Catalase in the Heat-Induced Chilling Tolerance of Cold-Stored Hybrid Fortune Mandarin Fruits

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Hybrid Fortune mandarins developed chilling injury (CI) upon cold storage, unless the fruits were conditioned at 37 °C for 3 days before they were held at low temperature. This heat treatment induced 2.5-, 1.2-, and 1.4-fold increases in the activities of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD), respectively, and reduced the activity of glutathione reductase (GR). The differences in the activities afforded by the heat treatment were, in general, maintained during cold storage. However, SOD levels in nonconditioned Fortune fruits exhibiting CI were similar to those of conditioned fruits stored for 0 or 6 weeks at 2 °C. No difference between APX activity in the conditioned and nonconditioned fruits stored for 6 weeks at 2 °C was found. The data indicate that CAT may be a major antioxidant enzyme operating in the heat-induced chilling tolerance of cold-stored Fortune mandarin fruits.

Keywords: *Ascorbate peroxidase; catalase; citrus; chilling injury; Fortune mandarin; glutathione reductase; high-temperature conditioning; hydrogen peroxide; superoxide dismutase*

INTRODUCTION

Because hybrid Fortune mandarin is a chilling-sensitive cultivar, exposure to low temperatures during storage or on the tree can severely damage the fruit. Chilling injury (CI) is manifested as pitting, necrosis, and rind staining along the peel of the fruit. However, holding the fruit at very low temperatures may be the most desired practice to maintain fruit quality after storage and extend the market period, allowing long-distance transport and control of Mediterranean fruit fly by quarantine treatments. Thus, physiological and molecular characteristics that improve low-temperature stress tolerance are of great interest to the citrus industry. Despite the fact that the chilling sensitivity of this cultivar changes considerably during the season, its cold-induced damage may be overridden, at any stage of maturity, by conditioning the fruits for 3 days at 37 °C before they are stored at 2 °C (Lafuente et al., 1997).

There is increasing evidence that chilling elevates the level of active oxygen species (AOS) (Wise and Naylor, 1987), which damage cellular components (Elstner, 1991; McKersie, 1991) and that acclimation to chilling may be partly related to an enhanced antioxidant system that would prevent the accumulation of AOS (Prasad 1996). This system involves both lipid-soluble antioxidants (α-tocopherol and β-carotene) and water-soluble reductants (ascorbate and glutathione) and enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2) (Zhang et al., 1995). Athough most of the studies have been done in plant vegetative tissues, there is evidence that chilling may impose oxidative stress in fruits such as cucumbers (Hariyadi and Parkin, 1991) and pears (Ju et al., 1994). Also, it has been suggested that mitochondria may be a major source of superoxide in chilling-sensitive green bell pepper fruits exposed to low temperature (Purvis et al., 1995).

In previous papers we have suggested that the oxidative stress may be involved in cold-induced peel damage of harvested citrus fruit. We have found that fruits of Clementine and Clemenules mandarins, which are chilling-tolerant cultivars, have a more efficient antioxidant enzyme system than the chilling-sensitive Fortune cultivar (Sala, 1998) and that the peroxidase activity was lower in nonconditioned fruits than in high-temperature-conditioned Fortune mandarins (3 days at 37 °C), which did not show peel damage throughout the cold storage period (Martínez-Téllez and Lafuente, 1997). On the other hand, we have shown that conditioning fruits of this cultivar at high temperature may increase the polyamine content in the flavedo, although it is not clear that the heat-induced increase in these radical-scavenger antioxidant compounds is related to the acclimation at chilling (Gonzalez-Aguilar et al., 1998). Wang (1995a,b, 1996) has shown that the tolerance to chilling of zucchini squash may be increased by conditioning the fruits at 15 °C and that this pretreatment reduced the chilling-induced decline in CAT, SOD, or APX activities and increased the GR activity. Prasad (1997) also investigated the mechanism of chilling acclimation and the role of antioxidant enzymes in inducing chilling tolerance in pre-emergent maize seedlings. This author did not observe changes in SOD and APX, but the acclimated seedlings had higher CAT, GR, and guaiacol peroxidase activities compared with those of nonacclimated seedlings during low-temperature stress conditions. Furthermore, he showed that CAT plays a major role in inducing the chilling tolerance. This result differs from that of Dat et al. (1998) in mustard seedlings. Little is known about the role of the

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endogenous antioxidant systems on the acclimation of citrus fruits at chilling.

The aim of the present work is (a) to investigate the effect of the high-temperature conditioning on the activity of the enzymes of the antioxidant system of cold-stored Fortune mandarins and (b) to determine whether the high-temperature-induced changes in the activity of those enzymes are linked to the induced tolerance to CI.

