Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection

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Abstract

When plants are exposed to stressful environmental conditions, the production of Reactive Oxygen Species (ROS) increases and can cause significant damage to the cells. Antioxidant defenses, which can detoxify ROS, are present in plants. A major hydrogen peroxide detoxifying system in plant cells is the ascorbate-glutathione cycle, in which, ascorbate peroxidase (APX) enzymes play a key role catalyzing the conversion of H2O2 into H2O, using ascorbate as a specific electron donor. Different APX isoforms are present in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisome, and cytosol. The expression of APX genes is regulated in response to biotic and abiotic stresses as well as during plant development. The APX responses are directly involved in the protection of plant cells against adverse environmental conditions. Furthermore, mutant plants APX genes showed alterations in growth, physiology and antioxidant metabolism revealing those enzymes involvement in the normal plant development.

Keywords: ascorbate peroxidase, antioxidant system, reactive oxygen species, abiotic stress, mutant plants.
Ascorbate Peroxidase in Plants

Ascorbate peroxidase (APX) (EC 1.11.1.11) belongs to the class I heme-peroxidases that is found in higher plants, chlorophytes (Takeda et al., 1998, 2000), red algae (Sano et al., 2001), and members of the protist kingdom (Shigeoka et al., 1980; Wilkinson et al., 2002). APX and other peroxidase sequences from all kingdoms of life are stored in the database Peroxibase (Oliva et al., 2009), which also provides a series of bioinformatics tools useful for analyzing the peroxidases stored sequences.

Genomic and cDNA APX sequences were obtained from a great variety of plant species, showing that APX are widely distributed in the vegetal kingdom. These enzymes are encoded by small gene families in these organisms (Passardi et al., 2007). The different isoforms are classified according to their subcellular localization. Soluble isoforms are found in cytosol (cAPX), mitochondria (mitAPX) and chloroplast stroma (sAPX), while membrane-bound isoforms are found in microbody (including peroxisome and glyoxisome) (mAPX) and chloroplast thylakoids (tAPX). The presence of organelle-specific targeting peptides and transmembrane domains found in the N- and C-terminal protein regions determine the final subcellular localization of the isoenzyme (Shigeoka et al., 2002; Teixeira et al., 2004, 2006).

Plant chloroplastic APX (chlAPX) isoenzymes encoding genes are divided into two groups. The first group comprises single genes encoding two isoenzymes through a post-transcriptional alternative splicing regulation. This group includes genes from spinach (S. oleracea), tobacco (N. tabacum), pumpkin (Cucurbita sp) and ice plant (M. crystallium). In the second group, individual genes codify different isoenzymes which are individually regulated. This group includes genes from Arabidopsis, rice, and tomato. The mechanism of alternative splicing in chlAPX has been studied in spinach (Ishikawa and Shigeoka, 2008) and the results showed that alternative splicing is fundamental for controlling the expression of stromal (sAPX) and thylakoid (tAPX) isoenzymes. This regulation occurs in a tissue-dependent manner.

Ascorbate peroxidases have been partially characterized in some plant species. In spinach, the APX family is formed by genes encoding one cytosolic and two chloroplastic (sAPX and tAPX membrane) isoenzymes, one targeted to microbody membrane and an unknown putative cytosol-soluble isoenzyme (Ishikawa et al., 1995, 1996, 1998). In cowpea, four cDNAs were isolated and characterized, corresponding to putative cytosolic, peroxisomal and chloroplastic (thylakoid and stromal) APX isoforms (D’Arcy-Lameta et al., 2006). Six loci encoding APX were identified in Eucalyptus grandis and their subcellular localizations were indicated by prediction programs. Among the six isoforms, three were putatively identified as cytosolic, one as a putative peroxisomal protein and two predicted to be associated with chloroplasts (Teixeira et al., 2005). In tomato, seven members were identified, three cytosolic, two peroxisomal, and two chloroplastic (Najami et al., 2008). In the model plant Arabidopsis thaliana, the presence of nine APX genes was described, two chloroplastic, one thylakoid-bound and one member whose product is targeted to both chloroplast stroma and mitochondria (Chew et al., 2003); the intracellular localization of an additional member is yet unknown. In addition, three cytosolic and three microsomal proteins were also described (Mittler et al., 2004; Narendra et al., 2006; Panchuk et al., 2002). In another important model plant, rice, the APX gene family comprises eight members, viz. two cytosolic, two peroxisomal, two chloroplastic (stromal and thylakoid-bound) and two mitochondrial ones (Teixeira et al., 2004, 2006). Recently, a new protein has also been identified as functionally associated with APX in rice, the APX-R (Ascorbate peroxidase-related) (Lazzarotto et al., 2011). Detailed analyses of evolution and structure of APX-R genes indi-
cate that these genes correspond to a new class of heme-peroxidases (Lazzarotto et al., 2011).

