Unravelling molecular responses to moderate dehydration in harvested fruit of sweet orange (*Citrus sinensis* L. Osbeck) by using a fruit-specific ABA-deficient mutant

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ABSTRACT

Water stress affects many agronomic traits that may be regulated by the phytohormone abscisic acid (ABA). Within these traits, loss of fruit quality becomes important in many citrus cultivars that develop peel damage in response to dehydration. To study peel dehydration transcriptional responsiveness in harvested citrus fruit and the putative role of ABA in this process, we have performed a comparative large-scale transcriptional analysis of water-stressed fruits of the wild-type 'Navelate' orange (*Citrus sinesis* L. Osbeck) and its spontaneous ABA-deficient mutant 'Pinalate', which is more prone to dehydration and to develop peel damage. Major changes in gene expression occurring in the wild-type line were impaired in mutant fruit. Gene ontology analysis revealed the ability of 'Navelate' fruits to induce the 'response to water deprivation' and 'di-, tri-valent inorganic cation transport' biological processes, as well as the repression of the 'carbohydrate biosynthesis' process in the mutant. Exogenous ABA triggered relevant transcriptional changes and repressed the 'protein ubiquitination' process although it could not fully rescue the physiological behaviour of the mutant. Overall, results indicate that dehydration responsiveness requires ABA-dependent and independent signals, and highlight that the ability of citrus fruits to trigger molecular responses against dehydration is an important factor in reducing their susceptibility to develop peel damage.

KEYWORDS

ABA-deficient mutant fruit, abiotic stress, abscisic acid (ABA), citrus, gene expression, microarray, peel damage, water stress

INTRODUCTION

Plant growth, crop agricultural productivity and quality are adversely affected by both biotic and abiotic stress factors. The effect of water stress on physiological and molecular responses of model plants has been largely described (Bray *et al.*, 2000; Bartels and Sunkar, 2005; Seki *et al.*, 2007). However, in spite of the relevance of this environmental factor on fruit quality, knowledge of these mechanisms in fruits is limited. Nevertheless, transcriptomic studies conducted in grapes indicate that genes, gene categories, and regulatory elements are differently affected by dehydration occurring before or after harvesting the fruit and also by the stress severity (Grimplet *et al.*, 2007; Rizzini *et al.*, 2009; Deluc *et al.*, 2009; Zamboni *et al.*, 2010).

Studies conducted in plants show that water stress causes removal of water from cytoplasm to extracellular space causing a reduction in the cytosolic and vacuolar volumes and an alteration of reactive oxygen species homeostasis, which originates accumulation of toxic substances but also the production of signal transduction molecules (Miller *et al.*, 2010). Accumulation of sugars, poly-alcohols, amino acids, amines and ABA in response to water stress have been demonstrated in the model plant *Arabidopsis thaliana* and in a number of important horticultural crops (Bartels and Sunkar, 2005; Seki *et al.*, 2007). Since these metabolites function as osmolytes, antioxidants, scavengers and/or signalling molecules that can help plants to tolerate abiotic stresses, changes in their homeostasis are thought to be associated with the maintenance of structure and function of cellular component networks. Therefore, the metabolic pathways of these compounds have been largely investigated (Seki *et al.*, 2007) although regulatory networks and cross-talk between their components need further investigation (Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Deregulation of these water stress metabolites and/or

responsive genes can be finally manifested as cellular damaged tissues (Alférez *et al.*, 2008). Moreover, mechanisms occurring in grape berries dehydrated after harvest (Grimplet *et al.*, 2007; Zamboni *et al.*, 2010) or in berries from water-stressed vines (Deluc *et al.*, 2009) indicated that dehydration may have a profound effect on the expression of genes associated with the biosynthesis of relevant compounds that ultimately impact fruit quality. Functional characterization of the stress-induced genes also highlights the relevance of the secondary metabolism, which may be affected by the rate and intensity of dehydration (Rizzini *et al.*, 2009). Furthermore, it should be also considered the relevance of fruit surface properties in the dehydration of detached fruits.

The tight relationship between ABA and dehydration is well known (Bartels and Sunkar, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007), although ABA-independent pathways may also operate in response to dehydration (Riera *et al.*, 2005). Plant hormone mutants have been extensively used to elucidate signal transduction pathways and to define the involvement of hormones in physiological processes. Focusing on ABA, natural and induced knockout and overexpressing mutants of biosynthetic and signalling transduction genes in *Arabidopsis* (Armstrong *et al.*, 1995; Koornneef *et al.*, 2004) and other plant species (Pena-Cortes *et al.*, 1989; Groot and Karssen, 1992; Schwartz *et al.*, 1997; Burbidge *et al.*, 1999) have been characterized. However, the availability of artificially generated mutants is very uncommon in woody plants. Therefore, the access to spontaneous fruit hormone mutants is of particular scientific interest. A spontaneous fruit-specific ABA-deficient mutant from the wild-type 'Navelate' orange (*Citrus sinensis* L. Osbeck), named 'Pinalate', has been described (Rodrigo *et al.*, 2003). 'Pinalate' orange presents distinctive yellow-coloured fruit because of a partial blockage of the carotenoid biosynthetic pathway, causing a fruit-

specific ABA-deficiency. Moreover, harvested 'Pinalate' fruit shows higher dehydration and much higher susceptibility than its parental to develop peel depressions, which in advanced stages become bronze and necrotic (Alférez *et al.*, 2005; Sala *et al.*, 2005). This physiological disorder, known as 'non-chilling peel pitting' (NCPP), 'rind breakdown' or 'rind staining' (Agustí *et al.*, 2001; Lafuente and Sala, 2002), occurs in many citrus cultivars at temperatures above 11 ºC, with water stress being an important causal factor in both attached and detached fruits (Alférez *et al.*, 2003; Lafuente and Zacarías, 2006). Therefore, because of its higher susceptibility to develop NCPP and to dehydration, and its fruit-specific ABA deficiency, 'Pinalate' fruit is a valuable experimental system to understand the involvement of ABA in the molecular mechanisms underlying the response of citrus fruits to water stress causing eventually peel damage.

In the last decade, 'omics' tools have been widely used to characterize regulatory networks involved in plant abiotic stress responses (Urano *et al.*, 2010). Numerous transcriptomic studies have been conducted to analyze model and crop plants transcriptome under various stress conditions, and have identified thousands of stress-responsive genes (Vij and Tyagi, 2007). Genome-wide studies have been also carried out in fruits with the aim of characterizing ripening or their responses to several stresses or hormone treatments (Maul *et al.*, 2008; Ziliotto *et al.*, 2008; Liu *et al.*, 2009) but information on changes occurring in the transcriptome of water-stressed fruits is limited to grapes (Grimplet *et al.*, 2007; Rizzini *et al.*, 2009; Deluc *et al.*, 2009). Over the past years, the Spanish Citrus Functional Genomic Project (CFGP) has generated useful tools for citrus transcriptomic research. Citrus cDNA microarrays have been developed in this Consortium (Forment *et al.*, 2005; Martínez-Godoy *et al.*, 2008), and the latest generation contains 21081 (20K) putative citrus unigenes, which offers a good

representation of the citrus genome. In the framework of the CFGP, important insights in citrus biology have been already achieved (Cercós *et al.*, 2006; Gandía *et al.*, 2007; Agustí *et al.*, 2008; Alós *et al.*, 2008; Huerta *et al.*, 2008; Brumós *et al.*, 2009; Ballester *et al.*, 2011). Global changes in gene expression in response to drought have been characterized in citrus seedlings (Gimeno *et al.*, 2009). However, in spite of the relevance of dehydration in fruit quality, a large-scale transcriptomic profile of citrus fruit in response to this stress has not been conducted so far.

With the aim of characterizing molecular mechanisms involved in the response of harvested citrus fruits to dehydration and the potential role of ABA in this process, as well as to elucidate the possible relationship existing between these two components and the occurrence of NCPP, a large-scale transcriptional analysis in the flavedo of 'Navelate' and its mutant 'Pinalate' oranges has been conducted by using the CFGP 20K microarray. To that end, fruits from both cultivars were stored at a temperature and RH causing moderate water stress and the appearance of peel damage. In addition, transcriptomic changes occurring in 'Pinalate' fruit treated with ABA were examined.

