripts and ABA levels

To clarify the mechanism of action of *FaABI1* in the regulation of strawberry fruit ripening, several ripening-related physiological parameters were measured, including fruit firmness, solid soluble concentrations, anthocyanin contents, and ABA levels, and ABA-responsive and ripening-related genes, such as *ABI3*, *ABI4*, *ABI5*, *PYR1*, *SnRK2*, *ABRE1*, chalcone synthase (*CHS*), polygalacturonase (*PGI*), pectate lyase (*PLI*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*), were also examined (Salentijn et al., 2003; Fujii et al., 2009; Chai et al., 2011; Jia et al., 2011; Wei et al., 2011) in both RNAi and OE fruit. The results showed that firmness (Fig. 5A) declined in RNAi fruit but increased in OE fruit; both anthocyanin (Fig. 5B) and solid soluble contents (Fig. 5C) were up-regulated in RNAi but down-regulated in OE fruit, compared with the corresponding control fruit. Gas chromatography-mass spectrometry analysis showed that ABA content was not significantly altered in RNAi fruit but increased in OE fruit compared with the corresponding control fruit (Fig. 5D).

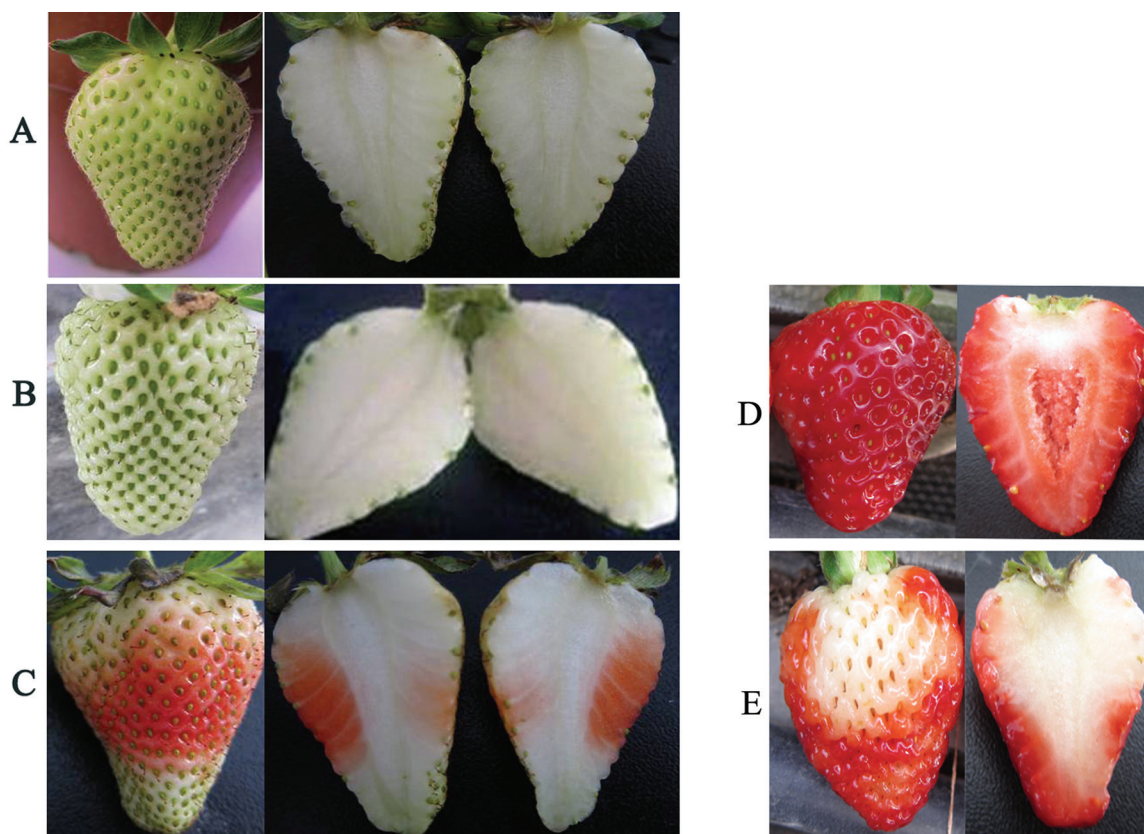


Fig. 3. The phenotypes of virus-induced *FaABI1* gene silencing and *FaABI1* overexpression in strawberry fruit. (A) Degreening fruit attached to the plant were infiltrated with *Agrobacterium* containing TRV alone (control fruit for RNAi), TRV carrying a fragment of *FaABI1* (RNAi fruit), PBI121 alone (control fruit for OE), or PBI121 carrying the coding sequence of *FaABI1* (overexpressing fruit). (B and C) Photographs of fruit were taken for control (B) and RNAi (C) 5 d after infiltration. (D and E) Photographs were taken for control (D) and overexpressing fruit (E) 12 d after infiltration.

Transcriptional analysis of the *FaNCEDI* gene key to ABA biosynthesis showed that the mRNA expression level of the *NCEDI* gene was up-regulated in OE fruit compared with that in control fruit (Fig. 5E). Real-time PCR analysis showed that the majority of genes, including *ABI3*, *SnRK2*, *AREB1*, *PGL1*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*, were significantly up-regulated in RNAi fruit (Fig. 6A); in contrast, together with the *PL1* gene, they were all markedly down-regulated in OE fruit (Fig. 6B). Notably, a small fraction of genes, such as *ABI4* and *ABI5*, were markedly down-regulated in RNAi fruit (Fig. 6A) but showed no significant alterations in expression level in OE fruit (Fig. 6B). Taken together, the observed regulation of expression of firmness (*PGL1* and *PL1*)-, sugar (*SnRK2*)-, and pigment (*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*)-related genes through the *FaABI1*-mediated signaling pathway was consistent with a negative role for PP2C in ABA signalling during fruit ripening.

The molecular basis of a negative regulator of FaABI1: FaPYR1 is an ABA receptor

The PYR1–PP2C–SnRK2 core signaling pathway of ABA action has been established in *Arabidopsis* (Fujii *et al.*, 2009). Given that *FaPYR1* acts as a positive regulator in strawberry

fruit ripening (Chai *et al.*, 2011), further determination of the receptor nature of *FaPYR1* protein contributes to understanding the molecular basis of the negative regulator *FaABI1*.

Isothermal calorimetry analysis using 15 mM recombinant *FaPYR1* protein and 1 mM S-ABA showed that each purified *FaPYR1* protein molecule could bind approximately one molecule of ABA, and the dissociation constant (K_d) was 87.5 μ M (Fig. 7). The stoichiometry (N) with a binding ratio of 1:1 between *FaPYR1* and ABA demonstrated that strawberry fruit *FaPYR1* could be an ABA receptor.

Discussion

The type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening

In plants, PP2C protein, serving as a negatively regulated hub in ABA signalling, was first integrated into the canonical ABA signalling network by genetics and structural biology in *Arabidopsis* (Fujii *et al.*, 2009). Although several studies previously reported that ABA plays an important role in strawberry fruit ripening (Jia *et al.*, 2011) and PP2C may be involved in the fruit maturation based on the results of

