

RESEARCH PAPER

The *Citrus* ABA signalosome: identification and transcriptional regulation during sweet orange fruit ripening and leaf dehydration

Paco Romero, María T. Lafuente and María J. Rodrigo*

Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Consejo Superior de Investigaciones Científicas, Av. Agustín Escardino, 7, 46980, Paterna-Valencia, Spain

*To whom correspondence should be addressed. E-mail: mjrodrigo@iata.csic.es

Received 23 February 2012; revised 8 May 2012; accepted 11 May 2012

Abstract

The abscisic acid (ABA) signalling core in plants include the cytosolic ABA receptors (PYR/PYL/RCARs), the clade-A type 2C protein phosphatases (PP2CAs), and the subclass III SNF1-related protein kinases 2 (SnRK2s). The aim of this work was to identify these ABA perception system components in sweet orange and to determine the influence of endogenous ABA on their transcriptional regulation during fruit development and ripening, taking advantage of the comparative analysis between a wild-type and a fruit-specific ABA-deficient mutant. Transcriptional changes in the ABA signalosome during leaf dehydration were also studied. Six *PYR/PYL/RCAR*, five *PP2CA*, and two subclass III *SnRK2* genes, homologous to those of *Arabidopsis*, were identified in the *Citrus* genome. The high degree of homology and conserved motifs for protein folding and for functional activity suggested that these *Citrus* proteins are *bona fide* core elements of ABA perception in orange. Opposite expression patterns of *CsPYL4* and *CsPYL5* and ABA accumulation were found during ripening, although there were few differences between varieties. In contrast, changes in expression of *CsPP2CA* genes during ripening paralleled those of ABA content and agreed with the relevant differences between wild-type and mutant fruit transcript accumulation. *CsSnRK2* gene expression continuously decreased with ripening and no remarkable differences were found between cultivars. Overall, dehydration had a minor effect on *CsPYR/PYL/RCAR* and *CsSnRK2* expression in vegetative tissue, whereas *CsABI1*, *CsAHG1*, and *CsAHG3* were highly induced by water stress. The global results suggest that responsiveness to ABA changes during citrus fruit ripening, and leaf dehydration was higher in the *CsPP2CA* gene negative regulators than in the other ABA signalosome components.

Key words: Abscisic acid (ABA), Citrus, fruit ripening, gene expression, leaf dehydration, PP2CA, PYR/PYL/RCAR, receptor, signalling, SnRK2

Introduction

A number of studies have attempted to obtain a deeper insight into the cellular and molecular responses to ABA in plants, comprising the perception, signalling, metabolism, and transport of this phytohormone (Nambara and Marion-Poll, 2005; Verslues and Zhu, 2007; Kang *et al.*, 2010; Kuromori *et al.*, 2010; Antoni *et al.*, 2011). Natural or induced plant mutants of ABA biosynthetic and signalling genes have been extensively used to elucidate the involvement of ABA in several physiological processes (Karszen

et al., 1983; Peña-Cortés *et al.*, 1989; Groot and Karssen, 1992; Armstrong *et al.*, 1995; Schwartz *et al.*, 1997; Galpaz *et al.*, 2008; Sun *et al.*, 2012). However, less information is available about the effects of this plant regulator on non-climacteric fruit performance and physiology (Rodrigo *et al.*, 2006; Zhang *et al.*, 2009a; Giribaldi *et al.*, 2010; Chai *et al.*, 2011; Jia *et al.*, 2011).

In woody plants, artificially generated mutants are less affordable, but spontaneous mutants are more widely found (Koorneef

et al., 2004). A spontaneous fruit-specific ABA-deficient mutant from the 'Navelate' orange (*Citrus sinensis* L. Osbeck), named 'Pinalate', has been biochemically characterized (Rodrigo *et al.*, 2003). 'Pinalate' orange presents distinctive yellow-coloured fruit because of a partial blockage of the carotenoid biosynthetic pathway, causing, consequently, a fruit-specific ABA deficiency. During natural ripening, the onset of fruit degreening is delayed in 'Pinalate' as compared with its wild-type cultivar (Rodrigo *et al.*, 2003). Moreover, the sensitivity to ABA and the molecular responses to fruit dehydration during post-harvest storage have been shown to be impaired in this mutant, which suggested that the ABA perception system may fail in sensing the phytohormone (Romero *et al.*, 2012). Therefore, the fruit-specific ABA-deficient 'Pinalate' orange offers an exceptional experimental system to investigate the role of endogenous ABA in the regulation of the hormone perception system components during citrus fruit ripening.

Several pieces of evidence support that multiple ABA receptors perceive the ABA signal outside and inside the cells, this perception being tissue specific (Finkelstein *et al.*, 2002). The PYR/PYL/RCAR soluble proteins (Ma *et al.*, 2009; Park *et al.*, 2009), belonging to the START protein superfamily (Klingler *et al.*, 2010), and the downstream complex composed of protein phosphatase type 2C (PP2CA) and SNF1-related kinases family 2 (SnRK2) proteins (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Hirayama and Umezawa, 2010), have been shown to regulate the well-known ABA responses in the model plant *Arabidopsis thaliana*. Thus, the ABA signalling core is composed of the cytoplasmic ABA receptors (PYR/PYL/RCAR) and the clade A PP2CAs as negative regulators (Gosti *et al.*, 1999; Merlot *et al.*, 2001), and a number of protein kinases, including the subclass III of the SnRK2s, as positive regulators of the pathway (Yoshida *et al.*, 2002). The PYR/PYL/RCAR proteins contain a ligand-binding pocket in a cavity that closes after ABA binding through conformational changes of two conserved β -loops that serve as a gate and a latch. ABA binding to the receptors is enhanced when PYR/PYL/RCAR proteins are bound to their negative regulator PP2CAs (Ma *et al.*, 2009; Melcher *et al.*, 2009; Park *et al.*, 2009). This new conformation locks the receptor in a closed structure and inhibits the PP2CA active site (Melcher *et al.*, 2009; Santiago *et al.*, 2009a). Consequently, SnRK2 is released and can phosphorylate downstream proteins or transcription factors that trigger the expression of ABA-responsive genes (Umezawa *et al.*, 2010). Some investigations have been conducted on ABA signalling core components at the transcriptional and functional levels. In general, concomitant with increases in ABA, positive effectors (PYR/PYL/RCAR and SnRKs) were transcriptionally repressed whereas negative regulators (PP2CAs) increased, together modulating downstream signalling and, consequently, physiological ABA responses in model and crop plants (Huai *et al.*, 2008; Park *et al.*, 2009; Santiago *et al.*, 2009b; Umezawa *et al.*, 2009; Nishimura *et al.*, 2010; Szostkiewicz *et al.*, 2010; Sun *et al.*, 2011). Currently, limited information is available in non-climacteric fruit (Jia *et al.*, 2011; Chai *et al.*, 2011; Li *et al.*, 2012) and there is no report analysing the expression of this set of genes as a whole.

In this study, 13 genes belonging to the PYR/PYL/RCAR, PP2CA, and SnRK2 families have been identified in sweet orange.

In order to obtain a deeper insight into the modulation of the ABA signalling components during fruit development and ripening of this non-climacteric fruit, as well as the relationship existing between these components and the changes in the endogenous ABA accumulation during these processes, the expression of the ABA signalosome components has been investigated in fruits of 'Navelate' orange and its ABA-deficient mutant 'Pinalate' during different developmental stages. Moreover, expression analysis of the ABA signalling core elements was performed in detached leaves from both cultivars subjected to dehydration. This has allowed a comparative analysis between fruit and vegetative tissue, providing further insights into the role of the different ABA signalosome genes, and has helped to decipher whether the key genes in this system are common or tissue specific.

Materials and methods

Plant material and colour measurement

Fruits of 'Navelate' (*C. sinensis* L. Osbeck) orange and its spontaneous ABA-deficient mutant 'Pinalate' were randomly harvested at six different ripening stages from adult trees grown at 'The Spanish Citrus Germoplasm Bank' at the Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain), and immediately delivered to the laboratory. The trees were the same age, grown in the same experimental orchard, and subjected to the same standard cultural practices. The six sampling periods were chosen based on previous reports describing colour evolution in citrus fruit during ripening (Rodrigo *et al.*, 2004) and were defined as: immature green, IG; mature green I, MI; mature green II, MII; breaker, Bk; coloured, C; and full coloured, FC. Thus, fruits of both cultivars were hand harvested on the same day and their colour was measured (Supplementary Table S1 available at *JXB* online) using a Minolta CR-330 on three locations around the equatorial plane of the fruit and expressed as the *a/b* Hunter ratio (Stewart and Wheaton, 1972), which is classically used for colour measurement in citrus fruit. This ratio is negative for green fruit and positive for orange fruit, while a zero value corresponds to yellow fruit at the midpoint of the colour break period. Flavedo (the outer coloured part of the peel) tissue samples were collected from the total surface of fruits, frozen and homogenized to a fine powder in liquid nitrogen, and kept at -80°C for later analysis. Three biological replicates of five fruits each were collected at each sampling period.

