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Artigo/Article

ABA accumulation in dehydrated roots: role of carotenoids and aerial tissues

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SUMMARY

It is widely accepted that abscisic acid (ABA) accumulation in leaves of dehydrated plants is supported by root-sourced ABA. However, this model is questioned due to the low availability of carotenoids (ABA precursors) in roots and by the fact that leaves synthetize their own ABA upon dehydration. To shed light in this process, the relative contribution of roots and leaves to ABA pool during dehydration was evaluated. Roots of stem-girdled Carrizo citrange seedlings showed a 50% reduction in ABA accumulation compared to roots of intact plants upon dehydration, being unable to increase ABA after a subsequent stress. Results suggest that ABA is transported from leaves to roots, a fact furtherly confirmed by feeding experiments with labelled ABA (*d6-ABA*) applied to the leaves and detected in both unstressed and dehydrated roots. Data also revealed that the limited content of carotenoids in roots decreased upon dehydration, taking several weeks to recover to unstressed basal levels. The relative contribution of carotenoids to root ABA biosynthesis was challenged in light-grown *Citrus macrophylla* roots (with high-carotenoid availability) submitted to osmotic stress, which were in fact unable to accumulate ABA. Contrastingly, a rapid upregulation of several key genes involved in carotenoid and ABA synthesis (*CsPSY3a*, *CsβCHX1*, *CsβCHX2*, and *CsNCED1*) was observed suggesting that carotenoids are rather being redirected to other pathways or even converted in other metabolites. Overall, these findings indicate that ABA accumulation in roots is sustained by ABA produced in leaves rather than by *in situ* biosynthesis from precursors.

Index terms: abscisic acid, basipetal transport, citrus, long-distance communication, water stress.

Acúmulo de ABA em raízes desidratadas: a função dos carotenoides na parte aérea

RESUMO

É amplamente aceito que o acúmulo de ácido abscísico (ABA) em folhas de plantas desidratadas é proveniente da produção de ABA nas raízes. No entanto, este modelo é questionado devido à baixa disponibilidade de carotenoides (precursores de ABA) nas raízes e pelo fato de que as folhas sintetizam seus próprios ABA após a desidratação. Para esclarecer esse processo, avaliou-se a contribuição relativa das raízes e folhas no acúmulo de ABA durante a desidratação. As raízes de mudas de citrange Carrizo, com caule anelado, mostraram uma redução de 50% no acúmulo de

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ABA em comparação com as raízes de plantas intactas após desidratação, sendo incapaz de aumentar a ABA após um estresse subsequente. Os resultados sugerem que o ABA é transportado de folhas para raízes, fato confirmado adicionalmente por experiências de alimentação com ABA rotulado (*d6-ABA*) aplicado às folhas e detectado em raízes não-estressadas e desidratadas. Os resultados também revelaram que a concentração limitada de carotenoides nas raízes diminuiu após a desidratação, levando algumas semanas para recuperar os níveis basais não estressados. A contribuição relativa dos carotenoides para a biossíntese de ABA nas raízes foi avaliada ainda em raízes expostas a luz durante o crescimento de *Citrus macrophylla* (com alta disponibilidade de carotenoides), submetidas a estresse osmótico, que de fato não conseguiram acumular ABA. Em contraste, observou-se uma rápida regulação positiva de vários genes chave envolvidos na síntese de carotenoides e ABA (*CsPSY3a*, *CsβCHX1*, *CsβCHX2* e *CsNCED1*) sugerindo que os carotenoides são mais redirecionados para outras vias ou mesmo convertidos em outros metabólitos. Em geral, esses achados indicam que o acumulo de ABA em raízes é sustentada pela produção de ABA nas folhas e não pela biossíntese in situ pelos precursores.

Termos de indexação: ácido abscísico, transporte basipetal, citros, comunicação à longas distâncias, estresse hídrico.