APX isoenzymes are labile in the absence of AsA. Thus, high level of endogenous AsA is essential to effectively maintain the antioxidant system that protects plants from oxidative damage (Asada, 1992; Shigeoka et al., 2002). Under special conditions in which the concentration of AsA is lower than 20 μM, the APX activity is quickly lost, this making the chlAPX the least stable isoform. Both cAPX and mAPX have half-inactivation times of around one hour or more, while that for mitAPX and chlAPX is less than 30 seconds (Chen and Asada, 1989; Miyake et al., 1998; Ishikawa et al., 1998; Leonardis et al., 2000).

APX enzyme Responses Under Abiotic Stress

The expression of APX encoding genes is modulated by various environmental stimuli, such as drought and salt stress, high light, high and low temperatures, pathogen attacks, H2O2 and abscisic acid (Zhang et al., 1997; Yoshimura et al., 2000; Agrawal et al., 2003; Fryer et al., 2003; Menezes-Benavente et al., 2004; Teixeira et al., 2006; Rosa et al., 2010; Bonfaci et al., 2011). Furthermore, the transcriptional expression of APX genes is tissue and developmental stage dependent (Agrawal et al., 2003; Teixeira et al., 2006).

Salt stress

Plants are greatly affected by salinity, which causes alteration in nutrient uptake, accumulation of toxic ions, osmotic stress, and oxidative stress (Verslues et al., 2006). Consequently, salinity results in molecular damage, growth arrest, and even cell death (Wang et al., 2008). Salt stress induces the production of ROS, and the response of APX genes to this condition is tissue and developmental stage regulated. When the response of major antioxidant enzymes transcripts was analyzed for different developmental stages in salt stressed rice, cAPX was up-regulated in 11-day-old seedlings, while in 6-week-old plants salt had no significant effect on this gene (Menezes-Benavente et al., 2004). In addition to APX expression alteration, discrimination on CAT transcript accumulation was also noticed in the basal region of rice leaves under salinity (Yamane et al., 2010). Concerning the APX rice isoforms, induction was observed for the OsAPX1, OsAPX4, OsAPX6 and OsAPX7 genes, whereas cytosolic OsAPX2 gene expression was not altered by salinity (Yamane et al., 2010).