MATERIALS AND METHODS

Plant material and ABA treatment

Full mature fruits of 'Navelate' (*Citrus sinensis* L. Osbeck) orange and its spontaneous ABA-deficient mutant 'Pinalate' were randomly harvested from adult trees grown in experimental orchards under normal cultural practices at 'The Spanish Citrus Germoplasm Bank' at Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain). After harvest, fruits without any damage or visual defects were immediately delivered to the laboratory. To test whether application of ABA modified the postharvest response of 'Pinalate' fruit to dehydration, fruits from both cultivars were divided into two groups. The first group was treated with ABA (Sigma-Aldrich, St. Louis, MO, USA) by dipping the fruits for 1 min in an aqueous solution of 1mM ABA containing 0.7% ethanol to dissolve the hormone, while fruits of the second group were just treated with water containing 0.7% ethanol by following the same procedure. Fruits were dried at room temperature and then stored in the dark at 12 ºC and 70-75% RH for up to 6 weeks. The ABA treatment was repeated every 2 weeks to ensure high ABA levels during fruit storage. Likewise, 'Pinalate' and 'Navelate' control fruits were dipped into 0.7% ethanol at these times. Periodically, flavedo (outer coloured part of the peel) samples were collected from the total surface of fruits, frozen and homogenized in liquid nitrogen, and kept at -80 ºC for later analysis. Three biological replicates, each consisting of 5 fruits, were collected at each sampling period.

Peel damage incidence and water loss measurement

A visual rating scale from 0 (no peel damage) to 4 (severe damage), based on surface necrosis and intensity of peel browning, was used to evaluate the incidence of NCPP in fruits stored at 12 ºC and 70-75% RH. The average NCPP index was calculated by

summing the products of the number of fruits in each category by the value assigned to each category in the rating scale, and then dividing the resulting sum by the total number of fruits evaluated. In citrus fruit, water is lost mainly through the peel surface. Therefore the cumulative percentage of fruit weight loss occurring during storage was expressed per cm^2 of fruit surface area. Fruit surface was estimated by using the Turrel's tables after measuring the diameter and height of the fruits (Turrel, 1946). Results are the means of 3 replicates of 10 fruits each ± SE.

RNA isolation, **cDNA labelling and microarray hybridization**

Total RNA was extracted from frozen flavedo samples by a modified method of the previously described by Rodrigo *et al*. (2004), as reported by Ballester *et al.* (2006). Total RNA was treated with Ribonuclease-free DNase (Ambion/Applied Biosystems, Austin, TX, USA) following the manufacturer's instructions for removing possible genomic DNA contaminations. Thereafter, the amount of RNA was measured by spectrophotometric analysis (Nanodrop, Thermo Fisher Scientific, Madrid, Spain) and its quality was verified by agarose gel electrophoresis and ethidium-bromide staining. cDNA synthesis and purification, dye coupling, and labelled-cDNA purification were accomplished according to the method described by Forment *et al*. (2005). cDNA samples were Cy5-labelled and co-hybridized with a Cy3-labelled cDNA reference pool from a mixture containing equal amounts of RNA from all experimental samples assayed. The use of this reference sample has been widely used in *Citrus* transcriptomic research since it represents a powerful tool for reducing the number of hybridizations to make all the possible pairwise comparisons between samples (Agustí *et al.*, 2008). Microarray hybridization and slide washes were performed by a modified method of that proposed by Forment *et al*. (2005) as described by Ballester *et al.* (2011). The

cDNA microarrays used were developed in the framework of the CFGP (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/), and contained 21081 putative unigenes (20K) isolated from 52 cDNA libraries of citrus generated from a wide range of varieties, developmental and fruit ripening stages, and from different tissues subjected to biotic and abiotic stress conditions (Martínez-Godoy *et al.*, 2008).

Microarray data acquisition and analysis

Hybridized microarrays were scanned by using a GenePix 4000A scanner (Axon Instruments, Sunnyvale, CA, USA) equipped with GenePix Pro 6.0 image acquisition software (Axon Instruments), following manufacturer's instructions to adjust the channels intensity ratio to 1.0 and the percentage of saturated spots close to 1%. Non-homogeneous and aberrant spots were discarded. Only spots with a background-subtracted intensity greater than 2-fold the mean of background intensity were used for normalization and further analysis. In order to compensate labelling differences among samples and other non-biological sources of variability, results were normalized by using Print-Tip-Lowess method, included in the Acuity 4.0 software (Axon Instruments), by using background subtracted median values and an intensity-based Lowess function within and among microarrays. Thereafter, differentially expressed genes for all possible pairwise comparisons were determined by applying the Significant Analysis of Microarrays (SAM) program (Tusher *et al.*, 2001) from the TM4 Microarray Software Suite (Saeed *et al.*, 2003). Genes that satisfied a statistical threshold (False Discovery Rate) lower than 0.01 were identified as differentially expressed genes. FatiGO+ (Babelomics, http://bioinfo.cipf.es/), developed by Al-Shahrour *et al.* (2004), was used to identify biological processes significantly under- or over-represented in a particular set of differentially expressed genes relative to a

reference group containing all genes present in the microarrays having an *Arabidopsis* homologous. Gene ontology analysis for induced and repressed genes was independently performed applying a Fisher two tailed test with a *p*-value lower than 0.05. In this analysis, the specificity of the biological process increases with the GO level from 3 to 9. Multivariate analyses as Principal Component (PCA) and Hierarchical Cluster Analysis (HCA) (ANOVA test, Benjamini-Hochberg FDR < 0.05) were performed by using the MultiExperiment Viewer (MeV) tool of TM4 Microarray Software Suite (Saeed *et al.*, 2003).

qRT-PCR expression analysis

Reverse transcription followed by quantitative polymerase chain reaction analysis (qRT-PCR) was performed to validate microarray results and to examine the time-course expression pattern of selected genes along fruit storage by using a LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany) equipped with LightCycler SW 1.5 software. A two-step qRT-PCR assay was designed as suggested by Udvardi *et al*. (2008). cDNAs were synthesized from all analyzed samples by using 400 U of SuperScript III RT (Invitrogen, Paisley, United Kingdom) in presence of 0.5 μg of Oligo(dT) 20-mer (Invitrogen) and 10 U of Ribonuclease Inhibitor (Invitrogen) according to manufacturer's instructions. Gene-specific primers were designed using DNAMAN 4.03 software (Lynnon BioSoft, Quebec, Canada). Both synthesized cDNA and the primer pairs were thereafter incubated with LightCycler 480 SYBR Green I Master (Roche Diagnostics) at 95 ºC for 10 min followed by 40 cycles at 95 ºC for 10 s, 60 ºC for 5 s and 72 ºC for 10 s. Forward (F) and reverse (R) sequences for specific primers and correlation coefficients (r^2) between the $\log_2$-transformed expression values as measured by microarray and RT-PCR analyses for each gene are shown in Table 1.

To rule out non-specific amplified products, melting curve analysis were performed and the reaction products were sequenced. To transform fluorescent intensity measurements into relative mRNA levels, a 2-fold dilution series of a mixture containing an equal amount of each cDNA sample was used and standard curves were constructed for all studied genes. Reference genes *CsACT* (F 5'-TTAACCCCAAGGCCAACAGA-3'; R 5'-TCCCTCATAGATTGGTACAGTATGAGA-3'), *CsEF1α* (F 5'-ATTGACAAGCGTGTGATTGAGC-3'; R 5'-TCCACAAGGCAATATCAATGGTA-3'), *CsGAPDH* (F 5'-CGTCCCTCTGCAAGATGACTCT-3'; R 5'-GGAAGGTCAAGATCGGAATCAA-3') and *CsTUB* (F 5'-GCATCTTGAACCCGGTAC-3'; R 5'-ATCAATTCGGCGCCTTCAG-3'), whose constitutive expression along fruit storage was confirmed by using geNorm program (Vandesompele *et al.*, 2002), were used for data normalization. Statistical analysis (Pair Wise Fixed Reallocation Randomisation Test) was carried out by using the Relative Expression Software Tool (REST, http://rest.gene-quantification.info) (Pfaffl, 2001). Each sample was analyzed in triplicate and mean ratios ± SE were calculated.

ABA analysis

ABA analysis was performed as described by Lafuente *et al*. (1997). ABA was extracted from 1 g fresh weight (FW) frozen flavedo with 80% acetone containing 0.5 g L^{-1} citric acid and 100 mg L^{-1} of butylated hydroxytoluene. After centrifugation, the supernatant was diluted in 3 serial dilutions in ice-cold TBS (6.05 g L^{-1} Tris, 8.8 g L^{-1} NaCl and 0.2 mg L^{-1} $MgCl_2$) adjusted to pH 7.8 with 6N HCl. Three samples for each dilution were analyzed by an indirect ELISA method using the ABA-4'-BSA conjugate that was synthesized as previously reported by Weiler (1980) with some modifications (Norman *et al.*, 1988). The results are the means of 3 replicate samples ± SE.

