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ABA-deficiency and molecular mechanisms involved in the dehydration response and ripening of citrus fruit

Dissertation submitted in partial fulfilment of the requirements for obtaining the degree of International Doctor (Ph. D.) in Biotechnology

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Las Dras. María Teresa Lafuente Rodríguez, Investigador Científico del Consejo Superior de Investigaciones Científicas (CSIC) con destino en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA), y Dª María Jesús Rodrigo Esteve, Científico Titular del citado Instituto:

CERTIFICAN:

Que D. **Francisco Romero Gascón**, Licenciado en Biología por la Universidad de Valencia y Postgraduado en Biología Molecular Celular y Genética por la misma Universidad, ha realizado bajo nuestra dirección el trabajo que, con el título '**ABA-deficiency and molecular mechanisms involved in the dehydration response and ripening of citrus fruit**', presenta para optar al grado de Doctor Internacional en Biotecnología por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firman el presente certificado en Paterna, a de del 2012.

Dra. María Teresa Lafuente Rodríguez Dra. María Jesús Rodrigo Esteve

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Que D. **Francisco Romero Gascón**, Licenciado en Biología por la Universidad de Valencia y Postgraduado en Biología Molecular Celular y Genética por la misma Universidad, ha realizado bajo su tutela el trabajo que, con el título '**ABA-deficiency and molecular mechanisms involved in the dehydration response and ripening of citrus fruit**', presenta para optar al grado de Doctor Internacional en Biotecnología por la Universidad Politécnica de Valencia.

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Dr. José Gadea Vacas

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ABSTRACT

The aim of this work has been to unravel the influence of the phytohormone ABA in the molecular mechanisms underlying the postharvest dehydration response and the development and ripening of citrus fruit, taking advantage of the spontaneous fruit-specific ABA-deficient 'Pinalate' mutant, which is more prone to dehydration and to develop non-chilling peel pitting (NCPP) than its wild-type 'Navelate' orange. Results of the comparative transcriptomic analysis between fruit of both cultivars stored under moderate water stress (70-75% RH, 12 ºC) favouring the occurrence of NCPP highlighted the ability of parental fruit to induce early molecular responses, including both ABA-dependent and independent genes, aimed to reduce water loss and their detrimental effects. ABA application to mutant fruit increased hormone levels and modulated relevant transcriptomic changes related to protein ubiquitination, although did not substantially modify either dehydration rate or NCPP incidence. Additionally, the ABA perception system components in *Citrus* were identified and their regulation under developmental and stressful conditions increasing ABA in reproductive and vegetative tissues of both cultivars was investigated. Six PYR/PYL/RCAR ABA receptors, five PP2CA negative regulators, and two subclass III SnRK2 downstream protein kinases showed conserved motifs for protein folding, interaction and functionality. Minor differences in the regulation of the ABA receptors and the *CsSnRK2*s were found, whereas *CsPP2CA*s levels were lower in the mutant fruit. In addition, ABA receptors and *CsSnRK2*s gene expression patterns depended on the tissue, the stress severity and the source of the ABA signal from a developmental or stressful stimulus, whilst *CsPP2CA*s displayed a consistent pattern. Overall results suggest that the ABA-deficient mutant fruit may sense ABA although the hormone signal could be impaired because reduced

*CsPP2CA*s levels causing altered water stress response and higher NCPP susceptibility.

RESUMEN

El objetivo de este trabajo ha sido estudiar la influencia de la hormona ácido abscísico (ABA) en los mecanismos moleculares implicados en la respuesta a la deshidratación y en la maduración de los frutos cítricos, haciendo uso de un mutante espontáneo de la naranja 'Navelate', llamado 'Pinalate', que presenta una deficiencia en ABA específica de fruto y es propenso a la deshidratación y a desarrollar 'colapso de la corteza' (NCPP, del inglés Non-Chilling Peel Pitting). Los resultados del análisis transcriptómico comparativo entre los frutos de ambas variedades almacenados en condiciones de estrés hídrico moderado (70-75% HR y 12 ºC), que favorecen el desarrollo de NCPP, reveló la capacidad de los frutos del parental para inducir respuestas moleculares tempranas, incluyendo genes dependientes e independientes de ABA, dirigidas a reducir la pérdida de agua y los efectos adversos de este estrés. La aplicación de ABA a los frutos del mutante incrementó los niveles de la hormona y moduló cambios transcriptómicos relevantes relacionados con la ubiquitinación de proteínas, aunque no modificó sustancialmente ni la tasa de deshidratación ni la incidencia del NCPP. De forma complementaria, se han identificado los componentes del sistema de percepción del ABA en *Citrus* y se ha investigado su regulación durante el desarrollo del fruto y bajo condiciones de estrés hídrico en frutos y hojas de ambas variedades, situaciones que causan aumento en ABA. Seis receptores de ABA (PYR/PYL/RCAR), cinco reguladores negativos (PP2CA) y dos proteínas quinasas (SnRK2) mostraron motivos conservados para el plegamiento, la interacción y la funcionalidad proteica. El análisis transcripcional apenas mostró diferencias entre 'Navelate' y 'Pinalate' en la regulación de los receptores de ABA y las *CsSnRK2*s, mientras que los niveles de *CsPP2CA*s se mantuvieron más bajos en los frutos deficientes en ABA. Además, los patrones de expresión de los receptores de ABA y las *CsSnRK2*s dependieron del tejido, la severidad del estrés y el estímulo inductor de la

acumulación de ABA (la maduración del fruto o el estrés hídrico), mientras que las *CsPP2CA*s mostraron un mismo patrón de respuesta independientemente del proceso y el tejido analizado. Los resultados globales sugieren que los frutos de 'Pinalate' deficientes en ABA pueden percibir el ABA, aunque la transducción de la señal hormonal pueda estar alterada debido a los niveles reducidos de las *CsPP2CA*s causando, consecuentemente, una respuesta deficiente al estrés hídrico y una mayor susceptibilidad al NCPP.

RESUM

L'objectiu d'aquest treball ha sigut estudiar la influència de l'hormona àcid abscísic (ABA) en els mecanismes moleculars implicats en la resposta a la deshidratació i la maduració dels fruits cítrics, fent ús d'un mutant espontani de la taronja 'Navelate', anomenat 'Pinalate', que presenta una deficiència en ABA específica de fruit i és propens a la deshidratació i a desenvolupar 'ratat' (NCPP, de l'anglès Non-Chilling Peel Pitting). Els resultats de l'anàlisi transcriptomic comparatiu entre els fruits d'ambdós varietats emmagatzemats en condicions d'estrès hídric moderat (70-75% HR i 12 ºC), que afavoreixen el desenvolupament de NCPP, revelaren la capacitat dels fruits del parental per a induir respostes moleculars primerenques, incloent gens dependents i independents d'ABA, dirigides a reduir la pèrdua d'aigua i els efectes adversos de l'estrès. L'aplicació d'ABA als fruits del mutant incrementà els nivells de l'hormona i va modular canvis transcriptomics rellevants relacionats amb la ubiquitinació de proteïnes, encara que no va modificar substancialment ni la taxa de deshidratació ni la incidència del NCPP. De manera complementària, s'han identificat els components del sistema de percepció del ABA en *Citrus* i s'ha investigat la seua regulació durant el desenvolupament del fruit i sota condicions d'estrès hídric en fruits i fulles d'ambdós varietats, situacions que causen l'augment en ABA. Sis receptors d'ABA (PYR/PYL/RCAR), cinc reguladors negatius (PP2CA) i dos proteïnes quinases (SnRK2) mostraren motius conservats per al plegament, la interacció i la funcionalitat proteica. L'anàlisi transcripcional no va mostrar diferències entre 'Navelate' i 'Pinalate' en la regulació dels receptors d'ABA i les *CsSnRK2*s, mentre que els nivells de *CsPP2CA*s es van mantenir més baixos en els fruits deficients en ABA. A més, els patrons d'expressió dels receptors d'ABA i les *CsSnRK2*s van dependre del teixit, la severitat de l'estrès i l'estímul inductor de l'acumulació d'ABA (la maduració del fruits o l'estrès hídric), mentre que les *CsPP2CA*s

mostraren un mateix patró de resposta independentment del procés i del teixit analitzat. Els resultats globals suggereixen que els fruits de 'Pinalate' deficients en ABA poden percebre l'ABA, encara que la transducció del senyal hormonal puga estar alterada debut als reduïts nivells de les *CsPP2CA*s causant, conseqüentment, una resposta deficient a l'estrès hídric i una major susceptibilitat al NCPP.

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ABBREVIATIONS

ABA: Abscisic acid
ABAR: ABA-receptor
ABF: ABA binding factor
ABRE: ABA responsive elements
ANOVA: Analysis of variance
AREBF: ABA-responsive element binding factor
Bk: Breaker
BR: Brassinosteroids
C: Coloured
CDPK: Ca^{2+}-dependent protein kinases
CFGP: Citrus Functional Genomic Project
CHLH: H subunit of the magnesium-protoporphyrin IX chelatase
DPA: Dihydrophaseic acid
FC: Full coloured
FCA: Flowering time control protein
FDR: False discovery rate
FH: Freshly harvested
FW: Fresh weight
GA: Gibberellins
GCPR: G-protein-coupled receptor
GGPP: Geranylgeranyl pyrophosphate
GO: Gene ontology
HCA: Hierarchical cluster analysis
IAA: Indol-acetic acid / Auxin
IG: Immature green
IPP: Isopentenyl pyrophosphate
JA: Jasmonic acid
LEA: Late-embryogenesis-abundant
MAPK: Mitogen-activated protein kinases
MEP: 2C-methyl-D-erythritol-4-phosphate
MI: Mature green I
MII: Mature green II

MoCo: Molybdenum cofactor
NCED: Nine-*cis*-epoxycarotenoid dioxygenase
NCPP: Non-chilling peel pitting
PA: Phaseic acid
PCA: Principal component analysis
PP: Protein phosphatase
PP2CA: Clade A protein phosphatase 2C
PYL: PYR-like
PYR: Pyrabactin Resistance
RCAR: Regulatory components of ABA receptors
REST: Relative expression software tool
RH: Relative humidity
ROS: Reactive oxygen species
RPK1: Receptor protein kinase 1
RT-qPCR: Reverse transcription quantitative polymerase chain reaction
SA: Salicylic acid
SAM: Significant analysis of microarrays
SnRK: Sucrose non-fermenting 1-related protein kinase
ZEP: zeaxanthin epoxidase

1. INTRODUCTION

1.1. THE HORMONE ABSCISIC ACID: PHYSIOLOGICAL ROLES

The hormone abscisic acid (ABA) was discovered by two independent groups when trying to isolate endogenous plant regulators (Ohkuma *et al.*, 1963; Cornforth *et al.*, 1965). Ohkuma et al. (1963) were looking for compounds promoting leaf abscission in cotton plants and, therefore, they named the identified compound as Abscissin II. On the other hand, the aim of Cornforth et al. (1965) was to isolate biological compounds responsible for bud dormancy. Their search resulted in the identification of a compound present in sycamore leaves able to inhibit wheat embryo germination, which was named Dormin. Later analyses revealed that abscissin II and dormin was the same chemical compound, an isoprenoid-derived molecule, which was renamed as ABA (Cracker and Abeles, 1969; Addicott and Lyon, 1969). Although ABA was firstly identified in plants, several researches provided evidences for its presence in algae and fungi, which suggested that the origin of this hormone was prior to the division of the kingdoms (Nambara and Marion-Poll, 2005).

The phytohormone ABA is involved in the regulation of growth and development in plants but also plays important roles as endogenous messenger in biotic and abiotic stress responses (Fig. 1). Thus, ABA is a key regulator of fundamental developmental processes such as seed dormancy and germination, senescence, leaf and fruit abscission, and fruit ripening. Moreover, it plays a dominant role in the regulation of stomatal movements and enhancing drought tolerance in response to the osmotic stresses that result from water deprivation, salinity or freezing. These developmental and stress signals result in changes in ABA levels, followed by relevant changes in gene expression and adaptive physiological responses.

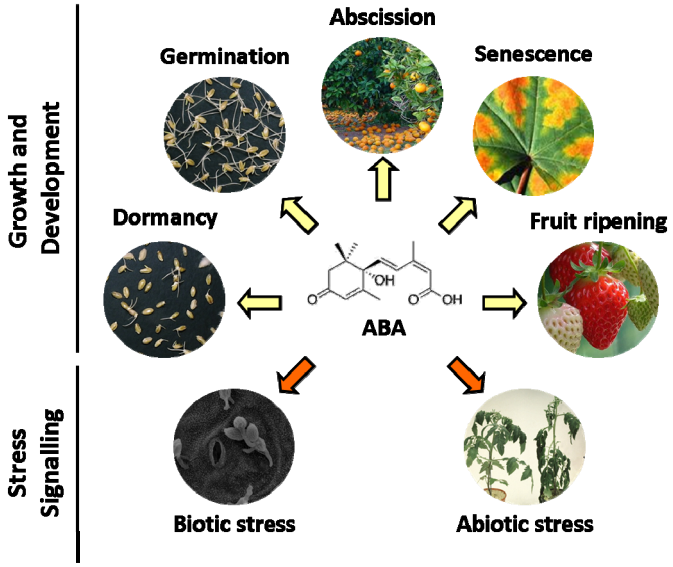

Figure 1. Schematic roles of abscisic acid (ABA), which controls a broad variety of crucial activities related to plant growth, development and survival.

1.1.1. ROLE OF ABA IN PLANT GROWTH AND DEVELOPMENT

The hormone ABA is required in plants to control and fine-tune growth and developmental processes under non-stressful conditions. Studies carried out in *Arabidopsis* demonstrated that ABA regulates germination and is crucial for seed development, and its effect depends on the tissue and concentration (Finkelstein *et al.*, 2002). Thus, the hormone prevents seed abortion and promotes embryo growth in early embryogenesis (Frey *et al.*, 2004; Kim *et al.*, 2012), whereas it induces seed dormancy and promotes seed desiccation tolerance at the late stages of this process (Karssen *et al.*, 1983). Other reports have also suggested a key function for ABA during seed development, inhibiting precocious germination and the induction of primary dormancy (Nambara *et al.*, 2010). Seed dormancy and germination are complex processes controlled by a large number of genes. Despite of the crucial role of ABA (Finkelstein *et al.*, 2002), the involvement of other plant regulators in these processes has been also

described (Kucera *et al.*, 2005). Whereas ABA is a positive regulator of dormancy induction and a negative regulator of germination (Karssen *et al.*, 1983), gibberellins (GA) release dormancy, promotes germination and counteract ABA effects (Debeaujon and Koornneef, 2000). Similarly, ethylene and brassinosteroids (BR) promote seed germination and also counteract ABA inhibitory effects (Kepczynski and Kepczynska, 1997).

The involvement of ABA in the regulation of abscission has been broadly accepted (Roberts *et al.*, 2002). It has been suggested that ABA stimulates abscission in leaves, seeds and flowers through ethylene-associated senescence in particular tissues (Cracker and Abeles, 1969; Aneja *et al.*, 1999; Roberts *et al.*, 2002; Trivellini *et al.*, 2011). In agreement, Gómez-Cadenas et al. (1996; 2002) pointed out the key role of ethylene as activator of leaf abscission in water-stressed citrus trees, and suggested that ABA was the primary stress signal responsible for the increase in ethylene. Work performed in citrus fruit further revealed a crosstalk between ABA and ethylene for triggering fruit abscission (Gómez-Cadenas *et al.*, 2000; Agustí *et al.*, 2008). Results obtained in mature sweet cherry, which are very prone to abscission, suggested, however, that the ABA increase observed at late stages of fruit ripening was more likely related to senescence than to the abscission process itself (Blanusa *et al.*, 2006). In contrast, Botton et al. (2011) showed that, besides the senescence-driven abscission of ripe apple fruits, the physiological drop occurring during early stages of fruit development was regulated by a sugar-ABA crosstalk.

Among physiological roles of ABA, it is also relevant the involvement of this hormone in the ripening of both climacteric and non-climacteric fruits. Auxins (IAA), GA, cytokinins and ethylene have been also involved at various stages of fruit development and maturation (Nitsch, 1970), although the role of ethylene in this process merits a special mention. The relationship existing between ABA and

ethylene in the development and ripening of climacteric fruits, such as nectarine and tomato, is well known (Ziliotto *et al.*, 2008; Zhang *et al.*, 2009b) since several reports suggested that the ABA accumulation just before the peak of ethylene production triggers ethylene biosynthesis responsible for climacteric fruit ripening (Zhang *et al.*, 2001; Zhang *et al.*, 2009a; Bastías *et al.*, 2011). The role of ABA in non-climacteric fruit is less clear in spite of several reports have correlated the increase in ABA levels during fruit ripening with the increase in sugars content and the decrease in organic acids or fruit colouration, which are classical traits occurring during fruit maturation (Giribaldi *et al.*, 2010; Chai *et al.*, 2011; Ren *et al.*, 2011; Gambetta *et al.*, 2011; Li *et al.*, 2012b).

The involvement of ABA in the ripening process of citrus, a non-climacteric fruit, has been also studied (Lafuente *et al.*, 1997; Alférez and Zacarías, 1999; Rodrigo *et al.*, 2003; Rodrigo *et al.*, 2006). Within the context of the crosstalk between ABA and ethylene, it has been suggested that ethylene would regulate the initiation of citrus fruit degreening, whereas ABA controls the rate of the process (Alférez and Zacarías, 1999). In agreement, Rodrigo et al. (2003) showed that ABA deficiency did not modify the initiation of chlorophyll loss, although the rate of degreening was lower in an ABA-deficient citrus mutant fruit. Recent comparative transcriptomic analysis between wild-type orange and this ABA-deficient mutant fruit has revealed that a high number of biological processes were equally modulated in both cultivars during fruit ripening, but also have highlighted a set of ABA-dependent biological responses that were down-regulated in the ABA-deficient fruit (Romero *et al.*, 2010). Together, these findings have highlighted a relevant role of ABA in the process of fruit ripening in *Citrus*.

1.1.2. DUAL ROLE OF ABA UNDER STRESS CONDITIONS

Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses. Hormone signalling pathways, such as those regulated by ABA, salicylic acid (SA), jasmonic acid (JA) and ethylene, play key roles in the crosstalk between biotic and abiotic stress signalling (Fujita *et al.*, 2006). Focusing on ABA, it is well known that changes in this hormone levels can modulate the closure of stomata and hence water loss in response to abiotic stress conditions. The stomatal closure not only leads to water conservation but also serves as a defence mechanism in preventing pathogen invasions, thereby acting as a scaffold for crosstalk between biotic and abiotic stress responses involving ABA action (Lee and Luan, 2012). Within this context, Fujita et al. (2006) suggested an antagonistic interaction between ABA-mediated abiotic stress signalling and disease resistance. This relationship may simply suggest that plants have developed strategies to avoid simultaneously producing proteins that are involved in abiotic stress and disease resistance responses.

1.1.2.1. ABIOTIC STRESSES.

Drought and high salinity stresses may provoke a strong increase of ABA levels in the plant, resulting in major changes in gene expression and physiological adaptive responses (Zhu, 2002). Other abiotic stresses such as cold and freeze partially share downstream signalling components involved in this stress response (Shinozaki and Yamaguchi-Shinozaki, 2000; Seki *et al.*, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). In salt stress, ABA contributes to the Salt Overly Sensitive (SOS) pathway, in which ion homeostasis is regulated through Ca^{2+} signal and membrane-bound proton transporters (Halfter *et al.*, 2000; Liu *et al.*, 2000), increasing the levels of second messengers (Ca^{2+} and 1,4,5-inositol triphosphate) and producing reactive oxygen species (ROS) that amplify

the stress signal (Pei *et al.*, 1997; Pei *et al.*, 2000). In contrast, molecular response to cold stress mainly involves ABA-independent signals, activating chaperone proteins, detoxification enzymes, and enzymes for osmoprotectants synthesis (Yamaguchi-Shinozaki and Shinozaki, 2006).

Within the context of the present work, water stress is one of the most important abiotic factors affecting plant growth, development and survival. Water stress causes removal of water from the cytoplasm to the extracellular space, causing a reduction in the cytosolic and vacuolar volumes. This provokes an altered ROS homeostasis and, consequently, the production of toxic substances and signal transduction molecules (Miller *et al.*, 2010). On the other hand, water stress causes the accumulation of sugars, poly-alcohols, amino acids and amines, which function as osmolytes, antioxidants, scavengers and/or signalling molecules that help plants to tolerate dehydration stress (Bray, 1993; Ingram and Bartels, 1996; Bray *et al.*, 2000; Bartels and Sunkar, 2005; Seki *et al.*, 2007). In addition, water stress promotes the accumulation of ABA through the induction of ABA biosynthetic genes, such as *ZEP* (zeaxanthin epoxidase) and *NCED* (Nine-*cis*-epoxycarotenoid dioxygenase). In turn, ABA stimulates the expression of ABA signalling genes, such as protein phosphatases (PPs) and bZIP transcription factors, which amplify the stress signal. Most of the mechanisms involved in drought tolerance are based on osmotic adjustment and protection of cellular structures from the effects of dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006; Seki *et al.*, 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). ABA play a key role in the prevention of detrimental effects caused by dehydration since it regulates the synthesis of dehydrins and other LEA proteins, which are known to act as chaperons protecting proteins and membranes under this stress conditions (Ingram and Bartels, 1996; Verslues and Bray, 2006). In addition, ABA modulates ion-chelating proteins and/or transporters that trigger ions

sequestration/mobilization into the vacuole, playing an important role in retaining water inside the cell (Zhu, 2002; Geiger *et al.*, 2009).

Although the tight relationship between ABA and dehydration is well established, it is noteworthy that ABA-independent pathways may also operate in response to water stress (Riera *et al.*, 2005; Wilkinson and Davies, 2010). In addition, many water-stress responsive genes are specifically regulated by ABA (Bartels and Sunkar, 2005), whereas others are commonly modulated by ABA and JA (Nemhauser *et al.*, 2006; Huang *et al.*, 2008). IAA, cytokinin, ethylene, BR and GA also modulate the expression of a wide number of common drought-related genes, which suggests a complex interplay among different signalling pathways during water stress response (Huang *et al.*, 2008).

Stomatal closure is one of the most important physiological responses to prevent water loss through transpiration. In a very simplified view, stomata movements are regulated by transport events. Stomatal opening is driven by hyperpolarization of the guard cell membrane, which is caused by H^+-ATPase-dependent proton efflux. This activates K^+-inward-rectifying channels (Lebaudy *et al.*, 2007) and induces solute influx followed by water uptake, which turns turgid the guard cells and opens the stoma. Under water stress, ABA content increases and anion channels are activated, throwing anions out of the cell and starting depolarization of the membrane (Geiger *et al.*, 2009; Geiger *et al.*, 2011). In turn, ABA inhibits the activity of the proton pump ATPase and the K^+-inward-rectifying channels to prevent from hyperpolarization (Merlot *et al.*, 2007; Sato *et al.*, 2009). Membrane depolarization also leads to activation of the K^+-outward–rectifying channel to throw out K^+ (Lebaudy *et al.*, 2007), contributing to decrease the osmotic pressure. This decrease leads to a reduction of turgor potential, closing the pore and decreasing evapotranspiration rate (Israelsson *et al.*, 2006; Kim *et al.*, 2010; Hubbard *et al.*, 2012).

The regulation of stomatal movements also involves JA, BR, SA, ethylene, IAA, and cytokinin signalling. ABA, JA, BR and SA have been described as positive regulators of stomatal closure, whereas IAA and cytokinins are positive regulators of stomatal opening. Thus, interaction of cytokinin or IAA with ABA inhibits the ABA-mediated stomatal closure, while interaction between ABA and SA, JA or BR positively regulates this process (Dodd, 2003; Acharya and Assmann, 2009).

ABA can also modulate root growth promoting primary root elongation in order to counteract water balance when the stress persists, (Sharp, 2002; Sharp *et al.*, 2004). Complementary, ABA has been implicated in inhibiting the development of lateral roots under water stress conditions, which might represent an adaptive response from the plant by restricting root horizontal proliferation and benefiting primary root growth, having this way, better chances to search for new and deeper water resources (De Smet *et al.*, 2003; Xiong *et al.*, 2006).

Dehydration is one of the most important stresses affecting agricultural crop productivity and quality. Specifically, water loss during postharvest handling and storage reduces external quality and hence commercial value of fresh fruit. However, the study of the molecular mechanisms underlying fruit dehydration has been limited to a few sets of genes involved in secondary metabolism and ABA signalling and biosynthesis (Schwartz *et al.*, 1997; Burbidge *et al.*, 1999; Alférez *et al.*, 2008; Bonghi *et al.*, 2012; Loyola *et al.*, 2012). In order to get an overview of the biological processes playing a role in the fruit stress tolerance, transcriptomic research has been performed in grapes. These studies indicated that molecular responses were differently regulated by dehydration occurring before or after harvesting the fruit and also by the level of stress severity (Rizzini *et al.*, 2009; Deluc *et al.*, 2009; Zamboni *et al.*, 2010; Bonghi *et al.*, 2012).

Within the context of *Citrus* fruit and the detrimental effects caused by dehydration, it is interesting to note that many citrus cultivars are prone to develop peel depressions affecting both the inner (albedo) and the outer part (flavedo) of the peel, which becomes bronze and necrotic as the disorder progresses (Alférez *et al.*, 2005; Lafuente and Zacarías, 2006; Alférez *et al.*, 2010). This physiological disorder, known as non-chilling peel pitting (NCPP) (Fig. 2), rind breakdown, or rind staining (Agustí *et al.*, 2001; Lafuente and Sala, 2002), occurs at temperatures higher than those causing chilling injury, and is enhanced by dehydration in both attached and detached citrus fruits (Alférez *et al.*, 2003; Alférez and Burns, 2004; Lafuente and Zacarías, 2006).

Figure 2. Non-chilling peel pitting damage in several *Citrus* cultivars. From the left, 'Navelina' orange, 'Clemenules' mandarin, and 'Marsh' grapefruit.

1.1.2.2. BIOTIC STRESS.

Stoma is a natural opening in the plant and has been recognized as a major point of entry to internal tissues for plant pathogenic bacteria (Melotto *et al.*, 2008; Lee and Luan, 2012). In agreement, stomatal closure has been reported to be part of the early-stage plant innate immune response to restrict microbe invasion. Moreover, the ABA signalling pathway in guard cells has been connected with the rapid stomatal closure upon bacterial perception (Kaliff *et al.*, 2007; Zeng *et al.*, 2010). Melotto et al. (2006) showed that pathogen-associated molecular

pattern (PAMP), which is recognized by plants and triggers plant innate immunity, induces stomatal closure. ABA-biosynthetic (*aba3-1*) and insensitive (*ost1-2*) mutants failed to induce PAMP-mediated stomatal closure, which suggested that this process required an active ABA signal transduction (Melotto *et al.*, 2006). Therefore, it was proposed that ABA plays a relevant role in pre-invasive defence against bacteria and has a positive effect on disease resistance.

In addition to its action in stomatal closure, ABA affects pathogen responses by interacting with other hormones that have been classically associated with plant defence mechanisms (Alvarez *et al.*, 1998). ABA is connected to the SA, JA and ethylene signalling pathways. Thus, it has been reported that ABA suppress both the SA-dependent disease resistance (Yasuda *et al.*, 2008) and the JA- and ethylene-dependent induction of defence-related genes (Anderson *et al.*, 2004). Therefore, in late-stage response, ABA exerts both the resistance suppression and the promotion of the susceptibility to microbe infection.

Overall, the role of ABA in biotic stress is not straightforward owing to its multifaceted function depending on the different tissues, developmental stages of the plant, and the kind of pathogen (Mauch-Mani and Mauch, 2005; de Torres-Zabala *et al.*, 2007; Ton *et al.*, 2009). Under controversial evidences pointing to either the repression or the promotion of disease resistance by ABA, Ton et al. (2009) proposed an integrative model in which ABA played a stimulatory role in plant defence during early stages of pathogen invasion and a mostly suppressive influence at later colonization stages.

1.2. REGULATION OF ABA LEVELS

The endogenous ABA content, which ranges from nanomolar to micromolar values depending on the tissue and the environmental conditions, is

determined by the balance among biosynthesis, catabolism, release from inactive conjugates, and transport rates.

