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Involvement of lycopene in the induction of tolerance to chilling injury in grapefruit

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ABSTRACT

Grapefruit are among the more sensitive *Citrus* varieties likely to develop chilling injury (CI) symptoms during postharvest storage at low temperatures. Comparative observations of the incidence of CI in fruit of white Marsh (MSH) and red Star Ruby (SR) grapefruit during postharvest storage at 2 °C plus 7 days at 20 °C to simulate shelf-life revealed that (1) the former was consistently more sensitive to CI, developing cold damage uniformly throughout the whole rind surface, and (2) more strikingly, CI symptoms in fruit of SR grapefruit were restricted to the yellow areas of the rind and the red-colored zones were almost absent of cold damage. This tolerance to CI in red flavedo was associated with high carotenoid (×2) and lycopene (×14) contents, as compared with yellow-colored flavedo. Absence of chilling damage in red areas of SR grapefruit rind was confirmed by cellular ultrastructure observations, in which these epidermal cells were intact, with a well-defined structure and compact vacuoles filled with content. Cells of yellow-colored tissue developing CI, were collapsed, with a contracted vacuole and shrinking organelles. To explore whether the tolerance to CI in red areas of grapefruit rind was due to an elevated lycopene concentration, chemical and environmental stimulation of this carotenoid was performed in fruit of both grapefruit varieties. Application of the inhibitor of the lycopene cyclase activity, CPTA (2-(4-chlorophenylthio) triethylamine hydrochloride) induced red coloration, increased lycopene accumulation (×32) and significantly delayed development of CI symptoms in the rind the CI-sensitive MSH. Bagging of SR grapefruit enhanced a homogenous red coloration and substantially induced lycopene accumulation (×75). CI symptoms in bagged fruit were notably delayed and reduced, as compared with non-bagged yellow fruit, upon subsequent storage at 2 °C for up to 58 days and 7 days at 20 °C. Analysis of the expression of ethylene biosynthetic genes (*ACS1*, *ACS2* and *ACO*) revealed a significant induction in chilling-damaged tissue of both varieties that was almost absent in red chilling-tolerant tissue. Similarly, accumulation of transcripts of the ethylene receptors *ETR1* and *ETR3* were also associated with chilling damage, but a cold factor appears to also mediate the expression of these genes. Taken together, our results indicate that high lycopene concentration appears to be responsible for the induction of tolerance to chilling in the red-colored areas of the flavedo of grapefruit during postharvest storage at low temperatures.

1. Introduction

Grapefruit (*Citrus paradisi* Macf.) are among the most susceptible *Citrus* species to develop CI when stored at temperatures below 8–10 °C (Schirra, 1992; Schirra et al., 1998). CI symptoms in grapefruit are initially manifested as small brown pits on the fruit surface that progressively increase in size, forming shrunken brown spots and depressed areas of different shape, that may affect large surface areas of the rind after prolonged cold storage periods (Schirra, 1992; Schirra et al., 1998; Lafuente and Zacarías, 2006). Susceptibility to development of CI may depend on the grapefruit variety, growing conditions or the harvest season, as it has been demonstrated that grapefruit harvested early and later in the season are more susceptible to CI than mid-season fruit (Schirra et al., 1998; Dou, 2005).

In order to induce cold tolerance in CI-sensitive citrus fruit, various postharvest treatments have been investigated over the years. Temperature conditioning treatments are among the most

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effective systems to induce cold tolerance during low temperature exposure (Hatton and Cubbedge, 1981; Porat et al., 2000; Biolatto et al., 2005). Conditioning treatments in grapefruit are carried out at either 21 °C for 3 days of at 16 °C for 7 days, although the induced tolerance to CI appears to be better with the latter treatment (Hatton and Cubbedge, 1981). Hot water dip treatments (Rodov et al., 1995; Ghasemnezhad et al., 2008), intermitent warming (Davis and Hofmann, 1973), and rinsing and brushing at temperatures of 60 °C for a few seconds (Porat et al., 2000) have been also reported to be effective in inducing chilling tolerance. Moreover, other non-thermal treatments, such as waxing or modified atmosphere packaging (Porat et al., 2000), thiabendazole or imazalil dip application (Schirra et al., 2000) or various combined methods (Rodov et al., 2000) may induce cold stress tolerance, to different extents.

Biochemical and molecular mechanisms involved in chilling-tolerance in *Citrus* fruit have been extensively studied. Genes differentially expressed by temperature-conditioning potentially related to chilling tolerance have been identified in different *Citrus* fruit (Sanchez-Ballesta et al., 2003; Sapitnitskaya et al., 2006; Maul et al., 2008). Results indicated that a complex interplay of different metabolic pathways operate in the induction of cold tolerance (lipid metabolism, metallothioneins, oxidative stress, dehydrins, osmoprotectants, defense responses, among others). Each heat treatment appears to selectively induce the expression of a specific set of genes, and also to modify the expression of chilling-induced transcripts (Maul et al., 2011).