INTRODUCTION

Plant responses to cope with abiotic stress occur at physiological, biochemical and molecular levels. Under water deficit, the most detrimental condition for crop production, abscisic acid (ABA) levels increase inducing several tissue-specific responses including stomatal closure and modifications in root architecture (Luo et al., 2014; Gómez-Cadenas et al., 2015). Although *de novo* synthesis of ABA is the main source of this hormonal burst, conjugation, catabolism and transport from other tissues also modulate the amount of this phytohormone (Boursiac et al., 2013). In this sense, ABA transport via xylem sap has been the base of most long-distance transport models that propose that the ABA produced in roots induce stomatal closure in leaves (Zhang et al., 1987; Zhang & Davies, 1987; Puértolas et al., 2013). On the other way, ABA phloem-transport has been largely recognized in herbaceous plants, which could export ABA to roots from shoots (Hoad, 1967, 1978; Davenport et al., 1977).

Despite most tissues and organs are able to synthetize ABA, the ability of the roots to increase ABA levels during dehydration has been questioned due to the low availability of ABA precursors (carotenoids) in this tissue, which usually are present in a 0.1% of the levels found in leaves (Ruiz-Sola et al., 2014). Indeed, the predominant plastid (organelles where carotenoids are accumulated) type present in roots are proplastids and leucoplasts, which are not specialized in carotenoid accumulation (Li & Yuan, 2013). Carotenoid biosynthesis involves the fusion of two molecules of geranylgeranyldiphosphate (GGPP) by the action of a phytoene synthase (PSY) enzyme, a light-responsive and rate-limiting step in carotenoid pathway (Toledo-Ortiz et al., 2010). Several successive desaturations and isomerizations lead to the production of lycopene, a key branching point in the pathway in which lycopene is converted into ε,β-carotenoids (α-carotene and lutein) or alternatively, in the β,β-carotenoids, rendering β-carotene. β-carotene is further hydroxylated to yield β-cryptoxanthin and zeaxanthin by the action of another key enzyme in the carotenoid synthesis, β-carotene hydroxylase (βCHX) (Nambara & Marion-Poll, 2005). Zeaxanthin is then converted into violaxanthin and neoxanthin, which in their *cis* configuration, are the specific substrates of 9-*cis*-epoxycarotenoid dioxygenase (NCED), a bottleneck enzyme in the ABA pathway which renders xanthoxin (Thompson et al., 2007b). ABA is finally produced by two consecutive oxidations of the xanthoxin molecule in the cytosol (Nambara & Marion-Poll, 2005).

Several evidence such as the over-expression of PSY (Lindgren et al., 2003), ABA-deficient mutants (Borel et al., 2001) and the use of carotenoid inhibitors such as norflurazon (NFZ, Ollas et al. 2013), suggested that carotenoid amount limits the downstream accumulation of ABA in response to water deficit. However, other results showed that an enhanced levels of these precursors does not necessary increase the ABA content (Arango et al., 2014). In this sense, the over-expression of βCHX in carrot roots (Arango et al., 2014) or *PSY* in potato tubers (Ducreux et al., 2005) did not increased the ABA content in belowground tissues, whereas the use of NFZ did not prevent ABA accumulation in citrus roots during dehydration (Ollas et al., 2013). The influence of the transport from other tissues appears to be more relevant in sustaining ABA increase rather than the *de novo* synthesis during adverse conditions (Ren et al., 2007; Ikegami et al., 2009).

In the current study, *de novo* synthesis of ABA in roots and the possible basipetal transport of ABA produced in leaves were analyzed for its contribution to

sustain ABA increase in roots upon water stress. To test these, several experiments were carried out including 1) the interruption of basipetal transport, 2) the exogenous application of a labelled ABA analogue and 3) the ability of detached roots with different levels of initial carotenoids to synthetize ABA. Analysis of ABA, carotenoids as well gene expression changes of key steps in the ABA pathway in roots during dehydration are showed.

