RESEARCH PAPER

Transcriptional regulation of *SIPYL*, *SIPP2C*, and *SISnRK2* gene families encoding ABA signal core components during tomato fruit development and drought stress

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Abstract

In order to characterize the potential transcriptional regulation of core components of abscisic acid (ABA) signal transduction in tomato fruit development and drought stress, eight *SIPYL* (ABA receptor), seven *SIPP2C* (type 2C protein phosphatase), and eight *SISnRK2* (subfamily 2 of SNF1-related kinases) full-length cDNA sequences were isolated from the tomato nucleotide database of NCBI GenBank. All *SIPYL*, *SIPP2C*, and *SISnRK2* genes obtained are homologous to *Arabidopsis AtPYL*, *AtPP2C*, and *AtSnRK2* genes, respectively. Based on phylogenetic analysis, *SIPYL*s and *SISnRK2*s were clustered into three subfamilies/subclasses, and all *SIPP2C*s belonged to PP2C group A. Within the *SIPYL* gene family, *SIPYL1*, *SIPYL2*, *SIPYL3*, and *SIPYL6* were the major genes involved in the regulation of fruit development. Among them, *SIPYL1* and *SIPYL2* were expressed at high levels throughout the process of fruit development and ripening; *SIPYL3* was strongly expressed at the immature green (IM) and mature green (MG) stages, while *SIPYL6* was expressed strongly at the IM and red ripe (RR) stages. Within the *SIPP2C* gene family, the expression of *SIPP2C*, *SIPP2C3*, and *SIPP2C4* increased after the MG stage; *SIPP2C1* and *SIPP2C5* peaked at the B3 stage, while *SIPP2C2* and *SIPP2C6* changed little during fruit development. Within the *SISnRK2* gene family, the expression of *SISnRK2.2*, *SISnRK2.3*, *SISnRK2.4*, and *SISnRK2C* was higher than that of other members during fruit development. Additionally, most *SIPYL* genes were down-regulated, while most *SIPP2C* and *SISnRK2* genes were up-regulated by dehydration in tomato leaf.

Key words: ABA signal transduction, drought, expression analysis, fruit development and ripening, *SIPP2C*, *SIPYL*, *SISnRK2*, tomato.

Introduction

Abscisic acid (ABA) plays important roles in various aspects of plant growth and development, such as adaptation to different biotic and abiotic stresses, seed maturation and germination, as well as fruit development and ripening. ABA has been shown to regulate tomato fruit set with other plant hormones, such as gibberellin and auxin (Vriezen *et al.*, 2008; Nitsch *et al.*, 2009). ABA-deficient tomato mutants with only 25% of wild-type ABA levels have a decreased total fruit weight and average fruit weight, but accumulate 30% more carotenoids in ripening fruit and release more ethylene when stored at room temperature (Galpaz *et al.*, 2008). Increased ABA levels have also been

observed during the onset of fruit ripening in many species, suggesting the involvement of ABA during this process (Vendrell and Buesa, 1989; Buta and Spaulding, 1994; Kojima, 1996; Kondo and Tomiyama, 1998; Zhang *et al.*, 2009a, b; Sun *et al.*, 2010). In cooperation with other phytohormones such as ethylene, ABA may promote fruit ripening in both climacteric (Jiang *et al.*, 2000; Zhang *et al.*, 2009a) and non-climacteric fruit (Sun *et al.*, 2010). ABA can also enhance pigment accumulation and post-harvest qualities in both citrus fruit and grapes (Alferez *et al.*, 2005; Cantin *et al.*, 2007). Taken together, these findings indicate that the physiological functions of ABA are relevant

throughout the whole process of fruit development and ripening.

With the advantage of molecular biotechnology, the functions of many genes involved in ABA biosynthesis and metabolism have been investigated in many species (Schwartz et al., 1997; Krochko et al., 1998; Chernys and Zeevaart, 2000; Rodrigo et al., 2006; Ren et al., 2010a), including tomato (Galpaz et al., 2008; Nitsch et al., 2009; Zhang et al., 2009a) which is considered a model system for studying climacteric fruit ripening (Barry and Giovannoni, 2007). However, the integrated ABA signal transduction pathway and its mechanism are not clearly defined. Although some ABA receptor-like proteins had been reported (Shen et al., 2006; Pandey et al., 2009), their functions in transducing ABA signals are still being debated (Muller and Hansson, 2009; Wu et al., 2009; Ren et al., 2010b).