The ABA biosynthesis starts in plastids with the production of C5-isopentenyl pyrophosphate (IPP) from glyceraldehyde-3-phosphate and pyruvate, through the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway. IPP is converted to a C20 product named geranylgeranyl pyrophosphate (GGPP), which leads to the synthesis of the carotene phytoene (C40) by condensation of two molecules of GGPP. Desaturation and isomerisation convert phytoene in lycopene, which subsequently goes through cyclization and hydroxylation to yield β,β-carotene and finally the xanthophyll zeaxanthin. Zeaxanthin is converted to antheraxanthin and later to violaxanthin by the same enzyme, zeaxanthin epoxidase (ZEP or ABA1). Then, *trans*-violaxanthin is transformed into *trans*-neoxanthin by a neoxanthin synthase (ABA4). Two putative isomerases might transform *trans*-violaxanthin and *trans*-neoxanthin into the nine-*cis* isomers although they have not been yet characterized. These nine-*cis*-isomers are cleaved by the NCED enzymes into a C15 product named xanthoxin and a C25 apocarotenoid metabolite. Then xanthoxin is translocated to the cytoplasm and reduced to abscisic aldehyde by the ABA2 reductase. Finally, this aldehyde is oxidized to convert its functional group in a carboxylic acid. This step is catalyzed by the aldehyde oxidase (AAO3), which requires a molybdenum cofactor (MoCo or ABA3) for its functionality (reviewed in Nambara and Marion-Poll, 2005; Wasilewska *et al.*, 2008; Rodríguez-Concepción, 2010; Farré *et al.*, 2010; Ruiz-Sola and Rodríguez-Concepción, 2012).

The up-regulation of several ABA biosynthetic genes is a common response to all ABA-mediated stress and developmental signals. In these responses, the induction of the *NCED* genes has been largely considered as the first committed step of the ABA biosynthesis. In *Arabidopsis*, Tan et al. (2003)

reported that this gene family is composed of nine members and that only five of them (*AtNCED2*, *AtNCED3*, *AtNCED5*, *AtNCED6* and *AtNCED9*) were targeted to plastids and likely involved in ABA production, showing a complex pattern of localized expression. Thus, *AtNCED2* and *AtNCED3* accounted for the total NCED activity in lateral roots, whereas *AtNCED2*, *AtNCED3*, *AtNCED5* and *AtNCED6* were expressed in flowers. *AtNCED5* and *AtNCED6* transcripts prevailed in anthers and pollen, respectively, whereas *AtNCED3* was the most stress-induced gene in leaves (Tan *et al.*, 2003). In addition, all these genes, except *AtNCED2*, were expressed in seeds (Tan *et al.*, 2003), although only *AtNCED6* and *AtNCED9* were required for the ABA synthesis that controlled seed dormancy and germination (Lefebvre *et al.*, 2006; Martínez-Andújar *et al.*, 2011). Recently, Frey et al. (2012) showed the involvement of *AtNCED5* in the induction of seed dormancy, plant growth and water stress tolerance.

These genes have been also studied in a high number of both climacteric and non-climacteric fruits. Studies performed in tomato, avocado, peach and persimmon indicated that *NCED* genes expression was induced as ripening progressed, showing a maximum before the ethylene production (Chernys and Zeevaart, 2000; Leng *et al.*, 2009; Zhang *et al.*, 2009a; Sun *et al.*, 2012b). In non-climacteric fruits such as strawberry, watermelon, sweet cherry and grape, *NCED* genes were mainly induced in response to water stress and ABA application, and transcripts accumulation paralleled ABA increase during fruit ripening (Zhang *et al.*, 2009a; Ren *et al.*, 2010; Ji *et al.*, 2012; Li *et al.*, 2012b). In citrus fruit, the expression of the *NCED* gene family has been also characterized. Rodrigo et al. (2006) showed that *CsNCED1* and *CsNCED2* displayed different expression patterns in response to leaf dehydration and during fruit ripening of sweet orange. Complementary, Agustí et al. (2007) indicated that *CcNCED3* (named *CsNCED1* in sweet orange) and *CcNCED5* (homolog to *CsNCED2*) genes were

preferentially induced in leaves and ripening fruits of Clemenules mandarin, respectively. In agreement, Kato et al. (2006) showed that *CitNCED2* (*CsNCED2*) and *CitNCED3* (*CsNCED1*) displayed a complex expression pattern that was not conserved among varieties and tissues.

Together with ABA biosynthesis, ABA levels are regulated by the catabolism of the active form of ABA. Thus, stress-induced rises in ABA also regulate gene expression of the ABA 8'-hydroxylase, a cytochrome P450 monooxygenase that catalyze the transformation of ABA in 8'-hydroxy ABA (Cutler and Krochko, 1999), which is spontaneously isomerized to phaseic acid (PA) (Krochko *et al.*, 1998). PA is further reduced to dihydrophaseic acid (DPA), by a soluble reductase (Gillard and Walton, 1976).

Another mechanism for reducing the pool of active ABA into the cell after a stress stimulus consists in the conjugation of ABA with a glucosyl ester (ABA-GE) group and its recruitment into the vacuole. When ABA is required after a stress or developmental signal, this storage of ABA-GE is hydrolyzed by β-glucosidases localized in the endoplasmic reticulum, contributing to the increase in the concentration of active ABA in the cell (Lee *et al.*, 2006). This mechanism has been proposed as an alternative, complementary to the induction of ABA biosynthetic genes, by which plants would rapidly adjust the ABA levels in order to quickly respond to changing environmental conditions (Lee *et al.*, 2006).

Previous studies suggested that ABA was produced in the roots and transported from there to the aerial part of the plant (Wilkinson and Davies, 2002). Complementary, it has been suggested that JA is needed for ABA increase in citrus roots under drought stress conditions since initial burst of JA could lead to the induction of ABA-biosynthetic genes (de Ollas *et al.*, 2012). In contrast, works in *Arabidopsis* plants indicated that vascular tissues localized in the shoot were major tissues providing dehydration-induced ABA pools (Endo *et al.*, 2008;

Ikegami *et al.*, 2009). Despite ABA is synthesized and metabolized in these tissues, it is well known that it acts in stomatal responses of distant guard cells. Therefore, plants need intercellular transport of the hormone. In last years, two plasma membrane-type ABC transporters with ability to bind and transport ABA have been described in *Arabidopsis* (Kang *et al.*, 2010; Kuromori *et al.*, 2010). The AtABCG25 exporter was a plasma membrane-localized protein mainly expressed in vascular tissues. Loss-of-function mutants presented hypersensitivity to ABA in germination and seedling establishment, and overexpressing plants presented a slower rate of water loss by transpiration. Therefore, it seems that AtABCG25 transported ABA from the vascular tissue to foliar cells (Kuromori *et al.*, 2010). In the case of the AtABCG40 transporter, it has been described that this protein was able to bind ABA, was localized in the plasma membrane, and had ability to transport ABA into foliar cells across the lipid bilayer. Consequently, Kang et al. (2010) suggested that AtABCG40 function was to import ABA into de cell. More recently, Kanno et al. (2012) have reported that a nitrate transporter had ability to bind and transport ABA from vascular tissues. Knockout mutants of this ABA-importing transporter (*AIT1*) displayed low sensitivity to applied ABA during *Arabidopsis* seed germination, whereas overexpression of *AIT1* resulted in ABA hypersensitivity. These findings indicated that ABA transport is complex and could present redundancy among their components.

The high number of key points for regulating availability of active ABA into the cell, together with the newly discovered soluble intracellular ABA receptors (explained in Section 1.4), suggest a modular network by which ABA levels, and hence signal transduction, might be rapidly regulated into the cell to elicit a timely response.

1.3. PHYSIOLOGICAL EFFECTS OF THE ABA DEFICIENCY

Plant hormone mutants have been used extensively to elucidate biosynthetic pathways and to define the involvement of hormones in physiological processes. Focusing on ABA, natural and induced knockout mutants of biosynthetic genes have been characterized (Armstrong *et al.*, 1995; Schwartz *et al.*, 1997; Xiong *et al.*, 2001; Seo *et al.*, 2004; González-Guzman *et al.*, 2004; Barrero *et al.*, 2006; North *et al.*, 2007; Fan *et al.*, 2009; Frey *et al.*, 2012).

The mutation of the ZEP/ABA1 enzyme of the ABA biosynthetic pathway caused ABA-deficient mutants that displayed, even under well-watered conditions, wilty phenotypes and reduced size of the leaves, inflorescences and flowers (Barrero *et al.*, 2005). Physiological characterization of the *Arabidopsis aao3* mutant showed an ABA-deficient phenotype, osmotolerance in germination and wilty leaves (Seo *et al.*, 2004; González-Guzman *et al.*, 2004; Barrero *et al.*, 2006). In addition, Seo et al. (2004) demonstrated that these mutants (AAO1-AAO4) displayed reduced or not affected dormancy because a partial redundancy among genes. Similarly, *aba3* mutant plants, which lack the MoCo required for AAO activity, showed ABA-deficiency and increased transpiration rate, as well as impaired cold-gene regulation (Xiong *et al.*, 2001). Moreover, it has been reported that *aba3* mutants failed increasing JA levels, even when *NCED* genes were overexpressed in this genetic background (Fan *et al.*, 2009), and that the levels of SA increased whilst biomass and relative leaf water content decreased in these mutants (Asensi-Fabado and Munné-Bosch, 2011).

The redundancy existing among the *NCED* gene family members has been confirmed by combining different knockout *nced* genes (Frey *et al.*, 2012). The most severe phenotype among these mutations (*nced3/nced5*) showed reduced seed dormancy and vegetative growth, together with increased water loss and decreased ABA levels under normal and stress conditions (Frey *et al.*, 2012).

Similar approaches have been developed in horticultural crops. *NCED* gene was silenced by RNAi in strawberry fruit reducing ABA levels, which impaired response of ABA-downstream signalling genes and showed uncoloured phenotype that was rescued by exogenous treatment with the hormone (Jia *et al.*, 2011). By using the same strategy Sun et al. (2012a) reduced NCED activity, which led to down regulation of major genes encoding cell wall catabolic enzymes in tomato. It has been also demonstrated that ABA-deficiency in *sitiens* tomato mutant fruit (Tal and Nevo, 1973; Taylor and Tarr, 1984) led to increased cuticle permeability, which was correlated with disease resistance (Curvers *et al.*, 2010). On the other hand, it has been demonstrated that ABA-deficiency in the double mutant *notabilis/flacca* tomato (Tal and Nevo, 1973; Taylor and Tarr, 1984) strongly correlates with reduced cell size, plant growth, leaf surface area, drought-induced wilting and ABA-related gene expression (Nitsch *et al.*, 2012). In agreement, the mutation of the *ZEP* gene in tomato fruits (named *high pigmentation 3*, *hp3*) caused ABA-deficiency and the enlargement of the plastid compartment size probably by increasing plastid division, which enabled a higher storage capacity of carotenoids pigments (Galpaz *et al.*, 2008).

Because of the complexity of obtaining artificially-generated mutants in woody plants, the access to spontaneous hormone mutants is of particular scientific interest. In citrus, a spontaneous fruit-specific ABA-deficient mutant from the 'Navelate' orange (*Citrus sinensis* L. Osbeck), named 'Pinalate' (Fig. 3), was biochemically characterized (Rodrigo *et al.*, 2003). 'Pinalate' orange presented distinctive yellow-coloured fruit because a partial blockage at the ζ-carotene desaturase step of the carotenoid biosynthetic pathway, which caused the accumulation of uncoloured carotenes, the decrease of xanthophylls content and a fruit-specific ABA-deficiency (Rodrigo *et al.*, 2003). Physiological comparative approaches indicated that ABA deficiency in the mutant might be

responsible for the higher transpiration rate (Alférez *et al.*, 2005) and higher sucrose content (Holland *et al.*, 2005) as compared to its parental. Furthermore, 'Pinalate' fruit displayed higher susceptibility to develop NCPP at 12 ºC at high relative humidity (85-90% RH) than 'Navelate' (Alférez *et al.*, 2005). In this regard, it was also demonstrated that the enzymes superoxide dismutase and catalase might be involved in the lower susceptibility of parental fruit to develop NCPP (Sala *et al.*, 2005) and that the enzymes phenylalanine ammonia-lyase and glucanase may be good biochemical markers of NCPP (Sala *et al.*, 2005; Sanchez-Ballesta *et al.*, 2008). In addition, during natural ripening, the rate of fruit degreening was lower in 'Pinalate' as compared to its wild-type cultivar (Alférez and Zacarías, 1999; Rodrigo *et al.*, 2003). High throughput transcriptional analysis during fruit ripening revealed a number of common biological processes between both cultivars, but also ABA-dependent processes lacking in the mutant (Romero *et al.*, 2010). Therefore, the fruit-specific ABA-deficient 'Pinalate' orange offered an exceptional experimental system to investigate the involvement of endogenous ABA in the water stress response of citrus fruit, as well as its relationship with the development of NCPP. Moreover, the comparative analysis of parental and mutant fruit would help to understand the role of endogenous ABA in the regulation of the hormone-perception system components during citrus fruit ripening and dehydration.

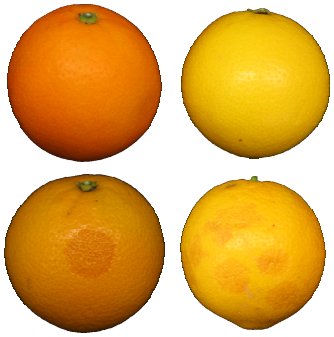

Figure 3. Differential phenotype and susceptibility of 'Navelate' (left) and its fruit-specific ABA-deficient mutant 'Pinalate' (right) fruit to NCPP. Upper fruits were freshly harvested (FH) and lower fruits were stored for 3 weeks at 12 ºC and 70-75% RH.

1.4. ABA SIGNALLING CASCADE

The ABA signalling pathway has been largely investigated, and a number of ABA-responsive genes and interacting proteins have been identified (Bartels and Sunkar, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). However, how ABA is perceived has been controversial and no consensus was achieved until 2009, when a family of cytosolic proteins was identified as ABA receptors by using different approaches (Park *et al.*, 2009; Ma *et al.*, 2009; Santiago *et al.*, 2009b; Nishimura *et al.*, 2010). These proteins have the ability to perceive the hormone and to start the signal transduction. Signal follows downstream in the pathway through the ABA-dependent receptor-mediated inhibition of the negative regulators, which allows the release of positive effectors and, subsequently, the switch on/off of transcription factors and proteins that modulate transcriptional and/or physiological responses (Fig. 4). Thus, through physicochemical properties of the signalling components and reversible phosphorilation processes, the ABA signalling cascade can be summarized in four simple steps that go from the hormone perception to gene expression (Klingler *et al.*, 2010; Weiner *et al.*, 2010; Joshi-Saha *et al.*, 2011).

1.4.1. HORMONE PERCEPTION: ABA RECEPTORS

To understand a hormone signalling pathway it is critical to know how the hormone is perceived and how its signal is transduced. In the case of ABA, several evidences support that multiple ABA receptors perceive the hormone signal outside and inside the cells, being this perception tissue- and developmental stage-specific (Finkelstein *et al.*, 2002) and dependent on the physiological process and on the stress imposed (Szostkiewicz *et al.*, 2010). Following a brief description of the different ABA receptors identified up to now is provided.

1.4.1.1. FCA: Flowering time control protein

The first putative ABA receptor identified in *Arabidopsis thaliana* was FCA (Flowering time control protein) (Razem *et al.*, 2006). FCA is a nuclear RNA-binding protein previously characterized as a flowering time regulator (Macknight *et al.*, 1997). Razem et al. (2006) proposed a signalling pathway in which FCA bound with high affinity and in a stereoespecific binding manner to (+)-ABA, hence inhibiting the association of FCA with FY (Flowering locus Y). The inhibition of the complex composed of FCA-FY by ABA enhanced the accumulation of the floral repressor FLC (Flowering locus C), which consequently delayed the flowering process. However, *FCA* loss-of-function mutants did not show the expected phenotypes in any of the common ABA-regulated physiological responses related to germination, stomatal regulation or primary root development. Therefore, subsequent works questioned the role of FCA as an ABA receptor (Risk *et al.*, 2008) and, finally, authors had to retract on the model proposed (Razem *et al.*, 2008).

1.4.1.2. CHLH: Magnesium-protoporphyrin IX chelatase

In a new attempt to found ABA-binding proteins, Zhang et al. (2002) identified a second putative ABA receptor isolated from broad bean leaves by an affinity chromatography. Analysis of this protein (named ABA Receptor, ABAR) revealed a high homology degree to the H subunit of the magnesium-protoporphyrin IX chelatase (CHLH) of *Arabidopsis thaliana*, which contained a chloroplast signal peptide in its sequence that localized this protein in this organelle (Shen *et al.*, 2006). In addition to its role producing Mg-protoporphyrin-IX, CHLH/ABAR had been also previously described as a key element in the plastid-to-nucleus signalling (Mochizuki *et al.*, 2001). The CHLH/ABAR binding to ABA was confirmed and its function was explored by using RNAi and overexpressing transgenic lines (Shen *et al.*, 2006). ABA-insensitive phenotypes in seed germination, post-germination growth and ABA-induced stomatal closure were found in the knockout mutants, whereas overexpressing CHLH/ABAR plants showed opposite phenotypes and hypersensitivity to the hormone (Shen *et al.*, 2006). Moreover, they found that downstream ABA-related signalling genes were differently regulated in these mutants. Within this context, Shang et al. (2010) proposed a model for the ABA signal transduction: CHLH/ABAR bound to ABA recruit WRKY proteins, negative regulators of the pathway, hence releasing the ABA-dependent transcription factors from a constitutive blockage and allowing the expression of ABA-responsive genes. Nevertheless, the role of this protein as ABA receptor has been also controversial since native protein lacked ABA binding ability in barley, and knockout mutants in this plant displayed wild-type phenotypes in the post-germination and stomatal closure responses (Müller and Hansson, 2009). Further support for the involvement of this protein in the ABA perception arrived from studies performed in fruits of agronomic interest. Thus, *CHLH/ABAR* transcript accumulation negatively correlated with the increment of

ABA along ripening of sweet cherry, and the hormone treatment significantly decreased the expression of this gene (Ren *et al.*, 2011). Moreover, *CHLH/ABAR* gene was silenced by RNAi in strawberry fruit (Jia *et al.*, 2011), and the resulting mutant displayed altered ABA, sugars and antocyanins content, as well as impaired regulation of ABA-responsive genes during fruit maturation. Moreover, ABA treatment did not rescue the uncoloured phenotype of this mutant. Together, these results suggested a role for CHLH/ABAR in the regulation of the ABA signal but also reinforced the idea of the involvement of ABA in the ripening of non-climacteric fruits.

1.4.1.3. GCPR: G-protein-coupled receptors

A third group of proteins that merits mention as potential ABA receptors is the family of G-protein-coupled receptors (GCPRs). The GPCRs are proteins anchored to plasma membrane through a seven-transmembrane domain that interacts with the intracellular heterotrimeric G protein complexes. It has been shown that the ligand binding to the GCPR triggers the conversion of the inactive G-heterotrimeric complex Gα-GDP/Gβγ to an active form in which the GDP of the Gα-subunit has been changed by the energetic GTP in a classical signalling cascade. Then, Gα-GTP and/or the dimeric Gβγ subunit can transduce the signal to downstream effectors (McCudden *et al.*, 2005). Liu et al. (2007) reported that a GCPR (GCR2) interacts with the Gα subunit GPA1 to mediate well known ABA responses in *Arabidopsis*. In addition, ABA binding to GCR2 led to the dissociation of the GCR2-GPA1 complex. Overexpressing mutants displayed ABA hypersensitivity and those showing loss-of-function presented ABA-insensitive phenotypes in all the hormone responses. These results suggested GCR2 as a positive regulator in the ABA signalling. Nevertheless, several groups found opposite results to those reported by Liu et al. (2007), which ought to question

the functionality of this protein as a new ABA receptor (Gao *et al.*, 2007; Guo *et al.*, 2008; Risk *et al.*, 2009). Thereafter, two new GCPR proteins (GTG1 and GTG2) were predicted by *in silico* analysis as potential ABA receptors (Pandey *et al.*, 2009). Although double knockout mutant showed ABA-insensitive phenotypes in germination, growth, stomatal closure and expression of ABA-responsive genes, GTG1 and GTG2 showed a very low affinity to ABA in binding assays. Nevertheless, both GTG1 and GTG2 bound to GPA1, which was thought as a negative regulator of the GTGs activity in this new model of ABA signalling (Pandey *et al.*, 2009).

Therefore, even taking into account this high controversy, all these findings conduced to highlight the relevance of the negative regulation loops for the fine-tuning of the ABA signalling pathway. This is in agreement with the signal transduction models proposed for other phytohormones such as IAA, GA and ethylene, in which the blockage or degradation of constitutive repressors allows a rapid and specific physiological response (Dharmasiri *et al.*, 2005; Ueguchi-Tanaka *et al.*, 2005; Kepinski and Leyser, 2005).

1.4.1.4. PYR/PYL/RCAR proteins: cytosolic receptors

Recent breakthrough studies have revealed the existence of soluble cytosolic proteins with ability to bind ABA. This last group of ABA receptors is composed of the PYR/PYL/RCAR proteins, which have been independently identified by different research groups in *Arabidopsis* (Park *et al.*, 2009; Ma *et al.*, 2009; Santiago *et al.*, 2009b; Nishimura *et al.*, 2010). By one hand, Park et al. (2009) identified PYR1 through a chemical genetic strategy in which a new synthetic ABA agonist, called pyrabactin, was used. Therefore, this family was named PYR/PYL (*Pyrabactin Resistance* and *PYR1-Like*). In an alternative approach, Santiago et al. (2009b) identified PYL5, PYL6 and PYL8 proteins in a yeast two-hybrid screen in which HAB1 protein phosphatase 2C (PP2C) was used as bait.

Similarly, Ma et al. (2009) identified PYL9/RCAR1 and PYL8/RCAR3 by using the same strategy but a different protein phosphatase (ABI2) as bait. This group named this family as regulatory components of ABA receptors (RCAR). Independently, Nishimura et al. (2010) performed an *in vivo* strategy and indentified nine PYR/PYL/RCAR proteins as major ABI1-interactors.

In *Arabidopsis*, the PYR/PYL/RCAR family is composed of 14 members of small proteins that belong to a superfamily of soluble ligand-binding proteins defined as START/BetV I superfamily (Klingler *et al.*, 2010). Binding assays determined that these proteins directly interact with ABA (Ma *et al.*, 2009; Miyazono *et al.*, 2009; Santiago *et al.*, 2009b; Szostkiewicz *et al.*, 2010), although they displayed different affinities to the hormone according to their oligomeric state (Dupeux *et al.*, 2011b) and stereospecificity (Santiago *et al.*, 2009b). Thus, it was demonstrated that monomeric receptors showed higher affinity to bind ABA than those with a dimeric structure, and that the same PYR/PYL/RCAR binds with different affinity to *plus* or *minus* stereoisomer of ABA. Moreover, biochemical analyses revealed that PYR/PYL/RCAR proteins directly bind clade A PP2Cs (Park *et al.*, 2009; Ma *et al.*, 2009; Santiago *et al.*, 2009b; Nishimura *et al.*, 2010), which are well known negative regulators of the pathway (Rodríguez *et al.*, 1998a; Gosti *et al.*, 1999; Merlot *et al.*, 2001; Saez *et al.*, 2004).

Beside this, genetic evidence also supported the role of PYR/PYL/RCAR proteins as positive regulators of the pathway. Park et al. (2009) demonstrated that loss-of-function mutants (*pyr1/pyl1/pyl4* and *pyr1/pyl1/pyl2/pyl4*) showed insensitive phenotypes to ABA in germination and root growth, and impaired ABA-induced stomatal closure and expression of ABA-responsive genes. In addition, overexpression of *PYL5* (Santiago *et al.*, 2009b) and *PYL9* (Ma *et al.*, 2009) displayed hypersensitivity to the hormone in similar responses.

Together, biochemical and genetic analyses demonstrated that these PYR/PYL/RCARs were ABA intracellular receptors able to control different aspects of ABA signalling and physiology. In spite of their role as positive regulators of the ABA signal, expression analysis performed in seedlings and plants of *Arabidopsis* revealed that most of the *PYR/PYL/RCAR* genes were down-regulated by both ABA treatment and stress-induced ABA levels (Table 1) (Szostkiewicz *et al.*, 2010).

Table 1. Transcriptional profiling of *PYR/PYL/RCAR* ABA receptors upon ABA treatment and stress conditions that increase endogenous ABA levels in seedlings and leaves of *Arabidopsis*. The numbers are the ratio of expression levels between treated and control samples.

	RCAR / PYR gene									
Treatment	*RCAR11 / PYR1*	*RCAR12 / PYL1*	*RCAR14 / PYL2*	*RCAR13 / PYL3*	*RCAR10 / PYL4*	*RCAR8 / PYL5*	*RCAR9 / PYL6*	*RCAR2 / PYL7*	*RCAR3 / PYL8*	*RCAR1 / PYL9*
ABA[a]	0.37	0.41	1.03	1.57	0.04	0.18	0.14	1.29	0.48	1.66
Drought[b]	1.30	0.91	0.44	1.60	0.19	7.72	1.64	0.94	0.29	0.87
Osmotic[c]	0.45	0.40	0.32	1.57	0.09	0.51	0.33	0.99	0.47	0.87
Salt[d]	0.59	0.65	0.53	1.43	0.13	0.38	0.17	1.30	0.43	1.06

Based on Genevestigator database and adapted from Szostkiewicz et al., 2010. Orange and green shading indicates significant ($P < 0.01$) up and down regulation, respectively. [a]Seedling treated with 10 mM ABA. [b]Dehydrated leaves for 7 days. [c]Plants treated with 300mM Mannitol. [d]Plants treated with 150 mM NaCl.

Recent breakthroughs on the ABA-response signalling mechanisms have been particularly relevant to agriculture since they have provided a deeper insight into the molecular events involved in stress tolerance and developmental processes. Thus, the discovery of the PYR/PYL/RCAR ABA receptors encouraged new researches aimed to improve drought hardiness in horticultural and crop plants (Chai *et al.*, 2011; Sun *et al.*, 2011; Kim *et al.*, 2012; Li *et al.*, 2012a). In rice, 13 putative orthologues to *PYR/PYL/RCAR* genes were identified, and plants overexpressing *OsRCAR5* showed hypersensitivity to ABA during seed germination and growth (Kim *et al.*, 2012). In tomato, eight genes homologues to the PYR/PYL/RCAR family were transcriptionally analyzed during fruit development and ripening, and also in vegetative tissue in response to stress (Sun *et al.*, 2011).

Such investigation demonstrated that genes were both regulated under stress and during non-stressful conditions, giving support to the role of this hormone in fruit developmental processes. In non-climacteric fruit such as strawberry, the involvement of these receptors in fruit ripening process was also undertaken. Thus, Chai et al. (2011) showed that silencing *PYR1* gene delayed strawberry fruit ripening, altered ABA content and sensitivity, and reduced the transcript levels of a set of ABA-responsive genes. In grape, three *PYR/PYL/RCAR* genes have been isolated (Li *et al.*, 2012a), although only one of them showed the ability to bind ABA and to inhibit the phosphatase activity of ABI1. Further studies revealed that grape ABA signalling cascade consists of at least seven ABA receptors showing organ and stress specificity (Boneh *et al.*, 2012b).

Overall, limited information is available about the involvement of the ABA perception system in the developmental or stress responses in horticultural and crop plants and, up to now, there is no report analyzing the expression of this set of genes as a whole in non-climacteric fruits.

1.4.2. CLADE-A PROTEIN PHOSPHATASES 2C (PP2CA): NEGATIVE REGULATORS

The reversible phosphorilation of proteins is a fundamental mechanism to modulate cellular processes. Protein phosphatases provide modulations of phosphoregulation by reversing the action of protein kinases. PPs have been grouped in two major classes based on their substrate specificity: protein tyrosine phosphatases and serine/threonine phosphatases. This last group has been divided in two subcategories (PP1 and PP2) based on enzymological criteria. In addition, PP2s have been further distinguished by their metal requirements. PP2C-type protein phosphatases, which are the largest family of PP2s, are composed of monomeric enzymes whose activity depends on Mg^{2+} or Mn^{2+}

(Rodríguez, 1998). The 76 members of this family identified in *Arabidopsis* fall into 10 clades (A-J) (Schweighofer *et al.*, 2004). Among them, at least six of the nine members of the clade A (PP2CAs) have been involved in the regulation of the ABA signal transduction.