MATERIALS AND METHODS

Stem girdling experiment

One-year-old seedlings of Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) potted in 2.5-L plastic bags filled with a mixture of peat moss, perlite and vermiculite (80:10:10; v:v:v) and watered three times a week with 0.5 L of a half-strength Hoagland solution were acclimated for four months in greenhouse (day/night, 25±3.0/18±2.0 °C). Girdling was performed by removing 5 mm-wide segment of bark just above the root-stem junction two days before the beginning of the experiment. After that period, water stress treatments were performed by transplanting the seedlings to dry perlite. Three treatments were established: water-stressed girdled (WS+GD) plants; water-stressed non-girdled (WS) plants, whereas a subset of plants was kept under well-watered conditions (CT) throughout the experimental period. In water-stressed (WS+GD and WS) treatments plants were dehydrated for three days, rehydrated for three additional days and thereafter, dehydrated again for a second period of two-days. Samples of leaves and roots were taken at 3, 6 and 8 days after the stress beginning.

Foliar application of ABA-analogue

Deuterium-labelled ABA (d_6-ABA) was foliar-applied to Carrizo citrange seedlings similar to those indicated above. A solution containing 50 μM d_6-ABA in distilled water with 0.5% (v:v) of Triton X-100 was sprayed three times in a week avoiding runoff to the substrate and roots. After treatment (day 7), plants were a) transplanted to dry perlite (WS) or b) kept under well-water conditions (WW). After 24 h of stress treatment, leaves and roots were collected and the presence of d_6-ABA was assessed by UPLC-MS/MS (see details in hormone analysis section).

Detached roots experiments. Seeds of Citrus Macrophylla (*Citrus macrophylla* Wester) were peeled and disinfected for 10 min in a 2% (v:v) sodium hypochlorite solution containing 0.1% (v:v) Tween-20 and then rinsed three times with sterile distilled water. In 25×150 mm culture tubes 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 was added. The pH was set at 5.7 ± 0.1 with 0.1N NaOH and then tubes were autoclaved. The medium was then solidified with agar at 9.0 g.L^{-1} (Pronadisa, Madrid, Spain). Seeds were individually sown in each tube and then were kept in darkness at 25 °C for two weeks. Thereafter, tubes containing germinated plants were transferred to a growth chamber (16 h photoperiod, photosynthetically active radiation of 150 μmol m^{-2} s^{-1} and constant temperature of 25 °C). Under this condition shoots received radiation whereas 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 a) shoots were excised with a scalpel 2 mm below to the root-shoot junction and roots were kept in the light (LT) or b) seedlings were kept intact maintaining roots in the dark and shoots light-exposed (CT). Plants under the two conditions were left for 3 weeks period in which LT roots developed a strong coloration. Thereafter, a osmotic stress was imposed by transferring plant material to a solution containing PEG-6000 to achieve water potential values of -1.5 MPa to induce ABA accumulation. During the dehydration period, roots were kept in the darkness and with the same conditions of temperature as indicated above. Samples material were collected at 0; 4 and 8 h after the stress onset, rinsed with distilled water, immediately frozen in liquid nitrogen, grounded into a fine powder and stored at -80 °C until analyses.

Hormone analysis. Abscisic acid were extracted and analyzed as described in Ollas et al. (2013) and Manzi et al. (2016). Briefly, 0.2 g of sample was extracted in 2 mL of distilled H_2O after spiking with 100 ng of d_6-ABA. After centrifugation at 10.000 × *g* at 4 °C, supernatant was recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified extract was partitioned twice against 3 mL of di-ethyl ether and then the organic layer was evaporated. The dry residue was resuspended in a 9:1 (v:v) H_2O:MeOH solution, filtered and injected into a HPLC system. Chromatographic conditions, metabolite monitoring and ABA quantification is described in (Manzi et al., 2016). In the labelling experiment samples

were similar extracted but no internal standards were spiked d_6-ABA. Relative quantification was performed by the comparison of areas of each sample.