Recently, two separate research teams reported a new type of ABA receptor named PYR/PYL/RCAR (Ma et al., 2009; Park et al., 2009). In this newly discovered ABA signal transduction pathway, there are three core components: ABA receptors PYR/PYL/RCARs (hereafter the 'PYLs'), negative regulators named PP2Cs (type 2C protein phosphatases), and positive regulators termed SnRK2s (subfamily 2 of SNF1-related kinases). The PYLs, which belong to the START protein superfamily, can sense and bind to ABA by their ligand-binding pockets (Ma et al., 2009; Melcher et al., 2009; Nishimura et al., 2009; Park et al., 2009). After binding to ABA, PYL closes two highly conserved β -loops, which function as a 'gate' and 'latch', around the entry of the ligand-binding pocket and forms a 'gate-latch' interface, which in turn binds to the PP2C active site and inhibits PP2C from dephosphorylating SnRK2 (R Yoshida et al., 2006; Fujii et al., 2009; Melcher et al., 2009). Finally, SnRK2 is then activated and can phosphorylate downstream effectors, such as the basic leucine zipper transcription factors ABFs/AREBs, thus switching on the transcription of ABA-responsive genes (Kobayashi et al., 2005; Furihata et al., 2006).

Among the three ABA core components, 13 PYLs (Ma et al., 2009; Park et al., 2009), nine group A PP2Cs (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998; Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006; T Yoshida et al., 2006; Nishimura et al., 2007; Umezawa et al., 2009) and 10 SnRK2s (R Yoshida et al., 2002, 2006; Hrabak et al., 2003; Boudsocq et al., 2004; Belin et al., 2006; Fujita et al., 2009) have been identified in *Arabidopsis*. Many homologous genes belonging to the PYL, PP2C, and SnRK2 gene families in other species, such as *Oryza sativa* and *Zea mays*, have also been identified (Miyazaki et al., 1999; Gonzalez-Garcia et al., 2003; Huai et al., 2008; Umezawa et al., 2010). The functions of genes mentioned above in response to ABA treatment and abiotic stresses, such as drought, have been investigated at the protein and transcriptional levels (Miyazaki et al., 1999; Huai et al., 2008; Li et al., 2009; Park et al., 2009; Umezawa et al., 2009). However, due to the different aims of these studies, expression patterns of *PYL*, *PP2C*, and *SnRK2* genes were not investigated systematically as a whole; meanwhile, little attention has been paid to their functions in fruit.

In this study, 23 genes belonging to the *PYL*, *PP2C*, and *SnRK2* gene families were identified in tomato. Their expression patterns were also investigated during fruit development and under drought stress. From the results obtained in this work, it is hoped to characterize the potential transcriptional regulation of core components of ABA signal transduction in tomato fruit development and drought stress, and deepen the understanding of ABA's biological effects in those processes.

Materials and methods

In silico analysis

The *PYL*, *PP2C*, and *SnRK2* full-length cDNA sequences of *Arabidopsis*, *Z. mays*, *O. sativa*, and tomato were obtained from the nucleotide database of NCBI (<http://www.ncbi.nlm.nih.gov/nucleotide/>) as reported in previous studies (Tao et al., 2004; Yuasa et al., 2007; Huai et al., 2008; Ma et al., 2009; Melcher et al., 2009; Park et al., 2009; Umezawa et al., 2010). To identify new homologues in tomato, the cDNA sequences of *PYL*, *PP2C*, and *SnRK2* gene families in *Arabidopsis*, *Z. mays*, *O. sativa*, and tomato were subjected to blastn searches (<http://blast.ncbi.nlm.nih.gov/>) against the nucleotide collection database in tomato. The open reading frames (ORFs) were determined by using NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

Phylogenetic analysis

Deduced amino acid sequences of SIPYLs, SIPP2Cs, and SLSnRK2s were aligned with the homologous proteins in *Arabidopsis*, *Z. mays*, and/or *O. sativa* using ClustalX 2.0.12 software in the default setting. The alignment results were edited and marked using BOXSHADE 3.21 software (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic trees were constructed by using the Neighbor-Joining (N-J) method in MEGA 4.0.2 software with the bootstrap analysis setting at 1000 replicates for evaluating the reliability of different phylogenetic groups. Tree files were viewed and edited using MEGA 4.0.2 software.