The first evidence of the role of PP2CAs in the ABA signalling pathway arrived from loss-of-function *abi1* and *abi2* mutants (Rodríguez *et al.*, 1998a; Gosti *et al.*, 1999; Merlot *et al.*, 2001), whose phenotype provoked to name this subfamily as ABA insensitive. The other members of the PP2CAs were clustered in three further subfamilies. HAB1 and HAB2 were identified by homology to ABI proteins, and their null mutants displayed hypersensitivity to ABA (Rodríguez *et al.*, 1998b). In a genetic screening searching for mutants with enhanced ABA response in germination and post-germination, *AHG1* and *AHG3* were identified and named hypersensitive to germination (Nishimura *et al.*, 2004; Yoshida *et al.*, 2006b; Nishimura *et al.*, 2007). Finally, a family of three members (*HAI1*, *HAI2* and *HAI3*) showing a highly ABA-induced response was described (Leonhardt *et al.*, 2004).

Genetic evidence was crucial to elucidate the role of these proteins as negative regulators of the ABA signalling pathway. Loss-of-function mutants from *ABI1, HAB1* and *AHG3*, revealed hypersensitive phenotypes to the hormone in germination, growth, stomatal closure and expression of ABA-responsive genes (Saez *et al.*, 2004; Leonhardt *et al.*, 2004; Saez *et al.*, 2006; Kuhn *et al.*, 2006; Yoshida *et al.*, 2006b). The hypersensitive mutant *ahg1-1*, displayed a strong phenotype in germination and post-germination growth but no evident responses in adult plants. In contrast, *35S:AHG3* and *35S:HAB1* overexpressing lines showed ABA insensitivity in seed germination and growth, as well as increased transpiration rates in adult plants (Saez *et al.*, 2004; Kuhn *et al.*, 2006), mainly due to reduced stomatal closure (Kuhn *et al.*, 2006). In agreement with this, and

contrary to findings mentioned above for PYR/PYL/RCAR ABA receptors, public *Arabidopsis* databases and results from several works (Nishimura *et al.*, 2007; Rubio *et al.*, 2009; Szostkiewicz *et al.*, 2010) indicated that these negative regulators of the ABA signal cascade are highly induced in response to ABA application and/or environmental stresses increasing endogenous ABA levels (Table 2). Additionally, it was found overlapping functions among the members of several PP2CA subfamilies. For instance, double mutant *ahg1-1/ahg3-1* exhibited a stronger phenotype than the single mutant lines in germination and post-germination growth, although functional differences in germination efficiency or seed dormancy were already observed when analyzing the singles mutants (Nishimura *et al.*, 2007).

Table 2. Transcriptional profiling of PP2CAs upon ABA treatment and stress conditions that increase endogenous ABA levels in seedlings and leaves of *Arabidopsis*. The numbers are the ratio of expression levels between treated and control samples.

	PP2CA gene								
Treatment	***ABI1***	***ABI2***	***HAB1***	***HAB2***	***AHG1***	***AHG3***	***HAI1***	***HAI2***	***HAI3***
ABA[a]	12.82	59.67	7.72	3.34	1.26	9.65	11.66	10.61	10.67
Drought[b]	1.83	12.56	16.00	4.24	74.91	8.74	15.93	15.40	10.59
Osmotic[c]	9.99	14.18	7.52	2.58	15.52	8.44	11.40	8.59	9.43
Salt[d]	5.57	6.19	3.16	1.69	5.27	5.56	10.99	9.96	8.02

Based on Genevestigator database and adapted from Szostkiewicz et al., 2010. Orange shading indicates significant ($P < 0.01$) up regulation. Values higher than 10-fold inductions are highlighted in red. [a]Seedling treated with 10 mM ABA. [b]Dehydrated leaves for 7 days. [c]Plants treated with 300mM Mannitol. [d]Plants treated with 150 mM NaCl.

Because of the relevance of ABA regulating the dehydration response, the elucidation of the ABA signalling cascade can be used to improve benefits in commercial agriculture. Seiler et al. (2011) found that development of barley grain under drought was ABA-controlled through PYL/PP2CA-mediated activation of the ABI5 transcription factor. Sun et al. (2011) performed a transcriptional

study in which the relationship between ABA and the *PP2CA*s gene-regulation was highlighted in different organs, developmental stages and in response to water stress. Studies performed in grape identified six putative *PP2CA*s and revealed that their expressions in leaves and roots were highly regulated by abiotic ABA-increasing stresses such as high salt concentration, cold and drought (Boneh *et al.*, 2012a; Boneh *et al.*, 2012b), and that sugar and exogenous ABA regulated these and other ABA-signalling genes during ripening of this non-climacteric fruit (Gambetta *et al.*, 2011). Therefore, recent works in crops of agronomic value attempt to find potential targets for biotechnological research.

1.4.3. PROTEIN KINASES INVOLVED IN THE ABA SIGNALLING CASCADE

Protein kinases are a wide family of proteins whose cellular function is to phosphorilate other proteins, or even auto-phosphorilate themselves, to turn on their functionality. Among the high number of families sharing this function, a group of calcium-independent protein kinases (SnRK2s) merits a special mention as important factors in the ABA signalling (Yoshida *et al.*, 2002; Fujii *et al.*, 2007; Fujii and Zhu, 2009; Fujita *et al.*, 2009) since their interactions with the negative regulators PP2CAs have been reported (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Hirayama and Umezawa, 2010). Several protein kinases from other families have been also involved in ABA signalling, such as the receptor-like kinase 1 (RPK1) (Osakabe *et al.*, 2005), the mitogen-activated protein kinases (MAPK) (Jammes *et al.*, 2009), and a number of Ca^{2+}-dependent protein kinases (CDPK) (Choi *et al.*, 2000; Mori *et al.*, 2006; Zou *et al.*, 2010). Because of the high number of reports describing the integration of the ABA signal through the PYR/PYL/RCAR receptors and the downstream complex composed of the PP2CAs and the SnRK2s, this section will be focused on the SnRK2 family.

Sucrose non-fermenting 1-related protein kinases family (SnRKs) in *Arabidopsis* is composed of 38 proteins divided in three groups (SnRK1, SnRK2 and SnRK3) accordingly to their sequence similarity and functional domains (Hrabak *et al.*, 2003). The group of SnRK2 is composed of 10 plant-specific proteins, which present a characteristic D-rich C-terminal domain that is essential for transducing the ABA signal (Yoshida *et al.*, 2006a). Among them, three proteins classified into the subclass III, named SnRK2.2, SnRK2.3 and SnRK2.6, are strongly activated by ABA in *Arabidopsis*. The structure and functionality of this subclass of SnRK2s is conserved in rice (Kobayashi *et al.*, 2004), and their corresponding orthologues have been classified into the same subfamily on the basis of results obtained from *in silico* analyses of tomato and grape (Sun *et al.*, 2011; Boneh *et al.*, 2012a).

The first subclass III SnRK2 protein identified was described as a guard cell-specific kinase implicated in the ABA-induced stomatal closure in *Vicia faba* (Li *et al.*, 2000). By genetic screen looking for proteins that displayed a reduced ability to close stomata in response to drought stress, the orthologue found in *Arabidopsis* was named open stomata 1 (OST1/SnRK2.6/SRK2E) (Mustilli *et al.*, 2002). The *ost1* null mutants showed high insensitivity to ABA-mediated stomatal closure (Yoshida *et al.*, 2002; Mustilli *et al.*, 2002). The analysis of the OST1/SnRK2.6-related kinases, SnRK2.2 and SnRK2.3, revealed that all members of this subclass played relevant roles in mediating ABA signalling during seed dormancy, germination and growth, as well as regulating gene expression in response to ABA (Fujii *et al.*, 2007). It is interesting to note that *SnRK2.2* and *SnRK2.3* single mutants did not display a clear ABA response phenotypes, whereas the double mutant *snrk2.2/snrk2.3* showed a strong ABA-insensitive phenotype in the processes mentioned above, which suggested a marked redundancy between these genes (Fujii *et al.*, 2007). The analysis of the triple mutant *snrk2.2/snrk2.3/snrk2.6* revealed extreme insensitive phenotypes to all ABA-

mediated responses, which led to classify SnRK2.2/SnRK2.3/SnRK2.6 proteins as central positive regulators in ABA signalling (Fujii and Zhu, 2009; Fujita *et al.*, 2009). Therefore, these results suggested that these proteins had redundant functions and that subclass III SnRK2-mediated protein phosphorylation is absolutely essential for ABA signalling (Fujii and Zhu, 2009; Fujita *et al.*, 2009).

Functionality of the subclass III SnRK2s has been confirmed in crops of agronomic interest such as rice (Kobayashi *et al.*, 2004) and maize (Li *et al.*, 2009), but not in fruits. Nevertheless, several reports have already analyzed the expression pattern of these genes in tomato fruits (Sun *et al.*, 2011) and in grape (Boneh *et al.*, 2012a). Results obtained in tomato fruit showed that *SnRK2*s were transiently induced by exogenous ABA and highly expressed at the most immature stages, concomitantly with lowest endogenous ABA levels (Sun *et al.*, 2011). However, water stress-induced ABA content in tomato leaves had little effect on the transcript levels of these genes. In agreement, water stress slightly changed gene expression of the subclass III *SnRK2*s in grape leaves (Boneh *et al.*, 2012a).

1.4.4. ABA SIGNALLING INTEGRATION

The ABA signalling pathway has been considered a very complex network since it involves numerous proteins (Bartels and Sunkar, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). The identification of the core elements of the pathway, PYR/PYL/RCAR-PP2CA-SnRK2 (also named ABA-signalosome), has simplified the integration of these components in a signal transduction mainly regulated by a negative feedback loop (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Fujii and Zhu, 2009). This breakthrough was possible due to the elucidation of the crystallographic structures of each component and of the putative complexes formed in absence/presence of the hormone (Melcher *et al.*, 2009; Santiago *et al.*, 2009a; Weiner *et al.*, 2010; Dupeux *et al.*, 2011a).

The PYR/PYL/RCAR protein structures consist of a central seven-stranded β-sheet flanked by two α-helixes highly conserved among species (Ma *et al.*, 2009; Santiago *et al.*, 2009b; Chai *et al.*, 2011; Sun *et al.*, 2011; Kim *et al.*, 2012). This β-sheet produces a cavity that contains a ligand-binding pocket that closes after ABA binding through conformational changes of two conserved β-loops that serve as *gate* and *latch* (Park *et al.*, 2009; Ma *et al.*, 2009; Melcher *et al.*, 2009; Santiago *et al.*, 2012). Comparison of the catalytic core of the different *Arabidopsis* PP2CAs with that of their orthologues in other plant species, indicated that critical active-site residues are highly conserved (Rodríguez, 1998; Schweighofer *et al.*, 2004; Xue *et al.*, 2008; Sun *et al.*, 2011; Boneh *et al.*, 2012a). Structural protein analyses further demonstrated that PP2CAs shared a similar folding pattern formed by a central sandwich of two β-sheets enclosed by two α-helices at each side of the catalytic site, which contains 3 Mn^{2+} or Mg^{2+} ions (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Yin *et al.*, 2009; Dupeux *et al.*, 2011a). Subclass III SnRK2s contains a kinase catalytic domain at the N-terminus with a well-conserved activation loop, and the C-termini rich in aspartic residues (Belin *et al.*, 2006; Yoshida *et al.*, 2006a). Moreover, it has been demonstrated that phosphorylation of conserved serine residues in the activation loop play a crucial role in SnRK2s function (Belin *et al.*, 2006; Boudsocq *et al.*, 2007).

In *Arabidopsis*, it has been shown that, in the absence of ABA, PP2CAs constitutively interact with subclass III SnRK2s through the D-rich C-terminal domain (Yoshida *et al.*, 2006a; Umezawa *et al.*, 2009; Fujii and Zhu, 2009) inactivating them by dephosphorylation of serine residues located in the activation loop (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Fujii and Zhu, 2009). Moreover, it has been demonstrated that ABA binding to the receptors is enhanced when PYR/PYL/RCAR proteins are bound to their negative regulators PP2CAs (Park *et al.*, 2009; Ma *et al.*, 2009; Melcher *et al.*, 2009; Dupeux *et al.*,

2011a; Santiago *et al.*, 2012). After ABA binding, the receptor locks in a closed structure that inhibits the PP2CA active site (Melcher *et al.*, 2009; Santiago *et al.*, 2009b). Therefore, ABA-induced PYR/PYL/RCAR-mediated inhibition of PP2CAs releases SnRK2s from their constitutive blockage, allowing its activation by phosphorylation (Mustilli *et al.*, 2002; Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Fujii and Zhu, 2009) and the ABA signal transduction downstream in the pathway (Fig. 4). Thus, SnRK2s positively regulate ABA response by phosphorylation and activation of ABF/AREB bZIP transcription factors that bind to ABA responsive elements (ABRE) (Kobayashi *et al.*, 2005; Fujita *et al.*, 2009; Yoshida *et al.*, 2010). It is also noticing that SnRK2s have been involved in the phosphorylation of different proteins, such as SLAC1 and KAT1 ion channels and NADPH oxidases in guard cells, associated with the control of ABA-induced stomata closure (Geiger *et al.*, 2009; Sirichandra *et al.*, 2009).

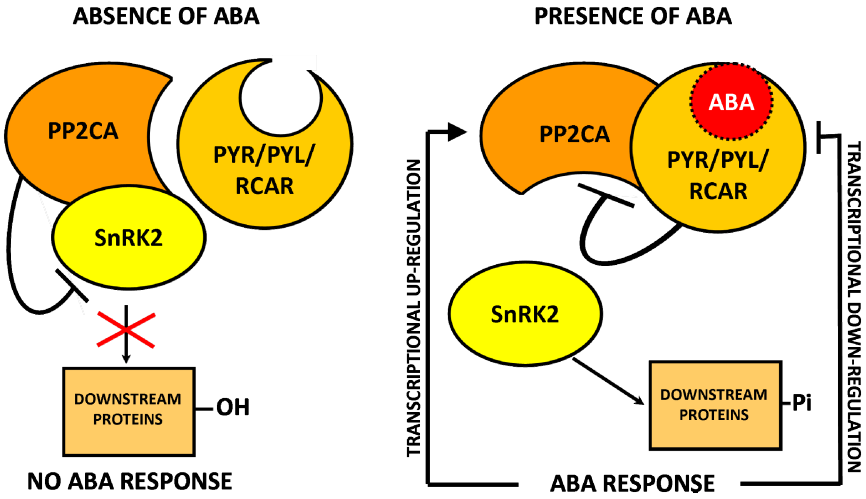

Figure 4. **Integration of the ABA signalling cascade.** Model for ABA-dependent PYR/PYL/RCAR-mediated inhibition of PP2CA activity and the consequent release of the SnRK2 for allowing the downstream ABA signalling. Adapted from Park *et al.* 2009. Details of the model are explained in the text.

In addition to these new structural insights, transcriptional profiling of the several components of the ABA-signalosome fits with the negative-feedback regulatory mechanism previously described by Merlot et al. (2001) for PP2CAs transcripts regulation. Thus, it was proposed that exogenous ABA or stress-induced rises in the hormone levels would induce the initial ABA-mediated PYR/PYL/RCAR inactivation of PP2CAs. This inactivation would turn on ABA-responsive genes and be later attenuated by the combination of both the ABA-induced down-regulation of PYR/PYL/RCARs expression and the up-regulation of the PP2CAs, which would restore the initial conditions. Therefore, the dual response of PP2CAs to ABA (regulated negatively by the input ABA and positively by the ABA signalling output) provides a dynamic and precise mechanism to adjust the adaptive response of plants to the strength and duration of the stress (Figure 4) (Vlad *et al.*, 2009; Santiago *et al.*, 2009b).

2. OBJECTIVES

The main objective of this PhD dissertation has been to characterize the involvement of ABA in the molecular mechanisms underlying the dehydration response and in the development and ripening of citrus fruit, taking the advantage of a fruit-specific ABA-deficient mutant, named 'Pinalate', from the wild type cultivar 'Navelate'.

This main purpose can be divided in the following partial objectives:

1. To characterize the molecular mechanisms involved in the response of harvested citrus fruits to dehydration and the potential role of ABA in this process, as well as elucidating the possible relationship between these two components (dehydration and ABA) and the development of peel damage during fruit storage at non-chilling temperature.

2. To identify the ABA perception system components in *Citrus* and to obtain a deeper insight into the transcriptional modulation of these elements under developmental and water stressful conditions increasing ABA in reproductive and vegetative tissues.

3. RESULTS

3.1. CHAPTER 1

Unravelling molecular responses to moderate dehydration in harvested fruit of sweet orange (*Citrus sinensis* L. Osbeck) 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.

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'-ATTGACAAGCGTGTGATT GAGC-3'; R 5'-TCCACAAGGCAATATCAATGGTA-3'), *CsGAPDH* (F 5'-CGTCCCTCTGCA AGATGACTCT-3'; R 5'-GGAAGGTCAAGATCGGAATCAA-3') and *CsTUB* (F 5'-GCATCT TGAACCCGGTAC-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.

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 *Arabidopsis*	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 transcription 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	

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 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)

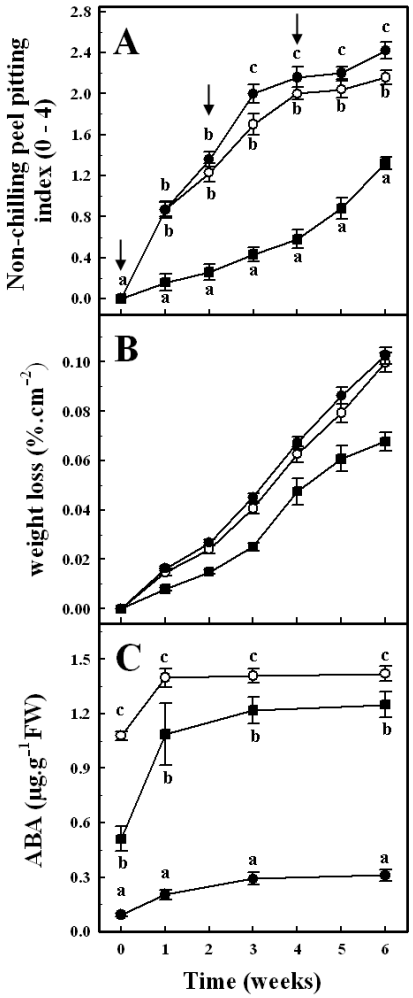

Figure 1. Non-chilling peel pitting index (A), percentage of fruit weight loss per surface area (B) and ABA content in the flavedo (C) of 'Navelate' (squares) and 'Pinalate' (circles) fruits treated (white) or not (black) with ABA and stored for up to 6 weeks at 12 ºC and 70-75% RH. The arrows indicate 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.

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).

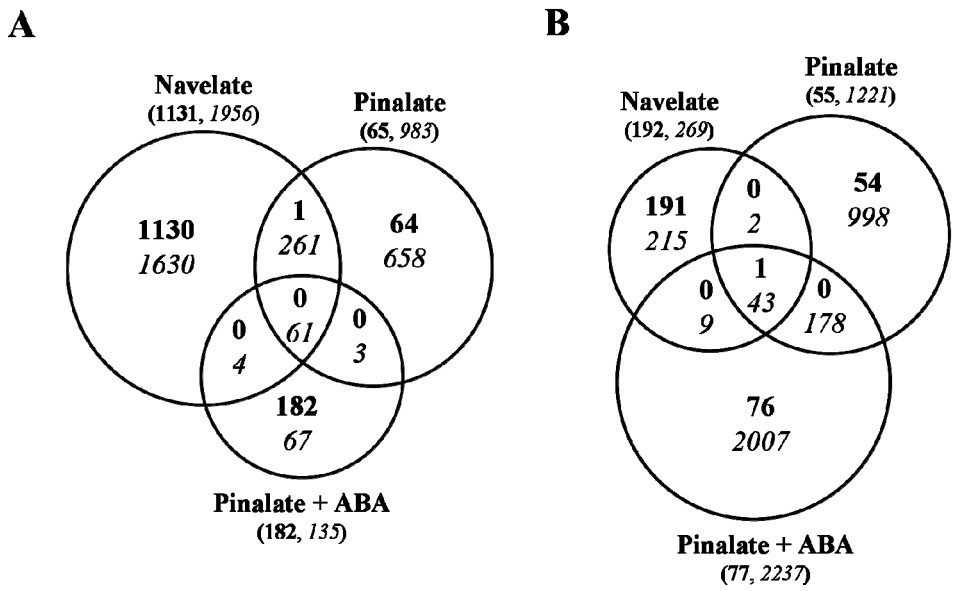

Figure 2. Venn diagrams showing differentially expressed genes (SAM analysis, FDR < 0.01) in the flavedo of 'Navelate', 'Pinalate' and ABA-treated 'Pinalate' fruits stored at 12 ºC and 70-75% RH for 1 (A) and 3 (B) weeks. Expression levels of up- (**bold**) and down-regulated (*italics*) genes in these fruits were compared to those of FH 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.

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.

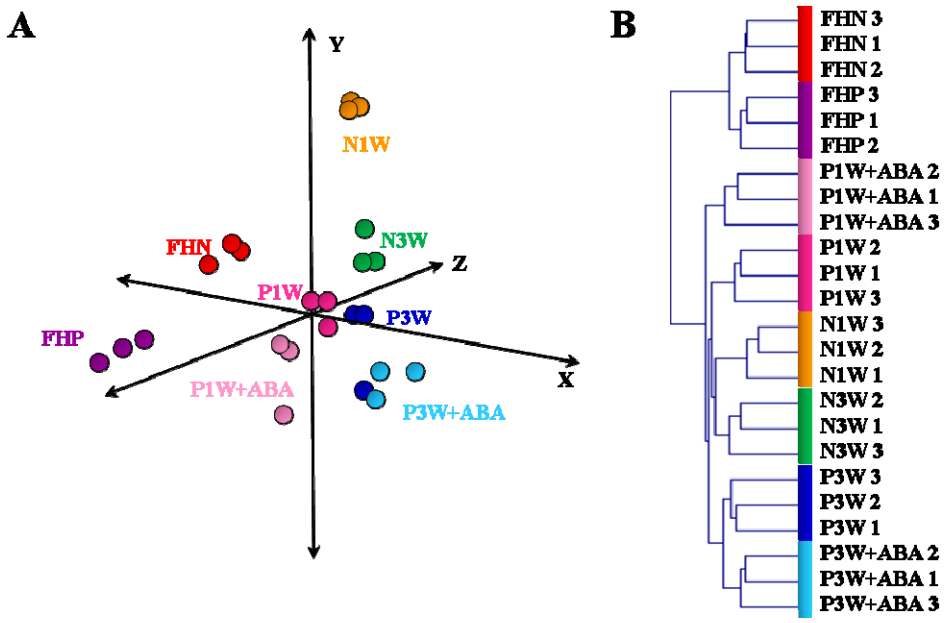

Figure 3. (A) Principal Component (PCA) and (B) Hierarchical Cluster Analysis (HCA) of flavedo large-scale transcriptional profiles of 'Navelate' (N), 'Pinalate' (P) and ABA-treated 'Pinalate' (P+ABA) fruits stored for one (1W) and three weeks (3W) at 12 ºC and 70-75% RH respect to FH fruits. Colours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

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).

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 FH fruits. Arrows indicate enriched biological processes (FatiGO+, $P \leq 0.05$) 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			↓

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).

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 FH fruits; P3W < FHP, genes repressed in 'Pinalate' fruits stored for 3 weeks respect to FH fruits; P3W+ABA < FHP, genes repressed in ABA-treated 'Pinalate' fruits stored for 3 weeks respect to FH 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

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).

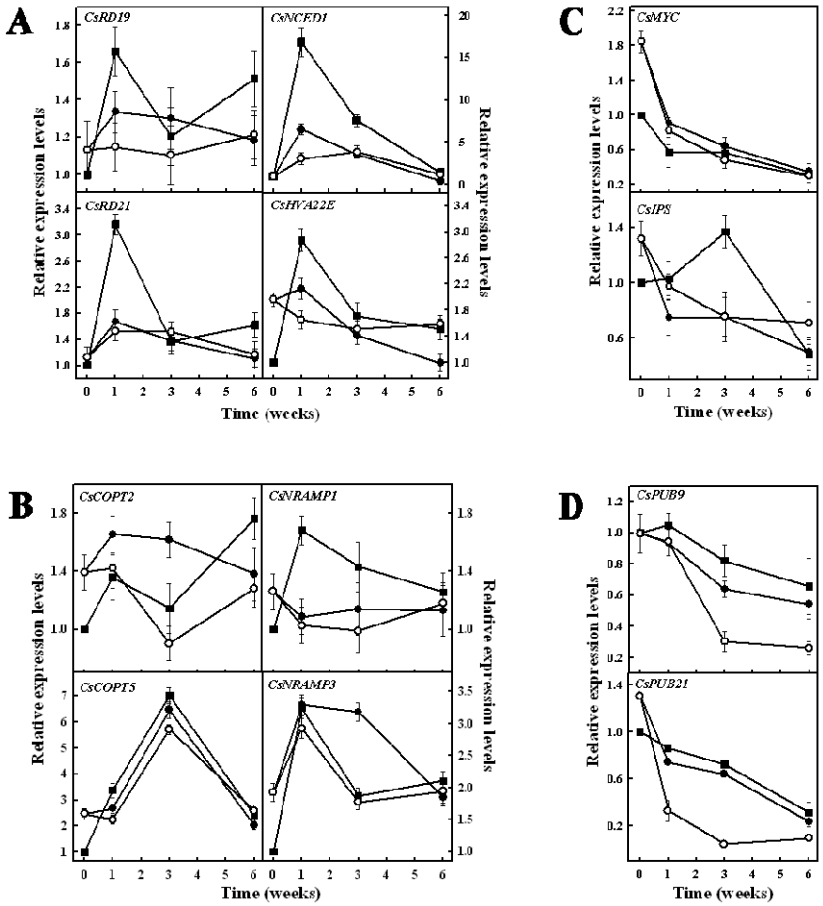

Figure 4. Real time qRT-PCR expression analysis for candidate genes selected from microarrays analysis. Relative transcript abundance for selected genes belonging to 'Water deprivation' (A), 'Di-, tri-valent inorganic cation transport' (B), 'Carbohydrate biosynthesis' (C) and 'Protein ubiquitination' (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 FH 'Navelate' fruits and expressed as relative values. Data are the mean values of three biological replicates ± SE.

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 to postharvest dehydration. 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 required as cofactors of superoxide dismutases that may contribute to the lower susceptibility of 'Navelate' fruit to develop NCPP (Sala *et al.*, 2005). It is known that an excess of metals may lead to the disruption of cellular processes and finally to cell death, and that the prevention of such harmful effects require the participation of metal-binding proteins and transporters (Puig *et al.*, 2007). Thus, the higher increase in the expression levels of iron and copper transporters detected in the wild-type fruit (Fig. 4B), suggests that the impaired ability of the ABA-deficient mutant to regulate metal homeostasis could be relevant for its higher susceptibility to dehydration and NCPP.

Most of the differentially expressed genes were down-regulated in the mutant by 3 weeks (Fig. 2B) and grouped into numerous biological processes (Table 2), being 'carbohydrate biosynthesis' the most specific. This is in agreement with previous results showing a higher reduction in soluble sugars and starch in 'Pinalate' respect to parental fruits during development of NCPP (Holland *et al.*, 2005), and highlights the interplay between ABA and sugars in plants. This process grouped not only genes involved in the metabolism of soluble sugars and starch but also in the metabolism of cell wall polysaccharides and putative regulatory elements, such as a MYC transcription factor and a gene (*CsIPS*) involved in regulating the levels of inositol-3-phosphate, which constitutes a node for the crosslink among several signalling pathways (Kaur and Gupta, 2005). The CsMYC transcription factor displays a 63% of identity with the ABA-responsive AtMYC2, which triggers the slow adaptive response of *Arabidopsis* to dehydration (Abe *et al.*, 2003; Bartels and Sunkar, 2005) and, therefore, the *CsMYC* transcript might be involved in the tolerance of citrus fruit to water stress. Nevertheless, this *Citrus* gene appears not to be a limiting step in this process since its expression levels continuously decreased in the ABA-deficient mutant but also in the parental fruit.