Carotenoid analysis

Carotenoids were extracted as quantified as described in (Alós et al., 2014). In brief, 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 4 °C. The aqueous phase was re-extracted with chloroform until it was colourless. 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 N_2 stream and kept at -20 °C until analysis. Chromatographic conditions, standards and calibration curves were previously described in (Alós et al., 2014).

Quantitative real-time PCR

Gene expression was determined as described in Manzi et al. (2016). Briefly, total RNA was isolated from root of detached experiment using RNeasy Plant Mini Kit (Qiagen, Madrid, Spain). One µg of DNA-free transcripts were reverse-transcribed using Primescript RT reagent with oligo(dT) primer (Takara Bio, Inc. Japan) in a total volume of 20 µL. 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) to a final volume of 10 µL. The primers employed for the amplification of each gene were: *Phytoene synthase3a* (*CsPSY3a*), F: 5′-AATGCATTTGTGTAAGCCCTGCT-3′, R: 5′- TGTCCCTAAAAGGCTTGATGTGTAAATTG-3′; β-*carotene hydroxylase1* (*CsβCHX1*), F: 5′-GGCTCATAAAGCTCTGTGGC-3′, R: 5′-CCAGCACCAAAACAGAGACC-3′; β-*carotene hydroxylase2* (*CsβCHX2*), F: 5′-AGAAGAGGAAACCGAAGAGCTTGAG-3′, R: 5′-GATTGCTGCAACAAGGTAGGTTTGT-3′ and *9-cis-epoxycarotenoid dioxygenase1* (*CsNCED1*) F: 5′-AATGCTTGGGAAGAGCCTGAG-3′, R: 5′-AGTGGACTCGCCGGTCTTTAG-3′. For expression measurements, StepOne™ Software v2.3 and expression levels relative to values of a reference sample (non-stressed harvest time at 0 h in within treatment was set at one) were calculated using the Relative Expression Software Tool (REST). Actin gene was used as housekeeping as previously indicate for citrus tissues (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 ($p \leq 0.05$).

RESULTS AND DISCUSSION

Abscisic acid accumulation in plants is crucial to trigger a wide range of responses to face several adverse conditions. Root-to-shoot ABA transport via xylem sap would be responsible for induce stomatal closure in leaves (Zhang et al., 1987; Zhang & Davies, 1987; Puértolas et al., 2013). However, ABA transport through phloem has been demonstrated in seminal works during the 1960's and 1970's, demonstrating that basipetal transport takes also place (Hoad, 1967, 1978; Davenport et al., 1977). In order to evaluate the involvement of the shoots on ABA accumulation in roots, plants were stem-girdled and water-stressed during two consecutive periods. Dehydration increased ABA content in a similar extent in leaves of intact (WS) and girdled (WS+GD) plants in the two periods of water stress (day 3 and 8, Figure 1A). However, ABA content in roots of girdled plants (WS+GD) was only 50% (475±28 ng.g^{-1}) compared to that levels found in roots of intact ones (WS) after the first period of water stress (960±78 ng.g^{-1}, Figure 1B). This result demonstrated a strong influence of the aerial tissues on the ABA accumulation in roots, a fact which was exacerbated after the second period of dehydration. In this second phase, roots of girdled plants (WS+GD) were unable to increase the ABA levels (204±10 ng.g^{-1}) whereas roots of intact plants (WS) notably increased ABA content similar to levels achieved during the first period of dehydration (870±78 ng.g^{-1}, Figure 1B).

