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

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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) (Fuji 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 (Fuji et al., 2009). In this model, the early reported PP2Cs served 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), first integrated into the canonical ABA signalling network by reversible phosphorylation (Fuji 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 Ca\(^{2+}\)-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 (Mg\(^{2+}\) dependent) (Cohen and Cohen, 1989; Sopory and Giraudat, 1997; Gosti et al., 1999; Merlot et al., 2001), which is a universal mechanism for regulating diverse biological functions in eukaryotes (Smith, 1996).

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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 SIPY1L, SIPY1L2, 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 FaABI1 gene in strawberry fruit were performed. The results showed that PP2C1 encoded by FaABI1 is a negative regulator of strawberry fruit ripening.

Materials and methods

Plant material

Strawberry (Fragaria‘ananassa’ cv. Camarosa) was grown in a glasshouse (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 days 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 FaABI1 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 A260/A280 ratios.

To clone the FaABI1 gene, an Arabidopsis PP2C protein ABI1 (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′-ATGGAGGAGCATGCTTCACGAGC-3′; reverse, 5′-TCACAGTTGCTCTTTGACTTTCC-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 FaABI1 were designed based on the determined sequences (forward, 5′-CAGCGAGTTAAAGTGGGAGAG-3′; reverse, 5′-AGGG CAAGAGTTGAAGATAAG-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 from Dr Liu Yu-le, Qinghua University. A 584 bp cDNA fragment near the 5′ end of FaABI1 was amplified using appropriate primers (forward, 5′-GGTGAACCATTCACCAACAG-3′; reverse, 5′-GGGTCAACTCTGGCTCTCC-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-FaABI1 was used for Agrobacterium-mediated infiltration. The pTRV1 and pTRV2 vectors (Liu et al., 2002) were kind gifts from Dr Liu Yu-le, Qinghua University. A 584 bp cDNA fragment near the 5′ end of FaABI1 was amplified using appropriate primers (forward, 5′-GGTGAACCATTCACCAACAG-3′; reverse, 5′-GGGTCAACTCTGGCTCTCC-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-FaABI1 was used for Agrobacterium-mediated infiltration. The pTRV1 and pTRV2 vectors (Liu et al., 2002) were kind gifts from Dr Liu Yu-le, Qinghua University. A 584 bp cDNA fragment near the 5′ end of FaABI1 was amplified using appropriate primers (forward, 5′-GGTGAACCATTCACCAACAG-3′; reverse, 5′-GGGTCAACTCTGGCTCTCC-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-FaABI1 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′-ATGGAGGAGCATGCTTCACGAGC-3′; reverse, 5′-TCACAGTTGCTCTTTGACTTTCC-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).

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

| Genes | Sequences for real-time PCR | Sources of primers |
|-------|----------------------------|--------------------|
| ABI1  | Sense: 5'-CAAGAGCCACATTGTGCGTGC-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-TGGAATACCTGAGGTTTTCA-3' | |
| SnRK2 | Sense: 5'-GCACTTCGCTACAGAGCTG-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-AGGAATCTGAGGTTGGATGATT-3' | |
| ABI3  | Sense: 5'-CGGGCCTGTGTAGTACGCGGA-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-TGCACTTCGCTACAGAGCTG-3' | |
| ABI4  | Sense: 5'-TCCTCTACACCAAGCTTCTT-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-ACCTTCGCTACACTGTTGCT-3' | |
| ABI5  | Sense: 5'-GGACGTGGCAATGGTGCG3-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-AGGCCCCCCTCTTCC-3' | |
| Actin | Sense: 5'-TGGGTGTGGCTGAGTATG-3' | Chai et al. (2011); Jia et al. (2011) |
|       | Antisense: 5'-CAAGTGGCAATGGTGCG3-3' | |
| DFR   | Sense: 5'-ACGAAGTGATAAAGCCAACA-3' | GenBank accession number AB201755 |
|       | Antisense: 5'-TTTTCTGAGCAATGGGAGG-3' | |
| CHI   | Sense: 5'-GGTGGTGACAGAAGATGC-3' | Gene 28250 locus in https://strawberry.plantandfood.co.nz/index.html |
|       | Antisense: 5'-CCCAAACGGCACGAAATG-3' | GenBank accession number HQ290318.1 |
| F3H   | Sense: 5'-CTGACTCCCTTGCTGTCTTTT-3'. | GenBank accession number EF441273 |
|       | Antisense: 5'-CTGGAGCAAGGTTGAGGTTG-3' | |
| PG1   | Sense: 5'-CGGCGCCTGTATTAGTCCC-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-GGAAGCTGGCAATGGTCG-3' | |
| PL1   | Sense: 5'-TGGGTGTGGCTGAGTATG-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-CCCAAACGGCACGAAATG-3' | |
| NCED1 | Sense: 5'-TGGGTGTGGCTGAGTATG-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-CCCAAACGGCACGAAATG-3' | |
| ABRE1 | Sense: 5'-TGGGTGTGGCTGAGTATG-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-CCCAAACGGCACGAAATG-3' | |
| CHS   | Sense: 5'-CGGCGCCTGTATTAGTCCC-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-GGAAGCTGGCAATGGTCG-3' | |
| PG1   | Sense: 5'-CGGCGCCTGTATTAGTCCC-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-GGAAGCTGGCAATGGTCG-3' | |
| NCED1 | Sense: 5'-TGGGTGTGGCTGAGTATG-3' | GenBank accession number EF441274 |
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|       | Antisense: 5'-GGAAGCTGGCAATGGTCG-3' | |
| F3H   | Sense: 5'-CTGACTCCCTTGCTGTCTTTT-3'. | GenBank accession number EF441274 |
|       | Antisense: 5'-CTGGAGCAAGGTTGAGGTTG-3' | |
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|       | Antisense: 5'-CCCAAACGGCACGAAATG-3' | |
| CHS   | Sense: 5'-CGGCGCCTGTATTAGTCCC-3' | GenBank accession number EF441274 |
|       | Antisense: 5'-GGAAGCTGGCAATGGTCG-3' | |

