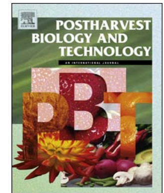
Postharvest Biology and Technology 133 (2017) 26–35

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio



Unravelling the role of abscisic acid in chilling tolerance of zucchini during postharvest cold storage

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ARTICLE INFO

Keywords:
Abscisic acid
Tungstate
Zucchini
Postharvest
Chilling
Cold tolerance

ABSTRACT

Abscisic acid (ABA) is a key phytohormone in the regulation of most stress responses, which especially involve dehydration such as drought, high salinity, and low temperature. Under these stress conditions, plants accumulate ABA that triggers a response to cope with the adverse environment. In this study, we investigated the implication of ABA in the acquisition of chilling tolerance in zucchini during fruit postharvest. For that, we have analyzed ABA biosynthesis and response in fruit of two varieties with different chilling tolerance, 'Natura' (cold tolerant) and 'Sinatra' (cold sensitive). The results have showed that the biosynthesis of ABA was induced in 'Natura' fruit, with an increase of the abscisic aldehyde oxidase activity (AAO), and consequently an accumulation of ABA during the first day of exposition to cold. The levels of ABA showed a significant negative correlation with weight loss and chilling damage, and a positive correlation with firmness. Transcriptional analysis of some genes involved in ABA signaling and response, such as the ABA receptors PYL1 and PYL4, the transcription factors AI5L2/ABF3, NAC072/RD26, ATHB7, bHLH112, and the enzymes KAT2 as well as HDA6, a deacetylase involved in histone modification, presented different expression levels between varieties, showing 'Natura' the highest expression in most cases at least at short-term storage. Finally, to corroborate the role of ABA in cold tolerance, 5 mM of sodium tungstate (an inhibitor of ABA biosynthesis) was applied to 'Natura' fruit and 0.5 mM of ABA was applied to 'Sinatra' fruit and their postharvest behaviour at low temperature was followed. The treatment with tungstate induced cold-sensitivity in 'Natura' fruit, whereas ABA-treated 'Sinatra' fruit improved their quality during the storage at 4 °C. The results obtained point to ABA as a phytohormone responsible for the acquisition of postharvest cold tolerance.

1. Introduction

The plant hormone abscisic acid (ABA) plays a crucial role in different processes of plant development and it is a central regulator of the resistance and adaptive response against several environmental stresses, especially those that induce dehydration such as drought, high salinity, and low temperature (Mehrotra et al., 2014; Sah et al., 2016). Under those unfavourable conditions, plants accumulate ABA, and this phytohormone activates the expression of many stress-related genes involved in the regulation of gene expression, signal transduction, and the acquisition of stress tolerance to withstand the adverse environment. This response involves first perception of the signal and then activation of ABA-induced genes. During signal perception, different ABA receptors have been described, belonging to PYR/PYL/RCAR (PYRabactin resistance/PYrabactin resistance-Like/Regulatory Component of ABA Receptor) family protein, that includes monomeric (PYR1, PYL1,-3) and dimeric receptors (PYL4-6, PYL8-10), having the monomeric receptors a higher affinity for ABA (Dupeux et al., 2011; Finkelstein, 2013; Pizzio et al., 2013). These receptors form a stable complex with ABA and a group-A protein phosphatases 2C (PP2C) that inactivates the phosphatase activity and enables the autophosphorilation of sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2), thus starting the ABA response (Finkelstein, 2013). ABA-induced genes contain in their promoter region a consensus domain known as the ABA responsive element (ABRE) (Fujita et al., 2011). The transcription factors (TFs) that bind to these ABRE elements are the ABF/AREB (ABRE Binding Factor/ABA-Responsive Element Binding Factor) family members; that encode a basic-domain leucine zipper

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http://dx.doi.org/10.1016/j.postharvbio.2017.07.004
Received 11 May 2017; Received in revised form 4 July 2017; Accepted 5 July 2017
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(bZIP) in their sequence (Yoshida et al., 2010).

During fruit development, it has been well established that ABA plays an important role on regulation of ripening in both climacteric and non-climacteric fruit such as tomatoes, strawberries, and sweet cherries (Chen et al., 2016a; Teribia et al., 2016; Zhang et al., 2009). In climacteric fruit, an increase of ABA content preceding an induction of ethylene production has been detected, whereas in non-climacteric fruit ABA appears to have a stronger role than ethylene in maturation (McAtee et al., 2013; Osorio et al., 2013; Vallarino et al., 2015). Recently, a large accumulation of ABA during fruit maturation and ripening has been described in squash (*Cucurbita pepo* L.), a non-climacteric species, as well as an induction of fruit ripening by exogenous application of this phytohormone (Chen et al., 2016b). However, the relationship between ABA metabolism and the behaviour of the fruit during postharvest storage has not been studied in depth.

Postharvest handling of many fruit usually includes storage at temperatures that slow down their respiratory rate to prevent deterioration and ripening, which means that fruit have to cope with several stresses before being brought to the consumer. Low but non-freezing temperatures (0–15 °C) induce in several tropical or subtropical fruit damages that are known as chilling injury (CI). Zucchini fruit, due to its subtropical origin, is susceptible to develop this physiological disorder. In addition, the fruit of zucchini are harvested in an immature stage, making them more susceptible to suffer dehydration and develop CI due to their accelerated metabolism (Mohammed and Brecht, 2003). Several studies have shown that chilling susceptibility in zucchini is variety dependent (Carvajal et al., 2011; Megías et al., 2016). These authors selected 'Natura' and 'Sinatra' as the commercial varieties with the most contrasting behaviour, being 'Natura' the most cold tolerant variety and 'Sinatra' the most sensitive (Carvajal et al., 2015a; Palma et al., 2014a, 2014b). In fruit of other species such as table grapes and pineapple, ABA induces physiological responses that help to diminish the damages that appear after postharvest cold storage (Cantín et al., 2007; Zhang et al., 2015). However, other studies show the opposite role of ABA respect to chilling sensitivity, as is the case in mandarins (Gosalbes et al., 2004). Therefore, the aim of this work has been to unravel the implication of ABA in the response to cold during the postharvest storage of zucchini fruit. For this purpose, ABA biosynthesis and response in 'Natura' and 'Sinatra' fruit were studied in order to compare and contrast the behaviour of these two varieties.