In addition, water stress experiments in vegetative tissue were carried out in detached mature leaves. To that end, leaves were collected, weighed, and allowed to dehydrate in storage chambers under continuous light at 22°C . Control non-stressed leaves were kept in the chambers at 90% relative humidity (RH) with petioles in distilled water, whereas stressed leaves were dehydrated by placing them on filter paper at 50–55% RH. The weight of the leaves was monitored periodically and tissue was collected after 0.5, 1, 3, 6, and 24 h. Three biological replicates of four leaves were used for each time period. Leaves were frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C until analysis.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from frozen flavedo and leaf samples by a method modified from that previously described by Rodrigo *et al.* (2004) and Ballester *et al.* (2006), as reported in Romero *et al.* (2012). Total RNA was treated with RNase-free DNase (Applied Biosystems) following the manufacturer's instructions. Thereafter, the amount of RNA was measured by spectrophotometric analysis and its quality was verified by agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription followed by quantitative PCR analysis (qRT-PCR) was performed as described previously by Romero *et al.* (2012) to examine the time course of gene expression patterns during fruit ripening and leaf dehydration. Briefly, a two-step qRT-PCR assay was designed as suggested by Udvardi *et al.* (2008). The cDNAs from

all biological replicates were synthesized from 2 µg of total RNA by using SuperScript III RT (Invitrogen) in the presence of an oligo(dT) 20-mer (Invitrogen) and an RNase inhibitor (Invitrogen) according to the manufacturer's instructions. Gene-specific primers were designed using DNAMAN 4.03 software (Lynnon BioSoft) and incubated, in a LightCycler 480 Instrument (Roche Diagnostics), with the cDNA samples and LightCycler 480 SYBR Green I Master (Roche Diagnostics) at 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s. Forward (F) and reverse (R) sequences for specific primers and the amplicon size for each gene are shown in Supplementary Table S2 at *JXB* online. The occurrence of non-specific amplified products was ruled out after performing a melting curve analysis and sequencing the reaction products. Fluorescent intensity measurements were transformed into relative mRNA levels by using standard curves constructed for all studied genes. The reference genes *CsACT*, *CsGAPDH*, and *CsTUB* (Supplementary Table S2), whose constitutive expression during fruit ripening was confirmed by using the geNorm program (Vandesompele *et al.*, 2002), were used for data normalization. Statistical analysis (pairwise fixed reallocation randomization test) was carried out by the $\Delta\Delta Ct$ method using the Relative Expression Software Tool (REST, <http://rest.gene-quantification.info>) (Pfaffl, 2001). Validation experiments were performed previously to ensure that the efficiency of the target and housekeeping genes was relatively equivalent. Relative expression levels for all flavedo samples were referred to that obtained in MI 'Navelate' fruits and those of vegetative samples were relative to that found in freshly harvested 'Navelate' leaves. In addition, in order to compare absolute gene expression values, amplicons of each gene were cloned in the pGEMT vector (Promega) and used to generate standard curves by serial dilutions. Data were then normalized by using the above-mentioned housekeeping genes. Three

biological samples for each sampling period, tissue, and variety were analysed in duplicate and mean ratios were calculated.

Statistical design

Results are the means of three replicate samples \pm SE. A mean comparison using Tukey's test was performed to determine if means values were significantly different ($P \leq 0.05$).

ABA analysis

ABA was extracted from 1 g fresh weight (FW) of frozen flavedo and leaves with 80% acetone containing 0.5 g l⁻¹ citric acid and 100 mg l⁻¹ butylated hydroxytoluene as previously described by Lafuente *et al.* (1997). After centrifugation, the supernatant was diluted in three serial dilutions in ice-cold TRIS-buffered saline (TBS; 6.05 g l⁻¹ TRIS, 8.8 g l⁻¹ NaCl, and 0.2 mg l⁻¹ MgCl₂ at pH 7.8) and three samples for each dilution were analysed by the indirect enzyme-linked immunosorbent assay (ELISA) reported by Walker-Simmons (1987). The ABA-BSA-(4, conjugate) was synthesized as previously reported by Weiler (1980) with some modifications (Norman *et al.*, 1988). The results are the means of three biological replicates of five fruit each \pm SE.

Sequence analyses, alignment, and phylogenetics

Sequence similarity comparisons between *A. thaliana* and *C. sinensis* proteins were performed by BLASTP in the Phytozome v7.0 database (www.phytozome.org; www.citrusgenomedb.org). A search for amino acid sequences of *Arabidopsis* PYR/PYL/RCAR, PP2CA, and SnRK2 proteins was carried out using the National Centre for Biotechnology

Table 1. Comparison of PYR/PYL/RCAR, clade-A PP2C, and subclass III SnRK2 genes between *Arabidopsis thaliana* and *Citrus sinensis*

<i>Arabidopsis thaliana</i>				<i>Citrus sinensis</i>				Comparison with <i>A. thaliana</i>		
Gene	Code	Introns	Amino acids	Citrus gene	Genome code	Introns	Amino acids	Most similar	Identity	Match/aligned
<i>AtPYR1</i>	AT4G17870	0	191	<i>CsPYR1</i>	<i>orange1.1g046151m</i>	0	209	PYR1	74.0%	130/175
<i>AtPYL1</i>	AT5G46790	0	221		<i>orange1.1g046151m</i>					
<i>AtPYL2</i>	AT2G26040	0	190	<i>CsPYL2</i>	<i>orange1.1g046697m</i>	0	187	PYL2	72.0%	121/168
<i>AtPYL3</i>	AT1G73000	0	209		<i>orange1.1g046697m</i>					
<i>AtPYL4</i>	AT2G38310	0	207	<i>CsPYL4</i>	<i>orange1.1g026007m</i>	0	245	PYL4	78.0%	136/174
<i>AtPYL5</i>	AT5G05440	0	203	<i>CsPYL5</i>	<i>orange1.1g038201m</i>	0	201	PYL5	70.0%	111/158
<i>AtPYL6</i>	AT2G40330	0	215		<i>orange1.1g026007m</i>					
<i>AtPYL7</i>	AT4G01026	2	211		<i>orange1.1g043944m</i>					
<i>AtPYL8</i>	AT5G53160	2	188	<i>CsPYL8</i>	<i>orange1.1g028067m</i>	2	214	PYL8	75.0%	137/183
<i>AtPYL9</i>	AT1G01360	2	187	<i>CsPYL9</i>	<i>orange1.1g043944m</i>	2	186	PYL9	86.0%	143/167
<i>AtPYL10</i>	AT4G27920	2	183		<i>orange1.1g028067m</i>					
<i>AtPYL11</i>	AT5G45860	0	161		<i>orange1.1g038201m</i>					
<i>AtPYL12</i>	AT5G45870	0	159		<i>orange1.1g038201m</i>					
<i>AtPYL13</i>	AT4G18620	0	164		<i>orange1.1g038201m</i>					
<i>AtABI1</i>	AT4G26080	3	434	<i>CsABI1</i>	<i>orange1.1g008880m</i>	4	550	ABI1	68.0%	227/391
<i>AtABI2</i>	AT5G57050	3	383		<i>orange1.1g008880m</i>					
<i>AtAHG1</i>	AT5G51760	3	416	<i>CsAHG1</i>	<i>orange1.1g013591m</i>	3	440	AHG1	40.0%	170/424
<i>AtAHG3</i>	AT3G11410	3	399	<i>CsAHG3</i>	<i>orange1.1g015135m</i>	2	412	AHG3	66.0%	258/390
<i>AtHAB1</i>	AT1G72770	4	406	<i>CsHAB1</i>	<i>orange1.1g009083m</i>	4	544	HAB1	57.0%	206/362
<i>AtHAB2</i>	AT1G17550	3	511		<i>orange1.1g009083m</i>					
<i>AtHAI1</i>	AT5G59220	3	413		<i>orange1.1g036852m</i>					
<i>AtHAI2</i>	AT1G07430	2	442		<i>orange1.1g036852m</i>					
<i>AtHAI3</i>	AT2G29380	2	362	<i>CsHAI3</i>	<i>orange1.1g036852m</i>	3	408	HAI3	64.0%	223/348
<i>AtSnRK2.2</i>	AT3G50500	8	369	<i>CsSnRK2.2</i>	<i>orange1.1g017860m</i>	8	365	SnRK2.2	82.0%	297/362
<i>AtSnRK2.3</i>	AT5G66880	8	361		<i>orange1.1g017936m</i>					
<i>AtSnRK2.6</i>	AT4G33950	9	362	<i>CsSnRK2.6</i>	<i>orange1.1g017936m</i>	8	363	SnRK2.6	88.9%	317/352