MATERIALS AND METHODS

Plant Materials and Treatments. Fortune mandarin fruits (*Citrus clementina* Hort ex Tanaka × *Citrus reticulata* Blanco) were harvested at random from 20-year-old trees. The trees were grafted onto Satsuma mandarin (*Citrus unshiu* Marc) and sour orange (*Citrus aurantium* L.) rootstock and grown at Sagunto, Valencia, Spain.

Fortune mandarin fruits were selected and randomly divided into two lots. The first lot was immediately stored at 80−85% relative humidity (RH) and 2 °C for up to 8 weeks, whereas the second lot was conditioned at 37 °C and 90−95% RH for 3 days before they were transferred to a cold storage room at 2 °C and 80−85% RH for up to 8 weeks. In each lot, fruits were randomly divided in three replicates of 27 fruits. CI symptoms were evaluated weekly, and three replicate samples of four fruits of each group were sampled after 0, 14, 28, 42, and 56 days at 2 °C to determine changes in enzyme activities. Flavedo (the colored outer layer of skin) tissue was separated from the whole fruit and cut into small pieces, and representative 1 g samples were frozen in liquid nitrogen and stored at −70 °C for enzyme assays.

CI Index. The degree of CI was evaluated according to the method described previously by Lafuente et al. (1997). A rating scale based on surface necrosis and intensity of browning was used: 0 = no pitting; 1 = slight; 2 = medium; 3 = severe pitting. The CI index, which expresses the severity of injury, was determined by summing the products of the amount of fruits showing pitting in each category by the value assigned to this category in the rating scale and dividing this sum by the total number of fruits examined.

Enzyme Assays. One gram fresh weight of frozen flavedo tissue was pulverized in a mortar and pestle with 10 mL of 100 mM potassium phosphate buffer, pH 6.8 at 4 °C. The homogenate was centrifuged at 27000*g* for 15 min at 4 °C twice and the supernatant used for the CAT assay. Catalase activity was determined at 25 °C according to the method of Kar and Mishra (1976). One unit of CAT was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2/min at 25 °C.

APX was extracted from 1 g of the flavedo tissue with 10 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 1 mM ascorbic acid, and 1% polyvinylpolypyrrolidone (PVPP) at 4 °C. The homogenate was centrifuged at 27000*g* for 15 min at 4 °C twice, and the supernatant was used for the APX assay. The activity was determined according to the method of Asada (1984). One unit of APX was defined as the amount of enzyme that oxidized 1 μmol of ascorbate/min at room temperature.

GR was extracted from 1 g of the flavedo tissue with 10 mL of 100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C. The homogenate was centrifuged at 27000*g* for 15 min at 4 °C twice and the supernatant used to assay GR spectrophotemetrically according to the method of Smith et al. (1988). The activity of the GR solution used for the standard curve was determined according to the procedure of Carlberg and Mannervik (1985). One unit of GR was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH/min.

SOD was extracted from 1 g of flavedo tissue with 10 mL of 50 mM potassium phosphate buffer, pH 7.8, containing 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC) at 4 °C, and the homogenate was centrifuged at 27000*g* for 15 min at 4 °C twice. According to Droillard et al. (1989), the supernatant crude plant extracts were used to assay SOD spectrophotometrically by using the method of Oberley and Spitz (1986). The superoxide radicals were generated by xanthine−xanthine oxidase, and nitro blue tetrazolium (NBT) was used as an indicator of superoxide radical production. One unit of SOD was defined as the amount of enzyme that gave half-maximal inhibition.

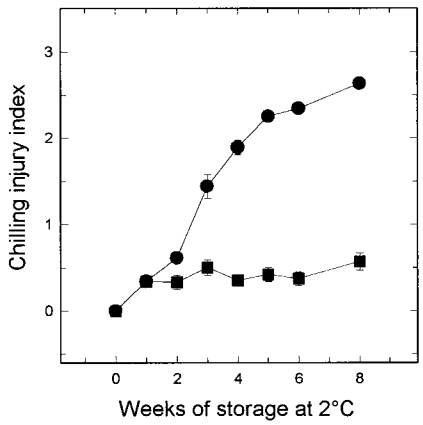

Figure 1. CI index of Fortune mandarins fruits conditioned for 0 (●) and 3 days (■) at 37 °C and 90−95% RH and stored for up to 8 weeks at 2 °C. Each value is the mean of three replicate samples ± SE.

Specific activities of all enzymes were expressed as units per milligram of protein. Protein was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Statistical Design. Experimental data are the mean ± standard error (SE) of three replicates of the determinations for each sample. ANOVA analysis were performed by means of the BMDP 7D program (Dixon, 1988), using the Duncan multiple-range test at 5% level for means comparison.

