Involvement of aerial organs on the ABA accumulation in roots of Citrus plants under water deficit

TESIS DOCTORAL

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HACEN CONSTAR QUE:

Matías Jesús Manzi Fraga, Ingeniero Agrónomo por la Universidad de la República de Uruguay y Máster en Técnicas Cromatográficas Aplicadas por la Universitat Jaume I, ha realizado la bajo su dirección el trabajo que, titulado '***Involvement of aerial organs on the ABA accumulation in roots of Citrus plants under water deficit***' reúne las condiciones necesarias para optar al grado de Doctor.

Dr. Aurelio Gómez Cadenas **Dr. Vicent Arbona Mengual**

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ABBREVIATIONS

AAO3 - ABA aldehyde oxidase3
ABA - Abscisic acid
ABA-GE - ABA glycosyl ester
ABA2 - Xanthoxin dehydrogenase
AIC - Ailsa Craig
AOG - UDP-glucosyltransferase
βCHX - β-carotene hydroxylase
βGluc - β-glucosidase
βLCY - β-lycopene cyclase
BR - Brassinosteroids
CKs - Cytokinins
CRTISO - Carotenoid isomerase
CYP707a - ABA 8'-hydroxilase
DHJA - Dehydrojasmonic acid
DPA - Dihydrophaseic acid
DW - Dry weight
ET – Ethylene
flc – *flacca*
FW - Fresh weight
GAs - Gibberellins
GGPP - Geranylgeranyl pyrophosphate
FAO - Food and Agricultural Organization
IPP - Isopentenyl pyrophosphate
JAs – Jasmonates
JA-Ile - Jasmonoyl-isoleucine
LCY - Lycopene cyclase
LUK – Lukullus
NCED - 9-*cis*-epoxycarotenoid dioxygenase
not – *notabilis*
NSY - Neoxanthin synthase
PA - Phaseic acid
PAR - Photosynthetically active radiation
PEG - Polyethylene glycol
PDS - Phytoene desaturase
PSY - Phytoene synthase
ROS - Reactive oxygen species
RWC - Relative water content
SA - Salicylic acid
SL – Strigolactones
TW - Turgid weight

VPD - Vapor pressure deficit
WT - Wild type
XantDH - Xanthoxin dehydrogenase
ZEP - Zeaxanthin epoxidase
ZDS - ζ-carotene desaturase
Z-ISO - ζ-carotene isomerase
$^{1}O_2$ - Singlet oxigen
[$^{2}H_6$]-ABA (or ***d_6*-ABA**) - deuterium-labelled ABA
[$^{2}H_3$]-PA - Deuterium-labelled PA
[$^{13}C_6$]-SA - Labelled salicylic acid

SUMMARY

Water deficit is one of the most detrimental conditions for plant growth and survival. However, plants have developed several strategies to cope with this condition including the rapid change in signaling compounds such as hormones. Abscisic acid (ABA) is one of the most important hormones which concentration increases upon plant dehydration. Among other effects, ABA accumulation induces stomatal closure to avoid water loss and modifies root architecture to maintain water uptake. For years, it has been assumed that dehydration is perceived by roots which stimulate the ABA increase which is further transported via transpiration stream to leaves to regulate the stomatal aperture. In this thesis, this model is questioned since several lines of evidence indicate that roots could not sustain larger increases of ABA.

In chapter I, it is demonstrated that roots of water-stressed Citrus plants are a sink of ABA rather than a source. Stem-girdled Citrus plants show that leaves are crucial in sustaining the ABA increase in dehydrated roots. Feeding experiment with deuterated ABA evidence that this molecule is basipetally transported from leaves to roots under unstressed conditions and this transport is enhanced upon dehydration. Interestingly, a detailed examination of root carotenoids reveals that the content of the ABA precursors decreases upon dehydration, suggesting that the low content of these precursors could be limiting the ABA accumulation in roots. Therefore, in Chapter II high levels of carotenoids in detached roots are induce to evaluate the ABA accumulation without the involvement of the leaf-sourced ABA. Data indicate that detached roots are unable to increase ABA despite the increased amounts of precursors. This physiological incapacity is independent of numerous changes in gene expression of both biosynthetic pathways: carotenoid and ABA. Finally, results from chapter III, where leaves are maintained at different relative water contents, demonstrate that leaf-dehydration is needed to induce the ABA rise in leaves and roots, and that the mere dehydration of roots is not sufficient to induce ABA accumulation in this organ. Results assign a key role for leaves in sensing the adverse conditions.

Globally, this thesis provides evidence that roots of Citrus plants are not an important source of ABA under water deficit and conversely, leaves sustain the accumulation of ABA in those dehydrated roots. Furthermore, data present here suggest that leaves are key not only in providing ABA to roots but also in triggering the water stress responses which lead to the ABA accumulation in both, leaves and roots.

RESUMEN

La sequía es una de las situaciones más perjudiciales para el crecimiento y la supervivencia de las plantas. Sin embargo, las plantas han desarrollado diversas estrategias para afrontar dichas condiciones, incluyendo la rápida señalización mediada por diversas fitohormonas. Entre ellas, el ácido abscísico (ABA) es una de las principales involucradas en la respuesta a la deshidratación de las plantas, induciendo el cierre estomático, restringiendo la pérdida de agua en hojas o modificando la arquitectura de las raíces para mantener la absorción de agua. Se ha popularizado que las raíces son el órgano capaz de detectar la deshidratación en el substrato, lo que lleva a un incremento en los niveles de ABA, que es posteriormente transportado vía xilema a las hojas, donde induce el cierre de estomas. Sin embargo, numerosas evidencias cuestionan este modelo debido a que entre otras razones, las raíces son incapaces de acumular elevados niveles de ABA. En este contexto, en esta tesis se ha estudiado la capacidad de las raíces en sintetizar localmente ABA y la implicación del transporte desde tejidos aéreos.

En el capítulo I, se demuestra que las raíces de plantas de cítricos sometidas a deshidratación reciben ABA proveniente desde las hojas. Las raíces de plantas con el tallo anillado, que imposibilita el trasporte basípeto por el floema, son incapaces de mantener elevados niveles de ABA en condiciones de deshidratación. Además, experimentos de transporte de ABA deuterado de hojas a raíces evidencian que el ABA es capaz de ser transportado de forma basípeta, y este transporte es más eficiente en condiciones de sequía. Además, el déficit hídrico induce un marcado descenso en los niveles de carotenoides en raíces, lo que podría convertirlos en el factor limitante en el mantenimiento de la síntesis local de ABA. Por ese motivo, en el capítulo II se inducen en raíces separadas de la parte aérea elevados niveles de carotenoides. Dichas raíces son incapaces de acumular ABA en condiciones de deficiencia hídrica, a pesar de la inducción de numerosos genes involucrados en la síntesis de carotenoides y de ABA. Estos resultados evidencian una limitada capacidad de sintetizar ABA localmente y la necesidad del aporte de ABA desde la parte aérea. Finalmente, los resultados en el capítulo III, donde se mantiene la canopia de plantas de cítricos a diferentes niveles de humedad mientras se deshidratan las raíces de forma similar, demuestran el papel de la parte aérea en inducir la acumulación de ABA en raíces. Así, la deshidratación de las raíces no es condición suficiente para desencadenar la acumulación de ABA en dicho órgano y ésta

solo se produce si las hojas se deshidratan. De esta forma, las hojas jugarían un papel fundamental en la percepción de las condiciones ambientales adversas.

En definitiva, esta tesis presenta evidencias que demuestran que bajo condiciones de deficiencia hídrica, las raíces de las plantas cítricas no constituyen una fuente relevante de ABA y por el contrario, la hoja es el órgano responsable de mantener la acumulación de ABA en las raíces deshidratas. Además, la información presentada en esta tesis sugiere que las hojas juegan un papel clave en detectar el déficit hídrico y por tanto en desencadenar la acumulación de ABA en hojas y en raíces.

1. INTRODUCTION

1.1 Citrus worldwide importance

Citrus are considered one of the most important fruit worldwide. Reports indicate that Citrus fruit are being consumed since 22nd century BC in China but the exact origin is still under debate since recent evidence suggests that the true origin of citrus species cultivated today comes from Australia, New Caledonia and New Guinea rather than the tropical and subtropical areas of Southeast Asia such as India and Malay Archipelago as it was believed (Liu *et al*., 2012).

Different species integrate the Citrus genus but species from other closely-related genus from the Rutaceae family are also commonly named as Citrus. There is no consensus about the common ancestral species but it is believed that current species and cultivars have arisen from inter-specific hybridization among parentals Citron (*Citrus medica*), Pumelo (*C. maxima* B. Osb) and mandarin (*C. reticulata* Bl.) and also spontaneous mutations (García-Lor *et al*., 2012). Domestication of citrus has led to the development of a worldwide industry comprising several species including sweet orange (*Citrus sinensis* L Osb.), mandarin (*C. reticulata* Bl.), lemon (*C. limon*) and grapefruit (*C. paradisi* Macf.) among others. In commercial citrus plantations, grafting is usually performed using different scion-rootstock combinations that affect plant development, fruit quality and confer adaptive advantages to different environments (Allario *et al*., 2013; Pedroso *et al*., 2014). In this sense, different citrus genotypes and related genera are commonly used as rootstocks such as trifoliate orange (*Poncirus trifoliata* L. Raf.), Citrange hybrids (Carrizo and Troyer; *C. sinensis* x *P. trifoliata*), Macrophylla (*C. macrophylla* W.) or Cleopatra mandarin (*C. reshni* Hort. Ex Tan.; García-Lor *et al*., 2012; Wu *et al*., 2015).

According to FAO, world citrus fruit production was estimated in 135: Tm last 2013-14 season, with an average production of around 14 Tm/ha. The top citrus producing countries are China, Brazil, USA, India, Mexico and Spain accounting for two thirds of the total world production. Spain is ranked in the sixth place with about 6.3: Tm/year from its 330.000 ha, accounting 4.7% of the worldwide production (Food and Agriculture Organization of the United Nations). Most Spain citrus production (77%) is exported as a fresh produce (Food and Agriculture Organization of the United Nations). Inside Spain, Valencian Community takes a special relevance since it accounts for 60% of the total country area and contributes with 76% of total country exports (GVA, 2015).

Citrus fresh consumption represents about 70% of the total production which is preferred by its characteristic flavor, taste and accessibility. Citrus are also recognized by their nutritional contribution and health-promoting properties (Liu *et al*., 2012). The average citrus intake is about 25 kg/person/year which makes it one of the most important sources of several nutrients, including vitamins C, B (thiamin, pyridoxine, niacin, riboflavin, pantothenic acid, and folate), E (tocopherols and tocotrienols) and provitamin A compounds (carotenoids) as well as phenolic compounds, pectins, limonoids and dietary fibers, contributing to the prevention of diverse chronic diseases (Zou *et al*., 2016)

Citrus crop is produced worldwide in tropical and subtropical regions being exposed to different climatic conditions that notably influence not only nutritional composition (Liu *et al*., 2012) but also plant agronomical behavior (Guo *et al*., 2006). As a response, plants have developed an intricate bund of mechanisms to cope with unfavorable conditions and to acclimate to different environments (Han *et al*., 2009; Argamasilla *et al*., 2013)

1.2. Plant abiotic stresses

Plant abiotic stress is a relative young scientific discipline within the plant physiology, which intends to understand how adverse environmental factors interfaces with plants and how these respond to this interaction through constitutive or adaptive traits (Blum, 2015a). In 1972, Levitt defined several terms concerning abiotic stress and stress resistance. Using an analogy from civil engineering, Levitt defined *stress* as a heavy load (adverse condition) placed in the center of a steel beam positioned on columns on its two edges. The beam will develop the *strain*, which could be plastic (irreversible) o elastic (reversible), being the magnitude of this strain proportionally to the magnitude and duration of the stress and the position of the load on the beam. In biological systems, apart from the concept introduced by Levitt regarding the constitutive parameters of the beam, other aspects should be taken into consideration (Blum, 2015a). In this sense, plants could present an *adaptive* strain tolerance, which is developed in response to a *signal* created by the strain. Plants also could avoid certain induced strains by exerting several responses (Blum, 2015a).

Economic implications of the stress are especially relevant in agronomical crops. From this point of view, plant abiotic stress is defined as the effect of adverse environmental

conditions that constrain or limit plant growth and, therefore, impact on crop yield and productivity (Boyer, 1982). The gap between yields obtained under optimal and suboptimal conditions is usually considered as a magnitude of the stress. However, this largely differs from the concept employed in controlled experimental conditions where other parameters such as plant survival or seed germination are also relevant (Dolferus, 2014).

Research in this discipline has been focused on understanding how plants exhibit abiotic stress responses, a process considered crucial to minimize decreases in production and potentiate crop survival (Hirayama and Shinozaki, 2010). With that aim, plants developed several strategies to reduce the impact of adverse conditions, enhancing the tolerance linked to morphological, physiological and biochemical changes that may lead to a decrease in the stress exposure, limit damage or repair possible damaged systems (Potters *et al*., 2007). Based on it, researchers intend to shed light into tolerance-associated mechanisms and develop strategies to improve plant ability to cope with different stresses. Indeed, it is expected that the occurrence of adverse environmental conditions will increase in the near future due to climate change (Ahuja *et al*., 2010), which represents a threatening scenario for plant resources, biodiversity and global food security (Ahuja *et al*., 2010).

1.2.1 Plant tolerance to abiotic stress

Plants have developed several mechanisms to cope with abiotic stress that involve the activation of different biochemical pathways leading to the production of either defensive compounds or morphological changes. Certain mechanisms are linked to a basal response that results in an adaptation in order to prevent damage from repetitive harmful conditions. This confers tolerance to geographical and temporal adverse conditions. As a result, there was a conservation of certain genome modifications resulting in improved physiological and metabolic defenses towards adaptation to different stress conditions (Clarke *et al*., 2004). Thus, basal tolerance or adaptation is linked to a long evolution process that involves permanent changes over several generations within a population or species. Most relevant cases involves those naturally occurring plants living under extreme conditions which are usually named as halophytes, xerophytes, thermophiles, among others (Minocha *et al*., 2014).

On the other side, induced tolerance or acclimation is displayed by plants to cope with temporary adverse conditions by triggering different mechanisms (Gepstein and Glick, 2013). The magnitude of plant responses to stress depends on the time of exposure and on the intensity of the adverse conditions (Syvertsen and Garcia-Sanchez, 2014), which evidences the existence of a fine-tuning to allow sensing and responding to different types and levels of stress (Claeys *et al*., 2014). Acclimation involves a cascade of events starting with the stress perception and resulting in the expression of target genes (Pastori and Foyer, 2002). This leads to the activation of several pathways, which often are induced under different stress situations (cross-tolerance), allowing acclimation to a wide range of stresses (Pastori and Foyer, 2002). These responses involve the production of several metabolites such as osmoprotectants, antioxidants and phytohormones (Peleg and Blumwald, 2011).

1.2.3. Water stress

The most relevant abiotic stresses affecting plants are drought, salinity, waterlogging, extreme temperatures, high light, and heavy metals, but it is also common that, at least, two different adverse conditions occur simultaneously (Chaves *et al*., 2009; Hirayama and Shinozaki, 2010; Sreenivasulu *et al*., 2012). Among them, drought is by far the most important abiotic stress condition, which severely constrains yield leading to important economic losses in different crops (Cattivelli *et al*., 2008; Medici *et al*., 2014). Plants experience drought when water supply becomes scarce and/or when the transpiration rate becomes higher than the rate of water uptake from roots (Reddy *et al*., 2004; Cattivelli *et al*., 2008). Nowadays and, under the future scenario of climate change, breeders have identified that the improvement of yield and crop productivity under drought conditions is one of the main goals to achieve stable yields but also to encourage the introduction of new crops in dry/arid environments (Cattivelli *et al*., 2008). Apart from timing and intensity, plant response to drought is strongly dependent on the genotype (Cattivelli *et al*., 2008; Katerji *et al*., 2008). Breeders and researchers use the tolerance variability with the aim of identifying physiological and morphological characteristics, but also biochemical and molecular changes associated with water stress tolerance (Cattivelli *et al*., 2008). In general, plants subjected to dehydration have developed strategies to increase water uptake and limit water losses (Kudoyarova *et al*., 2011) such as increasing root water

conductance (Comas *et al*., 2013) or decreasing stomatal aperture (McAdam and Brodribb, 2015). It has been demonstrated that a higher production of reactive oxygen species (ROS) takes place under water deficit (Noctor *et al*., 2014); therefore, plants also develop mechanisms to repair any possible damage such as the induction of ROS-scavenging enzymes (Osakabe *et al*., 2014a). Some of the above mentioned strategies to prevent damage or acclimate to water stress are directly triggered by changing water status of the different tissues (Ache *et al*., 2010; McAdam *et al*., 2016) but other changes are mediated by plant hormones, which are responsible for the adjustment and maintenance of plant tolerance and survival (Fujita *et al*., 2011; Peleg and Blumwald, 2011; Bartoli *et al*., 2013).

1.3. Plant hormones

Plant hormones, or phytohormones, are a diverse group of natural metabolites with a low molecular weight that act at micromolar or even lower concentrations and exert a crucial role on different physiological and developmental processes of the plant´s life cycle (Piotrowska and Bajguz, 2011). There are different groups of phytohormones that notably vary in molecular structure and functional properties being named as abscisic acid (ABA), gibberellins (GAs), cytokinins (CKs), ethylene (ET), auxins, jasmonates (JAs), salicylic acid (SA), and the recently discovered brassinosteroids (BR) and strigolactones (SL; Piotrowska and Bajguz, 2011). Most of these compounds exhibit biological activity triggering or modulating a very complex signaling network at cellular level. Research on mutants, inhibitors of biosynthesis or action have contributed to enlarge the current knowledge about the extensive and intricate functions of phytohormones (Gómez-Cadenas *et al*., 2014). Indeed, metabolic pathways for the biosynthesis, transport, conjugation and catabolism of the most important phytohormones have been successfully characterized. Moreover, specific receptors and signal transduction pathways have been characterized and recent evidences reveals an intricate interconnection and crosstalk among different phytohormones (Gómez-Cadenas *et al*., 2014).

Plant hormones are involved in the control of all developmental stages of plant growth including germination, seed and bud dormancy, flowering, fruit ripening and senescence among others (Kato *et al*., 2006; Piotrowska and Bajguz, 2011; Nitsch *et al*., 2012; Ye *et al*., 2012). Phytohormones also play a key role in triggering responses to a wide range of adverse environmental conditions which is essential to improve or achieve stress tolerance

(Kurepin *et al*., 2015). In this sense, modifications in phytohormones concentration is one of the first changes that takes place under stress conditions which may lead to stress avoidance or acclimation (Peleg and Blumwald, 2011; Golldack *et al*., 2013). However, the exact role of phytohormones under each stressful condition needs to be considered in the context of a tissue or even at the cellular level (Piotrowska and Bajguz, 2011). In this sense, particular changes in hormone concentration are associated to specific biochemical or metabolic process that takes place in certain tissues such as occurs with the role of ABA in leaf stomatal closure (Bauer *et al*., 2013). Nevertheless, plant responses to stressful situations involve a coordination at the entire plant level, where hormone transport from different plant tissues plays a crucial role (Crawford *et al*., 2010; Shkolnik-Inbar and Bar-Zvi, 2010).

1.3.1. Abscisic acid, discovery and main roles

Abscisic acid is one of the five classical plant hormones that regulates many aspects of plant growth and development but ABA also plays an important role in plant response to adverse conditions and therefore is commonly named the 'stress hormone' (Freundl *et al*., 2000; Kuromori and Shinozaki, 2010; Shatil-Cohen *et al*., 2011). From its discovery in the 1960's, ABA was considered a potent growth inhibitor. ABA was firstly isolated by Ohkuma *et al*. (1963) whereas its structure was also reported in 1965 by Ohkuma and Addicott group (Dörffling, 2015). During that decade, ABA was extracted and identified in multiple laboratories and since it was closely linked to dormancy researchers called it 'Dormidin' meanwhile other groups revealed its role in fruit abscission, naming it as 'Abscisin II' (Cutler *et al*., 2010). A later accurate identification of this substance revealed that both functions were carried out by the same molecule and after deliberations *Abscisic Acid* was chosen as the most suitable name (Addicott and Lyon, 1969). Since then, biochemical, molecular and genomic approaches were developed with the aim of elucidating the ABA biosynthetic and catabolic pathways, identifying any possible ABA transporters and shedding some light into the complex signaling components associated to the ABA response (Hirayama and Shinozaki, 2007). Moreover, the discovery of the ABA receptors and other genes involved in ABA downstream signaling cascade constituted an important milestone that deepened the understanding of ABA mode of action (Sreenivasulu *et al*., 2012).

ABA is ubiquitous in plant cells but its levels change depending on the plant's growth and developmental stage. Recently, transcriptome analysis showed that more than 10% of the *Arabidopsis* genome was transcribed when plants were exposed to ABA, showing a similar number of up or downregulated genes (Nemhauser *et al*., 2006), revealing the existence of widely diverse functions of ABA in plants (Cutler *et al*., 2010). ABA effects on plants were initially counterpoised to those exerted by plant growth promoters such as GAs and Auxins, since ABA was related to growth inhibition processes (Cutler *et al*., 2010). Among them, seed dormancy is one of the best examples of how plants integrate the environmental cues to induce dormancy but also to prevent the germination during adverse periods (Rodríguez-Gacio *et al*., 2009). Similarly, induction of bud dormancy in deciduous trees mediated by photoperiod shortening is accompanied by ABA accumulation, also inducing growth cessation in the apical bud and the correlative inhibition of the lateral shoot formation (Zheng *et al*., 2015). Leaf and cell growth are also affected by ABA which has a direct influence in plant size and biomass accumulation (Hancock *et al*., 2011; Nitsch *et al*., 2012). ABA also plays a key role on fruit growth and abscission and enhances fruit color in certain species with relevant consequences in pollinator attraction and therefore, in plant reproduction (Cantín *et al*., 2007; Nitsch *et al*., 2012; Soto *et al*., 2013).

Although ABA mediates the response to biotic stress during pathogen attack (Cutler *et al*., 2010; Lee and Luan, 2012) it is mainly recognized by its active role in the response to abiotic stresses (Ye *et al*., 2012). Plants subjected to stressful conditions usually modify ABA concentration in different tissues and cells (Piotrowska and Bajguz, 2011). Stomatal closure is one of the best recognized physiological consequences of many of these stresses such as drought, salinity, heavy metals and others (Ye *et al*., 2012) and indeed it is recognized as an accurate parameter to indicate changes in the ABA content (Aliniaeifard and van Meeteren, 2013; Dodd, 2013; Lee *et al*., 2013). ABA also regulates plant growth under water deprivation; ABA is a negative regulator of shoot growth (Ye *et al*., 2012) but also stimulates root development or modifies its architecture in order to achieve wetter zones, helping to maintain water uptake and status in the whole plant (Cutler *et al*., 2010). ABA is also involved in the accumulation of compatible osmolytes and in the synthesis of dehydrins and other LEA proteins, which are crucial in the acclimation to water deficit maintaining cell turgor and protecting proteins and membranes (Cutler *et al*., 2010; Kurepin *et al*., 2015).

1.3.2. ABA biosynthesis

1.3.2.1. Synthesis of ABA precursors: carotenoid pathway

The concentration of ABA in a specific tissue during a given period of time results from the tightly coordination of biosynthesis, conjugation, transport and catabolism (Ye *et al*., 2012). After its identification as an isoprenoid in the 1960´s (Fig. 1) biochemical approaches and the use of carotenoid-deficient mutants suggested that ABA biosynthesis in plants takes place from carotenoid precursors (Liotenberg *et al*., 1999). In plants, ABA biosynthesis follows the 'indirect' pathway which involves the isopentenyl pyrophosphate (IPP), a C_5 molecule which is the precursor of all terpenoids and other plant hormones such as GAs (Ye *et al*., 2012; Fig. 1). Carotenoid biosynthesis takes place entirely in the plastid using IPP derived from glyceraldehyde 3-phosphate and pyruvate (Liotenberg *et al*., 1999). IPP is converted into geranylgeranyl pyrophosphate (GGPP), a C_{20} product by IPP isomerase and GGPP synthase enzymes (Liotenberg *et al*., 1999). GGPP constitutes a key branching point from isoprenoid biosynthesis in plants whose synthesis is controlled by different GGPP synthase isoforms (Thabet *et al*., 2012). The first committed step of carotenoid biosynthesis, catalyzed by phytoene synthase (PSY) is the head-to-head condensation of two molecules of GGPP to form phytoene (C_{40}). Successive desaturation and isomerization mediated by the enzymes phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) are needed to form lycopene (Cazzonelli and Pogson, 2010), a linear carotenoid and key branching point in the carotenoid pathway. From this point, carotenoid biosynthesis is divided into the β,ε- and β,β- branches by the action of two lycopene cyclases (LCY), εLCY and βLCY (Cazzonelli and Pogson, 2010). In the former, lycopene is transformed into α-carotene and subsequently into lutein, a typical carotenoid from green sun-exposed tissues (Ramel *et al*., 2012). The β,β-branch, which at the end provides precursors for ABA synthesis, involves the conversion of lycopene into β-carotene by the formation of two β-rings at each end of the molecule (Frey *et al*., 2006). Both β-rings of β-carotene are then hydroxylated into β-cryptoxanthin and zeaxanthin by the enzyme β-carotene hydroxylase (βCHX) leading to the production of xanthophylls, key ABA precursors (Liotenberg *et al*., 1999). Zeaxanthin is the first carotenoid of the pathway involved in the 'xanthophyll cycle' which plays multiple relevant functions in photosystems providing tolerance to excess light and preventing photoinhibition (Demmig *et al*., 1988; Schwarz *et al*., 2015). Zeaxanthin is then converted to violaxanthin by two successive epoxidation

reactions via antheraxanthin by zeaxanthin epoxidase enzyme (ZEP; Chen and Gallie, 2012). Violaxanthin could be then converted into neoxanthin by means of the neoxanthin synthase (NSY; North *et al*., 2007; Neuman *et al*., 2014).

1.3.2.2. ABA biosynthetic pathway

In contrast to the carotenoid pathway which occurs only in plastids, ABA biosynthesis from xanthoxin takes place both in the plastids and cytosol (Fig. 1). First steps occur from the carotenoid isomers neoxanthin and violaxanthin, both in the configuration 9-*cis*, that serves as substrate for the 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes to synthetize xanthoxin (Nambara and Marion-Poll, 2005; North *et al*., 2007). The contribution of each of these substrates to the ABA accumulation remains to be elucidated, but the relative importance of 9-*cis*-neoxanthin and 9-*cis*-violanxanthin in ABA biosynthesis could be tissue-specific (Rodrigo *et al*., 2006). Nevertheless, NCED activity is considered the main bottleneck step in the ABA synthesis (Nambara and Marion-Poll, 2005) and, probably for this reason, several NCED genes are present in different species: five in Arabidopsis (Wang *et al*., 2015; Zhang *et al*., 2015) and two in citrus (Rodrigo *et al*., 2006; Agustí *et al*., 2007). However, only some of them are strongly linked to ABA accumulation (Wang *et al*., 2015) whereas the others seem to play an secondary role (Rodrigo *et al*., 2006). Xanthoxin is then extruded to the cytosol and oxidized into abscisic aldehyde by a short-chain alcohol dehydrogenase (Xanthoxin dehydrogenase, XantDH; González-Guzmán *et al*., 2002) which is then converted into ABA by the ABA aldehyde oxidase3 (AAO3), using a molybdenum cofactor activated by the molybdenum cofactor sulfurase (ABA3; Nambara and Marion-Poll, 2005). Alternatively, in plants where there is a shunt pathway in the abscisic aldehyde oxidation (AAO3) such as in the case of ABA-deficient tomato mutant *flacca* (*flc*), a substitute conversion to ABA-alcohol could still provide a significant amount of ABA (Rock *et al*., 1991).

1.3.2. ABA catabolism

As indicated above, ABA accumulation in tissues depends on several factors, in which biosynthesis is the main responsible for the rapid increase under stress conditions but also

catabolism could regulate the ABA homeostasis in plants (Priest *et al*., 2006). Main degradation pathway occurs via 8'-hydroxilation which renders 8'hydroxy-ABA (8'OH-ABA) in a reaction catalyzed by the cytochrome P450 monooxygenase ABA 8'-hydroxilase (CYP707a; Fig. 1). The 8'OH-ABA is spontaneously isomerized to phaseic acid (PA) and subsequently reduced to dihydrophaseic acid (DPA), the end-product of ABA degradation (Seiler *et al*., 2011; Pacifici *et al*., 2015). Regulation of ABA catabolism by plants is crucial to sustain the ABA-induced tolerance in plants exposed to detrimental conditions damage (Cai *et al*., 2015). In this sense, in roots of waterlogged plants ABA catabolism mediates a sharp decrease in the ABA levels (Chen *et al*., 2010) which in some species is needed to induce the shoot growth conferring tolerance to this condition (Van Der Straeten *et al*., 2001). In this sense, it is know that ABA is rapidly metabolized to PA under unstressed conditions and the amount of catabolic metabolites depends on the ABA levels (Ren *et al*., 2007). However, the type of the stress highly conditions the rate of ABA catabolism, being sharply triggered in plants subjected to waterlogging (Chen *et al*., 2010) and reduced under dehydration (Huang *et al*., 2008).

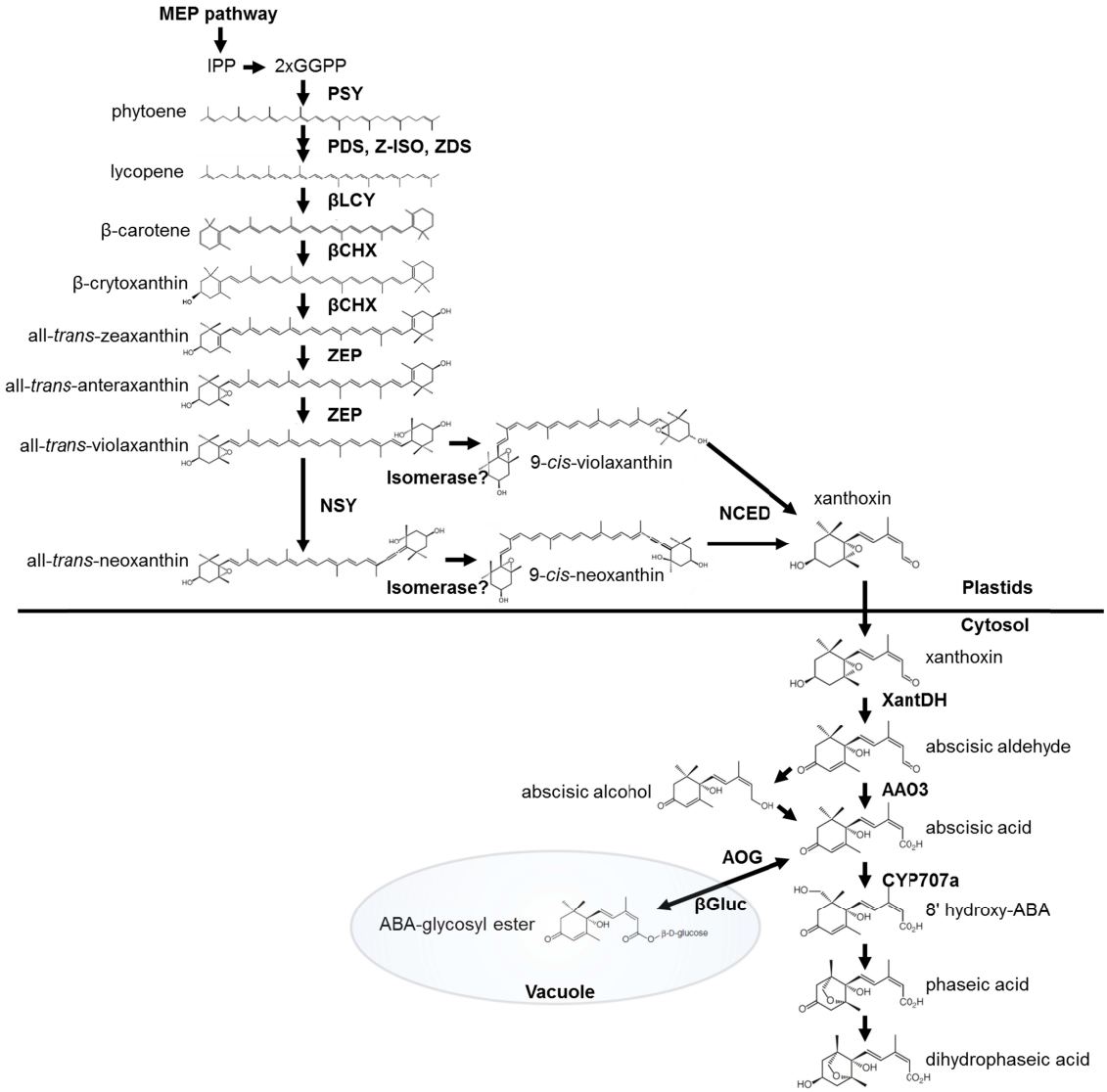

Figure 1. Biosynthesis, catabolism and conjugation of abscisic acid in plants. Enzymes are representing in bold; phytoene synthase (PSY); phytoene desaturase (PDS); ζ-carotene isomerase (Z-ISO); ζ-carotene desaturase (ZDS); βlycopene cyclase (βLCY); β-carotene hydroxylase (βCHX); zeaxanthin epoxidase enzyme (ZEP); neoxanthin synthase (NSY); 9-*cis*-epoxycarotenoid dioxygenase (NCED); xanthoxin dehydrogenase (XantDH); ABA aldehyde oxidase3 (AAO3); ABA 8'-hydroxilase (CYP707a); UDP-glucosyltransferase (AOG); β-glucosidase (βGluc). Isomerases are still uncharacterized.