Dehydration sharply reduced the levels of the β,β-carotenoids in roots of both water-stressed plants, from 1050±157 ng.g^{-1} in CT plants to 351±67 and 175±37 ng.g^{-1} in WS and WS+GD plants (Figure 2). This decrease in precursors seems to support the ABA increase after the

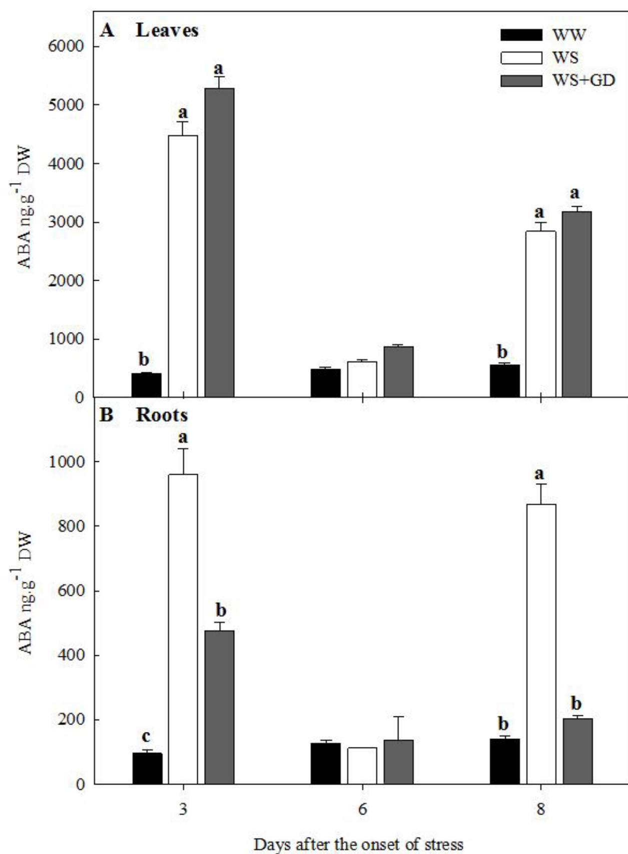

Figure 1. Abscisic acid accumulation in leaves (A) and roots (B) of intact or stem-girdled citrus plants subjected to two periods of water stress. WW: well-watered plants; WS water-stressed plants; WS+GD: water-stressed and girdled plants. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date (Tukey's test, p≤ 0.05).

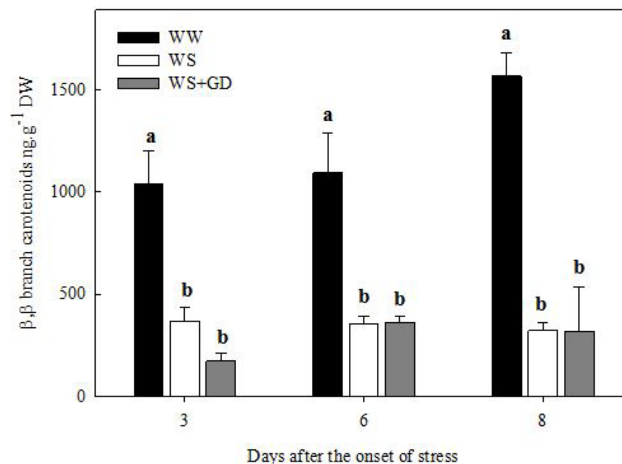

Figure 2. Total β,β-carotenoid content in roots of intact or stem-girdled citrus plants subjected to two periods of water stress. WW: well-watered plants; WS water-stressed plants; WS+GD: water-stressed and girdled plants. Data are means ± SE of at least 3 replicates. Different letters indicate significant differences between treatments at each date (Tukey's test, p≤ 0.05).

first period of dehydration (Figure 1B). However, this assumption needs to be reconsidered because after the second period of dehydration, levels of β,β-carotenoids remained low and unchanged (around 320 $ng.g^{-1}$) in intact (WS) and girdled (WS+GD) plants (Figure 2) but roots of the WS plants accumulated ABA (Figure 1B). Therefore, this data reinforce that aerial tissues strongly affect ABA accumulation in roots (Ren et al., 2007; Ikegami et al., 2009) whereas the β,β-carotenoids available in roots appears to have only a marginal influence on ABA accumulation.