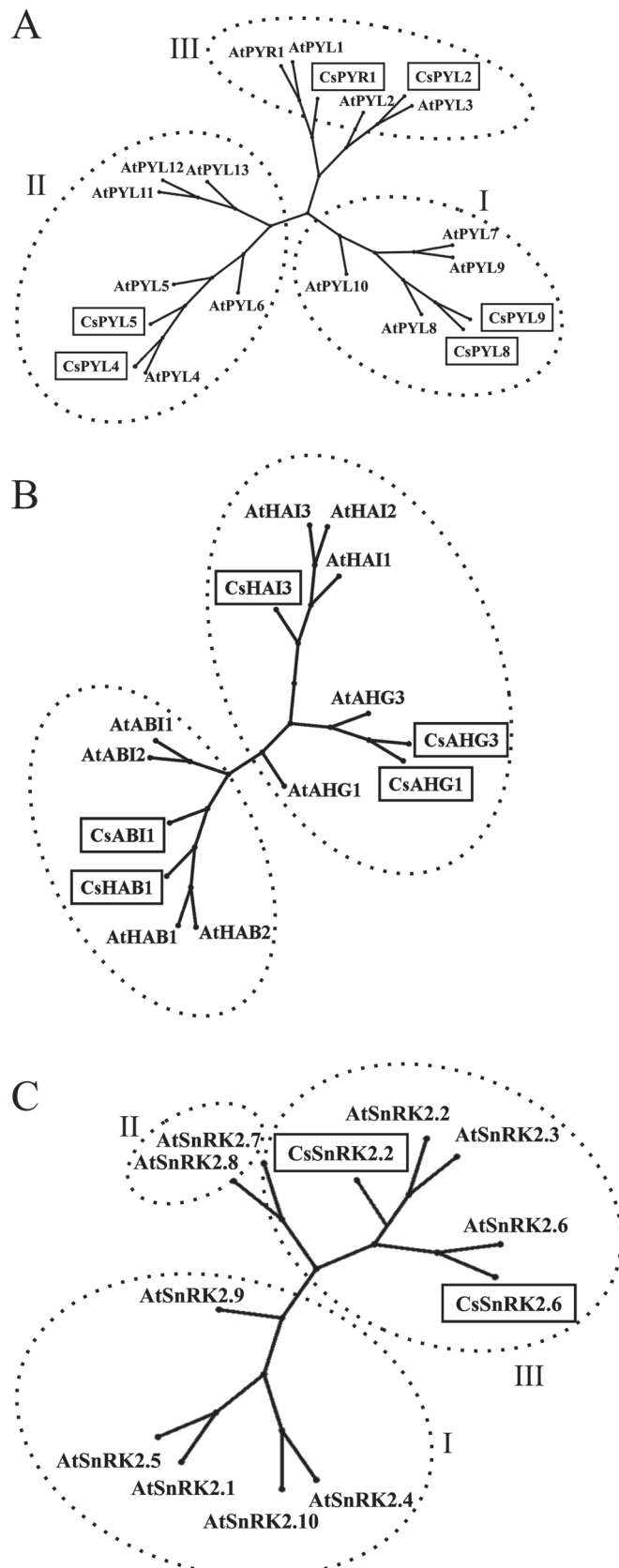


Fig. 1. Unrooted phylogenetic trees containing *Citrus sinensis* and *Arabidopsis thaliana* PYR/PYL/RCAR ABA receptors (A), clade-A PP2Cs (B), and SnRK2 protein kinases (C) obtained by using the Neighbor–Joining method in the PhyloWidget software and based on the protein sequence alignments. The gene code for each protein is detailed in Table 1.

Information (NCBI). Motif prediction was performed using whole protein sequences as input into the PSIPRED secondary structure prediction server. The tertiary structures of CsPYR1, CsPYL2, CsPYL5, CsPYL8, CsABI1, and CsSnRK2.6 proteins were modelled by using the I-Tasser program (Roy *et al.*, 2010), in which their corresponding *Arabidopsis* homologous crystallographic structures from the PDB database (3K90, 3KL1, 3QRZ, 3UQH, 3UJK, and 3UDB, respectively) were used as templates. Multiple sequence alignments of PYR/PYL/RCAR, PP2CA, and SnRK2 proteins were performed by using the default settings of the CLUSTALX 2.0 software and manually edited in GENEDOC (<http://www.nrbsc.org/gfx/genedoc/>). Based on these alignments, phylogenetic trees were constructed according to the Neighbor–Joining method using the PhyloWidget program. The reliability of the trees was established by conducting a 1000 bootstrap re-sampling.

Results

The PYR/PYL/RCAR family in Citrus sinensis

Genes encoding ABA receptors of *A. thaliana* were used as query to identify the orthologous proteins from *C. sinensis*. In the orange genome, only six proteins with homology to the 14 *Arabidopsis* PYR/PYL/RCAR proteins were found (Table 1). The genes *AtPYR1* and *AtPYL1* shared homology with the same orange locus (*orange1.lg046151m*) and showed 74% and 73% of identity, respectively, in 175 amino acid residues, which correspond to 84% of the protein length. Likewise, *AtPYL2* and *AtPYL3* shared homology to the *orange1.lg046697m* locus and showed 72% and 66% identity, covering 90% and 93% of the protein stretch, respectively. On the other hand, *AtPYL4* and *AtPYL6* were homologous to the protein encoded by the *Citrus* gene *orange1.lg026007m* and showed 78% and 62% identity, respectively. The *orange1.lg038201m* locus was the most similar to the *AtPYL5*, *AtPYL11*, *AtPYL12*, and *AtPYL13* genes and displayed 70, 62, 62, and 56% identity at the protein level, respectively. Genes *AtPYL8* and *AtPYL10* showed homology to *orange1.lg028067m* (75% and 74% identity in 183 and 158 amino acid residues, respectively) and *AtPYL7* and *AtPYL9* to *orange1.lg043944m* (79% and 86% identity, respectively). Taking into account their highest identity with *Arabidopsis* proteins, *Citrus* genes were named *CsPYR1*, *CsPYL2*, *CsPYL4*, *CsPYL5*, *CsPYL8*, and *CsPYL9*, respectively (Table 1). The analysis of the genomic structure of all *Citrus* PYR/PYL/RCAR genes revealed that only *CsPYL8* and *CsPYL9* genes showed the predicted introns. This is in concordance with the fact that only *Arabidopsis AtPYL7*, *AtPYL8*, *AtPYL9*, and *AtPYL10* contained putative intron regions. The intron number of *CsPYL8* and *CsPYL9* was also coincident with that of the *Arabidopsis* homologous genes (Table 1).

In order to assess the degree of conservation of the ABA receptors in *Citrus*, amino acid sequences were aligned and the START-like domain was compared. Sequences were, in general, highly conserved between proteins of both species (Supplementary Fig. S1A at JXB online). The latch and gate loops of *Citrus* proteins were identical to those described in *Arabidopsis*, and the functional sites for ABA binding and interaction with PP2Cs were also perfectly conserved in all *Citrus* proteins. No important differences between *Citrus* ABA-binding regions and those of *Arabidopsis* were found, with the exception of an insert of 17 amino acids inside ABA-binding region 2 of the *CsPYL8* protein. Furthermore, the alignment of the predicted secondary structure

of AtPYR1 with the *Citrus* sequences showed that most of the elements described in this protein matched with the highly conserved regions of the *Citrus* homologues. High similarity in the number and location of α -helices and strands forming β -sheets was also found between all *Citrus* PYR/PYL/RCAR proteins and their *Arabidopsis* homologues (data not shown). In addition, the predicted tertiary structure of CsPYR1, CsPYL2, CsPYL5, and CsPYL8 showed that two helical segments and seven strands forming a β -sheet formed a cavity for ligand binding highly similar to that found in their respective *Arabidopsis* homologues (Supplementary Fig. S2A–D). Phylogenetic analysis further showed that the *Citrus* ABA receptors were distributed in the three main subfamilies proposed by Ma *et al.* (2009) in *Arabidopsis*, and two representative *Citrus* proteins were included in each subfamily (Fig. 1A): CsPYL8 and CsPYL9 belong to subfamily I, CsPYL4 and CsPYL5 to subfamily II, and CsPYR1 and CsPYL2 to subfamily III. In addition, a similarity matrix of the deduced amino acid sequences confirmed that proteins clustered into the same subfamily shared the highest percentage similarity among *Citrus* proteins (Supplementary Table S3).

Family of clade-A PP2C proteins in *Citrus sinensis*

In the *C. sinensis* genome, five proteins were identified with significant homology to the nine members of the clade-A PP2C family of *Arabidopsis* (Table 1). The *Citrus* gene *orange1.lg008880m* was the most similar to both components of the ABA-insensitive (ABI) subfamily PP2Cs, *AtABI1* and *AtABI2*. Nevertheless, since *AtABI1* showed higher identity (68%) than *AtABI2* (58%) to the *Citrus* protein, the gene was named *CsABI1*. The members of the ABA-hypersensitive germination (AHG) subfamily, *AtAHG1* and *AtAHG3*, showed homology (40% and 66% identity, respectively) to different loci of the *Citrus* genome, which were named *CsAHG1* (*orange1.lg013591m*) and *CsAHG3* (*orange1.lg015135m*), respectively. Both components of the *Arabidopsis* homologous to ABI subfamily, also named hypersensitive to ABA (*AtHAB1* and *AtHAB2*), shared homology to the same *Citrus* locus (*orange1.lg009083m*) and showed a very similar percentage identity (57% and 55%, respectively); therefore, the *Citrus* gene was named *CsHAB1*. Likewise, the three members of the *Arabidopsis* highly ABA-induced (HAI) PP2CA subfamily (*AtHAI1*, *AtHAI2*, and *AtHAI3*) shared homology to the same locus of *Citrus sinensis* and displayed 62, 57, and 64% identity, respectively, to the protein encoded by *orange1.lg036852m*, which consequently was named *CsHAI3*.