1.3.3. ABA Conjugation and hydrolysis

ABA could be conjugated at the carboxyl group with different compounds that are accumulated in the vacuole or in the apoplast (Dietz *et al*., 2000). Among them, ABA glucosyl ester (ABA-GE) is the most abundant deriving from the action of a cytosolic UDP-glucosyltransferase (AOG, Fig. 1; Priest *et al*., 2006). This is an inactive form and

was considered as a catabolite since there were no evidences of an ABA-GE cleavage to release free ABA (Priest *et al*., 2006). Indeed, no changes in ABA-GE levels were found during some stresses, which reinforced the inactivity of this molecule stored in vacuoles as an end product of ABA metabolism (Priest *et al*., 2006; Piotrowska and Bajguz, 2011). However, a few years later it was shown that ABA is actually released from ABA-GE by the action of a β-glucosidase (βGluc), being crucial in ABA homeostasis (Lee *et al*., 2006). ABA-GE is, therefore, an effective manner developed by plants to regulate active ABA levels. Nevertheless, it has been also proposed as an ABA transport form (Vilaró *et al*., 2006; López-Carbonell *et al*., 2009) since it was found at high concentration in the xylem sap of water stressed plants. Therefore, ABA-GE was suggested as a possible root-to-shoot symplastic transport molecule which could be then cleaved in the target cells to release ABA (Sauter *et al*., 2002).

1.4. ABA transport

ABA movement throughout the plant has been the focus of different works (Sauter *et al*., 2001; Seo and Koshiba, 2011) in order to shed light into the main tissues and organs responsible for its biosynthesis or accumulation in target cells (Hoad, 1995). ABA free form and ABA-GE are considered the mobile molecules for ABA long-distance transport (Pérez-Alfocea *et al*., 2011), although the real involvement of ABA-GE in this process still remains to be elucidated (Nambara and Marion-Poll, 2005; Piotrowska and Bajguz, 2011). Free ABA transport has been largely studied in diverse metabolic processes such as seed dormancy, where vegetative tissues provide ABA to the forming embryo during seed maturation (Kanno *et al*., 2010). Similarly, ABA is exported and imported from shoots to developing fruits via phloem where it induces fruit softening and pigment accumulation (Else *et al*., 2004; Luo *et al*., 2013).

ABA transport appears to be relevant under stress conditions such as salinity (Osakabe *et al*., 2014b) or water stress (Dodd, 2005). Information regarding root-to-shoot ABA transport under dehydration has been largely provided considering its crucial involvement in the stomatal closure process (Ye *et al*., 2012; Chen *et al*., 2013; Jones, 2015). Since roots are responsible for the water uptake they are believed to be in charge of sensing the decrease in soil water potential and therefore, in triggering water stress responses (Fig. 2; Zhang and Davies, 1989; Wilkinson and Davies, 2002; Schachtman and Goodger, 2008;

Tardieu *et al*., 2010; Jones, 2015; Tardieu *et al*., 2015; Blum, 2015b). However, the involvement of the root-sourced ABA on the stomatal closure is not conclusive (Pérez-Alfocea *et al*., 2011; Dodd, 2013) since it could be interplaying with other factors that simultaneously regulate the stomatal aperture such as hydraulic signals (Soar *et al*., 2006; Christmann *et al*., 2007; Tardieu *et al*., 2010; Jones, 2015; Blum, 2015b), leaf water potential (Dodd, 2013), hydraulic conductance (Kudoyarova *et al*., 2011; Rodríguez-Gamir *et al*., 2011; Dodd, 2013; Stevenick, 2013) or even other phytohormones (Pérez-Alfocea *et al*., 2011; Chen *et al*., 2013; Puertolas *et al*., 2014; McAdam *et al*., 2016).

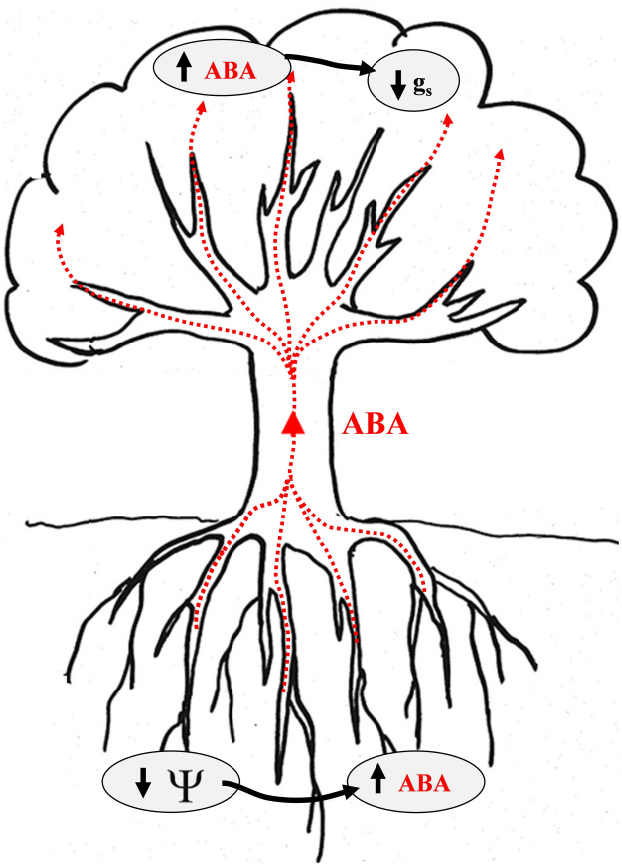

Figure 2. Schematic representation of the root-to-shoot model. Plant roots detected the decrease in soil water potential and triggers the ABA synthesis in this organ. ABA is transported via xylem sap to leaves inducing the stomatal closure to prevent water loss.

In the context of water deficit, two main factors act interfering with the supposed root-to-shoot ABA: the first relies on the ability of dehydrated leaves to increase ABA concentration in this organ. This was demonstrated using reciprocal grafting of ABA-

deficient mutants as rootstocks where leaves notably increased ABA concentration without any implication of the roots (Holbrook *et al*., 2002; Thompson *et al*., 2007). Moreover, ABA increase in dehydrated leaves could be triggered autonomously by the local conditions affecting the leaves (Aliniaeifard and van Meeteren, 2013; Bauer *et al*., 2013) even in the absence of roots (Pérez-Clemente *et al*., 2012). The second factor arises from the low efficiency of belowground tissues to accumulate ABA precursors (carotenoids). Accumulation of ABA precursors is highly responsive to light (Toledo-Ortiz *et al*., 2010) and carotenoid levels in dark-grown roots are almost negligible (0.1-0.2%) compared to those found in leaves (Ruiz-Sola *et al*., 2014; Walter *et al*., 2015). Indeed, plastids found in roots such as proplastids or leucoplasts are not specialized in carotenoid accumulation (Howitt and Pogson, 2006; Li and Yuan, 2013). Therefore, this shortage of carotenoids could limit the ability of roots to supply high levels of ABA to leaves where actually large amounts of the phytohormone are detected under water stress (Cornish and Zeevaart, 1985; Correia *et al*., 2014). Thus, taking these two proofs into consideration, the real relevance of the root-sourced ABA in inducing the stomatal closure has been questioned (Dodd, 2005; Goodger and Schachtman, 2010; Torres-Ruiz *et al*., 2014) as also was the ability of the roots to synthesize large amounts of this phytohormone under stressful conditions (Goodger and Schachtman, 2010; Sreenivasulu *et al*., 2012; Walter *et al*., 2015).

In this sense, evidences from herbaceous plants (Arabidopsis, tomato and bean seedlings) suggest that ABA could be basipetally transported (Cornish and Zeevaart, 1985; Vernieri *et al*., 2001; Ikegami *et al*., 2009; Waadt *et al*., 2014). Although this concept has not completely rooted among scientists, it could help to explain the high levels of ABA observed in water-stressed roots considering the limited availability of precursors in this organ (Ruiz-Sola *et al*., 2014). Moreover, the disruption of basipetal flow led to a reduction of ABA accumulation in dehydrated roots (Cornish and Zeevaart, 1985; Vernieri *et al*., 2001) suggesting a possible role of shoot-sourced ABA in water stress response.

Based on the previous background, the real contribution of the ABA produced in roots and its relationship with leaf-ABA during water stress still remains elusive. This work evaluates the global changes in ABA metabolism in roots of citrus plants during water stress with special emphasis on the carotenoid contribution to ABA accumulation and explores the possible role of the ABA transport between organs in this process.

2. OBJECTIVES

The main objective of this work consists in evaluating the involvement of different organs in ABA metabolism and water-stress perception in citrus plants.

To achieve this goal, four partial objectives were planned:

1. Demonstrate the ability of roots and shoots of *Citrus* plants to support ABA accumulation under dehydration and explore the changes in root-carotenoid levels in this condition.

2. Explore whether ABA basipetal transport from shoot-to-root is taking place and consequently, if it is contributing to the ABA accumulation during water stress.

3. Evaluate the real dependence of roots on ABA precursors (carotenoids) to synthesize ABA during dehydration

4. Explore whether the water status of leaves could be conditioning ABA accumulation in dehydrated roots.

3. RESULTS

3.1. Chapter I.

Root ABA Accumulation in Long-Term Water-Stressed Plants is Sustained by Hormone Transport from Aerial Organs.

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Abstract

The reduced pool of the abscisic acid (ABA) precursors, β,β-carotenoids, in roots does not account for the substantial increase of ABA content in response to water stress (WS) conditions, suggesting that ABA could be transported from other organs. Basipetal transport was interrupted by stem-girdling and ABA levels were determined in roots after two cycles of WS induced by transplanting plants to dry perlite. Leaf applications of isotope-labelled ABA and reciprocal grafting of ABA-deficient tomato mutants were used to confirm the involvement of aerial organs on root ABA accumulation. Disruption of basipetal transport reduced ABA accumulation in roots and this decrease was more severe after two consecutive WS periods. This effect was linked to a sharp decrease in the β,β-carotenoid pool in roots in response to water deficit. Significant levels of isotope-labelled ABA were transported from leaves to roots, mainly in plants subjected to water dehydration. Furthermore, the use of different ABA-deficient tomato mutants in reciprocal grafting combinations with wild-type genotypes confirmed the involvement of aerial organs in the ABA accumulation in roots. In conclusion, accumulation of ABA in roots after long-term WS periods largely relies on the aerial organs suggesting a reduced ability of the roots to synthesize ABA from carotenoids. Furthermore, plants are able to basipetally transport ABA to sustain high hormone levels in roots.

Keywords: ABA-GE; basipetal transport; carotenoids; drought; girdling; water deficit

Introduction

Plants have evolved different physiological, biochemical and molecular mechanisms to cope with abiotic stress. Under adverse conditions such as drought, abscisic acid (ABA) is the main phytohormone responsible for the regulation of key processes such as stomatal closure (Bright *et al*. 2006, Jiang and Hartung 2008), senescence (Finkelstein 2013) and root elongation and architecture (Luo *et al*. 2014, Thole *et al*. 2014). ABA accumulation in plant tissues is one of the first responses observed once water deficit is perceived. ABA synthesis is suggested to take place in several plant organs such as leaves and roots, but also in vascular tissues, and it can move to target cells through both xylem and phloem, allowing a two-way transportation between roots and shoots (Boursiac *et al*. 2013, Finkelstein 2013, Mittler and Blumwald 2015).

It is widely accepted that the reduction in soil water availability is perceived by roots, triggering ABA synthesis as a response to decrease root water potential (Puértolas *et al*., 2013), which is further transported via xylem sap to leaves (Correia *et al*. 2014, Dodd *et al*. 2009, Li *et al*. 2011, Wang *et al*. 2012). However, the physiological relevance of this root-sourced ABA on the stomatal closure has been questioned (Dodd 2005, Goodger and Schachtman 2010, Torres-Ruiz *et al*. 2014), since recent evidences indicate that ABA produced in leaves is sufficient to induce stomatal closure (Finkelstein 2013). Moreover, experiments using reciprocal grafting between wild-type (WT) and ABA-deficient mutants demonstrated that ABA produced in leaves is the main contributor to the total shoot hormone titer, meanwhile root-sourced ABA has a lesser contribution (Albacete *et al*. 2015, Christmann *et al*. 2007, Dodd *et al*. 2009, Holbrook *et al*. 2002). Similarly, detached Citrus shoots in absence of roots were able to accumulate ABA and to trigger several ABA-related responses under osmotic stress conditions (Pérez-Clemente *et al*. 2012).

De novo ABA synthesis is the main mechanism responsible for the accumulation of ABA pool in response to water deficit (Christmann *et al*. 2005). The biosynthesis of ABA precursors (C40 carotenoids) takes place in plastids (Fig. 1) where β-carotene is converted through several enzymatic steps into the β,β-xanthophylls, 9-*cis*-neoxanthin and 9-*cis*-violaxanthin (9-*cis*-epoxycarotenoids, Nambara and Marion-Poll 2005). The enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the conversion of both 9-*cis*-neoxanthin or 9-*cis*-violaxanthin into xanthoxin, in the rate limiting step of ABA biosynthesis (Wang *et al*. 2015). Xanthoxin is then extruded to the cytosol where the final

steps of ABA biosynthesis take place (Nambara and Marion-Poll 2005, Thompson *et al*. 2007). Additionally to the de novo synthesis and the transport among organs, active ABA pool is also determined by its catabolism to phaseic acid (PA) and subsequently to dihydrophaseic acid (DPA, Seiler *et al*. 2011) and also by the ABA turnover with conjugated forms, mainly as ABA-glycosyl ester (ABA-GE, Goodger and Schachtman 2010, Priest *et al*. 2006).

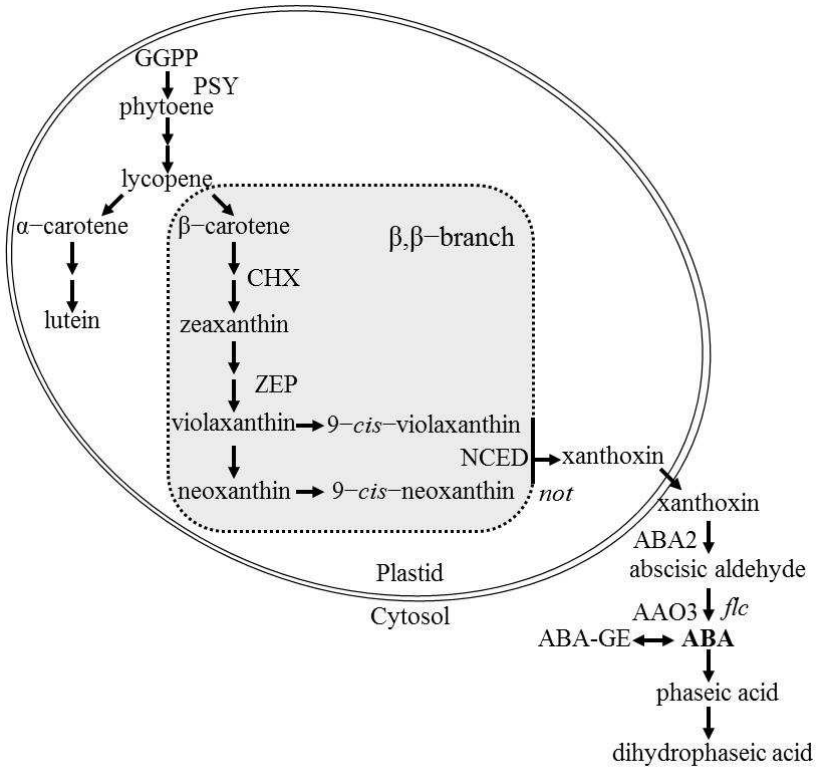

Figure 1. Schematic diagram of the biosynthesis pathway of carotenoids and abscisic acid. Dotted box indicates β,β−branch carotenes. Abbreviations GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; CHX, β-carotene hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ABA2, xanthoxin dehydrogenase; AAO3, abscisic aldehyde oxidase; ABA-GE, ABA-glycosyl ester; *not*, *notabilis*; *flc*, *flacca*. *not* and *flc* ABA-deficient mutants used in this work are indicated in the step affected.

The absence of a clear evidence of the contribution of root-sourced ABA in leaves have redirected the discussion to the real ability of the roots to synthesize this phytohormone under water deficit (Goodger and Schachtman 2010, Sreenivasulu *et al*. 2012, Walter *et al*. 2015). The synthesis of carotenoids (the ABA precursors) takes place in plastids, being a highly light-responsive process (Toledo-Ortiz *et al*. 2010). Indeed, carotenoid

accumulation in roots growing in dark conditions is almost negligible (0.1-0.2%) compared to that registered in green tissues (Ruiz-Sola *et al*. 2014, Walter *et al*. 2015). Furthermore, carotenoids are synthesized in several plant tissues; however, their accumulation depends on the plastid ability to sequester these pigments in defined sink structures (Li and Yuan 2013). In roots the main plastids are proplastids and leucoplasts which, in contrast to chloroplast and chromoplasts (primarily found in leaves and fruits), are not specialized in carotenoid accumulation (Howitt and Pogson 2006, Li and Yuan 2013).

Similar to its precursors, ABA concentration in leaves is notably higher (12 to 40 times) than those in roots under both normal and stressed conditions (Cornish and Zeevaart 1985, Correia *et al*. 2014). However, ABA accumulation in water-stressed roots is unexpected due to the limited carotenoid levels in this organ (Ruiz-Sola *et al*. 2014). This suggests that the ABA accumulation could partially depend on other sources rather than on de novo synthesis in the roots. Despite the possible limited ability of roots to synthesize ABA, this phytohormone displays a key role in this organ, being part of the early responses to water stress (root elongation and maintenance of hair density) that contribute to plant survival (Xu *et al*. 2013).

Recently, it has been suggested that ABA could be transported from the aerial organs to the roots (Ikegami *et al*. 2009, Waadt *et al*. 2014). Furthermore, other reports have demonstrated that ABA levels decrease in roots when the basipetal flow through the phloem is blocked or impaired (Cornish and Zeevaart 1985, Vernieri *et al*. 2001), indicating a direct influence of the aerial organs on ABA accumulation in the roots upon stress. However, these works did not explore the changes in carotenoids under different stress regimes and whether the reduced levels of these molecules in roots may be a limiting factor for *de novo* ABA synthesis during the water stress response. An additional level of complexity may be also considered since it has been speculated that the movement of ABA could take place as a conjugated form (Goodger and Schachtman, 2010). Results from herbaceous Arabidopsis, tomato and bean seedlings (Cornish and Zeevaart 1985, Ikegami *et al*. 2009, Vernieri *et al*. 2001) suggest the existence of a basipetal ABA transport under water stress conditions. Whether a similar mechanism may also operate in woody plants is currently unknown. In this case, investigations should consider the longer distance existing between green tissues and roots in woody plants and not only the structural and anatomical differences (Popko *et al*. 2010). To this respect,

Citrus has been successfully used as a tree model for studying plant responses to environmental stress (de Ollas *et al*. 2013, Gómez-Cadenas *et al*. 1996, Pérez-Clemente *et al*. 2012).

In the current study, an essential contribution of ABA synthesized in leaves to the final hormone pool in the roots upon water stress is hypothesized. Experiments designed to test this hypothesis include the inhibition of the basipetal transport by girdling and exogenous application of isotopically-labelled ABA to the leaves. Furthermore, changes in carotenoids in the roots during a water deficit period and subsequent recovery were analyzed. The basipetal ABA transport was further investigated in tomato plants by using ABA-deficient mutants. Reciprocal grafting of WT plants and ABA-deficient mutants confirmed the crucial shoot and root interaction in the transport and accumulation of ABA.

Results

Responses of citrus seedlings to water stress and recovery treatments

Water content. Carrizo citrange plants were subjected to two consecutive periods of severe water stress interrupted by a three day-period of water recovery. Previous to sampling, water status (RWC) was monitored. RWC of leaves and roots was similar between intact (WS) and stem-girdling (WS+GD) plants throughout the whole experimental period (Fig. 2). After the initial decline of water availability, RWC in leaves of WS and WS+GD plants dropped to 52.6±9.4% and 51.9±8.0%, respectively (day 3). After irrigation, the RWC in leaves of stressed plants was recovered to achieve values similar to that of non-stressed controls (day 6). After the second period of stress, RWC dropped again to 44.7±2.5% and 43.3±6.3% in WS and WS+GD, respectively (Fig. 2a). In roots, this parameter followed a pattern similar to that recorded in leaves. After the initial exposure to water deficit, RWC in roots of WS and WS+GD plants decreased in a similar way (day 3). After re-watering, RWC levels in leaves recovered to control values and, again, upon the second period of water shortage, RWC similarly decreased in WS and WS+GD (Fig. 2b). RWC in roots and leaves from control treatments (CT and CT+GD) remained unaltered throughout the experiment. These results indicate that the putative differences in hormonal content and other responses to water stress between WS and WS+GD plants are not likely due to differences in water content.

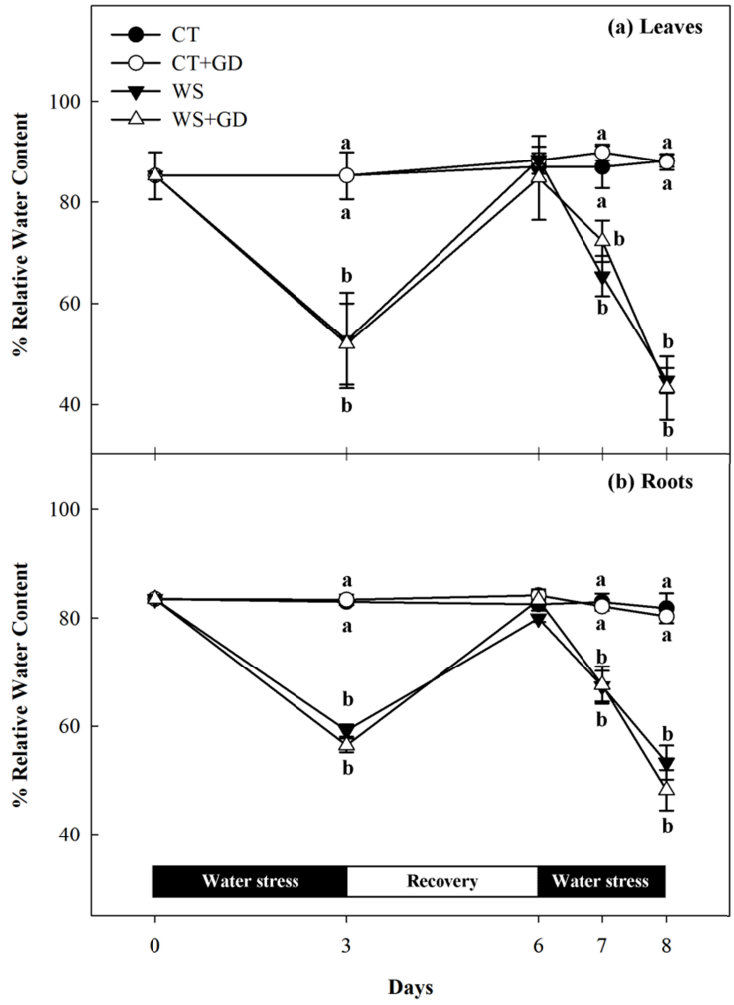

Figure 2. Relative water content (%) in leaves (a) and roots (b) of Citrus intact or stem-girdled plants subjected to two periods of water stress. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

ABA content. ABA content was determined in leaves and roots of control (CT and CT+GD) and water-stressed plants (WS and WS+GD; Fig. 3). Girdling did not induce differences in ABA content neither in leaves or roots. Moreover, it did not alter other stress-related hormones such as jasmonates (jasmonic acid and jasmonoyl-isoleucine) or salicylic acid at the initial date of sample collection (day 0, data not shown). ABA content in roots and leaves from both well-watered plants (CT and CT+GD) remained almost unchanged along the experiment without significant differences between girdled and non-girdled plants (Fig. 3). Contrastingly, in stressed plants, leaf ABA concentration increased 11-times respect to controls after three days of stress (Fig. 3a). Three days after rehydration (day 6) leaf ABA content dropped to control values. A second period of water

shortage induced a new ABA accumulation in leaves of girdled (WS+GD) and non-girdled (WS) plants (5-fold with respect controls at day 8) without significant differences between WS and WS+GD plants at any time point. However, stem-girdling modified the ABA profile in roots of stressed plants (WS+GD) respect to intact ones (WS). Hence, girdling reduced root ABA levels in WS plants (WS+GD) to 50% respect to non-girdled (WS) seedlings during the first stress period (day 3). After recovery, root ABA levels in both groups of plants returned to unstressed control values (Fig. 3b). During the second period of stress, there was a new marked boost in ABA content in roots of WS plants, reaching 868±63 ng g^{-1} at day 8, similar to those found after the previous water stress period (day 3). However, ABA content in roots of WS+GD plants remained unaltered at very low levels, similar to those observed in roots of control plants (Fig. 3b).

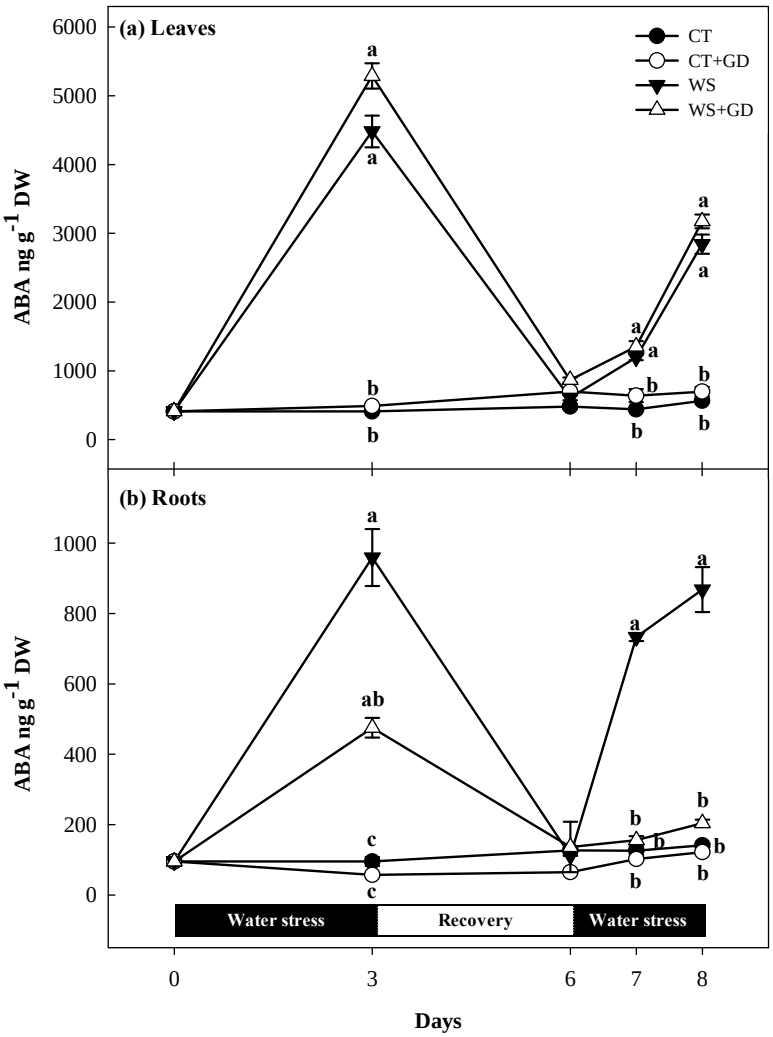

Figure 3. ABA accumulation in leaves (a) and roots (b) of Citrus intact or stem-girdled plants subjected to two periods of water stress. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

Carotenoid content and composition. In order to understand the differences in ABA levels in roots among treatments, the immediate metabolic precursors of the ABA biosynthetic pathway, the β,β-branch carotenoids, were quantified in citrus roots (Fig. 4). Main carotenoids identified were β-carotene, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, all-trans-neoxanthin and 9-*cis*-neoxanthin. Concentration of these carotenoids in roots of CT and CT+GD plants were similar and remained mostly unchanged throughout the experimental period, with total β,β-branch carotenoid levels ranging from 1241±156 to 1566±67 ng g^{-1}. By contrast, the pool of these carotenoids in roots of both WS and WS+GD plants markedly decreased (70 to 80% of the initial values) after the first period of water withdrawal (Fig. 4). After this drop, β,β-carotenoid levels remained at very low levels (ranging from 145±17 to 364±68 ng g^{-1}) in stressed roots, even after water rehydration. Thereafter, the second period of severe water stress did not alter the carotenoid content in roots of both, WS and WS+GD plants.

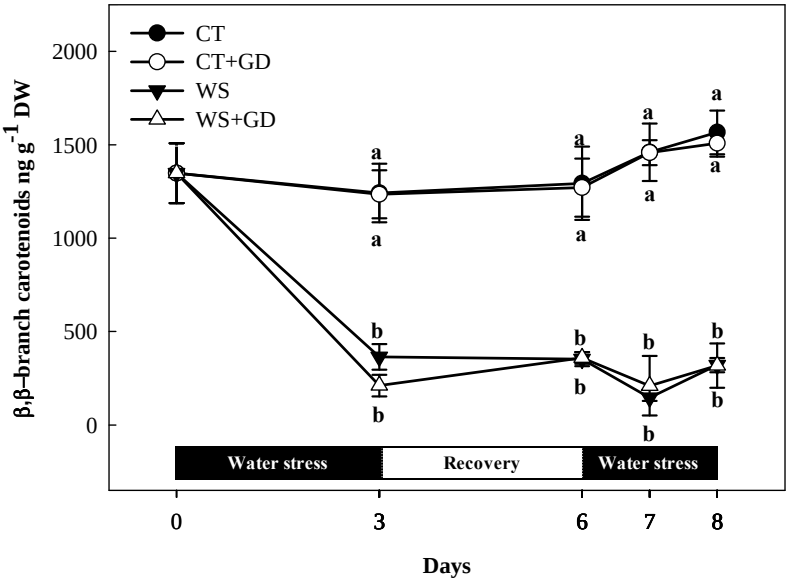

Figure 4. β,β-branch carotenoid accumulation in roots of Citrus intact or stem-girdled plants subjected to two periods of water stress. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

Content of ABA-GE and ABA catabolites. ABA-GE in roots of CT and CT+GD plants remained constant during the whole experiment period, ranging from 29.6±0.6 to 35.6±1.2 ng g^{-1} (Fig. 5a). On the other hand, ABA-GE levels in roots of WS followed a trend similar to that of free-ABA; with an increase during the first period of water shortage (61.7±2.3 ng g^{-1}), a reduction to unstressed levels after rehydration to increase again after

the second period of water withdrawal. Conversely, roots of stem-girdled (WS+GD) plants showed very low ABA-GE levels without differences with controls during the whole experimental period (Fig. 5a).

The concentration of the ABA catabolites, PA and DPA, followed a similar pattern in response to water shortage (Fig. 5b,c). Levels of both PA and DPA remained mostly unaltered and at low levels in roots of well-watered plants (CT and CT+GD). On the contrary, the content of both metabolites in roots increased in response to dehydration (WS and WS+GD), showing slight differences between stem-girdled and intact plants. Meanwhile, levels of DPA similarly increased in root of both types of plants after the first period of water deprivation (Fig. 5c), the content of PA in roots also increased but roots of girdled plants showed higher levels than those recorded in intact plants (Fig. 5b). Rehydration (day 6) decreased levels of both metabolites although DPA levels remained at higher levels than those in unstressed plants (CT and CT+GD). The second period of water shortage caused an additional increase in the levels of both catabolites in roots of water-stressed plants (WS and WS+GD). However, levels of DPA in roots of WS plants (day 8) were higher than those achieved in WS+GD plants (Fig. 5b).