Plant materials

Tomatoes (*Solanum lycopersicum* L. cv. Hongyu) were grown under standard greenhouse conditions (25 $\pm$ 5 °C and 70% humidity under a 14 h/10 h light/dark regime). Tomato seedlings, used for water stress treatments, were grown in plugs in a tissue culture room at 25 $\pm$ 2 °C with a 16 h light/8 h dark cycle. Fruit ripening stages were divided according to days after flowering (DAF) and fruit colour (Fig. 3): immature green (IM), 15 DAF; mature green (MG), 33 DAF; breaker 1 (B1), 38 DAF; breaker 2 (B2), 39 DAF; breaker 3 (B3), 40 DAF; breaker 4 (B4), 41 DAF; turning 1 (T1), 44 DAF; turning 2 (T2), 48 DAF; and red ripe (RR), 54 DAF. Five fruit were harvested at each stage, and the root, stem, and leaf used for organ-specific expression analysis were sampled from 25-day-old seedlings which were grown under normal soil moisture. All fruit and samples were immediately frozen with liquid nitrogen, powdered, mixed, and stored at -80 °C until further use.

Water stress treatments

The 25-day-old tomato seedlings were divided into three groups with 10 seedlings in each. Leaves of seedlings in group I, growing under normal soil moisture, were sampled as controls. Seedlings in group II were grown without watering for 4 d, and then the leaves were sampled when wilting. Group III was first left unwatered for 4 d as described for group II, and then were watered and incubated for 1 h before sampling the leaves. Leaves sampled from each group were immediately frozen with liquid nitrogen,

powdered, mixed, and stored at -80°C for further use. The entire experiment was repeated twice. For testing the effects of treatment, expression of *SINCE1* and *SICYP707A2* which encode key enzymes in ABA biosynthesis and degradation, respectively, was investigated as shown in Supplementary Fig. S7 available at *JXB* online.

ABA treatment

Twenty fruit at each stage of IM, MG, B3, and T2 were harvested, divided into two groups ($n=10$ per group), and immediately soaked in a $100\ \mu\text{mol l}^{-1}$ ABA (Sigma, A1049) water solution (group I) or distilled water (group II, control) for 10 min. The fruit were then placed in a tissue culture room at $25\pm 2^{\circ}\text{C}$ and 95% relative humidity. Absorbent cotton was placed on each fruit sepal and stalk. A 3 ml aliquot of $100\ \mu\text{mol l}^{-1}$ ABA water solution (group I) or 3 ml of distilled water (group II, control) was added to the absorbent cotton immediately and then every 6 h. After 12 h, all fruit were sampled, frozen with liquid nitrogen, powdered, mixed, and stored at -80°C for further use. For testing the effect of this method, the expression of *SINCE1* and *SICYP707A2* which encode key enzymes in ABA biosynthesis and degradation, respectively, was investigated in the MG stage (Supplementary Fig. S7 at *JXB* online).

Determination of ABA content

For ABA extraction, 1 g of flesh or leaf 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 C_{18} cartridge (Waters, Milford, MA, USA) to remove polar compounds, and then stored at -20°C for enzyme-linked immunosorbent assay (ELISA). The ELISA procedures were conducted according to the instructions provided by the manufacturer (China Agricultural University, Beijing, China). ABA was determined by Thermo Electron (Labsystems) Multiskan MK3 (PIONEER Co., China).