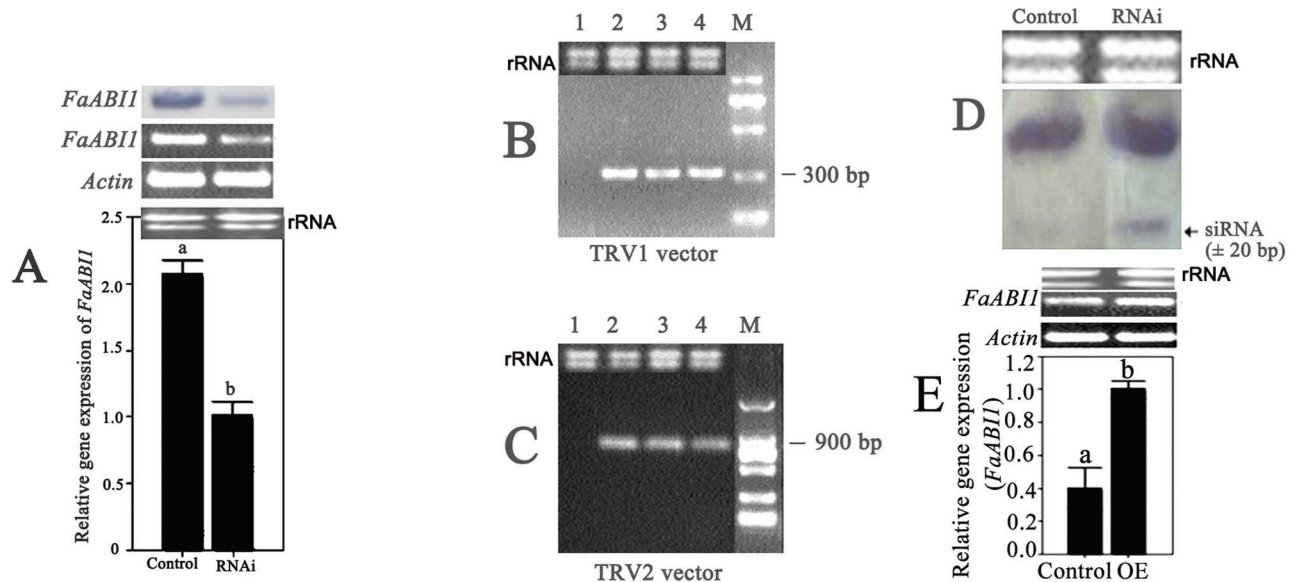


Fig. 4. Silencing and overexpression of the *FaABI1* gene in strawberry fruit at the molecular level. (A) Analysis of the *FaABI1* transcripts in control and RNAi fruit by northern blotting, semi-quantitative RT-PCR, and real-time PCR (from top to bottom). (B and C) Analysis of the transcripts of TRVs using RT-PCR. Five days after infiltration, the expression of virus vector genes [300 bp pTRV1 (B) and 900 bp pTRV2 (C)] was detected in fruit infiltrated with *Agrobacterium* containing TRV in white control fruit (lane 2, empty vector) and in two RNAi fruit (lanes 3 and 4), but not in fruit infiltrated with *Agrobacterium* alone (lane 1). (D) Detection of siRNA (~20 bp) specific to the *FaABI1* gene in control and RNAi fruit. (E) Analysis of the transcripts of *FaABI1* in control and overexpressing fruit using semi-quantitative RT-PCR and real-time PCR (from top to bottom). rRNA was the loading control for the RNA samples stained with ethidium bromide. *Actin* mRNA was used as an internal control. The error bars represent the standard error ($n=3$). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