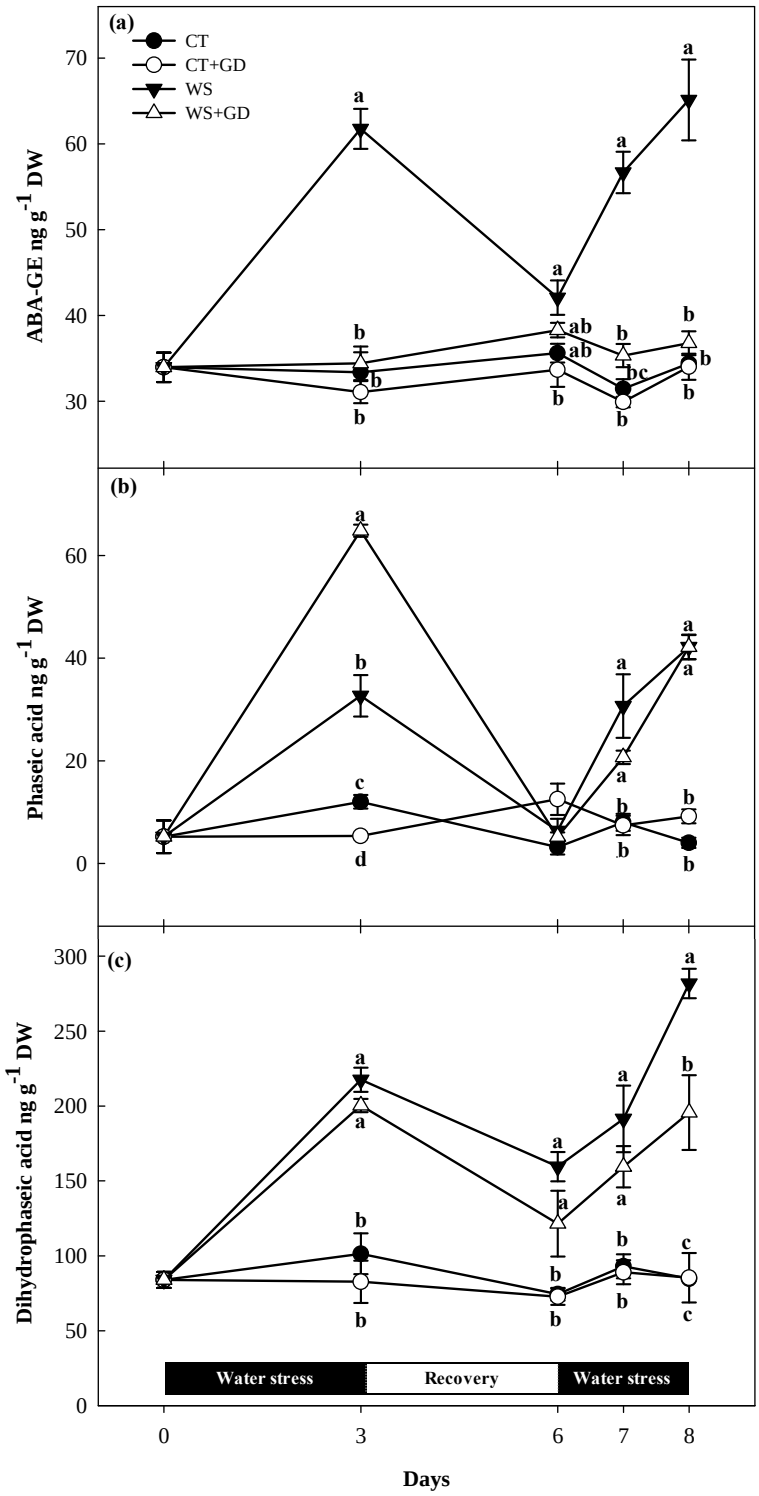

Figure 5. Accumulation of ABA-GE (a), phaseic acid (b) and dihydrophaseic acid (c) in roots of Citrus intact or stem-girdled plants subjected to two periods of water stress. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

ABA content in response to water stress in grafted tomato plants

Results indicate a potential transport of ABA from leaves to roots in water-stressed Citrus seedlings. To extend this knowledge to other plant species, we took advantage of the availability of two ABA-deficient tomato mutants (*flacca* and *notabilis*, from now on *flc* and *not*) which were grafted in reciprocal combinations with their respective WT genotypes (Ailsa Craig and Lukullus, from now noted as AIC and Luk). Based on preliminary experiments, a 24 h stress period was chosen as optimal since both ABA-deficient mutants exhibited a strong *wilty* phenotype and cannot withstand longer stress periods due to the severe decrease in the tissue water content (Fig. 6). Water stress-induced ABA changes in leaves and roots of grafted *flc* (Fig. 7) plants were similar to those in *not* ones (Fig. S1). Regardless the grafting combination, when WT genotypes were used as scions, a strong ABA accumulation was observed a few hours after the stress imposition. Hormone levels remained at very high levels for up to 24 h (AIC/AIC or AIC/*flc*; Fig. 7a). As expected, ABA levels in leaves of the deficient mutant did not increase upon water stress regardless the rootstock used (*flc*/AIC and *flc*/*flc*; Fig. 7a). Similarly, ABA content in roots of AIC/AIC strongly increased upon severe water stress conditions (Fig. 7b). However, in roots of *flc*/AIC combinations, ABA accumulated but to a lower extent (about 50% reduction after 8 h of water stress conditions) than in the roots of WT (AIC/AIC) plants (Fig. 7b). As expected, *flc*/*flc* plants showed a slight increase of ABA in roots upon stress imposition. However, the inability of the *flc* mutant to accumulate large amounts of ABA in roots was partially rescued by the AIC scion (AIC/*flc*). Thus, root ABA content in AIC/*flc* plants reached a maximum of 540±15 ng g^{-1} compared to 261±29 ng g^{-1} in stressed roots of *flc*/*flc* (Fig. 7b).

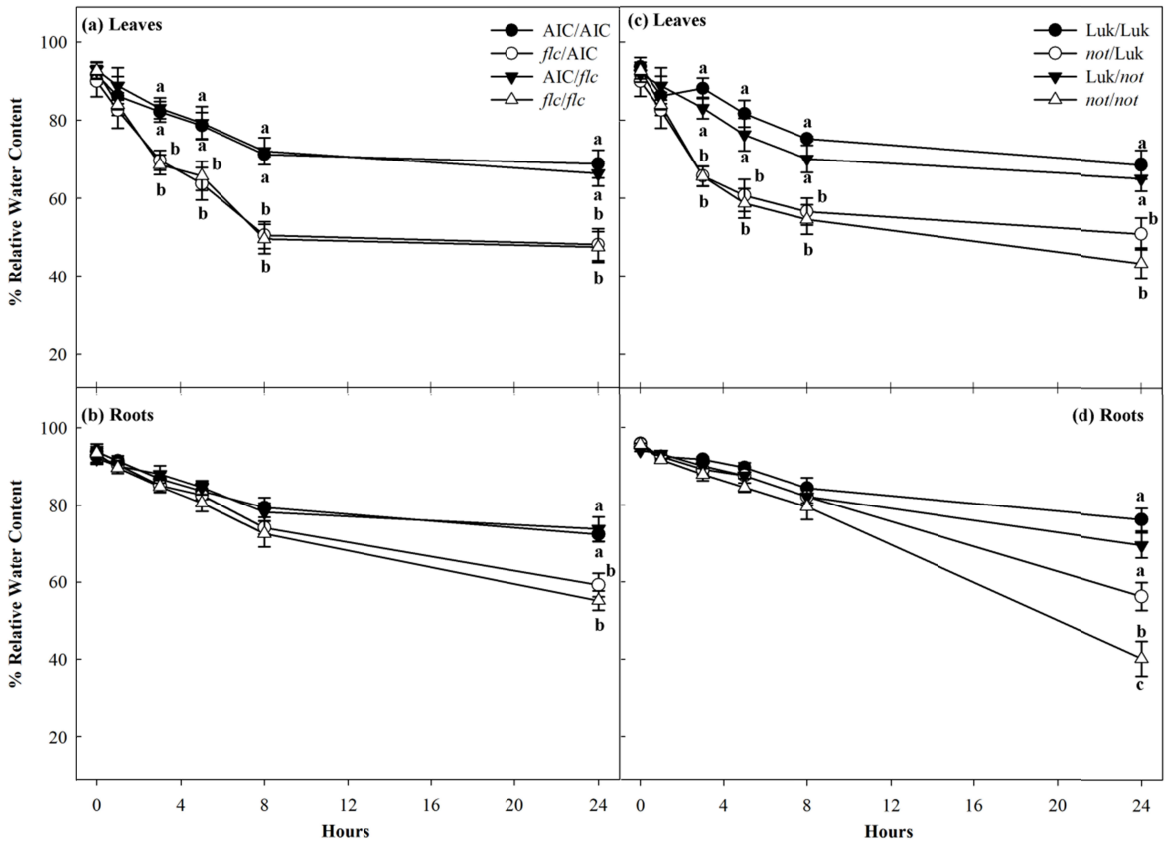

Figure 6. Relative water content (%) in leaves (a and c) and roots (b and d) of different tomato scion/stock combinations of *flacca* ABA-deficient mutant (*flc*) and wild-type Ailsa Craig (AIC; a and b) and *notabilis* ABA-deficient mutant (*not*) and wild-type Lukullus (Luk; c and d), respectively. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

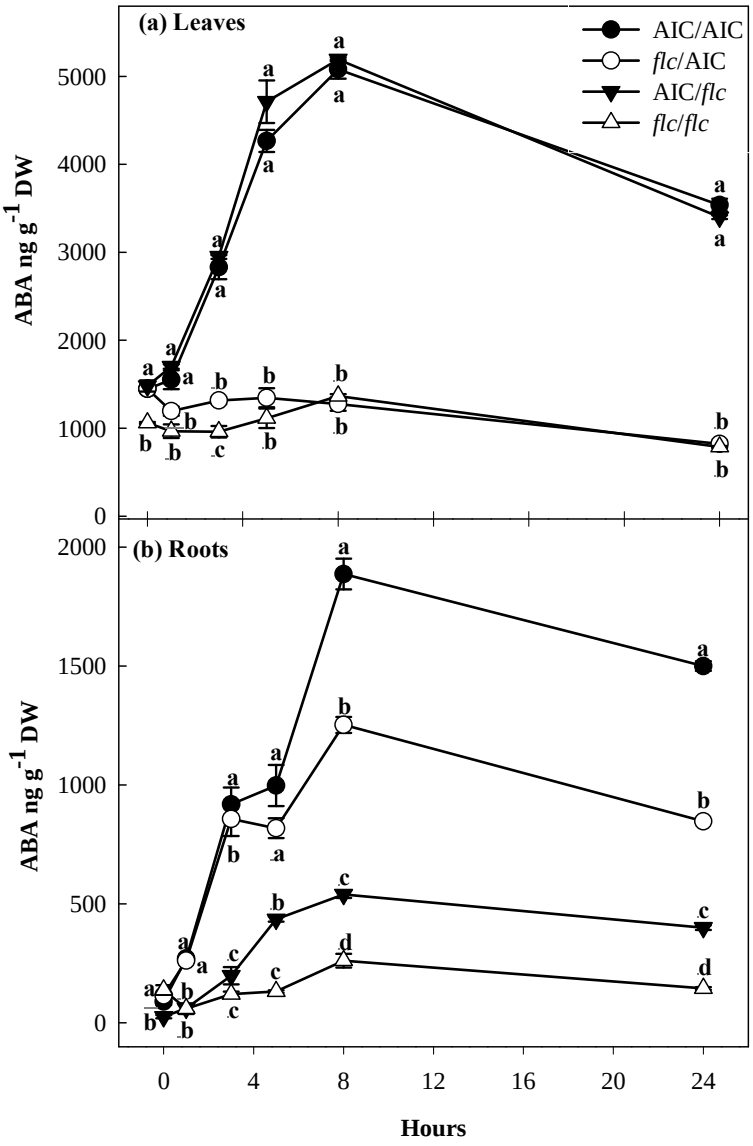

Figure 7. ABA content in leaves (a) and roots (b) of different tomato scion/stock combinations of *flacca* ABA-deficient mutant (*flc*) and wild-type Ailsa Craig (AIC). Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

Transport of labeled ABA from leaves to roots

To demonstrate that the water stress-induced ABA may be basipetally transported to the roots, a labelling experiment was performed. A solution containing both, 50 µM of [$^{2}H_{6}$]-ABA and [$^{13}C_{6}$]-SA was applied to leaves of Carrizo citrange seedlings and after a stress period of 24 h, both water-stressed and well-watered plants were harvested and labelled phytohormones analyzed in leaves and roots. Integrated peak areas of both compounds were normalised according to sample weight and results expressed as area g^{-1} tissue (Table

1). In sprayed plants, similar levels of [$^2H_6$]-ABA were found in leaves of well-watered and water-stressed plants (Table 1, Fig. S2). As expected, non-detectable ABA-labelled peak was observed in non-sprayed plants, neither in leaves nor in roots (data not shown). Roots of well-watered plants sprayed with [$^2H_6$]-ABA showed a clear peak of labelled hormone. Interestingly, roots of water-stressed plants showed labelled ABA levels 3-fold higher than those found in roots of non-stressed ones (Table 1). To check whether this was a common process affecting all exogenously applied metabolites or on the contrary, specific of ABA, labelled salicylic acid was also analyzed and it was found at similar levels in leaves of both CT and WS treatments while it was never detected in the roots.

Table 1. Relative amount (Peak areas per g^{-1} FW) of [$^2H_6$]-ABA and [$^{13}C_6$]-SA detected in leaves and roots of citrus plants after a [$^2H_6$]-ABA and [$^{13}C_6$]-SA mix was sprayed to the leaves. Plants were then maintained under well-watered conditions or subjected to water stress. Data are means ± standard error of 3 replicates. nd= non-detectable. Letters indicate significant differences within each organ ($p \leq 0.05$).

	[$^2H_6$]-ABA		**[$^{13}C_6$]-SA**	
	Leaves	Roots	Leaves	Roots
Control	1779±390 a	7976±297 b	104777±20889 a	nd
Water Stress	2218±357 a	23950±2120 a	91755±15170 a	nd

Discussion

In the conditions of severe water deficit imposed in this work, ABA content increased in leaves and roots of citrus plants. However, in roots of stem-phloem disrupted plants (WS+GD), ABA accumulation was severely impaired under stress conditions (Fig. 3b). These results confirm the relevance of the aerial organs in sustaining ABA accumulation in roots of stressed citrus plants, suggesting the existence of a shoot-to-root transport of ABA which is compromised when basipetal transport is interrupted by stem-girdling. Despite this reduction in root ABA levels, the interruption of the basipetal transport (WS+GD) did not affect hormone content in leaves (Fig. 3a), discarding the existence of a

signal derived from roots which could be affecting this phytohormone biosynthesis in leaves, as suggested in other species and named as ′accumulation message′ (Jackson 2002). ABA transport from shoot to root was also suggested in previous reports showing that in response to water deficit, stem-girdled plants accumulated higher ABA levels in the phloem sap above the incision whereas they were reduced below it (Liang *et al*. 1997). Moreover, using seedlings of *Lupinus albus* it was estimated that ABA moved from leaves to roots in response to salt stress (Wolf *et al*. 1990). Interestingly, basipetal movement of ABA is known to take place inside the roots itself (Chanson and Pilet 1982). Other results also showed altered ABA content in roots of stem-girdled seedlings of *Phaseolus vulgaris* (Vernieri *et al*. 2001) and tomato (Cornish and Zeevaart 1985), but since authors performed a single period of water stress, ABA reduction (compared to intact plants) was not as pronounced as that observed in the present work. Furthermore, our results using Citrus seedlings expand these previous observations and indicate that the basipetal ABA transport is relevant also in woody plants with a significant physical distance between leaves and roots. Moreover, the absence of communication between shoots and roots in girdled plants (WS+GD) impairs ABA accumulation in roots since its concentration is unable to increase after two consecutive periods of water withdrawal (Fig. 3b).

The above mentioned seems to be linked to a severe reduction in the carotenoid content in roots in response to the first period of water stress (Fig. 4). In this sense, the carotenoid precursors initially available in roots could partially support the burst of ABA during the first period of water withdrawal (day 3; Fig. 3b). However, the inability to accumulate ABA in roots of water-stressed girdled plants (day 8; Fig. 3b) is likely due to the absence of basipetal transport rather than to the reduction of carotenoids since stressed roots of intact plants accumulate large amounts of ABA that would rely on transport from the aerial organs. The severe decline observed in β,β-carotenoids content in water-stressed citrus roots in parallel with the increase in ABA suggests an increased flow of the biosynthetic pathways towards the synthesis of ABA (Walter *et al*. 2015). The role of the carotenoid precursors on ABA biosynthesis is well established since the overexpression of genes coding to different enzymes from the carotenoid pathway such as *phytoene synthase* (*PSY*, Cao *et al*. 2015) or *zeaxanthin epoxidase* (*ZEP*; Frey *et al*.1999), leads to a direct increase in ABA levels. However, the carotenoid content in roots (β,β-branch) is usually negligible compared to that in leaves (Ruiz-Sola *et al*. 2014, Thompson *et al*. 2007, Walter *et al*. 2015), partially explaining the reduced ability of the roots to sustain the ABA accumulation in response to long-term water stress. Taken together, data suggest the

existence of a close relationship between above and belowground organs to sustain the progressive ABA buildup in roots in response to water stress conditions.

This study also reveals that the contribution of root-sourced carotenoid as precursors of the ABA biosynthetic pathway appears to be limited to the initial stages of water deficit whereas the aerial organs are essential to sustain long-term root ABA accumulation. Furthermore, depletion of β,β-carotenoid levels in roots is a fast response upon severe stress imposition and their restoration to basal levels seems to require long periods under non-stressful conditions (at least longer than 3 days as shown in Fig. 4). Thus, to confirm this point, plants were subjected to a period of water stress and β,β-carotenoid content in roots periodically evaluated after re-watering. Results confirmed that the content of β,β-carotenoids restores upon re-watering but it requires at least 20 days to achieve levels similar to unstressed plants (Fig. S3). These results contrast with other works in *Arabidopsis thaliana* where abiotic stress conditions cause an increase in β,β-carotenoids content in roots as soon as 5 h after stress imposition (Ruiz-Sola *et al*. 2014), highlighting the existence of species-specific mechanisms to regulate ABA biosynthetic pathway under stress conditions. Moreover, the reduced ability of dark-grown organs to accumulate carotenoids appears to be related to the predominant type of plastid present in the roots (leucoplasts). Our results are also in agreement with a reduced and slow ability of this plastid type to de novo synthesize and accumulate carotenoids (Howitt and Pogson 2006, Li and Yuan 2013).

The contribution of aerial organs to maintain ABA increase in roots could be sustained by the basipetal transportation of ABA but also of ABA-GE, which was proposed as a mobile form (Goodger and Schachtman 2010, Jiang and Hartung 2008). In roots of stressed Citrus plants, ABA-GE levels follow a pattern of accumulation similar to that of ABA (Fig. 5). Therefore, results are in agreement with the classical statement that ABA-GE is an end product to the ABA metabolism (Neill *et al*. 1983), implying that under water stress ABA-GE is accumulated as a mechanism to regulate ABA levels (Priest *et al*. 2006). However, when roots are unable to receive shoot-sourced ABA and, as a result, ABA levels are reduced (WS+GD, day 3, Fig. 3b), plants seem to prioritize the accumulation of the free active form instead of ABA-GE, with levels of conjugate remaining similar to those found in well-watered plants (day 3, Fig. 5a). From these results, it seems clear that ABA-GE levels in Citrus roots are regulated by the availability of free ABA. However, the ABA catabolic pathway, which involves the hydroxylation of ABA into 8'-OH-ABA that

spontaneously cyclizes rendering PA and subsequently DPA, appears to regulate the ABA homeostasis in an ABA-independent mechanism triggered under water stress, similar to what was previously proposed (Priest *et al*. 2006, Sreenivasulu *et al*. 2012). In this sense, high levels of PA and DPA were found in both roots of intact and girdled plants (Fig. 5b,c), indicating that catabolism is activated irrespectively of the amount of ABA in the tissue. This is in agreement with previous results obtained in ABA-deficient *aba2-3* Arabidopsis mutant in which even when negligible levels of ABA are present, catabolism continues actively, favouring the accumulation of DPA (Priest *et al*. 2006).

The existence of an ABA basipetal transport was confirmed by the application of labelled ABA ([$^{2}H_{6}$]-ABA) to citrus leaves, which was subsequently detected in the roots (Table 1). Some earlier research did not find labeled ABA in roots after applying it to shoots (Chanson and Pilet 1982). This could be attributed to the low sensitivity of the available equipment in that time but also to the different amount of labelled compound applied. However, this work supports the idea that ABA mobilization takes place under normal conditions as was previously estimated in seedlings of Lupinus (Wolf *et al*. 1990). Indeed, Chen *et al*. (2002), working with unstressed tomato plants found that ABA concentration in roots of WT/ABA-deficient mutant was 20% higher than that in roots of mutant/mutant plants and, complementarily, roots of ABA-deficient mutant/WT combinations had ABA levels 15% lower than those in WT/WT plants. Moreover, dehydration notably enhances the ABA movement, suggesting therefore that the active shoot-to-root transport of this hormone is also stress-dependent. Indeed, it was suggested that salt stress stimulates ABA export from leaves to phloem, the tissue responsible for the basipetal transport to the roots (Wolf *et al*. 1990). Furthermore, this ABA transport appears to be highly specific since the co-sprayed [$^{13}C_{6}$]-SA did not result in detectable amounts of this metabolite in roots. Hence, the existence of free ABA transport from leaves to roots is confirmed although transport of conjugated forms cannot be ruled out (Burla *et al*. 2013, Jiang and Hartung 2008, Goodger and Schachtman 2010).

ABA transport from shoots to roots was also confirmed in tomato plants and therefore, it appears to be a conserved mechanism among different species (Fig. 7, Fig. S1) as reported in Phaseolus (Vernieri *et al*. 2001) and in Arabidopsis (Ikegami *et al*. 2009) plants under water deficit, and in Lupinus under salt stress conditions (Wolf *et al*. 1990). In the experimental design described in this work, water deficit increases ABA levels in leaves of WT plants irrespective of the genotype used as a rootstock (WT or ABA-deficient

mutant), suggesting that the WT ability to synthesize ABA is not impaired by the rootstock deficiency. On the contrary, aerial organs influenced ABA accumulation in the roots as was previously described in tomato (Chen *et al*. 2002). Under water stress, if ABA increase in (WT) leaves is sufficient, ABA accumulation in roots of ABA-deficient mutants occurs (2- and 4.5-fold in AIC/*flc* and Luk/*not*, respectively), contrasting with the inability of mutant/mutant plants to accumulate ABA in roots (Fig. 7b, Fig. S1). The same influence of the aerial part over the roots was confirmed in mutant/WT combinations, where ABA content in WT roots was markedly reduced due to the failure of the leaves to synthesize ABA. Therefore, both scion/rootstock combinations further support the idea that ABA is actively transported from green aerial organs to sustain the hormone accumulation in roots in response to water stress. Moreover, results for the two genotypes tested (*flc* and *not*) were similar despite the different step of ABA biosynthesis that they have mutated. This reinforces the notion that free ABA is the molecule directly involved in basipetal transport rather than other precursors or ABA-related metabolites. Furthermore, the influence of leaf ABA on root hormone accumulation is noticeable after 3 to 5 h of stress (Fig. 7, Fig. S1), indicating a fast trigger of this mechanism, as previously described in Arabidopsis seedlings (Ikegami *et al*. 2009, Waadt *et al*. 2014).

Since ABA content in a tissue reflects a tight balance among the biosynthesis, catabolism, conjugation and transport (Boursiac *et al*. 2013), our data suggest that these mechanisms are operating not only under water deficit but also in well-watered plants as demonstrated with the application of [$^2H_6$]-ABA (Table 1). Furthermore, it can be speculated that under well-watered conditions β,β-carotenoids are accumulated in the roots to merely contribute to de novo synthesis of ABA during the initial stages of water deficit. However, it cannot be ruled out that this decrease in carotenoids could be also linked to other function such as the biosynthesis of other apocarotenoids in response to water stress such as strigolactones (Ha *et al*. 2014). On the other side, ABA-GE seems to play a role in ABA homeostasis, allowing ABA sequestration when tissue levels are elevated. However, when ABA accumulation is restricted plants prioritize the availability of the free form over the conjugated one. Overall, data presented in this work support that the basipetal transport mechanism ensures an unlimited supply of ABA in plant roots in response to water stress conditions when the local pool of β,β-carotenoids is depleted.

Materials and methods

Citrus experiments

One-year-old Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) plants were used for the different assays. Seedlings were acclimated for at least four months before each experiment in a greenhouse under natural photoperiod and day/night temperatures of 25±3.0°C / 18±2.0°C. Plants were grown in 2.5-L plastic pots filled with a mixture of peat moss, perlite and vermiculite (80:10:10) as substrate and watered three times a week with 0.5 L of a half-strength Hoagland solution (Arbona *et al*. 2009). Homogeneously-sized plants of about 80 cm in height were used for each experiment.

Stem girdling experiment. Two days before the beginning of the experiments, a set of Carrizo seedlings were stem-girdled (removing a 5 mm-wide segment of bark and phloem tissues) immediately above the root-stem junction. After that period, a subset of girdled and non-girdled (control) plants were subjected to water stress treatments by transplanting them to dry perlite. This procedure does not induce any significant change in hormonal pattern derived from the transplanting (unlike those induced by damage or wounding) and allows an homogeneous dehydration among treatments as previously demonstrated in de Ollas *et al*. (2013) and Gómez-Cadenas *et al*. (1996). The rest of plants were set as controls and regularly watered. Therefore, four groups of plants were established: well-watered girdled (CT+GD) plants; water-stressed girdled (WS+GD) plants; well-watered non-girdled plants (CT); and water-stressed non-girdled (WS) plants. Water-stressed (WS+GD and WS) plants were kept in dry perlite for three days, and then rehydrated for three additional days. Thereafter (day number 6), a second period of two-day water shortage was imposed to the previously stressed plants by transplanting them again to dry perlite.

Deuterium-labelled ABA treatments. Foliar application of deuterium-labelled ABA ([$^{2}H_{6}$]-ABA) was performed on Carrizo citrange seedlings. A solution containing 50 µM [$^{2}H_{6}$]-ABA (pKa=4.75) and 50 µM labelled salicylic acid [$^{13}C_{6}$]-SA (pKa=3.01) in distilled water with 0.5% (v/v) of Triton X-100 was sprayed on the leaves three times in a week solely to the leaves. Both ABA (Liang *et al*. 1997) and SA (Bonnemain *et al*. 2013)

molecules can be transported by the phloem. After treatment, plants were protected from direct sunlight to facilitate foliar absorption of the applied chemicals. Pots were covered with a plastic appliance to avoid runoff to the substrate and roots. Three groups of treatments were established: a) plants sprayed with labelled hormones that were transplanted to dry perlite at day 7; b) plants identically sprayed but properly irrigated after day 7 and; c) well-watered plants sprayed with a mock solution containing only water and triton X-100. After 24 h of stress treatment, plants were harvested and the presence of [$^{2}H_6$]-ABA and [$^{13}C_6$]-SA was determined in roots and leaves by UPLC-MS/MS (see details in hormone analysis section).

Tomato grafting experiments

Tomato (*Solanum lycopersicum* Mill.) seeds of ABA-deficient mutants flacca (*flc*, which is impaired in the oxidation of ABA-aldehyde to ABA; Taylor *et al*. 1988) and notabilis (not, which carries a null mutation in the ABA biosynthesis gene *LeNCED1* (Burbidge *et al*. 1999) and their respective wild type genotypes Ailsa Craig (AIC) and Lukullus (Luk), were germinated in vermiculite as in the previously mentioned greenhouse conditions and nutrient solution. After the development of 3-4 true leaves, plants were grafted just above the cotyledons to generate the following scion/stock combinations: *flc*/AIC; AIC/*flc*; AIC/AIC; *flc*/*flc*; *not*/Luk; Luk/*not*; Luk/Luk and *not*/*not*. Grafted plants were then covered with a plastic sheet (50% shade) to protect them from excessive light intensity and to maintain a high relative humidity, avoiding wilting of ABA-deficient mutants. This plastic cover was progressively opened every day and finally removed ten days after grafting. Plants were then transplanted to 2.5 L pots containing 100% perlite and cultivated for other 4 weeks. Only homogenous plants were used for the experiments. Plants of each scion/stock combination were transplanted to dry perlite. Based on preliminary assays, samples were taken at 0 (CT); 1; 3; 5, 8 and 24 h after water stress imposition.

Sample extraction and storage

Roots and leaves of at least three independent plants for both Citrus and tomato experiments were collected at each sample date. Samples were rinsed with distilled water

and immediately frozen in liquid nitrogen, converted into a fine powder and storage at -80ºC or lyophilized until further analysis.

Relative water content

Relative water content (RWC) of leaves and roots was monitored during the stem girdling and recovery experiments and tomato experiments. Leaves and roots were weighted for fresh weight (FW). Then, tissues were hydrated to full turgor by maintaining them in distilled water for 24 h in the dark to assay turgid weight (TW). Afterwards, samples were dried at 70ºC during three days for dry weight (DW). The RWC was calculated as RWC= ((FW-DW)/(TW-DW))*100.

Hormone analysis

Abscisic acid and related metabolites (PA and DPA) were extracted and analyzed as described in de Ollas *et al*. (2013) with slight modifications. Briefly, 0.2 g of lyophilized plant material was extracted in 2 mL of distilled H2O after spiking with 100 ng of [$^{6}H_{2}$]-ABA and 50 ng of [$^{2}H_{3}$]-PA. After centrifugation at 10.000 × *g* at 4°C, supernatants were recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 mL of di-ethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The dry residue was then resuspended in a 9:1 H2O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a HPLC system (Waters Alliance 2695, Waters Corp., Milford, MA). Separations were carried out on a C18 column (Kromasil 100, 5 µm particle size, 100 × 2.1 mm, Scharlab, Barcelona, Spain) using a linear gradient of MeOH and milliQ H2O supplemented with 0.1% acetic acid at a flow rate of 300 µL min^{-1}. ABA-GE determination was performed as Neill *et al*. (1983) with some modification. The aqueous fraction containing ABA conjugates was hydrolyzed by adding 2.0 mL of NaOH 0.1 M and placed at 60ºC for 30 min. Samples were then cooled, acidified and spiked with [$^{2}H_{6}$]-ABA before partitioned as described above and ABA-GE was estimated as a ABA. Metabolites were monitored at m/z: ABA 263>153; [$^{2}H_{6}$]-ABA 269>159; PA 279>139; [$^{2}H_{3}$]-PA 282>147 and DPA at 281>171. Compounds were quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass,

Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. All data were acquired and processed using Mass Lynx v4.1 software. In the labelling experiments samples were similar extracted but no internal standards were spiked ([$^{2}H_6$]-ABA or [$^{13}C_6$]-SA). [$^{13}C_6$]-SA was monitored at m/z 143>99. Limits of detection were 0.58 ng g^{-1} and 0.30 ng g^{-1} for ABA and SA, respectively. Relative quantification was performed by the comparison of areas of each sample.

Carotenoid analysis

Carotenoid pigments were extracted as previously described by Alós *et al*. (2014) with some minor modifications. All operations were performed at 4ºC under dim light to prevent photodegradation, isomerization and structural changes of carotenoids. Briefly, lyophilized samples (0.6-0.8 g) were extracted with a solution containing 4 mL MeOH and 3 mL of Tris–HCl (50 mM, pH 7.5) by sonication. Chloroform (8 mL) was added to the mixture, stirred and centrifuged at 4ºC. The aqueous phase was re-extracted with chloroform until it was colourless. The combined chloroform extracts were dried under vacuum at 40°C and saponified overnight at room temperature with a methanolic KOH solution (50% w,v). Four mL of miliQ H2O and four ml petroleum ether:diethyl ether (9:1, v/v) mix were added to the saponified solution. After shaking the upper phase (containing the saponified carotenoids) was transferred to a volumetric flask. Repeated re-extractions (4–6 times) with 4 mL of the ethereal mix were performed until the hypophase was colourless. Afterwards, samples were dried under N_2 and kept at −20°C until HPLC analysis. Chromatographic conditions, standards and calibration curves were previously described in (Alós *et al*. 2014).

Statistical analysis

Mean ± standard errors of the different parameters are shown in figures. Analysis of variance (ANOVA) was used to determine the statistical significance. Mean comparisons were performed by Tukey's test ($p \leq 0.05$). Every experiment was repeated at least three times throughout the season.

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Disclosures

The authors have no conflicts of interest to declare.

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Supplementary Information

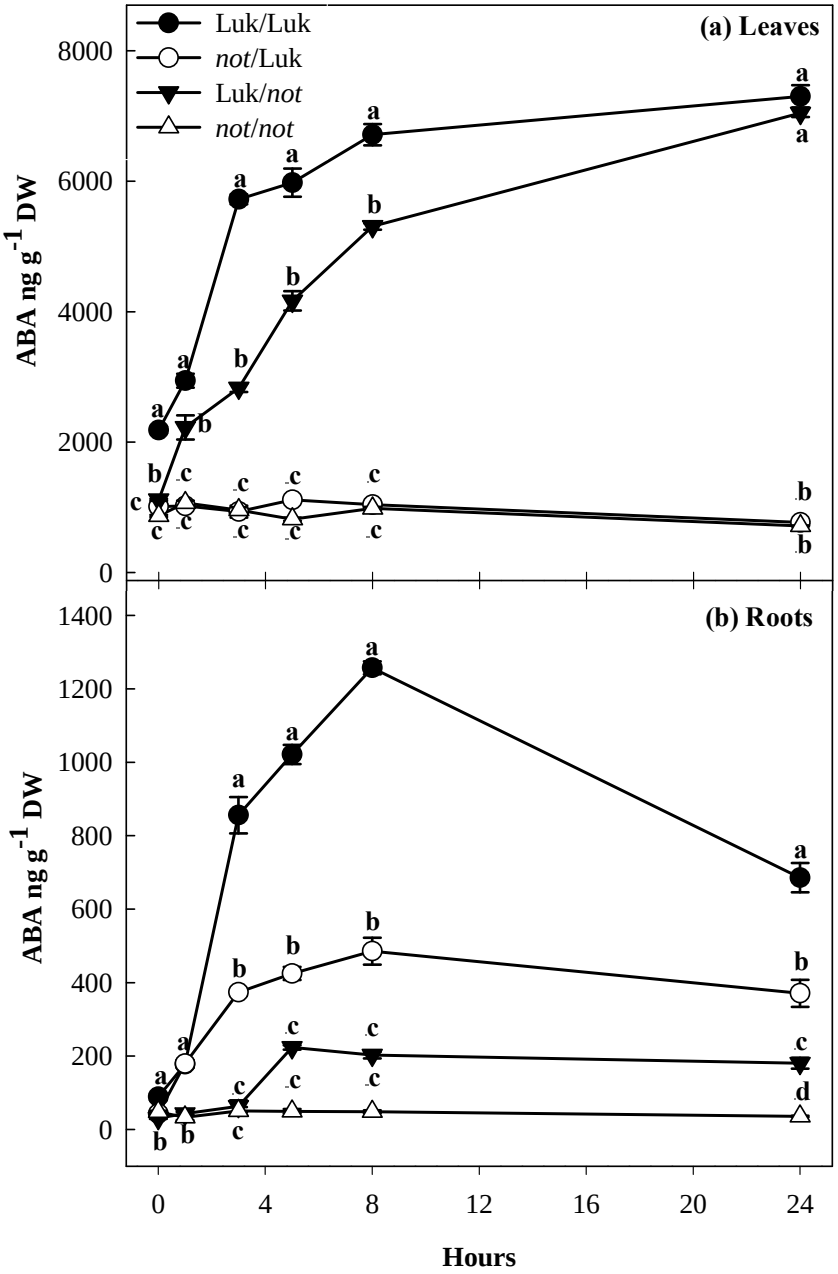

Fig. S1. ABA content in leaves (a) and roots (b) of different tomato scion/stock combinations of *notabilis* ABA-deficient mutant (*not*) and wild-type Lukullus (Luk). Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

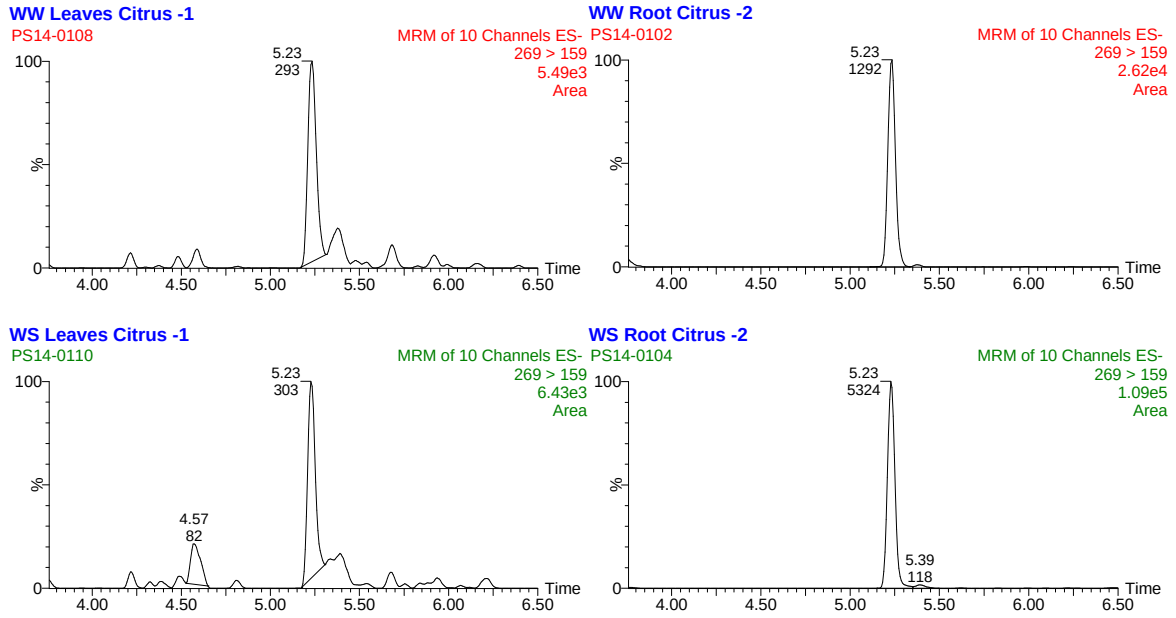

Fig. S2. Chromatograms obtained from the UPLC-MS/MS of the transition 269>159 corresponding to [$^2H_6$]-ABA molecule in leaves and roots of Citrus plants, either well-watered or water-stressed. Identification of [$^2H_6$]-ABA was performed by the specific transitions and the comparison with the retention time of the [$^2H_6$]-ABA standard.