Quantitative real-time PCR analysis

Total RNA was isolated from tomato samples using the hot borate method (Wan and Wilkins, 1994). Genomic DNA was eliminated using an RNase Free DNase I kit (Takara, Dalian, China) according to the manufacturer's recommendations. The quality and quantity of every RNA sample were assessed by agarose gel electrophoresis. The cDNA was synthesized from the total RNA using the PrimeScriptTM RT reagent kit (Takara) according to the manufacturer's instructions. Primers used for real-time PCR, designed using Primer 5 software, are listed in Supplementary Table S1 at *JXB* online. The *SAND* gene (SGN-U573169) encoding the SAND protein was selected as an internal control gene according to Exposito-Rodriguez *et al.* (2008), and the stability of its expression was tested in preliminary studies shown in Supplementary Fig. S6. All primer pairs were tested by PCR. A single product of the correct size for each gene was confirmed by agarose gel electrophoresis and double-strand sequencing (Invitrogen, Beijing, China). The amplified fragment of each gene was subcloned into the pMD18-T vector (Takara) and used to generate standard curves by serial dilution. The real-time PCR was conducted using a Rotor-Gene 3000 system (Corbett Research, Australia) with SYBR Premix Ex TaqTM (Takara). Each 20 μl reaction contained 0.8 μl of primer mix (containing 4 μM of each forward and reverse primer), 1.5 μl of cDNA template, 10 μl of SYBR Premix Ex TaqTM (2 $\times$) mix, and 7.7 μl of water. Reactions were carried out under the following conditions: $95^{\circ}\text{C}/30\ \text{s}$ (one cycle); $95^{\circ}\text{C}/15\ \text{s}$, $60^{\circ}\text{C}/20\ \text{s}$; $72^{\circ}\text{C}/15\ \text{s}$ (40 cycles). Relative fold expression changes were calculated using the relative two standard curves method with Rotor-Gene 6.1.81 software.

Results

Gene isolation and analysis

The eight *SIPYL*, seven *SIPP2C*, and eight *SISnRK2* full-length cDNA sequences from tomato were isolated and designated as *SIPYL1*–*SIPYL8*; *SIPP2C1*–*SIPP2C6* and *SIPP2C7*; and *SISnRK2.1*–*SISnRK2.7* and *SISnRK2C*, respectively (Fig. 1). Among them, *SIPYL1*–*SIPYL8*, *SIPP2C3*–*SIPP2C6* and *SISnRK2.1*–*SISnRK2.7* were not previously reported.

The deduced amino acid sequence lengths of *SIPYL*s, *SIPP2C*s, and *SISnRK2*s were 181–231, 281–546, and 336–362, respectively (Fig. 1; Supplementary Figs S1–S3 at *JXB* online). The similarities of the deduced amino acid sequences within each gene family were 35.06–84.13% for *SIPYL*s (Supplementary Table S2), 15.41–79.80% for *SIPP2C*s (Supplementary Table S3), and 60.75–82.13% for *SISnRK2*s (Supplementary Table S4). Based on multiple alignments of these protein sequences (Fig. 1; Supplementary Figs S1–S3), most of the functional residues or domains were observed to be well conserved within each gene family. Based on the phylogenetic analysis (Fig. 1; Supplementary Fig. S5), the *SIPYL* and *SISnRK2* protein families each clustered into three subfamilies/subclasses: *PYL* subfamily I, *SIPYL1*, *SIPYL2*, and *SIPYL3*; *PYL* subfamily II, *SIPYL4*, *SIPYL6*, and *SIPYL7*; *PYL* subfamily III, *SIPYL5* and *SIPYL8* (Fig. 1A); *SnRK2* subclass I, *SISnRK2.1*, *SISnRK2.2*, and *SISnRK2.6*; *SnRK2* subclass II, *SISnRK2.5*, *SISnRK2.7*, and *SISnRK2C*; and *SnRK2* subclass III, *SISnRK2.3* and *SISnRK2.4* (Fig. 1C; Supplementary Fig. S5B). Subclasses/subfamilies were named according to Ma *et al.* (2009) and Umezawa *et al.* (2010). All *SIPP2C*s belonged to *PP2C* group A (Fig. 1B; Supplementary Fig. S5A).

Expression of *SIPYL*, *SIPP2C*, and *SISnRK2* genes in tomato during fruit development and ripening

Compared with other genes of the *SIPYL* gene family (Fig. 2A), *SIPYL1* and *SIPYL2* were expressed at high levels throughout the process of fruit development and ripening, and their expression patterns could be clearly divided into three periods: early development, breaker, and ripening. Both of these genes peaked at the B3 stage. Other genes, such as *SIPYL3*, *SIPYL5*, *SIPYL7*, and *SIPYL8*, generally followed a decreasing expression pattern; among them, *SIPYL3* was strongly expressed at the IM and MG stages. *SIPYL6* was expressed strongly at the IM and RR stages, while *SIPYL4* was consistently expressed at extremely low levels at all maturity stages.