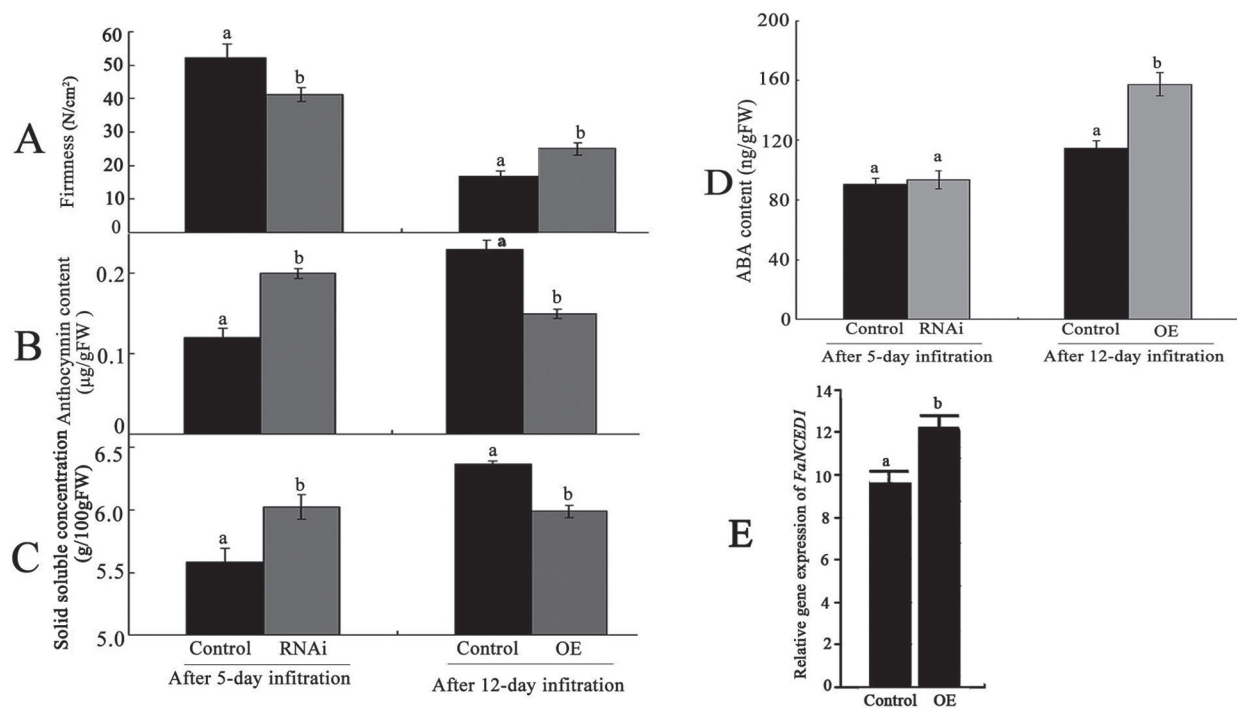


Fig. 5. Alteration of *FaABI1* expression affects several physiological parameters and *FaNCED1* transcripts in RNAi and OE fruit. The physiological parameters included (A) fruit firmness, (B) anthocyanin contents, (C) solid soluble concentrations, and (D) ABA contents. (E) *FaNCED1* transcripts in control and OE fruit. RNAi, *FaABI1*-silenced fruit, in which *FaABI1* was down-regulated by 50%; OE, *FaABI1*-overexpressing fruit, in which *FaABI1* was up-regulated by 60%. The error bars represent the standard error ($n=3$). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