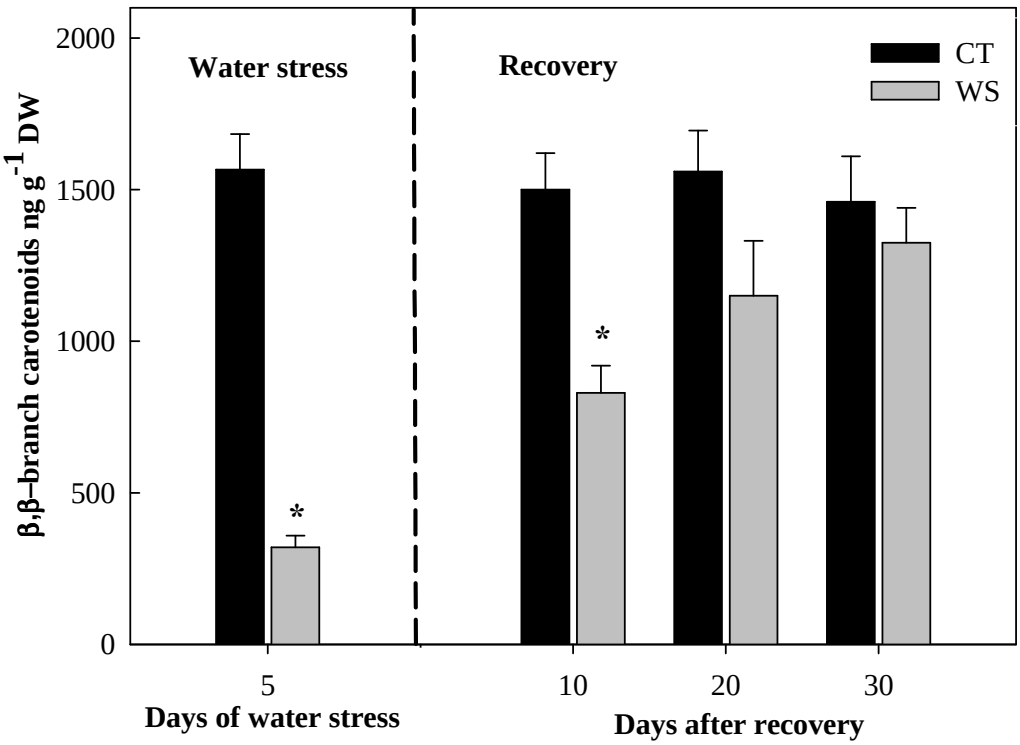

Fig. S3. Carotenoid accumulation of the ABA precursors, β,β-branch, in roots of Citrus plants after a five-day period of water stress and subsequent water rehydration. Data are means ± SE of at least 3 replicates. Asterisks indicate significant differences between treatments at each date ($p \leq 0.05$).

3.2. Chapter II.

Abscisic Acid Accumulation in Water-Stressed *Citrus* Roots is Independent of Carotenoid Availability and Involves Aerial Organs

Matías Manzi, Joanna Lado, María Jesús Rodrigo, Vicent Arbona, and Aurelio Gómez-Cadenas

Abstract

Sustained abscisic acid (ABA) accumulation in citrus roots upon dehydration depends on the transport from aerial organs. Under this condition, the contribution of the β,β-carotenoids (ABA precursors) to the *de novo* synthesis of ABA in roots needs to be clarified since their low availability in this organ precludes a limited contribution. To accomplish that, detached citrus roots were exposed to light in order to increase their carotenoid content and subsequently dehydrated to trigger ABA accumulation. Stress imposition sharply decreased the pool of β,β-carotenoids but, unexpectedly, no concomitant rise in ABA content was observed. Contrastingly, roots of intact plants with lower levels of carotenoids showed a similar decrease but a significant accumulation of ABA. Furthermore, upon dehydration both types of roots showed similar upregulation of the key genes involved in biosynthesis of carotenoids and ABA (*CsPSY3a*; *CsβCHX1*; *CsβCHX2*; *CsNCED1*; *CsNCED2*), demonstrating a conserved transcriptional response triggered by water stress. Thus, the sharp decrease in root carotenoid levels in response to dehydration should be related to other stress-related signals instead of contributing to ABA biosynthesis. In summary, ABA accumulation in dehydrated-citrus roots largely relies on the presence of the aerial organs and it is independent of the amount of available root β,β-carotenoids.

Keywords: *gene expression, jasmonoyl-isoleucine (JA-Ile), 9-cis-epoxycarotenoid dioxygenase (NCED), shoot-to-root transport, osmotic stress, water deficit*.

Introduction

Plants have evolved mechanism to cope with abiotic stress by triggering different mechanisms at physiological, biochemical and molecular levels. Under adverse conditions such as water deficit, abscisic acid (ABA) levels increase and trigger several tissue-specific responses including stomatal closure in leaves and modifications in root shape and architecture (Gómez-Cadenas *et al*., 2015; McAdam *et al*., 2015). This ABA accumulation takes place in most plant organs, including vascular tissues, leaves and roots. De novo synthesis of ABA is the main mechanism responsible for the accumulation of ABA upon water stress (Gómez-Cadenas *et al*., 2015). ABA derives from carotenoids, which are synthesized and accumulated in plastids (Nambara and Marion-Poll, 2005). Carotenogenesis involves the conversion of two molecules of geranylgeranyldiphosphate (GGPP) by the activity of phytoene synthase (PSY), which is considered a key regulatory step in the carotenoid biosynthesis. Several successive desaturations and isomerizations lead to the production of lycopene, a key branching point in the pathway. In the ε,β-branch (predominant in green tissues), lycopene is converted into α-carotene and subsequently into lutein. Alternatively, in the β,β-branch, lycopene is converted into β-carotene and further hydroxylated to yield β,β-xanthophylls, which are the ABA precursors. β-carotene hydroxylase (βCHX) enzyme is responsible for the conversion of β-carotene into β-cryptoxanthin and subsequently into zeaxanthin, which is then converted into violaxanthin in a reaction mediated by the enzyme zeaxanthin epoxidase (ZEP). Violaxanthin could be then converted into neoxanthin (Nambara and Marion-Poll, 2005). 9-*cis*-isomers of both violaxanthin and neoxanthin are the specific substrates of 9-*cis*-epoxycarotenoid dioxygenase (NCED) that catalyzes a reaction rendering xanthoxin in a bottleneck step in the ABA pathway (Thompson *et al*., 2007). This molecule is subsequently exported from the plastids to the cytosol where it is oxidized to ABA (Nambara and Marion-Poll, 2005; Thompson *et al*., 2007). This oxidation takes place in two steps; first, xanthoxin is converted into abscisic aldehyde by ABA2, an enzyme belonging to short-chain dehydrogenase/reductase family (XantDH). Then, this compound is finally oxidized by the abscisic aldehyde oxidase (AAO3) into ABA (Nambara and Marion-Poll, 2005). Additionally, ABA catabolism takes place by the action of CYP707A subfamily of P450 monooxygenases to yield the two main catabolic products of ABA, phaseic acid (PA) and subsequently dihydrophaseic acid (DPA; Nambara & Marion-Poll 2005). Complementarily, ABA levels could be modulated by conjugating the molecule to a sugar to form ABA-glycosyl ester (ABA-GE), in a reversible manner, in reactions catalyzed by

ABA O-glycosyl transferase (AOG) and β-glycosidase (βGluc) enzymes (Priest *et al*., 2006; Seo and Koshiba, 2011).

Different lines of evidence including over-expression of key genes for carotenoid biosynthesis such as *PSY* (Lindgren *et al*., 2003) and *ZEP* (Park *et al*., 2008), ABA-deficient mutants (Borel *et al*., 2001), and the use of inhibitors such as norflurazon (NFZ, De Ollas *et al*. 2013), indicate that levels of carotenoids are limiting in the downstream accumulation of ABA in response to water deficit. However, there are also contradictory evidence demonstrating that an enhanced amount of carotenoids does not necessarily increase the levels of ABA (Arango *et al*., 2014; Cao *et al*., 2015), indicative of the existence of an intricate mechanism governing ABA biosynthesis in different plant species and tissues (Schwarz *et al*., 2015). In fact, the over-expression of *βCHX* in carrot roots (Arango *et al*., 2014) or *PSY* in potato tubers (Ducreux *et al*., 2005) did not result in an increase of ABA levels in belowground tissues, whereas pharmacological inhibition of phytoene desaturase did not prevent ABA accumulation in citrus roots upon water stress imposition (De Ollas *et al*., 2013). This accumulation of ABA in roots independently of the carotenoid levels could be explained by the influence of other potential sources of ABA rather than de novo synthesis in stressed root tissues (Ikegami *et al*., 2009; Manzi *et al*., 2015; McAdam *et al*., 2015; Ren *et al*., 2007).

It has been previously demonstrated that in roots of citrus plants subjected to water deficit ABA levels increase concomitantly to the reduction β,β-carotenoid concentration (Manzi *et al*., 2015), suggesting that accumulation of ABA (and/or other downstream metabolites) is directly fed by carotenoid precursors (Walter *et al*., 2015; Welsch *et al*., 2008). Thus, the initial accumulation of ABA in response to water stress would be supported by the local carotenoid pool (Manzi *et al*., 2015; Ren *et al*., 2007). However, under longer or reiterative periods of dehydration, ABA accumulation in roots appears not to be further sustained from this source (Manzi *et al*., 2015; Ren *et al*., 2007), and aboveground tissues seem to play a role in sustaining hormonal increase in roots as demonstrated in several species such as maize (Ren *et al*., 2007), Arabidopsis (Ikegami *et al*., 2009) and citrus (Manzi *et al*., 2015). Indeed, this influence also takes place under non-stressful conditions (Manzi *et al*., 2015; McAdam *et al*., 2015). Under these circumstances, ABA transport from aboveground tissues becomes essential to sustain its accumulation in roots and consequently, any interruption of basipetal transport severely limits hormone accumulation in roots (Ikegami *et al*., 2009; Manzi *et al*., 2015), as observed in detached

roots where ABA content upon dehydration sharply decreased compared to that in intact plants (Ren *et al*., 2007). Furthermore, carotenoid content in roots is usually around 0.1% of that found in leaves (Ruiz-Sola *et al*., 2014; Walter *et al*., 2015), related to the predominant plastid type in roots (proplastids or leucoplasts), which are not specialized in carotenoid accumulation (Li and Yuan, 2013). Moreover, carotenoid biosynthesis is highly responsive to light, being more active in light-exposed tissues (Toledo-Ortiz *et al*., 2010) and consequently, recovery of carotenoid levels in dark-growing roots after dehydration is a slow process that takes several weeks of regular watering. Therefore, it seems unlikely that carotenoids could sustain ABA buildup under consecutive events of dehydration (Manzi *et al*., 2015).

Other evidence suggested that apart from NCED, PSY could be an important enzymatic bottleneck in ABA biosynthesis in roots under certain stress conditions (Thompson *et al*., 2007). Based on this, an increase in the carotenoid content in roots could allow the sustained ABA accumulation in this tissue (Li *et al*., 2008; Welsch *et al*., 2008).

In this work, the objective was to clarify the real contribution of root carotenoids to de novo synthesis of ABA in this organ under water stress conditions. To accomplish that, the entire root system of young citrus seedlings was detached from plants several weeks before dehydration stress was imposed. In parallel, carotenoid accumulation in detached roots of citrus was induced by light exposure before the stress onset. ABA production under stress was studied in illuminated and obscured detached roots and results were compared with those obtained in roots of intact plants. The involvement of different important genes in carotenoid and ABA metabolism was assessed by studying their transcription profile in a time-course experiment.

Results

Effect of light- and dark-growing conditions on pigment content in detached citrus roots after light preconditioning treatments

In this experimental setup, detached roots were illuminated to increase the amount of ABA precursors (carotenoids). The ability of roots to synthetize ABA was evaluated in the two groups of detached roots (LT and DK) and compared to that of intact CT plants (with roots kept in the dark and shoots exposed to light). During the three-week period that

preceded stress imposition, roots showed relevant differences in coloration. DK roots were mainly white (uncolored) whereas LT roots showed an intense green coloration (Fig. 1).

Figure 1. Plant material used in the experiment. Roots were detached and cultivated for 3 weeks in the dark (DK) or under light (LT). In the middle, intact plants (with illuminated shoots and obscured roots) used as a control (CT).

Roots from CT plants showed a white coloration similar to DK roots. These visual differences were mirrored by changes in chlorophyll and carotenoid content. Total chlorophylls were lower in DK roots (25.5±0.7 $\mu g.g^{-1}$), representing a 50% of those measured in CT roots (52.1±1.2 $\mu g.g^{-1}$, Fig. 2a). By contrast, detached roots exposed to light markedly increased the chlorophyll content by 5.5 times compared to CT roots. The highest chlorophyll concentration was found in CT shoots (1955±70 $\mu g.g^{-1}$) being 6.8 times higher than that in LT roots (Fig. 2a). Similarly, carotenoid content was modified in response to light; compared to levels in CT roots, total carotenoid content was induced 5.5-fold in LT roots and reduced by 45% in DK ones (Fig. 2b). As expected, carotenoid

levels in leaves were much higher than in roots, being the carotenoid content in LT roots only 7.7% of the value found in leaves of CT plants (Fig. 2b).

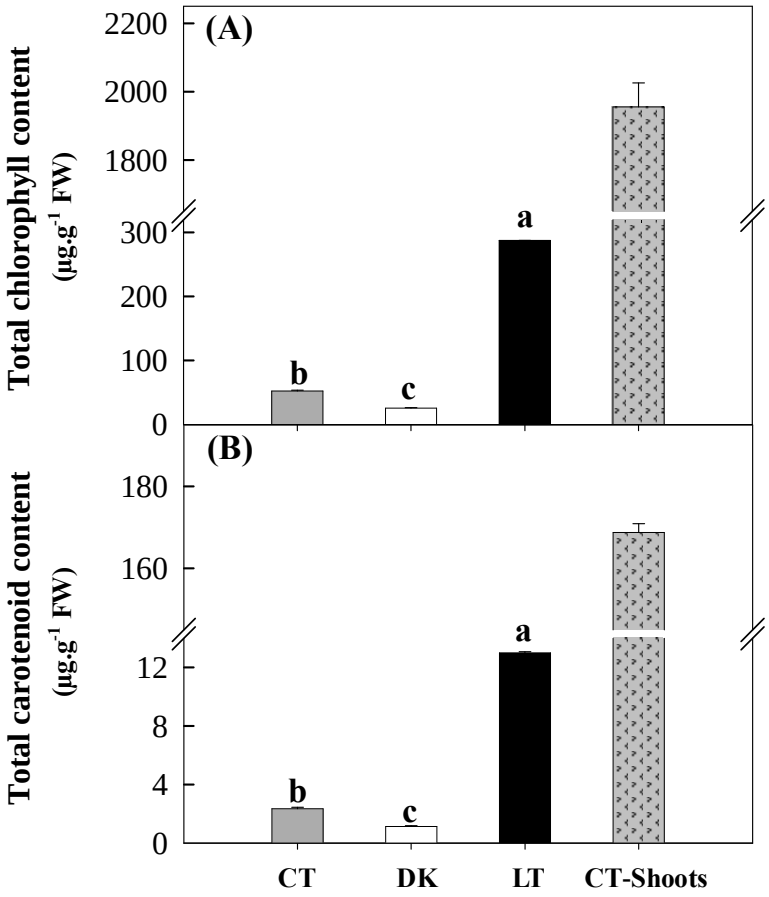

Figure 2. Total chlorophyll (A) and carotenoid (B) content in roots and shoots of Citrus seedlings. Roots of intact plants (CT) grown in the darkness; detached roots grown during three weeks into dark conditions (DK) or under illumination (LT); Shoots of intact plants (CT-Shoots) grown under illumination. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments within roots (Tukey´s test, $p \leq 0.05$).

Relative water content in response to osmotic stress

Detached roots (DK and LT) and intact CT plants were exposed to PEG-6000 (-1.5 MPa) treatment for 8 h to induce osmotic stress and ABA accumulation. Stress induced progressive decreases in RWC without differences among treatments. The initial RWC levels (95.5 % in all treatments at 0 h) decreased to values of 91.0 after 8 h of stress (Fig. 3). Therefore, any difference found among treatments may not be ascribable to differences in water availability in roots since all showed similar decreases in RWC levels.

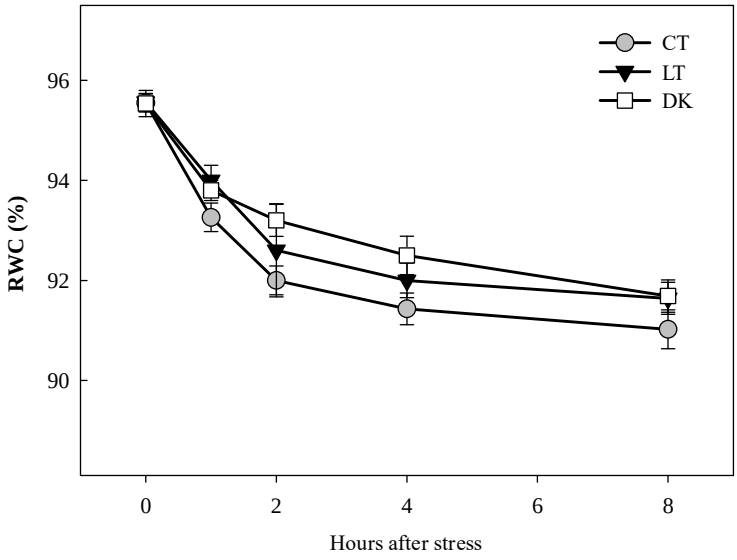

Figure 3. Relative water content in *Citrus* roots exposed to dehydration (-1.5 MPa). Intact plants (CT); detached roots grown during three weeks into dark conditions (DK) or under illumination (LT). Data are means ± SE of at least 3 replicates. The absence of letters indicates no significant differences among treatments (Tukey´s test, $p \leq 0.05$).

Variation in ABA and JA-Ile levels in response to osmotic stress

ABA and JA-Ile concentrations were determined in LT, DK and CT roots. ABA content in LT and DK roots was extremely low throughout the experiment without showing any change in response to the PEG treatment (Fig. 4a). Contrastingly, ABA levels in CT roots increased after 4 h of PEG treatment, reaching a 60-fold increase after 8 h of stress compared to initial levels. Indeed, even before stress onset (0 h), CT roots had 7.3±0.4 $ng.g^{-1}$ ABA whereas in DK and LT roots, ABA levels were 0.3±0.1 and 0.6±0.2 $ng.g^{-1}$, respectively. Similarly, a 14-fold ABA increase was observed in shoots of CT after 8 h of stress (Supplementary Table S1). Despite the differences found in root ABA content, dehydration induced a similar pattern of JA-Ile accumulation among the three treatments; JA-Ile levels transiently increased after 1 h of PEG exposure and then progressively returned to basal levels (Fig. 4b). The maximum JA-Ile was recorded after 1 and 2 h of stress being higher in DK and CT roots than in LT roots. Indeed, JA-Ile content in LT roots returned to basal levels as soon as 2 h after stress onset whereas in DK and CT roots it did it after 8 h of stress.

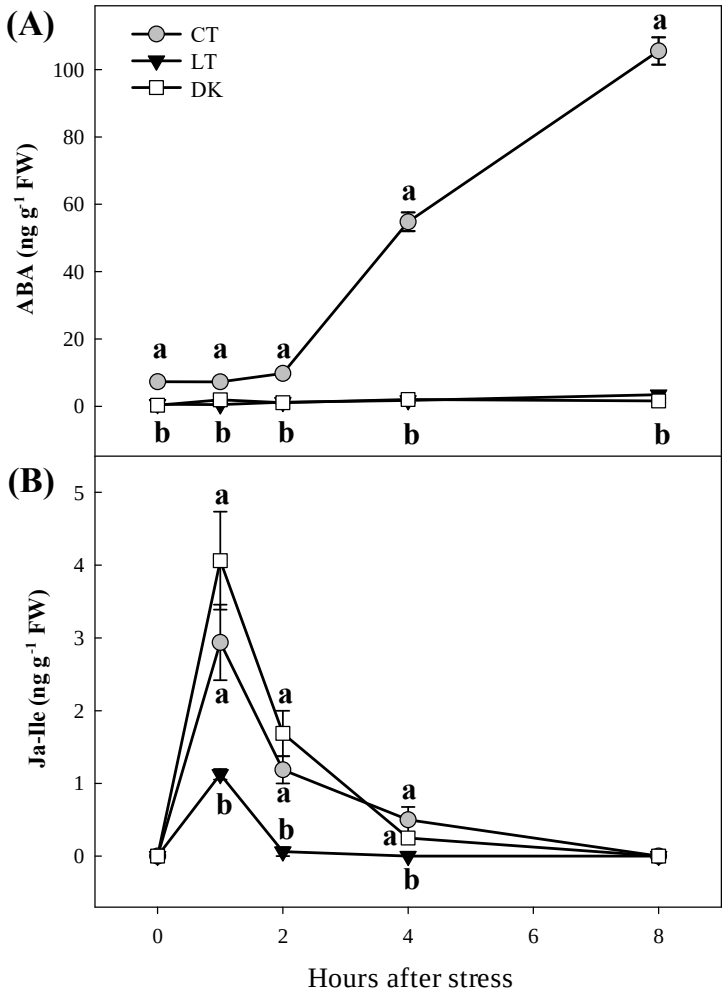

Figure 4. ABA (A) and JA-Ile (B) content in *Citrus* roots exposed to dehydration (-1.5 MPa). Roots of intact plants (CT) grown in the darkness; detached roots grown during three weeks into dark conditions (DK) or under illumination (LT). Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments within roots (Tukey´s test, $p \leq 0.05$).

Changes in β,β-carotenoid content in response to osmotic stress

In order to understand the potential role of carotenoid pool on ABA biosynthesis, β,β-branch carotenoids were quantified in roots upon dehydration. Among them, β-carotene and trans-violaxanthin were found in LT, DK and CT roots whereas 9-*cis*-neoxanthin was detected in LT and DK roots and β-cryptoxanthin only in LT roots (Fig. 5). Initial unstressed levels of total β,β-carotenoids were higher in roots exposed to light (LT) than in roots growing in the dark (DK). In all cases, PEG-induced osmotic stress drastically reduced the content of all β,β-carotenoids although at different rates (Fig. 5). Thus, this reduction was more pronounced in roots in the dark, being 92% and 73% respect of the initial values in CT and DK roots, respectively after 2 h whereas in LT roots, only a 25%

reduction was found (Fig. 5). Regarding individual carotenoids, a severe reduction in β-carotene, the most abundant β,β-carotenoid, was detected as soon as 1 h after the onset of stress in all treatments. Interestingly, after 8 h of stress, it was undetectable in DK roots or showed extremely low levels in LT and CT roots (Fig. 5). A similar pattern was found for trans-violaxanthin, the second most abundant β,β-carotenoid, showing a 75% and 85% reduction respect to initial values in LT/CT and DK roots, respectively, after 8 h of stress. Similarly, 9-*cis*-neoxanthin levels were undetectable after stress was imposed in DK and after 8 h in LT roots (Fig. 5). Interestingly, a minor content of β-cryptoxanthin was detected only in LT roots after 2 h of stress and then decreased by 40% at 8 h. In shoots, levels of β,β-carotenoids in CT plants decreased (30%) after 8 h of dehydration with respect to the initial values (Supplementary Table S1).

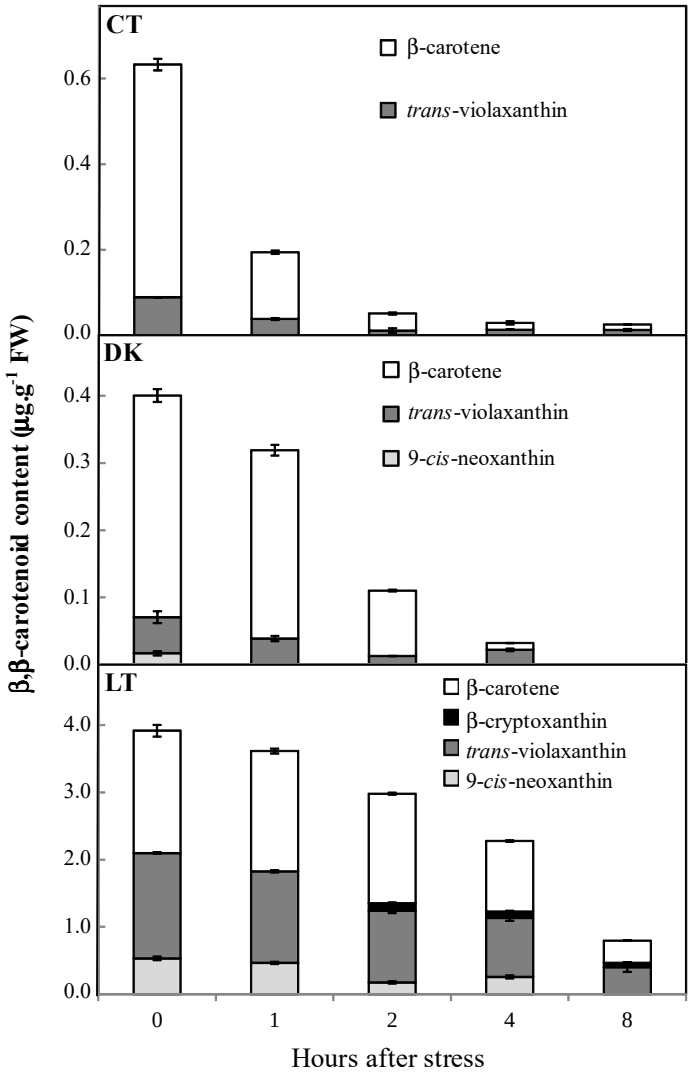

Figure 5. β,β-carotenoid content in *Citrus* roots exposed to dehydration (-1.5 MPa). Roots of intact plants (CT) grown in the darkness; detached roots grown during three weeks into dark conditions (DK) or under illumination (LT). Data are means ± SE of at least 3 replicates.

Changes in the expression of genes involved in carotenoid biosynthesis in response to osmotic stress

Expression levels of genes involved in three key steps of carotenoid biosynthesis (*CsPSY1*, *CsPSY3a*, *CsPSY3b*, *CsβCHX1*, *CsβCHX2* and *CsZEP*) were determined in dehydrated excised roots and intact plants (Fig. 6). Data represent the relative variations in transcript abundance at each time compared to the initial unstressed (0 h) values. As a general trend, stress upregulated most of the key genes involved in carotenoid biosynthesis. Among the three PSYs identified in Citrus (Peng *et al*., 2013), *CsPSY3a* showed a transient strong upregulation, reaching a 50-fold increase in DK roots after 2 h; a 15-fold increase in LT roots after 4 h; and a 10-fold increase in shoots of CT plants after 2 h (Fig. 6). Conversely, *CsPSY1* transcripts only slightly increased (2.1-fold) upon stress in DK roots whereas in LT and CT roots the expression level remained almost unchanged throughout the experiment. For all samples analyzed the expression of the *CsPSY3b* was practically undetectable (data not shown). Interestingly, different basal levels of *CsPSY* transcripts were found in non-stressed plants (0 h) among tissues and growing conditions: *CsPSY1* transcript abundance in DK roots was 20-fold lower than that found in CT and LT treatments, and the expression in shoots was 5.6-fold higher than in CT roots (Supplementary Fig. S1). At the initial time the *CsPSY3a* expression in LT roots was 4.3-fold higher than in CT and DK roots (Supplementary Fig. S1).

Transcript levels of both *βCHX* genes gradually increased in response to dehydration in roots from all treatments (Fig. 6). *CsβCHX1* was upregulated up to 46-fold in DK roots, and to 5.3-, 24-, and 6.5-fold in LT roots, CT roots and shoots, respectively after 8 h of stress. *CsβCHX2* showed a similar upregulation pattern to that of *CsβCHX1* but showing more pronounced changes in CT roots and shoots. Gene transcripts also increased (14-fold) in DK roots after 8 h of stress. Transcript abundance of *CsβCHX1* and *CsβCHX2* also varied in response to light in a tissue-dependent manner (Supplementary Fig. S1). Relative expression of *CsβCHX1* was higher in unstressed LT (33-fold increase) and DK (6.0-fold) roots compared to those of CT plants. Furthermore, transcript abundance in CT shoots was 2.5-fold higher than in CT roots (Supplementary Fig. S1).

Among the carotenoid biosynthetic genes, *CsZEP* was the less responsive to the PEG treatment; transcript levels remained unaltered in LT and CT roots whereas a 7.3-fold increase was found in DK roots after 4 and 8 h of stress conditions (Fig. 6). In shoots, *CsZEP* was downregulated after 8 h of stress. Moreover, its expression was also higher in

illuminated LT roots and CT shoots compared to unstressed dark roots (CT and DK, Supplementary Fig. S1).

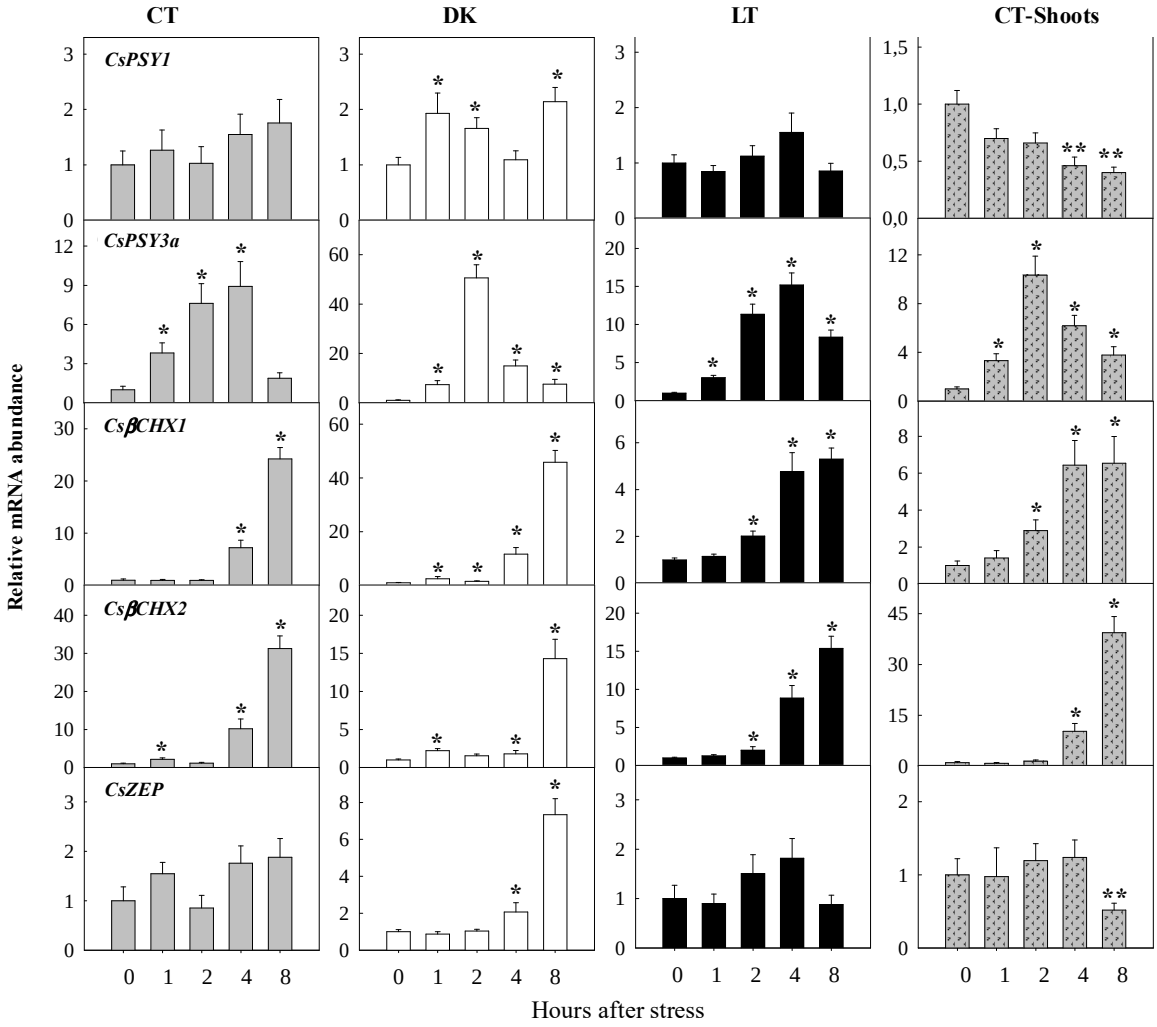

Figure 6. Relative mRNA abundance of carotenoid biosynthetic genes in *Citrus* roots and shoots exposed to dehydration (-1.5 MPa). Roots of intact plants (CT) grown in the darkness; Detached roots grown during three weeks into dark conditions (DK); or under illumination (LT); Shoots of intact plants (CT-Shoots) grown under illumination. Data are means ± SE of 4 replicates. ***** denotes significant upregulation compared to the initial unstressed level (=1). ****** denotes significant downregulation compared to the initial unstressed level (=1); Tukey´s test, $p \leq 0.05$.