Statistics

A mean comparison using the Tukey´s test and Statgraphics.5.1 Software (Manugistics, Inc.) was performed to determine significant differences at $p \leq 0.05$ in NCPP, fruit weight loss per surface area and ABA levels between samples of ‘Navelate’ and ‘Pinalate’ fruits, treated or not with ABA, during fruit storage at 12 ºC and 70-75% RH.

RESULTS

Susceptibility of ‘Navelate’ and the ABA-deficient mutant ‘Pinalate’ fruit to non-chilling peel pitting and dehydration and influence of exogenous ABA

The susceptibility of fruits of the ABA-deficient mutant ‘Pinalate’ to NCPP was much higher than that of fruits of its parental ‘Navelate’ (Fig. 1A). Peel pitting was already visible by 1 week in stored ‘Pinalate’ fruits, while in ‘Navelate’ fruits the incidence of the disorder was barely detected. This difference between mutant and wild-type fruits was much more evident as storage progressed, reaching the highest difference by 3 weeks, when mutant fruits showed about a 5-fold higher NCPP index than the parental fruits (Fig. 1A). By this period, the weight loss per surface area in mutant fruits was twice that of ‘Navelate’ fruits (Fig. 1B). ABA level in the flavedo of freshly harvested (FH) ‘Pinalate’ fruits was about 5-fold lower than in ‘Navelate’ fruits (Fig. 1C). A rapid increase in the ABA content occurred in ‘Navelate’ peel by 1 week, while it remained at low levels in ‘Pinalate’ fruits along storage (Fig. 1C). By the end of the experiment (6 weeks), ABA content in parental fruits was about 4-fold higher than in the mutant. In this context, it is noteworthy that ABA-treated ‘Pinalate’ fruits had even slightly higher phytohormone levels than the wild type from the beginning of the experiment (Fig. 1C) but the treatment had little effect on reducing the susceptibility of the mutant to NCPP (Fig. 1A) or its dehydration rate (Fig. 1B). Likewise, exogenous ABA did not significantly modify the severity of NCPP or weight loss per surface area in wild-type fruits (Fig. S1)

Comparative transcriptional profiling during storage conditions inducing moderate water stress

Considering the sharply increase in ABA content in ‘Navelate’ oranges by 1 week, and also the marked difference in NCPP index between varieties by 3 weeks, both time-points were selected for microarray hybridizations to compare changes in transcriptional profiling of both genotypes with respect to FH fruits. The above mentioned results indicate that applying ABA did not rescue the phenotype of the mutant. In order to determine whether increasing endogenous ABA levels in the mutant may simulate the molecular responses induced by moderate water stress in the wild-type phenotype, ABA-treated ‘Pinalate’ fruits were also included in the transcriptome analysis. Venn diagrams summarize the number of differentially expressed genes (SAM, FDR < 0.01) in fruits stored for 1 (Fig. 2A) or 3 (Fig. 2B) weeks respect to FH fruits.

Major changes in the number of differentially expressed genes occurred by 1 week in ‘Navelate’ fruits (Fig. 2A) and by 3 weeks in ‘Pinalate’ (Fig. 2B). This effect was even more marked in the ABA-treated fruits (Fig. 2B). It is also noteworthy that repression prevailed in both cultivars along whole storage. Major inductions (1131 genes) occurred in parental fruits by 1 week, while a small set of up-regulated genes was found in both ‘Pinalate’ fruits treated or not with ABA (182 and 65, respectively) (Fig. 2A). Likewise, ‘Navelate’ showed the highest number of down-regulated genes by 1 week (1956). The expression of 322 of them also decreased in ‘Pinalate’, although this number was reduced (65) when ABA was applied (Fig. 2A). By 3 weeks (Fig. 2B), the number of induced (192) and repressed (269) genes in the flavedo of ‘Navelate’ fruits was less remarkable. By contrast, a high increment in the number of down-regulated genes was observed in ‘Pinalate’ (1221) and this effect was enhanced by applying ABA (2237) (Fig. 2B).

Principal Component (PCA) and Hierarchical Cluster Analysis (HCA) were performed to validate the repeatability of the microarray data across replications and to cluster

samples according to their global gene expression profile. ANOVA test revealed that 1471 genes, from a total of 21081, showed differential expression and were used for PCA and HCA. In all conditions, the transcriptional profile of the 3 separate RNA replicate samples were tightly clustered (Fig. 3A). On the other hand, PCA revealed marked differences in gene expression patterns between FH and stored fruits (X axis, explaining 44 % of the total variation), and also between FH fruits of both genotypes (variation Y and Z axes = 18.8 %, Fig. 3A). 'Pinalate' (P) fruits stored for 1 week (1W) were distributed in the middle of the three axes, close to mutant fruits stored for 3 weeks (P3W). By this period, fruits of 'Navelate' (N1W) were clustered in the upper part of the Y axis and far from those stored for 3 weeks (N3W). ABA-treated 'Pinalate' fruits stored for 3 weeks (P3W+A) grouped together, far from both P3W and P1W+A fruits (Fig. 3A). HCA confirmed results obtained by PCA. 'Navelate' and 'Pinalate' FH fruits were separately clustered in an independent branch from the stored samples, which were grouped by storage period (Fig. 3B). Interestingly, P1W+A fruits clustered into an independent group.

Functional categorization of differentially expressed genes

Gene ontology analysis identified biological processes significantly under- or over-represented in the sets of differentially expressed genes selected from the SAM analysis. This analysis revealed that repressed genes in 'Navelate' fruit stored for 1 week were enriched in biological processes related to biopolymer, heterocycle and RNA metabolism, and to cellular biosynthesis with respect to FH fruits, while induced genes were enriched in the response to water deprivation and the di-, tri-valent inorganic cation transport processes (Table 2). However, the differentially expressed genes in 'Navelate' fruits stored for 3 weeks were not statistically grouped in any biological

process. Likewise, no biological process was over-represented in either ‘Pinalate’ or ‘Pinalate + ABA’ fruits stored for 1 week. In contrast, the down-regulated genes in the mutant fruits stored for 3 weeks, treated or not with ABA, were statistically enriched in the same processes. Among these processes, responses to biotic and abiotic stimulus, including light, temperature, jasmonic acid, wounding and to other organism, as well as processes related to energy derivation and carbohydrate biosynthesis were identified. Interestingly, the inhibition of ‘protein ubiquitination’, associated with protein degradation, was the unique biological process differentially affected by the ABA treatment in mutant fruits (Table 2).

Genes belonging to the most relevant and specific biological processes (higher GO levels) are shown in Table 3. Among genes belonging to ‘water deprivation’ biological process, genes involved in ABA synthesis and perception (*NCED1*, *ZEP* and *PP2C*), ABA-responsive genes (*HVA22E*, *Lea5* and *ADH*) and ABA-dependent transcription factors (*HB7*, *NAC4* and *ABF4*) were found. Furthermore, genes included in this process encoded aquaporins, vacuolar proton-pump, and other proteins playing protective roles against dehydration (Table 3). Within the inorganic cation transport process, iron transporters and chelators, several copper transporters and two calcium-dependent transporter proteins were identified (Table 3). It is also noteworthy to highlight that the most specific process (‘carbohydrate biosynthesis’) repressed in both ‘Pinalate’ and ‘Pinalate’ fruits treated with ABA, included not only biosynthesis-related genes but also genes related to cell-wall metabolism, a *MYC* transcription factor and an inositol-3-phosphate synthase (Table 3). The unique biological process affected by exogenous ABA in ‘Pinalate’ fruits (‘protein ubiquitination’) included 6 genes belonging to a super-family of E3-ubiquitin ligases involved in protein degradation and with high similarity to plant U-box domain-containing proteins (PUB) of *Arabidopsis* (Table 3).