Within the *SIPP2C* gene family (Fig. 3A), *SIPP2C*, *SIPP2C3*, and *SIPP2C4* generally followed an increasing expression pattern after the MG stage; among them, *SIPP2C* was expressed at a much higher level than the other genes. *SIPP2C1* and *SIPP2C5* were both strongly expressed during the breaker stages and peaked at stage B3. Compared with the other genes, *SIPP2C2* and *SIPP2C6* were expressed at low levels and changed little during the whole process of fruit development and ripening.

Fig. 1. Sequence alignments and phylogenetic trees of the *PYL*, *PP2C*, and *SnRK2* gene families. Genes studied are marked with black diamonds. Amino acid sequences are shown only for functional residues and domains. Residue positions are marked with the numbers nearby. Conserved residues are marked with black or grey shading. Phylogenetic trees are shown only with their topological structures. (A) Sequence alignment and phylogenetic tree of the *PYL* family. Residues forming the ligand-binding pocket are marked with black triangles. The gate and latch domains are indicated. Functional residues and domains are based on reports by Melcher *et al.* (2009) and Santiago *et al.* (2009). (B) Sequence alignment and phylogenetic tree of the *PP2C* family. Residues interacting with ABA, PYLs, and Mn/Mg ions are marked with black triangles, asterisks, and white triangles, respectively. Phosphatase sites are marked with black circles. Functional residues and domains are based on studies by Melcher *et al.* (2009) and Santiago *et al.* (2010). (C) Sequence alignment and phylogenetic tree of the *SnRK2* family. Potential phosphatase sites are marked with black circles according to Umezawa *et al.* (2009). Functional residues and domains are noted according to Yoshida *et al.* (2006) and Yuasa *et al.* (2007). (D) Sequence alignment of C-terminal regions of subclass III *SnRK2*s. Functional domains are noted according to Yoshida *et al.* (2006) and Huai *et al.* (2008).

The genes within the *SISnRK2* family (Fig. 4A) could be clearly divided into three groups according to their expression levels. *SISnRK2.2*, *SISnRK2.3*, *SISnRK2.4*, and *SISnRK2C* belonged to the first group with high expression levels at all maturity stages; among them, *SISnRK2C* expression continued to increase after the MG stage and had two peaks at the B3 and T2 stages. Other genes followed a decreasing expression pattern from the IM to MG stages, after which they were expressed differently, with *SISnRK2.3* and *SISnRK2.4* peaking at the B3 stage. Moreover, *SISnRK2.6* and *SISnRK2.7* were moderately expressed, while *SISnRK2.1* and *SISnRK2.5* were expressed at a low level.

Expression of *SIPYL*, *SIPP2C*, and *SISnRK2* genes in response to exogenous ABA treatment in tomato fruit

All tested genes within the *SIPYL* family (Fig. 5A) were up-regulated by exogenous ABA at the IM stage, but the influence was slight at the MG stage. At the B and T stages, different *SIPYL* genes showed different responses to ABA treatment. For example, *SIPYL2* was consistently up-regulated, while

SIPYL8 was consistently down-regulated by ABA throughout development.

Almost all *SIPP2C* genes were up-regulated by exogenous ABA at the IM and MG stages (Fig. 5B). At the B and T stages, *SIPP2C* genes showed variable responses to ABA treatment. Generally, *SIPP2C*, *SIPP2C2*, and *SIPP2C6* were up-regulated, while *SIPP2C3* and *SIPP2C4* were down-regulated. *SIPP2C1* and *SIPP2C5* showed almost no response to ABA treatment.

Within the *SISnRK2* gene family (Fig. 5C), all tested genes were up-regulated except for *SISnRK2.4* at the IM and MG stages. At the B stage, most of the *SISnRK2* genes showed a slight response to exogenous ABA, and all *SISnRK2* genes were down-regulated at the T stage, except for *SISnRK2.3* and *SISnRK2.4* which showed no significant changes.

Expression of *SIPYL*, *SIPP2C*, and *SISnRK2* genes in vegetative organs

All *SIPYL* genes were found to be expressed in all vegetative organs, except for *SIPYL4* which was very weakly expressed in leaf (Fig. 2B). The highest combined

Fig. 2. Expression of *SIPYL* genes. (A) Expression of *SIPYL* genes during fruit development. Genes with low expression are shown at the top. Maturity stages: immature green (IM), mature green (MG), breaker 1 (B1), breaker 2 (B2), breaker 3 (B3), breaker 4 (B4), turning 1 (T1), turning 2 (T2), red ripe (RR). (B) Combined expression levels of *SIPYL* genes in tomato vegetative organs and in response to dehydration. Vegetative organs: root (Rt), stem (St), leaf (L), dehydrated leaf (DL), rehydrated leaf (RL). (C) Colour-coded expression levels of *SIPYL* genes.

expression of *SIPYL* genes is in the leaf, followed by the root and then the stem (Fig. 2B).