Teixeira et al. (2006) reported that three rice APX genes, OsAPX2, OsAPX7, and OsAPX8, showed altered transcript levels in response to NaCl treatment. The expression of OsAPX2 and OsAPX7 was increased, whereas OsAPX8 transcript accumulation was strongly suppressed in plants undergoing salt stress (Teixeira et al., 2006). The transcript level of OsAPX8 was slightly decreased by salinity in the basal region of rice leaves (Yamane et al., 2010). On the other hand, OsAPX8 expression in rice roots was enhanced by all NaCl concentrations tested (150, 200 and 300 mM), and OsAPX7 expression was down-regulated by 300 mM NaCl. This discrepancy in regulation for the OsAPX genes might be due to differences in cultivars, organs, plant age and growth conditions (Hong et al., 2007). An increase in rice cytosolic APX2 gene transcript levels after treatment with salt has previously been shown by our group (Menezes-Benavente et al., 2004; Teixeira et al., 2006). In accordance, transgenic Arabidopsis plants over-expressing cytosolic OsAPXb (OsAPX2) showed higher tolerance to NaCl than those over-expressing cytosolic OsAPXa (OsAPX1) (Lu et al., 2007). A similar increment in salt stress tolerance was also observed in transgenic tobacco over-expressing the Arabidopsis cAPX gene (Badawi et al., 2004) and also in tobacco plants over-expressing a Solanum lycopersicum thylakoid-bound ascorbate peroxidase gene (StAPX) (Sun et al., 2010b). Transgenic tobacco plants that simultaneously expressed CuZnSOD, APX, and DHAR in chloroplasts presented increased protection against salt induced injury (Lee et al., 2007b). Transgenic tobacco BY-2 cells with 50 and 75% lower cAPX activity showed higher intracellular content of ROS. On the other hand, the tobacco cells showed a potential enhancement in tolerance to heat and salt stress, perhaps by induction of stress-related gene expression. However, no substantial differences were observed in the activity levels of the other antioxidant enzymes (Ishikawa et al., 2005).

In barley, the transcript level of peroxisomal APX gene (HvAPX1) increased significantly under salt stress (Shi et al., 2001). However, Arabidopsis apx3 knockout mutants exposed to normal or stressful conditions did not present disturbed growth or development. In these plants, other antioxidant enzymes possibly compensate the lack of the peroxisomal isoform (Narendra et al., 2006). In contrast, the overexpression of a Populus peroxisomal APX (PpAPX) gene in transgenic tobacco improved salt tolerance at the vegetative stage and plants were more resistant to oxidative damage induced by methyl viologen (MV) and, in addition, the plants had longer roots (Li et al., 2009). Lin and Pu (2010) studied changes in enzymes involved in ROS scavenging in sweet potato plants tolerant and sensitive to salinity. After exposure to salinity (450 mM NaCl), APX activity increased in plants at 24 and 48 h, and this response was higher in a salt-stress tolerant genotype than in the salt sensitive ones. The expression of cAPX, mAPX and chlAPX in response to salinity was tissue specific and dependent on stress duration (Lin and Pu, 2010). Taken together, these studies put in evidence that salt stress causes disturbances in antioxidant gene expression by producing alterations in the transcriptional pattern in several plant species, and that the expression of distinct APX isoform may result in redox homeostasis regulation in each cellular compartment.
Temperature stress

Extreme temperatures affect the growth, yield and quality of plant production. ROS levels tend to increase if plants are exposed to stressful conditions such as low or high temperatures (Mittler et al., 2004; Scandalios, 2005). In potato tubers, the transient accumulation of cAPX mRNA after storage at low-temperature was greater than after high-temperature storage, showing that APX expression was induced in response to low temperature (Kawakami et al., 2002). Likewise, the two rice cAPX (OsAPX1 and OsAPX2) genes were induced after rice plants were exposed to low temperatures. Furthermore, OsAPX3, OsAPX4, OsAPX6 and OsAPX7 were also significantly induced, while OsAPX8 were repressed after 24 h under low temperature (unpublished data). The sweet potato cAPX gene was highly induced in leaves after exposure to high temperature (Park et al., 2004). In cucumber plants submitted to heat treatment, the activities of cAPX, sAPX and mAPX increased after an initial slight decline during the course of the experiment. The expression of sAPX followed a similar pattern (Song et al., 2005). In response to cold, the expression of a peroxisomal APX gene increased slightly in Arabidopsis (Zhang et al., 1997).