Changes in the expression of genes involved in ABA biosynthesis and catabolism in response to osmotic stress

Expression of genes involved in ABA biosynthesis such as the two *NCEDs* described in Citrus (Rodrigo *et al*., 2006), Xanthoxin dehydrogenase (*CsXantDH* an ortholog of

Arabidopsis ABA2) and abscisic aldehyde oxidase (*CsAAO3*) were determined in roots and shoots in response to dehydration (Fig. 7). Osmotic stress upregulated *CsNCED1* and *CsNCED2* levels in all type of roots and in shoots. Indeed, *CsNCED1* transcript abundance increased 701-, 155-, 157- and 280-fold in roots of DK, LT, CT and in shoots of CT, respectively, compared to initial levels. Upregulation of the *CsNCED2* gene was more moderate, reaching 14.5-, 20.8-, 3.6- and 10.9-fold increases in roots of DK, LT, CT and in CT shoots, respectively (Fig. 7). Interestingly, *CsNCED1* transcript levels at 0 h were higher in both light-exposed tissues (shoots and LT roots) compared to CT roots. The opposite trend was observed for *CsNCED2* since these light-exposed tissues showed a reduced expression compared to CT roots (Supplementary Fig. S1).

CsXantDH expression levels in DK transiently increased after 1 h of dehydration (2.6-fold) and returned to initial levels in the following hours (Fig. 7). However, in detached LT roots, levels of *CsXantDH* transcripts were unaltered by the osmotic stress and only a slight decrease was detected after 8 h. On the other side, downregulation of *CsXantDH* occurred in both roots and shoots of CT plants after an initial transient upregulation, reaching 27% and 38% of the initial values after 8 h of stress, respectively. As the final step in the ABA biosynthesis, *CsAAO3* gene showed a transient upregulation (2.5-fold) in DK roots, similar to those found for *CsXantDH* (Fig. 7). In LT roots, *CsAAO3* gene expression remained unchanged during the first 4 h of stress and decreased a 50% after 8 h; also following a similar pattern to that of *CsXantDH* gene. Interestingly, the expression of this gene was progressively reduced in roots of CT plants being 3 times lower than the initial levels after 8 h. *CsAAO3* transcript levels in shoots increased after 1 h of stress to finally return to unstressed levels (Fig. 7). Furthermore, both *CsXantDH* and *CsAAO3* genes were upregulated in LT roots compared to DK and CT roots (Supplementary Fig. S1).

CsCYP707a gene, coding an enzyme involved in ABA catabolism, was strongly upregulated in the three types of roots (58-, 69- and 26-fold in DK, LT and CT, respectively) and in shoots of CT plants (103-fold) as soon as 1 h after the dehydration onset. Interestingly, levels of *CsCYP707a* transcripts decline after this initial increase but remained at levels higher than in the unstressed plants (Fig. 7).

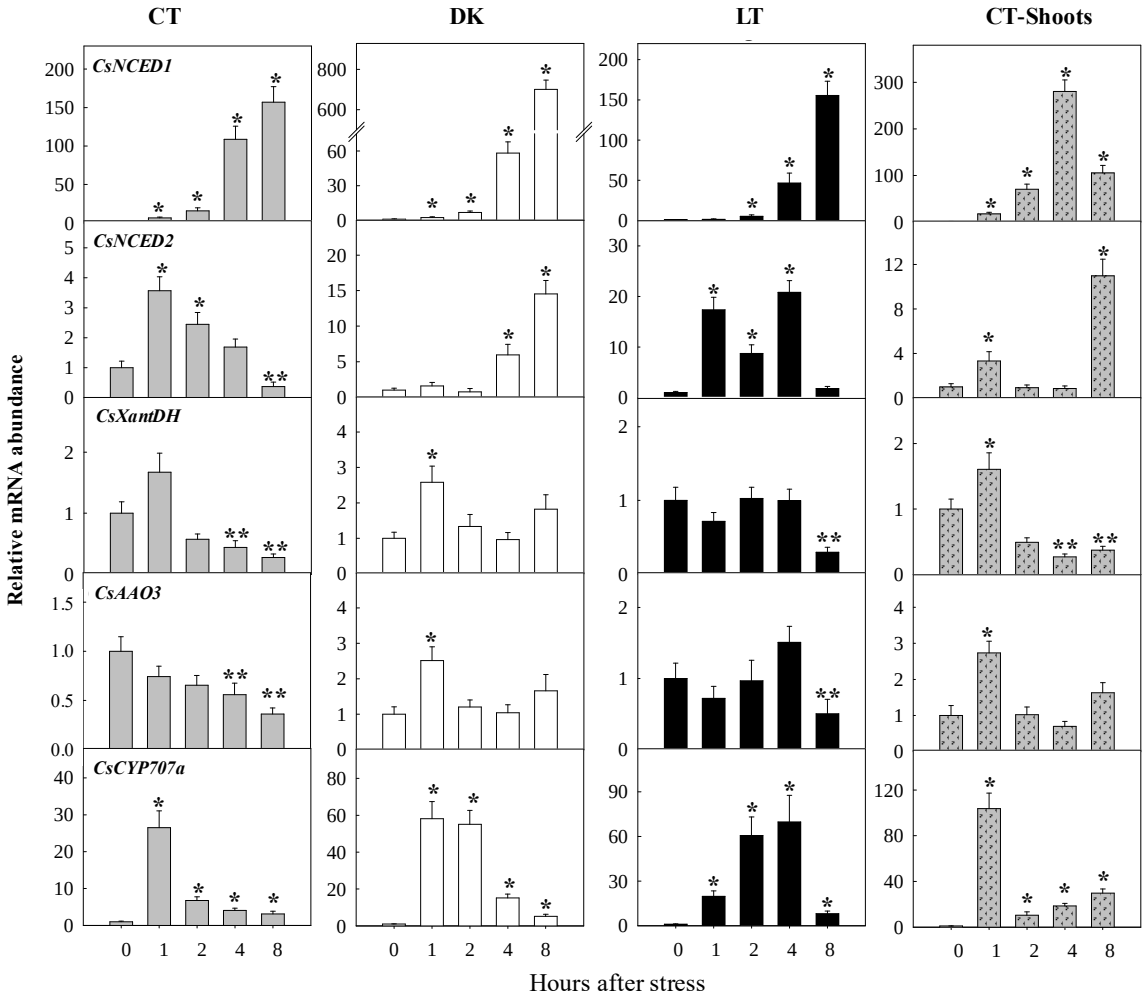

Figure 7. Relative mRNA abundance of ABA biosynthetic genes in *Citrus* roots and shoots exposed to dehydration (-1.5 MPa). Roots of intact plants (CT) grown in the darkness; Detached roots grown during three weeks into dark conditions (DK); or under illumination (LT); Shoots of intact plants (CT-Shoots) grown under illumination. Data are means ± SE of 4 replicates. ***** denotes significant upregulation compared to the initial unstressed level (=1). ****** denotes significant downregulation compared to the initial unstressed level (=1); Tukey´s test, $p \leq 0.05$.

Discussion

Plant growth and development and subsequent crop productivity are severely affected by water restriction. In this context, ABA plays a crucial role triggering several physiological and molecular responses to cope with this adverse condition in different plant tissues (Gómez-Cadenas *et al.*, 2015; McAdam *et al.*, 2015). Evidences demonstrated that aboveground tissues are crucial to sustain the accumulation of ABA in roots since this plant hormone is basipetally transported through the phloem in plants exposed to water

shortage (Manzi *et al*., 2015; Ren *et al*., 2007; Waadt *et al*., 2014) or high salinity (Waadt *et al*., 2014; Wolf *et al*., 1990). In addition, the contribution of roots to ABA accumulation upon water stress appears to be limited, which could be related to the low levels of carotenoids in this tissue (Ruiz-Sola *et al*., 2014). However, it has been suggested that pre-existing levels of precursors in this tissue should be sufficient to sustain the local accumulation of ABA (Walter *et al*., 2015). In this sense, the rapid basipetal flow of ABA from shoots to roots (Manzi *et al*., 2015; McAdam *et al*., 2015) could be masking the real ability of belowground tissues to synthetize ABA. Based on this evidence, it could be hypothesized that the reduced ability of roots to sustain the ABA accumulation in response to consecutive periods of water deficit (Manzi *et al*., 2015; Ren *et al*., 2007) could be overcome by increasing carotenoid availability.

Taking advantage of the capability of citrus roots for developing green coloration under light conditions (Fig. 1) we investigated the effect of an increased content of carotenoids on ABA biosynthesis. Carotenoid accumulation is highly responsive to light (Toledo-Ortiz *et al*., 2010) and, as expected, the exposure of roots to direct light increased (5.5-fold) the content of these pigments (Fig. 1 and 2b). Light also stimulated chlorophyll synthesis (Fig. 2a) which denotes an active conversion of typical root proplastids into chloroplasts typically associated to aboveground green tissues (Li and Yuan, 2013). In this sense, 65% of the carotenoid increase in roots was explained by the accumulation of ε,β-carotenoids (mainly lutein, Supplementary Fig. S2), but levels of β,β-carotenoids also substantially increased (Fig. 5). This was the case of β-carotene (3.3-fold higher in LT roots) and trans-violaxanthin (Fig. 5), which are typical carotenoids found in photosynthetic tissues (Lado *et al*. 2015a). Therefore, as hypothesized, in roots exposed to light total β,β-carotenoids amounts were significantly higher (6.1-fold) than in dark-incubated roots (Fig. 5 and Supplementary Fig. S2).

Despite this higher carotenoid availability in LT roots, dehydration experiments showed unexpected results: both detached roots (DK and LT) were unable to accumulate ABA (Fig. 4a), irrespective of the amount of β,β-carotenoids available and even in the presence of 9-*cis*-noexanthin (Fig. 5), the native substrate of NCEDs (Nambara and Marion-Poll, 2005). On the contrary, root and shoot tissues from intact plants accumulated ABA upon stress (Fig. 4a and Supplementary Table S1). Since roots under the three experimental conditions showed similar degrees of dehydration (Fig. 3) and also similar transient increases in JA-Ile content (Fig. 4b), which is considered a previous and necessary signal

to trigger ABA accumulation in water-stressed citrus roots (De Ollas *et al*., 2013), differences in ABA content in roots appear to be associated to the presence of the aerial part. Therefore, these data reinforce the idea that the aerial tissues plays a pivotal role in ABA accumulation in water-stressed roots (Ikegami *et al*., 2009; Manzi *et al*., 2015; Ren *et al*., 2007; Vernieri *et al*., 2001; Waadt *et al*., 2014). Taken together, ABA accumulation in response to dehydration in roots of intact plants with low levels of carotenoids (CT), and the incapability of carotenoid-rich LT roots to show a similar response, indicates that ABA accumulation is not directly linked to carotenoid availability in roots. Moreover, data suggest that its accumulation occurs also independently of the ability for de novo ABA biosynthesis in this organ. A plausible explanation is that plants have efficiently evolved to use shoots as a rapid source of ABA that is distributed to belowground tissues in response to water deficit. In this sense, our previous work (Manzi *et al*., 2015) and others (McAdam *et al*., 2015; Waadt *et al*., 2014; Wolf *et al*., 1990) demonstrated that ABA is continuously transported from shoots to roots. This process seems important to maintain basal ABA levels under non-stressed conditions (Fig. 4a) but also could provide elevated amounts of ABA to the roots by enhancing transport right after the stress onset (Ikegami *et al*., 2009; Manzi *et al*., 2015; Waadt *et al*., 2014). Thereby, presence of shoots (as a carotenoid-rich and active ABA-synthesizing tissue) ensures an unlimited supply of ABA to roots (Supplementary Table S1).

Classical models postulate that, in response to soil-borne stress such as drought or salinity, ABA would be produced in roots and subsequently transported to the aerial tissues via xylem sap (Christmann *et al*., 2005). However, results obtained in this work partially disagree with those extended evidences and also contrast with those results in which detached roots accumulate ABA upon stress (Borel *et al*., 2001; Ren *et al*., 2007). Metabolic differences existing at the moment of stress initiation and right after root detachment could explain variability in ABA content. It is possible that some complementary source of ABA (such as the conjugated ABA-GE) could be supporting any initial ABA increase when stress is applied immediately after root detachment (Manzi *et al*., 2015; Ren *et al*., 2007). Thus, in this work, detached roots were grown for three weeks before the stress imposition to likely exhaust any possible conjugated source of ABA. Results showed in this work expand previous findings indicating a reduced contribution of roots to the total ABA bulk in this tissue that was previously demonstrated in intact tomato (Thompson *et al*., 2007), maize (Ren *et al*., 2007) and Arabidopsis (Ikegami *et al*., 2009). Recently, Manzi *et al*. (2015) demonstrated that roots are unable to

sustain increasing ABA levels by de novo synthesis using reciprocal grafting of ABA-deficient tomato mutants.

Despite that the carotenoid pool available in roots does not influence the ABA production, dehydration induces a sharp decrease in carotenoid levels in all types of roots, which even leads to a complete depletion of some specific carotenoids (Fig. 5 and Supplementary Fig. S2). Although carotenoids in green tissues play several well-known functions (Ramel *et al*. 2012a) and their levels remained mainly unaltered by water stress (Supplementary Table S1), the role of β,β-carotenoids in roots still remains to be elucidated since evidences indicate that they are not directly contributing to the rise of ABA levels. However, it is likely that carotenoids in roots are being degraded or transformed into a different signal possibly involved in triggering a stress-related response. Despite ABA, several carotenoid derivatives such strigolactones (Ha *et al*., 2014) or nor-isoprenoids volatiles (Avendaño-Vázquez *et al*., 2014; Rubio-Moraga *et al*., 2014) could be formed through the action of different enzymes (CCDs, NCEDs, etc). In this sense, it was recently demonstrated that overexpression of PSY in Arabidopsis leaves, increased the C13-apocarotenoid glycosides by the action of CCD4 (Lätari *et al*., 2015). In addition, carotenoids can quench molecules of ROS generated during stress counteracting their harmful effects on physiology (Ramel *et al*. 2012a; Lado *et al*. 2016) The oxidation of carotenoids renders different derivatives such as short-chain volatile aldehydes or ketones (β-ionone or β-cyclocitral) that could act as stress signals (Ramel *et al*. 2012b). Moreover, certain ROS such as $^1O_2$ react with β-carotene rendering compounds that stimulate the auto-oxidation of other carotenoids (Fiedor *et al*., 2005). This could explain in part the sharp and severe carotenoid decrease observed upon dehydration (Fig. 3 and Supplementary Fig. S2). In the same sense, it has been recently suggested that yet uncharacterized carotenoid derivatives (different from ABA and SL) are needed to induce lateral root formation in Arabidopsis (Van Norman *et al*., 2014). Overall, the rapid decrease in carotenoid levels in stressed roots suggests that they take part in drought-stress responses in citrus plants, such as the detoxification of ROS and the production of different signaling molecules (McQuinn *et al*., 2015; Nisar *et al*., 2015).

Dehydration activates the carotenoid biosynthetic pathway as indicated by the upregulation of several key genes involved in this process (*CsPSY3a*, *CsBCHX1* and *CsBCHX2*; Fig. 6). However, the increased expression of these genes in detached roots was not mirrored by ABA accumulation (Fig. 4a) or an increase in the carotenoid pool

(Fig. 5). This suggests that in roots, this upregulation is part of a conserved response to water deficit but not directly related to the de novo ABA synthesis. Other mechanisms such as post-transcriptional regulation of the enzymes (Seo *et al*., 2000) could explain these results but it remains to be elucidated.

Nevertheless, it has been suggested that PSY could be a crucial enzyme in carotenoid accumulation (Maass *et al*., 2009), being also identified as a potential bottleneck for ABA biosynthesis in roots (Li *et al*., 2008). Although information exists on the expression of *CsPSY1*, *CsPSY2* and *CsPSY3* in several tissues such as leaves, fruit and flowers (Peng *et al*., 2013), no data could be found related to roots. Based on expression profile and sequence homology compared to other species (Walter *et al*., 2015), *CsPSY2* was renamed as *CsPSY3a* and *CsPSY3* as *CsPSY3b*, suggesting that *CsPSY3a* replaces a paralog PSY1/PSY2 which has been lost (Walter *et al*., 2015). *CsPSY1* expression in roots and leaves remained mainly unchanged during dehydration, resembling the response described in roots of monocots such as maize *ZmPSY1* (Li *et al*., 2008) and rice *OsPSY1* (Welsch *et al*., 2008). Indeed, its major role seems to be related to the accumulation of carotenoids in light-exposed citrus tissues such as leaves, flowers and fruits (Peng *et al*., 2013; Walter *et al*., 2015), which is in accordance to the higher expression levels in shoots compared to roots (Supplementary Fig. S1). Unlike *CsPSY1*, dehydration markedly upregulated *CsPSY3a* in all root types and also in the shoots (Fig. 6), suggesting that it plays a key regulatory role in stimulating carotenoid biosynthesis in response to water deficit. These results resemble the response of *ZmPSY3*, which is a specific gene induced in maize plants under water stress (Li *et al*., 2008) or *SlPSY3* induced in tomato roots under phosphorous starvation (Walter *et al*., 2015), suggesting a conserved transcriptional regulatory mechanism of carotenoid biosynthesis under different stresses. Moreover, light upregulates PSY in aboveground tissues such as citrus fruit (Lado *et al*. 2015b) and in Arabidopsis seedlings (Toledo-Ortiz *et al*., 2010), but also in carrot roots growing under light conditions (Fuentes *et al*., 2012). Interestingly, *CsPSY3a* expression was upregulated by light in unstressed roots but not in shoots (Supplementary Fig. S1), contrasting with previous reports suggesting PSY gene might be stress- but not light-responsive in Arabidopsis (Ruiz-Sola *et al*., 2014). Abundance of *CsPSY3b* transcripts in both roots and shoots was below detection limits, suggesting that it has a limited role in these tissues, in agreement with previous findings in citrus (Peng *et al*., 2013).

The β-carotene hydroxylation pathway and especially *CsβCHX1* gene expression has been deeply studied in several citrus aerial tissues such as fruit, flowers and leaves (Lado *et al.* 2015b), but no information on its expression in roots could be found in the literature. A second gen, *CsβCHX2* (orange1.1g027413m), which showed a 65% protein identity with a βCHX from *Vitis vinifera* (Young *et al*., 2012) and 58% identity with the Arabidopsis protein (AT4G25700) was included in this work. Both *CsβCHX1* and *CsβCHX2* showed a similar gradual upregulation in response to stress in both shoots and roots (Fig. 6). Moreover, *CsβCHX1* was strongly upregulated by light in LT roots whereas *CsβCHX2* showed only a slight increase in transcript levels (Supplementary Fig. S1), suggesting that *CsβCHX1* might control β-cryptoxanthin accumulation in LT roots (Fig. 5).

ZEP was previously involved in the tolerance of Arabidopsis to adverse conditions such as dehydration and salinity (Park *et al*., 2008); however, this response appears to be species- and tissue-specific (Schwarz *et al*., 2015). It was recently demonstrated that in leaves of Arabidopsis, abundance of ZEP enzyme decreased in response to drought whereas increased in roots (Schwarz *et al*., 2015). Expression data included in this work do not allow linking *CsZEP* to any particular role in ABA as no relevant changes were detected, except in DK roots (Fig. 6). Interestingly, light upregulates its expression in shoots and more importantly in roots (Supplementary Fig. S1), which likely contributes to increase xanthophyll levels in these tissues (Fig. 2b and 5). Indeed, it was previously demonstrated that under water deficit, the expression of *NpZEP* in *Nicotiana plumbaginifolia* was more pronounced in detached roots compared to intact plants (Borel *et al*., 2001). This suggests that expression of *CsZEP* is related to light and aerial organs, where ZEP plays a key role in the xanthophyll cycle (Park *et al*., 2008), rather than directly related to ABA metabolism (Frey *et al*., 2006). It could be hypothesized that upregulation of carotenogenic genes in response to water stress could provide substrates for other carotenoid-derived signals rather than ABA, as previously discussed.

Dehydration upregulated ABA biosynthetic genes *CsNCED1* and *CsNCED2* in roots and shoots as soon as 1 h after the stress onset (Fig. 7). *CsNCED1* is considered crucial for ABA accumulation in leaves and roots of citrus (Agustí *et al*., 2007; Rodrigo *et al*., 2006). However, the enhanced expression of these genes in detached roots upon dehydration was not reflected by an increase in ABA levels (Figs. 4a and 7). Moreover, the availability of the specific substrate 9-*cis*-neoxanthin and *trans*-violaxanthin (Fig. 5), which could turn into 9-*cis*-violaxanthin by action of a yet undiscovered 9-*cis*-isomerase (Nambara and

Marion-Poll, 2005; Neuman *et al*., 2014), was not sufficient to ensure ABA accumulation. Therefore, upregulation of *CsNCED* genes in roots and the availability of its native substrate do not seem enough to sustain ABA accumulation in roots. Interestingly, *CsNCED1* transcript abundance increased in response to light (shoots and roots; Supplementary Fig. S1). Nevertheless, a rapid upregulation of this gene in shoots has been traditionally associated to ABA production in this tissue (Christmann *et al*., 2005; Soar *et al*., 2006) and seems to be key in sustaining ABA transport to the roots (Manzi *et al*., 2015). On the other side, light-induced downregulation of *CsNCED2* in roots and shoots (Supplementary Fig. S1) is in accordance to its subsidiary role in sunlight-exposed tissues (Rodrigo *et al*., 2006).

Downstream *CsNCED* enzymes encoded by *CsAAO3* and *CsXantDH* genes are not considered rate-limiting steps and basal levels of those enzymes are believed to be sufficient to readily convert their substrates subsequently to ABA (González-Guzmán *et al*., 2002). Indeed, upregulation of *AAO3* gene induced by dehydration had no impact on the levels of protein but it did not impair the ability to accumulate ABA (Seo *et al*., 2000). However, both *CsAAO3* and *CsXantDH* genes were upregulated in LT roots (Supplementary Fig. S1), resembling the induction observed in carotenogenic genes by light-exposure, suggesting therefore an interaction between carotenoid and ABA biosynthetic pathways in this tissue. Likewise, it was recently demonstrated an intricate feedback regulation among carotenoids which regulates the expression of other carotenoid biosynthetic genes in the pathway (Kachanovsky *et al*., 2012). Nevertheless, it seems that water stress induces several conserved molecular responses irrespective of substrate availability, such as *CsCYP707a* gene, which showed a similar expression pattern among all roots (Fig. 7), in spite of the relevant differences existent in ABA content (Fig. 4a). To this respect, only ABA-accumulating CT roots were able to increase PA levels (Supplementary Fig. S3).

Data presented in this work showed that the mere presence of ABA-precursors (carotenoids) in the roots does not preclude phytohormone biosynthesis in this organ. Indeed, in response to dehydration ABA accumulated in roots of intact plants, despite containing low amounts of β,β-carotenoids, whereas detached carotenoid-rich roots were unable to accumulate ABA. Indeed, the levels β,β-carotenoids in LT roots (Fig. 5) largely exceed the ABA content commonly found in dehydrated roots of CT plants (Fig. 4a). This work, therefore, demonstrates that citrus roots are unable to de novo synthetize ABA to

sustain long-term hormone accumulation, irrespective of substrate availability. Moreover, dehydration upregulates specific genes from the carotenoid and ABA biosynthetic pathways in roots as occurs in shoots but this molecular mechanism does not result in ABA accumulation in this organ. In this sense, previously described ABA transport from aerial tissues to roots (Manzi *et al*., 2015; McAdam *et al*., 2015; Waadt *et al*., 2014) seems to be the main source for hormone accumulation in roots.

Materials and methods

Plant material

After removing coats, seeds of Citrus Macrophylla (*Citrus macrophylla* Wester) were disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 as a wetting agent and then rinsed three times with sterile distilled water. Seeds were sown individually in 25×150 mm culture tubes containing 25 mL of germination medium consisting of Murashige and Skoog (MS) salt solution, 100 $mg.L^{-1}$ *myo*-inositol, 1.0 $mg.L^{-1}$ pyridoxine-HCl, 0.2 $mg.L^{-1}$ thiamine-HCl, 0.5 $mg.L^{-1}$ nicotinic acid, 0.2 $mg.L^{-1}$ glycine and 30 $g.L^{-1}$ sucrose. The pH was set at 5.7 ± 0.1 with 0.1N NaOH before autoclaving. The medium was solidified with agar at 9.0 $g.L^{-1}$ (Pronadisa, Madrid, Spain). The cultures were maintained in darkness at 25ºC for two weeks. At this point, tubes containing plants were transferred to a growth chamber under a 16 h photoperiod of photosynthetically active radiation (PAR) of 150 µmol m^{-2} s^{-1}, and at a constant temperature of 25ºC. Roots were kept in the dark by covering the lower part of the tube with an aluminum foil. After three weeks, (height of 6-8 cm), plants were divided into three groups: in the first, shoots were excised with a scalpel 2 mm below to the root-shoot junction. This group was kept in the complete darkness (DK) by covering the tube rack with an opaque box; in the second set, shoots were also removed but roots were kept in the light (LT); finally, a third group of plants (CT) was left intact, maintaining roots in the dark and shoots light-exposed. To exclude any side-effect of wounding, plants were kept in these conditions for 3 weeks before stress imposition, period in which LT roots developed a strong coloration. Osmotic stress was imposed by transferring plant material to a solution containing PEG-6000 to achieve water potential values of -1.5 MPa. During the dehydration period, roots were kept in the darkness and with the same conditions of temperature as indicated above. Sample material was collected at 0; 1; 2; 4 and 8 h after

the stress onset. It was rinsed with distilled water, immediately frozen in liquid nitrogen, ground into a fine powder and stored at -80ºC until analyses.

Relative water content

Relative water content (RWC) of roots was monitored at each harvest time. Leaf and root samples were immediately weighed after collection (FW) and then hydrated to full turgor by maintaining them in distilled water for 24 h in the dark to assay turgid weight (TW). Samples were therefore desiccated at 70ºC for two days to obtain dry weight (DW). Three replicates were used for each treatment. The RWC was calculated as RWC= ((FW-DW)/(TW-DW)) ×100.

Hormone analysis

ABA and jasmonoyl-isoleucine (JA-Ile) were extracted and analyzed essentially as described in (De Ollas *et al*., 2013) with slight modifications. Briefly, 0.2 g of dry plant material was extracted in 2 ml of distilled H_2O after spiking with 25 µl of a 2 $mg.L^{-1}$ solution of d_6-ABA and dehydrojasmonic acid (DHJA) as internal standards. After centrifugation at 10.000 × *g* at 4°C, supernatants were recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 mL of di-ethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The dry residue was then resuspended in a 9:1 H_2O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a UPLC system (Waters Acquity SDS, Waters Corp., Milford, MA) interfaced to a TQD triple quadrupole (Micromass Ltd. Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source.

Separations were carried out on a Gravity C_{18} column (50 × 2.1 mm, 1.8-µm, Macherey-Nagel GmbH, Germany) using a linear gradient of MeOH and H2O supplemented with 0.1% acetic acid at a flow rate of 300 $\mu L.min^{-1}$. Specific transitions for ABA/d_6-ABA (263>153/269>159) and JA-Ile/DHJA (322>130/211>59) were monitored in negative ionization mode. Quantitation of plant hormones was achieved by external calibration with known amounts of pure standards using Masslynx v4.1 software.

Pigment identification and quantification

Plant pigments were extracted as previously described (Alós *et al*., 2014; Rodrigo *et al*., 2006). The chlorophyll (*a*+*b*) content was determined by measuring the absorbance at 644 and 662 nm and calculated as described in (Alós *et al*., 2014). After chlorophyll measurements the ethereal pigment solution was dried and saponified using a 10:90 (v:v) methanol:KOH solution. Carotenoids were extracted and samples dried under a N2 stream and kept at -20 ºC until analysis. Carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model 2998 photodiode array detector, and Empower software (Waters). A C_{30} carotenoid column (250 × 4.6 mm, 5 μm, YMC Europe GmbH, Germany) coupled to a C_{30} guard column (20 × 4.0 mm, 5 μm; YMC Europe GmbH, Germany) was used. Samples were prepared for HPLC by dissolving carotenoid extracts in a $CHCl_3$:MeOH:acetone solution (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl tert-butyl ether was used for carotenoid separation (Alós *et al*., 2014). The carotenoid peaks were integrated at their individual maxima wavelength and their contents were calculated using calibration curves for β-cryptoxanthin (Extrasynthese), lutein (Sigma), neoxanthin, violaxanthin isomers, zeaxanthin (Extrasynthese) and α- and β-carotene (Sigma). Determinations were carried out in duplicate and all procedures were performed on ice and under dim light to prevent isomerization and photodegradation of carotenoids.

Quantitative real-time PCR

Total RNA was isolated from roots and leaves at each sampling date using RNeasy Plant Mini Kit (Qiagen, Madrid, Spain) and subsequently treated with RNase-free DNase (Promega Biotech Ibérica, SL. Madrid, Spain) in order to remove genomic DNA contamination. The transcripts present in 1 μg of total RNA were reverse-transcribed using Primescript RT reagent with oligo(dT) primer (Takara Bio, Inc. Japan) in a total volume of 20 μL. For amplification, 1:5 first-strand cDNA (containing approximately 100 ng of cDNA) was used. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a StepOne™ Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) instrument, using the SYBR- Green I Master kit (Roche, Madrid, Spain). The reaction mixture contained 1 μL of cDNA solution, 1 μM of each gene-specific primer pair and 5 μL of SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA)

including dye, uracil-DNA glycosylase and AmpliTaq® DNA polymerase to a final volume of 10 µL. The primers employed for the amplification of each gene are described in (Supplementary Table S2). The protocol for all the genes analyzed consisted of 10 min at 95ºC for pre-incubation, followed by 40 cycles of 10 s at 95ºC for denaturation, 10 s at 60ºC for annealing and 20 s at 72ºC for extension. Fluorescence intensity data were acquired during the extension time. Amplicon specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, StepOne™ Software v2.3 and expression levels relative to values of a reference sample were calculated using the Relative Expression Software Tool (REST; Pfaffl *et al*. 2002). Normalization was performed using the expression levels of the actin gene based on previous housekeeping selection for citrus tissues (Alós *et al*., 2014). For all genes analyzed, the reference sample was the expression value obtained at the non-stressed harvest time (0 h) in each treatment and set at one.

Statistical analysis

Samples were analyzed by triplicate. Mean and standard error (±) are shown in results. Analysis of variance (ANOVA) was used to determine the statistical significance. Post-hoc mean comparisons were performed by Tukey´s test ($p \leq 0.05$).

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Disclosures

The authors have no conflicts of interest to declare.

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Supplementary Information

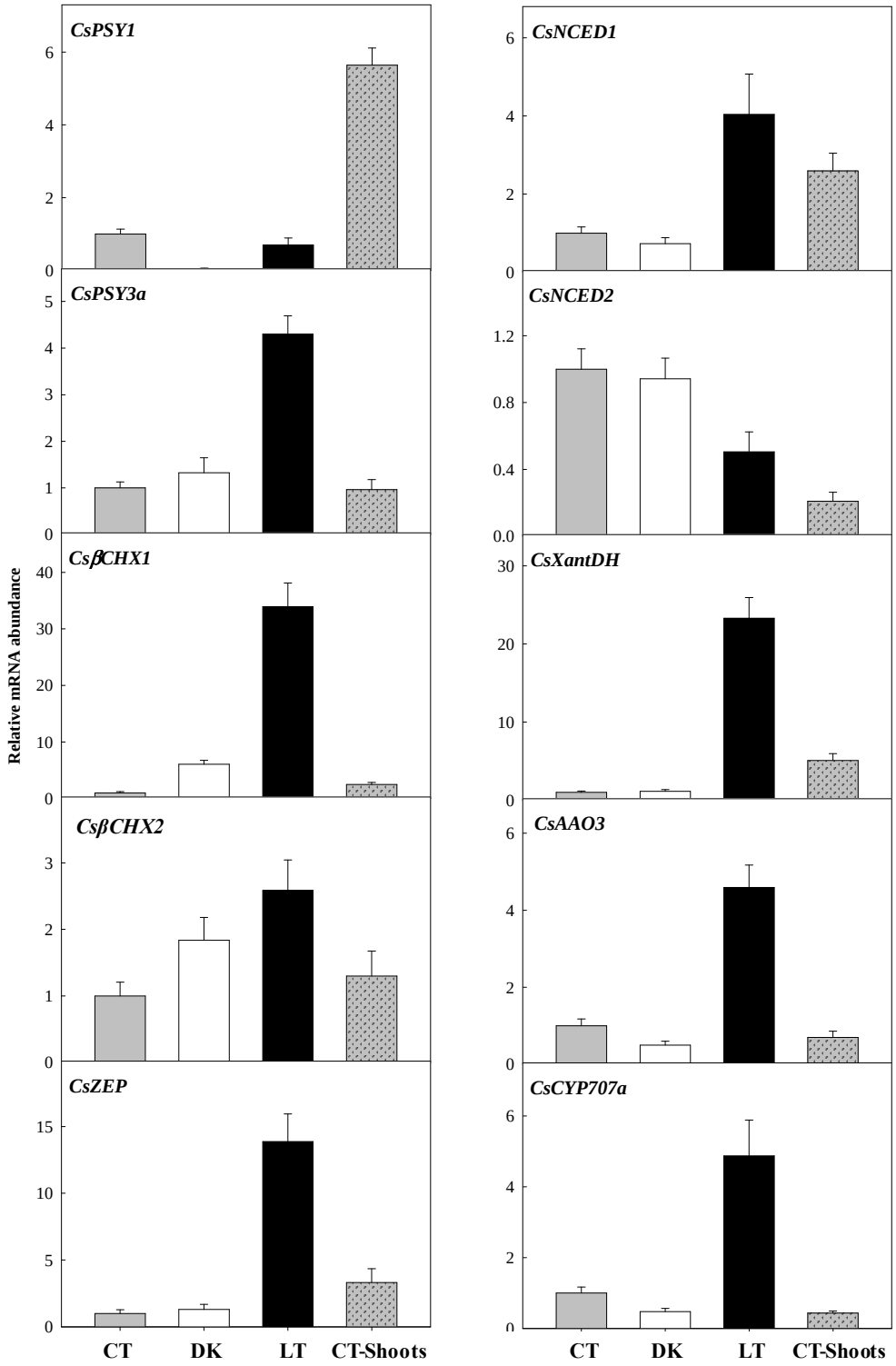
Supporting information Table S1. ABA, β,β-carotenoid and total carotenoid content in shoots of CT plants in response to PEG.