In order to confirm whether the shoots are able to be a source of ABA to the roots, an ABA-labelled analogue (d_6-ABA) was sprayed to leaves and then plants were dehydrated (WS) or kept under well-water conditions (WW). ABA was found in roots of WW and WS plants, being three fold higher in the latter compared to those levels found in WW roots (Figure 3). These results confirm that ABA is indeed basipetally transported from shoot to roots even under non-stressed conditions as was suggested in other herbaceous species including Pisum and tomato (Ikegami et al., 2009; McAdam et al., 2016), and also that this transport is notably stimulated under water deprivation. Therefore, results demonstrate that ABA transport from shoots to roots is occurring in a woody plant such as Citrus, being in opposition to that reports indicating an acropetal movement of ABA from roots to shoots (Zhang et al., 1987; Puértolas et al., 2013).

The above results also minimize the real importance of the direct precursors in roots to sustain ABA biosynthesis. Despite the low ability of the plastids present in roots to synthetize and accumulate carotenoids (Li & Yuan, 2013), the contribution of these precursors to the ABA increase in this tissue has never been questioned. To evaluate this contribution, roots were detached in order to avoid the ABA incoming from shoots and exposed to light to increase the levels of carotenoids in roots (LT). By its side, intact plants with roots in the dark and illuminated shoots were used as control (CT). Total carotenoid content increased in roots exposed to light (LT) by 5.5-times

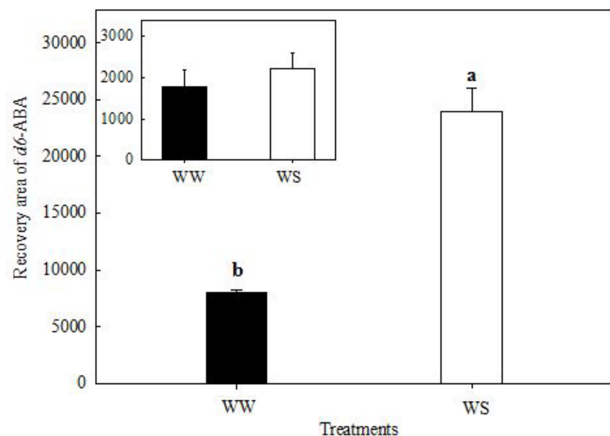

Figure 3. Relative amount (Peak areas per g-1 FW) of deuterium ABA (d_6-ABA) detected in leaves (inset) and roots of citrus plants after exogenous application of d_6-ABA to the leaves. Plants were then maintained under well-water (WW) conditions or subjected to water stress (WS). Data are means ± standard error of 3 replicates. Letters indicate significant differences (Tukey's test, $p \leq 0.05$).

(13±0.1 μg.g^{-1}) compared to that roots maintained in the dark (CT, 2.4±0.1 μg.g^{-1}) as shown at 0 h (Figure 3B). In spite of the initial basal differences in the carotenoid content between these two types of roots, carotenoid levels sharply decreased upon dehydration (Figure 3B), similarly to that results obtained previously in girdled plants (Figure 2). Strikingly, after the exposure to dehydration only those roots of intact plants (CT) accumulated ABA (up to 105±4.5 ng.g^{-1}) whereas in carotenoid-rich detached roots (LT), ABA levels kept low and unchanged (3.4±0.9 ng.g^{-1}, Figure 3A). These unexpected results evidenced that the availability of carotenoids in roots is not related to the ABA accumulation. Therefore, these results reinforce the idea that shoots are an essential source of ABA to roots, and also evidence that this transport takes place shortly after dehydration (Figure 3A). Moreover, carotenoid degradation in roots upon water stress suggests that these compounds are involved in other processes different from ABA biosynthesis that could be related to their antioxidant capacity (Fiedor et al., 2005; Ramel et al., 2012; Lado et al., 2016), or to a signaling process (Fiedor et al., 2005; Arango et al., 2014; Visentin et al., 2016).