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

been recently characterized in *Arabidopsis* (Park *et al.*, 2009; Ma *et al.*, 2009), between mutant and wild-type fruits under water stress conditions.

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

citrus fruit through proteins involved in the ubiquitin-proteasome system machinery.

In conclusion, the comparative transcriptional analysis between 'Navelate' and its mutant 'Pinalate' fruits highlights the ability of parental fruit to develop responses to reduce water loss and other detrimental consequences caused by this stress. These responses involve the 'water deprivation' and the 'di-, tri-valent inorganic cation transport' biological processes, which include both ABA-dependent and independent genes. The alteration of these responses in the mutant fruit suggests their relevance for the prevention of peel damage in citrus fruit. Likewise, repression of the 'carbohydrate biosynthesis' process occurred specifically in 'Pinalate' fruits, which showed higher susceptibility to NCPP. Overall, results suggest that the sensitivity/response to ABA may be impaired in the ABA-deficient mutant fruit and reveals molecular mechanisms triggering the response to water stress in citrus fruit.

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SUPPLEMENTARY MATERIAL

Table S1. Representative set of ABA-regulated genes whose expression did not significantly (SAM, FDR < 0.01) change in 'Pinalate' fruits after ABA treatment respect to FH 'Pinalate' fruits.

Citrus unigene (CFGP)	Homolog in *A. thaliana*	Most similar protein	Fold expression values (Log_2)			
			1 week		3 weeks	
			- ABA	+ ABA	- ABA	+ ABA
aC04015B01SK_c	AT4G38900	Putative bZIP transcription factor	-0.33	-0.12	-0.29	-0.28
aC04028A10SK_c	AT1G32640	MYC transcription factor	-2.64	-2.07	-2.92	-3.17
aCL111Contig1	AT1G77120	Alcohol dehydrogenase	-0.28	-0.06	-0.02	-0.11
aCL172Contig2	AT5G25610	RD22-like protein	1.05	0.91	1.56	n.d.
aCL1923Contig1	AT1G32640	Transcription factor AtMYC	0.47	0.61	0.37	0.72
aCL2272Contig1	AT4G26080	Protein phosphatase-2C	0.31	0.19	0.19	0.12
aCL2763Contig1	AT3G20310	Ethylene-responsive transcription factor 3	-0.07	-0.22	0.27	0.15
aCL3553Contig1	AT1G45249	Putative ripening-related bZIP protein	-0.50	-0.28	-0.39	-0.40
aCL4559Contig1	AT5G52300	Low-temperature-induced 65 kDa protein	-0.36	0.25	-0.28	-0.10
aCL474Contig1	AT3G19290	Putative ripening-related bZIP protein	0.62	0.61	0.12	0.26
aCL474Contig2	AT3G19290	Putative ripening-related bZIP protein	0.12	-0.23	0.61	0.26
aCL5131Contig1	AT2G36270	Abscisic acid insensitive 5	n.d.	0.58	0.47	n.d.
aCL6186Contig1	AT3G15730	Phospholipase D alpha 1 precursor	-0.17	-0.38	-0.60	-0.65
aCL7631Contig1	AT3G56850	BZIP transcription factor	0.31	0.03	0.45	0.24
aIC0AAA65DA09RM1_c	AT2G26300	G protein alpha subunit	0.43	0.48	0.39	0.52
aIC0AAA88DA08RM1_c	AT4G27410	NAC domain-containing protein 68	-0.06	-0.39	0.25	0.40

n.d. not detected

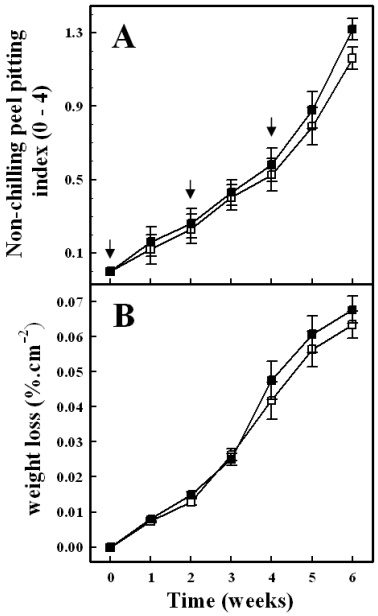

Figure S1. Non-chilling peel pitting index (A) and percentage of fruit weight loss per surface area (B) of 'Navelate' (squares) fruits treated (white) or not (black) with ABA and stored for up to 6 weeks at 12 ºC and 70-75% RH. The arrows indicate when ABA was applied. Results are the means of three biological replicates of 10 fruits each ± SE. Mean separation was performed by applying Tukey's test. No significant differences ($p \leq 0.05$) between samples for the same storage period were found.

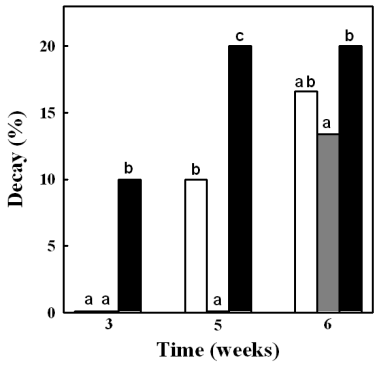

Figure S2. Percentage of decay in 'Navelate' (white), 'Pinalate' (grey) and ABA-treated 'Pinalate' (black) fruits stored at 12 ºC and 70-75% RH. Different letters for the same storage period indicate significant differences according to Tukey's test (*P*-value ≤ 0.05).

3.2. CHAPTER 2

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

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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 in 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*, 5 *PP2CA* and 2 subclass III *SnRK2* genes, homologous to those of *Arabidopsis*, were identified into the *Citrus* genome. The high homology degree and conserved motifs for protein folding and for functional activity suggested that these *Citrus* proteins are bona-fide core elements of the 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. Contrary, changes in *CsPP2CA*s expression during ripening paralleled those of ABA content and concurred with relevant differences between wild-type and mutant fruit transcripts accumulation. *CsSnRK2*s 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. Global results suggest that responsiveness to ABA changes during citrus fruit ripening and leaf dehydration was higher in the *CsPP2CA*s negative regulators than in the other ABA-signalosome components.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a critical endogenous messenger in the adaptive responses of plants to environmental stresses (Bartels and Sunkar, 2005) and also plays a role in the biotic stress responses (Ton *et al.*, 2009). This hormone is also crucial for the regulation of a number of physiological processes under non-stressful conditions (Finkelstein *et al.*, 2002; Gómez-Cadenas *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007; Kim *et al.*, 2012). The involvement of ABA in the ripening of both climacteric and non-climacteric fruits has been also studied (Lafuente *et al.*, 1997; Alférez and Zacarías, 1999; Zhang *et al.*, 2001; Rodrigo *et al.*, 2003; Zhang *et al.*, 2009a; Sun *et al.*, 2010; Bastías *et al.*, 2011; Jia *et al.*, 2011). In climacteric fruits, ABA accumulates just before the peak of ethylene production, triggering ethylene biosynthesis responsible for fruit ripening (Zhang *et al.*, 2009b). The role of ABA in non-climacteric fruits is less clear although several evidences correlate the increase in ABA levels during ripening with the activation of pathways related to the ripening process (Agustí *et al.*, 2007; Giribaldi *et al.*, 2010; Chai *et al.*, 2011; Ren *et al.*, 2011; Gambetta *et al.*, 2011). These findings indicate a relevant role of ABA in the process of fruit ripening. However, although the knowledge of conventional physiology and biochemistry of ABA underlying fruit development has been highly improved in the last years, the regulatory mechanisms of ABA action in non-climacteric fruit ripening are less known.

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 (Karssen *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; Jia *et al.*, 2011; Chai *et al.*, 2011).

In woody plants, artificially generated mutants are less affordable but spontaneous mutants are more broadly found (Koornneef *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 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 to its wild-type cultivar (Rodrigo *et al.*, 2003). Moreover, the sensitivity to ABA and the molecular responses to fruit dehydration during postharvest storage have been shown to be impaired in this mutant, which suggested that ABA perception system may fail 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 evidences support that multiple ABA receptors perceive the ABA signal outside and inside the cells, being this perception tissue-specific (Finkelstein *et al.*, 2002). The PYR/PYL/RCAR soluble proteins (Park *et al.*, 2009; Ma *et al.*, 2009), belonging to the START protein superfamily (Klingler *et al.*, 2010), and the downstream complex composed of PP2CA and 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 by the cytoplasm ABA receptors (PYR/PYL/RCAR), the clade A protein phosphatases type 2C (PP2CAs) as negative regulators (Gosti *et al.*, 1999; Merlot *et al.*, 2001), and a number of protein kinases, including the subclass III of the SNF1-related kinases family 2 (SnRK2), as positive regulators of the pathway (Yoshida *et al.*, 2002). The PYR/PYL/RCAR proteins contain a ligand-binding pocket into a cavity that closes after ABA binding through conformational changes of two conserved β-loops that serve as *gate* and *latch*. ABA binding to the receptors is enhanced when PYR/PYL/RCAR proteins are bound to their negative regulator PP2CAs (Park *et al.*, 2009; Ma *et al.*, 2009; Melcher *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 transcriptional and functional levels. In general, concomitant with ABA rises, positive effectors (PYR/PYL/RCAR and SnRKs) were transcriptionally repressed whereas negative regulators (PP2CAs) increased, modulating together downstream signalling and, consequently, physiological ABA responses in model and crop plants (Huai *et al.*, 2008; Park *et al.*, 2009; Umezawa *et al.*, 2009; Santiago *et al.*, 2009b; 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 analyzing 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 get 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 'Navelate' orange and its ABA-deficient mutant 'Pinalate' fruits 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' (*Citrus sinensis* L. Osbeck) orange and its spontaneous ABA-deficient mutant 'Pinalate' were randomly harvested at 6 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 attending to 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 the same day and their colour was measured (Table S1) 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 zero value corresponds to yellow fruit at the midpoint of colour break period. Flavedo (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 ºC for later analysis. Three biological replicates of 5 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 ºC. Control non-stressed leaves were kept in the chambers at 90% 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 ºC until analysis.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from frozen flavedo and leaf samples by a modified method of 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 Ribonuclease-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 polymerase chain reaction analysis (qRT-PCR) was performed as described previously by Romero *et al*. (2012) to examine time-course gene expression patterns along 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 presence of Oligo(dT) 20-mer (Invitrogen) and Ribonuclease Inhibitor (Invitrogen) according to 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 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 the amplicon size for each gene are shown in Table S2. 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. Reference genes *CsACT, CsGAPDH* and *CsTUB* (Table S2), whose constitutive expression along fruit ripening 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 ΔΔCt 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 target and housekeeping genes were 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 FH 'Navelate' leaves. In addition, in order to compare absolute gene expression values, amplicons of each gene were cloned in pGEMT vector (Promega) and used to generate standard curves by serial dilutions. Data were then normalized by using the housekeeping genes previously mentioned. Three biological samples for each sampling period, tissue and variety were analyzed in duplicate and mean ratios were calculated.

Statistical design

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

ABA analysis

The ABA was extracted from 1 g fresh weight (FW) frozen flavedo and leaves with 80% acetone containing 0.5 g l^{-1} citric acid and 100 mg l^{-1} of butylated hydroxytoluene as previously described by Lafuente *et al*. (1997). After

centrifugation the supernatant was diluted in 3 serial dilutions in ice-cold TBS (6.05 g Tris l^{-1}, 8.8 g l^{-1} NaCl and 0.2 mg l^{-1} $MgCl_2$ at pH 7.8) and 3 samples for each dilution were analyzed by the indirect 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 3 biological replicates of 5 fruit each ± SE.

Sequence analyses, alignment and phylogenetics

Sequence similarity comparisons between *Arabidopsis thaliana* and *Citrus sinensis* proteins were performed by BLASTP in the Phytozome v7.0 database (www.phytozome.org; www.citrusgenomedb.org). Search for amino acids sequences of *Arabidopsis* PYR/PYL/RCAR, PP2CA and SnRK2 proteins were carried out using the National Centre for Biotechnology Information (NCBI). Motive prediction was performed using whole protein sequences as input into the PSIPRED secondary structure prediction server. Tertiary structure of CsPYR1, CsPYL2, CsPYL5, CsPYL8, CsABI1 and CsSnRK2.6 proteins were modelled by using I-Tasser program (Roy *et al.*, 2010), in which their corresponding *Arabidopsis* homologous crystallographic structures from 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 neighbour-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 *Arabidopsis thaliana* were used as query to identify the orthologous proteins from *Citrus sinensis*. In the orange genome, only 6 proteins with homology to the 14 *Arabidopsis* PYR/PYL/RCAR proteins were found (Table 1). The genes *AtPYR1* and *AtPYL1* shared homology to the same orange locus (*orange1.1g046151m*) and showed a 74 and 73% of identity, respectively, in 175 amino acid residues, which correspond to the 84% of the protein length. Likewise, *AtPYL2* and *AtPYL3* shared homology to the *orange1.1g046697m* locus and showed a 72 and 66% of identity, covering the 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 *orange1.1g026007m* and showed a 78 and 62% identity, respectively. The *orange1.1g038201m* locus was the most similar to the *AtPYL5*, *AtPYL11*, *AtPYL12* and *AtPYL13* genes and displayed a 70, 62, 62 and 56% identity at the protein level, respectively. Genes *AtPYL8* and *AtPYL10* showed homology to *orange1.1g028067m* (75 and 74% identity in 183 and 158 amino acid residues, respectively) and *AtPYL7* and *AtPYL9* to *orange1.1g043944m* (79 and 86% identity, respectively). Attending to 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 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 the *Arabidopsis* homologous genes (Table 1).

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

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

In order to assess the conservation degree 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 (Fig. S1A). The *latch* and *gate* loops of *Citrus* proteins were identical to those described in *Arabidopsis* and the functional sites for ABA-binding and PP2Cs interaction were also perfectly conserved in all *Citrus* proteins. No important differences between *Citrus* ABA-binding regions and those of the *Arabidopsis* were found, with the exception of an insert of 17 amino acids inside the ABA-binding region 2 of 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* homologous. High similarity in the number and location of α-helices and strands conforming β-sheets was also found between all *Citrus* PYR/PYL/RCAR proteins and their *Arabidopsis* homologous (data not shown). In addition, predicted tertiary structure of CsPYR1, CsPYL2, CsPYL5 and CsPYL8 showed that 2 helical segments and 7 strands forming a β-sheet conformed a cavity for ligand binding highly similar to that found in their respective *Arabidopsis* homologous (Fig. S2A-D). Phylogenetic analysis further showed that the *Citrus* ABA receptors were distributed along the three main subfamilies proposed by Ma *et al*. (2009) in *Arabidopsis*, and 2 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, similarity matrix of deduced amino acid sequences confirmed that proteins clustered into the same subfamily shared the highest percentage of similarity among *Citrus* proteins (Table S3).

Family of clade-A PP2C proteins in Citrus sinensis

In the *Citrus sinensis* genome 5 proteins were identified with significant homology to the 9 members of the clade-A PP2C family of *Arabidopsis* (Table 1). The *Citrus* gene *orange1.1g008880m* 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* (*orange1.1g013591m*) and *CsAHG3* (*orange1.1g015135m*), 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 (*orange1.1g009083m*) and showed very similar percentage of identity (57 and 55%, respectively); therefore the *Citrus* gene was named *CsHAB1*.

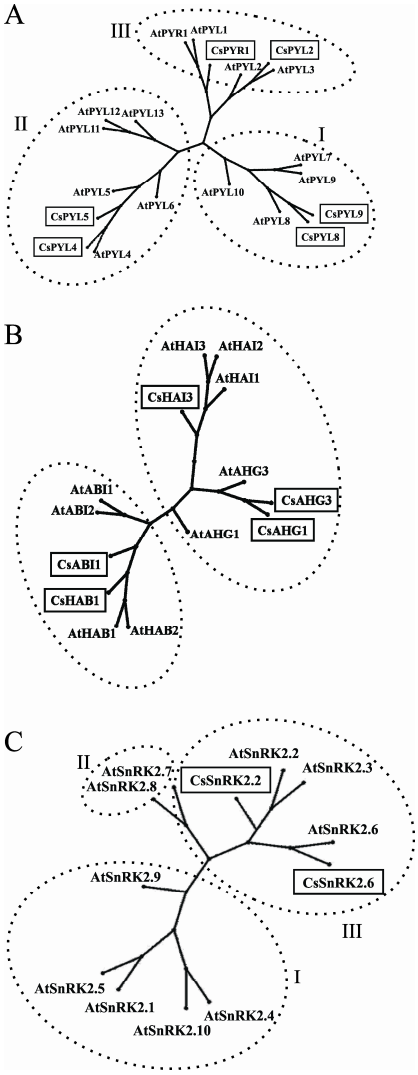

Figure 1. Unrooted phylogenetic trees containing *Citrus sinensis* and *Arabidopsis thaliana* PYR/PYL/RCAR ABA receptors (A), clade-A PP2Cs (B) and SnRK2s protein kinases (C) obtained by using the neighbour-joining method in PhyloWidget software and based on the protein sequence alignments. The full name for each protein is detailed in Table 1.

Likewise, the 3 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 a 62, 57 and 64% of identity, respectively, to the protein encoded by *orange1.1g036852m*, 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 to the protein alignments, the PP2C-like domain was highly conserved through all proteins sequences although the length of all *Citrus* PP2CAs proteins was longer than the *Arabidopsis* homologous (Fig. S1B). Metal binding sites described in *Arabidopsis* were also identified in *Citrus* proteins and phosphatase activity regulatory sequences were identical for all proteins analyzed. The predicted secondary structure of AtABI1 matched with 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 compared each protein with its *Arabidopsis* homologous (data not shown). Predicted tertiary structure of CsABI1 was performed by using the crystallographic structure of AtABI1 as template, and revealed a high similarity degree in protein folding between species (Fig. S2E). In addition, the phylogenetic tree performed 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 percentages of similarity among *Citrus* proteins sequences were found between CsABI1 and CsHAB1, and among CsAHG1, CsAHG3 and CsHAI3 proteins (Table S4). Furthermore, representative genes of each group were identified and, as expected, each *Citrus* protein was clustered nearby to its corresponding *Arabidopsis* homologous.

The ABA-related subclass III SnRK2 proteins in Citrus

Among sucrose non-fermenting related protein kinases 2 (SnRK2s) of *Arabidopsis,* the proteins belonging to the subclass III, SnRK2.2, SnRK2.3 and SnRK2.6, have been related to ABA signalling. A BLAST search in *Citrus sinensis* genome revealed that 2 different loci (*orange1.1g017860m* and *orange1.1g017936m*) shared homology with these ABA-related SnRK2s (Table 1). The protein encoded by the gene *orange1.1g017936m* showed its highest identity (90%) to AtSnRK2.6, whereas *orange1.1g017860m* protein showed 82% of 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* homologous. Amino acid alignment of the *Arabidopsis* subclass III SnRK2s and their corresponding *Citrus* homologous showed a highly conserved kinase domain between both species (Fig. S1C). Furthermore, ATP binding and the activation loop regions as well as the ATP binding and the proton acceptor active sites were identical. By 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 (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 homologous (data not shown). Likewise, tertiary structure of CsSnRK2.6 was predicted by using crystallographic structure of AtSnRK2.6 as template, and protein folding was highly conserved between species (Fig. S2F). Phylogenetic analysis further revealed that *Arabidopsis* ABA-related SnRK2s proteins (AtSnRK2.2, AtSnRK2.3 and AtSnRK2.6) grouped in an independent branch (subclass III) to the other proteins belonging to this family, and the *Citrus*

homologous (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%) of similarity when their sequences were compared (Table S5).

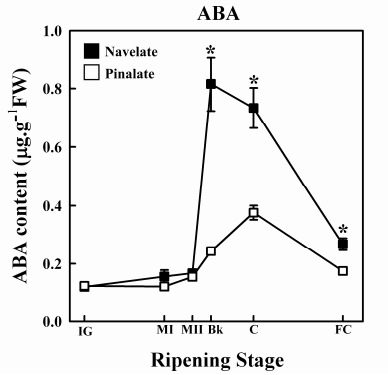

Figure 2. ABA content in the flavedo of 'Navelate' (black) and 'Pinalate' (white) fruit during development and ripening (Immature Green, IG; Mature Green I, MI; Mature Green II, MII; Breaker, Bk; Coloured, C; Full Coloured, FC). Results are the means of three biological replicates of 5 fruits each ± 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 6 *Citrus PYR/PYL/RCAR*, 5 clade-A *PP2C* and 2 *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) while 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 Bk stage and the ABA content in the mutant was more than 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 only remarkable differences were 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 Bk and C stages, and increased again at FC stage to levels similar to IG. In the mutant fruit, the maximum expression levels of the *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 parental at intermediate ripening fruit stages (MI, MII, Bk and C) and showed a minimum at 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 stage in 'Navelate' and 'Pinalate' fruit, decreased dramatically to minimum levels at Bk and C stages, respectively, and then increased again to higher levels at FC stage. It should be mentioned that the repression of *CsPYL4* at Bk was 2-fold higher in the ABA-deficient mutant.

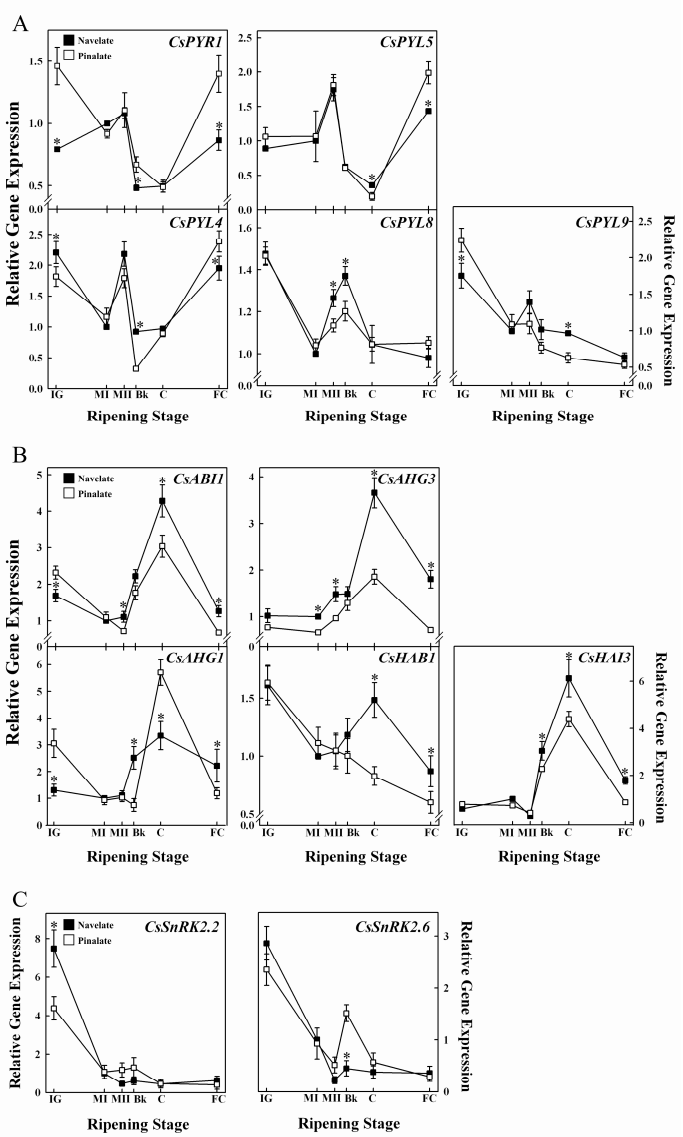

Figure 3. Relative gene expression analysis by qRT-PCR of *Citrus PYR/PYL/RCAR* ABA receptors (A), clade-A PP2Cs (B) and subclass III SnRK2s (C) in 'Navelate' (black) and 'Pinalate' (white) fruits during fruit development and ripening (Immature Green, IG; Mature Green I, MI; Mature Green II, MII; Breaker, Bk; Coloured, C; Full Coloured, FC). Expression values are relative to transcript levels obtained in MI 'Navelate' fruits. Values are mean ratios ± SE from three biological samples for each sampling period and variety analyzed 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.

Overall, in spite of the differences observed between varieties, *CsPYR1*, *CsPYL4* and *CsPYL5* genes showed a consistent pattern in which the minimum transcript levels were coincident with the highest ABA levels. On the other hand, *CsPYL8* and *CsPYL9* genes 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 FC stage (Fig 3A). Moreover, absolute gene expression analysis further revealed similar levels of *CsPYL4* and *CsPYL5* transcripts, whereas the most expressed gene during fruit development and ripening was *CsPYL9*. It is also interesting that expression of *CsPYR1* and *CsPYL8* genes remained at very low levels and *CsPYL2* was not detected in any of the fruit samples analyzed (Table S6).

The analysis of the *Citrus* clade-A *PP2C* genes revealed a differential regulation between both varieties. Although *CsABI1, CsAHG3* and *CsHAI3* transcripts accumulation followed a similar pattern peaking at 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*s 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' displayed a transient increase at C stage. In general, the highest expression levels of the *Citrus PP2CA* genes were observed at the C stage (Fig. 3B and Table S6), concurring 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, transcript levels of *CsAHG3* at C stage almost

doubled that of *CsHAB1* and *CsHAI3*, and showed more than 14-fold accumulation than the other genes of this family (Table S6).

Transcriptional analysis of *Citrus SnRK2*s 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* (Table S6).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 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 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 about 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 rise in weight loss preceded that of ABA and significant increases in ABA in response to dehydration only occurred when the leaves reached a 10 % of weight loss (Fig. 4).

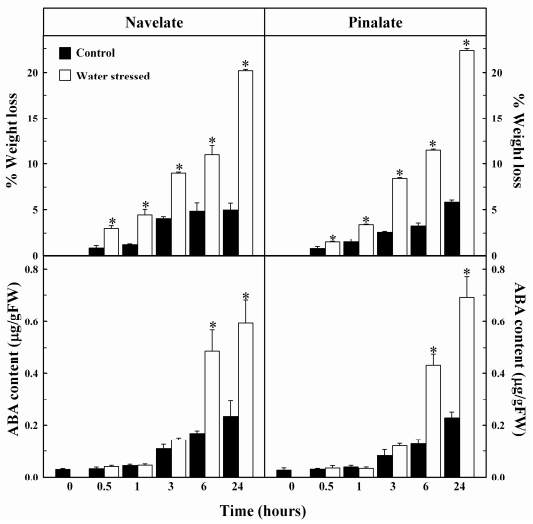

Figure 4. Effect of water stress on weight loss and ABA content in 'Navelate' and 'Pinalate' detached leaves. Changes in control samples are represented as black bars and in water-stressed leaves as white bars. Results are the means of three biological replicates of 4 leaves each ± SE. Significant differences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

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 a 3- and a 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). Contrary to that found in flavedo samples, *CsPYL2* expression was detected in leaves and results showed that this gene was down-regulated by water stress.

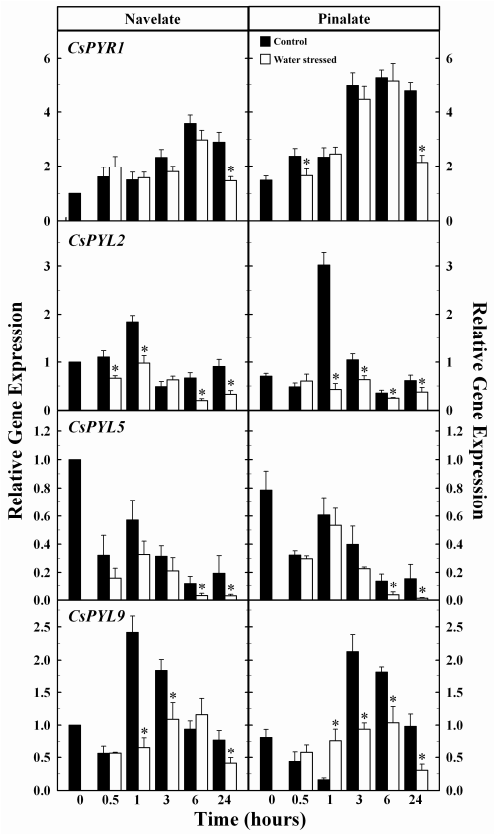

Figure 5. Relative gene expression analysis by qRT-PCR of *Citrus PYR/PYL/RCAR* ABA receptors in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. Results are the means of three biological replicates of 4 leaves each ± SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analyzed period are indicated by an asterisk.