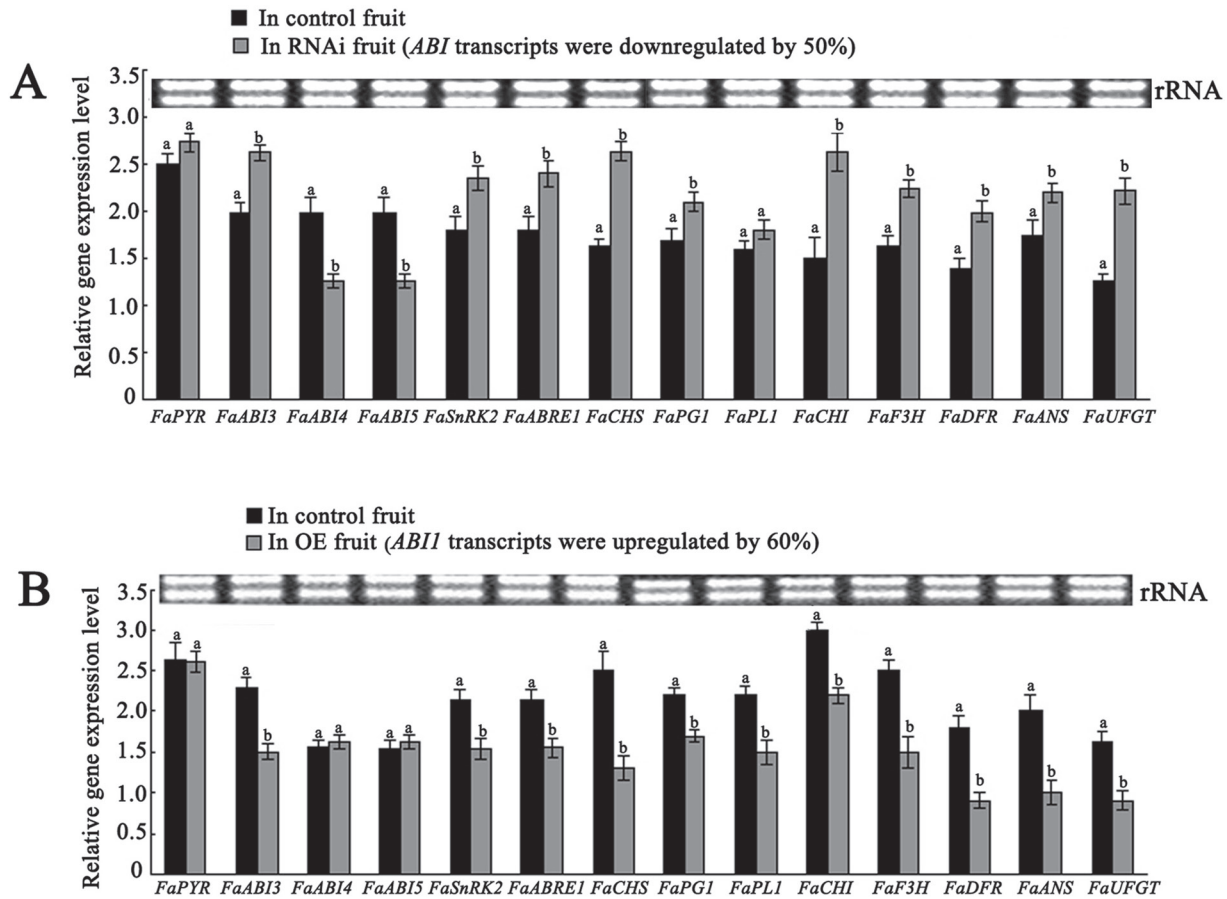


Fig. 6. Alteration of *FaABI1* expression affects transcripts of a set of ABA-responsive and ripening-related genes in RNAi and overexpressing fruit. (A) The mRNA expression levels of ABA signalling and ripening-related genes in RNAi fruit, in which the *FaABI1* transcript was down-regulated by 50%. (B) The mRNA expression levels of ABA signalling and ripening-related genes in overexpressing fruit, in which the *FaABI1* transcript was up-regulated by 60%. *Actin* mRNA was used as an internal control. The error bars represent the standard error ($n=3$). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

transcriptional analyses (Gambetta *et al.*, 2010; Sun *et al.*, 2011), the defined function of PP2C in fruit ripening remains unclear.

In the present study, it was found that the levels of *FaABI1* mRNA expression decrease rapidly during strawberry fruit development (Fig. 2), suggesting that this PP2C protein may negatively regulate fruit development. Importantly, the down- and up-regulation of *FaABI1* mRNA expression levels in DG strawberry fruit promotes and inhibits ripening, respectively (Fig. 3), indicating that the PP2C protein is a negative regulator of fruit ripening. Furthermore, this notion is also confirmed by transcriptional analysis of a set of both ABA signalling and ripening-related genes such as *ABI3*, *SnRK2*, *ABRE1*, *PG1*, *PL1*, and *CHS* in *FaABI1* RNAi and OE fruit (Fig. 6). The analysis showed that the transcripts of the positive ABA signalling genes, including *ABI3*, *SnRK2*, and *ABRE1*, and firmness/pigment-related genes, including *CHS* and *PG1*, are up-regulated significantly in the RNAi fruit, in which the mRNA expression level of *FaABI1* is down-regulated by 50%; while they are down-regulated remarkably in the OE fruit, in which the mRNA expression level of *FaABI1* is up-regulated by 60% (Fig. 6). These results were consistent

with a negative role for PP2C in ABA signalling during fruit ripening. Taken together, this study has provided physiological and molecular evidence to demonstrate that the type 2C protein phosphatase *FaABI1* is a negative regulator of strawberry fruit ripening.

The receptor nature of FaPYR1 lays the molecular foundation for FaABI1 serving as a negative regulator in strawberry fruit ripening

In *Arabidopsis*, the ABA–PYR1–PP2C–SnRK2 core signalling pathway of ABA action has been established, by which ABA signal transduction consists of a double-negative regulatory mechanism, namely ABA-bound PYR inhibits PP2C activity and PP2Cs inactivate SnRK2s (Fujii *et al.*, 2009). According to this ABA action model, in the absence of ABA, the signalling transduction is blocked by PP2C activity; in the presence of ABA, the signalling transduction is relayed by ABA-bound PYR-inhibited PP2C activity. In a previous study and the present study, it is shown that in green strawberry fruit, low ABA levels (Chai *et al.*, 2011) and the resultant relatively high levels of PP2C expression might block