Hours	ABA (ng g^{-1} FW)	β,β-carotenoids (μg g^{-1} FW)	Total carotenoids (μg g^{-1} FW)
0	10.3 ± 1.3	66.2 ± 2.6	168.7 ± 5.4
1	7.1 ± 1.0	44.1 ± 1.7 *	115.8 ± 2.1 *
2	14.9 ± 1.2	76.4 ± 5.6	184.7 ± 8.4
4	65.8 ± 1.0 *	54.0 ± 1.7 *	154.6 ± 3.9 *
8	140.8 ± 2.4 *	46.6 ± 1.0 *	134.5 ± 1.7 *

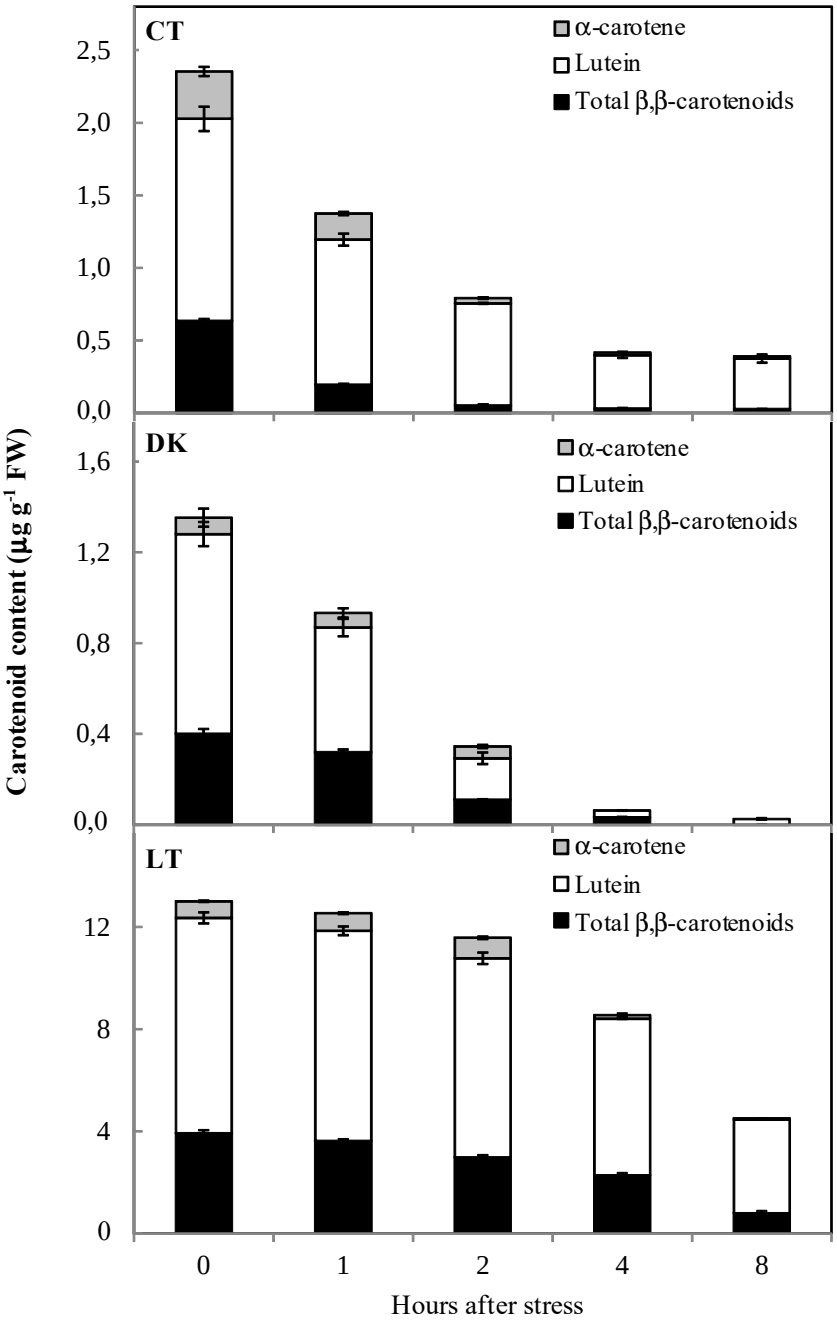
* denotes statistical differences ($p<0.05$) compared to the levels at the unstressed time 0h.

Supporting information Table S2. Sequences of the primers used for qRT-PCR. The genes analysed were: *Phytoene synthase 1* (*CsPSY1*), *Phytoene synthase 3* (*CsPSY3a* and *3b*), *β-carotene hydroxylase 1* (*CsβCHX1*), *β-carotene hydroxylase 2* (*CsβCHX2*), *Zeaxanthin epoxidase* (*CsZEP*), *9-cis-epoxycarotenoid dioxygenase1* (*CsNCED1*), *9-cis-epoxycarotenoid dioxygenase2* (*CsNCED2*), *Xanthoxin dehydrogenase* (*CsXantDH*), *Abscisic aldehyde oxidase* (*CsAAO3*), *ABA 8´-hydroxylase* (*CsCYP707a*), and *actin* (*CsActin*).

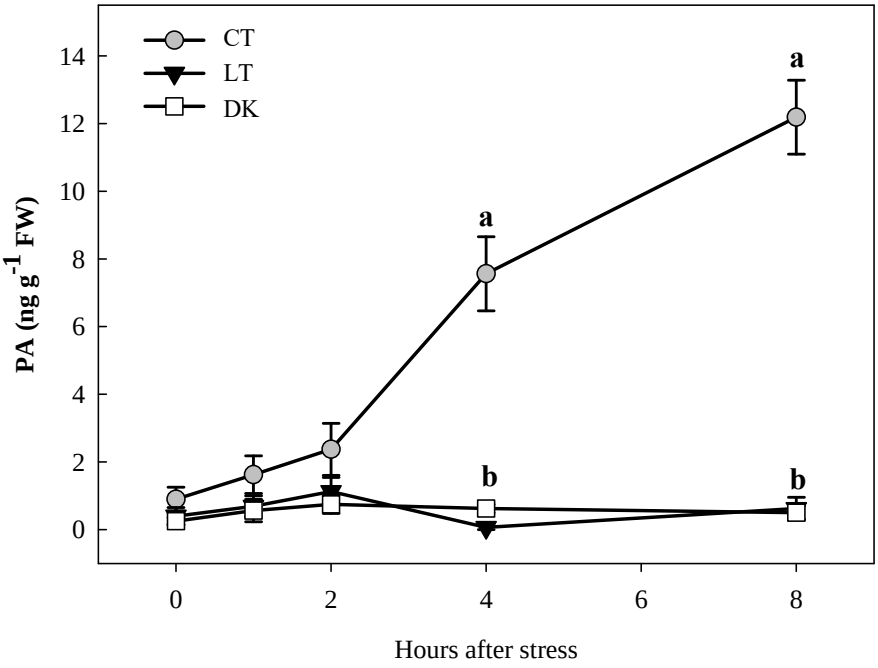
Citrus gene	Locus	Direction	Primer sequence (5'-3')	Amplicon size (bp)
CsPSY1	**orange1.1g044623m**	F	GGTCGTCCATTTGATATGCTTG	111
		R	CCTAAGGTCCATCCTCATTCCT	
CsPSY3a	**orange1.1g016696m**	F	AATGCATTTTGTGTAAGCCCTGCT	148
		R	TGTCCCTAAAAGGCTTGATGTGTAAATTG	
CsPSY3b	**orange1.1g036368m**	F	CAACGCTACTTACATGGGCTCTGTA	145
		R	TCTAAAAGGCTTGATGTCTAGCGGG	
CsβCHX1	**orange1.1g020892m**	F	GGCTCATAAAGCTCTGTGGC	210
		R	CCAGCACCAAAACAGAGACC	
CsβCHX2	**orange1.1g027413m**	F	AGAAGAGGAAACCGAAGAGCTTGAG	182
		R	GATTGCTGCAACAAGGTAGGTTTGT	
CsZEP	**orange1.1g005770m**	F	GCAGCTATGAGAGAGCTAGG	117
		R	GACAACGGTCCAAGTCCAAC	
CsNCED1	**orange1.1g007379m**	F	AATGCTTGGGAAGAGCCTGAG	147
		R	AGTGGACTCGCCGGTCTTTAG	
CsNCED2	**orange1.1g007291m**	F	GTGAATCGCAACAGCCTTGG	164
		R	GGCAAGAAAAATGGCTCGCC	
CsXantDH	**orange1.1g023573m**	F	GTGGAGCTACTGGGATTGGA	186
		R	GGTCAACAGCAGAGCAAACA	
CsAAO3	**orange1.1g000629m**	F	CACTTAGGTCAACCCCTGGA	174
		R	CCCGAACTTTTTCCAACAGA	
CsCYP707a	**orange1.1g038621m**	F	CTTTGGCACCCATGAAAGAT	264
		R	TCAATGTTGCACTGCTCTCC	
CsActin	**orange1.1g037845m**	F	CCCTTCCTCATGCCATTCTTC	105
		R	CGGCTGTGGTGGTAAACATG	



Supporting information Fig. S1. Relative expression of genes involved in the carotenoid and ABA biosynthesis pathway at the unstressed time (0 h). Values are referenced to CT roots (=1). Roots of intact plants (CT) grown in the darkness; detached roots grown during three weeks into dark conditions (DK); or under illumination (LT); Shoots of intact plants (CT-Shoots) grown under illumination. Data are means ± SE of 4 replicates.



Supporting information Fig. S2. Total carotenoid accumulation in roots in response to dehydration. Data are means ± SE of at least 3 replicates.



Supporting information Fig. S3. Phaseic acid accumulation in roots in response to dehydration. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments (Tukey´s test, $p \leq 0.05$).

3.3. Chapter III.

Leaf dehydration is needed to induce ABA accumulation in leaves and roots of citrus plants

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Abstract

Roots are the main organ involved in the water uptake; hence, it has been assumed that they are the organ sensing soil dehydration and triggering water stress responses at the entire plant level, including abscisic acid (ABA) accumulation. However, several lines of evidence demonstrate that leaves directly adjust their water status (and induce ABA) in response to changes in the evaporative demand. To evaluate the influence of leaf water status on ABA accumulation in roots, the canopy of citrus plants was subjected to different levels of humidity while roots were kept dehydrated. Leaves and roots of completely dehydrated plants accumulated ABA whereas plants with turgid leaves did not accumulate ABA in leaves or roots. Furthermore, in dehydrated roots, the expression of several key genes involved in carotenoid and ABA biosynthetic pathways such as *CsβCHX1* and *CsNCED1* was strongly affected by the water status of leaves. Results demonstrate that leaves are not only a relevant source of ABA for roots during dehydration but also that leaf-dehydration is needed to trigger ABA-induced responses in roots.

Keywords: ABA transport, gene expression, drought, 9-*cis*-epoxycarotenoid dioxygenase (NCED), shoot-to-root transport, water stress.

Abbreviations: ABA, abscisic acid; ABA-GE, ABA-glycosyl ester; AOG, ABA O-glycosyl transferase; βCHX, β-carotene hydroxylase; βGluc, β-glycosidase; CYP707a, ABA 8'-hydroxylase; DPA, dihydrophaseic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PEG, polyethylene glycol; PSY, phytoene synthase; RAB, Responsive to ABA; RD, Responsive to Dessication; RWC, relative water content; VPD, vapor pressure deficit; ZEP, zeaxanthin epoxidase.

Introduction

Abscisic acid (ABA) accumulation is one of the first responses displayed by plants to cope with different abiotic stress conditions such as water deficit, which severely affects plant growth and development and compromises crop productivity and plant survival (Gómez-Cadenas *et al*., 2015). A widely accepted model suggests that the increased ABA levels in water-stressed plants coordinate water relations throughout the entire plant. In this scenario, dehydration induces root ABA biosynthesis which is then translocated via xylem sap to leaves, inducing stomatal closure and minimizing water loss (Zhang and Davies 1989; Jones 2015). Under this model, roots not only act as a water uptake organ but also play a crucial role in detecting the decrease in soil water potential and consequently, in triggering ABA accumulation (Christmann *et al*. 2007; Dodd *et al*. 2008a). However, ABA levels in roots or in xylem sap hardly mirrored stomatal closure in leaves (Dodd *et al*. 2008b; Puertolas *et al*. 2014; Carvalho *et al*. 2016). Moreover, grafting experiments using ABA-deficient tomato mutants have revealed that leaf ABA accumulation could take place independently of root-sourced ABA (Holbrook *et al*. 2002; Manzi *et al*. 2015). Indeed, it was recently demonstrated that guard cells are able to autonomously close stomata in response to changes in air relative humidity due to the existence of a complete drought-responsive ABA biosynthetic pathway in these cells (Bauer *et al*. 2013). Thus, leaf ABA accumulation appears to depend not only on root exposure to dehydration but also on shoot perception of water deficit (Ikegami *et al*. 2009; Blum 2015).

The incomplete understanding of the relationship between changes in root water potential and stomatal closure via ABA transport from root-to-shoot have led to alternative explanations such as hydraulic signals (Soar *et al*. 2006b; Christmann *et al*. 2007; Tardieu *et al*. 2010; Blum 2015; Jones 2015). Water potential gradients generate the driving force to transport water through the soil-plant-atmosphere continuum system where leaves play a crucial role in detecting the environmental changes such as relative humidity or vapor pressure deficit (VPD; Christmann *et al*. 2013) and in consequence, modulating the levels of ABA and stomatal conductance (Waadt *et al*. 2014). Indeed, in several plant species, stomatal closure seems to be primarily under the control of hydraulic signals whereas ABA prevails after leaf turgor loss (Christmann *et al*. 2007; McAdam and Brodribb 2014). Besides the differences between iso- and anisohydric behaviors in regulating the stomatal closure and the water loss (Tombesi *et al*. 2015), root water uptake depends on the water potential gradient between leaves and roots (Blum 2015). In this process, leaves play a key

role on the adaptation to the changes in evaporative demand, adjusting therefore, the water status of the entire plant (Waadt *et al*. 2014). In this sense, it is clearly established that leaves are able to rapidly adapt to daily fluctuations from environmental stimuli (Soar *et al*. 2006b; Caldeira *et al*. 2014) whereas root exposition to soil drying is a slow and gradual process under natural conditions. Thus, it could be stated that leaves generate its own ABA which could be therefore responsible for these physiological changes in the leaves (Soar *et al*. 2006b).

In addition, several lines of evidence demonstrate that ABA is basipetally transported from aerial tissues to roots under well-watered conditions, questioning also the root-to-shoot ABA transport model (Waadt *et al*. 2014; Manzi *et al*. 2015), a process that is remarkably favored by dehydration (Ikegami *et al*. 2009; Manzi *et al*. 2015) and other adverse conditions (Wolf *et al*. 1990; Vernieri *et al*. 2001). The low ability to accumulate ABA precursors (carotenoids) in roots under natural conditions suggests an ABA transport to sustain long-term root ABA accumulation (Ren *et al*. 2007; Manzi *et al*. 2015). Thus, besides the existence of a small and transient accumulation of ABA in detached roots (Ren *et al*. 2007; Ikegami *et al*. 2009) or in stem-girdled plants (Cornish and Zeevaart 1985; Vernieri *et al*. 2001; Manzi *et al*. 2015), the massive root ABA accumulation under water stress is supported by the transport from aerial organs (Ren *et al*. 2007; Manzi *et al*. 2015). Indeed, artificially-increased levels of carotenoid in detached roots failed to sustain the ABA rise under dehydration (Manzi *et al*., unpublished data), suggesting that roots are not specialized in synthesizing large amounts of ABA.

Moreover, leaves can sense and autonomously respond to dehydration elevating ABA levels even in the absence of roots (Pérez-Clemente *et al*. 2012) whereas ABA production in detached roots or stem-girdled plants is severely impaired (Ikegami *et al*. 2009), emphasizing the role of leaves on ABA accumulation in both organs. In stressed roots, ABA accumulation is delayed compared to the leaves (Christmann *et al*. 2005) which is coincident with a latter upregulation of rate limiting genes in the ABA pathway, such as 9-*cis*-epoxycarotenoid dioxygenase (NCED; Soar *et al*., 2006b).

The accepted paradigm claims that roots are able to ‘measure’ the soil water status (Zhang and Davies 1989; Tardieu *et al*. 2010; Blum 2015; Jones 2015; Tardieu *et al*. 2015) and in consequence, induce diverse stress responses (including ABA accumulation) at the whole plant level under water deficit. However, available experimental data reveal that leaves could also be exerting an active role in perceiving water deficit and triggering

physiological and biochemical responses (Ikegami *et al*. 2009). Determining the exact role of leaves on sensing water deficit is a difficult task under natural conditions, since experiments may involve dehydration of leaves keeping the roots in a constant level of humidity.

Dehydration experiments were designed in this work to explore whether leaves participate in sensing water deficit stress at the whole plant level, and particularly if canopy influence ABA accumulation in roots. The approach followed included time-course assays in which water deficit in roots was applied with different relative humidity levels at the canopy. Hormonal and transcriptional changes in the ABA biosynthetic pathway in response to water stress were explored in leaves and roots.

Materials and methods

Plant material and growing conditions

One-year-old Carrizo citrange (*Citrus sinensis* L. Osb. × *Poncirus trifoliate* L. Raf.) seedlings were used for the different assays. Plants were acclimated for at least 4 months before each experiment in a greenhouse under natural photoperiod and day/night temperatures of 25±3.0ºC/18±2.0ºC. Plants were grown in 2.5 L plastic pots filled with a mixture of peat moss, perlite and vermiculite (80:10:10) as substrate and watered three times a week with 0.5 L of a half-strength Hoagland solution (Manzi *et al*., 2015). Homogeneously sized plants of about 80 cm in height were used for each experiment.

Defoliation experiments. Seedlings were transplanted to dry perlite to cause plant dehydration. After three days submitted to this condition, three treatments were performed: 1) a subset of these water-stressed plants was fully defoliated (WS+100%Def); 2) other group of plants was partially defoliated discarding 50% of the leaves (WS+50%Def); and 3) a last subset of plants remained with intact leaves (WS). After defoliation, all plants were kept for two extra days under stress conditions. Also, a set of well-watered plants (WW) was used as a control along the experiment. Based on preliminary assays, defoliation was performed after three days of dehydration since an early defoliation highly influenced the water availability in roots due to the different number of leaves, and therefore, affected ABA content in this tissue. Root samples were harvested after 3 and 5 days of dehydration.

Bagging experiments. Intact Carrizo seedlings were transferred to a plastic basin where roots were submerged in a -1.5 MPa PEG 6000 solution whereas canopy was subjected to different treatments. In the first set of plants, canopy was covered with a transparent plastic to increase the relative humidity around the plant. These plants were regularly sprayed with distilled water to maintain a thin layer of water on their leaves (WS+100% RH) and relative humidity over 80% throughout the experiment. A second set of plants was also covered but no water was applied to leaves. Relative humidity was maintained at 60% throughout the experimental period (WS+60% RH). A third set of plants was also dehydrated but neither covered nor water was sprayed to the leaves (CT). Relative canopy humidity was about 40% in this treatment. Temperature during the experiment was similar among treatments (24±2.0ºC). Roots and leaves were collected at 0 (prior to stress), 2, 4, 8 and 24 h from the onset of stress.

Sample extraction and storage

In both experiments, samples of at least three plants were collected at each sample date, rinsed with distilled water, immediately frozen in liquid nitrogen, grounded into a fine powder and stored at –80ºC or lyophilized until analysis.

Relative water content

The relative water content (RWC) of leaves and roots was monitored in both experiments. Leaves and roots were weighed to determine fresh weight (FW). Then, tissues were hydrated to full turgor by maintaining them in distilled water for 24 h in the dark to assess turgid weight (TW). Afterwards, samples were dried at 70ºC during 3 d for dry weight (DW). The RWC was calculated as RWC= [(FW –DW)/(TW – DW)] × 100.

Hormone analysis

ABA and dihydrophaseic acid (DPA) were extracted and analyzed essentially as described in Manzi *et al*. (2015) with slight modifications. Briefly, 0.2 g of plant material was extracted in 2 mL of distilled H_2O after spiked with 100 ng of d_6-ABA. After centrifugation at 10.000 × *g* at 4°C, supernatants were recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 mL of di-ethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The aqueous phase containing the ABA-glycosyl ester (ABA-GE) was hydrolyzed by adding 2.0 mL of 0.1 M

NaOH and placed at 60ºC for 30 min. Samples were then cooled, acidified and spiked with d_6-ABA before partitioning as described above. ABA-GE was estimated as ABA. The dry residue was then resuspended in a 9:1 H_2O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a HPLC system (Waters Alliance 2695, Waters Corp., Milford, MA). Separations were carried out on a Gravity C18 column (50 × 2.1 mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) using a linear gradient of MeOH and H2O supplemented with 0.1% acetic acid at a flow rate of 300 µL.min^{-1}. ABA, d_6-ABA and DPA were monitored at *m*/*z* 263>153, 269>159 and 281>171, respectively. Hormones were then quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. All data were acquired and processed using Mass Lynx v4.1 software.

Quantitative real-time PCR

Relative mRNA abundance of key carotenoid and ABA biosynthetic pathway genes was determined as described as follow. Total RNA was isolated from roots and leaves at each sample date, using RNeasy Plant Mini Kit (Qiagen, Madrid, Spain) and subsequently treated with RNase-free DNase (Promega Biotech Ibérica, SL. Madrid, Spain) according to the manufacturer in order to remove genomic DNA contamination. The transcripts present in 1 µg of total RNA were reverse-transcribed using Primescript RT reagent with oligo(dT) primer (Takara Bio, Inc. Japan) in a total volume of 20 µL. One microliter of a five times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a StepOne™ Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) instrument, using the SYBR- Green I Master kit (Roche, Madrid, Spain). The reaction mixture contained 1 µL of cDNA, 1 µM of each gene-specific primer pair and 5 µL of SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) including dye, uracil-DNA glycosylase and AmpliTaq® DNA polymerase to a final volume of 10 µL. Analyzed genes were: *phytoene synthase1* (*CsPSY1*), *phytoene synthase3a* (*CsPSY3a*), *β-carotene hydroxylase1* (*CsβCHX1*), *β-carotene hydroxylase2* (*CsβCHX2*), *zeaxanthin epoxidase* (*CsZEP*), *9-cis-epoxycarotenoid dioxygenase1* (*CsNCED1*), *9-cis-epoxycarotenoid dioxygenase2* (*CsNCED2*), *ABA O-glycosyl transferase* (*CsAOG*), *β-glycosidase* (*CsβGluc*), *ABA 8'-hydroxylase* (*CsCYP707a*), *Responsive to Dessication29* (*CsRD29*), *Responsive to Dessication22* (*CsRD22*) and

Responsive to ABA (*CsRAB18*). The primers employed for the amplification of each gene are described in (Supplementary Table S1). The comparative C_T method was used to relative quantify each amplified product in the samples. The threshold cycle (C_T) was automatically determined with default parameters. For expression measurements, StepOne™ Software v2.3 and expression levels relative to values of a reference sample were calculated using the Relative Expression Software Tool (REST). Normalization was performed using the expression levels of the actin gene as housekeeping (Alós *et al*. 2014). The specificity of RT-qPCR reactions was determined by melt curve.

Statistical analysis

Samples were analyzed at least by triplicate and means ± standard errors are shown in results. Analysis of variance (ANOVA) was used to determine the statistical significance. Mean comparisons were performed by Tukey´s test ($p \leq 0.05$).

Results

Defoliation experiments in dehydrated plants

To explore the role of leaves on ABA accumulation in roots, defoliation experiments were carried out in dehydrated plants. Firstly, intact plants were transferred to dry perlite to induce a homogeneous dehydration (Fig. 1a). After three days, RWC in the roots of water-stressed plants decreased until 58.6±2.1% compared to 81.6±0.2% obtained in well-watered plants (WW, Fig. 1a). At this point, defoliation was performed to dehydrated plants, and plants kept under stress for two more days. At day five, RWC in roots was even lower than before but it is important to highlight that all dehydrated plants reached similar values (Fig. 1a).

ABA content was measured in dehydrated roots subjected to these different levels of defoliation (Fig. 1b). After three days of water withdrawal, a 15.8-fold increase in root ABA concentration was observed in comparison to WW plants. At day 5, after defoliation treatments were performed, root ABA in WS and partially defoliated plants (WS+50% Def) remained at high levels. However, in roots of fully defoliated plants (WS+100% Def) ABA decreased 30% compared to WS plants (Fig. 1b).

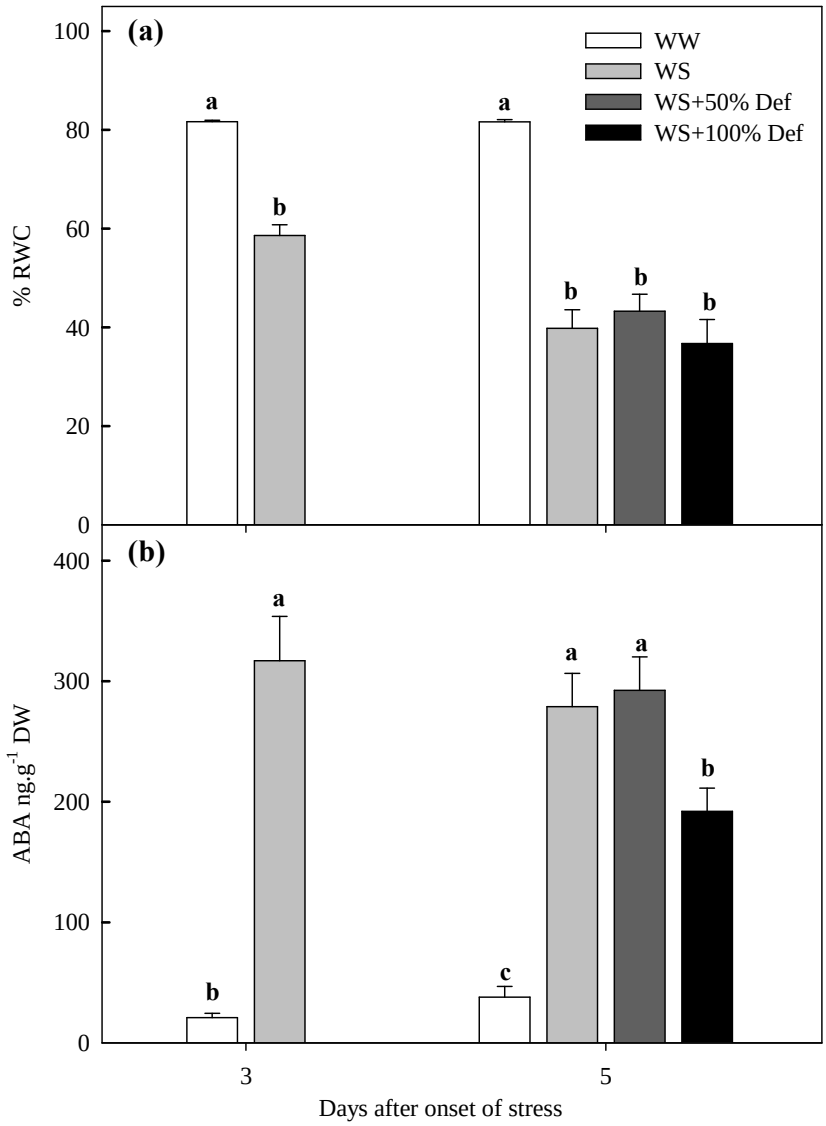

Figure 1. RWC (a) and ABA (b) content roots of defoliated *Citrus* plants. WW: well-watered plants; WS: water-stressed intact plants; WS+50%Def: water-stressed plants with removal 50% of leaves after three days of water deficit; WS+100%Def: water-stressed plants with total removal of leaves after three days of water deficit. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments (Tukey´s test, $p \leq 0.05$).

Effect of different canopy relative humidity levels on dehydrated roots

Relative water content

To explore the relative contribution of leaves to sense water stress and triggering ABA accumulation, plants were subjected to the same root dehydration intensity (-1.5 MPa PEG solution) but in this case, leaves were maintained under different relative humidity conditions. In plants that were fully covered and leaves were periodically sprayed with

distilled water (WS+100% RH) there were no changes in RWC, remaining this parameter around 80% throughout the experiment (Fig. 2a). However, in leaves of covered plants without water sprayed (WS+60% RH), RWC decreased from 82.3±2.6 to 51.6±3.0% after 24 h of dehydration (Fig. 2a). RWC in CT plants had a similar behavior and values decreased to 49.1±2.1% after 24 h of stress. Besides these differences observed in leaves, RWC decreased similarly in roots (from 95% to around 80% after 24 h of stress) of all treatments irrespective of the relative humidity existing in aerial organs (Fig. 2b). Consequently, the experiment setup allowed us to dehydrate roots at similar levels whereas there were clear differences in leaf water availability.

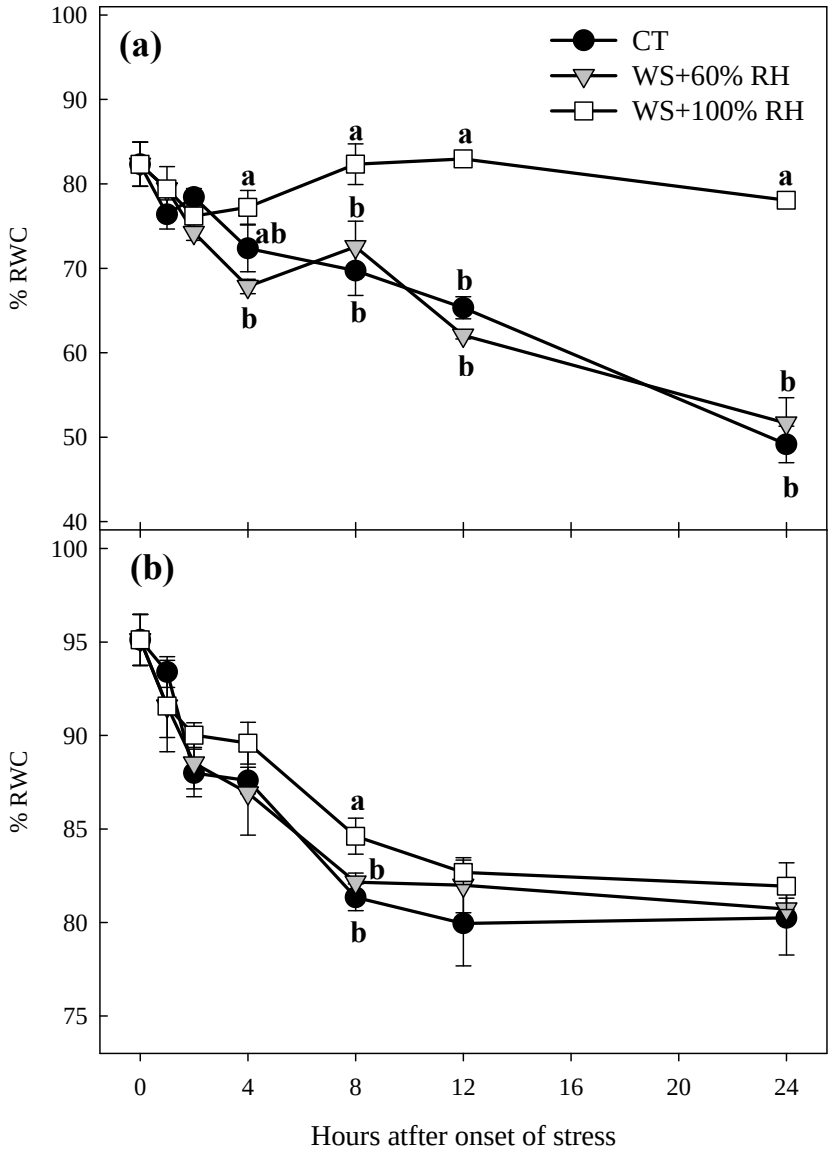

Figure 2. RWC content in leaves (a) subjected to different relative humidity and roots (b) exposed to osmotic dehydration (-1.5 MPa). CT: leaves and roots dehydrated; WS+60% RH: roots dehydrated and leaves at 60% RH; WS+100% RH: roots dehydrated and leaves in at 100% RH. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments at each point (Tukey´s test, $p \leq 0.05$).

ABA and ABA metabolites

ABA content in leaves of CT plants increased after 2 h of dehydration and showed a sharp rise (29-fold) after 8 h (Fig. 3a). Partially dehydrated leaves (WS+60% RH) showed a similar but more moderate response, with a 13-fold increment compared to initial levels. By contrast, leaves of WS+100% RH treatment showed only slight changes and the lowest levels of ABA during the initial 8 h of stress, increasing moderately after 24 h of dehydration (Fig. 3a). Root ABA accumulation followed a pattern similar to that observed in leaves: CT plants showed the highest values, reaching 4-fold increase after 24 h of dehydration compared to the initial values (Fig. 3d). Besides, ABA content in WS+60% RH roots increased by 2.5-fold in response to 24 h of water stress. On the contrary, dehydration caused only minor changes in ABA content in WS+100% RH roots, with a slight and transient decrease in hormone levels after 4 h of stress and a slight increase after 24 h (Fig. 3d. This pattern of hormonal variation is compatible with a hormonal response in roots delayed with respect to that in leaves.