These results were corroborated in Arabidopsis by overexpressing a putative peroxisomal membrane-bound APX from barley, resulting in an increased tolerance to higher temperature treatment (Shi et al., 2001). Furthermore, the overexpression of chloroplastic tAPX in tobacco plants improved the tolerance to chilling stress combined with high light intensity (Yabuta et al., 2002). On the other hand, Arabidopsis plants lacking tAPX had enhanced tolerance to heat stress (Miller et al., 2007). Recently, Sato et al. (2011) showed that transgenic rice plants overexpressing a cytosolic APX1 gene (OsAPXa) which exhibited higher APX activity in spikelets than in wild type (WT) plants, sustained higher levels of APX activity under cold stress, resulting in enhanced cold tolerance at the booting stage.

Plants with enhanced tolerance to multiple environmental stresses were obtained through induced expression of CuZnSod and APX genes. Sod and APX genes were expressed in chloroplasts of potato plants under the control of an oxidative stress inducible promoter - SWPA2. These plants showed enhanced tolerance to MV and when exposed to 42 °C for 20 h, the photosynthetic activity of these transgenic plants decreased by only 6%, whereas in non-transformed (NT) plants it decreased by 29% (Tang et al., 2006). Sweet potato plants expressing both CuZnSod and APX in chloroplasts through the inducible promoter also showed higher tolerance to MV-mediated oxidative stress and chilling stress (Lim et al., 2007). The tolerance to high and low temperature stresses was studied in tobacco plants overexpressing a tomato tAPX gene. The overexpression of chloroplastic APX played a significant role in H$_2$O$_2$ detoxification and in minimizing photooxidative damage during temperature stress. The transgenic plants showed a higher photochemical efficiency of photosystem II when compared to WT plants under cold and heat stresses (Sun et al., 2010a). These results put in evidence that the manipulation of the antioxidative mechanism in chloroplasts may be applied in the development of plants with increased tolerance to multiple environmental stresses.

High light stress

Plants exposed to excessive light can suffer photo-inhibition, serious damage to the photosynthetic apparatus, and degradation of photosynthetic proteins (Demmig-Adams and Adams, 1992). Light stress can also lead to ROS accumulation and antioxidant enzymes activation (Mittler, 2002). The responses of APX isoenzymes to photooxidative stress were studied in spinach leaves during high light stress. cAPX activity and transcripts increased during high light stress, however protein levels were not altered. The activities of chlAPX isoforms showed a gradual decrease, while the other isoenzymes showed no significant variation in transcript and protein levels, as well as activities (Yoshimura et al., 2000). In wheat, a mutant line showing decreased tAPX activity presented reduced photosynthetic activity and biomass accumulation when growing under high-light intensity, suggesting that TAPX is essential for photosynthesis (Danna et al., 2003). Single mutants of Arabidopsis lacking tAPX or sAPX presented higher levels of H$_2$O$_2$ and oxidized proteins than WT plants when exposed to high light and MV stresses. The strongest effect of photooxidative stress was observed in plants lacking TAPX, these showing increased H$_2$O$_2$ accumulation and oxidized proteins (Maruta et al., 2010).

Double mutants deficient in two APX genes, thylakoid-bound and a cytosolic one (tylapx/apx1), resulted in different signals in Arabidopsis plants, such as late flowering, low protein oxidation during light stress and enhanced accumulation of anthocyanins (Miller et al., 2007). Mutants lacking a functional copy of TAPX, sAPX or both, were characterized in Arabidopsis under photooxidative stress during germination. The stress led to chloroplast bleaching in sapx single-mutant and tapx/sapx double-mutant plants, while the greening process of WT and tapx plants was partially impaired (Kangasjarvi et al., 2008). When mature leaves of tapx/sapx double mutants were submitted to short-term photooxidative stress induced by high light or MV treatment, the plants showed susceptibility (Kangasjarvi et al., 2008). These results indicate that the APXs isoenzymes are indispensable under environmental stresses in different species, especially under light stress conditions.