All *SIPP2C* genes were also found to be expressed in all vegetative organs, except for *SIPP2C2* which was consistently expressed at low levels in all vegetative organs (Fig. 3B). The highest combined expression of *SIPP2C* genes is in the leaf, followed by the root and then the stem (Fig. 3B).

Within the *SISnRK2* gene family (Fig. 4B), *SISnRK2.1* was only expressed in the root, while all the other *SISnRK2* genes were expressed in all vegetative organs. The highest combined expression of *SISnRK2* genes is in the leaf, followed by the root and then the stem (Fig. 4B).

Expression of *SIPYL*, *SIPP2C*, and *SISnRK2* genes in response to dehydration in tomato leaf

Within the *SIPYL* gene family (Fig. 2B), all genes except for *SIPYL1* and *SIPYL8* were down-regulated by dehydration. *SIPYL1* was up-regulated by dehydration, while *SIPYL8* showed no response. After rehydration for 1 h, most of the *SIPYL* genes returned to their normal expression levels.

All genes within the *SIPP2C* gene family (Fig. 3B) were significantly up-regulated by dehydration, except for *SIPP2C6* which showed no response. After rehydration for 1 h, expression of all *SIPP2C* genes decreased but was still higher than their normal expression levels.

Within the *SISnRK2* gene family (Fig. 4B), all genes were up-regulated by dehydration, except for *SISnRK2.7* which showed nearly no response. After rehydration for 1 h, all *SISnRK2* genes decreased to levels at or lower than their normal expression levels.

Variations in ABA content during tomato fruit development and ripening and in response to dehydration in leaf

ABA content was determined during fruit development and ripening. As shown in Fig. 6, ABA was at its highest level at the IM stage and decreased to the lowest level at the MG stage. It increased and peaked again at the B3 stage, then decreased thereafter toward the RR stage. The ABA content was also determined in response to dehydration in tomato leaf. As shown in Fig. 6, the ABA content in dehydrated leaf increased to ~5 times that of the control leaf. After rehydration for 1 h, the ABA content of the leaf sharply decreased to 40% of that of the dehydrated leaf, but it was still higher than that of the control leaf.

Discussion

The mechanism of fruit ripening has long been studied, and ethylene has been demonstrated to be an important regulator of climacteric fruit (Barry and Giovannoni,

Fig. 3. Expression of *SIPP2C* genes. (A) Expression of *SIPP2C* genes during fruit development. Fruit colour and relative size at each stage are shown below the graph. (B) Combined expression levels of *SIPP2C* genes in tomato vegetative organs and in response to dehydration. (C) Colour-coded expression levels of *SIPP2C* genes.

Fig. 4. Expression of *SISnRK2* genes. (A) Expression of *SISnRK2* genes during fruit development. Genes with low expression levels are shown at the top. (B) Combined expression levels of *SISnRK2* genes in tomato vegetative organs and in response to dehydration. (C) Colour-coded expression of *SISnRK2* genes.

2007). However, the mechanism of non-climacteric fruit ripening is still unclear. Other plant hormones or effectors have also been shown to be involved in regulating fruit development and ripening (Vriezen *et al.*, 2008; Nitsch *et al.*, 2009). Among them, ABA has pleiotropic biological effects and correlates with multiple processes during fruit ripening (Vendrell and Buesa, 1989; Buta and Spaulding, 1994; Kojima, 1996; Kondo and Tomiyama, 1998; Alferez *et al.*, 2005; Cantin *et al.*, 2007). Therefore, in recent years, our laboratory has committed to studying the biological functions of ABA in the regulation of fruit development and ripening (Zhang *et al.*, 2009a, b; Ren *et al.*, 2010a, b; Sun *et al.*, 2010). However, the molecular mechanism of how ABA regulates fruit ripening has still not been fully clarified. Encouragingly, the ABA signal transduction pathway has been established by identification of three core components: PYL/RCAR, PP2C, and SnRK2 (Ma *et al.*, 2009; Park *et al.*, 2009). This breakthrough provided new leads for investigating the issue mentioned above.