The conjugated form of the hormone, ABA-GE, also showed a different accumulation among organs and treated plants (Fig. 3b and 3e). Leaf ABA-GE levels gradually increased (two-fold) after 24 h of the onset of dehydration in CT plants. On the other side, in 60% and 100% RH leaves, ABA-GE content initially decreased after 4 h of dehydration to further increase at 24 h (Fig. 3b). By contrast, ABA-GE content in CT roots transiently increased after 2 h but drastically decreased afterwards. In WS+60% RH and WS+100% RH roots, there was a rapid drop in ABA-GE content (Fig. 3e).

Dehydration also affected the content of the main ABA catabolic product, dihydrophaseic acid (DPA, Fig. 3c and 3f). In CT leaves, DPA content increased 2.3-fold after 8 h of dehydration to return to unstressed levels (0 h) after 24 h (Fig. 3c). Similarly, DPA levels transiently increased in leaves of WS+60% RH plants, re-establishing initial levels after 8 h whereas WS+100% RH plants showed a second phase of DPA content increase (2.8 fold) after 24 h (Fig. 3c). Root DPA levels were similar in plants under the different defoliation treatments; a transient 2-fold increase of catabolite concentration was detected after 2 and 4 h of dehydration to finally recover unstressed basal levels (Fig. 3f).

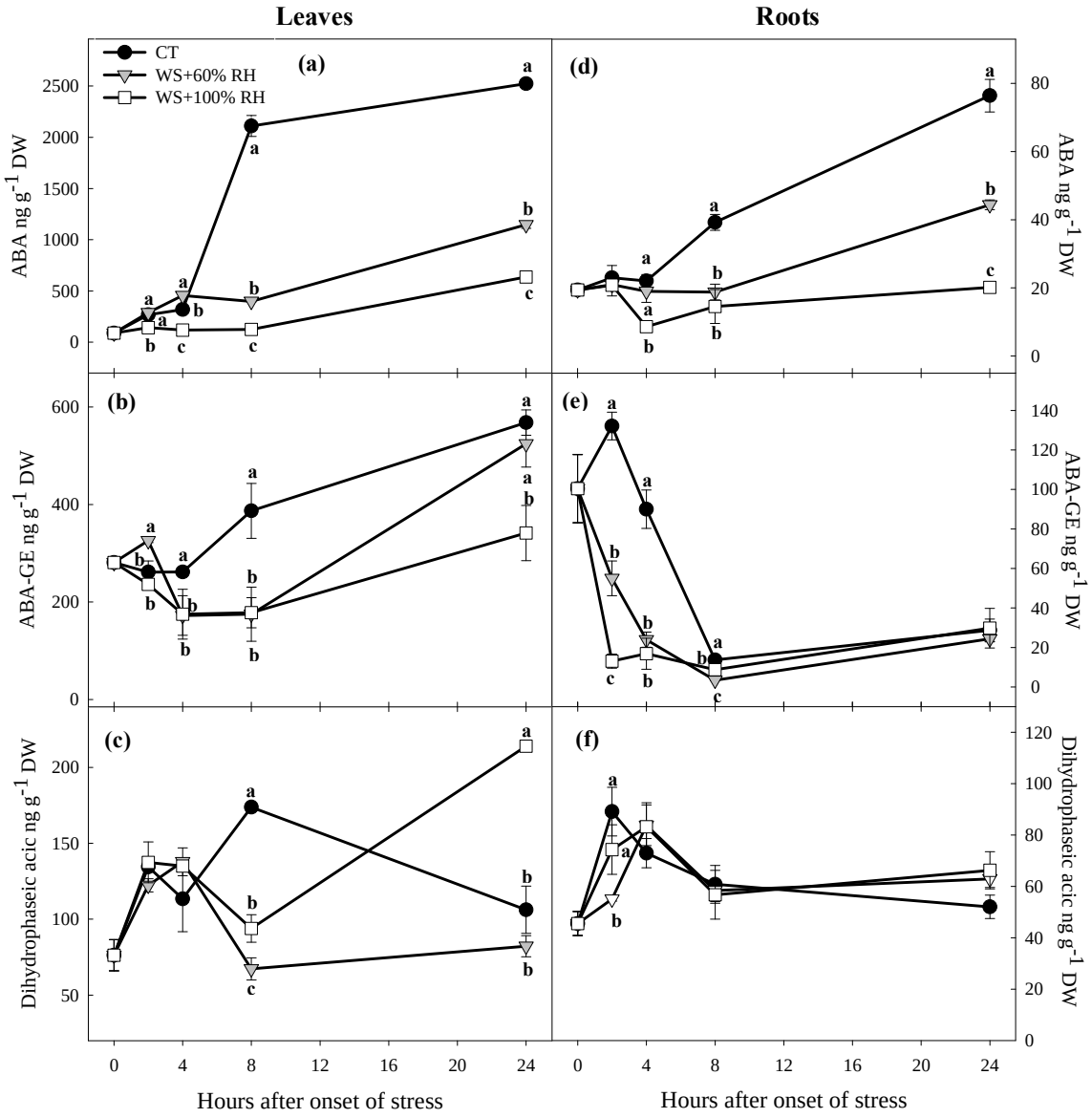

Figure 3. ABA (a, c), ABA-GE (b, d) and DPA (c, e) content in leaves subjected to different relative humidity and roots exposed to osmotic dehydration (-1.5 MPa). CT: leaves and roots dehydrated; WS+60% RH: roots dehydrated and leaves at 60% RH; WS+100% RH: roots dehydrated and leaves in at 100% RH. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences among treatments at each point (Tukey´s test, $p \leq 0.05$).

Expression of genes involved in the carotenoid biosynthetic pathway

The expression of key genes for the synthesis of carotenoids, the ABA precursors, was followed in leaves and roots from CT and partially dehydrated WS+60% RH and WS+100% RH plants. Relative abundance of *CsPSY1* transcripts in leaves and roots of plants under the different stress treatments was similar among plants under different

treatments (Fig. 4). In leaves, *CsPSY1* was gradually upregulated, being this increase earlier in CT plants (up to 3.5-fold) and slightly delayed in WS+60% RH and WS+100% RH treatments (Fig. 4a). In roots, *CsPSY1* transcript abundance remained unchanged until 8 h of dehydration when a transient increase was observed in CT plants whereas in WS+60% HR and WS+100% HR plants a decrease was detected after 24 h of stress (Fig. 4f). The evaluation of *CsPSY3a* expression revealed a notable upregulation in all types of leaves (Fig. 4b) but specially marked in the root tissue under water stress, reaching up to 12.5 fold increase in WS+100% RH plants (Fig. 4g).

CsβCHX genes were upregulated in leaves of all treated plants throughout the experiment (Fig. 4c and 4d). *CsβCHX1* expression increased by dehydration up to 21.9-fold in CT leaves, up to 11.7-fold in partially dehydrated leaves (WS+60% RH) and only 5-fold in WS+100% RH leaves (Fig 4c). A similar pattern was observed in roots (Fig. 4h); CT plants showed a sharp and continuous *CsβCHX1* upregulation (up to 11.6-fold), whereas in WS+100% RH roots this increase was less important (Fig. 4h). By contrast, expression of *CsβCHX2* followed different patterns in leaves and roots. In leaves, *CsβCHX2* was upregulated upon treatment mainly in CT and in WS+100% RH plants (Fig. 4d). In roots, *CsβCHX2* was downregulated at final stages of the experiment, showing a 60% decrease in WS+60% RH plants after 8 h whereas a similar decrease was detected in CT and WS+100% RH plants at 24 h of stress (Fig. 4i). Regarding *CsZEP* expression, changes in leaves were more relevant than in roots (Fig. 4e and 4j). In leaves, *CsZEP* was similarly upregulated among plants under the three conditions (Fig. 4e). In roots, *CsZEP* expression was slightly induced in CT plants after two h of stress whereas remained almost unchanged in roots of the WS+60% RH and WS+100% RH plants (Fig. 4j).

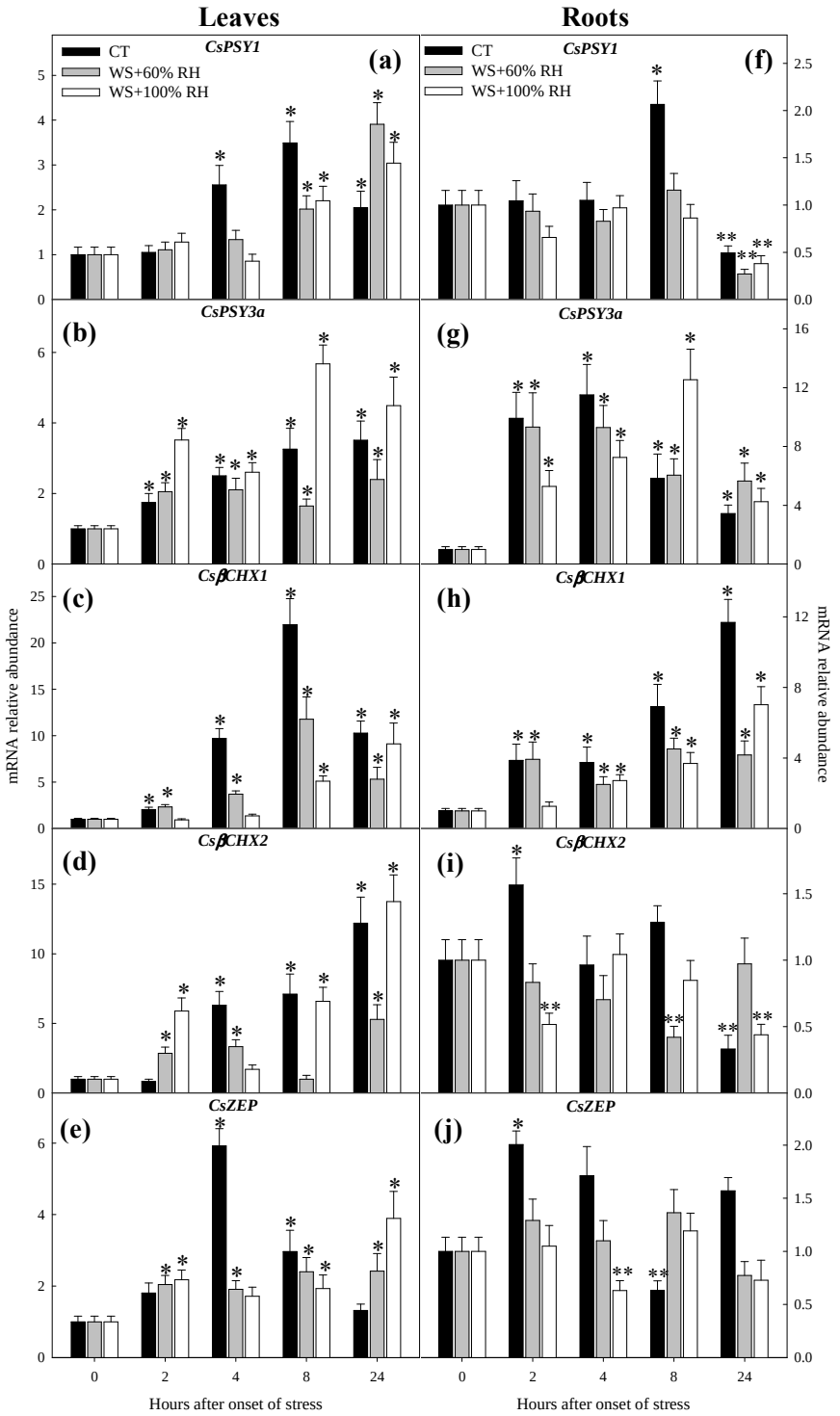

Figure 4. Relative mRNA abundance of genes involved in the carotenoid biosynthetic pathway (*CsPSY1*: a, f; *CsPSY3a*: b, g; *CsβCHX1*: c, h; *CsβCHX2*: d, i and *CsZEP*: e, j), in leaves subjected to different relative humidity and roots exposed to osmotic dehydration (-1.5 MPa). CT: leaves and roots dehydrated; WS+60% RH: roots dehydrated and leaves at 60% RH; WS+100% RH: roots dehydrated and leaves in at 100% RH. Data are means ± SE of 4 replicates. Data are means ± SE of 4 replicates. ***** denotes significant upregulation compared to the initial unstressed level (=1) within each treatment. ****** denotes significant downregulation compared to the initial unstressed level (=1) within each treatment; Tukey´s test, $p \leq 0.05$.

Expression of genes involved in the biosynthesis, conjugation and catabolism of ABA

ABA biosynthetic pathway was affected by treatments in both, leaves and roots (Fig. 5). The most remarkable changes occurred in *CsNCED1* that was sharply upregulated in leaves and roots of CT plants (Fig 5a and 5f). *CsNCED1* transcriptional changes were more moderate in dehydrated roots than in leaves (Fig. 5a and 5f). After 8 h of stress, *CsNCED1* expression increase (100-fold and 24-fold in CT leaves and roots, respectively). However this upregulation was less pronounced in WS+60% RH plants (27- and 6.5-fold in leaves and roots, respectively) and almost imperceptible in WS+100% RH plants (5- and 2-fold, respectively; Fig. 5a and 5f). Although *CsNCED2* expression was completely different from that of *CsNCED1* also displayed notable differences between both organs (Fig. 5b and 5g). This gene was markedly downregulated (by more than 90%) in leaves of CT and WS+60% RH plants, showing an uneven oscillation in WS+100% RH plants (Fig. 5b). Contrastingly, expression of *CsNCED2* was transiently upregulated after two h of stress in roots of all treated plants with a subsequent decline (Fig. 5g). The expression of *CsAOG* was stimulated in leaves and roots under the three conditions upon stress (Fig. 5c and 5h). In leaves, an early upregulation occurred after two h of stress reaching the highest levels in CT plants after 24 h (86-fold increase), intermediate in WS+60% RH plants (44-fold) and lowest in WS+100% RH plants (22-fold; Fig. 5c). In roots, expression of *CsAOG* was also upregulated but to a lesser extent compared to leaves, and no clear differences were found among plants under different treatments (Fig 5h). *CsβGluc* expression was much less affected in leaves than in roots. In leaves, expression levels remained almost unchanged during the experimental period in plants under the three conditions (Fig. 5d). By contrast, *CsβGluc* was mostly upregulated in roots reaching maximum increases of 8.9-, 5.3- and 6.2-fold in CT, WS+60% RH and WS+100% RH plants, respectively (Fig. 5i). Regarding ABA catabolism, *CsCYP707a* expression in leaves was drastically downregulated (achieving a 99% of decrease) in plants under the three stress conditions (Fig. 5e), differing to that response observed in roots where a transient upregulation was observed in dehydrated plants to finally achieve levels lower than the initial (60% decrease) in plants subjected to the three different treatments (Fig. 5j).

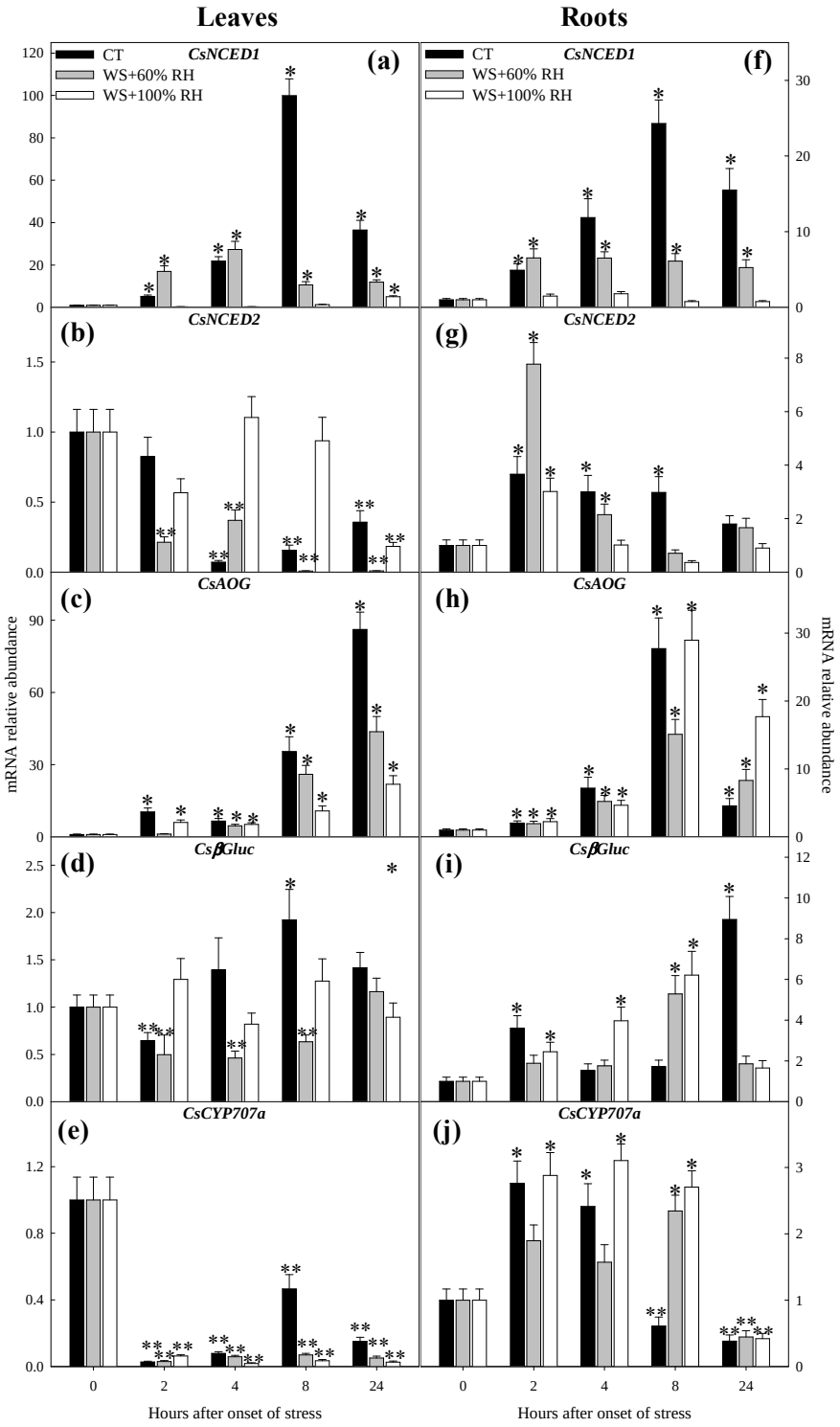

Figure 5. Relative mRNA abundance of genes involved in the ABA biosynthetic pathway (*CsNCED1*: a, f and *CsNCED2*: b, g); ABA conjugation (*CsAOG*: c, h and *CsβGluc*: d and i); and ABA catabolism (*CsCYP707a*: e, j), in leaves subjected to different relative humidity and roots exposed to osmotic dehydration (-1.5 MPa). CT: leaves and roots dehydrated; WS+60% RH: roots dehydrated and leaves at 60% RH; WS+100% RH: roots dehydrated and leaves in at 100% RH. Data are means ± SE of 4 replicates. ***** denotes significant upregulation compared to the initial unstressed level (=1) within each treatment. ****** denotes significant downregulation compared to the initial unstressed level (=1) within each treatment; Tukey´s test, $p \leq 0.05$.

Expression levels of ABA- and desiccation-responsive genes

Stress gradually upregulated all ABA-responsive genes in leaves of plants under the three conditions, contrasting with the changes observed in roots (Fig. 6). *CsRAB18* was highly upregulated in leaves of CT and WS+60% RH plants up to 95- and 23-fold, respectively, whereas in WS+100% RH plants only a slight increase was detected (Fig. 6a). In roots, *CsRAB18* was transiently downregulated (50%) in WS+60% RH and WS+100% RH plants during the initial 2-8 h of stress. During this period, *CsRAB18* remained irresponsive to stress in CT plants. After 24 h of stress, expression of *CsRAB18* increased in plants under the three conditions (Fig 6d). Regarding *CsRD29* gene, a gradual upregulation was detected in leaves of plants subjected to the three different humidity conditions, being higher in CT (up to 45-fold) and WS+60% RH (up to 52-fold) than in WS+100% RH (up to 7.5-fold) plants (Fig 6b). In roots, *CsRD29* was also upregulated although to a lesser extent compared to leaves (Fig. 6e); *CsRD29* was importantly upregulated (up to 15-fold) in CT plants whereas the upregulation in WS+60% RH and WS+100% RH roots was more moderate (up to 11- and 7-fold, respectively; Fig. 6e). *CsRD22* expression increased upon stress in leaves of plants under the three conditions, achieving higher values in CT (3.7-fold) and WS+60% RH (3.2-fold) plants than in WS+100% RH (2.5-fold) plants (Fig. 6c). By contrast, *CsRD22* transcript levels remained almost unchanged in response to dehydration in roots of plants under the three conditions (Fig. 6f).

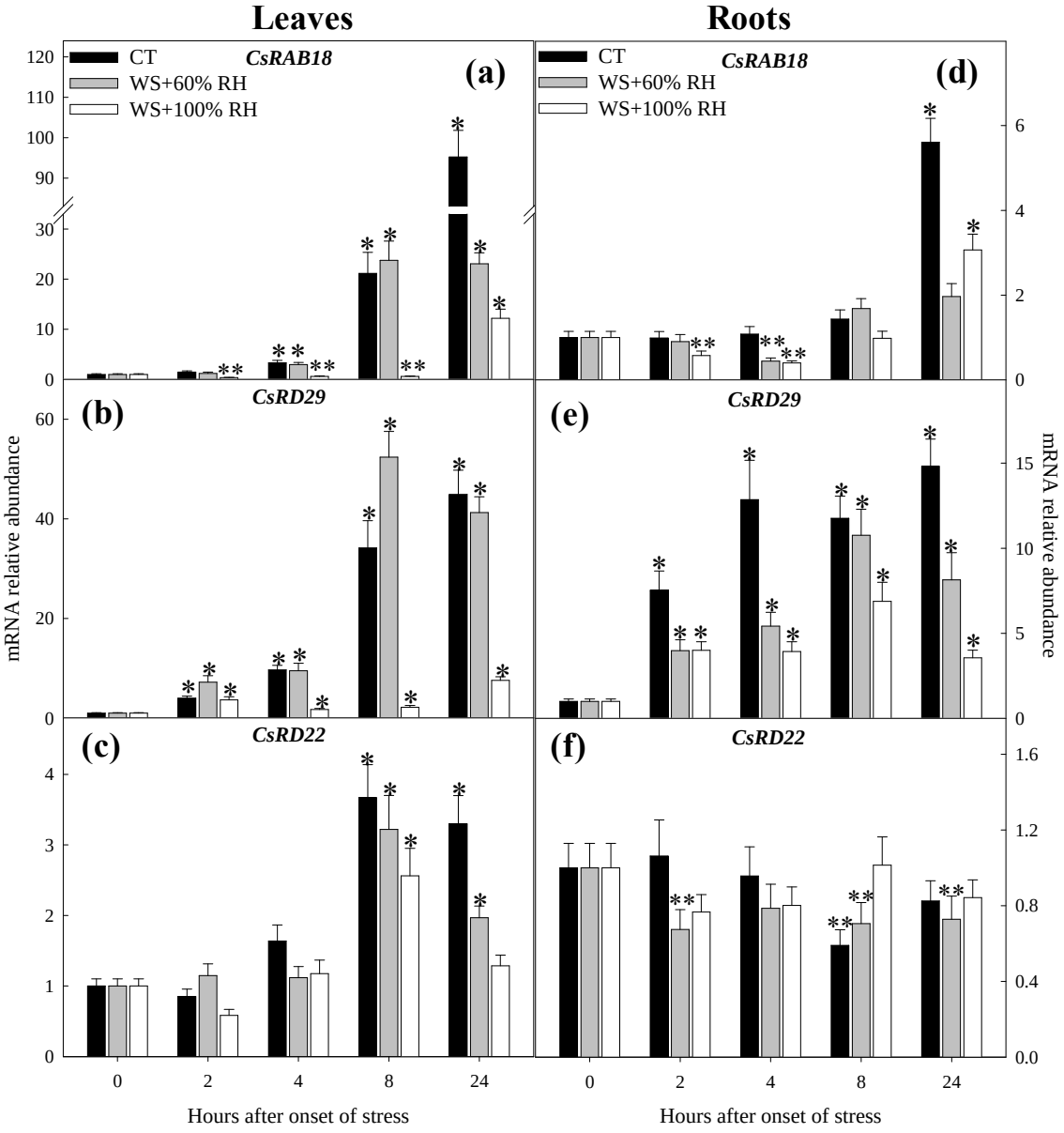

Figure 6. Relative mRNA abundance of Responsive to ABA (*CsRAB18*: a, d), Responsive to desiccation29 (*CsRD29*: b, e), Responsive to desiccation22 (*CsRD22*: c, f) genes in leaves subjected to different relative humidity and roots exposed to osmotic dehydration (-1.5 MPa). CT: leaves and roots dehydrated; WS+60% RH: roots dehydrated and leaves at 60% RH; WS+100% RH: roots dehydrated and leaves in at 100% RH. Data are means ± SE of 4 replicates. ***** denotes significant upregulation compared to the initial unstressed level (=1) within each treatment. ****** denotes significant downregulation compared to the initial unstressed level (=1) within each treatment; Tukey´s test, $p \leq 0.05$.

Discussion

It is widely accepted that soil water shortage induces ABA accumulation in most plant tissues. During this process, it is assumed that roots are in charge of sensing the decrease in soil water availability and consequently trigger ABA biosynthesis, which is then transported to leaves and induces stomatal closure (Zhang and Davies 1989; Davies *et al*. 2005; Blum 2015). However, experimental evidences using ABA-deficient mutants revealed that stomatal closure takes place independently of the root-sourced ABA (Holbrook *et al*. 2002; Thompson *et al*. 2007), a response that could be affected by the surrounding conditions of the canopy (Bauer *et al*. 2013; Aliniaeifard and van Meeteren 2013). Results in this work showed that ABA levels increase in leaves and roots of plants subjected to water deficit; however, the complete removal of leaves in WS+100%Def plants decreased (at least 30%) ABA accumulation in roots (Fig. 1b). This corroborates previous findings indicating that leaves are responsible for fueling high amounts of ABA to stressed-roots (Ikegami *et al*. 2009; Waadt *et al*. 2014; Manzi *et al*. 2015). Interestingly, partial canopy removal did not affect the ABA levels in roots, suggesting that leaves provide a substantial source of this phytohormone and the presence of a few green organs could sustain ABA accumulation in water-stressed roots at similar levels to those of intact plants (Fig. 1b).

In this work, we also explored the role of leaves in triggering water stress responses occurring in roots, focusing in ABA metabolism. Results question the ability of dehydrated roots to synthesize relevant amounts of ABA (Ren *et al*. 2007; Manzi *et al*. 2015; McAdam *et al*. 2016). When roots and leaves were dehydrated or partially dehydrated (CT and WS+60% RH; Fig. 2a and 2b), ABA levels increased in both organs, but contrastingly, when only roots were dehydrated, ABA accumulation was much lower in both leaves and stressed roots (Fig. 3a and 3d). Thus, these data also challenge the accepted role of roots as the unique and first stress sensor, suggesting that leaves could be playing a more protagonist role in this process.

These results not only indicate that ABA accumulation does not depend on root exposure to drought but also suggest that shoot can perceive water deficit (Ikegami *et al*. 2009; Blum 2015). Therefore, turgor loss would be the critical parameter triggering ABA accumulation in leaves (Zeevaart and Creelman 1988). Furthermore, these results evidence that roots accumulate ABA only after leaf hormone concentration has increased (Fig. 3a and 3d), highlighting a predominant role of leaves in a tightly coordinated response to

water stress. Results shown here may be explained by the soil-plant-air continuum model, in which the evaporative demand determines the water flow through the plant by exerting a water potential gradient that decreases from roots to shoots (Christmann *et al*. 2013). Leaves directly face with rapid changes in evaporative demand whereas roots are subjected to slower changes in soil water potential (Caldeira *et al*. 2014). Thus, leaves would integrate the external stimuli and increase ABA local production to regulate the water status in the whole plant. In agreement with this, Soar *et al*. (2006b) demonstrated in a series of time-course experiments in *Vitis vinifera* that the induction of several genes involved in ABA biosynthesis, such as *VvNCED1*, take place specifically in leaves whereas no transcriptional changes occurred in roots of dehydrated plants. Similarly, in dehydrated maize plants, ABA increased in roots and xylem sap before any rise in *ZmNCED* gene expression, suggesting that leaves rather than roots are firstly involved in the water-stress responses (Ernst *et al*. 2010).

Despite it is assumed that there is a tight interaction between both organs which finally determines ABA accumulation in both tissues (Soar *et al*. 2006a), results obtained here suggest that root dehydration barely influences the ABA concentration in leaves since no ABA accumulation was detected in plants with stressed-roots and turgid leaves (WS+100% RH, Fig. 3a). In this line, evidence from PRD assays, in which a portion of roots is allowed to dehydrate to accumulate ABA that would be further transported to leaves, could help explain these results. Under this irrigation system, ABA frequently remains unaltered in citrus leaves when the half wet roots are able to supply enough water to cover the atmospheric demand (Romero-Conde *et al*. 2014). Indeed, results from common bean indicate that stomatal closure and water potential in leaves are correlated with the total water provided by roots, discarding any possible involvement of a chemical signal such as ABA originated only in the dry roots (Wakrim *et al*. 2005). In fact, a strong correlation between ABA levels in aboveground tissues and availability of water in both wet and dry roots under PRD system was suggested in apple trees (Liu *et al*. 2008; Einhorn *et al*. 2012). Consequently, these lines of evidence agree with results presented here; ABA levels increase in leaves only after they experience a decrease in water availability, irrespective of the dehydration level occurring in roots (Fig. 2c). In spite of this, in certain occasions a transient ABA increase could be detected in dry roots under PRD system, part of a rapid response induced by local dehydration (Puertolas *et al*. 2014) with minimal implication of leaves (Thompson *et al*. 2007). Dehydration could, therefore, induce local ABA accumulation as observed during the initial stages of water withdrawal

which could be partially supported by the available local ABA precursors as it was suggested in detached roots (Borel *et al*. 2001; Ren *et al*. 2007; Thompson *et al*. 2007) or stem-girdled plants (Manzi *et al*. 2015). ABA accumulation in roots could also be supported by its release from ABA-GE (Priest *et al*. 2006), as suggested also in this work by the sharp decrease in this molecule in roots of all treated plants (Fig. 3c).

Apart from this interaction, there are many transcriptional changes in both tissues in response to water deficit which also appear to depend on the leaf water status. Several key carotenoid (Fig. 4) and ABA (Fig. 5) biosynthetic genes were upregulated in response to dehydration in leaves and roots and the magnitude of these changes seems to be regulated by the level of leaf dehydration. Therefore, there was a stimulation of almost all carotenoid biosynthetic genes evaluated (*CsPSY1*, *CsPSY3a*, *CsβCHX1* and *CsβCHX2*) in leaves and roots in response to water stress (Fig. 4). Among them, *CsPSY3a* and *CsβCHX1* appear to be more stress-responsive in both organs whereas *CsPSY1* and *CsZEP* showed only minor changes as described in a previous work (Manzi *et al*., unpublished results; Fig. 4). PSY3 and βCHX1 appears to play a key role in stimulating root-carotenoid biosynthesis during dehydration in several species, such as Citrus and maize (Li *et al*., 2008; Manzi *et al*., unpublished results) whereas ZEP seems to be more related to other stress responses such as the activation of the xanthophyll cycle (Seo *et al*., 2000). Leaf water status also modulated transcriptional changes in this organ. Particularly *CsPSY1*, *CsβCHX1* and *CsβCHX2* were upregulated in CT plants compared to WS+100% RH leaves (Fig. 4a, 4c and 4d). This agrees with the preponderant activity of PSY1 in light-exposed tissues rather than in roots (Fig. 4a and 4f; Walter *et al*., 2015; Manzi *et al*., unpublished results). In roots, *CsβCHX1* expression was higher in CT plants upon stress (Fig. 4h), suggesting that leaf water status could be influencing β-carotene hydroxylation in both roots and shoots, assuring the biosynthesis of xanthophylls to feed the ABA biosynthesis. Indeed, it was suggested that certain steps in the carotenogenic pathway such as β-carotene hydroxylation in tomato (Thompson *et al*. 2007) and phytoene biosynthesis in maize (Li *et al*. 2008) could be limiting the ABA increase. However, previous works have shown that in citrus roots the increase in carotenoid availability does not determine a rise in ABA levels under dehydration (Manzi *et al*., unpublished results).

ABA biosynthesis was also affected by leaf water status and *CsNCED1* was notably upregulated in CT leaves (100-fold) and roots (24-fold) whereas only minor changes were observed in leaves (5-fold) and roots (1.8-fold) of WS+100% RH plants (Fig. 5a and 5f).

CsNCED1 gene encodes for the bottleneck step in the ABA biosynthetic pathway (Nambara and Marion-Poll 2005) and therefore, a higher expression is usually associated with dehydration and consequently with the increase in ABA levels (Thompson *et al*. 2007) as observed in CT leaves and roots (Fig. 3a and 3d). Interestingly, leaf turgor markedly impacted the expression of *CsNCED1* in both organs whereas the dehydration of roots showed a marginal influence on this gene expression as observed in WS+100% RH plants (Fig. 5a and 5f).