In Arabidopsis leaves, high light treatment induced the expression of cytosolic APX2, which has its expression restricted to bundle sheath cells of the vascular tissue (Fryer et al., 2003). In Arabidopsis, APX1 knockout plants showed suppressed growth and development, altered stomatal responses and induction of heat shock proteins.
during light stress. The inactivation of cytosolic APX resulted in the alteration of several transcripts involved in different functions. In transgenic APX1 plants kept under optimal conditions, the transcripts encoding APX enzymes were not elevated. However, during light stress, certain enzymes were induced in knockout-APX1 plants (Pnueli et al., 2003). In another study (Davletova et al., 2005) with APX1-deficient Arabidopsis plants the observation was that the entire chloroplastic H$_2$O$_2$-scavenging system collapsed, H$_2$O$_2$ levels increased and protein oxidation occurred in leaves subjected to a moderate light stress, suggesting that the absence of cytosolic APX1 resulted not only in the accumulation of H$_2$O$_2$ but also in damage to specific proteins in leaf cells. On the other hand, rice plants double silenced for cytosolic APXs up-regulated other peroxidases, making these transgenic plants able to survive under stress, such as salt, heat, high light and MV, similar to NT plants. The antioxidative compensatory mechanism exhibited by the silenced plants was associated with increased expression of Gpx genes. The transcript levels of OsCatA and OsCatB and the activities of CAT and guaiacol peroxidase (GPOD; type III peroxidases) were also up-regulated. In contrast, none of the other isoforms of OsAPX were up-regulated under normal growth conditions. These results suggested that signaling mechanisms triggered in rice could be distinct from those proposed for Arabidopsis (Bonifacio et al., 2011).

**Drought stress**

Drought stress in plants leads to severe effects such as reduction in vegetative growth and cell division. As a consequence of drought stress several changes occur inside the cell, including changes in gene expression levels, synthesis of molecular chaperones, and activation of enzymes involved in the production and removal of ROS (Mahajan and Tuteja, 2005). In two cowpea (Vigna unguiculata) cultivars, one drought-tolerant and the other drought-sensitive, APX activity was 60% higher in tolerant plants cultivated under control conditions. In response to drought stress, a higher increase in transcript levels of cytosolic and peroxisomal APX genes was observed in the sensitive cultivar (D’Arcy-Lameta et al., 2006). Chloroplastic APX genes expression was stimulated earlier in the tolerant cultivar when submitted to drought stress. These data suggest the capacity of these enzymes to efficiently detoxify ROS at their production site (D’Arcy-Lameta et al., 2006). Relative APX transcript levels showed distinct changes in two genotypes of wheat exposed to mild water deficit. Cytosolic APX1 expression levels increased in both genotypes, while cytosolic APX2 was up-regulated only in the drought-tolerant genotype. The transcript level of thylakoid APX increased in the drought-tolerant genotype, while stromal APX2 showed higher expression levels in the drought-sensitive cultivar (Secenji et al., 2010).

APX gene expression patterns in rice were studied after 15 days of drought stress. In marked contrast with the experiments with wheat, thylakoid APX (OsAPX8) expression was down-regulated in this condition, while the OsAPX1, OsAPX2, OsAPX5, OsAPX6 and OsAPX7 genes were up-regulated. The peroxisomal OsAPX3 gene was not affected, while OsAPX4 was slightly but significantly down-regulated by this treatment (Rosa et al., 2010). This discrepancy could be due to distinct responses of APX genes in different species and to different magnitudes of stress. In Arabidopsis, APX1 protein and mRNA accumulated during combination of heat and drought stress. A cytosolic APX1-deficient mutant accumulated more H$_2$O$_2$ and was more sensitive to stress combination than WT plants when exposed to heat and drought stress combined. In contrast, plants deficient in thylakoid APX were not more sensitive to this stress combination than APX1-deficient mutant or WT plants. The cytosolic APX1 gene may thus play a key role in the acclimation of plants to combined stress such drought and heat (Koussevitzky et al., 2008). Indeed, when the overexpression of cytosolic APX was studied in tobacco chloroplasts, its overexpression protected the plant from several oxidative stresses, including drought and polyethylene glycol-induced stress (Badawi et al., 2004). Plants overexpressing other antioxidant enzymes in different species showed increased tolerance to various stresses, including drought resistance. The overexpression of a Populus peroxisomal ascorbate peroxidase (PpAPX) gene in transgenic tobacco improved drought resistance in these plants (Li et al., 2009). The overexpression of tomato (Solanum lycopersicum) thylakoid-bound APX (StAPX) gene in tobacco plants enhanced the tolerance of these plants to salt and osmotic stress (Sun et al., 2010b).