It is interesting to note that all genes of the clade-A PP2C from both *Arabidopsis* and *Citrus* contained introns, although the intron number for most of the genes was different between species (Table 1). Concerning the protein alignments, the PP2C-like domain was highly conserved throughout all proteins sequences although the length of all *Citrus* PP2CA proteins was longer than that of the *Arabidopsis* homologues (Supplementary Fig. S1B at JXB online). Metal-binding sites described in *Arabidopsis* were also identified in *Citrus* proteins, and phosphatase activity regulatory sequences were identical for all proteins analysed. The predicted secondary structure of *AtABI1* matched the most conserved regions of the alignment. Secondary structures were

also predicted for the *Citrus* clade-A PP2Cs, and similar sizes and location of the different motifs were observed when each protein was compared with its *Arabidopsis* homologue (data not shown). Prediction of the tertiary structure of *CsABI1* was performed by using the crystallographic structure of *AtABI1* as template, and it revealed a high degree of similarity in protein folding between species (Supplementary Fig. S2E). In addition, the phylogenetic tree constructed with *Arabidopsis* and *Citrus* PP2CAs showed that *Citrus* proteins fitted into the two groups described by Schweighofer *et al.* (2004) for these *Arabidopsis* proteins (Fig. 1B). Accordingly, the highest percentage similarity among *Citrus* protein sequences was found between *CsABI1* and *CsHAB1*, and among *CsAHG1*, *CsAHG3*, and *CsHAI3* proteins (Supplementary Table S4). Furthermore, representative genes of each group were identified and, as expected, each *Citrus* protein was clustered near to its corresponding *Arabidopsis* homologue.

The ABA-related subclass III SnRK2 proteins in *Citrus*

Among SnRK2s of *Arabidopsis*, the proteins belonging to subclass III, SnRK2.2, SnRK2.3, and SnRK2.6, have been found to be related to ABA signalling. A BLAST search in the *C. sinensis* genome revealed that two different loci (*orange1.lg017860m* and *orange1.lg017936m*) shared homology with these ABA-related SnRK2s (Table 1). The protein encoded by the gene *orange1.lg017936m* showed the highest identity (90%) to *AtSnRK2.6*, whereas *orange1.lg017860m* protein showed 82% identity to *AtSnRK2.2*. Therefore, these *Citrus* genes were named *CsSnRK2.6* and *CsSnRK2.2*, respectively. Gene structure analysis revealed that the number of introns in *CsSnRK2.6* and *CsSnRK2.2* genes was very similar to that found in their *Arabidopsis* homologues. Amino acid alignment of the *Arabidopsis* subclass III SnRK2s and their corresponding *Citrus* homologues showed a kinase domain highly conserved between both species (Supplementary Fig. S1C at JXB online). Furthermore, the ATP-binding and the activation loop regions as well as the ATP-binding and the proton acceptor active sites were identical. In contrast, osmotic stress and ABA-responsive domains were less conserved, even among *Arabidopsis* proteins. The secondary structure predicted for *AtSnRK2.6* showed that α -helices and β -strands matched with the most conserved regions in the protein alignment (Supplementary Fig. S1C). Additionally, the secondary structure predicted for *CsSnRK2.2* revealed a high consensus in the number and location of the putative functional motifs when compared with its respective homologue (data not shown). Likewise, the tertiary structure of *CsSnRK2.6* was predicted by using the crystallographic structure of *AtSnRK2.6* as template, and protein folding was highly conserved between the species (Supplementary Fig. S2F). Phylogenetic analysis further revealed that *Arabidopsis* ABA-related SnRK2s proteins (*AtSnRK2.2*, *AtSnRK2.3*, and *AtSnRK2.6*) grouped in a branch (subclass III) independent from the other proteins belonging to this family, and the *Citrus* homologues (*CsSnRK2.6* and *CsSnRK2.2*) were also clustered into this group (Fig. 1C). It should be also mentioned that these *Citrus* proteins displayed a high percentage (82%) similarity when their sequences were compared (Supplementary Table S5).

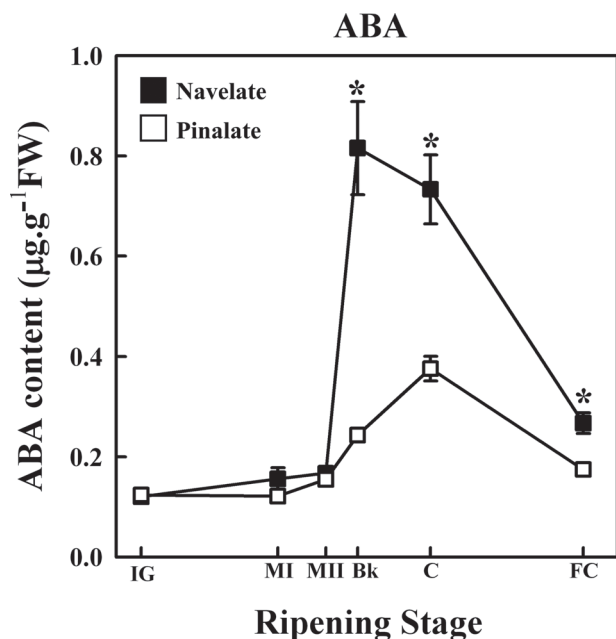


Fig. 2. ABA content in the flavedo of 'Navelate' (black) and 'Pinalate' (white) fruit during development and ripening (immature green, IG; mature green I, MI; mature green II, MII; breaker, Bk; coloured, C; full coloured, FC). The results are the means of three biological replicates of five fruits each \pm SE. Significant differences ($P \leq 0.05$) in ABA content between 'Navelate' and 'Pinalate' flavedo samples for the same maturity stage are indicated by an asterisk.

Transcriptional regulation of PYR/PYL/RCAR, PP2CA, and subclass III SnRK2 genes during orange fruit development and ripening: influence of endogenous ABA levels

In order to investigate the regulation of the ABA signalling core during citrus fruit development and ripening, and its relationship with endogenous ABA levels, the expression analysis of the six *Citrus* PYR/PYL/RCAR, five clade-A PP2C, and two SnRK2 genes was carried out together with the ABA measurement in the flavedo of fruits of 'Navelate' and its ABA-deficient mutant 'Pinalate'.

Six ripening stages were selected covering from IG to FC fruits. As expected, no difference in ABA content between 'Navelate' and the 'Pinalate' mutant was found while the fruits remained green (IG, MI, and MII stages), whereas the differences between parental and mutant fruit increased thereafter with fruit ripening (Fig. 2). The flavedo of parental fruits reached the highest ABA levels at the Bk stage, and the ABA content in the mutant was >3-fold lower. The ABA levels in 'Pinalate' fruit peaked at the C stage but the concentration was half of that reached in the parental fruits at the same ripening stage. In the flavedo of FC fruits from both varieties, an important decrease in ABA content was observed, but levels in the parental fruit remained higher than in the mutant (Fig. 2).

In spite of the differential ABA accumulation in 'Navelate' and 'Pinalate' flavedo during ripening, only minor differences were observed in the expression pattern of most of the PYR/PYL/RCAR genes between both cultivars (Fig. 3A) and

remarkable differences were only observed in the expression profile of *CsPYR1*. In parental fruit, *CsPYR1* transcript levels fluctuated during ripening, reaching a maximum at MII and a minimum at the Bk and C stages, and increased again at the FC stage to levels similar to those at IG. In the mutant fruit, the maximum expression levels of *CsPYR1* were found at the IG and FC stages, reaching levels almost 2-fold higher than in 'Navelate'. Nevertheless, the *CsPYR1* transcript level and profile of mutant fruit was similar to that of the parent at intermediate ripening fruit stages (MI, MII, Bk, and C) and showed a minimum at the C stage (Fig. 3A). The evolution of *CsPYL4* and *CsPYL5* transcripts was similar to that described above since the expression of both genes peaked at MII in 'Navelate' and 'Pinalate' fruit, decreased dramatically to minimum levels at Bk and C, respectively, and then increased again to higher levels at FC. It should be mentioned that the repression of *CsPYL4* at Bk was 2-fold higher in the ABA-deficient mutant. Overall, in spite of the differences observed between varieties, *CsPYR1*, *CsPYL4*, and *CsPYL5* showed a consistent pattern in which the minimum transcript levels were coincident with the highest ABA levels. On the other hand, *CsPYL8* and *CsPYL9* displayed the maximum expression at the IG stage. A transient increase in the *CsPYL8* expression levels occurred at the Bk stage, which was higher in 'Navelate' fruit, and accumulation of *CsPYL8* and *CsPYL9* decreased to reach minimum levels at the FC stage (Fig. 3A). Moreover, absolute gene expression analysis further revealed similar levels of *CsPYL4* and *CsPYL5* transcripts, whereas the gene most expressed during fruit development and ripening was *CsPYL9*. It is also interesting that expression of *CsPYR1* and *CsPYL8* remained at very low levels and *CsPYL2* was not detected in any of the fruit samples analysed (Supplementary Table S6 at JXB online).

The analysis of the *Citrus* clade-A PP2C genes revealed a differential regulation between both varieties. Although *CsAB11*, *CsAHG3*, and *CsHAI3* transcript accumulation followed a similar pattern, peaking at the C stage in both 'Navelate' and 'Pinalate' fruit, the relative expression levels reached by the parental fruit were higher than those reached by the ABA-deficient mutant (Fig. 3B). Interestingly, *CsAHG1* showed a similar expression profile to that described above for *Citrus* PP2CA genes, but the transcript levels at the C stage were 2-fold higher in 'Pinalate' than in 'Navelate'. On the other hand, *CsHAB1* was the only PP2CA gene whose expression decreased continuously in 'Pinalate' during fruit ripening while in 'Navelate' it displayed a transient increase at the C stage. In general, the highest expression levels of the *Citrus* PP2CA genes were observed at the C stage (Fig. 3B; Supplementary Table S6 at JXB online), agreeing with higher levels of ABA in both varieties. Interestingly, *CsHAB1* showed the highest transcript accumulation in both varieties at the beginning of the experiment, followed by *CsAHG3* and *CsHAI3*. However, only in 'Navelate' fruit were the transcript levels of *CsAHG3* at the C stage almost double those of *CsHAB1* and *CsHAI3*, and showed >14-fold accumulation compared with the other genes of this family (Supplementary Table S6).