Dehydration also upregulated several genes from the carotenoid and ABA biosynthetic pathways such as *CsPSY3a*, *CsβCHX1*, *CsβCHX2* and *CsNCED1* in both types of roots (Figure 4). The expression of the *CsPSY3a* increased 15- and 9-fold after 4 h of stress and 8-and 2-fold after 8 h in LT and CT roots, respectively (Figure 4A). The expression of *CsβCHX1* and *CsβCHX2* showed a similar pattern between treatments being strongly upregulated in CT roots after 8 h of stress (Figure 4B,C). In spite of the differences found in the stimulation of these genes between the two types of roots, these upregulation could not be related to changes found in carotenoid levels, despite that PSY and βCHX are crucial during the carotenogenesis (Rodrigo et al., 2003; Li et al., 2008). Indeed, whereas the expression of these genes increased in response to dehydration (Figure 4A-C), levels of carotenoids decreased (Figure 5B). Therefore, results suggest that gene upregulation is a conserved stress-induced response; however, ABA synthesis is impaired in roots irrespective of carotenoid availability. Similarly, *CsNCED1* was upregulated in both types of roots being this increase similar between them after 8 h of stress (156-fold). In spite of the relevance of CsNCED1, which catalyzes the bottleneck step in the ABA biosynthesis (Nambara & Marion-Poll, 2005), *CsNCED1* upregulation could only be linked to the ABA increase found in CT roots (Figure 5A). Besides the high levels of carotenoids and the sharp upregulation of *CsNCED1* gene, LT roots were unable to accumulate ABA, revealing that ABA biosynthesis in roots is limited and confirming that the main source of ABA are the shoots, as evidenced in CT roots. Therefore, the data present here questioned previous evidence that directly link the expression of the *NCED* genes with the changes in ABA levels during the stress conditions in the roots (Thompson et al., 2007a; Ahrazem et al., 2012). Overall, the expression of *CsPSY3a*, *CsβCHX1*, *CsβCHX2* and *CsNCED1* genes in roots appears to be a conserved response induced by dehydration rather than directly contributing to *de novo* synthesis of carotenoids and ABA.

In summary, results showed here evidence that ABA accumulation in roots is mainly supported by the contribution of the ABA produced in aerial tissues, which is transported via phloem downward from shoot-to-roots. This basipetal movement of ABA occurs under unstressed conditions and is enhanced by dehydration. Moreover, the upregulation of several key genes in ABA biosynthesis in roots such as *CsNCED1* reflects a response induced by dehydration rather than contributing to ABA increase. The role of carotenoids in the roots to sustain ABA biosynthesis is questioned and a novel function for these compounds is currently explored.