An increased ethylene production during exposure of citrus fruit to chilling temperatures has been observed in many varieties (McCollum and McDonald, 1991; Lafuente et al., 2001; Ghasemnezhad et al., 2008). The increment of ethylene is associated with an induction of *ACO* and *ACS* gene expression during cold storage (Zacarías et al., 2003; Maul et al., 2008) while temperature conditioning treatments down-regulated expression of *ACO* gene (Maul et al., 2011). These results suggest that ethylene is involved in the response of citrus fruit to postharvest cold stress, but whether ethylene is a protective defense response or a cause of CI-induced damage is not fully understood (Lafuente and Zacarías, 2006).

Fruit coloration is one of the most important attributes determining fruit quality. In *Citrus*, fruit color is determined by the specific and particular carotenoid content and composition in the rind and pulp of the different varieties (Kato, 2012; Rodrigo et al., 2013). Carotenoids are C_{40} isoprenoid molecules playing a wide range of functions in plants, transferring energy to chlorophyll, as photoprotectors, dissipating excess light energy, displaying an important role in membrane stabilization (especially in chloro- and chromoplasts), and as powerful scavengers of reactive oxygen species (Britton, 2008). Moreover, carotenoids are also relevant for human nutrition and health, as specific carotenoids are the precursors of vitamin A (α- and β-carotene, and β-cryptoxanthin) and display potent antioxidant properties (lycopene) (Rao and Rao, 2007).

Among carotenoids, lycopene is an acyclic red carotene with 13 double bounds, 11 of which are conjugated and responsible for the high antioxidant capacity of the molecule (Di Mascio et al., 1989; Krinsky, 1989; Aizawa et al., 2011; Rodrigues et al., 2012). Increments in carotenoid content in fruit and vegetables have been associated with a higher tolerance to different stresses. For example, transgenic cells of sweet potato accumulating higher amount of β-carotene were able to grow under stressful salt conditions (Kim et al., 2012, 2013). An increased accumulation of lycopene in tomato has also been associated with a lower incidence of chilling damage (Whitaker, 1994) and damaged fruit showed four times lower lycopene than healthy fruit (Rugkong et al., 2011). Similarly, accumulation of other potent antioxidants (anthocyanins) in transgenic tomato led to a reduction in postharvest deterioration and disease incidence during cold storage (Zhang et al., 2013).

Since the development of CI has been associated with oxidative stress in citrus fruit (Sala, 1998; Sala and Lafuente, 1999) and carotenoids display important antioxidant properties, it is reasonable to conceive that these pigments may have an active role in the protection of fruit from cold stress. Accumulation of lycopene in *Citrus* fruit is an unusual feature, restricted to few species such as grapefruit, pummelo and sweet orange mutants (Liu et al., 2007; Alquezar et al., 2008, 2013; Pan et al., 2009). Thus the objective of the present study was to test the hypothesis that accumulation of lycopene in the rind of grapefruit may protect this tissue from CI development during cold storage. To that end, the incidence of CI was recorded in fruit of two grapefruit varieties with contrasting fruit coloration and carotenoid complement: the white grapefruit Marsh (MSH), that has been demonstrated to be almost devoid of carotenoids in rind and pulp, and the red grapefruit Star Ruby (SR) that is widely recognized by its intense red coloration in the pulp and is also able to develop red color in the rind due to the accumulation of lycopene (Alquezar et al., 2013). Moreover, to corroborate the potential protection to CI induced by this carotene, total carotenoids and especially lycopene content were increased in the rind of grapefruit by chemical (CPTA application) and environmental (fruit shading) manipulation.

2. Materials and methods

2.1. Plant material, treatments and storage conditions

Fruit of the white Marsh and the red Star Ruby grapefruit (*Citrus paradisi* Macf.) were harvested from adult trees from an orchard located in Moncada, Valencia, Spain. Mature fruit were harvested at the end of January, delivered to the laboratory, inspected to be free of damage and defects, selected by uniformity of size and external color and then classified in three replicate lots of 20 fruit each. Fruit from the different experiments were stored at 2 °C and 80–85% RH for up to 58 days with subsequent 1 week storage at 20 °C to simulate shelf-life conditions. At harvest and after 28 and 58 days of storage, the flavedo (colored part of the rind) was excised and immediately frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. At each sampling date, rind color was measured using a Minolta CR-330 colorimeter on three locations around the equatorial plane of the fruit, using three replicates of 10 fruit each. Color was expressed as the *a*/*b* Hunter ratio. The *a*/*b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at color break and is positive for orange to red colored fruit. Comparative evaluation of CI incidence in MSH and SR grapefruit harvested from the same orchard was done in two successive seasons.

Two different treatments were performed to induce red coloration in the rind of grapefruit. The first experimental approach was carried out using the inhibitor of lycopene cyclase activity, 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA), that promotes the accumulation of the red lycopene in plant tissues, including citrus fruit (Coggins et al., 1970; Yokoyama et al., 1972). Fruit of the white MSH grapefruit were harvested in December, delivered to the laboratory and dipped in 5000 mg/L CPTA (Sagechem, China) +0.1 % Triton X-100 solution for 30 s and dried at room temperature. Control fruit were dipped in with the same conditions without CPTA. Control and CPTA-treated fruit were maintained 4 days at 20 °C to stimulate pigment biosynthesis and accumulation, and then stored at 2 °C for up to 58 days and 7 days of shelf-life at 20 °C. In the second approach, SR fruit were covered at the end of July (55 mm diameter) with black plastic bags, to avoid direct sunlight exposure. Uncovered control fruit were located outside of the tree