Transcriptional analysis of *Citrus* SnRK2 genes revealed similar expression patterns between *CsSnRK2.2* and *CsSnRK2.6* genes, although the *CsSnRK2.6* transcript accumulation was at least 8-fold higher than that of *CsSnRK2.2* (Supplementary Table

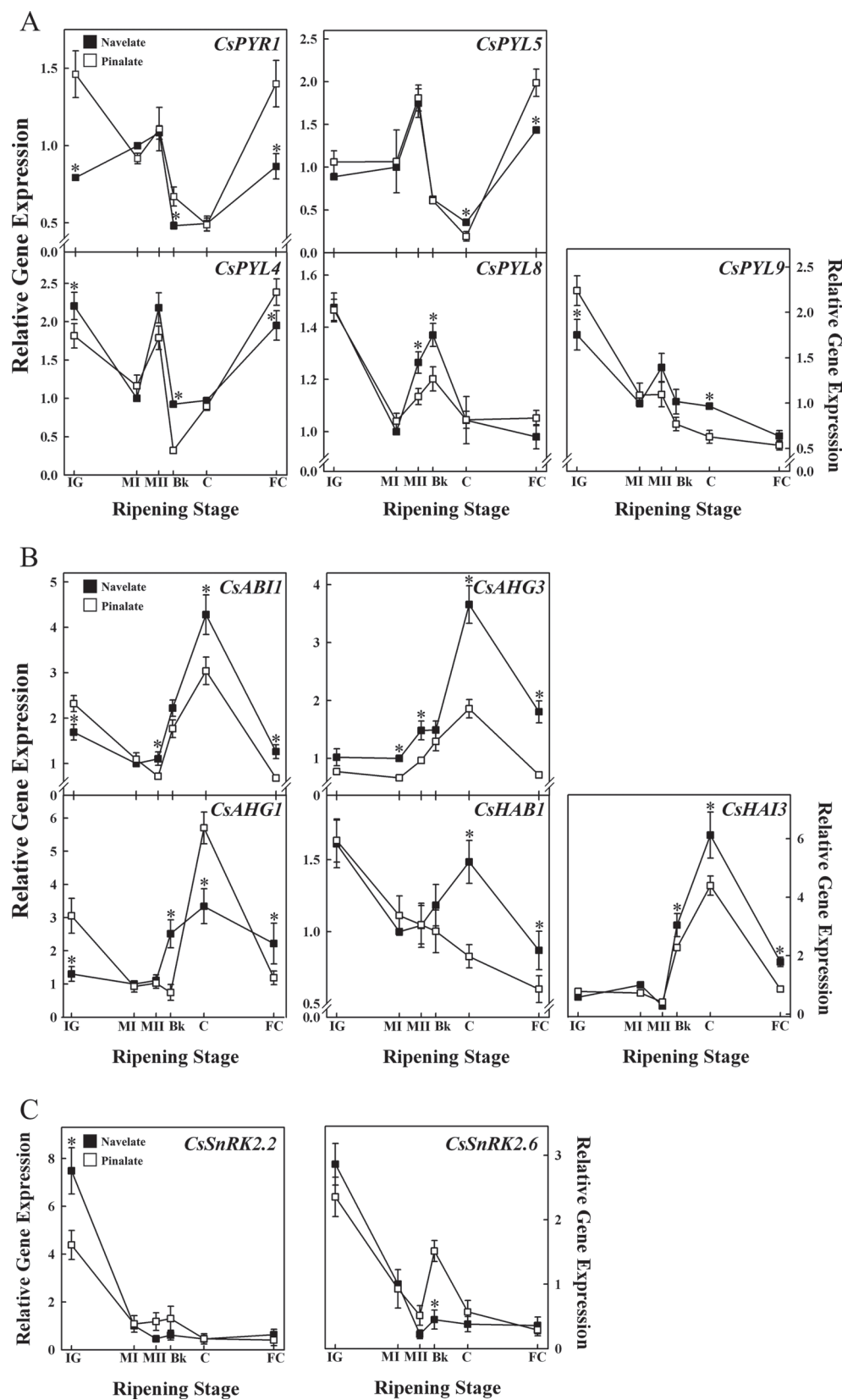


Fig. 3. Relative gene expression analysis by qRT-PCR of *Citrus* PYR/PYL/RCAR ABA receptors (A), clade-A PP2Cs (B), and subclass III SnRK2s (C) in 'Navelate' (black) and 'Pinalate' (white) fruits during fruit development and ripening (immature green, IG; mature green I, MI; mature green II, MII; breaker, Bk; coloured, C; full coloured, FC). Expression values are relative to transcript levels obtained in MI 'Navelate' fruits. Values are mean ratios \pm SE from three biological samples for each sampling period and variety analysed in duplicate. Significant differences ($P \leq 0.05$) in gene expression between 'Navelate' and 'Pinalate' flavedo samples for the same maturity stage are indicated by an asterisk.

S6 at *JXB* online). The highest transcript levels were found at the IG stage in fruits of both cultivars and decreased thereafter as ripening progressed (Fig. 3C). Differences between cultivars in *CsSnRK2.2* transcript accumulation were found at the IG stage, in which parental fruit showed 2-fold higher levels than the mutant. In contrast, similar relative transcript levels were found in *CsSnRK2.6* at this stage, although gene expression peaked at the Bk stage in 'Pinalate' fruit but not in 'Navelate' (Fig. 3C). It should be mentioned that the expression level of these genes bottomed out in both varieties at the MII stage, which was concomitant with the inductions in several *PYR/PYL/RCAR* genes.

Water stress-induced changes in ABA content and transcriptional regulation of ABA signalosome components in leaves

The evolution of ABA content and weight loss in 'Pinalate' and 'Navelate' leaves during the course of a water stress experiment was very similar for both genotypes. The ABA content increased ~20-fold in response to water stress by 24 h (Fig. 4), whereas a minor increase was observed in detached control leaves of both genotypes. Significant differences in ABA content between dehydrated and control leaves were observed after 6 h. Differences in weight loss between control and dehydrated leaves were observed from the beginning of the experiment (0.5 h). Therefore, the increase in weight loss preceded that of ABA, and significant increases in ABA in response to dehydration only occurred when the leaves reached a 10% weight loss (Fig. 4).

The accumulation of *CsPYR1* increased in both 'Pinalate' and 'Navelate' leaves after detachment, but no significant differences were found between control and water-stressed leaves until the end of the experiment (24 h) (Fig. 5). The most important increase occurred by 6 h, and 3- and 5-fold increases were found in 'Navelate' and 'Pinalate' leaves, respectively. Thereafter, the expression level remained almost constant in the control leaves but significantly decreased in water-stressed 'Pinalate' and 'Navelate' samples (Fig. 5). In contrast to that found in flavedo samples, *CsPYL2* expression was detected in leaves, and the results showed that this gene was down-regulated by water stress. The relative gene expression of *CsPYL5* decreased rapidly (0.5 h) after leaf detachment, and significant differences between control and water-stressed leaves were only found by 6 h and 24 h. On the other hand, *CsPYL9* gene expression sharply increased and reached a maximum by 1 h and 3 h in control 'Navelate' and 'Pinalate' leaves, respectively. The transcript level of this gene was, in general, lower in water-stressed leaves, and changes were less relevant. Moreover, *CsPYL4* and *CsPYL8* transcripts were not detected in fresh, detached, or water-stressed 'Pinalate' and 'Navelate' leaves. Interestingly, absolute expression showed that *CsPYL2* and *CsPYL9* were the most highly expressed genes in leaves, whereas *CsPYR1* and *CsPYL5* transcript accumulation remained at very low levels during the whole experiment (Supplementary Table S6 at *JXB* online).

The expression of the *CsPP2CA* genes increased after detachment in both control and stressed 'Navelate' and 'Pinalate' leaves, but such increases were, in general, substantially higher in the water-stressed leaves (Fig. 6). As shown in Fig. 6, dehydration

had an important impact, up-regulating the expression of both *CsAB11* and *CsAHG1* genes, which reached maximum levels by 3 h in water-stressed leaves. The effect of dehydration on *CsAHG3* gene expression was also evident, and important differences between control and stressed leaves were found by 3 h and 6 h after detachment. In contrast, dehydration had little effect on *CsHAB1* and *CsHAI3* transcript levels. Absolute gene expression analysis revealed that *CsHAB1* was the most expressed *CsPP2CA* in freshly harvested leaves, followed by *CsAHG3*, *CsHAI3*, and *CsAB11*. In contrast, the most expressed genes during dehydration of 'Navelate' and 'Pinalate' leaves were *CsAHG3*, *CsHAI3*, and *CsAB11* (Supplementary Table S6 at *JXB* online). As occurred in fruit, *CsAHG1* transcript accumulation remained at much lower levels in both varieties.