2. Materials and methods

2.1. Plant material and storage conditions

Experiment 1. Zucchini fruit (*Cucurbita pepo* L. morphotype *Zucchini*) of the commercial varieties 'Natura' (Enza Zaden) and 'Sinatra' (Clause-Tezier) were provided by FEMAGO S.L. After harvest, fruit were stored in a temperature-controlled chamber and in permanent darkness at 4 °C and 85–90% RH for 14 d.

Experiment 2. Freshly-harvested fruit of the same varieties used in experiment 1 were employed. Fruit of 'Natura' were treated with sodium tungstate (TS), an inhibitor of the abscisic aldehyde oxidase, and fruit of 'Sinatra' were treated with ABA. 'Natura' fruit were submerged at 20 °C for 20 min in 5 mM of TS, distilled water in the case of the control or 5 mM of TS plus 0.5 mM of ABA as a second control to discard non-specific effects of TS. For 'Sinatra', fruit were submerged at the same conditions described above in 0.5 mM of ABA or distilled water as the control. All fruit were then placed on desiccant paper and allowed to dry during 2 h before storage in a temperature-controlled chamber and in permanent darkness at 4 °C and 85–90% RH for 14 d.

In both experiments, three replicates were prepared per variety, treatment, and storage period, each consisting in 6 fruit of similar size. After storage, weight loss, chilling-injury index, firmness, and in the second experiment also cell-death rate were determined, after which the exocarp was separated, frozen, and pulverized in liquid nitrogen, and stored at −80 °C until use.

2.2. Weight loss and chilling-injury index

Fresh weight loss was evaluated for each condition studied and the percentage of weight loss was calculated as: % weight loss = $(W_i - W_f)/W_i \times 100$, being W_i the initial fruit weight and W_f the final fruit weight. Chilling-injury (CI) index was evaluated using a subjective scale of visual symptoms previously described by Martínez-Téllez et al. (2002): 0 = no pitting, 1 = slight (10% or less), 2 = medium (10–20%), and 3 = severe pitting (> 20%). CI index was determined using the following formula: Σ (pitting scale (0–3) × number of corresponding fruit within each class)/total number of fruit estimated.

2.3. Firmness

Firmness was measured in each peeled fruit by penetration assay employing a Fruit hardness tester FHT-803 (Silverado) with a 7.9 mm-diameter probe and results were expressed in Newtons (N).

2.4. Cell-death assay

Cell-death rate was assayed indirectly by estimation of trypan blue uptake according to Qu et al. (2009) with the modifications described in Carvajal et al. (2015b). Exocarp was separated with a vegetable peeler and 5 discs were taken from each replicate with an 11 mm diameter stainless-steel cork borer. Four replicates from each treatment were measured. Exocarp discs were submerged in 0.25% (w/v) trypan blue in petri dish and incubated on a platform shaker for 10 min. After that, the discs were rinsed with deionized water until no more blue stain was eluted and then dried by filter paper. The dry discs were weighed and homogenized in 50% (v/v) ethanol. After that the samples were centrifuged at 4 °C and 10,000 × *g* for 10 min and the absorbance of the supernatant was determined at 585 nm. Cell-death rate was expressed as percentage of trypan blue accumulation respect to the exocarp of freshly-harvested fruit.

2.5. Measurement of thiobarbituric acid reactive species (TBARS)

Malondialdehyde (MDA) content was determined using the TBARS procedure described by Heath and Packer (1968), with some modifications. Exocarp ground in liquid nitrogen was homogenized (1:4, w/v) in 20% (w/v) trichloroacetic acid (TCA), 0.2 mL of 4% (w/v) butylated hidroxytoluene was added during the process. The homogenate was centrifuged at 4 °C and 10,000 × *g* during 15 min. The supernatant was mixed with 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA in proportion 1:4 (v/v). The mixture was heated at 95 °C in a water bath for 30 min, cooled immediately in ice to stop the reaction, and centrifuged at 4 °C and 4,000 × *g* for 10 min. Absorbance of supernatant was measured at 532 and 600 nm. TBARS were calculated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm and using a standard curve of MDA. Results were expressed as μmol MDA kg^{-1} of fresh weight.

2.6. Abscisic acid content

ABA content was measured according to Palma et al. (2015). Exocarp tissue was ground in liquid nitrogen and homogenized in proportion 1:8 (w/v) with cold 100% methanol and 2% butylated hidroxytoluene (w/v). The homogenate was ultrasonicated for 25 min at 4 °C, stirred for 2 h at 4 °C, and centrifuged at 3000 × *g* for 10 min at 4 °C. After centrifugation, the supernatant was collected and the pellet was re-extracted with 1 mL of extraction solvent, and the extraction was repeated again. Then, supernatants were combined and dried completely under nitrogen stream, and redissolved in 2 mL of water. The pH of the aqueous phase was adjusted to 1.5–2.5 with 1 N HCl. ABA was

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