Expression profiles for selected genes by qRT-PCR analysis

Quantitative RT-PCR analysis was conducted to validate microarray gene expression data and to further characterize expression patterns of selected genes in fruits exposed to moderate water stress for up to 6 weeks. Comparison between the transcript abundance data obtained by the 20K microarray and by RT-PCR analysis with gene-specific primers revealed a high correlation for all selected genes with r^2 values between 0.90 and 0.98 (Table 1). Among genes belonging to 'response to water deprivation' biological process, the genes *CsRD19* and *CsRD21*, with homology to dehydration responsive genes of *Arabidopsis* (AT4G39090 and AT1G47128, respectively), the *CsHVA22E*, homologous to an ABA-inducible gene (AT5G50720), and the gene *CsNCED1* (AT3G14440), involved in ABA biosynthesis, were selected. A rapid and transient increase in relative expression levels of these genes was observed by 1 week in parental fruits. Interestingly, the relative expression level of *CsNCED1* also increased in the flavedo of 'Pinalate' fruit, but such increase was much lower than that occurring in 'Navelate'. Moreover, such increases were not induced by applying ABA to the mutant (Fig. 4A). Within the 'di-, tri-valent inorganic cation transport' biological process, *CsCOPT2* and *CsCOPT5* genes, with homology to copper transporters of *Arabidopsis* (AT3G46900 and AT5G20650, respectively), and *CsNRAMP1* and *CsNRAMP3*, homologous to iron transporter genes (AT1G80830 and AT2G23150, respectively), were selected. The expression levels of all these genes in FH mutant fruits were higher than in the parental fruits (Fig. 4B). However, a higher increase in their expression was detected in wild-type fruits exposed to moderate dehydration for 1 week than in mutant. From these genes, only the expression levels of *CsCOPT5* continued increasing in response to dehydration for up to 3 weeks. Accumulation of *CsNRAMP1* was, in

general, higher during storage in ‘Navelate’ fruits. In contrast, expression levels of *CsCOPT2* and *CsNRAMP3* were higher in ‘Pinalate’ fruits. Interestingly, the expression pattern of these two genes in mutant fruits treated with ABA was more similar to that of parental fruits than to the mutant fruits (Fig. 4B). On the other hand, citrus unigenes *CsIPS* and *CsMYC*, with homology to genes encoding a inositol-3-phosphate synthase (AT2G22240) and a MYC transcription factor (AT1G32640), respectively, were selected as representative genes of the ‘carbohydrate biosynthesis’ biological process. Both genes were repressed in the ABA-treated and non-treated ‘Pinalate’ fruits, though their expression levels in FH mutant fruits were higher than in ‘Navelate’ fruits (Fig. 4C). Expression levels of *CsMYC* transcription factor also decreased in the parental, while that of *CsIPS* increased from 1 to 3 weeks of storage (Fig. 4C). Genes *CsPUB9* and *CsPUB21* encoding proteins showing homology to E3-ubiquitin-ligases of *A. thaliana* involved in ABA (AT3G07360) and pathogen (AT5G37490) responses respectively, were selected among genes of the ‘protein ubiquitination’ biological process (Table 3). The rate of decrease in expression levels of both genes was similar in parental and mutant fruits but applying ABA had a marked effect on favouring repression (Fig. 4D).

DISCUSSION

The working hypothesis was that the ABA-deficiency may be an important factor for the high susceptibility of 'Pinalate' fruit to dehydration and to NCPP. To test this hypothesis and to understand the molecular mechanisms underlying both processes in citrus fruit, a comparative large-scale transcriptional analysis has been performed in harvested 'Navelate', 'Pinalate' and in ABA-treated 'Pinalate' fruits stored under conditions (12 ºC and 70-75% RH) causing moderate water stress and peel damage. The higher susceptibility to NCPP (Fig. 1A) and dehydration (Fig. 1B) observed in 'Pinalate' fruit agree with previous data showing that, under the same storage conditions, fruit weight loss and the decrease in water potential of the flavedo tissue was higher in fruits of the mutant (Alférez *et al.*, 2005).

Differential gene expression analysis (Fig. 2) further revealed the higher ability of 'Navelate' fruit to develop earlier molecular responses. These responses might contribute to reduce detrimental effects caused by dehydration and hence to the delay in peel damage development with respect to mutant fruit, which showed evident damage by 1 week. Thus, gene ontology analysis revealed that the most specific biological processes induced only in 'Navelate' fruit by 1 week were 'response to water deprivation' and 'di-, tri-valent inorganic cation transport' (Table 2), which fit into classical plant responses to water deficit and osmotic adjustment (Shinozaki *et al.*, 1998; Ramanjulu and Bartels, 2002). This result is also in concordance with previous findings showing that transport and abiotic stress-related genes are differentially regulated by dehydration in detached grape berries (Grimplet *et al.*, 2007; Rizzini *et al.*, 2009; Zamboni *et al.*, 2010). As expected, most of the genes belonging to the 'response to water deprivation' biological process (Table 3) were related to ABA. Thus, genes involved in ABA synthesis and perception (*NCED1*, *ZEP* and *PP2C*), ABA-dependent

transcription factors (*HB7*, *NAC4* and *ABF4*), and also genes encoding ABA-responsive proteins (*HVA22E*, *Lea5* and *ADH*) were identified, which highlights that the responses of 'Navelate' oranges to dehydration are modulated, at least in part, by the phytohormone. Among ABA-dependent genes belonging to this process, it is also worth mentioning those encoding proteins with homology to the plasma membrane PIP1B and PIP1E aquaporins as they play important roles adjusting osmotic potential in dehydrated plants (Shinozaki *et al.*, 1998; Shinozaki and Yamaguchi-Shinozaki, 2007). Therefore, and considering the fact that the number of stomata per surface area in fruits of both cultivars is similar (Alférez and Zacarías, unpublished data), the above results indicate a higher ability of 'Navelate' fruits to synthesize ABA, which controls stomata closure to reduce dehydration, and also to modulate ABA-related genes important for cell homeostasis and viability and hence for the reduction of peel damage. Other genes within this process (e.g. *CsRD19* and *CsRD21*) have not been classified as up-regulated by ABA in different plant systems (Koizumi *et al.*, 1993; Coupe *et al.*, 2003). From the results of the present work, it cannot be ruled out that they are ABA-dependent in citrus fruits since they were not induced by dehydration in the mutant. Nevertheless, genes within other categories like *CsCOPT5* and *CsNRAMP3* were induced by dehydration in both 'Navelate' and the ABA-deficient 'Pinalate' fruits. In addition, the expression of these genes did not increase either in 'Pinalate' fruits after the ABA treatment. Therefore, these results in citrus fruit might support previous findings suggesting the involvement of ABA-independent genes in the response to dehydration in plants (Riera *et al.*, 2005). In this context, it should be mentioned that the occurrence of alternative dehydration-responsive pathway(s) to minimize water-loss in plants under ABA deficiency has been reported (Wilkinson and Davies, 2010). Furthermore, it cannot be excluded that physico-chemical properties of the fruit surface may be altered in the

mutant since ABA may affect epicuticular wax biosynthesis in plants (Islam *et al.*, 2009) and also cuticle permeability, development and composition in fruits (Curvers *et al.*, 2010). Although the effect of different hormones on the synthesis or morphology of epicuticular waxes have been shown in citrus fruits (El-Otmani *et al.*, 1986; Cajuste *et al.*, 2010), that of ABA has not been described yet. Therefore, the availability of the spontaneous 'Pinalate' ABA-deficient mutant and its high susceptibility to dehydration encourages new investigations aimed to determine how ABA deficiency impacts the cuticle wax composition.

Besides the 'response to water deprivation' process, the inorganic cation transport appears to be operating in the lower susceptibility of 'Navelate' fruit to dehydration and NCPP. The transport and/or the sequestration of ions constitute a plant strategy to prevent water loss from the cytoplasm to the extracellular matrix and the subsequent osmotic stress originated by dehydration (Shinozaki *et al.*, 1998; Ramanjulu and Bartels, 2002). Prevention of water and osmotic stress has been mainly attributed to potassium, chloride and calcium ions. However, results obtained in the present work revealed that the 'di-, tri-valent inorganic cation transport' biological process, induced only in 'Navelate' fruit by 1 week, involved calcium (*ECA3* and *GNC1*), iron (*FER4*, *IRT1*, *NRAMP1* and *NRAMP3*) and copper chelators and transporters (*COPT1*, *COPT2*, *COPT5* and *SAG14*). Copper and iron cations are trace elements and, consequently, their concentration inside the cell might barely affect cell osmotic pressure. Therefore, an attractive possibility from the present results is that these metal transporters could play a role in the tolerance of citrus fruit to dehydration by modulating ABA-responsive pathways. This would be in concordance with previous findings indicating that these ions may affect the ABA-dependent signal transduction pathway in plants (Sudo *et al.*, 2008). Within the context of this work, it is noteworthy that iron and copper cations are

