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ABA accumulation in water-stressed *Citrus* roots does not rely on carotenoid content in this organ

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ABSTRACT

Sustained abscisic acid (ABA) accumulation in dehydrated citrus roots depends on the transport from aerial organs. Under this condition, the role 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 restricts its accumulation. To accomplish that, detached citrus roots were exposed to light (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 low levels of carotenoids) showed a similar decrease of ABA precursor together with a significant ABA accumulation. 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.

1. Introduction

Plant mechanisms to cope with abiotic stress occur 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 [1,2]. 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 [2]. ABA derives from carotenoids, which are synthesized and accumulated in plastids [3]. 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 (Supplemental Fig. S1). 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 [3]. 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 [4]. This molecule is subsequently exported from the plastids to the cytosol where it is oxidized to ABA [3,4]. This oxidation takes place in two steps;

Abbrevations: ABA, abscisic acid; ABA-GE, ABA-glycosylester; AAO3, abscisic aldehyde oxidase; AOG, ABA *O*-glycosyl transferase; DHJA, dehydrojasmonic acid; DPA, dihydrophaseic acid; GGPP, geranylgeranyldiphosphate; JA-Ile, jasmonoyl-isoleucine; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NFZ, norflurazon; PA, phaseic acid; PAR, photosynthetically active radiation; PEG, polyethylene glicol; PSY, phytoene synthase; RWC, relative water content; XantDH, xanthoxin dehydrogenase; ZEP, zeaxanthin epoxidase; βCHX, β-carotene hydroxylase; βGluc, β-glycosidase.

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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 [3]. 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; [3]). 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 ([5,6], Supplemental Fig. S1).

Different lines of evidence including over-expression of key genes for carotenoid biosynthesis such as PSY [7] and ZEP [8], ABA-deficient mutants [9], and the use of inhibitors such as norflurazon (NFZ, [10]), 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 [11,12], indicative of the existence of an intricate mechanism governing ABA biosynthesis in different plant species and tissues [13]. In fact, the over-expression of *βCHX* in carrot roots [12] or *PSY* in potato tubers [14] 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 [10]. These 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 [1,15–17].

It has been previously demonstrated that ABA accumulation in roots of citrus and Arabidopsis plants subjected to water deficit takes place after a transient increase of jasmonic acid (JA) and jasmonoyl-isolucine (JA-Ile; [10,18]). Interestingly, ABA increase occurs concomitantly to the reduction β,β-carotenoid concentration [17], suggesting that accumulation of ABA is directly fed by carotenoid precursors [19,20]. However, this contribution appears to be quite limited in time and only providing reduce amounts of ABA [16,17]. Moreover, under longer or reiterative periods of dehydration, ABA accumulation in roots exclusively relies on aboveground tissues as demonstrated in several species such as maize [16], Arabidopsis [15] and citrus [17]. Indeed, this input from aerial organs also takes place under non-stressful conditions [1,17]. Furthermore, carotenoid content in roots is usually around 0.1% of that found in leaves [20,21], related to the predominant plastid type in roots (proplastids or leucoplasts), which are not specialized in carotenoid accumulation [22]. Moreover, carotenoid biosynthesis is highly responsive to light, being more active in light-exposed tissues [23] and consequently, recovery of carotenoid levels in dark-growing roots after dehydration is a slow process that takes several weeks of regular watering [17].

Other pieces of work suggested that apart from NCED, PSY could be an important enzymatic bottleneck in ABA biosynthesis in roots under certain stress conditions [4]. Based on this, an increase in the carotenoid content in roots could allow the sustained ABA accumulation in this tissue [19,24].

The objective of this work was to depict 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 before dehydration stress was imposed. In parallel, carotenoid accumulation in detached roots of citrus was induced by light exposure before the stress onset with the aim of sustaining the ABA synthesis in roots. 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.

2. Material and methods

2.1. 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 mgL^{-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 0.1 N NaOH before autoclaving. The medium was solidified with agar at 9.0 gL^{-1} (Pronadisa, Madrid, Spain). The cultures were maintained in darkness at 25 °C 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 25 °C. Tubes were foil-wrapped at the bottom part to maintain the roots under dark conditions. After three weeks of growing (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 and to increase the carotenoid content in LT roots, plants were kept in these conditions for 3 weeks before stress imposition. 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. At least 20 individual roots or shoots were collected at each sample point. Material was rinsed with distilled water, immediately frozen in liquid nitrogen, ground into a fine powder and stored at −80 °C until analyses.

2.2. 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 70 °C for two days to obtain dry weight (DW). Three replicates were used for each treatment. The RWC was calculated as $RWC = ((FW - DW)/(TW - DW)) \times 100$.

2.3. Hormone analysis

ABA and jasmonoyl-isoleucine (JA-Ile) were extracted and analyzed essentially as described in [10] 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