The *CsSnRK2.2* gene showed a different gene expression profile in dehydrated and control leaves (Fig. 7). The transcript level of this gene transiently peaked by 0.5 h in 'Navelate' and 'Pinalate' water-stressed leaves, whereas the expression continuously increased from 0 h to 3 h in the control leaves. Interestingly, *CsSnRK2.2* expression was similar in control and stressed 'Navelate' and 'Pinalate' leaves by 6 h, but transcript accumulation was higher in leaves of the mutant at the end of the experiment. The *CsSnRK2.6* expression pattern barely differed between control and dehydrated leaves and was very similar in 'Navelate' and 'Pinalate'. The transcript levels of this gene continuously increased after detachment and reached a maximum by 3 h in 'Pinalate' and by 6 h in 'Navelate' leaves. It is also interesting to note that absolute gene expression of *CsSnRK2.6* was substantially higher than that of the *CsSnRK2.2* gene during the whole experiment in both varieties (Supplementary Table S6 at *JXB* online).

Discussion

The homologous genes of the ABA signalosome have been identified in this work for the first time in the *Citrus* genome in order to explore the relationship between the regulation of these components and the changes in the endogenous ABA levels occurring in citrus fruit during natural fruit ripening and in dehydrated leaves. A comparative transcriptional analysis of these genes has been performed between 'Navelate' orange fruit and its spontaneous fruit-specific ABA-deficient mutant 'Pinalate'. In this context, it is noteworthy that the link between ABA and the ripening process has been reported in non-climacteric fruits such as strawberry (Chai *et al.*, 2011), grapevine (Giribaldi *et al.*, 2010), sweet cherry (Ren *et al.*, 2011), and citrus (Lafuente *et al.*, 1997; Alférez and Zacarías, 1999; Rodrigo *et al.*, 2003; Gambetta *et al.*, 2011), although the molecular mechanism of how ABA regulates this process has not been fully established.

In silico analysis of the sweet orange (*C. sinensis*) genome database has revealed that proteins belonging to the ABA signalosome were less represented in *Citrus* as compared with *Arabidopsis*. Only six *PYR/PYL/RCAR*, five *PP2CA*, and two subclass III *SnRK2* genes were found in *Citrus* (Table 1), while in *Arabidopsis* there are 14 *PYR/PYL/RCAR* ABA receptors, nine clade-A *PP2Cs*, and several protein kinases, including three of the subclass III *SnRK2* (Merlot *et al.*, 2001; Yoshida *et al.*, 2002; R. Yoshida *et al.*, 2006; Ma *et al.*, 2009; Park *et al.*,

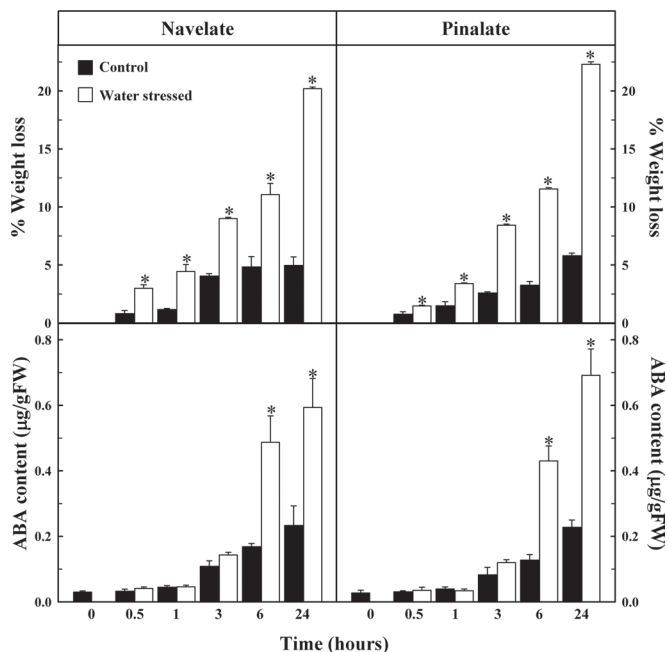


Fig. 4. Effect of water stress on weight loss and ABA content in 'Navelate' and 'Pinalate' detached leaves. Changes in control samples are represented as black bars and in water-stressed leaves as white bars. The results are the means of three biological replicates of four leaves each \pm SE. Significant differences ($P \leq 0.05$) in weight loss and ABA content between samples for the same analysed period are indicated by an asterisk.

2009). This is in concordance with the lower number of *PYR/PYL/RCAR* and *PP2CA* genes recently identified in tomato (Sun *et al.*, 2011) and in strawberry (Chai *et al.*, 2011). High percentage identity was observed between *Citrus* proteins and their homologues in *Arabidopsis*, as well as similar protein length and genetic structures (Table 1; Supplementary Tables S3–S5 at *JXB* online). Interestingly, the consensus motifs for functional protein folding, such as the gate and latch regions in *PYR/PYL/RCARs* (Melcher *et al.*, 2009) (Supplementary Figs S1A, Fig. S2), and for phosphatase activity in *PP2CAs* (Weiner *et al.*, 2010) (sequences underlined in Supplementary Fig. S1B) were identified in *Citrus*. D-rich C-terminal domain II, which has been shown to be essential for ABA signal transduction (R. Yoshida *et al.*, 2006), was also fully conserved in *Citrus* SnRK2s proteins (number 4 in Supplementary Fig. S1C). Phylogenetic analysis revealed that *Citrus* *PYR/PYL/RCARs* were clustered together with their homologues in accordance with the distribution proposed by Ma *et al.* (2009), in which *Arabidopsis* ABA receptors were divided into three main subfamilies. In fact, two representative genes of each group were identified in the *Citrus* genome (Fig. 1A). The *Citrus* clade-A *PP2Cs* were clustered close to their respective homologues (Fig. 1B) and arranged in two separate branches as previously described by Schweighofer *et al.* (2004) in the phylogenetic analysis of *Arabidopsis* *PP2Cs*. Furthermore the two *Citrus* kinases, *CsSnRK2.2* and *CsSnRK2.6*, were classified into subclass III of *AtSnRK2s* (Fig. 1C), whose components have been related to ABA signalling (Fujii and Zhu, 2009). Therefore, the sweet orange proteins encoded by *CsPYR/PYL/*

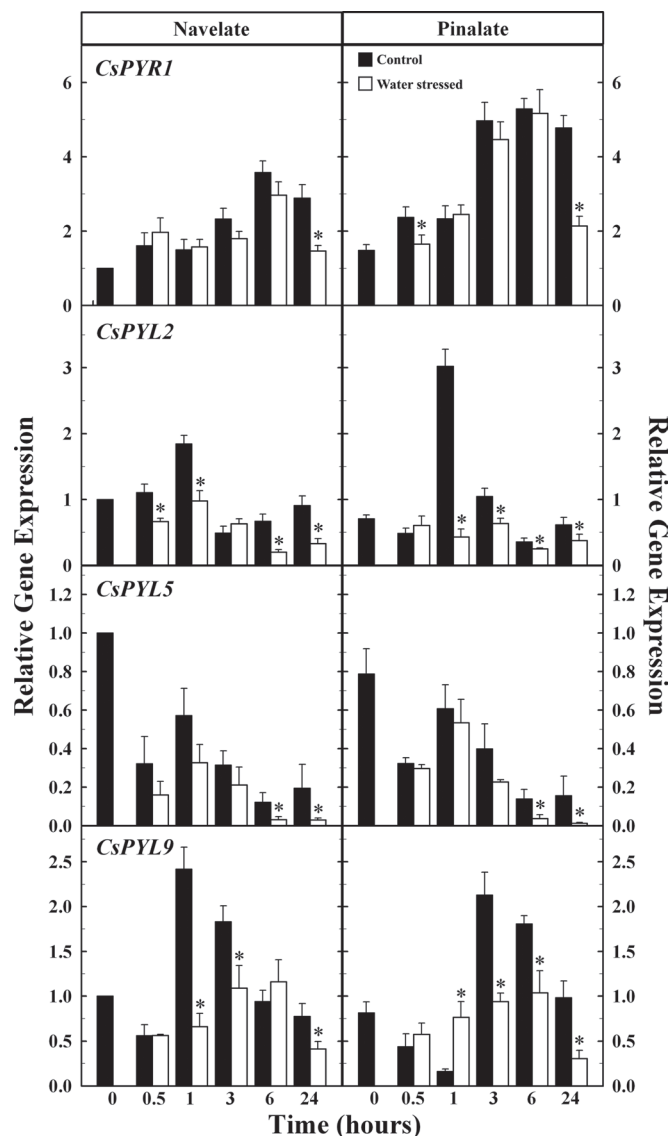


Fig. 5. Relative gene expression analysis by qRT-PCR of *Citrus* *PYR/PYL/RCAR* ABA receptors in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. The results are the means of three biological replicates of four leaves each \pm SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analysed period are indicated by an asterisk.

RCAR, *CsPP2CA*, and *CsSnRK2* genes identified in this work might function as the core elements of the ABA perception and signalling pathway.