495 required as cofactors of superoxide dismutases that may contribute to the lower
496 susceptibility of 'Navelate' fruit to develop NCPP (Sala *et al.*, 2005). It is known that an
497 excess of metals may lead to the disruption of cellular processes and finally to cell
498 death, and that the prevention of such harmful effects require the participation of metal-
499 binding proteins and transporters (Puig *et al.*, 2007). Thus, the higher increase in the
500 expression levels of iron and copper transporters detected in the wild-type fruit (Fig.
501 4B), suggests that the impaired ability of the ABA-deficient mutant to regulate metal
502 homeostasis could be relevant for its higher susceptibility to dehydration and NCPP.
503 Most of the differentially expressed genes were down-regulated in the mutant by 3
504 weeks (Fig. 2B) and grouped into numerous biological processes (Table 2), being
505 'carbohydrate biosynthesis' the most specific. This is in agreement with previous results
506 showing a higher reduction in soluble sugars and starch in 'Pinalate' respect to parental
507 fruits during development of NCPP (Holland *et al.*, 2005), and highlights the interplay
508 between ABA and sugars in plants. This process grouped not only genes involved in the
509 metabolism of soluble sugars and starch but also in the metabolism of cell wall
510 polysaccharides and putative regulatory elements, such as a MYC transcription factor
511 and a gene (*CsIPS*) involved in regulating the levels of inositol-3-phosphate, which
512 constitutes a node for the crosslink among several signalling pathways (Kaur and Gupta,
513 2005). The CsMYC transcription factor displays a 63% of identity with the ABA-
514 responsive AtMYC2, which triggers the slow adaptive response of *Arabidopsis* to
515 dehydration (Abe *et al.*, 2003; Bartels and Sunkar, 2005) and, therefore, the *CsMYC*
516 transcript might be involved in the tolerance of citrus fruit to water stress. Nevertheless,
517 this *Citrus* gene appears not to be a limiting step in this process since its expression
518 levels continuously decreased in the ABA-deficient mutant but also in the parental fruit.
519 Expression analysis showed that *CsIPS* transcript levels also decreased in 'Pinalate'

fruit for up to 6 weeks but transiently increased in the wild-type phenotype when the highest difference in NCPP between both varieties was observed (Fig. 4C, 3 weeks). This result suggests a higher availability of the second messenger inositol-3-phosphate in the wild type, which might favour putative signalling pathways involved in the protection of fruit against detrimental effects caused by water stress and NCPP, whereas these pathways might be impaired in the ABA-deficient mutant. The above results, together with the high number of down-regulated genes belonging to the 'carbohydrate biosynthesis' process in mutant fruit, and the well known protective roles of sugars against osmotic and water stresses in plants (Bartels and Sunkar, 2005; Seki *et al.*, 2007), suggest that the repression of this biological process is relevant for the susceptibility of citrus fruit to such stresses leading to peel damage. The repression of this process was also associated with the enhancement of NCPP in 'Navelate' fruits exposed to a different stress (Establés-Ortiz *et al.*, 2009), indicating the relevance of carbohydrate metabolism in the convergence of the mechanisms underlying NCPP.

The interpretation of results derived from the application of plant growth regulators to hormone-deficient mutants may be complex as these treatments may fail to recover the wild-type phenotypes. Different examples can be found in the literature in fruits (Sandhu *et al.*, 2011) and also in seedlings (Mahouachi *et al.*, 2011) in spite of the ability of seedling plants to use foliar- or roots-applied hormones and to translocate them to almost all plant parts (Mäkelä *et al.*, 1996). Results from ABA treatment on 'Navelate' fruits suggests that endogenous levels of the phytohormone might be sufficient to trigger cellular processes coping with dehydration and further consequences related to peel damage in the wild-type orange since NCPP index and weight loss were not significantly affected by the ABA application (Fig. S1). Interestingly, application of ABA increased the hormone content in the flavedo of

545 ‘Pinalate’ mutant fruit to levels that were always slightly higher than those of the
546 parental, triggered changes in the expression of thousands of genes, and repressed the
547 ‘protein ubiquitination’ biological process. However, it did not modify either the
548 expression levels of a subset of ABA-regulated genes (Bartels and Sunkar, 2005) (Table
549 S1) or rescue the wild-type phenotype since exogenous ABA slightly affected the
550 incidence of NCPP and did not modify the cumulative weight loss of mutant fruits.
551 Therefore, these results, together with the obtained by multivariate and qRT-PCR
552 analyses (Fig. 3 and 4), indicate that exogenous ABA modulates gene expression in
553 ‘Pinalate’ fruits but it is not fully effective either redirecting the mutant transcriptome
554 towards that of the parental fruit or recovering its phenotype. These results might be
555 unexpected but there are several examples showing that ABA did not rescue normal
556 phenotype in ABA-deficient mutants (Busk and Pagès, 1998). In addition, plants may
557 be less sensitive to exogenous ABA under normal conditions than to the stress-induced
558 rises in endogenous ABA (Imay *et al.*, 1995). In agreement with these ideas, Mahouachi
559 *et al*. (2011) reported that ABA treatment did not stimulate physiological responses of
560 papaya seedlings exposed to drought, whereas treatments favouring the rise of
561 endogenous ABA levels were able to trigger physiological responses coping with
562 dehydration. Taking together these ideas and that ‘Pinalate’ has reduced ABA levels
563 during the whole period of development and ripening (Rodrigo *et al.*, 2003), it cannot
564 be ruled out the possibility of an altered ABA-perception system in ‘Pinalate’ fruit, as
565 reported in other hormone-deficient mutants (Guo and Ecker, 2004), or some defect in
566 the ABA signalling transduction pathway that would impair its responses to the ABA
567 treatment. Therefore, it would be interesting to further investigate whether there are
568 differences in the regulation of the ABA-signalling components, which have been

569 recently characterized in *Arabidopsis* (Park *et al.*, 2009; Ma *et al.*, 2009), between
570 mutant and wild-type fruits under water stress conditions.
571 In spite of the relevance of plant sensitivity for triggering hormone-responses, Hoth *et*
572 *al*. (2002) found that treating seedlings of the *Arabidopsis* ABA-insensitive mutant
573 *abi1-1* with ABA induced relevant changes in the expression of genes and processes
574 regulated by the hormone although, as expected, it did not rescue the typical ABA-
575 insensitive phenotype. The modulation of protein ubiquitination was observed by these
576 authors after ABA treatment. Interestingly, this was the only biological process down-
577 regulated by exogenous ABA in 'Pinalate', which suggests the involvement of protein
578 degradation in the ABA-signalling network in citrus fruits. In this context, it is also
579 noteworthy to mention different reports associating this biological process with ABA-
580 signalling/responses in the model plant *Arabidopsis* (López-Molina *et al.*, 2003; Zhang
581 *et al.*, 2005; Luo *et al.*, 2006; Ryu *et al.*, 2010). The six *Citrus* genes grouped into
582 'protein ubiquitination' biological process encoded plant U-box (PUB) domain-
583 containing proteins with E3-ubiquitin ligase activity. Three of them (*PUB9*, *PUB17*,
584 *PUB43*) have been related to ABA (Samuel *et al.*, 2008; Raab *et al.*, 2009; Ni *et al.*,
585 2010) and the others (*PUB21*, *PUB24* and *PUB29)* to cell death signalling and plant
586 defence responses to biotic stress (Libault *et al.*, 2007). In concordance with that, it was
587 found that rots developed earlier (3 weeks) and with higher incidence during storage in
588 ABA-treated mutant fruits respect to non-treated mutant or parental fruits (Fig. S2).
589 Real-time expression analysis of *CsPUB9* and *CsPUB21* genes further revealed an
590 enhanced repression of transcript levels in ABA-treated 'Pinalate' fruit, which further
591 confirm that the protein ubiquitination process may be negatively regulated by ABA
592 treatment in mutant fruit. Therefore, these results suggest a crosslink between ABA and

593 the modulation of defence responses in citrus fruit through proteins involved in the
594 ubiquitin-proteasome system machinery.
595 In conclusion, the comparative transcriptional analysis between ‘Navelate’ and its
596 mutant ‘Pinalate’ fruits highlights the ability of parental fruit to develop responses to
597 reduce water loss and other detrimental consequences caused by this stress. These
598 responses involve the ‘water deprivation’ and the ‘di-, tri-valent inorganic cation
599 transport’ biological processes, which include both ABA-dependent and independent
600 genes. The alteration of these responses in the mutant fruit suggests their relevance for
601 the prevention of peel damage in citrus fruit. Likewise, repression of the ‘carbohydrate
602 biosynthesis’ process occurred specifically in ‘Pinalate’ fruits, which showed higher
603 susceptibility to NCPP. Overall, results suggest that the sensitivity/response to ABA
604 may be impaired in the ABA-deficient mutant fruit and reveals molecular mechanisms
605 triggering the response to water stress in citrus fruit.
606
607

SUPPLEMENTARY MATERIAL

Table S1. Representative ABA-regulated genes impaired in ‘Pinalate’ mutant fruit after ABA treatment.