The *CsPYL5* relative gene expression decreased rapidly (0.5 h) after leaf detachment and significant differences between control and water-stressed leaves were only found by 6 and 24 h. On the other hand, *CsPYL9* gene expression sharply increased and reached a maximum by 1 and 3 h in control 'Navelate' and 'Pinalate' leaves, respectively. The transcripts 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 (Table S6).

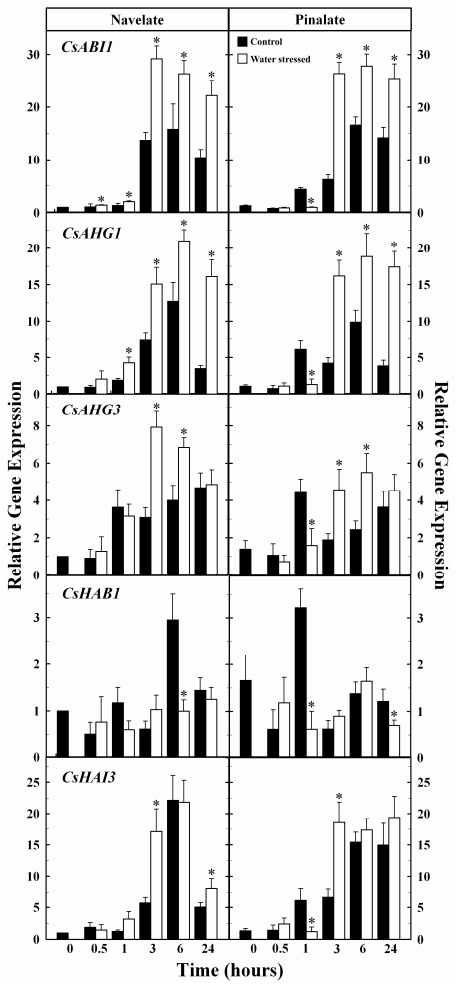

Figure 6. Relative gene expression analysis by qRT-PCR of *Citrus* clade-A PP2Cs in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. Results are the means of three biological replicates of 4 leaves each ± SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analyzed period are indicated by an asterisk.

The expression of the *CsPP2CA*s 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 *CsABI1* 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 and 6 h after detachment. Contrary, dehydration had little effect on *CsHAB1* and *CsHAI3* transcript levels. Absolute gene expression analysis revealed that *CsHAB1* was the most expressed *CsPP2CA* in FH leaves followed by *CsAHG3*, *CsHAI3* and *CsABI1*. In contrast, the most expressed genes during dehydration of 'Navelate' and 'Pinalate' leaves were *CsAHG3*, *CsHAI3* and *CsABI1* (Table S6). 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). 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 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 *CsSnRK2.2* gene along the whole experiment in both varieties (Table S6).

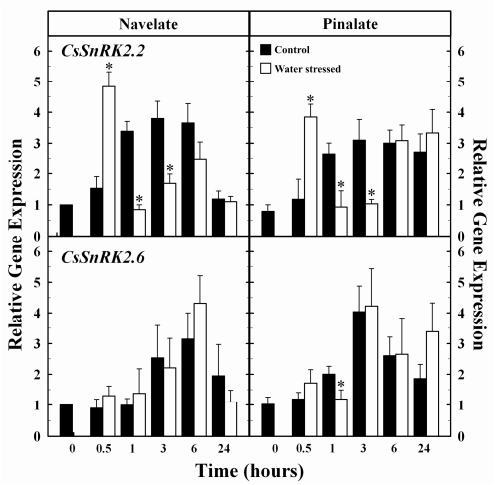

Figure 7. Relative gene expression analysis by qRT-PCR of *Citrus* subclass III SnRK2s in control (black) and water-stressed (white) 'Navelate' and 'Pinalate' leaves. Results are the means of three biological replicates of 4 leaves each ± SE. Significant differences ($P \leq 0.05$) in gene expression between samples for the same analyzed period are indicated by an asterisk.

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 existing 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 like 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 sweet orange (*Citrus sinensis*) genome database has revealed that proteins belonging to the ABA-signalosome were less represented in *Citrus* as compared to *Arabidopsis*. Only 6 *PYR/PYL/RCAR*, 5 *PP2CA* and 2 subclass III *SnRK2* genes were found in *Citrus* (Table 1) while in *Arabidopsis* it is composed by 14 PYR/PYL/RCAR ABA receptors, 9 clade-A PP2Cs and several protein kinases, including 3 of the subclass III SnRK2 (Merlot *et al.*, 2001; Yoshida *et al.*, 2002; Yoshida *et al.*, 2006a; Park *et al.*, 2009; Ma *et al.*, 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 percentages of identity were observed between *Citrus* proteins and their homologous in *Arabidopsis*, as well as similar protein length and genetic structures (Table 1 and Table S3-S5). Interestingly, the consensus motifs for functional protein folding, such as *gate* and *latch* regions in PYR/PYL/RCARs (Melcher *et al.*, 2009) (Fig. S1A

and Fig. S2), and for phosphatase activity in PP2CAs (Weiner *et al.*, 2010) (sequences underlined in Fig. S1B) were identified in *Citrus*. D-rich C-terminal domain II, which has been shown to be essential for ABA signal transduction (Yoshida *et al.*, 2006a), was also fully conserved in *Citrus* SnRK2s proteins (number 4 in Fig. S1C). Phylogenetic analysis revealed that *Citrus* PYR/PYL/RCAR were clustered together with their homologous accordingly with the distribution proposed by Ma *et al.* (2009), in which *Arabidopsis* ABA receptors were divided in 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 homologous (Fig. 1B) and arranged in two separated 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 the 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/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 that no important differences in most of the *CsPYR/PYL/RCAR* expression profiles, although 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 transcript levels of these genes were concomitant with the highest ABA levels in 'Navelate'

and 'Pinalate' fruits, whereas their expressions peaked before the ABA rise (Fig. 2 and Fig. 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 receptors gene expression may be concomitant with the rise in ABA during non-climacteric fruit ripening. In this context, it should be pointed out that gene expression of *PYR/PYL/RCAR*s 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, 3 different expression patterns were observed among *PYR/PYL/RCAR*s: 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 (Table S6). Secondly, *CsPYL8* transcript level peaked when ABA rose during fruit ripening and, finally, *CsPYL9* continuously decreased as ripening progressed, although it slightly increased 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 the subfamily I (Fig. 1A). In this context, it is interesting to note that tomato genes belonging to this subfamily (*SlPYL1*, *SlPYL2* and *SlPYL3*) 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 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 (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 (Table S6). It is also interesting that the expression of *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, some tomato genes such as *SlPYL5*, belonging to the same subfamily that *CsPYL2* (subfamily III, Fig. 1A), were almost undetectable during fruit ripening (Sun *et al.*, 2011). Therefore, gene expression levels pointed out the relevance of *CsPYL4*, *CsPYL5* and *CsPYL9* genes in the ABA perception during fruit development and ripening.

Clade-A PP2Cs function as negative regulators of the ABA signalling pathway and their expressions are highly induced by ABA in plants (Merlot *et al.*, 2001; Saez *et al.*, 2004; Yoshida *et al.*, 2006b; 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; Vlad *et al.*, 2009; Santiago *et al.*, 2009b; 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 re-setting of the ABA transduction pathway offers a dynamic mechanism to modulate the ABA response (Santiago *et al.*, 2009b). The expression pattern of the *CsPP2CA*s analyzed in this work mostly paralleled the ABA accumulation in 'Navelate' and 'Pinalate' fruit during ripening (Fig. 2 and Fig.3B) and, interestingly, the *CsPP2CA*s up-regulation 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 *SlPP2C1* and *SlPP2C5* transcripts peaked with the increment in ABA occurring during fruit ripening, while any of the *SlPYR/PYL/RCAR* genes analyzed were negatively related to the accumulation of those *SlPP2C*s (Sun *et al.*, 2011). Therefore, it would be interesting to further investigate 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 analyzing the relationship between the expression of the *CsPP2CA*s and the endogenous ABA accumulation along *Citrus* fruit ripening. Gene expression levels of *CsABI1*, *CsAHG3* and *CsHAI3* peaked at 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 C stage, although 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 and Table S6), suggest an important effect of ABA content on the *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*s were more responsive to ABA compared to ABA receptors and suggested a higher sensitivity of these negative regulators to ABA changes. Unexpectedly, although *CsAHG1* showed an expression pattern similar to the other *CsPP2CA*s, the transcript level at C stage was two-fold higher in the ABA-deficient mutant whereas the ABA content in 'Navelate' doubled that of 'Pinalate' (Fig. 2 and Fig. 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 (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). 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 (Fig. 2 and Fig. 3C), although transcript accumulation of *CsSnRK2.6* gene was much higher than that of *CsSnRK2.2* in both varieties along fruit ripening (Table S6). As ripening progressed, however, *CsSnRK2.2* remained almost unchanged in 'Navelate' and 'Pinalate' fruits whereas *CsSnRK2.6* showed a transient increase at Bk stage only in the mutant fruit. Similar expression patterns were found in the climacteric tomato fruit. During ripening of tomato, SnRKs expression levels were high in the most immature stages and transiently increased with the rise in ABA (Sun *et al.*, 2011). In spite of differences found in *CsSnRK2.6* transcript levels between 'Navelate' and 'Pinalate' fruits, 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*s.

In order to get further insights on the role of the ABA-signalosome components and to understand whether the key genes are common or tissue-specific in *Citrus*, the expression analysis of these elements has been also performed in leaves exposed to dehydration. As indicated above, the deficiency in ABA of 'Pinalate' is fruit-specific and, consequently, we did not find relevant

differences in ABA content or weight loss between 'Navelate' and 'Pinalate' leaves. Since leaves are very prone to dehydration, special attention was paid to minimize water loss in control leaves. Under the experimental conditions used, water loss was always lower than 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 (Fig. 5, 6 and 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 (Table S6). Therefore, results obtained in 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 bottomed down when 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 of this gene in leaves (Table S6). Moreover, the expression profile of these genes did not mirror that of

ABA accumulation during leaves dehydration as occurred during fruit ripening. This differential regulation under physiological or stress conditions may provide a mean 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*s 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 as compared to the other *CsPYR/PYL/RCAR* genes and *CsPYL9* was highly expressed. Comparison between fruit and vegetative tissue has also revealed some tissue specificity: *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 6 PYR/PYL/RCAR ABA receptors, 5 PP2CAs and 2 subclass III SnRK2s. During sweet orange fruit development and ripening the expression pattern of some ABA receptors mirrored ABA content whereas *CsPP2CA*s paralleled the hormone accumulation, modulating together 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*s, negative regulators of the ABA signal transduction pathway, was much higher than in other components of the ABA-signalosome.

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SUPPLEMENTARY MATERIAL

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

		a/b ratio	
	Month	**Navelate**	**Pinalate**
Immature green (IG)	July	-0.89 ± 0.01	-0.89 ± 0.01
Mature green I (MI)	September	-0.72 ± 0.01	-0.72 ± 0.01
Mature green II (MII)	October	-0.41 ± 0.01	-0.50 ± 0.01
Breaker (Bk)	October	-0.13 ± 0.01	-0.31 ± 0.01
Coloured (C)	November	0.49 ± 0.01	0.10 ± 0.01
Full coloured (FC)	February	0.63 ± 0.01	0.12 ± 0.01

Values are mean of three replicates ± SD.

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

Citrus gene	Homologous in *A. thaliana*	Forward / Reverse	Sequence 5' → 3'	Amplicon size (bp)
CsPYR1	AT4G17870; Pyrabactin Resistance 1	F R	CCGGGGTAACTCAGGACGAG CGGAGACGGAACAGCTCTTG	198
CsPYL2	AT2G26040; PYR1-Like protein 2	F R	GGCCTATCATCGAAAGATACC CGATACAACCGTCACTTCTC	209
CsPYL4	AT2G38310; PYR1-Like protein 4	F R	CGAACGAAGACACCTGCGTG CGAGAACAGAACATGACCTG	168
CsPYL5	AT5G05440; PYR1-Like protein 5	F R	GCCCGGCGGTACATCACAAA GCCCGCAGCTAACAGCACG	184
CsPYL8	AT4G27920; PYR1-Like protein 8	F R	GCGGTGCATTTTGGACGCTTC GGCAAAGCCTTAGCAGAATCG	111
CsPYL9	AT1G01360; PYR1-Like protein 9	F R	CGGAGATCATCGACGGGAGA CGGTCCTGCACCGCCATCC	166
CsABI1	AT4G26080; ABA-insensitive 1	F R	GGTGACTGCAAAAGCACGAT GGGGCAACAGGTTCACCTCC	129
CsAHG1	AT5G51760; ABA-hypersensitive germination 1	F R	CGGGTAATGCGAATGCGGG CCCAACGCTCCCACACGCGCACG	170
CsAHG3	AT3G11410; ABA-hypersensitive germination 3	F R	GGGATGACTTCAGTTTGCGGTA CGGAGCTCTTCACTTTAATGGC	227
CsHAB1	AT1G72770; Hypersensitive to ABA 1	F R	CCGGAGTGTCTTCGAGGTGG GGCCATTCAAACAGTGGCTC	173
CsHAI3	AT5G59220; Highly ABA-induced 3	F R	GCCCCTGGCGGCCACTCC GGCCCACACATATAGAAACC	245
CsSnRK2.2	AT3G50500; SNF1-related protein kinase 2.2	F R	CTGTTCCAGACACTAATCCA GGCTACTCATAGTCTTTTCATCC	165
CsSnRK2.6	AT4G33950; SNF1-related protein kinase 2.6	F R	GAGCCAAAGAACTTCCGCAA GGGTTTCGGATCTCGGGTAT	139
CsALDH3	AT1G44170; Aldehyde Dehydrogenase 3 H1	F R	GGAGTGAGGAGGAGACGAAGA ACGAAGAGCGTCGACGATGTC	188
CsHVA22E	AT5G50720; Abscisic acid induced-like protein	F R	GCGGCATGGCTGGTTCTGC GCCTCGTGCTCCCCTTTCTT	178
CsACT	AT5G09810; Actin7. Structural constituent	F R	TTAACCCCAAGGCCAACAGA TCCCTCATAGATTGGTACAGTATGAGA	176
CsGAPDH	AT1G13440; Glyceraldehyde-3P dehydrogenase	F R	CGTCCCTCTGCAAGATGACTCT GGAAGGTCAAGATCGGAATCAA	204
CsTUB	AT1G75780; Tubulin1. Structural constituent	F R	GCATCTTGAACCCGGTAC ATCAATTCGGCGCCTTCAG	158

Table S3. Similarity matrix between *Citrus* and *Arabidopsis* PYR/PYL/RCAR proteins based on deduced amino acid and sequences alignment.

	AtPYR1	AtPYL1	AtPYL2	AtPYL3	AtPYL4	AtPYL5	AtPYL6	AtPYL7	AtPYL8	AtPYL9	AtPYL10	AtPYL11	AtPYL12	AtPYL13	CsPYR1	CsPYL2	CsPYL4	CsPYL5
AtPYR1	100																	
AtPYL1	77.50	100																
AtPYL2	39.00	35.80	100															
AtPYL3	35.20	31.70	56.50	100														
AtPYL4	51.40	44.00	39.20	31.10	100													
AtPYL5	39.70	33.20	36.40	28.70	45.40	100												
AtPYL6	46.90	42.00	37.60	27.00	57.20	40.90	100											
AtPYL7	31.10	30.70	27.80	27.20	31.70	31.20	29.80	100										
AtPYL8	42.10	41.00	31.70	30.20	48.40	37.10	41.70	41.80	100									
AtPYL9	30.00	28.90	31.30	28.70	32.00	32.70	30.10	81.30	44.00	100								
AtPYL10	40.80	39.60	32.60	27.70	47.70	34.70	42.90	39.80	70.30	42.00	100							
AtPYL11	36.40	36.50	38.80	30.10	39.50	38.50	39.70	36.30	33.60	36.30	35.30	100						
AtPYL12	36.80	34.40	37.30	29.20	37.70	39.20	38.70	34.50	32.50	34.50	32.90	81.10	100					
AtPYL13	36.20	32.50	32.70	27.80	34.00	33.60	36.40	34.70	29.30	34.70	32.50	63.10	66.00	100				
CsPYR1	73.40	65.20	37.00	34.00	48.20	33.50	43.50	32.60	43.90	33.50	43.40	37.50	36.20	33.80	100			
CsPYL2	43.00	38.50	66.70	63.60	41.30	39.40	36.10	32.00	37.80	33.00	34.30	39.70	39.20	36.10	43.30	100		
CsPYL4	50.80	45.60	36.80	29.10	68.30	46.00	56.70	29.40	47.80	31.90	43.30	41.10	39.30	36.90	46.60	39.30	100	
CsPYL5	52.10	46.90	41.30	34.40	65.50	46.20	55.20	36.00	45.50	36.20	44.80	43.60	40.00	37.60	47.40	46.40	68.70	100
CsPYL8	29.00	28.80	30.10	26.50	31.70	29.70	28.30	37.30	52.70	45.20	48.50	34.20	33.10	32.20	29.90	32.10	29.70	32.40
CsPYL9	32.00	32.90	35.20	31.70	38.40	33.30	32.30	45.20	50.30	52.40	47.90	37.90	35.30	34.10	37.10	38.40	32.80	37.00

Table S4. Similarity matrix between *Citrus* and *Arabidopsis* clade-A PP2C proteins based on deduced amino acid and sequences alignment

	AtABI1	AtABI2	AtAHG1	AtAHG3	AtHAB1	AtHAB2	AtHAI1	AtHAI2	AtHAI3	CsABI1	CsAHG1	CsAHG3	CsHAB1	CsHAI3
AtABI1	100													
AtABI2	79.30	100												
AtAHG1	39.00	37.60	100											
AtAHG3	44.30	47.80	43.30	100										
AtHAB1	45.50	52.20	36.70	36.80	100									
AtHAB2	51.40	57.30	39.50	41.40	71.30	100								
AtHAI1	40.50	45.10	39.60	55.70	37.10	39.70	100							
AtHAI2	42.00	44.50	38.60	55.30	37.80	39.70	59.10	100						
AtHAI3	46.20	46.30	43.10	59.80	40.40	42.40	66.60	66.50	100					
CsABI1	54.20	58.70	39.50	40.80	43.70	50.40	40.40	39.80	42.70	100				
CsAHG1	40.80	45.30	41.90	65.20	36.20	40.60	52.60	53.40	61.40	40.80	100			
CsAHG3	40.50	45.30	41.80	65.30	36.60	41.10	52.80	53.40	61.40	41.00	97.80	100		
CsHAB1	53.90	58.20	40.70	38.80	55.10	58.50	40.20	37.70	42.90	61.00	39.50	39.90	100	
CsHAI3	42.50	42.40	37.90	60.10	41.10	40.90	63.60	55.20	64.70	40.70	57.70	57.70	40.80	100

Table S5. Similarity matrix between *Citrus* and *Arabidopsis* subclass III SnRK2 proteins based on deduced amino acid and sequences alignment.

	AtSnRK2.2	AtSnRK2.3	AtSnRK2.6	CsSnRK2.2	CsSnRK2.6
AtSnRK2.2	100				
AtSnRK2.3	88.40	100			
AtSnRK2.6	74.40	75.50	100		
CsSnRK2.2	81.50	79.40	78.10	100	
CsSnRK2.6	74.70	85.30	88.90	82.00	100

Table S6. Absolute gene expression analysis by qRT-PCR of the ABA-signalosome components in 'Navelate' and 'Pinalate' varieties during fruit ripening and leaf dehydration. Values correspond to number of copies of each gene per µg of total RNA. Values are means of three biological replicates for each sampling period and variety.

			PYR/PYL/RCAR						PP2CA					SnRK2	
		Ripening Stage	PYR1	PYL2	PYL4	PYL5	PYL8	PYL9	ABI1	AHG1	AHG3	HAB1	HAI3	SnRK2.2	SnRK2.6
Fruit	Navelate	IG	9	n.d.	1566	1101	24	12757	692	267	3976	8714	806	16256	139325
		M1	11	n.d.	710	1239	16	7274	410	205	3897	5415	1385	2172	48701
		M2	12	n.d.	1548	2167	20	10136	452	227	5780	5649	386	993	10790
		BK	5	n.d.	656	772	22	7393	910	515	5805	6409	4217	1329	21883
		C	5	n.d.	691	440	17	7035	1755	685	14247	8034	8474	975	18380
		FC	10	n.d.	1386	1777	16	4633	517	455	7029	4707	2473	1362	17485
	Pinalate	IG	16	n.d.	1289	1313	23	16294	951	627	3005	8842	1081	9523	114626
		M1	10	n.d.	827	1323	17	7921	448	190	2590	6018	1000	2338	45145
		M2	12	n.d.	1271	2240	18	7959	293	211	3756	5669	570	2553	25048
		BK	7	n.d.	228	754	19	5599	724	153	5041	5424	3150	2823	73697
		C	5	n.d.	636	236	17	4575	1246	1170	7237	4482	6083	992	27681
		FC	15	n.d.	1694	2461	17	3890	278	243	2784	3253	1191	878	13993
		Time (h)	PYR1	PYL2	PYL4	PYL5	PYL8	PYL9	ABI1	AHG1	AHG3	HAB1	HAI3	SnRK2.2	SnRK2.6
Leaf	Navelate Control	0	2	14285	n.d.	60	n.d.	9701	941	78	5792	10897	1404	2081	89249
		0.5	3	15739	n.d.	19	n.d.	5438	994	74	5303	5543	2617	3188	80093
		1	3	26296	n.d.	34	n.d.	23444	1265	147	21048	12786	1721	7058	88636
		3	5	6959	n.d.	19	n.d.	17770	12915	575	17871	6708	8004	7915	226187
		6	7	9551	n.d.	7	n.d.	9128	14860	993	23212	32122	31092	7614	280793
		24	6	12976	n.d.	12	n.d.	7536	9828	273	27177	15686	7099	2477	173883
	Navelate Dehydrated	0.5	4	9502	n.d.	10	n.d.	5471	1303	159	7421	8349	2041	10075	116749
		1	3	13953	n.d.	20	n.d.	6409	1941	333	18297	6591	4435	1767	123039
		3	4	9016	n.d.	13	n.d.	10582	27461	1177	45915	11233	24000	3520	197336
		6	6	2834	n.d.	2	n.d.	11275	24788	1636	39621	10851	30651	5186	385560
		24	3	4713	n.d.	2	n.d.	4011	20881	1257	28182	13576	11416	2296	98070
	Pinalate Control	0	3	10088	n.d.	47	n.d.	7903	1225	84	8043	18094	1889	1658	91632
		0.5	5	6931	n.d.	19	n.d.	4243	757	60	6156	6784	2008	2464	106470
		1	5	43144	n.d.	36	n.d.	1548	4148	477	25649	35083	8803	5519	178654
		3	10	14910	n.d.	24	n.d.	20635	5914	329	10905	6800	9468	6457	361168
		6	11	5060	n.d.	8	n.d.	17515	15664	772	14101	15016	21672	6257	232467
		24	10	8800	n.d.	9	n.d.	9555	13371	300	21100	13213	21028	5650	166202
	Pinalate Dehydrated	0.5	3	8659	n.d.	18	n.d.	5579	858	87	4227	12878	3402	8018	153159
		1	5	6180	n.d.	32	n.d.	7428	961	104	9189	6770	1709	1934	106777
		3	9	9079	n.d.	14	n.d.	9139	24848	1262	26478	9791	26066	2160	377740
		6	10	3609	n.d.	2	n.d.	10084	26187	1470	31848	17913	24384	6420	236768
		24	4	5351	n.d.	1	n.d.	2968	23933	1359	26266	7678	27236	6948	302733