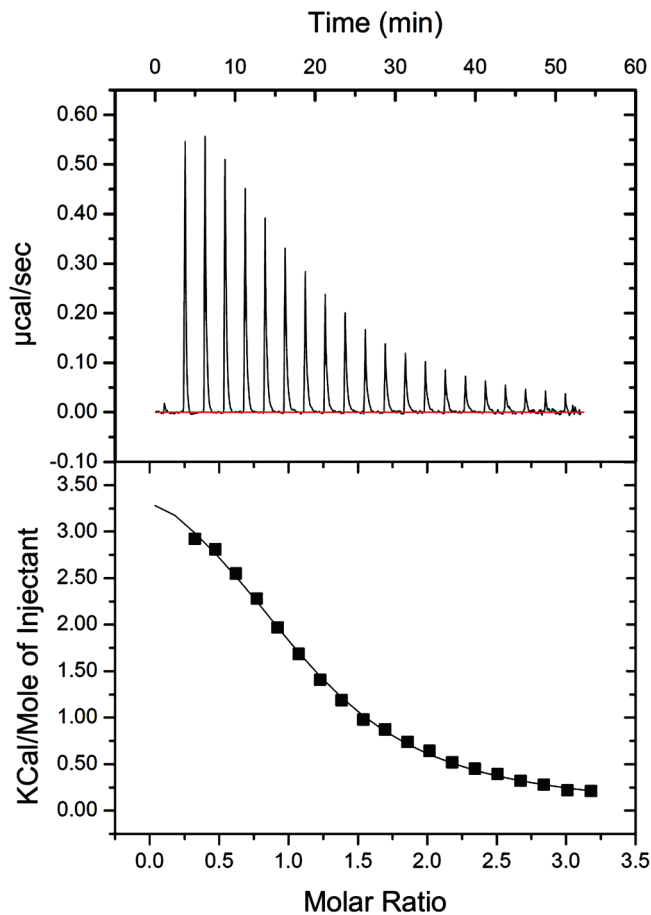


Fig. 7. Measurement of binding affinity between (+)-ABA and purified FaPYR1 protein using isothermal titration calorimetry (ITC). A typical and specific saturation curve with stoichiometry (N) ~1:1 was obtained, suggesting that one ABA molecule could bind per purified protein molecule with a dissociation constant (K_d) of 87.5 μ M.

ABA signalling transduction and, as a result, inhibit the burst of the ripening process at these stages. In red fruit, high ABA levels (Chai et al., 2011) and the resultant relatively low levels of PP2C expression might evoke ABA signalling transduction and thus promote the ABA-regulated ripening-related genes such as *CHS*, *PGI*, *PLI*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*, and finally lead to the fruit ripening.

The committed step of ABA action is initiated by ABA perception with its receptor. Thus, determination of the receptor nature of FaPYR1 contributes to understanding the negative mechanism of the FaABI1 protein. It was not only demonstrated previously that FaPYR is a positive regulator of strawberry fruit ripening (Chai et al., 2011), but the present study also validated that the binding of one ABA molecule with one purified FaPYR1 protein molecule was determined by stoichiometry (N) with a ratio of ~1:1 as determined by isothermal titration calorimetry analysis (Fig. 7), demonstrating that strawberry FaPYR1 is also an ABA receptor. It is interesting to note that Chai et al. (2011) found that down-regulation of FaPYR1 expression significantly promotes ABA accumulation in the white section of RNAi

fruit, and the ABA increased by feedback results from the blocked FaPYR1 signalling. Similarly, in the present study, up-regulation of *FaABI1* expression repressed ABA signalling in the white section of OE fruit, and consequently led to the feedback accumulation of ABA by an increase in *NCED1* expression. These results were consistent with a positive and negative role for PYR1 and PP2C, respectively, in ABA signalling during fruit ripening. The positive role of FaPYR1 together with the negative role of FaABI1 in regulation of fruit ripening may suggest that ABA perception and signalling transduction underlying PYR1–PP2C–SnRK2 might be a core mechanism in non-climacteric fruit. Reconstitution of an ABA–FaPYR1–FaABI1–FaSnRK2 signalling pathway *in vitro* will be an important task for future studies.

Acknowledgements

We would like to thank Professor Liu Yu-le for pTRV vectors. This work was supported financially by the National Key Basic Research ‘973’ Program of China (grant no. 2012CB126306), the National Science Foundation of China (grant nos 30971977 and 31272144), and the Newstar of Science and Technology supported by Beijing Metropolis (grant no. Z111105054511048).

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