Figure S1. Non-chilling peel pitting index and percentage of fruit weight loss of ‘Navelate’ fruits treated with ABA.

Figure S2. Percentage of decay in ‘Navelate’ and ‘Pinalate’ fruits along storage.

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TABLES

Table 1. Selected genes and primers used for quantitative RT-PCR analysis and comparison between Citrus 20K microarray and qRT-PCR gene expression data. Multiple linear regression analysis (r^2) was performed for each reported gene including samples from all comparisons and storage periods.

Gene	Citrus unigene (CFGP DB)	Most similar protein	Homolog in *A. thaliana*	Forward / Reverse	Sequence 5' → 3'	r^2
CsCOPT2	aCL7045Contig1	Copper transporter protein homolog	AT3G46900	F	GGGGGCCGACCTGAAGAAC	**0.98**
				R	CGCACTAGCCGCTAGAAAAG	
CsCOPT5	aCL1547Contig2	T1M15_50 protein	AT5G20650	F	GGAGGACAGGCGCGTCCG	**0.90**
				R	GCCGAGAATTTCCCGACGAC	
CsHVA22E	aC31106H02EF_c	Abscisic acid-induced-like protein	AT5G50720	F	GCGGCATGGCTGGTTCTGC	**0.91**
				R	GCCTCGTGCTCCCCTTTCTT	
CsIPS	aC31301D12EF_c	Inositol-3-phosphate synthase	AT2G22240	F	GGACACAGTGCAACAAGCCA	**0.95**
				R	CCCATCCTCCAAACACAATG	
CsMYC	aC04028A10SK_c	MYC transciption factor	AT1G32640	F	GCCTGAGTCCGGGGAGATAT	**0.92**
				R	CCCTCTCGAAGTAGGAGATC	
CsNCED1	aCL1933Contig1	N-cis-epoxycarotenoid dioxigenase 1	AT3G14440	F	CCACGATGATAGCTCATCCG	**0.93**
				R	CCACTTGCTGGTCAGGCACC	
CsNRAMP1	aIC0AAA15AB01RM1_c	Metal transporter Nramp1	AT1G80830	F	GCCACTGGGCAGCCCCAG	**0.93**
				R	CAGCTTGTCTTATCGGGCAC	
CsNRAMP3	aCL3476Contig1	Metal transporter Nramp3	AT2G23150	F	GGCTCTGAGCTTCTTATTGGC	**0.93**
				R	GGACACGGCCTTTCTTACTG	
CsPUB9	aCL8840Contig1	F21O3.7 protein	AT3G07360	F	AGCAAGAGCTGTGCGTGATG	**0.97**
				R	GCGAAGCATGCAAGAAACTCC	
CsPUB21	aC31304F06EF_c	Immediate-early fungal elicitor protein CMPG1	AT5G37490	F	AAGATCCGGTGACGACGACT	**0.90**
				R	GCACCCAACTTGATCCTGTGT	
CsRD19	aCL96Contig1	Cysteine proteinase	AT4G39090	F	GCACGACCGTAGGTTCACTAT	**0.93**
				R	GTCCGGCGGAACTCGGCC	
CsRD21	aCL23Contig3	Cysteine protease CP1	AT1G47128	F	GCCCTGAGAGCAACACTTGC	**0.90**
				R	GGGATAGTCATGTGGGCAGC	

Table 2. Functional categorization of differentially expressed genes in the flavedo of 'Navelate', 'Pinalate' and ABA-treated 'Pinalate' fruits stored at 12 ºC and 70-75% RH for 1 and 3 weeks respect to freshly harvested fruits. Arrows indicate enriched biological processes (FatiGO+, $p < 0.05$) in sets of significantly (SAM analysis, FDR < 0.01) induced (↑) or repressed (↓) genes into each condition.

			1 week	3 weeks	
GO Level	**GO Code**	**Biological Process**	**Navelate**	**Pinalate**	**Pinalate + ABA**
4	0043283	Biopolymer metabolic process	↓		
4	0044249	Cellular biosynthetic process	↓		
4	0006091	Generation of precursor metabolites and energy		↓	↓
4	0046483	Heterocycle metabolic process	↓		
4	0006800	Oxygen and reactive oxygen species metabolic process		↓	↓
4	0048583	Regulation of response to stimulus		↓	↓
4	0009753	Response to jasmonic acid stimulus		↓	↓
4	0051707	Response to other organism		↓	↓
4	0009314	Response to radiation		↓	↓
4	0009266	Response to temperature stimulus		↓	↓
4	0009415	Response to water	↑		
4	0009611	Response to wounding		↓	↓
5	0015980	Energy derivation by oxidation of organic compounds		↓	↓
5	0009416	Response to light stimulus		↓	↓
5	0009414	Response to water deprivation	↑		
5	0016070	RNA metabolic process	↓		
7	0016051	Carbohydrate biosynthetic process		↓	↓
7	0015674	Di-, tri-valent inorganic cation transport	↑		
9	0016567	Protein ubiquitination			↓

Table 3. Genes differentially expressed in the indicated comparisons and belonging to the most specific and relevant biological processes**.** N1W > FHN, genes induced in 'Navelate' fruits stored for 1 week respect to freshly harvested fruits; P3W < FHP, genes repressed in 'Pinalate' fruits stored for 3 weeks respect to freshly harvested fruits; P3W+ABA < FHP, genes repressed in ABA-treated 'Pinalate' fruits stored for 3 weeks respect to freshly harvested fruits. Asterisks refer to genes chosen for multiple linear regression and qRT-PCR analysis.

	Citrus unigene (CFGP DB)	Most similar protein	Homolog in *A. thaliana*
	N1W > FHN	**Response to water deprivation (GO level 5)**	
	aCL474Contig1	ABF4 ; Putative ripening-related bZIP protein	AT3G19290
	aC18012D10Rv_c	ADH ; Aldehyde dehydrogenase - putative	AT1G44170
	aCL8452Contig1	AVP1 ; Vacuolar H+-pyrophosphatase	AT1G15690
	aCL5941Contig1	HB7 ; Homeobox-leucine zipper protein	AT2G46680
	aCL5217Contig1	HK3 ; Histidine kinase	AT1G27320
*	aC31106H02EF_c	HVA22E ; Abscisic acid-induced-like protein	AT5G50720
	aCL9Contig16	LEA5 ; Late embryogenesis abundant protein	AT4G02380
	aCL35Contig5	NAC4 ; NAC domain protein	AT4G27410
*	aCL1933Contig1	NCED1 ; 9-cis-epoxycarotenoid dioxygenase 1	AT3G14440
	aCL3500Contig1	PIP1B ; Plasma membrane aquaporin	AT2G45960
	aC31502B11EF_c	PIP1E ; Aquaporin	AT4G00430
	aCL143Contig2	PP2C ; Protein phosphatase 2C	AT3G11410
*	aCL96Contig1	RD19 ; Cysteine proteinase	AT4G39090
*	aCL23Contig3	RD21 ; Cysteine protease CP1	AT1G47128
	aCL1551Contig1	ZEP ; Zeaxanthin epoxidase	AT5G67030
	N1W > FHN	**Di-, tri-valent inorganic cation transport (GO level 7)**	
	aC18018E02Rv_c	CNGC1 ; Cyclic nucleotide-gated calmodulin-binding ion channel	AT5G53130
	aC01009A02SK_c	COPT1 ; Copper transporter 1	AT5G59030
*	aCL7045Contig1	COPT2 ; Copper transporter protein homolog	AT3G46900
*	aCL1547Contig2	COPT5 ; T1M15_50 protein	AT5G20650
	aC04013B01SK_c	ECA3 ; Calcium-transporting ATPase3-endoplasmic reticulum-type	AT1G10130
	aKN0AAQ10YG21RM1_c	FER4 ; Ferritin	AT2G40300
	aC34108F04EF_c	IRT1 ; Root iron transporter protein	AT4G19690
*	aIC0AAA15AB01RM1_c	NRAMP1 ; Metal transporter Nramp1	AT1G80830
*	aCL3476Contig1	NRAMP3 ; Metal transporter Nramp3	AT2G23150
	aCL5880Contig1	SAG14 ; NtEIG-A1 protein	AT5G20230
	P3W < FHP **P3W+A < FHP**	**Carbohydrate biosynthetic process (GO level 7)**	
	aC31305H08EF_c	ADG1 ; ADP-glucose pyrophosphorylase small subunit	AT5G48300
	aCL5827Contig1	ADG1 ; Glucose-1-phosphate adenylyltransferase	AT5G48300
	aCL6121Contig1	CALS1 ; Putative callose synthase 1 catalytic subunit	AT1G05570
	aCL4673Contig1	CESA1 ; Cellulose synthase	AT4G32410
	aC03001C04Rv_c	CESA2 ; Cellulose synthase	AT4G39350
	aCL1466Contig1	CTL1 ; T20M3.12 protein	AT1G05850
	aCL18Contig7	CYP79A2 ; Cytochrome P450 79A2	AT5G05260
	aCL60Contig1	F9L11.8 ; Granule-bound starch synthase 1	AT1G32900
	aCL281Contig3	GAPB ; Glyceraldehyde-3-phosphate dehydrogenase B	AT1G42970
	aCL3226Contig1	GATL10 ; Glycosyl transferase-like protein	AT3G28340
	aCL1394Contig1	GMD2 ; GDP-mannose 4 -6 dehydratase 1	AT3G51160
	aCL381Contig1	GOLS2 ; Galactinol synthase	AT1G56600
*	aC31301D12EF_c	IPS2 ; Inositol-3-phosphate synthase	AT2G22240
	aC08005B05SK_c	KAM1 ; Xyloglucan galactosyltransferase KATAMARI 1	AT2G20370
*	aC04028A10SK_c	MYC2 ; MYC transcription factor	AT1G32640
	aCL4197Contig1	QUA2 ; Putative early-responsive to dehydration stress protein	AT1G78240
	aCL2181Contig1	SIP1 ; Raffinose synthase	AT5G40390
	P3W+A < FHP	**Protein ubiquitination (GO level 9)**	
*	aCL8840Contig1	PUB9 ; F21O3.7 protein	AT3G07360
	aC34202B10EF_c	PUB17 ; Avr9/Cf-9 rapidly elicited protein 276	AT1G29340
*	aC31304F06EF_c	PUB21 ; Immediate-early fungal elicitor protein CMPG1	AT5G37490
	aC31801H08EF_c	PUB24 ; F26K24.13 protein	AT3G11840
	aCL270Contig1	PUB29 ; Photoperiod responsive protein	AT3G18710
	aC05134D01SK_c	PUB43 ; Armadillo repeat-containing protein	AT1G76390