The results in Fig. 1 and in Supplementary Figs S1–S5 at JXB online showed that the *SIPYL*, *SIPP2C*, and *SISnRK2* genes identified in tomato are homologous to *AtPYL*, *AtPP2C*, and *AtSnRK2* in *Arabidopsis*, respectively. Additionally, most of their deduced amino acid sequences

are well conserved in functional residues or domains (Fig. 1; Supplementary Figs S1–S4) within each gene family. Therefore, it can be presumed that *SIPYL* genes encode ABA receptors in tomato. Meanwhile, it is worth noting the presence of an amino acid mutation (R146K) in the latch domain of *SIPYL7* (Fig. 1A; Supplementary Fig. S1), as previously reported (Melcher *et al.*, 2009), which may decrease the ability of *SIPYL7* to respond to ABA. Compared with other members of the *SIPP2C* family (Fig. 1B; Supplementary Fig. S2), the deduced amino acid sequences of *SIPP2C*, *SIPP2C3*, and *SIPP2C4* had significant differences in residues interacting with PYLs and ABA. However, as the *SIPP2C* gene was previously shown to encode an enzymatically active PP2C in tomato (Tao *et al.*, 2004), it can be presumed that all *SIPP2C* genes encode PP2Cs, but only *SIPP2C1*, *SIPP2C2*, *SIPP2C5*, and *SIPP2C6* may be involved in ABA signal transduction in tomato. Within the *SISnRK2* gene family, only *SISnRK2.3* and *SISnRK2.4* belonged to the SnRK2 subclass III (Fig. 1C; Supplementary Fig. S5B) and contained a D-rich C-terminal domain II (Fig. 1D; Supplementary Figs S3, S4) which was shown to be essential for transducing ABA signals (R Yoshida *et al.*, 2006). Therefore, *SISnRK2.3* and *SISnRK2.4* may encode ABA signal transduction core components in tomato,

Fig. 5. Gene expression in ABA-treated fruit. (A) Changes in expression of *SIPYL* genes in response to ABA in tomato fruit. (B) Changes in expression of *SIPP2C* genes in response to ABA in tomato fruit. (C) Changes in expression of *SLSnRK2* genes in response to ABA in tomato fruit. Bars in the positive quadrant indicate up-regulated gene expression, while those in the negative quadrant indicate down-regulated gene expression. Numbers/letters above/below the bars correspond to the last number/letter of the indicated gene family members.

while the other *SLSnRK2* genes may encode SnRK2. However, whether they can transduce ABA signals will still need to be explored in further studies.

The variations in expression of many genes mentioned above were associated with the process of fruit development and ripening (Figs 2A, 3A, 4A) and ABA content (Fig. 6). Therefore, it can be presumed that the ABA signal transduction core components are involved in the regulation of fruit development and ripening at the transcriptional level. Within the *SIPYL* gene family (Fig. 2A), *SIPYL1*, *SIPYL2*, *SIPYL3*, and *SIPYL6* were the major genes involved in the regulation of fruit development and ripening. Among them, *SIPYL1* and *SIPYL2* may have functional roles throughout the whole process of fruit development and ripening. Meanwhile, *SIPYL3* and *SIPYL6* may carry out regulatory functions mainly at the early development (IM and MG) stages and later ripening stages, respectively. Within the *SIPP2C* gene family (Fig. 3A), *SIPP2C1* and *SIPP2C5* were the main genes regulating both fruit development and

ripening processes. Of the *SLSnRK2* gene family (Fig. 4A), *SLSnRK2.3* and *SLSnRK2.4* may play key roles in the regulation of fruit development and ripening in all maturity stages, and *SLSnRK2.3* may play a more important role during the breaker stages.

Recent studies suggested that ABA may be involved in regulating the onset of fruit ripening (Zhang *et al.*, 2009a; Sun *et al.*, 2010). In this present work, it was also found that the ABA content peaked at the breaker stages (Fig. 6), corresponding to the peak of the highly expressed genes *SIPYL1*, *SIPYL2*, *SIPP2C1*, *SIPP2C5*, and *SLSnRK2.3* (Figs 2A, 3A, 4A). Therefore, these genes may also be involved in regulating fruit ripening onset.