The comparative transcriptional analysis between wild-type 'Navelate' fruit and its ABA-deficient mutant 'Pinalate' revealed no important differences in most of the *CsPYR/PYL/RCAR* expression profiles, although the transcript level of *CsPYR1* in IG and FC fruits was different between genotypes. This result suggests that the expression of this gene family might be developmentally regulated in *Citrus* and that changes in ABA content found in 'Pinalate' fruit during ripening may be sufficient for regulating *CsPYR/PYL/RCAR* gene expression. Indeed, the expression profiles of *CsPYR1*, *CsPYL4*, and *CsPYL5* suggest the involvement of ABA in their regulation since the lowest

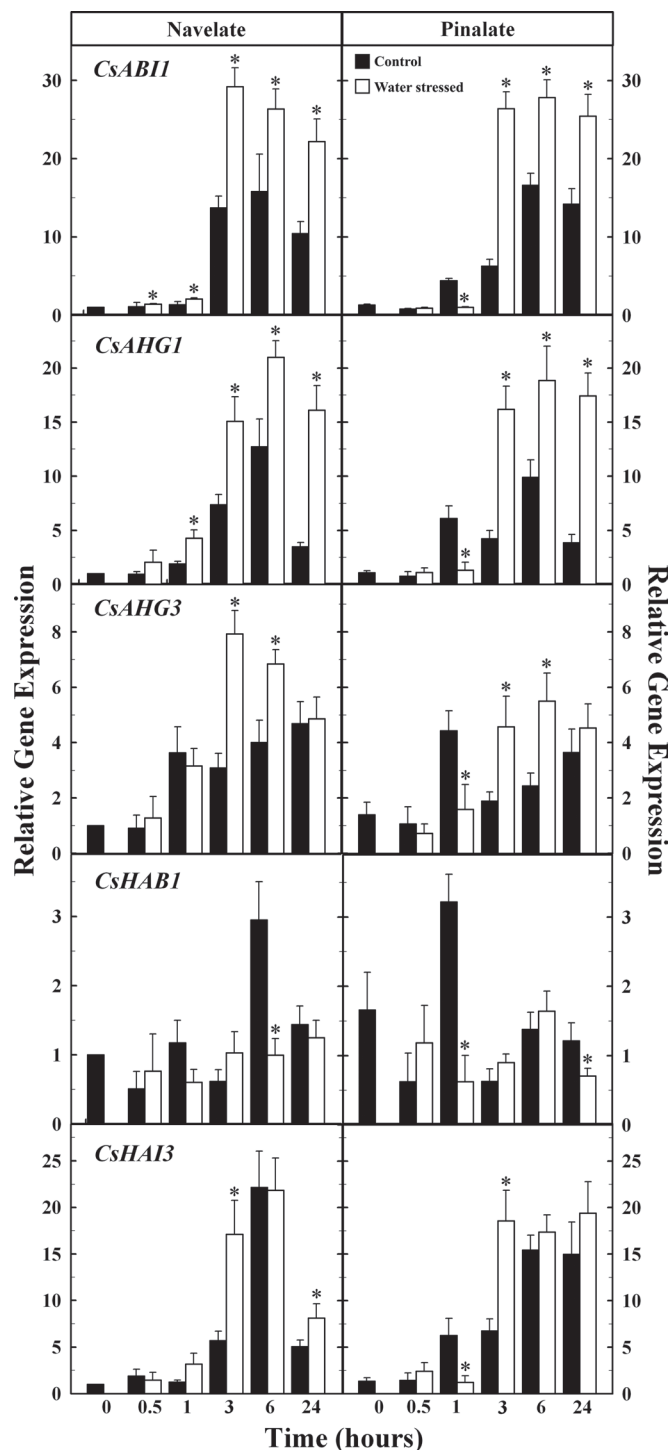


Fig. 6. Relative gene expression analysis by qRT-PCR of *Citrus* clade-A PP2Cs in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. The results are the means of three biological replicates of four leaves each \pm SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analysed period are indicated by an asterisk.

transcript levels of these genes were concomitant with the highest ABA levels in 'Navelate' and 'Pinalate' fruits, whereas their expressions peaked before the ABA increase (Figs 2, 3A). This result is in agreement with that found in sweet cherry showing

the concomitant down-regulation of the plastid ABA receptor magnesium chelatase (CHLH) and the increment in endogenous ABA during fruit ripening (Ren *et al.*, 2011). Overall, these results suggest that the reduction in ABA receptor gene expression may be concomitant with the increase in ABA during non-climacteric fruit ripening. In this context, it should be pointed out that the expression of *PYR/PYL/RCAR* genes is differentially affected by ABA treatment in seedlings of *Arabidopsis* (Santiago *et al.*, 2009b; Szostkiewicz *et al.*, 2010), and that the accumulation of *PYR/PYL/RCAR* transcripts may also parallel the increase in ABA during ripening of strawberry and tomato fruits (Chai *et al.*, 2011; Sun *et al.*, 2011). In this work, three different expression patterns were observed among *PYR/PYL/RCAR* genes: a first set of genes (*CsPYR1*, *CsPYL4*, and *CsPYL5*) showed their minimum transcript levels when the highest ABA content was detected in the flavedo of 'Navelate' and 'Pinalate' fruits. Although their expression patterns were similar, transcript accumulation of *CsPYR1* was much lower than that of *CsPYL4* and *CsPYL5* genes, which showed similar values (Supplementary Table S6 at JXB online). Secondly, the *CsPYL8* transcript level peaked when ABA increased during fruit ripening and, finally, *CsPYL9* continuously decreased as ripening progressed, although it increased slightly before the increment in ABA in both varieties (Fig. 3A). Interestingly, genes whose changes in expression did not mirror ABA accumulation during ripening (*CsPYL8* and *CsPYL9*) were clustered into subfamily I (Fig. 1A). In this context, it is interesting to note that tomato genes belonging to this subfamily (*SIPYL1*, *SIPYL2*, and *SIPYL3*) have been related to ABA changes during fruit development and ripening (Sun *et al.*, 2011), and functional activity for AtPYL8 and AtPYL9 proteins has been demonstrated by Ma *et al.* (2009) in vegetative tissues. It is noteworthy, however, that the ABA-binding region of *CsPYL8* showed an insert of 17 amino acids, which is not present either in *Arabidopsis* or in tomato sequences and might affect the ability of this protein to bind the hormone (Supplementary Fig. S1A). In addition, *CsPYL8* transcript levels were much lower than that of *CsPYL9*, which showed the highest transcript accumulation among *CsPYR/PYL/RCAR* genes (Supplementary Table S6). It is also interesting that the expression of the *CsPYL2* gene was not detected in fruits of both cultivars during ripening, which suggests that the expression of some ABA receptors could be tissue specific in *Citrus*. In agreement with this, some tomato genes such as *SIPYL5*, belonging to the same subfamily as *CsPYL2* (subfamily III, Fig. 1A), were almost undetectable during fruit ripening (Sun *et al.*, 2011). Therefore, gene expression levels indicated the relevance of *CsPYL4*, *CsPYL5*, and *CsPYL9* genes in ABA perception during fruit development and ripening.

Clade-A PP2Cs function as negative regulators of the ABA signalling pathway and their expression is highly induced by ABA in plants (Merlot *et al.*, 2001; Saez *et al.*, 2004; T. Yoshida *et al.*, 2006; Xue *et al.*, 2008; Li *et al.*, 2009). Within this context, a transcriptional negative feedback regulatory mechanism has been proposed for modulating the ABA responses (Merlot *et al.*, 2001; Melcher *et al.*, 2009; Santiago *et al.*, 2009b; Vlad *et al.*, 2009; Weiner *et al.*, 2010). Thus, the initial response to ABA implies the ABA-dependent PYR/PYL/RCAR-mediated inactivation of PP2CAs, which allows the release of SnRKs and hence the phosphorylation of ABA-dependent

transcription factors or other proteins. This ABA signal is later attenuated by the up-regulation of *PP2CA* and the down-regulation of *PYR/PYL/RCAR* genes in an ABA-dependent manner. Thus, the resetting of the ABA transduction pathway offers a dynamic mechanism to modulate the ABA response (Santiago *et al.*, 2009b). The expression pattern of the *CsPP2CA* genes analysed in this work mostly paralleled the ABA accumulation in 'Navelate' and 'Pinalate' fruit during ripening (Figs 2, 3B) and, interestingly, the up-regulation of *CsPP2CA* genes was also concomitant with the down-regulation of the *CsPYL4* and *CsPYL5* genes (Fig. 3A). Therefore, these results suggest that a transcriptional negative feedback regulatory mechanism might be modulating the ABA responses during *Citrus* fruit ripening. In tomato, however, only *SIPP2C1* and *SIPP2C5* transcripts peaked, with the increment in ABA occurring during fruit ripening, while all of the *SIPYR/PYL/RCAR* genes analysed were negatively related to the accumulation of those *SIPP2C* genes (Sun *et al.*, 2011). Therefore, it would be interesting to investigate further the functionality of these proteins through protein-protein interactions, which would help to unravel the involvement of these subfamilies in ABA perception in *Citrus*.