n.d. no detected

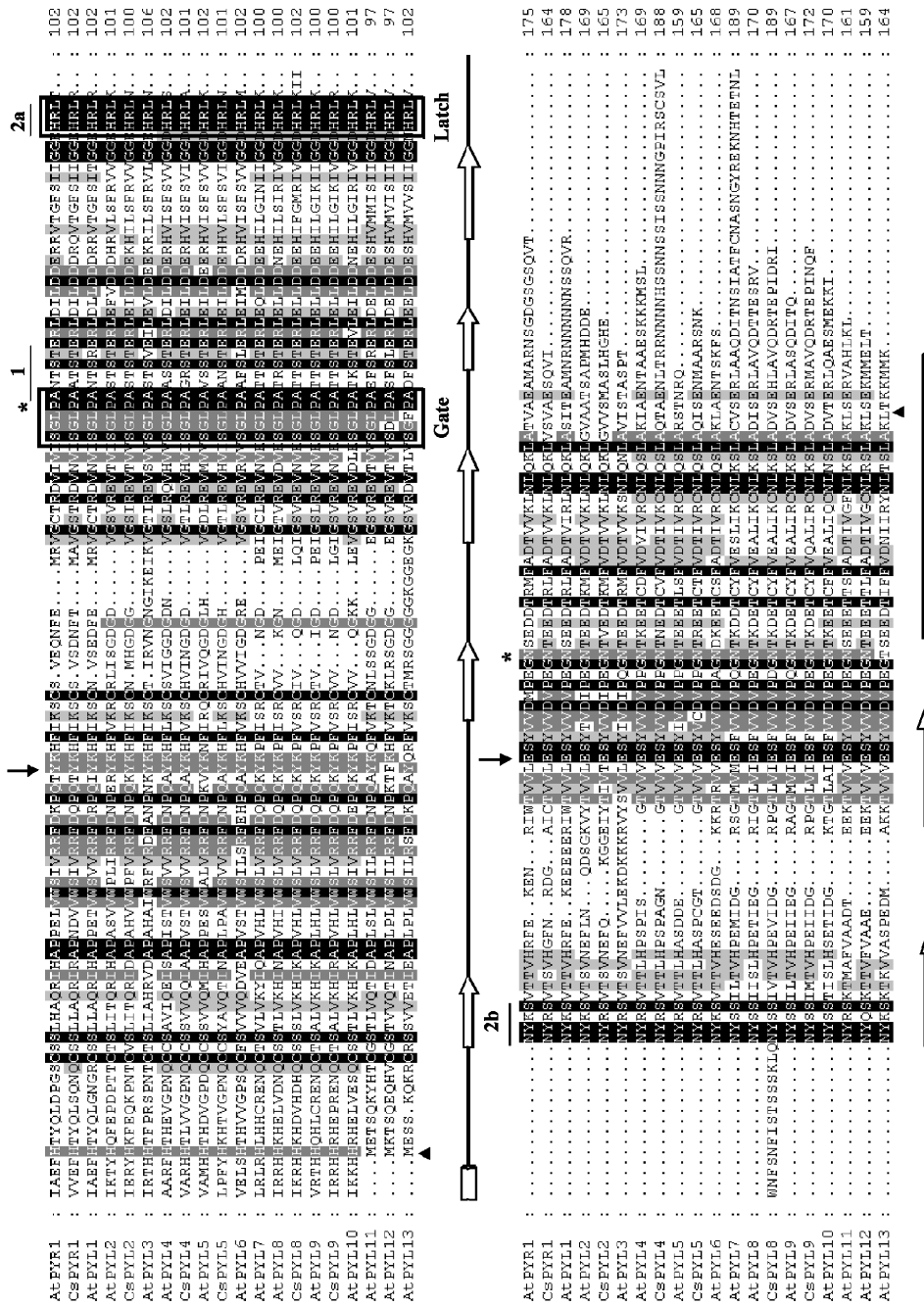

Figure S1A

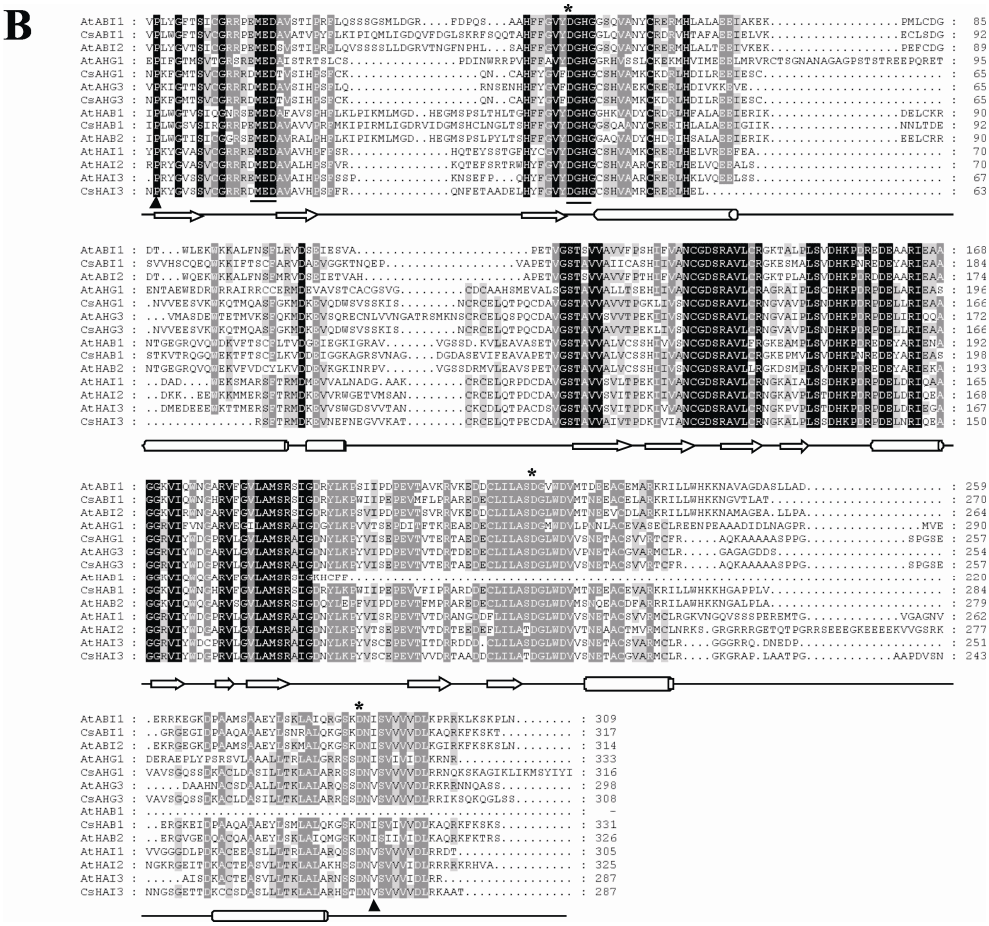

Figure S1B

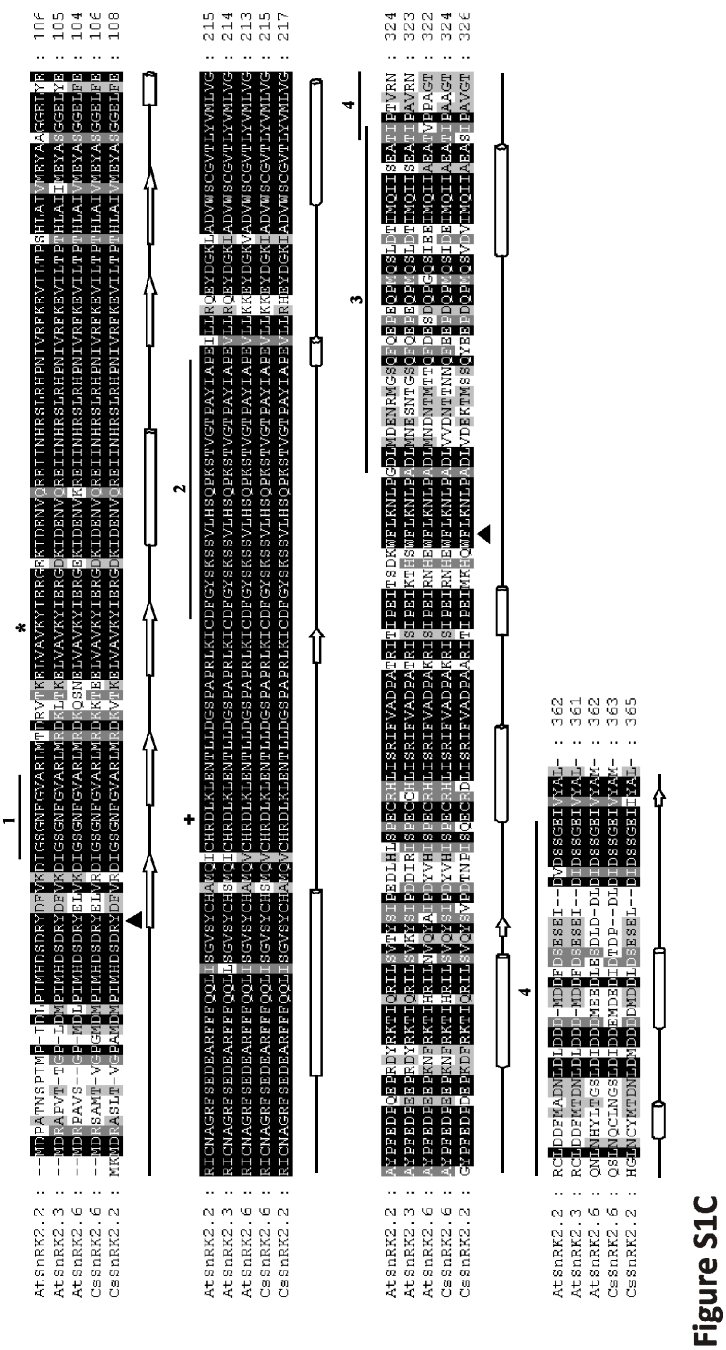

Figure S1C

Figure S1. Multiple sequence alignment of the *Arabidopsis thaliana* and *Citrus sinensis* ABA signalling core components. (A) START-like domain of the ABA-receptors, delimited by black triangles below the alignment. ABA-binding sites are indicated by arrows and PP2Cs interaction sites by asterisks. Boxes indicate *Gate* and *Latch* loops. Horizontal lines (1 and 2) above the alignment delimit the two ABA-binding regions. Line 2 is divided in 2a and 2b due to the insertion of 17 amino acid residues in CsPYL8 protein. (B) PP2C-like domain of the clade-A PP2Cs proteins, delimited by black triangles below the alignment. Metal-binding sites are pointed out by asterisks and phosphatase activity regulatory sequences are underlined. (C) Subclass III SnRK2s full protein sequences. Black triangles below the alignment delimit protein kinase domain, asterisk indicate ATP binding site and plus symbol highlights the proton acceptor active site. Horizontal lines are numbered as follows: 1, ATP binding region; 2, activation loop; 3, osmotic stress response domain I; and 4, ABA response and ABI1 binding domain II. Predicted secondary structures of AtPYR1 (A), AtABI1 (B) and AtSnRK2.6 (C) are reported below the corresponding alignment with alpha-helices as tubes, beta-strands as arrows and coiled regions as lines, as estimated by PSIPRED software. Dots indicate gaps in the amino acid sequences when compared with others for obtaining optimal alignments. Amino acids are numbered to the right of each line. Identical residues are in black, highly conservative are in dark grey and less conserved are in light grey.

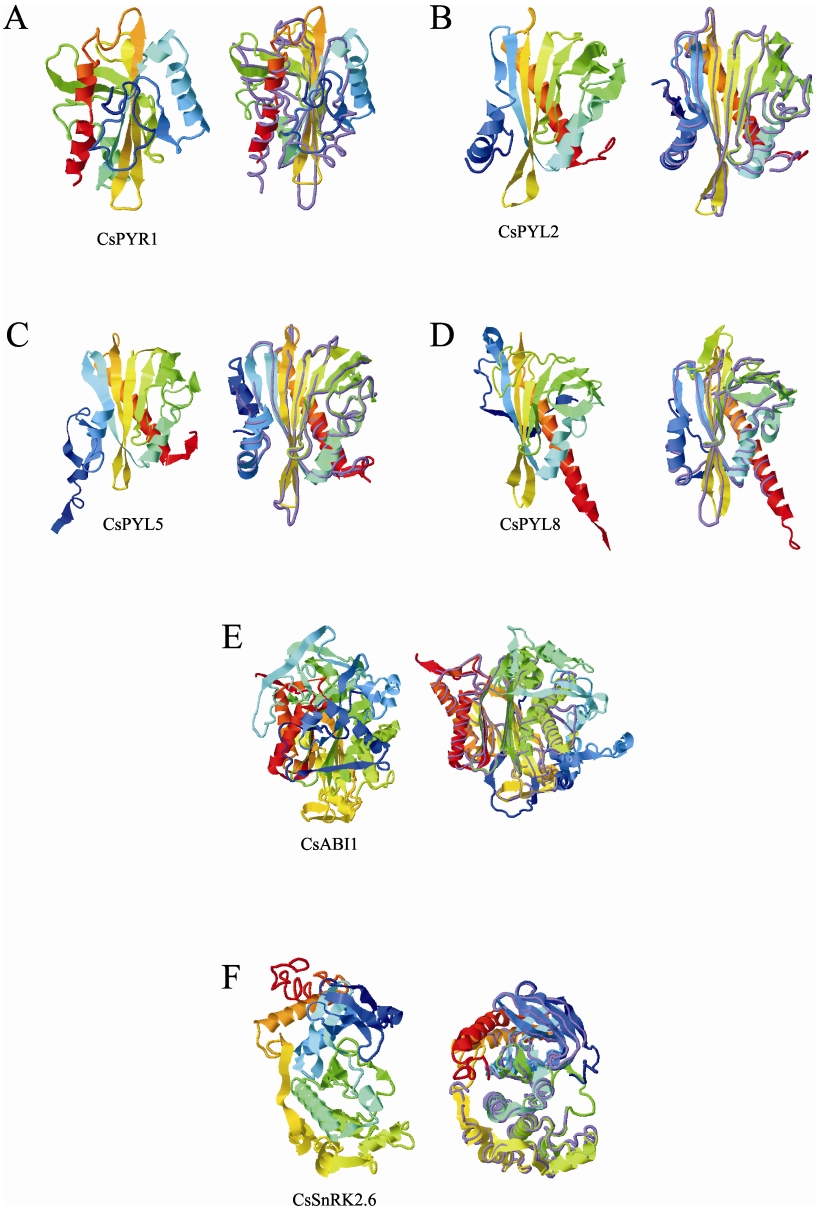

Figure S2. Predicted tertiary structure model of the ABA-signalosome components of *Citrus* by using the *Arabidopsis* available crystallographic structure of homologous proteins as templates in I-Tasser program (left). Overlay of the predicted structure with the corresponding *Arabidopsis* homologous (right). Query structures are shown in cartoon, while templates are displayed using purple trace. (A-D) PYR/PYL/RCAR ABA-receptors. (E) PP2CA. (F) SnRK2.

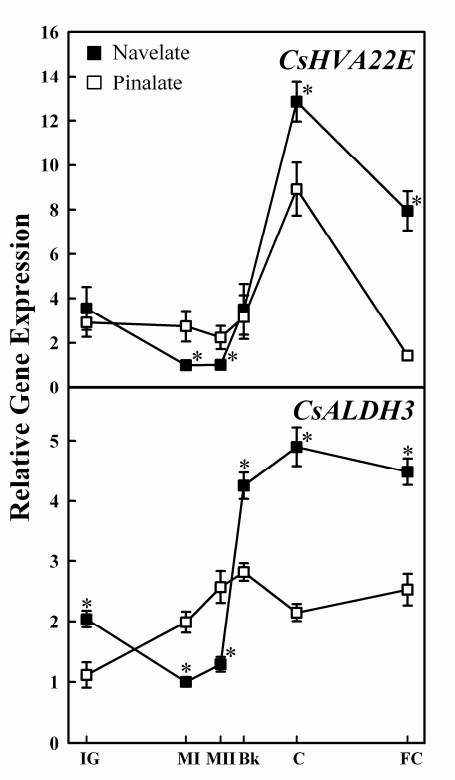

Figure S3. Gene expression analysis by RT-qPCR of *Citrus HVA22E* (upper panel) and *ALDH* (lower panel) in 'Navelate' (black) and 'Pinalate' (white) fruits during development and ripening stages (Immature Green, IG; Mature Green I, MI; Mature Green II, MII; Breaker, Bk; Coloured, C; Full Coloured, FC). Expression values are relative to transcript levels obtained in MI 'Navelate' fruits. Values are mean ratios ± SE from three biological samples for each sampling period and variety, and analyzed 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.

3.3. CHAPTER 3

Differential expression of the *Citrus sinensis* ABA perception system genes in postharvest fruit dehydration

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ABSTRACT

Water stress occurring during postharvest handling and storage is an important factor affecting external quality of fresh fruit and vegetables. The phytohormone abscisic acid (ABA) is a key regulator of the dehydration response in citrus fruit, which are prone to develop peel damage in response to water stress. To study the involvement of the ABA perception system in the dehydration response and its relationship with the occurrence of peel damage in citrus fruit, a comparative transcriptional analysis of the ABA signalling core components in water-stressed fruit of the wild-type 'Navelate' orange (*Citrus sinensis* L. Osbeck) and its spontaneous fruit-specific ABA-deficient mutant 'Pinalate', which is more susceptible to dehydration and to develop non-chilling peel pitting (NCPP), has been performed. Minor differences in ABA receptors (*CsPYR/PYL/RCAR*) and downstream protein kinases (*CsSnRK2*) transcripts levels were found in response to dehydration between wild-type and the ABA-deficient mutant fruit. Contrary, the expression of the ABA-mediated PYR/PYL/RCAR-inactivated clade A protein phosphatases 2C (*CsPP2CA*) was highly regulated by ABA content and showed significant differences between cultivars. Results obtained by ABA application suggest the involvement of these negative regulators in the impaired response of 'Pinalate' fruit to water stress. Overall, the higher responsiveness to ABA changes of the *CsPP2CA*s during citrus fruit dehydration highlights these components as key regulator control points in the response of citrus fruit to water stress.

INTRODUCTION

Water stress during postharvest is one of the most important factors reducing external quality and hence commercial value of fresh fruit. However, the study of the molecular mechanisms underlying fruit dehydration in horticultural crops has been limited to a few set of genes (Alférez et al., 2008; Bonghi et al., 2012; Loyola et al., 2012), and high throughput approaches have been only performed in grape and citrus fruit. Studies in grapes indicate that molecular responses are differently regulated by dehydration occurring before or after harvesting the fruit and also by the stress severity (Deluc et al., 2009; Rizzini et al., 2009; Bonghi et al., 2012). Transcriptomic research in citrus fruit has evidenced the relevance of the early molecular events for coping with water loss and the deleterious effects affecting external fruit quality caused by this stress (Romero et al., 2012b). In this regard, it should be mentioned that many citrus cultivars are prone to develop non-chilling peel pitting (NCPP), a physiological disorder enhanced by dehydration (Alférez et al., 2003; Alférez and Burns, 2004), and manifested as peel depressions affecting both the inner (albedo) and the outer part (flavedo) of the peel that becomes bronze as the disorder progresses (Alférez et al., 2005; Lafuente and Zacarías, 2006; Alférez et al., 2010). The tight relationship between ABA and dehydration is well established as this hormone plays a crucial role regulating stomatal closure and hence water loss (Bartels and Sunkar, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). Therefore, ABA may have an important effect on fruit such as citrus, which contain abundant stomata in their external tissues. Moreover, it has been demonstrated that ABA-independent pathways may also operate in response to dehydration in model plants (Riera et al., 2005). These observations have been extended recently to citrus fruit (Romero et al., 2012b).

Natural and induced mutants of ABA-biosynthetic and signalling transduction genes have been characterized in model and crop plants (Groot and Karssen, 1992; Schwartz et al., 1997). Since artificially generated mutants are less affordable in woody plants, the access to spontaneous hormone mutants is of particular scientific interest. Different works have attempted to unravel physiological (Alférez et al., 2005; Holland et al., 2005; Sala et al., 2005) and molecular (Sanchez-Ballesta et al., 2008; Romero et al., 2012b) mechanisms linking ABA, fruit dehydration and peel damage development in *Citrus* taking advantage of a spontaneous fruit-specific ABA deficient mutant from 'Navelate' orange (*Citrus sinensis* L. Osbeck), named 'Pinalate'. Fruit of this mutant presents a partial blockage of the carotenoid biosynthetic pathway, causing distinctive yellow-coloured fruit and a fruit-specific ABA-deficiency (Rodrigo et al., 2003), and is more prone to dehydration and to develop NCPP than its parental (Alférez et al., 2005). The study performed recently by Romero et al. (2012b) has highlighted the relevance of early molecular responses to dehydration, lacking in the mutant and including both ABA-dependent and independent genes, in the fruit susceptibility to NCPP. Results from this study also suggested that 'Pinalate' mutant could be partially insensitive to ABA but further research is needed to elucidate whether perception of ABA may be related to the higher susceptibility of this mutant to dehydration and NCPP.

As far as we know, the role of hormone perception on postharvest behaviour of citrus fruit is mainly restricted to ethylene (John-Karuppiah and Burns, 2010). Recent works on the ABA-response signalling mechanisms have been particularly relevant to agriculture since they provide a deeper insight into the molecular events involved in stress tolerance. Thus, the discovery of the pyrabactin-resistant (PYR/PYL/RCAR) soluble ABA receptors (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010) and the identification of

the clade A type 2C protein phosphatases and SNF1-related protein kinases subclass III complex (PP2CA-SnRK2) as their downstream elements in *Arabidopsis thaliana* (Umezawa et al., 2009) has encouraged new research aimed to improve drought hardiness in crop plants (Li et al., 2009; Chai et al., 2011; Sun et al., 2011; Boneh et al., 2012a; Boneh et al., 2012b; Kim et al., 2012; Li et al., 2012;). In citrus, ABA signalling core components have been recently identified and their transcriptional regulation has been studied during fruit development and ripening and also in vegetative tissue subjected to water stress (Romero et al., 2012a). Nevertheless, the effect of water stress on the transcriptional regulation of the ABA signalling core components has not been yet studied in any fruit.

Taking into account that ABA accumulation and the expression of its signal transduction components in citrus fruit could be differentially regulated under diverse physiological and stress conditions, and also that plants are less sensitive to exogenous ABA under normal conditions than to the stress-induced rises in endogenous ABA (Imay et al., 1995), the aim of this work was to investigate the transcriptional regulation of the ABA signalling core components in 'Navelate' and its ABA-deficient mutant 'Pinalate' fruit stored under conditions causing moderate water stress and NCPP (70-75% RH and 12 ºC). Additionally, to test ABA sensitivity of 'Pinalate' fruit, transcriptional analysis of these ABA signalling core components was performed in ABA-treated mutant fruit.

MATERIALS AND METHODS

Plant material and ABA treatment

Mature fruit of 'Navelate' (*Citrus sinensis* L. Osbeck) orange and its spontaneous ABA-deficient mutant 'Pinalate' were randomly harvested from trees grafted on Citrange carrizo rootstock. The trees were of the same age, grown in the same experimental orchard and subjected to standard cultural practices at 'The Spanish Citrus Germoplasm Bank' at Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain). After harvest, fruit without any damage or visual defects were immediately delivered to the laboratory. Fruit of the ABA-deficient mutant were divided into two groups, which were treated with ABA by dipping them for 1 min in an aqueous solution of 1mM ABA containing 0.7% ethanol to dissolve the hormone (group 1) or with water containing 0.7% ethanol (control solution) by following the same procedure (group 2). Likewise, 'Navelate' fruit were dipped in the control solution. Fruit from both cultivars were allowed to dry at room temperature and immediately stored 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 fruit were dipped into 0.7% ethanol at these times. Periodically, flavedo samples were collected from the total surface of fruit, frozen and homogenized in liquid nitrogen, and kept at -80 ºC for later analysis. The three groups composed of 'Navelate', 'Pinalate' and 'Pinalate' fruit treated with ABA contained the same number of fruit (90 fruit). Three biological replicates, each consisting of 5 fruit, were collected by 1, 3 and 6 weeks of storage. In addition, 3 biological replicates of 10 fruit each were used to estimate NCPP incidence and water loss.

Water loss and peel damage incidence

The susceptibility to dehydration of 'Navelate' fruit and its ABA-deficient mutant treated or not with ABA was evaluated by calculating the percentage of fruit weight loss along storage at 12 ºC and 70-75% RH. In addition, the incidence of these fruit to NCPP during storage was estimated by determining the percentage of fruit showing damage. The results are the means of three biological replicate samples of 10 fruit each ± S.E.M.

ABA analysis

The ABA was extracted from 1 g fresh weight (FW) frozen tissue with 80% acetone containing 0.5 g L^{-1} citric acid and 100 mg L^{-1} of butylated hydroxytoluene as previously described by Lafuente et al. (1997). After centrifugation, the supernatant was diluted in 3 serial dilutions in ice-cold TBS (6.05 g Tris L^{-1}, 8.8 g L^{-1} NaCl and 0.2 mg L^{-1} Mg Cl_2 at pH 7.8) and 3 samples for each dilution were analyzed by the indirect 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 replicate samples ± S.E.M.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from frozen flavedo samples as reported by Romero et al. (2012b). Total RNA was treated with Ribonuclease-free DNase following the manufacturer's instructions. The amount and integrity of RNA was measured by spectrophotometric analysis and by agarose gel electrophoresis and ethidium-bromide staining, respectively.

Reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR) was performed in a two-step qRT-PCR assay as previously described Romero et al. (2012b). Gene-specific primers sequences are detailed in Table 1.

Table 1. Primers designed for the gene expression analysis of the ABA-signalling core components by quantitative RT-PCR (qRT-PCR).

Citrus gene	Homologous in *A. thaliana*	Forward / Reverse	Sequence 5' → 3'	Amplicon size (bp)
CsPYR1	AT4G17870;	F	CCGGGGTAACTCAGGACGAG	198
	Pyrabactin Resistance 1	R	CGGAGACGGAACAGCTCTTG	
CsPYL2	AT2G26040;	F	GGCCTATCATCGAAAGATACC	209
	PYR1-Like protein 2	R	CGATACAACCGTCACTTCTC	
CsPYL4	AT2G38310;	F	CGAACGAAGACACCTGCGTG	168
	PYR1-Like protein 4	R	CGAGAACAGAACATGACCTG	
CsPYL5	AT5G05440;	F	GCCCGGCGGTACATCACAAA	184
	PYR1-Like protein 5	R	GCCCGCAGCTAACAGCACG	
CsPYL8	AT4G27920;	F	GCGGTGCATTTTGGACGCTTC	111
	PYR1-Like protein 8	R	GGCAAAGCCTTAGCAGAATCG	
CsPYL9	AT1G01360;	F	CGGAGATCATCGACGGGAGA	166
	PYR1-Like protein 9	R	CGGTCCTGCACCGCCATCC	
CsABI1	AT4G26080;	F	GGTGACTGCAAAAGCACGAT	129
	ABA-insensitive 1	R	GGGGCAACAGGTTCACCTCC	
CsAHG1	AT5G51760;	F	CGGGTAATGCGAATGCGGG	170
	ABA-hypersensitive germination 1	R	CCCAACGCTCCCACACGCGCACG	
CsAHG3	AT3G11410;	F	GGGATGACTTCAGTTTGCGGTA	227
	ABA-hypersensitive germination 3	R	CGGAGCTCTTCACTTTAATGGC	
CsHAB1	AT1G72770;	F	CCGGAGTGTCTTCGAGGTGG	173
	Hypersensitive to ABA 1	R	GGCCATTCAAACAGTGGCTC	
CsHAI3	AT5G59220;	F	GCCCCTGGCGGCCACTCC	245
	Highly ABA-induced 3	R	GGCCCACACATATAGAAACC	
CsSnRK2.2	AT3G50500;	F	CTGTTCCAGACACTAATCCA	165
	SNF1-related protein kinase 2.2	R	GGCTACTCATAGTCTTTTCATCC	
CsSnRK2.6	AT4G33950;	F	GAGCCAAAGAACTTCCGCAA	139
	SNF1-related protein kinase 2.6	R	GGGTTTCGGATCTCGGGTAT	

The occurrence of non-specific amplified products was ruled out after performing a melting curve analysis and sequencing the reaction products. 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). Expression levels were always referred to that obtained in freshly harvested (FH) 'Navelate' fruit. Three biological samples for

each sampling period and variety were analyzed in duplicate and mean ratios ± S.E.M. were calculated. In addition, in order to compare absolute gene expression values, amplicons of each gene were cloned in pGEMT vector (Promega) and used to generate standard curves by serial dilutions as described in Romero et al. (2012a). Data were then normalized by using the housekeeping genes *CsACT, CsGAPDH* and *CsTUB* (Table 1). Three biological replicates for each sampling period and variety were analyzed in duplicate and mean ratios were calculated.

Statistical design

Results are the means of three replicates samples ± S.E.M. A mean comparison using the Tukey's test was performed to determine if means values were significantly different ($P \leq 0.05$) between varieties at each sampling period.

RESULTS

Six *CsPYR/PYL/RCAR*, five *PP2CA* and two subclass III *SnRK2* genes, homologous to those of *Arabidopsis*, have been recently identified in *Citrus* as the core elements of the ABA perception system (Romero et al., 2012a). In the present work, the relationship between changes in endogenous ABA content and in gene expression levels of the ABA-signalosome components occurring in the flavedo of 'Navelate' oranges and of its spontaneous ABA-deficient mutant 'Pinalate' stored under conditions causing moderate water stress and NCPP (12 ºC and 70-75% RH) have been examined.

Transcriptional regulation of CsPYR/PYL/RCAR in the flavedo of 'Navelate' and its ABA-deficient mutant 'Pinalate' stored under conditions inducing moderate water stress

No significant differences were found in the relative expression levels of *CsPYR/PYL/RCAR* genes between the flavedo of FH parental and ABA-deficient mutant fruit (Fig. 1). Two different expression patterns were found among these genes in 'Navelate', 'Pinalate' and ABA-treated 'Pinalate' fruit exposed to moderate dehydration (Fig. 1). The expression levels of *CsPYR1*, *CsPYL8* and *CsPYL9* genes increased in both parental and mutant fruit and the most important increases occurred by the first storage week. Thereafter, the rate of increase in the accumulation of *CsPYR1* and *CsPYL9* transcripts in 'Navelate' and 'Pinalate' fruit non-treated with ABA was slower and no relevant changes were found in *CsPYL8* gene expression (Fig. 1). These patterns did not parallel that of weight loss, which continuously increased during the whole storage period (Table 2). In contrast, the most important increases in ABA levels occurred also in both cultivars by the first week (Table 2).

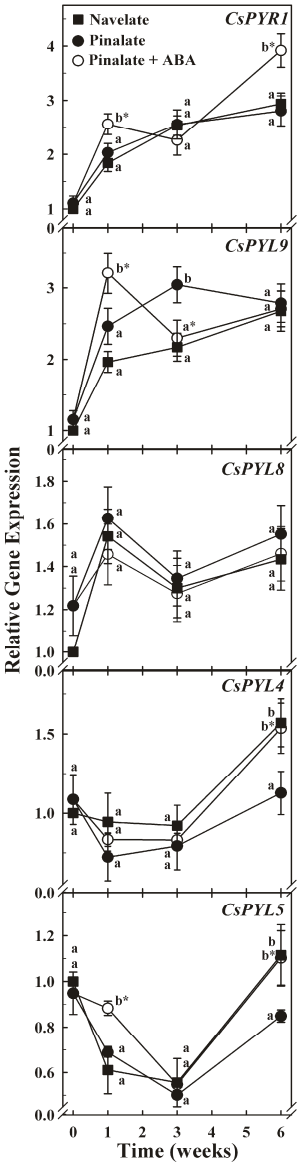

Figure 1. Relative gene expression analysis by qRT-PCR of *Citrus PYR/PYL/RCAR* ABA receptors in 'Navelate' (squares) and 'Pinalate' (circle) fruit, treated (white) or not (black) with ABA and stored under conditions causing moderate water-stress (70-75% RH and 12 ºC). Expression values are relative to transcript levels obtained in FH 'Navelate' fruit. Values are mean ratios ± S.E.M. from three biological replicates for each sampling period and variety analyzed in duplicate. Significant differences ($P \leq 0.05$) for the same storage period are indicated by different letters. Asterisks

indicate significant differences ($P \leq 0.05$) between 'Pinalate' fruit treated or not with ABA for the same storage period.

