Evaluation of Antioxidant Metabolism in Commercially Grown Citrus Genotypes in Florida

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Generation and excess accumulation of hydrogen peroxide (H$_2$O$_2$) is toxic to plants. To combat the ill effect of H$_2$O$_2$, plants have developed an array of enzymatic and non-enzymatic antioxidants. In the present investigation, H$_2$O$_2$ metabolism was studied in young and developing leaves of ‘Nagami’ kumquat [Fortunella margarita (Lour.) Swing], grapefruit (Citrus paradisi Macfad.), and sweet orange (Citrus sinensis Pers.). Lower H$_2$O$_2$ levels were detected in kumquat and appeared to be associated with higher catalase (CAT) activity than sweet orange and grapefruit. However, higher guaiacol peroxidase (POD) and ascorbate peroxidase (APOD) activity was observed in grapefruit and sweet orange than kumquat. Besides having higher POD and APOD activity, H$_2$O$_2$ levels were higher in grapefruit and sweet orange, which indicates inefficient removal of H$_2$O$_2$ in these genotypes. This might be the cause of poor availability of co-substrates (phenolics and ascorbate) required for POD and APOD activity. On the other hand, kumquat leaves with higher redox insensitive CAT activity efficiently removed H$_2$O$_2$. Thus, developing kumquat leaves were better protected from the ill effects of oxidative stress than grapefruit and sweet orange.

Reactive oxygen species [ROS: superoxide radical (O$_2^−$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), perhydroxyl radical (HO$_2$), and peroxynitrite (ONOO−)] are the unavoidable by-product of oxygen metabolism in aerobes (Imlay, 2008). In plants, photosynthetic and mitochondrial electron transport, lipid metabolism, and photosynthesis in glyoxysomes and peroxisomes chiefly contribute to the generation of these ROS (Mittler, 2002). Under abiotic and biotic stresses the rate of ROS generation is accelerated beyond the capacity of effective removal and cause substantial damage to nucleic acids, proteins, and lipid membranes (Mittler, 2002; Wojtaszek, 1997).

In contrast to superoxide radical and hydroxyl radical, hydrogen peroxide is less toxic, which is partly due to its longer half-life (10$^{−3}$ s) compared to O$_2^−$ (10$^{−6}$ s) and OH$^−$ (10$^{−9}$ s) (Bhattacharjee, 2005; Wojtaszek, 1997). The physiological function of H$_2$O$_2$ is concentration dependent: at low concentration it acts as a diffusible signaling molecule and enhanced stress tolerance against biotic and abiotic stresses and at higher concentration can cause plant cell death (Dut et al., 2000; Mittler, 2002).

Plants have evolved an extensive network of antioxidant enzymes such as catalases, peroxidases, and ascorbate peroxidases to control the concentration of ROS (Hossain et al., 2009). The combined action of these enzymes minimizes the adverse effect of H$_2$O$_2$ and, thus, prevent cellular injury (Pukacka and Ratajczak, 2005). Catalases are the major H$_2$O$_2$ degrading enzymes and dismutate H$_2$O$_2$ in water and oxygen. The low affinity of CAT (mM) and higher affinity of APOD (µM) for H$_2$O$_2$ suggests different roles of these two enzymes. CAT might be responsible for the removal of excess H$_2$O$_2$ from the system while APOD regulates the fine modulation of H$_2$O$_2$ for signaling during stress (Mittler, 2002). H$_2$O$_2$ can also be scavenged by class III plant peroxidases that belong to a large gene family and are coded by a network of at least 73 genes in Arabidopsis (Hiraga et al., 2001; Tognolli et al., 2002). It seems a critical balance is required between ROS production and subsequent metabolism for survival of plants under adverse conditions.

In the present investigation, we evaluated the antioxidant capacities of kumquat [Fortunella margarita (Lour.) Swing], grapefruit (Citrus paradisi Macfad.), and sweet orange (Citrus sinensis Pers.). The latter two are important commercial species of citrus grown in Florida, whereas kumquat is a minor crop with disease resistance characteristics (Khalaf et al., 2007)). Oxidative stress was assessed in terms of H$_2$O$_2$ generation and activities of CAT, POD, and APOD were measured.

Materials and Methods

PLANT MATERIAL AND CULTURE. ‘Nagami’ kumquat, grapefruit, and ‘Hamlin’ sweet orange plants were purchased from a commercial nursery (Harris Citrus Nursery, Lithia, FL) and maintained in 15 × 8 cm plastic pots containing Fafard Mix 4P (Southern Agricultural Insecticides Inc., Palmetto, FL). Mineral nutrition was provided periodically using Peters’ professional fertilizer (The Scotts Co., Marysville, OH) containing ammonium nitrate (30% to 60%), potassium nitrate (15% to 40%), potassium phosphate (10% to 30%), magnesium sulphate (0.5% to 1.5%), iron–EDTA (0.1% to 1%), manganese EDTA (0.1% to 1%), zinc EDTA (0.1% to 1.0%), copper EDTA (<0.1%), ammonium hexamethylenetetraamine tetrahydrate (<0.1%), and boric acid (<0.1% by weight). Plants were pruned 60 d before sampling and grown in environmental growth chambers (temperatures ranging from 23 to 32 °C and a relative humidity of 65% to 95%) to promote new growth and young leaves only 40% to 50% fully expanded.

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**H₂O₂ CONCENTRATION.** Leaf samples [0.5 g fresh weight (FW)] were homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 x g for 15 min at 4 °C. Supernatant (0.3 mL) was mixed with 1.3 mL potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) solution and incubated for 5 min before measuring oxidation product at A₅₀₀. The amount of H₂O₂ was calculated from a standard curve prepared from known concentrations of H₂O₂ and expressed as μmol·g⁻¹ FW (Velikova et al., 2000).