The availability of the fruit-specific ABA-deficient mutant 'Pinalate' has allowed analysis of the relationship between the expression of the *CsPP2CA* genes and endogenous ABA accumulation during *Citrus* fruit ripening. Gene expression levels of *CsAB11*, *CsAHG3*, and *CsHAI3* peaked at the C stage in both 'Navelate' and 'Pinalate' fruit, but transcript accumulation was always higher in parental fruit. Likewise, *CsHAB1* transcript levels peaked in 'Navelate' at the C stage, although the level continuously decreased in the ABA-deficient mutant fruit (Fig. 3B). These results, together with the fact that gene expression of *CsAHG3* and *CsHAI3* increased from 3- to 10-fold (Fig. 3B; Supplementary Table S6 at *JXB* online), suggest an important effect of ABA content on *CsPP2CA* gene expression. In agreement with this idea and with the lower differences found between cultivars in the *CsPYR/PYL/RCAR* transcriptional levels, Szostkiewicz *et al.* (2010) reported that *PP2CA* genes were more responsive to ABA compared with ABA receptors, and suggested a higher sensitivity of these negative regulators to ABA changes. Unexpectedly, although *CsAHG1* showed an expression pattern similar to that of the other *CsPP2CA* genes, the transcript level at the C stage was 2-fold higher in the ABA-deficient mutant whereas the ABA content in 'Navelate' was double that of 'Pinalate' (Figs 2, 3B). The increased expression of this negative regulator supports previous molecular data suggesting the impaired response of this mutant to ABA treatments and dehydration (Romero *et al.*, 2012). Moreover, the expression of well-known ABA-dependent genes is also strongly reduced in the mutant fruit during ripening (Supplementary Fig. S3), which further supports the idea of a reduced sensitivity of 'Pinalate' fruit to ABA.

It is well known that the release of SnRK2s by PP2CAs after ABA binding to PYR/PYL/RCARs allows these positive effectors to phosphorylate downstream transcription factors and proteins involved in the ABA response (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Hirayama and Umezawa, 2010). The results obtained in the present work revealed that both *CsSnRK2.2* and *CsSnRK2.6* genes reached their highest transcript levels at the most immature

stages, when the minimum ABA content was detected in both cultivars (Figs 2, 3C), although transcript accumulation of the *CsSnRK2.6* gene was much higher than that of *CsSnRK2.2* in both varieties during fruit ripening (Supplementary Table S6 at *JXB* online). As ripening progressed, however, *CsSnRK2.2* remained almost unchanged in 'Navelate' and 'Pinalate' fruits, whereas *CsSnRK2.6* showed a transient increase at the Bk stage only in the mutant fruit. Similar expression patterns were found in the climacteric tomato fruit. During ripening of tomato, expression levels of SnRKs were high in the most immature stages and transiently increased with the increase in ABA (Sun *et al.*, 2011). In spite of differences found in *CsSnRK2.6* transcript levels between 'Navelate' and 'Pinalate' fruits, the overall results suggest that the relationship between endogenous ABA content and the transcriptional regulation of these components of the ABA signalosome during *Citrus* fruit ripening is less relevant than that occurring for the *CsPP2CA* genes.

In order to gain further insights into the role of the ABA signalosome components and to understand whether the key genes are common or tissue specific in *Citrus*, expression analysis of these elements has also been performed in leaves exposed to dehydration. As indicated above, the deficiency in ABA of 'Pinalate' is fruit specific and, consequently, no relevant differences were found in ABA content or weight loss between 'Navelate' and 'Pinalate' leaves. Since leaves are very prone to dehydration, special attention was paid to minimizing the water loss in control leaves. Under the experimental conditions used, water loss was always <5% and 4-fold lower than in the water-stressed leaves. Thus, changes observed in ABA levels can be related to changes in weight loss. The attenuated expression profiles of some of the studied genes in control leaves suggest that the response of vegetative tissue to dehydration may depend on the severity of the stress imposed.

Gene expression changes of the three core components of the ABA signalosome in dehydrated leaves (Figs 5–7) were similar to those found in *Arabidopsis* (Santiago *et al.*, 2009; Szostkiewicz *et al.*, 2009). Overall, transcriptional profiling of these genes suggested that ABA increases caused by dehydration up-regulate the levels of all *CsPP2CA* and down-regulate some *PYR/PYL/RCAR* and *SnRK2* family members, such as *CsPYL2*, *CsPYL5*, *CsPYL9*, and *CsSnRK2.2*, whereas the relative levels of other members of these families, such as *CsPYR1* and *CsSnRK2.6*, remain fairly constant. Nevertheless, it is interesting to note that *CsPYL2* and *CsPYL9* were the most expressed genes in control and dehydrated leaves, suggesting that *CsPYL2* might play a key role in ABA responses in leaves but not in fruit, while *CsPYL9* could be relevant in both vegetative and reproductive tissues (Supplementary Table S6 at *JXB* online). Therefore, the results obtained in the leaf are complementary to those found in reproductive tissue. The high transcript accumulation of *CsPYL9* was down-regulated as ABA increased during both fruit ripening and leaf dehydration. Likewise, *CsPYR1* and *CsPYL5* gene expression was lowest when the highest ABA levels were achieved during fruit ripening and leaf dehydration, although transcript accumulation of *CsPYL5* in fruit was much higher than in vegetative tissue, suggesting a minor role for this gene in leaves (Supplementary Table S6). Moreover, the expression profile of these genes did not mirror that of ABA accumulation during leaf dehydration as

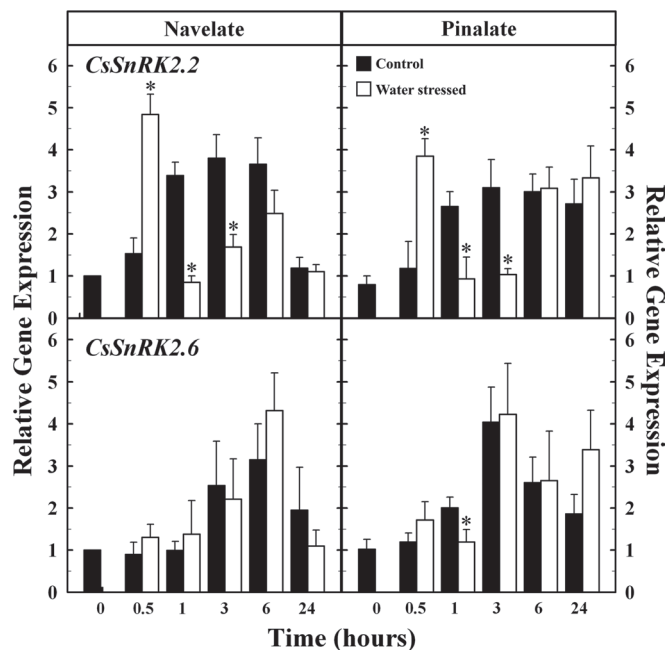


Fig. 7. Relative gene expression analysis by qRT-PCR of *Citrus* subclass III *SnRK2s* in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. The results are the means of three biological replicates of four leaves each \pm SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analysed period are indicated by an asterisk.

occurred during fruit ripening. This differential regulation under physiological or stress conditions may provide a means for the plant to cope with sustained high levels of ABA or to adjust the sensitivity of ABA perception and signalling. It is interesting to mention that some common responses in the ABA signalosome were observed between fruit and leaf tissues, such as the high sensitivity of the *CsPP2CA* gene components to ABA changes and *CsSnRK2.6* as the major subclass III *SnRK2* expressed gene. Moreover, in both tissues, *CsPYR1* transcript accumulation was very low compared with the other *CsPYR/PYL/RCAR* genes, and *CsPYL9* was highly expressed. Comparison between fruit and vegetative tissue has also revealed some tissue specificity: the *CsPYL2* gene was highly expressed in leaves but no expression was detected in fruit, whereas *CsPYL4* and *CsPYL8* transcripts were detected during fruit development but not in leaves subjected or not to water stress.

In summary, this work reports for the first time the identification of ABA signalling core components in *Citrus* comprising six PYR/PYL/RCAR ABA receptors, five PP2CAs, and two subclass III SnRK2s. During sweet orange fruit development and ripening, the expression pattern of some ABA receptors mirrored the ABA content, whereas that of *CsPP2CA* genes paralleled the hormone accumulation, together modulating ABA perception, downstream signalling, and, consequently, physiological ABA responses. Additionally, transcriptional analysis performed in water-stressed leaves revealed that some members of the PYR/PYL/RCAR family are tissue specific and that sensitivity to ABA changes in the *PP2CA* genes, which are negative regulators of the ABA signal transduction pathway, was much higher than in other components of the ABA signalosome.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Multiple sequence alignment of the *Arabidopsis thaliana* and *Citrus sinensis* ABA signalling core components.

Figure S2. Predicted tertiary structure model of the ABA signalosome components of *Citrus*.

Figure S3. Gene expression analysis of ABA-dependent downstream signalling genes.

Table S1. Colour evolution of 'Navelate' and 'Pinalate' fruit during ripening.

Table S2. Primers designed for the ABA signalling core component gene expression analysis by quantitative RT-PCR (qRT-PCR).

Table S3. Similarity matrix between *Citrus* and *Arabidopsis* PYR/PYL/RCAR proteins.

Table S4. Similarity matrix between *Citrus* and *Arabidopsis* PP2CA proteins.

Table S5. Similarity matrix between *Citrus* and *Arabidopsis* SnRK2 proteins.

Table S6. Absolute gene expression levels of ABA signalosome components during fruit ripening and leaf dehydration.

Acknowledgements

We thank Dr L. Navarro (IVIA, Spain) for allowing us to use the Spanish Citrus Germplasm Bank. Special thanks are also due to Dr L. Gonzalez-Candelas for his help with absolute gene expression analysis. The technical assistance of M. Sánchez-Hervás is also gratefully acknowledged. This work was supported by the Spanish Ministry of Science and Technology (Research Grants AGL2006-09496, AGL2009-11969, and AGL2009-11558) and by the Generalitat Valenciana (PROMETEO/2010/010). PR was the recipient of a fellowship from the Spanish Ministry of Science and Technology.

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