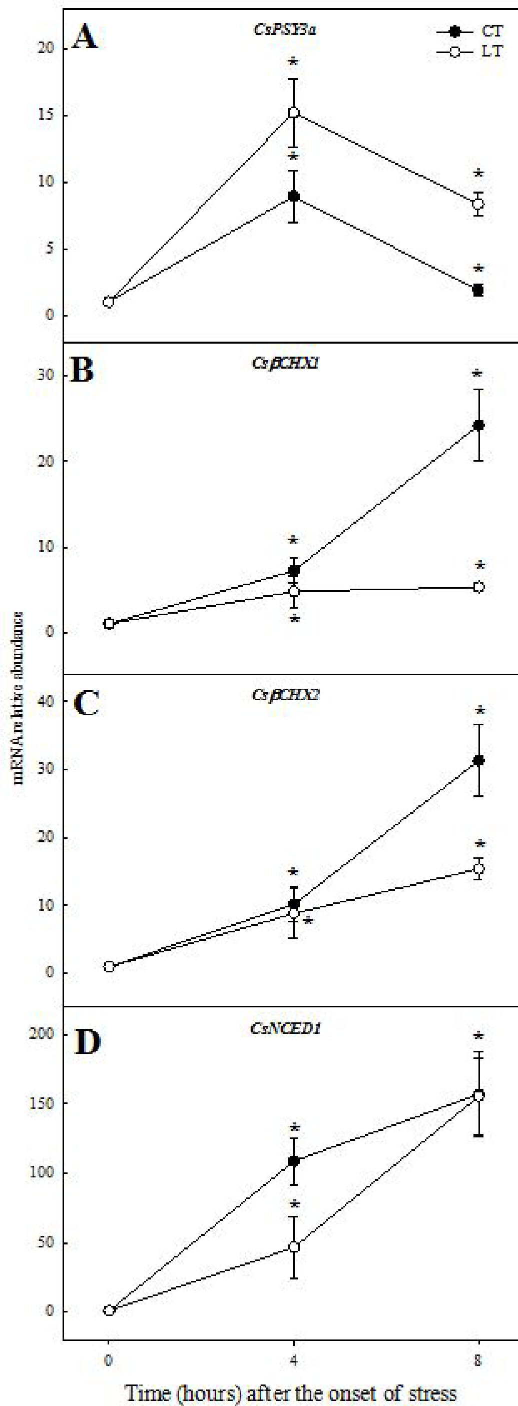

Figure 4. Relative mRNA abundance of carotenoid *CsPSY3a* (A), *CsβCHX1*(B) and *CsβCHX2* (C) and ABA biosynthetic gene *CsNCED1* (D) in *Citrus* roots exposed to dehydration (-1.5 MPa). Roots of intact plants grown in the darkness and light-exposed shoots (CT) and detached roots grown during three weeks under illumination (LT). Data are means ± SE of 4 replicates.* denotes significant upregulation compared to the initial unstressed levels within roots treatment (Tukey's test, p≤ 0.05).

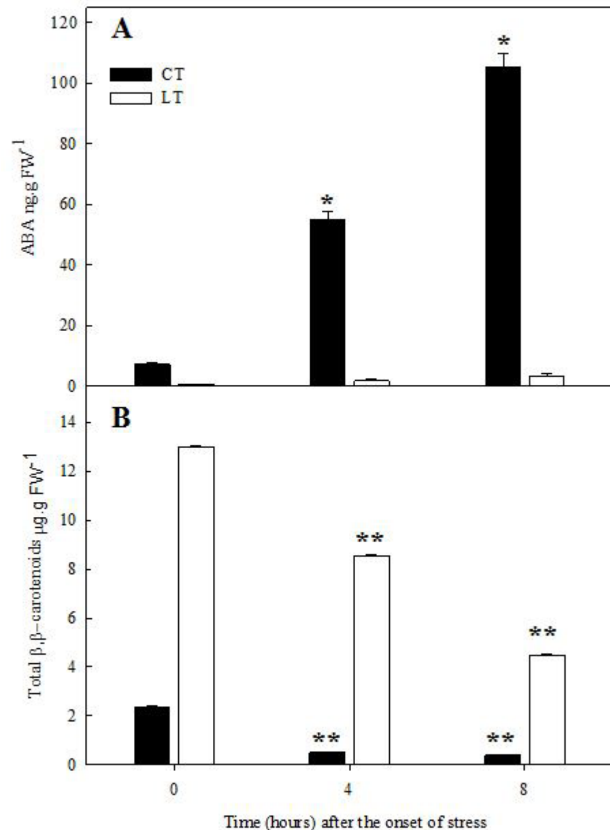

Figure 5. ABA (A) and total β,β-carotenoid (B) content in citrus roots exposed to dehydration (-1.5 MPa). Roots of intact plants grown in the darkness and light-exposed shoots (CT) and detached roots grown during three weeks under illumination (LT). Data are means ± SE of, at least 3, replicates.* and ** denotes significant increase and decrease, respectively, to the initial unstressed levels within roots treatment (Tukey's test, p≤ 0.05).

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