In spite of the differences found in the ABA content between parental and the ABA-deficient mutant fruit, and in the higher ability of parental fruit to increase the hormone levels under mild water stress (Table 2), significant differences in the expression levels of this set of genes (*CsPYR1*, *CsPYL8* and *CsPYL9*) were found only when analyzing the receptor *CsPYL9.* As shown in Fig. 1, the accumulation of this transcript was significantly higher in the ABA-deficient mutant by 3 weeks although such difference was lost after prolonged storage (6 weeks). Exogenous ABA had, in general, little effect on the expression of these genes (Fig. 1) in spite of the efficacy of the treatment increasing ABA content in the flavedo of mutant fruit (Table 2). The most relevant changes were observed in the expression levels of *CsPYR1* and *CsPYL9*, which were transiently increased by ABA application in 'Pinalate' fruit by 1 week. Final *CsPYL9* transcript levels in 'Navelate' and 'Pinalate' fruit treated or not with ABA were similar, whereas *CsPYR1* gene expression was significantly higher in the ABA-treated mutant fruit by 6 weeks (Fig. 1).

On the other hand, the expression of *CsPYL5* gene decreased in all cultivars for up to 3 weeks although it increased thereafter. The accumulation of *CsPYL4* transcript also decreased by 1 week in the mutant and this effect was not counteracted by ABA application (Fig. 1). Exogenous ABA delayed the sharp decline in *CsPYL5* transcript accumulation occurring by 1 week in 'Pinalate' fruit although expression levels of this gene were similar by this period in the mutant and parental fruit. Transcript levels of both *CsPYL4* and *CsPYL5* increased after 3 weeks in all conditions. As shown in Fig. 1, expression levels of both genes reached by 6 weeks in mutant fruit were significantly lower than those reached in the parental or in the ABA-treated 'Pinalate' fruit.

Table 2. Absolute gene expression analysis by qRT-PCR of the ABA-signalosome components, ABA content and percentage of weight loss in 'Navelate', 'Pinalate' and ABA-treated 'Pinalate' fruit during water stress conditions. Absolute gene expression values are means of three biological samples analyzed in duplicate and correspond to number of copies of each gene per µg of total RNA. ABA content and percentage of weight loss values are means of three biological replicates for each sampling period and variety. Significant differences ($P \leq 0.05$) for the same storage period are indicated by different letters. Asterisks indicate significant differences ($P \leq 0.05$) between 'Pinalate' fruit treated or not with ABA for the same storage period.

		Absolute gene expression levels											**ABA (µg/gFW)**	**% Weight loss**
		PYR/PYL/RCAR					***PP2CA***					***SnRK2***		
	Weeks	***PYR1***	***PYL9***	***PYL8***	***PYL4***	***PYL5***	***ABI1***	***HAB1***	***AHG3***	***HAI3***	***AHG1***	***SnRK2.2***	***SnRK2.6***	
Navelate	**0**	3a	3193a	30a	1365a	1341a	1787a	12382a	5753a	6341a	2825a	754a	3200a	0.512a	0.00a
	1	6a	6269a	47a	1289a	820a	2972ab	18574a	11277a	11782a	1416a	677a	2967a	1.086a	1.06a
	3	9a	6930a	40a	1257a	746a	1964a	9831a	9158a	10385a	1262a	544a	4046a	1.218a	3.31a
	6	10a	8541a	44a	2143a	1494a	2415a	14444a	10418a	18386a	2211a	1111a	2955a	1.250a	8.95a
Pinalate	**0**	4a	3681a	37a	1483a	1272a	1410b	10435b	5204b	3636b	2355b	1150b	3797a	0.093b	0.00a
	1	7a	7863a	50a	986a	924a	2470a	14057b	8782b	5949b	484b	1421b	3625a	0.203b	1.80b
	3	9a	9716b	41a	1082a	672a	1983a	10952a	6132b	5329b	568b	1953b	4444a	0.291b	4.97b
	6	10a	8898a	47a	1539b	1139b	1961a	8629a	7538b	8276b	796b	1011a	1873b	0.308b	11.31b
Pinalate + ABA	**1**	9b*	10247b*	44a	1138a	1185b*	3447b*	16464ab	10557ab*	8091c*	819b*	2139c*	5170b*	1.398c*	1.63b
	3	8a	7336a*	39a	1134a	735a	1949a	11552a	10080ab	7442c*	818ab	838c*	3850a	1.408c*	4.47b
	6	13b*	8639a	44a	2096a*	1479a*	2137a	13061a	9029ab	9735b	748b	970a	3909a*	1.420c*	11.00b

Absolute gene expression analysis further revealed that the least expressed gene was *CsPYR1,* followed by *CsPYL8* (Table 2). *CsPYL4* and *CsPYL5* transcripts accumulation was at least 15-fold higher than those of *CsPYR1* and *CsPYL8* genes and similar along whole storage (Table 2). The most expressed gene during fruit dehydration was *CsPYL9*, whose transcript accumulation was up to 10-fold higher than that of the other genes of this family (Table 2). In contrast, the expression of *CsPYL2* gene was not detected in the flavedo of fruit of any cultivar under these experimental conditions.

Transcriptional regulation of the CsPP2CAs-SnRK2 complex in the flavedo of 'Navelate' and its ABA-deficient mutant 'Pinalate' stored under conditions inducing moderate water stress

Relative gene expression levels of all *CsPP2CA*s were significantly higher in FH parental fruit than in the FH mutant (Fig. 2). Applying ABA to the mutant partially rescued the wild-type phenotype since significantly increased the accumulation of *CsPP2CA*s transcripts in 'Pinalate' fruit stored for 1 week under conditions causing moderate dehydration (see asterisks in Fig. 2). As result of these increases, some of the differences afforded by applying ABA were lost when gene expression levels were compared simultaneously with those of 'Navelate' fruit (see letters in Fig. 2).

As shown in Fig. 2, transcript levels of *CsABI1, CsHAB1* and *CsAHG3* sharply and transiently increased in 'Navelate' and 'Pinalate' fruit, treated or not with ABA, during the first storage week and rises in *CsHAB1* and *CsAHG3* were higher in the parental than in the mutant fruit. ABA application to 'Pinalate' fruit, which was effective increasing the hormone content in the flavedo (Table 2), significantly increased the expression levels of the *CsABI1* and *CsAHG3* genes by 1 week in 'Pinalate' fruit but not those of *CsHAB1* (Fig. 2).

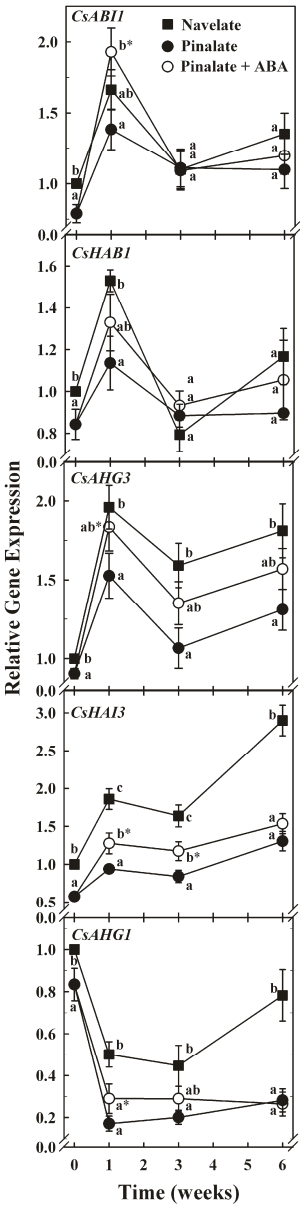

Figure 2. Relative gene expression analysis by qRT-PCR of *Citrus PP2CA* negative regulators in 'Navelate' (squares) and 'Pinalate' (circle) fruit, treated (white) or not (black) with ABA and stored under conditions causing moderate water-stress (70-75% RH and 12 ºC). Expression values are relative to transcript levels obtained in FH 'Navelate' fruit. Values are mean ratios ± S.E.M. from three biological replicates for each sampling period and variety analyzed in duplicate. Significant differences ($P \leq 0.05$) for the same storage period are indicated by different letters. Asterisks

indicate significant differences ($P \leq 0.05$) between 'Pinalate' fruit treated or not with ABA for the same storage period.

The accumulation of *CsABI1* and *CsHAB1* transcripts levelled off in the parental by 3 weeks, reached similar levels to those of mutant fruit treated or not with ABA, and remained almost steady thereafter in all conditions (Fig. 2). The expression levels of *CsAHG3* also decreased by 3 weeks in both varieties, although such decrease was lower in the parental. Therefore, transcript levels of this gene were significantly higher in the parental than in the mutant fruit by 3 and 6 storage weeks (Fig. 2).

The expression levels of *CsHAI3* increased during storage in both 'Navelate' and 'Pinalate' fruit (Fig. 2). This effect was much higher in wild-type fruit and hence *CsHAI3* expression levels were higher in the parental during the whole storage period and doubled those of the mutant fruit by the end of the experiment (Fig. 2). Treating 'Pinalate' fruit with ABA also favoured the accumulation of this transcript during storage although statistical differences with respect to control mutant fruit were lost by 6 weeks.

The gene *CsAHG1* was the unique *CsPP2CA* whose expression was down-regulated during the first storage week. By this period, a 2 and 4-fold decreases in transcript levels were found in parental and mutant fruit, respectively, and this major decrease in 'Pinalate' was slightly counteracted by applying ABA. Thereafter, transcript accumulation remained steady in 'Pinalate' fruit treated or not with ABA. However, *CsAHG1* gene expression increased after 3 weeks in 'Navelate' fruit and reached levels 4-fold higher than in mutant fruit by the end of the experiment (Fig. 2). Absolute transcript levels of this gene were similar to those of *CsABI1* and much lower than those of the other *CsPP2CA*s in both parental and mutant FH fruit (Table 2). Thus, *CsAHG3* and *CsHAI3* transcripts accumulation was similar and remained at higher levels than those of *CsABI1* and

AHG1, whereas *CsHAB1* was the most expressed *CsPP2CA* in FH fruit of both cultivars.

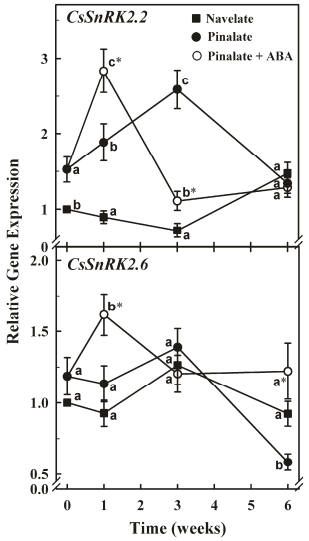

Figure 3. Relative gene expression analysis by qRT-PCR of *Citrus SnRK2* downstream protein kinases in 'Navelate' (squares) and 'Pinalate' (circle) fruit, treated (white) or not (black) with ABA and stored under conditions causing moderate water-stress (70-75% RH and 12 ºC). Expression values are relative to transcript levels obtained in FH 'Navelate' fruit. Values are mean ratios ± S.E.M. from three biological replicates for each sampling period and variety analyzed in duplicate. Significant differences ($P \leq 0.05$) for the same storage period are indicated by different letters. Asterisks indicate significant differences ($P \leq 0.05$) between 'Pinalate' fruit treated or not with ABA for the same storage period.

The expression levels of *CsSnRK2.2* and *CsSnRK2.6* genes, belonging to the citrus subclass III SnRK2 family, were higher in the FH ABA-deficient mutant fruit than in the parental, although significant differences were only found in *CsSnRK2.2* transcript accumulation (Fig. 3). These genes showed different patterns of changes during fruit storage (Fig. 3). The *CsSnRK2.6* gene expression barely increased by 3 weeks in both parental and mutant fruit. Thereafter, it decreased and reached final levels 2-fold lower in 'Pinalate'. Applying the hormone to the ABA-deficient mutant increased the expression level of this gene by 1 week and

avoided the sharp decrease occurring in 'Pinalate' fruit by 6 weeks (Fig. 3). The *CsSnRK.2.2* transcript accumulation reached a maximum by 3 weeks in 'Pinalate' fruit. A sharp decrease in gene expression was observed thereafter in this cultivar while transcript levels increased by 6 weeks in parental fruit (Fig. 3). As in the case of the *CsSnRK2.6* gene, exogenous ABA significantly induced the accumulation of the *CsSnRK.2.2* transcript by 1 week in 'Pinalate' respect to control mutant fruit. The expression levels of this gene sharply decreased thereafter in ABA-treated 'Pinalate' fruit and similar levels were found in all conditions by the end of the experiment (Fig. 3). In addition, absolute gene expression analyses revealed that the accumulation of the *CsSnRK2.6* protein kinase transcript was at least 2-fold higher than that of the *CsSnRK2.2* gene in freshly and dehydrated fruit of both cultivars (Table 2).

Incidence of NCPP in 'Navelate' fruit and its ABA-deficient mutant 'Pinalate'

Peel pitting was evident by 1 week and the percentage of damaged mutant fruit doubled that of the parental cultivar (Fig. 4). By this period, about 60% of mutant fruit displayed damaged, although NCPP severity was still very low in the mutant and almost undetectable in the parental fruit (data not shown). This difference between mutant and wild-type fruit was maintained as storage progressed. By the end of the experiment (6 weeks), all mutant fruit developed NCPP, whereas only a 60% of parental fruit displayed damage (Fig. 4). NCPP incidence in ABA-treated mutant fruit became significantly lower than that of the control non-treated mutant fruit after 3 weeks, but it was still much higher than that of the parental fruit (Fig. 4).

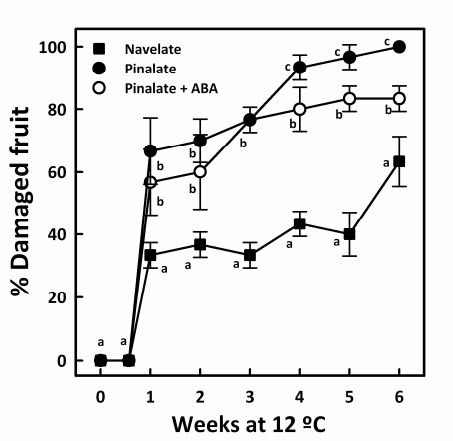

Figure 4. Non-chilling peel pitting incidence in 'Navelate' (squares) and 'Pinalate' (circle) fruit, treated (white) or not (black) with ABA, stored for up to 6 weeks at 12 ºC and 70-75% RH. Results are means of three biological replicates of 10 fruit each ± S.E.M. Mean separation was performed by applying Tukey's test. Different letters indicate statistical ($P \leq 0.05$) differences between varieties for the same storage period.

DISCUSSION

The hormone ABA has been involved in postharvest quality and stress tolerance of fruit (Serrano et al., 2004; Alférez et al., 2005; Cantín et al., 2007). The three core components of the ABA perception and signal transduction pathway have been recently identified in *Citrus* and the relationship existing between their transcriptional regulation and ABA accumulation during natural fruit ripening and severe leaf dehydration has been described (Romero et al., 2012a). However, the regulation of the ABA-signalosome in response to moderate dehydration occurring in fruit during postharvest handling and storage remains still unknown. In citrus fruit, water stress increases ABA and favours the occurrence of postharvest physiological disorders such as NCPP (Lafuente and Zacarías, 2006). Therefore, this research has focused on comparing the effect of moderate water stress on the transcriptional regulation of the ABA-signalosome in the flavedo of 'Navelate' and its fruit-specific ABA-deficient mutant 'Pinalate', which is more prone to dehydration and to develop NCPP (Alférez et al., 2005).

Results obtained have revealed that the expression of some ABA-signalosome elements are differentially regulated in the flavedo of harvested fruit of both cultivars under water stress conditions, which may fit with the lower ability of the mutant to increase ABA levels when stored at 70-75% RH and/or with its higher susceptibility to dehydration (Table 2). Thus, although minor differences were found in the expression levels of the ABA receptors (*CsPYR/PYL/RCAR*s) between 'Navelate' and 'Pinalate' fruit in response to dehydration for the same storage period (Fig. 1 and Table 2) differences in the negative regulators (*CsPP2CA*s) gene expression were relevant (Fig. 2 and Table 2). This is in agreement with previous findings showing that the different ability of both cultivars to increase ABA during fruit maturation has little effect on gene

expression of the positive regulators of the ABA signalling pathway (*CsPYR/PYL/RCAR*s and *CsSnRK2*s) (Romero et al., 2012a). In this context, it is noteworthy that rises in ABA occurring during fruit maturation were similar to those found in the present work in response to moderate dehydration. Furthermore, little changes in the regulation of both ABA receptors and downstream protein kinases were observed between non-stressed citrus leaves and those exposed to severe water stress conditions causing a 15-fold ABA increment and a 20% weight loss (Romero et al., 2012a). Therefore, results of the present work reinforce the idea that *PYR/PYL/RCAR* genes are barely affected by the endogenous ABA content in *Citrus*, independently of the tissue and the physiological or stressful conditions examined. Our results in *Citrus* agree with other found in tomato fruit treated with ABA and in *Vitis* and tomato leaves exposed to water stress (Sun et al., 2011; Li et al., 2012), although *PYR/PYL/RCAR* genes may be regulated by water stress and/or ABA content in *Arabidopsis* seedlings (Szostkiewicz et al., 2010).

Results presented herein also revealed that transcript levels of *CsPYL9*, which was the most expressed ABA receptor, continuously increased during fruit dehydration. In contrast, this gene was down-regulated by dehydration in leaves and during fruit ripening. These results, together with the fact that *CsPYL2* gene expression was detected in leaves (Romero et al., 2012a) but not in flavedo (Fig. 1), highlights the relevance of the differential regulation and tissue specificity expression of the different genes in this family and brings to question the effect of the stress severity on the modulation of the ABA perception and response. Such effect appears to be independent of the hormone content since rises in ABA levels during fruit ripening were similar to those found in response to moderate dehydration. In this regard, it is noticing that although some *CsPYR/PYL/RCAR* gene expression patterns were tightly related to ABA and mirrored the hormone

accumulation in both 'Navelate' and its ABA-deficient mutant 'Pinalate' during fruit ripening (Romero et al., 2012a), this behaviour was not fully conserved either in dehydrated flavedo (Fig. 1) or leaf tissues (Romero et al., 2012a). Therefore, transcriptional regulation of *Citrus* ABA receptors might be differentially modulated by tissue specificity and by the developmental or stress conditions responsible for the induction of the hormone signal.

Differences in the expression levels of the *CsPP2CA*s genes between 'Navelate' and 'Pinalate' fruit in response to dehydration were relevant, which is in concordance with the fact that they 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; Yoshida et al., 2006; Xue et al., 2008; Li et al., 2009). Thus, we found that moderate water stress is able to induce *CsPP2CA*s gene expression in 'Navelate' and 'Pinalate' fruit concomitantly with ABA accumulation (Fig. 2 and Table 2), which agrees with previous findings in *Arabidopsis* and tomato leaves and also with *PP2CA*s responses occurring in tomato and strawberry during fruit ripening (Szostkiewicz et al., 2010; Chai et al., 2011; Sun et al., 2011). It is noticing that all *CsPP2CA*s followed similar expression patterns in fruit of both cultivars during moderate water stress (Fig. 2), as it occurred during maturation (Romero et al., 2012a), although absolute transcript levels in the fruit-specific ABA-deficient mutant 'Pinalate' were significantly lower than in the parental fruit (Table 2). Likewise, non-dehydrated leaves displayed similar *CsPP2CA*s expression patterns than water-stressed leaves although transcript accumulation remained at lower levels in control samples, which might be associated with much lower ABA levels (Romero et al., 2012a). Therefore, it appears that the expression of the *CsPP2CA*s negative regulators is tightly regulated by the endogenous ABA content under both physiological and stressful conditions, independently of the tissue and the severity of the stress.

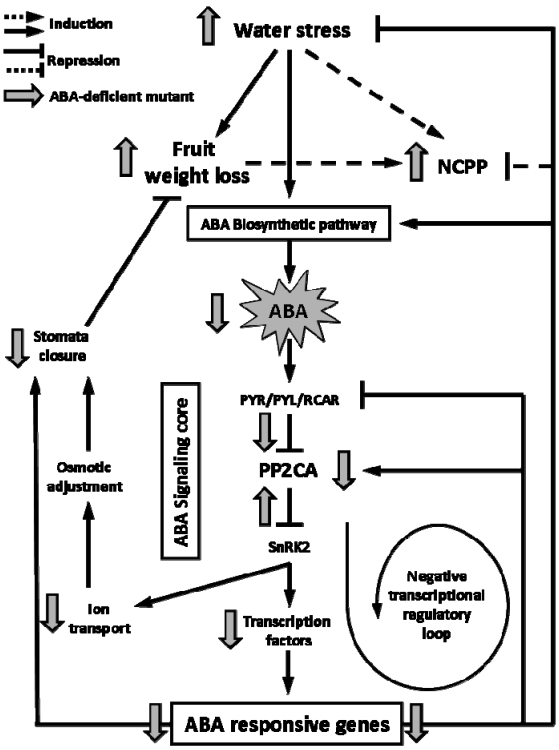

Figure 5. Proposed ABA signalling integration model in dehydrated citrus fruit and influence of ABA-deficiency: Water stress causes fruit weight loss and enhances the development of NCPP in citrus fruit. Stress signal promotes ABA-biosynthetic genes expression and, consequently, ABA accumulation. ABA-mediated PYR/PYL/RCAR-inactivation of the PP2CAs, negative regulators of the ABA signalling pathway, releases SnRK2 downstream protein kinases from their constitutive blockage, allowing its activation by phosphorilation and the transduction of the ABA signal downstream in the pathway. Thus, SnRK2s regulate the phosphorilation degree of transcription factors and ion channels, which modulate the expression of ABA-responsive genes and adjust cell osmotic pressure, respectively. These responses would cope with dehydration and the deleterious effects caused by this stress and would attenuate the ABA signal by a negative transcriptional regulatory loop that involves the repression of the ABA receptors and the induction of the PP2CA negative regulators. Grey arrows indicate the hypothesized molecular response of the ABA-deficient mutant fruit 'Pinalate'. In this mutant, water stress increases weight loss and NCPP incidence, but trigger a deficient accumulation of endogenous ABA. Consequently, the release of the SnRK2s, the subsequent downstream signal transduction including the osmotic adjustment, the water stress and NCPP responses, and the negative transcriptional loop that regulates the PP2CAs levels are attenuated. Therefore, this ABA-deficient mutant would result in an impaired ABA response to dehydration mainly caused by an inefficient ABA signalling. Dotted lines refer to interactions that have not been confirmed in model plants or in *Citrus* cultivars.

Among downstream protein kinases forming the *CsPP2CA*s-*SnRK2* complex, it should be highlighted that the *CsSnRK2.6* kinase was more expressed in fruit (Table 2) and leaves (Romero et al., 2012a) than the second kinase (*CsSnRK2.2*) identified in *Citrus,* and that changes in *CsSnRK2.6* gene expression in response to dehydration appears to be tissue-specific. Thus, this gene was down-regulated in both cultivars during fruit dehydration concomitantly with ABA rises (Table 2 and Fig. 3) as it occurred during fruit ripening (Romero et al., 2012a), but it was up-regulated by water stress in leaves (Romero et al., 2012a). Contrary, *CsSnRK2.2* gene expression continuously decreased during dehydration in both fruit (Table 2 and Fig. 3) and leaves (Romero et al., 2012a) and also during fruit ripening, concomitantly with ABA increases. It should be also mentioned that relevant differences in the expression pattern of this gene were found between 'Navelate' and 'Pinalate' fruit under water stress, which encourage further research to better understand the role of *CsSnRK2.2* gene in the differential response of parental and mutant fruit to dehydration.

Results obtained in a previous work by comparing transcriptomic profiling of dehydrated 'Navelate' and 'Pinalate' fruit suggested that the ABA perception system could be impaired in the mutant and that this might affect its water-stress responses and NCPP susceptibility (Romero et al., 2012b). Results from the present work also revealed that ABA application to 'Pinalate' fruit was effective modifying the transcriptional regulation of some ABA-signalling components. Thus, exogenous ABA mainly increased gene expression of the ABA receptors by 1 week and at the end of the storage (Fig. 1), whereas transcript levels of all components of the downstream complex (PP2CA-SnRK2) peaked by 1 week (Fig. 2 and 3). The treatment also increased the ABA content in the flavedo (Table 2) but it did not modify significantly ($P \leq 0.5$) the weight loss of this mutant (Table 2). Differences in NCPP incidence between ABA-treated and non-treated mutant fruit

were significant, however, after 3 storage weeks. Thus, the percentage of damaged fruit was reduced by the ABA treatment but it was still much higher in the ABA-treated 'Pinalate' than in the parental fruit (Fig. 4). These results may indicate that exogenous ABA partially rescues the susceptibility of 'Pinalate' fruit to NCPP, which would be in concordance with previous findings showing that water stress favours the occurrence of NCPP although other factors should be important for its development (Petracek et al., 1998; Ben Yoshua et al., 2001; Porat et al., 2004; Cajuste et al., 2007). Complementary, results found and summarized in the model proposed in Fig. 5 suggest that this mutant is not fully sensitive to the hormone. This behaviour could be explained by the occurrence of a negative transcriptional loop that regulates ABA receptors and *PP2CA*s expression levels, as suggested by Merlot et al. (2001) in *Arabidopsis* plants. Accordingly, overall results of the present work might suggest that ABA-reduction and, hence, the decreased expression levels of the *CsPP2CA* negative regulators in 'Pinalate' fruit could be in part responsible for its inefficient response to ABA-related signals and for its higher susceptibility to develop NCPP under moderate water stress.

In summary, results revealed the first evidence for the transcriptional regulation of the ABA-signalosome components in fruit subjected to water-stress conditions. Low differences in ABA receptors and downstream protein kinases transcript levels were found between 'Navelate' and its ABA-deficient mutant 'Pinalate' fruit in response to dehydration. Contrary, *CsPP2CA*s gene expression was substantially regulated by ABA content and showed significant differences between varieties. Moreover, it appears that transcriptional regulation of the ABA receptors and downstream protein kinases might be differentially affected by tissue specificity, the stress severity and the source of the ABA signal from a developmental or stressful stimulus. In contrast, *CsPP2CA*s negative regulators

have shown a consistent response among all studied conditions and tissues, which point out these components as potential targets to improve drought hardiness in citrus.

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4. GENERAL DISCUSSION

Drought is one of the major environmental causes of poor plant performance and limited crop yield worldwide (Yamaguchi-Shinozaki and Shinozaki, 2006). Numerous researches have been focused on understanding the molecular mechanisms underlying this stress in model and crop plants (Bray, 1993; Bray *et al.*, 2000; Seki *et al.*, 2002; Bartels and Sunkar, 2005). Among the water stress-induced compounds, the hormone ABA is one of the most relevant because it plays a key role in the modulation and activation of numerous processes, such as the stomata closure, to prevent water loss in the plant (Wilkinson *et al.*, 2012). Under drought conditions, ABA content is regulated by the induction of biosynthetic genes, and its signal transduction is modulated by the induction of signalling genes and ABA-responsive proteins or transcription factors (Bray, 1993; Ingram and Bartels, 1996; Shinozaki *et al.*, 1998; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006; Seki *et al.*, 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). During postharvest handling of fruits of agronomic interest, water stress is one of the most important factors reducing the external quality and hence the commercial value. However, the study of the molecular responses during dehydration in harvested fruit has been limited to a few set of genes (Alférez *et al*., 2008; Bonghi *et al*., 2012; Loyola *et al*., 2012), and global changes occurring in fruits under water stress conditions was only performed in grapes (Grimplet *et al.*, 2007; Rizzini *et al.*, 2009; Deluc *et al.*, 2009; Zamboni *et al.*, 2010).