FIGURE LEGENDS

Figure 1. Non-chilling peel pitting index (A), percentage of fruit weight loss per surface area (B) and ABA content in the flavedo (C) of 'Navelate' (squares) and 'Pinalate' (circles) fruits treated (white) or not (black) with ABA and stored for up to 6 weeks at 12 ºC and 70-75% RH. The arrows indicate when ABA was applied. Results are the means of three biological replicates of 10 fruits each ± SE. Mean separation was performed by applying Tukey's test. Significant differences ($p \leq 0.05$) in NCPP index and ABA content between samples for the same storage period are indicated by different letters. Significant differences ($p \leq 0.05$) in weight loss (panel B) between 'Navelate' and 'Pinalate' samples, treated or not with ABA, were found from the first week of storage while no statistical differences were found between control and ABA-treated 'Pinalate' fruits.

Figure 2. Venn diagrams showing differentially expressed genes (SAM analysis, FDR < 0.01) in the flavedo of 'Navelate', 'Pinalate' and ABA-treated 'Pinalate' fruits stored at 12 ºC and 70-75% RH for 1 (A) and 3 (B) weeks. Expression levels of up- (**bold**) and down-regulated (*italics*) genes in these fruits were compared to those of freshly harvested fruits from each variety. Numbers in brackets are the sum of all induced (**bold**) or repressed (*italics*) genes in each particular condition. The sizes of the circles are consistent with the total number of differentially expressed genes for each condition.

Figure 3. (A) Principal Component (PCA) and (B) Hierarchical Cluster Analysis (HCA) of flavedo large-scale transcriptional profiles of 'Navelate' (N), 'Pinalate' (P) and ABA-treated 'Pinalate' (P+ABA) fruits stored for one (1W) and three weeks (3W) at 12 ºC and 70-75% RH respect to freshly harvested (FH) fruits. Colours in PCA for

each condition are consistent with those in HCA. The three axes in PCA account 62.8% of the total variance among varieties and storage periods. Three biological replicates from each condition were used for both analyses.

Figure 4. Real time qRT-PCR expression analysis for candidate genes selected from microarrays analysis. Relative transcript abundance for selected genes belonging to 'Water deprivation' (A), 'Di-, tri-valent inorganic cation transport' (B), 'Carbohydrate biosynthesis' (C) and 'Protein ubiquitination' (D) biological processes differentially regulated in 'Navelate' (squares) and 'Pinalate' (circles) fruits treated (white) or not (black) with ABA and stored for up to 6 weeks at 12 ºC and 70-75% RH. Transcript levels for all conditions were referred to freshly harvested 'Navelate' fruits and expressed as relative values. Data are the mean values of three biological replicates ± SE.