To explore the correlations between gene expression and ABA content, the highly expressed genes were investigated in response to exogenous ABA treatment in fruit. The results in Fig. 5 indicated that at early developmental stages, especially at the IM stage, expression of most genes positively correlated with ABA accumulation. In contrast,

Fig. 6. ABA content at different maturity stages of tomato fruit and in leaves in dehydration conditions. Maturity stages: immature green (IM), mature green (MG), breaker 1 (B1), breaker 2 (B2), breaker 3 (B3), breaker 4 (B4), turning 1 (T1), turning 2 (T2), red ripe (RR). Leaves: non-treated leaf (L), dehydrated leaf (DL), rehydrated leaf (RL).

there was no clear correlation between gene expression and ABA content accumulation at the B and T stages. For example, *SIPP2C1* and *SIPP2C5*, which varied with the fruit ripening process and ABA content, had almost no response to exogenous ABA treatment (Fig. 5B); while *SIPP2C2* and *SIPP2C6* which changed little during different maturity stages (Fig. 3A) were up-regulated by exogenous ABA (Fig. 5B). However, direct experimental evidence is still needed to clarify the exact relationship between changes in expression of ABA signal transduction core components and endogenous ABA content.

Another interesting result worth noting was that, within the *SIPYL* gene family, the highly expressed genes *SIPYL1*, *SIPYL2*, and *SIPYL3* were on the same branch of the phylogenetic tree (Fig. 1A) and shared a high identity in their amino acid sequences (Supplementary Table S2 at JXB online). Therefore, it can be presumed that, in tomato plants which produce a typical fleshfruit, some ABA receptors may evolve to take on more tasks in regulating fruit development and ripening.

The expression patterns of genes encoding ABA signal transduction core components were also investigated in response to dehydration in tomato leaf. The results (Figs 2B, 3B, 4B) were similar to those found in *Arabidopsis* and *Z. mays* (Chak *et al.*, 2000; Santiago *et al.*, 2009), suggesting that members in each gene family of the ABA signal transduction core components had similar basic characteristics allowing them to respond to dehydration at the transcriptional level.

All the findings herein were based on analysis at the transcriptional level. However, protein accumulation may not always correspond with the mRNA levels (Kevany *et al.*, 2007). Additionally, regulation of ABA signal transduction occurs through protein–protein interactions between PYLs

and PP2Cs, and also between PP2Cs and SnRK2s (Fujii *et al.*, 2009; Kline *et al.*, 2010; Umezawa *et al.*, 2010). To better understand the molecular mechanism involving ABA in the regulation of fruit development and ripening, it is intended to investigate in further studies the functions of *SIPYL*s, *SIPP2C*s, and *SISnRK2*s at the protein level by utilizing the yeast two-hybrid method and RNA interference (RNAi) technology combined with proteomic analysis.

Conclusion

In this study, *SIPYL*, *SIPP2C*, and *SISnRK2* genes encoding the ABA signal core components from tomato were isolated *in silico* and confirmed to be homologous to the *Arabidopsis AtPYL*, *AtPP2C*, and *AtSnRK2* genes, respectively. The transcriptional regulation of these genes was determined by detecting the expression patterns and levels of *SIPYL*, *SIPP2C*, and *SISnRK2* genes, which varied during fruit development and ripening, as well as during dehydration in tomato leaf. Therefore, the ABA receptors and transduction pathway in tomatoes may function to regulate fruit development and response to drought stress.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Sequence alignment of the PYL family.

Figure S2. Sequence alignment of the PP2C family.

Figure S3. Sequence alignment of the SnRK2 family.

Figure S4. Sequence alignment of subclass III SnRK2s.

Figure S5. Phylogenetic trees of PP2C and SnRK families in tomato and *Arabidopsis*.

Figure S6. Expression of the *SAND* gene at different stages of maturity of tomato fruit.

Figure S7. Expression of *SINCE1* and *SICYP707A2* genes in dehydration-treated leaf and ABA-treated fruit.

Table S1. Real-time PCR primers used in this study.

Table S2. Similarity of *SIPYL*s based on deduced amino acids.

Table S3. Similarity of *SIPP2C*s based on deduced amino acids.

Table S4. Similarity of *SISnRK2*s based on deduced amino acids.

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