Contrastingly, the relative expression of *CsNCED2*, *CsAOG*, *CsβGluc* and *CsCYP707a* was similar in plants under different conditions (Fig. 5), which accounts for a dehydration-induced response rather than to a real contribution to the differences in root ABA accumulation (Fig. 2d). Indeed, *CsNCED2*, which plays a subsidiary role in the ABA biosynthesis (Rodrigo *et al*. 2006), was downregulated in leaves (Fig. 5a) whereas in roots its expression was induced in response to dehydration (Fig. 5a). *CsCYP707a* was also induced by water shortage in roots (Fig. 5j) in an ABA-independent manner (Fig. 2d), as previously reported (Priest *et al*. 2006; Manzi *et al*. 2015). DPA, a catabolic inactive product produced by an ABA 8'-hydroxylase encoded by the *CsCYP707a* gene, transiently increased in roots of all treated plants in response to dehydration (Fig. 3d), contrasting with the idea that ABA stimulates its own degradation (Thompson *et al*. 2007). Interestingly, a sharp decrease in *CsCYP707a* expression was observed in leaves of all kind of plants (Fig. 5e), which suggests that water turgor in leaves is not involved in the regulation of this gene.

Result showed here also reveals that under dehydration, the conjugation of the ABA could regulate the hormone metabolism. *CsAOG*, which encoded for an ABA O-glycosyl transferase enzyme that conjugates ABA (Seo and Koshiba 2011), was strongly upregulated linked to the water status in leaves (Fig. 5c), whereas *CsβGluc*, which encode for a glucosidase responsible for the ABA release from ABA-GE, showed an uneven response to the stress (Fig. 4d). This could explain the higher levels of ABA-GE measured in the CT plants (Fig. 3b). On the other side, expression of *CsAOG* and *CsβGluc* in roots (Fig. 5h and 5i) hardly explain the sharply decrease of ABA-GE in all treatments (Fig. 3e), since *CsAOG* was strongly upregulated in plants under the three conditions compared to *CsβGluc* (Fig. 5i).

Globally, results showed that, except for a few genes such as *CsNCED1* and *CsβCHX1*, gene expression barely help to explain the differences observed in ABA levels in plants

subjected to root dehydration and different RH in leaves. In this sense, other factors such as postranscriptional regulation could be taking place (Seo *et al*. 2000). Interestingly, both steps are suggested as key in controlling the ABA increase in roots, where *βCHX1* would become limiting only after the rise in NCED1 levels (Thompson *et al*. 2007).

Several ABA- and desiccation-responsive genes are usually upregulated in leaves of stressed plants (Shi *et al*. 2014) but lack of information exists for citrus plants. Expression of *CsRAB18*, the citrus ortholog for an Arabidopsis ABA-responsive LEA protein (Yamaguchi-Shinozaki and Shinozaki 2006), and *CsRD29* in leaves (Fig. 6a and 6b) mirrored ABA accumulation in plants under the different treatments (Fig. 3a), suggesting that in leaves ABA is essential for their induction, as previously reported (Yamaguchi-Shinozaki and Shinozaki 2006). In a different way, *CsRD22* induction in leaves occurs at the final stages of experiments (Fig. 6c), in agreement with the fact that transcription factors (MYC and MYB) binding RD22 promoter are activated only when a high accumulation of ABA takes places (Yamaguchi-Shinozaki and Shinozaki 2006). In roots however, *CsRAB18* and *CsRD22* genes appear irresponsive to ABA and dehydration conditions (Fig. 6d and 6f), whereas levels of *CsRD29* transcripts increased in response to stress (Fig. 6e). *CsRD29* expression was higher in roots of CT plants, which accumulates high amounts of ABA (Fig. 2d) but interestingly, similar upregulation was found in roots of WS+60% RH and WS+100% RH plants (Fig 6e) despite the different ABA levels between them. This suggests that, the expression of this gene is in part controlled by dehydration, but also by ABA, as previously suggested (Yamaguchi-Shinozaki and Shinozaki 2006). Globally, it seems that these genes better reflect drought-induced ABA changes in citrus leaves than in roots, where additional stress markers must be found to exactly evaluate those changes.

Overall, the results confirm that the presence of the aerial organs is crucial for sustaining the ABA accumulation in roots as previously stated (Ikegami *et al*. 2009; Manzi *et al*. 2015) and propose that leaves are potent sources of ABA. Since leaves must experience dehydration to observe a rise in root ABA levels, it appears that leaves are part of the mechanism sensing water deficit, supporting ABA accumulation at the entire plant. Indeed, water availability in leaves could modulate the expression of key genes for ABA biosynthesis, such as *CsNCED1* and *CsβCHX1* in roots. Data presented here could be useful to draw water saving strategies, since leaves not only limit the water loss but also appear to be directly involved in water stress responses in roots.

Author contributions

M.M., A.G-C. and V.A. designed research, M.M. and M.P-B. performed experiments, M.M., M.P-B., A.G-C., and V.A. analyzed data, M.M., A.G-C. and V.A. wrote the paper.

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Supplementary Information

Supplementary Table S1. Sequences of the primers used for qRT-PCR. The genes analysed were: *Phytoene synthase1* (*CsPSY1*), *Phytoene synthase3a* (*CsPSY3a*), *β-carotene hydroxylase1* (*CsβCHX1*), *β-carotene hydroxylase2* (*CsβCHX2*), *Zeaxanthin epoxidase* (*CsZEP*), *9-cis-epoxycarotenoid dioxygenase1* (*CsNCED1*), *9-cis-epoxycarotenoid dioxygenase2* (*CsNCED2*), *ABA O-glycosyl transferase* (*CsAOG*), *β-glycosidase* (*CsβGluc*), *ABA 8´-hydroxylase* (*CsCYP707a*), *Responsive to ABA* (*CsRAB18*), *Responsive to Dessication29* (*CsRD29*), *Responsive to Dessication22* (*CsRD22*), and *actin* (*CsActin*).

Citrus gene	Locus	Direction	Primer sequence (5'-3')	Amplicon size (bp)
CsPSY1	orange1.1g044623m	F	GGTCGTCCATTTGATATGCTTG	111
		R	CCTAAGGTCCATCCTCATTCCT	
CsPSY3a	orange1.1g016696m	F	AATGCATTTTGTGTAAGCCCTGCT	148
		R	TGTCCCTAAAAGGCTTGATGTGTAAATTG	
CsβCHX1	orange1.1g020892m	F	GGCTCATAAAGCTCTGTGGC	210
		R	CCAGCACCAAAACAGAGACC	
CsβCHX2	orange1.1g027413m	F	AGAAGAGGAAACCGAAGAGCTTGAG	182
		R	GATTGCTGCAACAAGGTAGGTTTGT	
CsZEP	orange1.1g005770m	F	GCAGCTATGAGAGAGCTAGG	117
		R	GACAACGGTCCAAGTCCAAC	
CsNCED1	orange1.1g007379m	F	AATGCTTGGGAAGAGCCTGAG	147
		R	AGTGGACTCGCCGGTCTTTAG	
CsNCED2	orange1.1g007291m	F	GTGAATCGCAACAGCCTTGG	164
		R	GGCAAGAAAAATGGCTCGCC	
CSAOG	orange1.1g022744m	F	CGGGTTCACTGTTTGGTCTT	176
		R	GCCTCGAGAGAAATGGATGT	
CsβGluc	orange1.1g046891m	F	GCAACTTTGCACATTCAGCA	136
		R	GTGCCAAACACAAATCCATC	
CsCYP707a	orange1.1g038621m	F	CTTTGGCACCCATGAAAGAT	264
		R	TCAATGTTGCACTGCTCTCC	
CSRAB18	orange1.1g027438m	F	GCATTGGCAGATGGCTTAAT	103
		R	GTTACCTCCCTCGCGTCTTT	
CSRD22	orange1.1g000107m.g	F	GTGCATTGTGTCGTGGATTC	142
		R	TCTCCGCATCTGATTTTTCC	
CSRD29	orange1.1g007806m	F	GCTCTATGCCAGCATCAACA	150
		R	CCTTCTCGCTGCCTGTATTA	
CsActin	orange1.1g037845m	F	CCCTTCCTCATGCCATTCTTC	105
		R	CGGCTGTGGTGGTAAACATG	

4. DISCUSSION

Plant growth and development and subsequently crop productivity are severely affected by water restriction. Previous works indicate that roots are crucial in sensing the soil water deficit and triggering the synthesis of ABA which is further transported to other organs such as leaves in a process known as the root-to-shoot ABA transport (Zhang *et al.*, 1987; Sauter *et al.*, 2002; Dodd *et al.*, 2008*a*; Carvalho *et al.*, 2016). This concept was introduced during the late 1980's (Zhang and Davies, 1987, 1989; Zhang *et al.*, 1987) and profusely studied afterwards. Based on this concept, research mostly evaluated how water restriction affected the concentration of ABA in xylem sap being this phytohormone responsible for the change in the stomatal conductance as proposed by Tardieu & Davies (1993). However, other research lines showed that stomatal closure occurs independently of the increase in xylem ABA concentration (Vernieri *et al.*, 2001; Dodd *et al.*, 2008*b*; Puertolas *et al.*, 2014; Carvalho *et al.*, 2016). Additionally, further evidence contrasted with the original model such as the ability of leaves to synthetize by themselves large amounts of ABA (Gómez-Cadenas *et al.*, 1996; Holbrook *et al.*, 2002; Christmann *et al.*, 2005) or the low ability of roots to accumulate carotenoids, the ABA direct precursors (Parry and Horgan, 1992; Ruiz-Sola *et al.*, 2014; Walter *et al.*, 2015).

Despite this controversy, it seems clear that ABA signaling in roots is crucial to cope with water scarcity since the hormone is involved in the modification of root architecture (Jung and McCouch, 2013; Boursiac *et al.*, 2013; Harris, 2015) and in the increase of root hydraulic conductance (Thompson *et al.* 2007; Ruiz-Lozano *et al.* 2009), maintaining therefore the water uptake (Sharp and LeNoble, 2002). In this PhD Thesis, we aimed to investigate the real ability of roots to accumulate ABA under water deficit and to explore the possible role of the aerial tissues in this process.

Initially, the possible contribution of aerial tissues to ABA accumulation in roots was explored by stem girdling experiments in citrus plants and by grafting experiments using ABA-deficient tomato mutants (Chapter I). Secondly, possible causes that explain the observed results were addressed by *in vitro* experiments where detached citrus roots and intact plants were grown under dark or light conditions to assure different levels of carotenoids. After stress induction, ABA production and the transcription of ABA biosynthetic and catabolic genes was measured (Chapter II). Thirdly, the potential contribution of aerial tissues to stress sensing was also explored by totally or partially enclosing the canopy in transparent bags in order to maintain water status in leaves whereas roots were dehydrated (Chapter III).

One of the most important results from this work showed that most of the ABA accumulated in roots of dehydrated plants is originated from aerial tissues, contrasting to the extensively accepted literature on the subject. The first approach used to evaluate the roots as a potential ABA sink for aerial tissues was based on interrupting the phloem transport by performing stem girdling. The ability of water-stressed roots to increase ABA levels was reduced by 50% when basipetal transport was disrupted, showing that communication with aerial tissues is essential to sustain ABA accumulation during water stress. Interestingly, after a consecutive second period of dehydration, stem-girdled plants were unable to increase ABA levels in the roots (Chapter I, Fig. 3b). Similar results were obtained when reciprocal grafting of ABA-deficient tomato mutant in combination with wild type revealed that root capacity to accumulate ABA under drought is deeply modulated by levels of the hormone achieved in the grafted aerial part (wild type; Chapter I, Fig. 7b and Supp. Information S1). These results suggest that dehydrated roots are able to partially increase ABA content maybe due to preexisting precursors or other sources present in roots. However, once these sources are exhausted ABA accumulation entirely relies on the contribution from aerial organs. These results are in accordance to previous observations in detached roots of maize seedlings (Ren *et al.*, 2007) or in bean stem-girdled seedlings which only showed a transient root-ABA accumulation (Vernieri *et al.*, 2001).

The inability of roots to sustain ABA accumulation during consecutive stress periods could be explained either by its intrinsic capacity to synthetize this phytohormone or by the absence of key precursors in this tissue. In this sense, a sharp decrease in β,β-carotenoids during dehydration suggested that carotenoids could be fueling ABA accumulation (Chapter I, Fig 4; Walter *et al.* 2015). Furthermore, the inability of roots to accumulate ABA in stem-girdled plants during the second period of dehydration appears to be linked to the depletion of β,β-carotenoid precursors during the first stress response. *In vitro* experiments, which were designed to specifically evaluate the contribution of root β,β-carotenoid to ABA accumulation during dehydration, also showed a decrease in root-carotenoid content upon dehydration (Chapter II, Fig. 5) indicating that this change is elicited by water stress.

According to results, it appears that carotenoids are needed to sustain ABA increase in root tissue and to corroborate this fact, *in vitro*-grown detached roots and intact plants differing on carotenoid levels were subjected to dehydration and ABA production was

followed (Chapter II). Despite that light-grown detached roots showed 5.5-fold more total carotenoids (and 10-fold more β,β-carotenoid) than dark-grown and control plants (Chapter II, Fig. 2 and Supp. Information S2), ABA content was not detected in this organ unless it is indeed joined to the aerial part. Previously, it was believed that the lack of precursors in this tissue could be limiting the ABA accumulation (Christmann *et al.*, 2005, 2007; Ren *et al.*, 2007; Ikegami *et al.*, 2009); however, results from *in vitro* experiments suggest that root-carotenoid do not directly contribute to ABA accumulation in this organ (Chapter II, Fig 4) since high-carotenoid roots were unable to accumulate ABA during dehydration (Chapter II, Fig. 4). Therefore, results demonstrate impairment in ABA biosynthesis in this tissue. Furthermore, the slow recovery of β,β-carotenoid pools to unstressed basal levels after dehydration (Chapter I, Supp. Information S3) remarks the unsuitable strategy of dependence on these precursors to sustain ABA increase in roots under water stress.

It is also interesting to note that, except for the merely increase in β-cryptoxanthin content, levels of all carotenoids sharply decreased under water deficit (Chapter II, Fig. 5), even those from the β,ε- pathway such as α-carotene and lutein (Chapter II, Supp. Information Fig. S2). This however, is opposite to the strong upregulation observed in the genes encoding for carotenoid biosynthesis (Chapter II, Fig. 6). In this sense, dehydration could be triggering processes in roots which involve an extensive transformation of carotenoid pool. Indeed, under stress conditions, carotenoid-derived signaling compounds such as SLs or other volatile norisoprenoid compounds, with different molecular weight and structure and diverse functions in plants, are actually produced (Rubio-Moraga *et al.*, 2014; Ha *et al.*, 2014; Avendaño-Vázquez *et al.*, 2014; Lätari *et al.*, 2015; Walter *et al.*, 2015). Furthermore, the similar structure among carotenoids (Fig. 1) could lead to an unspecific cleavage of these compounds and consequently, to a sharp reduction in their content. In this sense, it is believed that the neoxanthin synthase (NSY) function could be in fact exerted by a lycopene cyclase (LCY; Neuman *et al.* 2014) and that carotenoid cleavage dioxygenases (CCDs) are able to metabolize most of the carotenoids present in plants meanwhile co-oxidation of carotenoids could be taking place by the action of free radicals generated by other stress-induced reactions (Fleischmann and Holger, 2008). Moreover, non-enzymatic degradation of carotenoids often takes place in plant tissues in an unspecific manner and results in random products depending on the carotenoid precursor (Fleischmann and Holger, 2008). Based on this, it seems that carotenoids are either degraded or converted in other signaling compounds still uncharacterized

(Avendaño-Vázquez *et al.*, 2014) rather than contributing to the ABA accumulation in roots.

ABA levels in a specific tissue or organ are also regulated by catabolism and conjugation (Zhang *et al.*, 2006) and there is a tight coordination between these two pathways aimed to control ABA availability (Dong and Hwang, 2014). Results presented in this thesis support the idea that ABA catabolism is rapidly induced by dehydration in roots in an ABA-independent manner as observed in stem-girdled plants (Chapter I, Fig. 4a and 4b) and in canopy bagging experiments (Chapter III, Fig. 3f). In this sense, dehydrated roots showed a similar DPA accumulation pattern despite the different amounts of ABA (Chapter III, Fig. 3f). This response was associated to changes in gene expression, where the 8'-OH-ABA enzyme (*CsCYP707a*) was upregulated independently of the level of substrate (ABA) as observed in bagged leaves (Chapter III, Fig. 5j) and in detached roots from the *in vitro* experiment (Chapter II, Fig. 7). These results contrast with the idea that the rate of ABA catabolism is proportional to the ABA levels in a given tissue (Jia *et al.*, 1996), hence the more ABA is produced during stress conditions, the more ABA should be degraded (Zhang *et al.*, 2006; Thompson *et al.*, 2007*b*). However, other evidence demonstrated that the accumulation of ABA catabolic metabolites is triggered under water stress independently of the ABA levels as previously reported in ABA-deficient Arabidopsis plants (Priest *et al.*, 2006; Sreenivasulu *et al.*, 2012). Indeed, a high rate of ABA catabolism seems necessary to maintain high rates of ABA synthesis during stress (Zeevaart and Creelman, 1988). Nevertheless, we confirmed that ABA catabolism is triggered by water stress irrespective of the levels of substrate in roots of citrus plants.

Contrastingly, ABA-GE accumulation in roots appears to be dependent on the ABA content as previously suggested (Dietz *et al.*, 2000; Sauter *et al.*, 2002; Lee *et al.*, 2006). ABA-GE was accumulated in dehydrated roots displaying high levels of ABA but its accumulation was impaired in ABA non-accumulating roots (Chapter I, Fig. 5a). Interestingly, in leaf bagging experiments, root ABA-GE sharply decreased in all treatments (Chapter III, Fig 3e), even in those roots that accumulated ABA upon stress. Therefore, the exact involvement of ABA-GE under water deprivation is not clear yet but data suggest an active role of this molecule in ABA homeostasis as previously stated (Priest *et al.*, 2006; Okamoto *et al.*, 2009; Piotrowska and Bajguz, 2011; Li *et al.*, 2012). Since the release of ABA from ABA-GE is a fast process, involving only a single step, it could contribute to the fast increase in ABA levels rather than relying on *de novo*

synthesis which involves a long and complex pathway (Lee *et al.*, 2006; Schroeder and Nambara, 2006). Globally, evidence presented here supports the fact that ABA metabolism, in particular catabolism and conjugation, are finely tuned under water stress conditions to assure a fast release or depletion of ABA when needed (Li *et al.*, 2012). Indeed, using transgenic lines overexpressing *CYP707a* or *AOG* genes it was demonstrated that an increased activity in one of those pathways is compensated with a reduced activity in the other pathway (Dong and Hwang, 2014). Thus, analysis of these pathways must be considered in the context of ABA metabolism, including ABA synthesis and transport.

ABA transport among tissues and organs is also considered as a relevant mechanism to assure a desired cellular level of this phytohormone (Zhang and Davies, 1989; Hartung *et al.*, 2002; Davies *et al.*, 2005; Ikegami *et al.*, 2009; Dong and Hwang, 2014; Waadt *et al.*, 2014; Blum, 2015*b*). ABA-GE was thought to be transported from different organs especially from root-to-shoot under water scarcity (Sauter *et al.*, 2002; Jiang and Hartung, 2008; Goodger and Schachtman, 2010; Correia *et al.*, 2014). However, confirmation of this hypothesis is lacking and indeed, it appears unlikely that ABA-GE could be long-distance transported among tissues (Lee *et al.*, 2006; Dong and Hwang, 2014). For this reason, we investigated the possible existence of a basipetal ABA transport in woody citrus plants by the exogenous application of labeled ABA ([$^{2}H_{6}$]ABA) to the leaves. Results confirmed that ABA is indeed transported from leaves to roots in non-stressful conditions and that this transport is notably enhanced under water stress conditions (Chapter I, Table 1). The specific transportation of ABA was also proved since labelled SA was co-applied but no traces of this compound could be found in citrus roots (Chapter I, Table 1). These results are in agreement with several works that evidenced the possibility of ABA basipetal transport from aboveground organs to roots (Vernieri *et al.*, 2001; Hartung *et al.*, 2002; Ikegami *et al.*, 2009; McAdam *et al.*, 2016*a*).

Interestingly, during the late 1960's and the early 1970's, just after ABA identification, a series of investigations using exogenous application of [^{14}C]ABA concluded that ABA is transported throughout the phloem (Hoad, 1967, 1973) in both a basipetal and acropetal manner (Hocking *et al.*, 1972; Shindy *et al.*, 1973; Bellandi and Dorffling, 1974; Hartung, 1976; Davenport *et al.*, 1977). Some of these works clearly demonstrated that ABA produced in mature leaves could be transported to roots or apical tissues (Hocking *et al.*, 1972; Shindy *et al.*, 1973; Bellandi and Dorffling, 1974). In this sense, ABA applied to

apical buds was never found in lower plant organs but if exogenous ABA was applied to lower leaves, labelled ABA was found in roots (Bellandi and Dorffling, 1974). Those results are similar to our findings and remark that leaves are a great source of ABA and that phloem is involved in the ABA transport (Chanson and Pilet, 1982). However, those early investigations failed to understand the real importance of the basipetal ABA transport during water stress conditions. Thus, working with seedlings of *Phaseolus coccineus*, Hartung (1976) found that basipetal transport of ABA is reduced under water deficiency in order to sharply increase the ABA in leaves. However, other reports showed that ABA levels substantially increased in phloem of *Ricinus communis* and *Lupinus albus* seedlings under water deficiency (Hoad, 1973, 1978). Due to the lack of polarity of ABA transport (Davenport *et al.*, 1977), it could be found either in roots or in apical meristems. From our results, exogenous application of labeled ABA indicated that basipetal transport from shoot to root increased 3-fold under water deprivation (Chapter I; Table 1) similarly to salt-stressed seedlings of *Lupinus albus*, that showed a 5-fold increase (Wolf *et al.*, 1990). Interestingly, previous works were carried out on herbaceous or in woody species at the seedling stage, and therefore our data are relevant confirming this mechanism in a woody plant such as Citrus.

It is striking how those early works that demonstrated the crucial role of leaves in synthetizing ABA (Hoad, 1973, 1975; Milborrow *et al.*, 1973) and its transport to roots (Hocking *et al.*, 1972; Shindy *et al.*, 1973; Bellandi and Dorffling, 1974) were mostly ignored during the following decades. Indeed, the role of the leaves as a key organ in ABA biosynthesis during dehydration was mostly accepted by researchers after three decades due to the use of reciprocal grafting of tomato ABA-deficient mutants (Holbrook *et al.*, 2002). Our results are in accordance with those data since ABA levels in roots substantially decreased when there was a lack of support from aerial tissues. This happened in girdled plants (Chapter I, Fig. 3b), in ABA-deficient tomato plants (Chapter I, Fig. 7b and Supp. Information S1), and in defoliated citrus plants (Chapter III; Fig. 1b). Interestingly, the partial defoliation of plants demonstrated that leaves are a strong source of ABA able to sustain its accumulation in roots (Chapter III; Fig. 1b), as was previously suggested (Bellandi and Dorffling, 1974).

From an opposite point of view, an endless number of works which started during the late 1980's and early 1990's (Zhang and Davies, 1987; Zhang *et al.*, 1987; Tardieu and Davies, 1993) and continued to our days (Chen *et al.*, 2013; Jones, 2015; Tardieu *et al.*,

2015; Carvalho *et al.*, 2016) intended to decipher the relationship among soil water potential, ABA concentration in xylem sap and stomatal closure in a context of root-to-shoot ABA transport. Possible explanations for the little attention paid to transport across phloem rather than by xylem was presented in Hoad (1995) and remarks the difficulty of collecting sufficient uncontaminated volumes of phloem sap. Therefore, it appears that the theory indicating that ABA synthesis occurs in roots and the hormone is transported to leaves via xylem sap has prevailed over the evidence supporting the basipetal transport through the phloem. In fact, this basipetal-transport model has been mainly ignored and only sporadic references are found in the literature (Cornish and Zeevaart, 1985; Wolf *et al.*, 1990; Vernieri *et al.*, 2001; Ikegami *et al.*, 2009). However, both models do not need to be mutually excluding (Hoad, 1995), since ABA that reach the roots from the aerial tissues could be redistributed again to the aerial tissues mainly by the xylem sap as demonstrated under high salinity conditions (Hoad, 1975; Wolf *et al.*, 1990).

It is worth noting that roots are able to sustain a transient increase in ABA as an early response to dehydration. This could be masking the contribution of leaf-sourced ABA to this process. Additionally, the transcriptional response of ABA biosynthetic genes is similar in roots that are able and unable to accumulate ABA (Chapter II, Fig. 6 and 7), which could contribute to the general belief that ABA is actively produced in dehydrated roots. In this sense, some key steps from carotenoid and ABA pathway such as PSY and βCHX, as well as NCED, which are considered limiting in the root-ABA accumulation (Nambara and Marion-Poll, 2005; Thompson *et al.*, 2007*b*; Li *et al.*, 2008), were highly upregulated under dehydration. The change in gene expression upon stress was used to estimate the role of these genes (Ernst *et al.*, 2010; Aroca *et al.*, 2013) although it has been demonstrated that gene expression does not always match with the observed changes in the enzyme activity as reported for AAO3 in Arabidopsis (Seo *et al.*, 2000). Despite of this, it is interesting to note that changes in the expression of *CsNCED1* are usually correlated with the levels of ABA in roots, as observed in leaf bagging experiments (Chapter III; Fig. 5f), assuming a positive relationship between gene expression and ABA levels (Aroca *et al.*, 2013). However, other works disagree with this affirmation as occurred in *Vitis vinifera*, where no changes in *VvNCED1* expression was detected in roots of water-stressed plants (Soar *et al.*, 2006); in maize, where upregulation of genes such as *ZmNCED* and *ZmAAO3* occurs at later stages of water deficit (Ernst *et al.*, 2010); and in roots of lettuce, where *LsNCED* expression did not correlate with ABA levels (Aroca *et al.*, 2008). Overall, it appears that measuring only gene expression when working with

carotenoid and ABA pathways could lead to misleading interpretations. Therefore, it seems appropriate to recall that only a few works measured protein abundance or enzyme activity (Frey *et al.*, 2012) and some of them could not find any correlation between ABA levels and protein abundance (Endo *et al.*, 2008). Indeed, very low levels (or even no protein) of the carotenoid-related enzymes ZEP or VDE were detected in roots of *Nicotiana plumbaginifolia* besides the presence of their respective transcripts (North *et al.*, 2005).

The strong dependence of roots from ABA supplied by the leaves under dehydration led us to investigate whether leaves were playing a more active role in sensing water stress. Results from chapter III, where leaves were maintained at different relative water contents, demonstrate that leaf-dehydration is needed to induce the ABA rise in leaves and roots, and that the mere dehydration of roots is not sufficient by itself to induce ABA accumulation in this organ (Chapter III, Fig. 3d). The ability of leaves to increase ABA content independently of root water status is clearly established under conditions that lead to changes in atmospheric demand or daily fluctuations (Soar *et al.*, 2006; Caldeira *et al.*, 2014; McAdam *et al.*, 2016*b*). Similar results were found in Arabidopsis where the decrease in water availability in roots did not influence the ABA accumulation in leaves when leaf water turgor is maintained (Ikegami *et al.*, 2009). These results highlight a primordial role of leaves in synthetizing ABA under dehydration but also confer them a crucial role in sensing the adverse conditions as was also previously suggested (Ikegami *et al.*, 2009; Blum, 2015*b*). This indicates that leaf turgor is a critical parameter in triggering ABA accumulation in plants (Zeevaart and Creelman, 1988). Furthermore, Christmann *et al.* (2005) using transgenic plants expressing an ABA-inducible reporter gene, detected that water stress applied to the root system of Arabidopsis plants resulted in the increase of ABA pools in the shoot but not in the root. In this sense, an earlier work of Milborrow *et al.* (1973) demonstrated that detached roots are unable to synthetize large amounts of ABA even after a substantial 30% decrease in the root water content. Thus, evidence showed here demonstrated that leaves are not only a primary source of ABA for the roots, but also that leaf-dehydration is needed for the accumulation of this phytohormone in both leaves and roots.

Overall, data in this work demonstrate that under water deprivation ABA content increase in leaves and then it is transported to roots to sustain high levels of ABA in this organ, in accordance to previous work in herbaceous species or seedlings such as Arabidopsis,

maize and Lupinus (Wolf *et al.*, 1990; Ren *et al.*, 2007; Ikegami *et al.*, 2009; Waadt *et al.*, 2014). However, results presented here do not provide information whether this basipetal transport responds to any signal (likely from roots) which could be crucial for the enhancement in the ABA transport. In this sense, jasmonates could be involved in triggering the signal required to enhance the ABA basipetal transport since a transient increase in jasmonoyl isoleucine (JA-Ile), that was detected in the *in vitro* experiment (Chapter II, Fig. 4b), was previously demonstrated to be a necessary step for the increase in ABA levels in dehydrated citrus roots (De Ollas *et al.*, 2013) and Arabidopsis (de Ollas *et al.*, 2015).

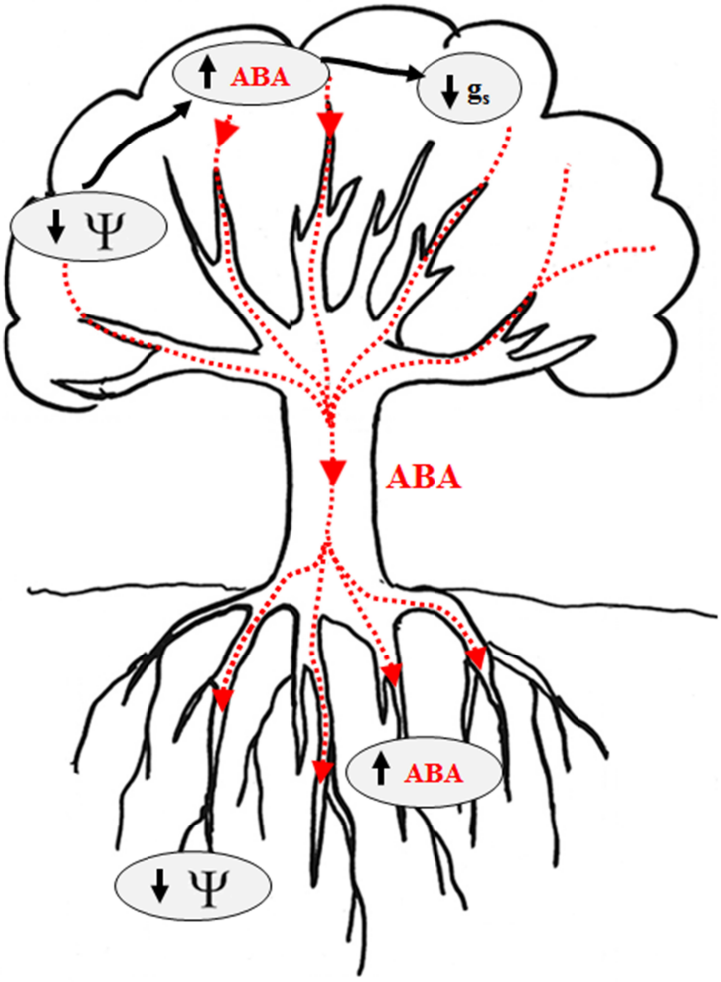

Figure 3. Proposed model of ABA accumulation in plants subjected to water deficit.

Based on the data presented in this work, a new model for ABA accumulation, transport, and the control of water relations under dehydration is presented in Fig 3. Under this

model, a drop in soil water availability leads to a reduction in water uptake and consequently in leaf water content. The reduction in water availability could take place even without an increase of ABA in this tissue, depending on the genotype and the environmental condition (Ache *et al.*, 2010); however, once certain threshold is achieved, ABA is rapidly synthesized. ABA induces the stomatal closure in leaves to avoid water loss (McAdam *et al.*, 2016*b*) and additionally, it is loaded into the phloem and basipetally transported to roots. In this sense, it was demonstrated that ABA is synthetized in phloem companion cells concomitantly with the increase of an ATP-binding cassette (ABC) transporter family in *Arabidopsis thaliana* ABCG25 (AtABCG25) expression in the same cells. This ABA exporter show efflux activity from inside to outside cells which is required to secrete ABA from the site of biosynthesis to reach the target cells in different tissues (Kuromori *et al.* 2014). Once in roots, ABA plays several relevant functions such as modification of root architecture (Jung and McCouch, 2013; Boursiac *et al.*, 2013; Harris, 2015) and root hydraulic conductivity (Thompson *et al.* 2007; Ruiz-Lozano *et al.* 2009) to increase water uptake (Aroca *et al.*, 2012). Once in roots, ABA could be degraded to catabolic metabolites such as DPA or even dumped into the xylem, where ABA could be recirculated to aerial tissues (Hocking *et al.*, 1972; Hoad, 1975; Wolf *et al.*, 1990).

5. CONCLUSIONS

1) Leaves constitute a relevant source of ABA in citrus plants under water stress conditions. Basipetal ABA transport through the phloem takes place in well-watered plants but it is notably enhanced under dehydration to sustain the long-term ABA accumulation in roots. This belowground organ is unable to accumulate substantial amounts of ABA during dehydration if basipetal transport from the canopy is disrupted.

2) Leaves play a key role in sensing the water deficit and triggering the ABA increase in both leaves and roots. A drop of the leaf water turgor is needed for root ABA accumulation which only takes place after the ABA rise in leaves. A decrease in root water availability under dehydration is not sufficient to induce ABA accumulation in this belowground organ.

3) The mere presence of β,β-carotenoids in roots is not sufficient to sustain the root-ABA accumulation under dehydration since detached roots with an increased availability of β,β-carotenoids were unable to accumulate ABA, suggesting that other mechanisms are operating.

4) ABA catabolism and conjugation appears to be involved in the ABA homeostasis in roots; catabolism is induced by dehydration irrespective of the ABA content in roots. Accordingly, expression of *CsCYP707a* gene is induced under this condition. However, no clear function of ABA-GE could be highlighted despite the strong changes observed in both metabolites and in the expression of main genes controlling their accumulation.

5) Other mechanisms apart from changes in gene expression operate in controlling ABA accumulation in roots. Several genes involved in carotenoid and ABA biosynthesis are modulated in response to dehydration in roots but these changes are not always linked to the ABA accumulation. Genes such as *CsPSY3a*, *CSβCHX1*, *CsβCHX2* and *CsNCED1* are substantially upregulated during water scarcity although no ABA increase in roots is detected.

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