In the present work, the availability of the sweet orange fruit-specific ABA-deficient mutant 'Pinalate' has allowed to get a deeper insight into the molecular mechanisms involved in the response of harvested citrus fruits to dehydration and the potential role of ABA in this process. Within this context, it is interesting to note the link existing between water stress and the occurrence of NCPP in citrus fruit (Alférez *et al.*, 2003; Lafuente and Zacarías, 2006). Although

dehydration is not a limiting step (Lafuente and Sala, 2002; Cajuste and Lafuente, 2007; Cajuste *et al.*, 2010), it enhances the development of this peel disorder (Alférez and Burns, 2004). 'Pinalate' fruit is prone to dehydration and much more susceptible to develop NCPP than its parental 'Navelate' (Alférez *et al.*, 2005). Therefore, the comparative high throughput transcriptional analysis of fruits from both cultivars stored under conditions causing moderate water stress and the development of NCPP (12 ºC and 70–75% RH) allowed investigating the putative relationship existing between ABA, dehydration and the occurrence of this peel disorder.

Functional categorization of differentially expressed genes between both cultivars indicated the ability of parental fruit to trigger early molecular responses for coping with dehydration and the deleterious effects caused by this stress (Fig. 2 and Table 2, Results Chapter 1). The biological processes induced in 'Navelate' fruit included ABA biosynthetic and signalling genes, ABA-dependent transcription factors, and genes encoding ABA-responsive proteins, but also ABA-independent genes such as cysteine-proteases and ion transporters (Table 2 and 3, Results Chapter 1). The lack of induction of these genes in 'Pinalate' fruits (Fig. 4 and Table 2, Results Chapter 1) suggested that the higher ability of 'Navelate' fruits to synthesize ABA and to modulate ABA-related genes was important for reducing water loss and for maintaining cell homeostasis and viability, hence reducing the incidence of peel damage.

The molecular responses displayed by 'Navelate' fruit fit into the classical responses induced in *Arabidopsis* plants subjected to water stress conditions, which showed the involvement of ion transporters, aquaporins, and chaperone and other protective proteins for maintaining cellular structures and to avoid water loss (Bray, 1993; Ingram and Bartels, 1996; Shinozaki *et al.*, 1998; Ramanjulu and Bartels, 2002). Those results also agreed with previous findings in

detached grape berries, showing that both ABA-dependent and independent genes were affected by dehydration occurring before or after harvesting the fruit and by the stress severity (Grimplet *et al.*, 2007; Rizzini *et al.*, 2009; Deluc *et al.*, 2009; Zamboni *et al.*, 2010). Likewise, our results were in concordance with those reported by Riera et al. (2005) suggesting the involvement of both ABA-dependent and independent signals for regulating the dehydration response in *Arabidopsis* plants. Wilkinson et al. (2010) suggested the existence of alternative dehydration-responsive pathways operating in plants under ABA-deficiency to minimize water loss. Moreover, it is well known that cuticle plays a key role in fruit dehydration (Islam *et al.*, 2009; Curvers *et al.*, 2010). However, results of the present work indicated that the lower ability of 'Pinalate' fruit to synthesize ABA was crucial for the impaired response of this mutant to water stress, which might be related to the higher susceptibility of this mutant to develop NCPP.

Results from the comparative transcriptomic assay further revealed that mutant fruits down-regulated the biosynthesis of carbohydrates at late stages of the storage, when NCPP incidence was much higher than in the wild-type fruits (Fig. 1 and Table 2, Results Chapter 1). Since sugars play protective roles against osmotic and water stresses in plants (Bartels and Sunkar, 2005; Seki *et al.*, 2007), the repression of this biological process might be relevant for the susceptibility of citrus fruit to such stresses leading to peel damage. This was in agreement with previous findings showing the higher reduction in soluble sugars and starch in 'Pinalate' than in 'Navelate' fruit during the development of NCPP (Holland *et al.*, 2005). Furthermore, the repression of this process was also associated with the enhancement of NCPP in 'Navelate' fruits exposed to a different postharvest stress (Establés-Ortiz *et al.*, 2009), indicating the relevance of carbohydrate metabolism in the convergence of the mechanisms underlying NCPP.

Results obtained after treating 'Navelate' fruits with ABA suggested that endogenous levels of the hormone might be sufficient to trigger cellular processes coping with dehydration and further consequences related to peel damage, since NCPP index and the percentage of weight loss were not significantly affected by ABA application in the wild-type fruits (Fig. S1, Results Chapter 1). In contrast, ABA treatment on mutant fruit increased the hormone content to levels even higher than those of the parental and repressed the expression of thousands of genes (Fig. 1 and 2, Results Chapter 1). This set of genes was enriched in genes encoding proteins with E3-ubiquitin ligase activity (Table 2 and 3, Results Chapter 1), which is involved in protein recycling (Rechsteiner, 1987). In spite of ubiquitination has been largely associated with the degradation of proteins involved in hormone-signal transduction pathways (Ueguchi-Tanaka *et al.*, 2005; Kepinski and Leyser, 2005), including those of ABA (López-Molina *et al.*, 2003; Zhang *et al.*, 2005; Luo *et al.*, 2006; Ryu *et al.*, 2010), an exhaustive analysis of the genes belonging to this process revealed that they were related to pathogen signals and infection. Accordingly, it was found that rots developed earlier in ABA-treated than in non-treated mutant fruit (Fig. S2, Results Chapter 1), which agreed with the proposed role of ABA in the resistance suppression and the promotion of the susceptibility to pathogen infection (Anderson *et al.*, 2004; Yasuda *et al.*, 2008; Ton *et al.*, 2009).

Results of the present work showed that water loss and NCPP incidence occurring in mutant fruit during storage were barely counteracted by the ABA application, and that there were no statistical differences between ABA-treated and control mutant fruits. Although plants are less sensitive to exogenous ABA than to stress-induced rises in endogenous ABA (Imay *et al.*, 1995), these results suggested that the ABA-deficient mutant 'Pinalate' might be partially insensitive to the hormone, being able to modulate gene expression but showing a fail in the

regulation of the dehydration response. These results encouraged new research focused on determining whether the genes encoding the proteins responsible for the ABA perception and signal transduction in citrus fruit were differentially regulated in 'Navelate' and 'Pinalate' genotypes.

Therefore, the homologous genes of the ABA perception system components, identified recently in *Arabidopsis* (Santiago *et al.*, 2009; Park *et al.*, 2009; Ma *et al.*, 2009; Nishimura *et al.*, 2010) so-called ABA-signalosome, were characterized for the first time in the *Citrus* genome. To unravel the relationship between endogenous ABA and the expression of these components, we performed a comparative transcriptional analysis between 'Navelate' and 'Pinalate' fruits and leaves subjected to conditions increasing ABA content. Thus, to study whether the expression of these genes was affected by the source of the ABA signal from stressful or developmental conditions, fruits from both cultivars were exposed to moderated water stress or harvested at different ripening stages. It is interesting to note herein that rises in ABA occurring during postharvest fruit dehydration were similar to those found during fruit development and ripening, and that ABA content in the ABA-deficient mutant 'Pinalate' was up to 4-fold lower than in 'Navelate' in both processes (Fig. 1, Results Chapter 1; Fig.2, Results Chapter 2). In addition, in order to identify the occurrence of some tissue specificity and to get further insights on the relevance of the stress severity for the regulation of the ABA-signalosome components, transcriptional analyses were also performed in leaves subjected to severe water stress (50-55% RH) (Fig. 4, Results Chapter 2). In this regard, it should be mentioned that basal ABA levels in 'Navelate' and 'Pinalate' leaves were similar and much lower than those detected in freshly harvested 'Navelate' fruits, although the hormone content in dehydrated leaves increased about 15-fold and

reached levels similar to those of the stored parental fruit (Fig. 4, Results Chapter 2 and Fig.1, Results Chapter 1).

The identification of the *Citrus* ABA-signalosome genes was performed by *in silico* analysis of the *Citrus* genome, which revealed that 6 *CsPYR/PYL/RCAR*, 5 *CsPP2CA* and 2 subclass III *CsSnRK2* genes encoded proteins with high homology degree to the *Arabidopsis* core elements of the ABA perception. It appeared that these proteins were less represented in *Citrus* than in *Arabidopsis* (Park *et al.*, 2009; Ma *et al.*, 2009) and *Vitis* (Boneh *et al.*, 2012a; Boneh *et al.*, 2012b). Nevertheless, a lower number of *PYR/PYL/RCAR*s and *PP2CA*s genes has been also found in tomato (Sun *et al.*, 2011) and strawberry (Chai *et al.*, 2011) compared to *Arabidopsis*. Amino acid alignments revealed that relevant motifs for functional protein folding, such as the *gate* and *latch* regions in PYR/PYL/RCARs (Melcher *et al.*, 2009), and for phosphatase activity in PP2CAs (Weiner *et al.*, 2010), were conserved in *Citrus* proteins (Supplementary Fig. S1A and S1B, Results Chapter 2). D-rich C-terminal domain II, which has been shown to be essential for ABA signal transduction (Yoshida *et al.*, 2006a), was also fully conserved in *Citrus* SnRK2s proteins (Supplementary Fig. S1C, Results Chapter 2). Results obtained from the phylogenetic analysis showed the distribution of these proteins close to their respective homologous in *Arabidopsis* (Fig. 1, Results Chapter 2). In addition, protein folding analysis revealed high similarity between the tertiary structure of *Arabidopsis* and *Citrus* proteins (Supplementary Fig. S2, Results Chapter 2), which further supported that these proteins might function as the core elements of the ABA perception in *Citrus*.

Transcriptional studies performed in 'Navelate' and 'Pinalate' fruits and leaves revealed that *CsPYR/PYL/RCAR* genes expression was barely affected by the endogenous ABA content in both tissues and cultivars. *CsPYR/PYL/RCAR* transcript levels found during fruit development and ripening were similar in both cultivars

in spite of ABA content in the ABA-deficient mutant was at least 2-fold lower than in the parental at coloured stages (Fig. 2 and 4, Results Chapter 2), which suggested that ABA accumulation found in the ABA-deficient fruit may be sufficient for regulating the expression of these genes.

Different expression patterns were found among genes belonging to this family, which is in agreement with previous findings in tomato fruits (Sun *et al.*, 2011). On one hand, *CsPYR1*, *CsPYL4* and *CsPYL5* expression patterns mirrored that of the ABA accumulation in both 'Navelate' and 'Pinalate' fruits during ripening (Fig. 2 and 3A, Results Chapter 2). The concomitant repression of these genes with the induction of ABA content was in concordance with the transcriptional response observed in *Arabidopsis* seedlings after osmotic and drought stresses (Szostkiewicz *et al.*, 2010). On the other hand, *CsPYL8* and *CsPYL9* gene expression decreased as ripening progressed, although a sudden increase was observed in both genes when maximum ABA content was achieved in parental and mutant fruits (Fig. 2 and 3A, Results Chapter 2). It is also interesting that *CsPYL2* transcripts were not detected in fruits of both cultivars during ripening, which suggested that the expression of some ABA receptors could be tissue specific in *Citrus*. In agreement with this, some tomato genes such as *SlPYL5*, belonging to the same subfamily as *CsPYL2* (subfamily III; Fig. 1A, Results Chapter 2), were almost undetectable during fruit ripening (Sun *et al.*, 2011). In spite of the expression of the ABA receptors was differently regulated during fruit ripening, the study of ABA-responsive genes, such as *CsHVA22E* and *CsALDH3*, showed expression patterns that mostly paralleled the ABA accumulation (Fig. S3, Results Chapter 2).

The transcriptional analysis of the ABA-signalosome components in water stressed fruits, which has been performed for the first time in this work, confirmed that the expression of most of the *CsPYR/PYL/RCAR* genes was barely

affected by the difference in ABA content between cultivars. Thus, little differences were observed between 'Navelate' fruit and its ABA-deficient mutant 'Pinalate' in spite of ABA content in the parental was 4-fold higher from the first week of postharvest storage (Fig. 1 and Table 2, Results Chapter 3). Interestingly, the expression patterns of these genes were not coincident with those found during fruit ripening in spite of the ABA content achieved was similar during both processes. In fact, whereas *CsPYR1*, *CsPYL9* and *CsPYL8* genes were repressed along ripening in fruit of both genotypes, they were induced concomitantly with ABA rises from the first week of storage under conditions causing moderate water stress. In contrast, *CsPYL4* and *CsPYL5* were down-regulated in both cultivars during fruit ripening and dehydration (Fig. 3A, Results Chapter 2; Fig. 1, Results Chapter 3). Therefore, these results suggested that the expression of some members of the *CsPYR/PYL/RCAR* family might be modulated by the source of the ABA signal from developmental or stressful conditions. In addition, ABA treatment on 'Pinalate' fruits transiently increased the expression of *CsPYR1*, *CsPYL9* and *CsPYL5* genes by the first week of postharvest storage although, thereafter, these genes followed similar expression patterns than non-treated mutant fruits. These transient inductions partially agreed with previous data reported by Szostkiewicz et al (2010) in *Arabidopsis* seedlings treated with ABA and with studies performed in ABA-treated tomato fruit (Sun *et al.*, 2011), and also reinforced the idea that ABA content found in 'Pinalate' fruits during ripening and dehydration might be sufficient to regulate the expression of this gene family in a manner similar to that of the parental.

Little changes in the regulation of ABA receptors were observed between non-stressed *Citrus* leaves and those exposed to water stress, where a 15-fold ABA increment and a 20% weight loss was observed (Fig. 5, Results Chapter 2). These results agree with the slight differences found in the expression pattern of

these receptors between 'Navelate' and 'Pinalate' dehydrated fruits. Differences in the transcripts accumulation of *CsPYL2* and *CsPYL9*, the most expressed *CsPYR/PYL/RCAR* genes in vegetative tissue, between stressed and non-stressed leaves were detected before the sharp increase in ABA (Fig. 4 and 5, Results Chapter 2), which suggested that these changes may be due to osmotic adjustments but not to ABA content. Contrary, *CsPYL5* continuously decreased as dehydration progressed and, in both cultivars, transcript levels in dehydrated leaves became significantly lower than in their controls only when ABA rose dramatically (6 hours; Fig. 4 and 5, Results Chapter 2). Taking together this result and the consistent repression of *CsPYL5* during fruit dehydration and ripening, these data suggest that the regulation of this gene might be tightly related to ABA accumulation in *Citrus*. Nevertheless, overall results indicated that most of the members of the *CsPYR/PYL/RCAR* family were barely affected by endogenous ABA content, independently of the tissue and the physiological or stressful conditions studied. This agreed with results found in ABA-treated tomato fruit and in *Vitis* and tomato leaves exposed to water stress (Sun *et al.*, 2011; Li *et al.*, 2012), while it differs with results obtained in *Arabidopsis* seedlings since *PYR/PYL/RCAR* genes appeared to be mainly regulated by both ABA and water stress (Szostkiewicz *et al.*, 2010).

Studies performed in *Citrus* leaves further allowed identifying tissue specificities. Thus, *CsPYL2* gene, whose transcripts were not detectable in fruits, was one of the most expressed genes in leaves (Supplemental Table 6, Results Chapter 2). Contrary, *CsPYL4* and *CsPYL8* genes were expressed in fruits but not in vegetative tissue. It is also noteworthy that *CsPYL9* continuously increased during moderate fruit dehydration in both 'Navelate' and 'Pinalate' cultivars (Fig. 1, Results Chapter 3), whereas this gene was down-regulated by dehydration in leaves (Fig. 5, Results Chapter 2). These results highlighted the relevance of the

tissue specificity and/or the stress severity in the modulation of the ABA perception in *Citrus*. Overall results suggested that the transcriptional regulation of the *Citrus* ABA receptors might be differentially modulated by tissue specificity and also by the physiological or stress conditions responsible for the induction of the hormone signal.

Clade A PP2Cs function as negative regulators of the ABA signalling pathway and their transcript levels are highly induced by ABA in plants (Merlot *et al.*, 2001; Saez *et al.*, 2004; Saez *et al.*, 2006; Yoshida *et al.*, 2006b; Xue *et al.*, 2008; Li *et al.*, 2009). Comparative transcriptional analyses between 'Navelate' fruit and its ABA-deficient mutant during ripening and postharvest dehydration revealed that the expression of the genes belonging to *CsPP2CA* family mostly paralleled ABA accumulation in both genotypes, independently of the process examined (Fig. 3B, Results Chapter 2; Fig. 2, Results Chapter 3). The relationship between dehydration and ABA is supported by the fact that transcript levels of the ABA biosynthetic gene *CsNCED1* increased transiently by the first week of storage promoting ABA synthesis (Fig.1 and 4A, Results Chapter 1), which agreed with results reported in harvested grapes and tomatoes (Sun *et al.*, 2010; Sun *et al.*, 2012a; Sun *et al.*, 2012b) and in vegetative tissues (Tan *et al.*, 2003; Loyola *et al*., 2012; Frey *et al*., 2012). Interestingly, *CsNCED1* followed an expression pattern highly similar to that displayed by the members of the *CsPP2CA* family in citrus fruit (Fig.2, Results Chapter 3). Accordingly, ABA-induced genes, such as *CsHVA22E* and *CsALDH3*, showed expression patterns that paralleled ABA accumulation during fruit ripening (Fig. S3, Results Chapter 2). However, transcripts levels of all these genes in the ABA-deficient mutant 'Pinalate' were lower than that of the 'Navelate' fruit, which suggested a strong effect of ABA content on the expression of the *CsPP2CA*s and the selected ABA-regulated downstream genes. Moreover, ABA application to 'Pinalate' fruit during storage enhanced the induction of

*CsPP2CA*s genes, which reached values close to those observed in the parental by the end of the experiment (Fig. 2, Results Chapter 3). Overall, this was in concordance with the studies performed in grapes and tomato fruits, in which *PP2CA*s transcript levels were highly induced concomitantly with ABA raises during fruit ripening or in response to ABA application (Sun *et al.*, 2011; Boneh *et al.*, 2012a). Results obtained from vegetative tissue studies agreed with those performed in fruits since *CsPP2CA*s gene expression paralleled ABA accumulation in both control and water-stressed leaves, and transcripts levels were much higher in dehydrated than in the control non-stressed leaves (Fig. 6, Results Chapter 2). Accordingly, dehydrated tomato leaves displayed higher transcript levels than those non-stressed (Sun *et al.*, 2011).

Within this context, *CsAHG1* protein phosphatase merits special mention because of its differential responsiveness to endogenous ABA during fruit development and dehydration. Among *CsPP2CA*s, *CsAHG1* was the only down-regulated by dehydration in both 'Navelate' and 'Pinalate' fruit (Fig. 2, Results Chapter 3). In addition, this was the only *CsPP2CA* whose fold induction was higher in the mutant than in the parental during fruit ripening in spite of the ABA content in 'Pinalate' was 2-fold lower than in 'Navelate' (Fig. 2 and 3B, Results Chapter 2). Moreover, *CsAHG1* was one of the most induced *PP2CA*s (about 20-fold) in dehydrated *Citrus* leaves (Fig. 6, Results Chapter 2), which agreed with data reported in *Arabidopsis* leaves (Szostkiewicz *et al.*, 2010). Therefore, it would be interesting to investigate further the involvement of this gene in the differential response of 'Navelate' and 'Pinalate' fruit to physiological or stress conditions causing an increase in ABA levels.

Overall, these findings indicated that the gene expression of the *CsPP2CA*s negative regulators was highly regulated by ABA content under both developmental and stressful conditions, independently of the tissue and the

severity of the stress applied. In agreement with this idea and with the lower differences found between cultivars in the *CsPYR/PYL/RCAR* gene expression, Szostkiewicz et al. (2010) reported that *PP2CA* genes are more responsive to ABA as compared with ABA receptors, and suggested a higher sensitivity of these negative regulators to ABA changes in plants.

Regarding to the concomitant induction of the *PP2CA*s gene expression levels and the ABA accumulation, a transcriptional negative feedback regulatory mechanism has been proposed for modulating the ABA responses in model plants (Merlot *et al.*, 2001; Santiago *et al.*, 2009; Melcher *et al.*, 2009; Vlad *et al.*, 2009; Weiner *et al.*, 2010). Thus, it was suggested that exogenous ABA or stress-induced rises in hormone levels would induce the initial ABA-mediated PYR/PYL/RCAR inactivation of PP2CAs. This allows the release of SnRK2s and hence the phosphorylation of ABA-dependent transcription factors, which finally would modulate the expression of ABA-responsive genes. Hormone signal would be later attenuated by the combination of both the ABA-induced down-regulation of *PYR/PYL/RCAR*s gene expression and the up-regulation of the *PP2CA*s transcript levels, hence restoring the initial conditions (Merlot *et al.*, 2001; Santiago *et al.*, 2009; Vlad *et al.*, 2009) (Fig. 4, Introduction Section). Thus, the resetting of the ABA signal transduction pathway would provide a dynamic and precise mechanism to adjust the adaptive response of the plant to the strength and duration of the stress.

The expression pattern of most of the *CsPP2CA* genes analysed in this work paralleled the accumulation of ABA in 'Navelate' and 'Pinalate' fruits and leaves. Interestingly, the up-regulation of these *CsPP2CA* genes during fruit ripening was also concomitant with the down-regulation of the *CsPYR1*, *CsPYL4* and *CsPYL5* genes. Similarly, *CsPYL4* and *CsPYL5* transcript levels bottomed down concomitantly with the transient induction of several *CsPP2CA*s by the first week

of fruit storage, when ABA rose in response to water stress. In vegetative tissue, it was found that *CsPYL5* gene expression decreased concomitantly with the highest hormone levels and the induction of most of the *CsPP2CA*s. Therefore, in spite of further research should be addressed at protein level for elucidating the interactions occurring among these components in *Citrus*, it can not be ruled out that this negative regulatory loop might be modulating the ABA responses during conditions causing the increase of ABA in *Citrus* fruits and leaves.

SnRK2s positively regulate ABA responses by the phosphorylation of ABF/AREB bZIP transcription factors that bind to ABA-responsive elements (Kobayashi *et al.*, 2005; Fujita *et al.*, 2009; Yoshida *et al.*, 2010). It is also well known that SnRK2s are able to phosphorylate ion channels and NADPH oxidases in guard cells, hence taking part in the control of the ABA-induced stomata closure (Geiger *et al.*, 2009; Sirichandra *et al.*, 2009). However, the activity of the subclass III SnRK2s is inhibited by their association with the PP2CAs. The ABA-mediated PYR/PYL/RCAR inactivation of PP2CAs, dissociates the PP2CA-SnRK2 complex and release SnRK2s for transducing the ABA signal downstream in the pathway (Santiago *et al.*, 2009; Vlad *et al.*, 2009). The functionality of the subclass III SnRK2s has been confirmed in *Arabidopsis* plants (Fujii and Zhu, 2009; Fujita *et al.*, 2009) and in crops such as rice (Kobayashi *et al.*, 2004) and maize (Li *et al.*, 2009) but not in fruits of agronomic interest. Nevertheless, the transcriptional analysis of these genes has been performed in tomato fruit (Sun *et al.*, 2011) and in grape plants (Boneh *et al.*, 2012a). Studies performed in tomato fruit showed that *SnRK2*s were transiently induced by exogenous ABA, and highly expressed at the most immature stages of the fruit ripening concomitantly with the lowest endogenous ABA levels. In contrast, water stress-induced ABA rises in tomato leaves provoked little changes in the transcript levels of these genes (Sun *et al.*,

2011). Accordingly, water stress slightly affected gene expression of the subclass III *SnRK2*s in grape (Boneh *et al.*, 2012a).

In concordance with results obtained during tomato fruit ripening, *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 (Fig. 2 and Fig. 3C, Results Chapter 2). Moreover, ABA treatment on mutant fruit triggered the transient induction of both genes by the first week of storage, which was also in agreement with tomato fruit results (Sun *et al.*, 2011). Nevertheless, as reported in the *CsPYR/PYL/RCAR* analyses, little differences were found in *CsSnRK2.2* and *CsSnRK2.6* genes expression between 'Navelate' and its ABA-deficient mutant 'Pinalate' during fruit ripening, which suggested that the low ABA content in mutant fruit might be sufficient for modulating the expression of these genes in a similar manner than in parental fruit during ripening process. During fruit dehydration, however, *CsSnRK2.6* did not show relevant differences between cultivars, whereas *CsSnRK2.2* peaked by 3 weeks only in 'Pinalate' fruit. Interestingly, no much difference was found in the transcriptional regulation of the *CsSnRK2*s between water-stressed and control non-stressed *Citrus* leaves in spite of the sharp increase in ABA levels occurring in dehydrated leaves (Fig. 4 and 7, Results Chapter 2). These results were also in concordance with that found in tomato leaves (Sun *et al.*, 2011) and grape studies (Boneh *et al.*, 2012a), and suggested that, in general, these positive effectors of the ABA signalling pathway might be differentially affected by the tissue, the stress severity and the source of the ABA signal from a physiological or stressful stimulus.

The elucidation of the ABA signalling core components and the molecular mechanisms by which ABA signal is transduced downstream in the pathway have encouraged new investigations addressed to understand the regulation of ABA-related processes in crops of agronomic interest (Chai *et al.*, 2011; Sun *et al.*,

2011; Li *et al.*, 2012; Kim *et al.*, 2012; Boneh *et al.*, 2012a; Boneh *et al.*, 2012b). Based on the results presented in this work, the PP2CAs appeared to be key regulator points in the ABA response of *Citrus* under developmental and stress conditions, independently of the tissue and the stress severity imposed. Therefore, the results encourage future research to attempt biotechnological engineering on these genes in order to improve drought hardiness in *Citrus*.

5. CONCLUSIONS

1. The comparative transcriptional analysis between 'Navelate' and its fruit-specific ABA-deficient mutant 'Pinalate' fruit during postharvest storage under mild water stress conditions (70-75% RH, 12 ºC) highlighted the ability of parental fruit to trigger responses to reduce water loss and other detrimental consequences caused by this stress, which may lead to the NCPP development. These responses involved the 'water deprivation' and the 'di-, tri-valent inorganic cation transport' biological processes, which included both ABA-dependent and -independent genes. The lack of these responses in the mutant fruit indicated their relevance for the prevention of water loss and the development of peel damage in citrus fruit. In addition, the repression of the 'carbohydrate biosynthesis' process occurred specifically in 'Pinalate' fruits.

2. Exogenous ABA did not rescue the impaired response of 'Pinalate' fruit to water stress, but modulated relevant transcriptomic changes. Genes related to different biological processes were identified, although only the 'protein ubiquitination' biological process was over-represented in the ABA-treated mutant fruit. An exhaustive analysis revealed that genes belonging to this process were related to the fruit-pathogen interactions.

3. The *Citrus* ABA signalling core components were identified for the first time. *In silico* analysis revealed that *Citrus* ABA-signalosome was composed of six PYR/PYL/RCAR ABA receptors, five PP2CAs negative regulators, and two subclass III SnRK2s downstream protein kinases. The high homology degree with *Arabidopsis* proteins and the presence of conserved motifs for functional protein folding and activity indicate that

these proteins are *bona fide* core elements of the ABA perception in *Citrus*.

4. Transcriptional analysis performed on 'Navelate' and 'Pinalate' fruits at different ripening stages and stored under mild water stress conditions, as well as in leaves submitted to severe water stress, revealed that genes encoding the ABA perception system components are differently regulated by the endogenous ABA content.

5. *CsPYR/PYL/RCAR* ABA receptors showed minor differences between cultivars, independently of the source of the ABA signal, the tissue and the stress severity. Nevertheless, some components of this family displayed tissue specificity, such as *CsPYL2*, *CsPYL4* and *CsPYL8*.

6. *CsPP2CA* negative regulators showed significant differences between 'Navelate' and the ABA-deficient mutant 'Pinalate' fruit, which revealed the high sensitivity and responsiveness of these components to ABA. Furthermore, these genes followed a consistent expression pattern and ABA-response independently of the physiological process responsible for ABA increases, tissue and severity of the stress imposed. The expression pattern of the *CsAHG1* during fruit ripening and dehydration bring to question the relevance of this particular gene in the ABA signalling pathway in citrus fruit and in the impaired response of the 'Pinalate' fruit.

7. Subclass III *CsSnRK2s*, positive effectors of the ABA signalling pathway, showed minor differences in gene expression between cultivars. The expression of these genes was differentially affected by the tissue, the

stress severity and the source of the ABA signal from a physiological or stressful stimulus.

8. Overall results indicate that the ABA-deficient mutant 'Pinalate' fruit may sense ABA, although the hormone signal could be impaired because of reduced *CsPP2CA*s levels causing altered water stress response and higher NCPP susceptibility.

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