**Heavy metals**

The contamination of soils with heavy metals is a serious environmental problem that limits crop production. Exposure at higher concentrations of heavy metals can increase the production of ROS and change antioxidant response (Gratão et al., 2005). Exposure of pea (Pisum sativum L.) plants to cadmium changed enzymatic and non-enzymatic antioxidant defenses, however, APX activity or accumulation of its transcripts were not significantly different (Romero-Puertas et al., 2007). However, it was observed that in coffee cells, the activity of APX was increased at the lower cadmium concentration. On the other hand, APX activity was not detectable in cells submitted to the higher cadmium concentration after 24 h of treatment (Gomes-Junior et al., 2006b).

An increase in APX activity was also observed in response to other heavy metals such as aluminum (Sharma and Dubey, 2007). In rice, the transcript levels of all OsAPX genes, except OsAPX6, were significantly increased after eight hours of 20 ppm aluminum exposure (Rosa et al., 2010). In pea plants, cAPX expression increased in the
shoots under aluminum treatment, but APX activity presented a significant decline at 10 μM aluminum in roots and shoots after 24 and 48 h of stress; at 50 μM aluminum treatment, however, APX activity did not show any significant changes (Panda and Matsumoto, 2010). Transgenic rice plants double silenced for APX1 and APX2 (APX1/2s plants) exhibited normal development and enhanced tolerance to a toxic concentration of aluminum (Panda and Matsumoto, 2010). In bean plants, the expression of cytosolic APX was induced both at mRNA and protein levels in leaves of de-rooted plants in response to iron overload. Likewise, transgenic tobacco plants with suppressed cytosolic APX levels were more sensitive to iron application than WT plants (Pekker et al., 2002). In coffee cells treated with nickel showed a rapid increase in APX activity, although the activity trends were slightly different between the two nickel concentrations tested (0.05 mM and 0.5 mM) (Gomes-Junior et al., 2006a). Transgenic tall fescue plants expressing the CuZnSOD and APX genes in chloroplasts were submitted to copper, cadmium or arsenic treatment. Of the metals tested, copper and cadmium increased SOD and APX activities in control and transgenic plants, with a higher increase observed in transgenic plants. In contrast, in leaves exposed to arsenic, both enzymes exhibited less activity when compared to other treatments and no significant differences were observed between control and transgenic plants (Lee et al., 2007a). These results emphasize the important role of APX and other antioxidant enzymes in H2O2 scavenging under toxic metals levels in the soil.

Conclusions

Ascorbate peroxidase is a key enzyme regulating ROS levels acting in different subcellular compartments (Figure 2). The expression of APX encoding genes is differentially modulated by several abiotic stresses in different plant species. All the data collected so far firmly indicate that APX isoforms play important and direct roles as protective elements against adverse environmental conditions. The diverse effects of knockdown or knockout of different APX genes on the plant growth, physiology and antioxidant metabolism indicate that APX may also regulate redox signaling pathways involved in plant development. These re-

Figure 2 - APX enzymes and the elimination of ROS excess in different subcellular compartments. H2O2 is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes. Abiotic and biotic stresses enhance H2O2 and chlAPX, mAPX, cAPX and mitAPX enzymes which can eliminate ROS excess in different subcellular compartments. The plasma membrane-NADPH oxidases also generate H2O2, which can cross membranes through aquaporin channels. Superoxide (O2·−), hydrogen peroxide (H2O2), monodehydroascorbate (MDHA).
sults emphasize the importance and complexity of the interactions of APX with other antioxidants in fine tuning the vegetal antioxidant metabolism.

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