**LEAF PREPARATION FOR ENZYME ASSAYS AND ISOFORM SEPARATION.** Leaf samples (0.5 g) were homogenized in 100 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA and 2% PVP in a pre-chilled pestle and mortar. The extraction buffer also contained 5 mM ascorbate and homogenate was centrifuged at 4 °C for 30 min at 15,000 g. An aliquot of each sample was made to 10 mM DTT to be used for catalase spectrophotometric assays. The supernatant was used for enzyme assays. All enzymes activities were measured in a final volume of 3 mL using various aliquots of the supernatants.

**CATALASE (CAT).** CAT activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H₂O₂, and enzyme. The decomposition of H₂O₂ was followed at 240 nm (E = 39.4 mM·cm⁻¹) (Cakmak and Marschner, 1992).

**PEROXIDASE (POD).** POD activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1.0 mM H₂O₂, 0.25 mM ascorbic acid and enzyme. The POD activity was determined by the increase in absorption at 470 nm due to guaiacol oxidation (E = 26.6 mM·cm⁻¹; Cakmak and Marschner, 1992).

**ASCORBATE PEROXIDASE (APOD).** APOD activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.05% guaiacol and enzyme. The activity of APOD was determined by the increase in absorption at 290 nm (E = 2.8 mM·cm⁻¹; Nakano and Asada, 1981).

**STATISTICAL ANALYSIS.** All experiments were repeated three times and 15 total replicates were used for all biochemical estimations. The standard error (SE) of each mean (n=15) was calculated and means were consider different when 2 × SE of the mean did not overlap.

**Results and Discussion**

Critical balance is required between H₂O₂ production and its subsequent removal for survival and fitness of plant cells (Mittler, 2002). Relatively lower levels of H₂O₂ were detected in developing kumquat leaves, which indicates the existence of a better H₂O₂ detoxification system than grapefruit and sweet orange (Table 1).

Higher H₂O₂ concentration may be toxic for both grapefruit and sweet orange and could react with O₂⁻ and transition metal ions (Fe²⁺ and Cu²⁺) to form highly toxic hydroxyl radicals via Haber–Weiss and Fenton reactions and therefore, increase oxidative load and cellular injury (Wojtaszek, 1997). We have dissected the role of individual components of H₂O₂ degrading machinery by analyzing the activities of CAT, POD, and APOD.

Catalase is an indispensable enzyme for stress tolerance in C₃ plants (Willekens et al., 1997). The highest CAT activity was detected in kumquat and was (44% to 61%) higher than sweet orange and grapefruit (Table 1). CAT exhibits poor affinity for H₂O₂ but has extremely high reaction rate, which removes bulk H₂O₂ from the kumquat leaves in an energy-efficient mode (Scandalios et al., 1997). In addition, there are reports that showed CAT-deficient plants were more sensitive to various environmental stresses (Willekens et al., 1997). In CAT-deficient tobacco and barley, elevated levels of H₂O₂ not only perturbed antioxidant redox state but also induced a cell death program (Dat et al., 2003; Desiken et al., 2003). These observations support the importance of CAT in amelioration of oxidative stress in kumquat leaves.

Plant peroxidases are versatile enzymes and use H₂O₂ as an electron acceptor during the oxidation of a vast variety of compounds (Yoshida et al., 2003). Various physiological functions are governed by peroxidases, such as removal of H₂O₂, auxin metabolism, lignin biosynthesis, and defense against pathogens and insects (Yoshida et al., 2003 and within). Guaiacol peroxidases (POD) are secretory peroxidases that belong to class III of the peroxidases family (De Gara, 2004). The class III PODs are glycoproteins and localized in cell walls and vacuoles (Almagro et al., 2009). Guaiacol peroxidase activity was highest (2.3-fold) in grapefruit and sweet orange and lowest in kumquat (Table 1). A very similar trend of APOD activity was observed in grapefruit and sweet orange (Table 1). APOD was the dominating H₂O₂ scavenging enzyme in this study, and exhibited higher activity than CAT and POD in all the genotypes. During the development of di-oxygenic photosynthesis, peroxidases were prefered over catalase due to their greater affinity (μM range) for H₂O₂ (De Gara, 2004) and only a few μM of H₂O₂ are required to inhibit photosynthetic carbon fixation by 50% (Kaiser, 1976). Thus, the activity of peroxidases maintains a low oxidative environment for photosynthesis. Surprisingly, besides having higher activity of both POD and APOD, the rate of H₂O₂ generation was 20-fold higher in grapefruit and sweet orange than kumquat (Table 1). This observation clearly indicated the functional dependence of these two enzymes (POD and APOD) on co-substrates (ascorbate and phenolics) and expected to be limiting in these citrus genotypes. PODs showed a broad range in their substrate requirements but are highly specific for phenols, especially for coniferyl alcohols (Morales and Ros Barcelo, 1997). APODs are part of the ascorbate dependent multi-enzyme operated ascorbate–glutathione cycle and water–water cycle, which are redox regulated and require a continuous supply of reducing power (NADH/NADPH) to detoxify ROS (Mittler, 2002). There is evidence that showed a severe decline in the ratios of ascorbic acid to dehydroascorbic acid and reduced glutathione to oxidized glutathione under oxidati-
tive stress that impaired the functioning of the APOD mediated 
\( \text{H}_2\text{O}_2 \) detoxification system (Gossett et al., 1994).

In summary, developing kumquat leaves were better protected 
from the ill effects of oxidative stress than grapefruit and sweet 
orange. This protection is mainly governed by the elevated levels 
of redox independent CAT.

**Literature Cited**