was used for RNA interference (RNAi). For overexpression, the 1641 bp cDNA was amplified using appropriate primers (forward, 5'-TCTAGAATCTGGAGACATGTGCTCAGAGCAG-3'; reverse, 5'-GAAGCTGGAGATATGTTGTTAC-3'), cloned into the pMD 19-T simple vector (TaKaRa), digested with Xba I and Sac I, and subsequently cloned into the binary expression vector PB121 cut with the same restriction enzymes. Agrobacterium strain GV3101 containing PBI121 or the PBI121 derivative PBI121-FaABI11 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 for detection of each parameter, 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 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 (1968, a, 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'-CAGGAAGGAGTTGAAAGATAAG-3'; reverse, 5'-AGGCCAGTGGCTACGAGAA-3'). For the small
interfering RNA (siRNA) test. siRNA was extracted from 10 g of flesh using the miReute 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 ABI1-specific probe corresponding to the FaABI1 region (forward, 5’-GGTAAACATCCATCCATCAAG-3’; reverse, 5’-GGGTCACACTGGCTCATTCC-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 RNA, 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 (FaABI1, forward, 5’-CAGCAGGTGAATGTTAGCG (reverse, GAGGACATGTCTCCAGCAG-3’); and FaABI1, BamHI site) 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 ATGGAGAAGCTT GTCGAC (BamHI site); and reverse, GGCGCGCCG CGCACATCTGGAGGATTAGCG (NotI site)] and cloned into the expression vector pET28a (Novagen, Germany). The modified fusion protein contains two His-tags at both the N-term and the C-term. BL21(DE3) cells transformed with the expression plasmid were grown in 1 litre of LB medium containing 50 µg ml–1 kanamycin to 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-NiTA 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-NiTA His Bind slurry and mixed gently by shaking at 4 °C for 60 min. The lysate–resin mixture was loaded on 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 microcalorimeter (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 FaABI1 gene

To clone the FaABI1 gene, the Arabidopsis PP2C protein ABI1 (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’-TCAAGATTTGCTCTTGAACTTTC-3’) were designed to amplify a coding sequence from ‘Camarosa’ strawberry fruit by RT–PCR. A 1641 bp cDNA homologous to Arabidopsis ABI1, named FaABI1, 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 FaABI1 protein on the NCBI protein blast website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 1B), indicating successful isolation of the FaABI1 gene encoding the putative strawberry PP2C.

To investigate further the members of the FaABI1 gene family in the strawberry genome, Southern blot analysis was performed using a probe corresponding to the FaABI1-coding cDNA sequence. Genomic DNA was digested with XhoI, XbaI, NcoI, and BamHI 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 FaABI1 gene family with multiple members.

Expression analysis of the FaABI1 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
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 \textit{FaABI1} gene is involved in strawberry fruit ripening, the mRNA expression levels of \textit{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 \textit{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 \textit{FaABI1} transcripts and fruit size and colour suggested that \textit{FaABI1} might negatively regulate strawberry fruit ripening.

Silencing of the \textit{FaABI1} gene promotes strawberry fruit ripening

To confirm the function of the \textit{FaABI1} gene in strawberry fruit development, a 584 bp cDNA fragment of the \textit{FaABI1}
gene (from 207 bp to 790 bp) was cloned based on the coding sequence isolated above and inserted into the TR V2 virus vector using the restriction enzymes Xba I and Sac I. A mixture of Agrobacterium tumefaciens strain GV3101 cultures containing pTR V1 and pTR V2 or pTR V2 carrying the 584 bp fragment of the FaABI1 gene (pTR V2-FaABI1 584) in a 1:1 ratio was inoculated into DG fruit using a syringe (Fig. 3A). Control fruit were inoculated with TR V 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 XbaI and a SacI 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 (PL1), 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).
Transcriptional analysis of the *FaNCED1* gene key to ABA biosynthesis showed that the mRNA expression level of the *NCED1* 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*, *PGI*, *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 (*PGI* and *PL1*), sugar (*SnRK2*), and pigment (*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*)-related genes through the *FaABI1*-mediated signalling 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* (*Fuji 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 (*Kd*) was 87.5 µM (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* (*Fuji 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.
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. 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.](image)
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 \textit{CHS}, \textit{PGI}, \textit{PL1}, \textit{CHI}, \textit{F3H}, \textit{DFR}, \textit{ANS}, and \textit{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 \textit{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 \textit{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–FuSnRK2 signalling pathway \textit{in vitro} will be an important task for future studies.

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