FIGURES

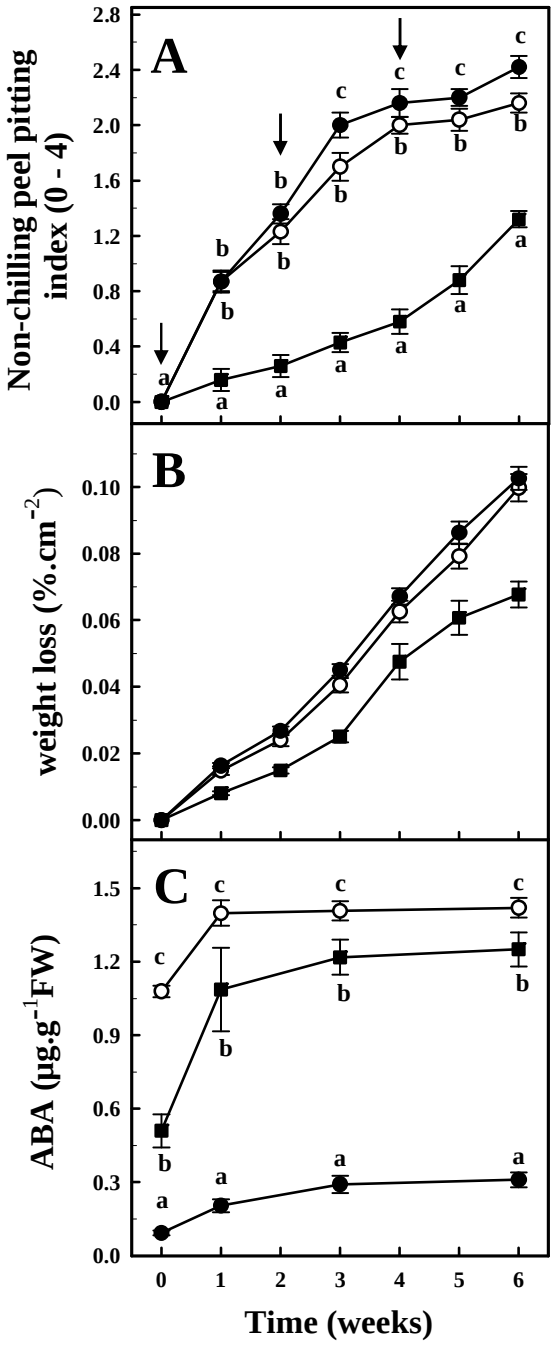

Figure 1

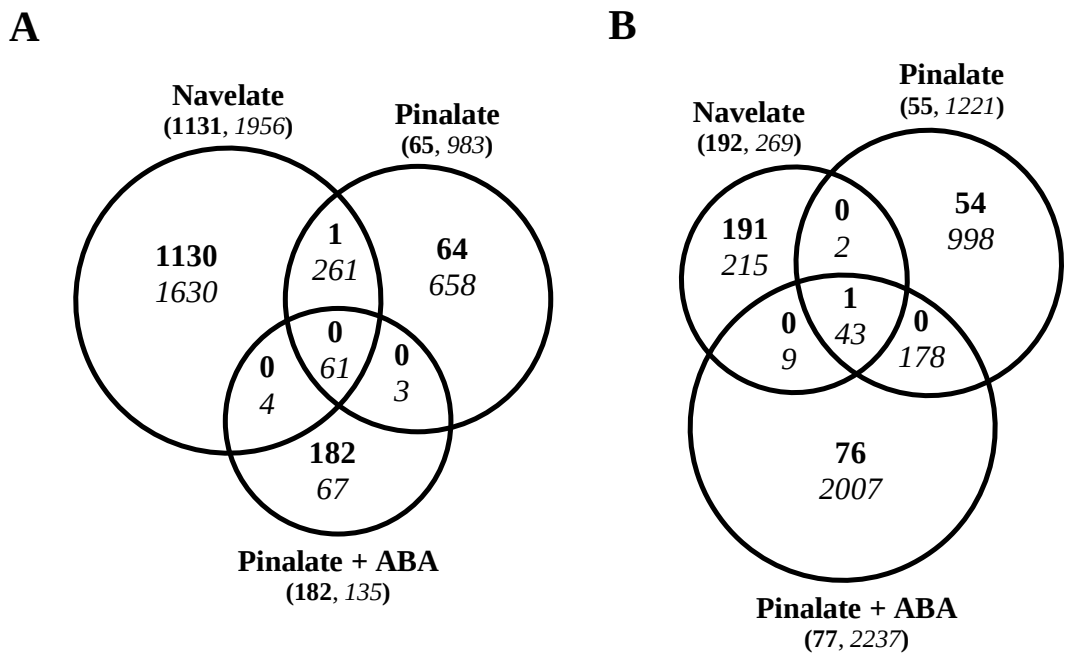
A
Navelate
(1131, 1956)
Pinalate
(65, 983)
Pinalate + ABA
(182, 135)
1130
1630
1
261
64
658
0
61
0
4
0
3
182
67
B
Navelate
(192, 269)
Pinalate
(55, 1221)
Pinalate + ABA
(77, 2237)
191
215
0
2
54
998
1
43
0
9
0
178
76
2007

Figure 2

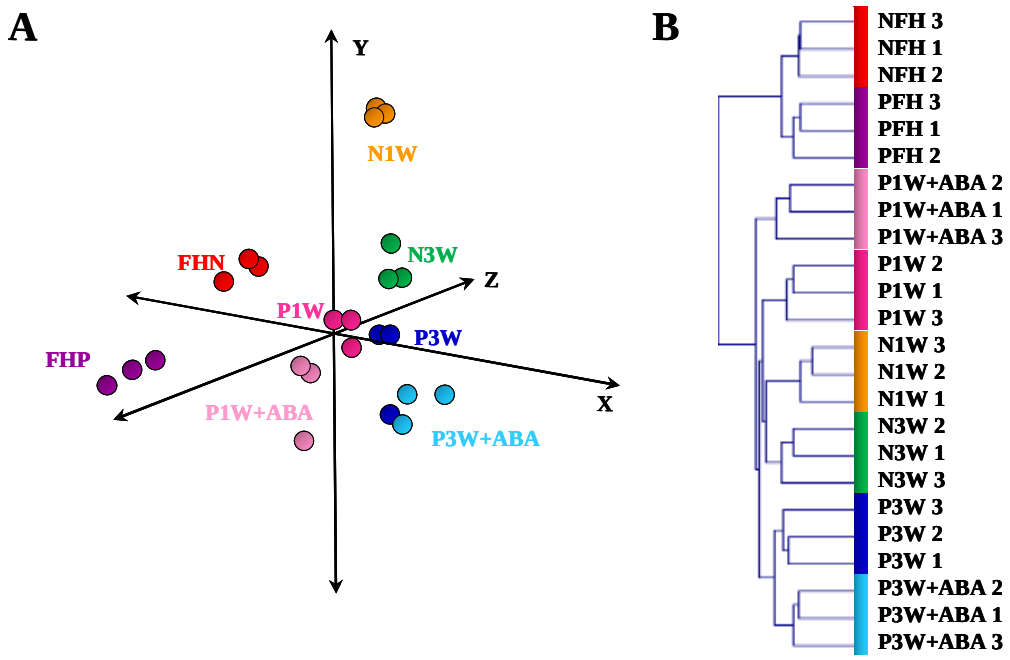
A
Y
N1W
N3W
Z
FHN
P1W
P3W
FHP
P1W+ABA
P3W+ABA
X
B
NFH 3
NFH 1
NFH 2
PFH 3
PFH 1
PFH 2
P1W+ABA 2
P1W+ABA 1
P1W+ABA 3
P1W 2
P1W 1
P1W 3
N1W 3
N1W 2
N1W 1
N3W 2
N3W 1
N3W 3
P3W 3
P3W 2
P3W 1
P3W+ABA 2
P3W+ABA 1
P3W+ABA 3

Figure 3

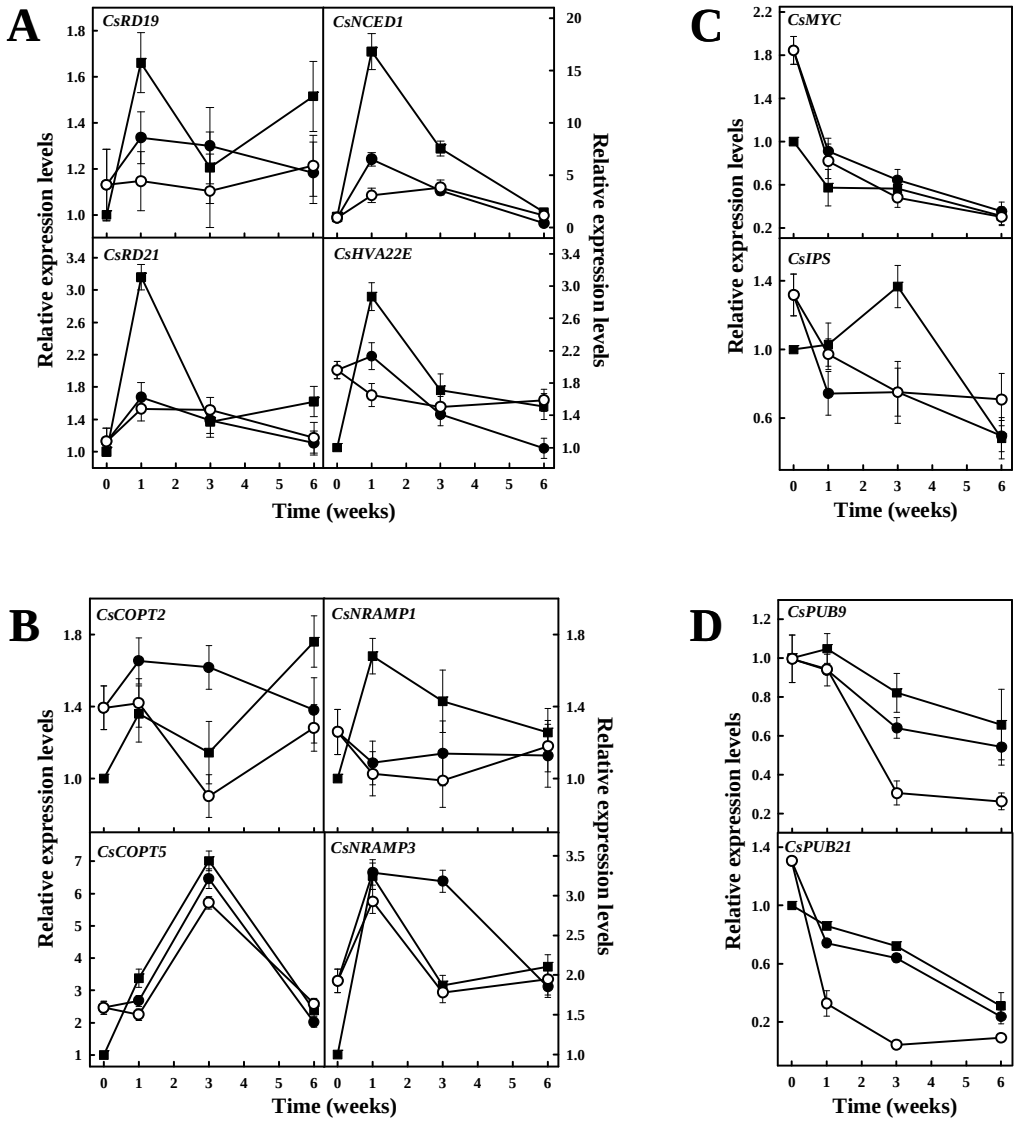
A
CsRD19
CsNCED1
CsRD21
CsHVA22E
Relative expression levels
Relative expression levels
Time (weeks)
C
CsMYC
CsIPS
Relative expression levels
Time (weeks)
B
CsCOPT2
CsNRAMP1
CsCOPT5
CsNRAMP3
Relative expression levels
Relative expression levels
Time (weeks)
D
CsPUB9
CsPUB21
Relative expression levels
Time (weeks)

Figure 4