RESULTS

Low-temperature storage of Fortune mandarin fruit induced CI, peel pitting being the primary symptom of this physiological disorder. Slight pitting appeared in nonconditioned fruit for up to 2 weeks at 2 °C (CI = 0.6), whereas in the heat-conditioned fruit insignificant pitting was observed (CI = 0.3). The CI index increased quickly thereafter in nonconditioned fruits but remained nearly constant in the conditioned fruits for up to 8 weeks of storage at 2 °C (Figure 1).

Heating Fortune mandarin fruits for 3 days at 37 °C before they were transferred to 2 °C induced a 2.5-fold increase in CAT activity (Figure 2). The CAT activity, in the conditioned and in the nonconditioned samples, declined during storage at 2 °C, but in the conditioned fruits was about twice that of nonconditioned fruits throughout the 8 weeks of exposure to low temperature. A slight increase in APX (1.2-fold increase) was also induced by heating the fruits at 37 °C (Figure 3). The activity afforded by the heat-conditioning treatment was maintained for up to 4 weeks at 2 °C. Thereafter, it decreased and no difference in APX activity between conditioned and nonconditioned fruits was found after 6 weeks. At the end of the storage period, however, APX activity still was about twice that of the nonconditioned mandarins (Figure 3). GR activity was reduced by heating the fruits for 3 days at 37 °C and, with one exception, was higher in the nonheated fruits along the storage period (Figure 4). This heat treatment induced a 1.4-fold increase in the activity of SOD (Figure 5). SOD activity in the flavedo of conditioned fruits was higher than in the nonconditioned fruits during the 8 weeks of

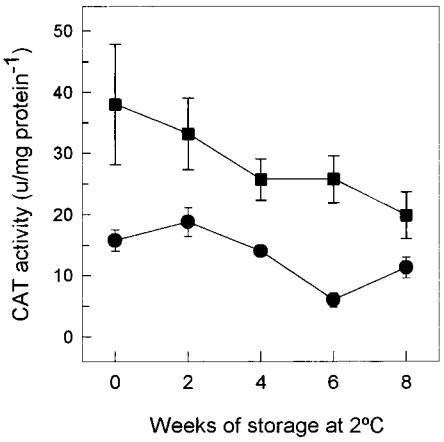

Figure 2. Changes in the activity of CAT of Fortune mandarin fruits conditioned for 0 (●) and 3 days (■) at 37 °C and 90−95% RH and stored for up to 8 weeks at 2 °C. Each value is the mean of three replicate samples ± SE.

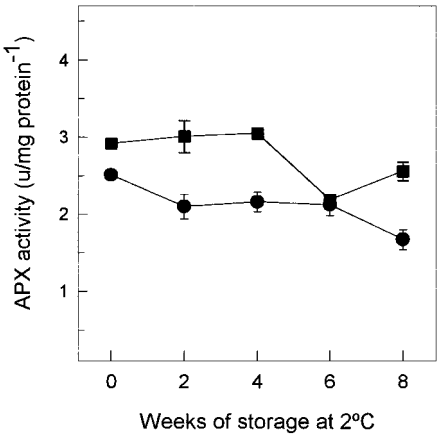

Figure 3. Changes in the activity of APX of Fortune mandarin fruits conditioned for 0 (●) and 3 days (■) at 37 °C and 90−95% RH and stored for up to 8 weeks at 2 °C. Each value is the mean of three replicate samples ± SE.

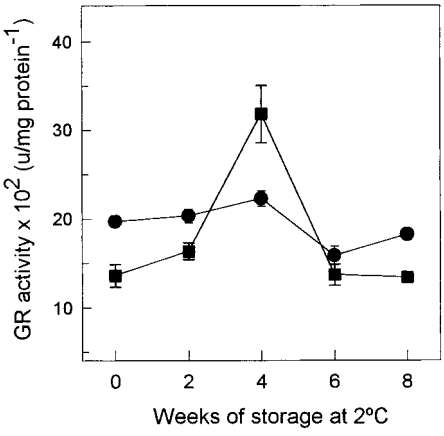

Figure 4. Changes in the activity of GR of Fortune mandarin fruits conditioned for 0 (●) and 3 days (■) at 37 °C and 90−95% RH and stored for up to 8 weeks at 2 °C. Each value is the mean of three replicate samples ± SE.

storage at 2 °C. However, the activity of this enzyme in nonconditioned Fortune fruits stored for 2 or 8 weeks at 2 °C was similar to that of conditioned fruits stored for 0 or 6 weeks (Figure 5).

DISCUSSION

As previously reported, Fortune mandarins developed chilling damage upon cold storage, unless the fruits

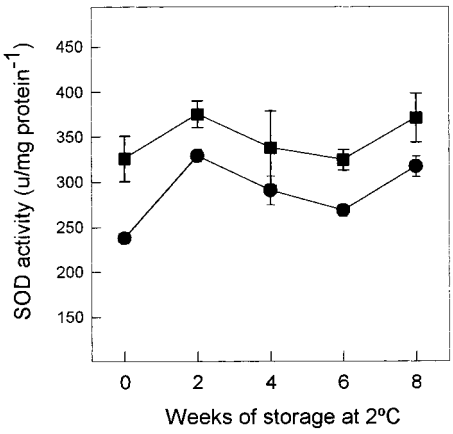

Figure 5. Changes in the activity of SOD of Fortune mandarin fruits conditioned for 0 (●) and 3 days (■) at 37 °C and 90−95% RH and stored for up to 8 weeks at 2 °C. Each value is the mean of three replicate samples ± SE.

were pre-exposed to 37 °C for 3 days (Lafuente et al., 1997). This result is in accordance with observations that temperature conditioning treatments conferred cold hardiness on a variety of fruits such as grapefruit (Chalutz et al., 1985), papaya (Chen and Paull, 1986), lemons (Houck et al., 1990), tomato (Lurie and Klein, 1991), and oranges (Schirra et al., 1997). Different biochemical and physiological mechanisms have been associated with chilling acclimation, although the basis of cold-induced tolerance remains unclear. Temperature acclimation at low temperature has been found to correlate with the accumulation of a number of heat shock proteins (HSPs) (Lafuente et al., 1991; Lurie and Klein, 1991). It may also involve increases in sugars (Purvis et al., 1979) and polyamine content (McCollum et al., 1991; González-Aguilar et al., 1998) and in the insaturation of fatty acids (Clarkson et al., 1980; Wang et al., 1992).

Oxidative stress has been considered to be a response of different plant tissues to chilling (Hariyadi and Parkin, 1991; Ju et al., 1994; Wang, 1995a), including citrus fruit peel (Sala, 1998). In the present work, we have focused our attention on the involvement of the activated oxygen-scavenging enzymes on the adaptive process induced by a high-temperature conditioning treatment at 37 °C against chilling on mandarin fruits. Heating Fortune mandarins for 3 days increased the activity of CAT, SOD, and APX, but GR activity was reduced. It is interesting to note that conditioning resulted in a greater induction in CAT activity (2.5-fold increase) than in the activity of APX (1.2-fold) and SOD (1.4-fold). Furthermore, the differences in the activities afforded by the heat treatment were, in general, maintained during cold storage. These results may indicate that the linked action of CAT, APX, and SOD may contribute, at least to some extent, to the avoidance of CI in conditioned Fortune mandarin fruits. The increase in SOD activity could enhance the ability of the flavedo to dismutate superoxide radicals, whereas the increase in CAT and APX activities would contribute to the elimination of hydrogen peroxide. It should be pointed out, however, that SOD levels in nonconditioned Fortune fruits increased during storage at 2 °C and fruits exhibited CI despite the activity of SOD, similar to the responses of conditioned fruits stored for 0 or 6 weeks at 2 °C. Therefore, the role of SOD in the defense mechanism induced by heating the fruits at 37 °C appears to be less important than that of APX and CAT.

In zucchini squash it has been also suggested that the acclimation to chilling, induced by conditioning the fruits at 15 °C, may involve modifications in the antioxidant system. This pretreatment did not induce an increase in SOD, CAT, or APX but reduced the decline of their activities after the squash had been held at low temperature (Wang, 1995a,b). The GR activity did not decrease in the untreated squash held at low temperature and was higher when it was temperature-conditioned (Wang, 1996).

At physiological pH, the dismutation of superoxide by SOD occurs at a rate constant of 2×10^9 M^{-1} s^{-1} (Elstner, 1982). Hydrogen peroxide can cross biological membranes because it has no unpaired electron, whereas the charged superoxide species can do so only very slowly. If hydrogen peroxide, which enters the cell cytoplasm, survives in sufficient concentrations to reach the plant nucleus, it could react with intracellular metal ions to originate hydroxyl free radical and result in considerable damage to plant (Halliwell and Gutteridge, 1990).

CAT is one of the enzymes that protect cells against AOS because it catalyzes the decomposition of hydrogen peroxide to form oxygen and water. Prasad (1997) found that CAT seems to play a major role in inducing acclimation to chilling stress in dark-grown maize seedlings. In mustard seedlings, however, it has been shown that a heat acclimation treatment resulted in decreased CAT activity during the period of induced thermoprotection (Dat et al., 1998). Our results agree with those of Prasad (1997) because the increase in CAT was the most marked change induced by high-temperature conditioning in the flavedo of Fortune mandarins.

In conclusion, our data collectively indicate that hydrogen peroxide appears to be involved in peel damage induced by cold stress in citrus fruits and that CAT may be a major antioxidant enzyme operating in the heat-induced chilling tolerance of cold-stored Fortune mandarin fruits.

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