CHARACTERISTICS OF THE PLANT ASCORBATE PEROXIDASE FAMILY

GRAŻYNA DĄBROWSKA[1*], ALEKSANDRA KATA[1], ANNA GOC[1],
MAGDALENA SZECHYŃSKA-HEBDA[2], AND EDYTA SKRZYPEK[2]

[1]*Department of Genetics, Nicolaus Copernicus University,*
ul. Gagarina 9, 87-100 Toruń, Poland
[2]*Polish Academy of Sciences, F. Górski Institute of Plant Physiology,*
ul. Niezapominajek 21, 30-239 Cracow, Poland

This paper reviews plant ascorbate peroxidases (APX), an important part of the antioxidative system, maintaining the balance and uninterrupted functioning of the plant cell. The main role of APXs is to control the hydrogen peroxide concentration in cells. In reaction the enzymes use ascorbate as an electron donor. The active site is highly conserved by every member of the APX family. APXs belong to class I of the superfamily of bacterial, fungal and plant peroxidases. All the isoforms differ from each other in molecular weight, optimal pH, stability, substrate specificity, localization and level of response to specific stress conditions. It is suggested, however, that the responsible genes originated from one common gene by multiple duplication events followed by natural selection.

Key words: Ascorbate peroxidase, gene evolution, oxidative stress, hydrogen peroxide, antioxidative system, ROS, photosynthetic electron transport, programmed cell death (PCD), nitric oxide, gene expression.

INTRODUCTION

Plants are not isolated entities, and must respond to external stimuli. Environmental conditions change and often are a source of stress to which the plant responds by excessive production of oxygen radicals. Reactive oxygen species (ROS) damage cell components such as DNA, proteins and lipids. It is known that an excess of free oxygen radicals leads to programmed cell death (PCD) (Pellinen et al., 2002; Vranova et al., 2002).

However, ROS are also utilized in various metabolic processes such as formation of lignin in the cell wall (Inze and Montagu, 1995), leaf and flower abscission, cell senescence, ripening of fruit and flowering (Mehlhorn et al., 1996).

In the course of evolution, organisms have developed complicated antioxidative systems to maintain the balance and efficient functioning of the cell. Two types of antioxidative systems, nonenzymatic and enzymatic, have been investigated. Tocopherols, glutathione, flavonoids, carotenoids and ascorbic acid belong to the former; the latter include superoxide dismutases, peroxidases (of ascorbate, glutathione, guaiacol), catalases and enzymes oxidizing and reducing ascorbate. Both antioxidant systems cooperate, forming an integral mechanism controlling the concentration of ROS in the organism. In this paper we cannot characterize the entire operation of this complex regulatory system. Here we present the ascorbate peroxidases (APXs), one of the most important groups of antioxidant enzymes of plants. So far at least five APX isoforms have been identified in plants: cytosolic isoforms, mitochondria isoforms, peroxisomal/glyoxysomal isoforms and chloroplastic isoforms. All the forms of APX are thought to function as scavengers of the H_2O_2 that is generated continuously in cells (Miyake and Asada, 1996). For instance, superoxide (O_2^-) is formed in the chloroplasts of photosynthetic organisms when insufficient CO_2 is available to balance electrons being generated by the photosystems; these excess electrons then reduce O_2 to O_2^-. The electron transport chains can also produce O_2 in the mitochondria. In both cases superoxide dismutase converts O_2^- to H_2O_2, which can then be removed by APX or catalase.

Expression of APX genes can be activated by specific factors such as pathogen attack, mechanical pressure, injury, UVB radiation (Rao et al., 1996), water deficiency (Mittler and Zilinskas, 1994), salt stress (Lopez et al., 1996; Mittova et al., 2004a,b;

*e-mail: browsk@uni.torun.pl

TABLE 1. Characteristics of plant ascorbate peroxidases

Isozyme	Molecular weight [kDa]	Active form	SKL at C-end	HSE in promoter	Heat induction
APX1	~27.5 Dependent on species	Homodimer	–	+	+
APX2	27.9 *A. thaliana* [34]	Homodimer	–	+	+
APX6	? Lack of complete sequence	Homodimer	–	–	No clear answer
APX3	31	Monomer	No	Similar sequence	+
APX4	32 [PDB]	Monomer	+	–	+
APX5	28.7 [PDB]	Monomer	No	Similar sequence	–
sAPX	30-33.5 Dependent on species	Monomer	–	–	No clear answer
tAPX	~46.1 *A. thaliana* [NCBI]	Monomer	–	–	No clear answer

PDB – Protein Data Base; NCBI – National Center for Biotechnology Information

Gomez et al., 2004), excess excitation energy (Karpinski et al., 1997; Yoshimura et al., 2000), too low or too high temperature, excess oxygen after a period of anoxia (Blokhina et al., 2001), atmospheric pollution (e.g., sulphur dioxide, nitrogen oxide and dioxide or ozone) (Tanaka et al., 1985; Mehlhorn et al., 1987; Kubo et al., 1995; Rao et al., 1996), excess metal ions (Vansuyt et al., 1997), deficiency of some mineral salts (e.g., phosphates), and herbicides (Cakmak and Marschner, 1992).

CHARACTERISTICS OF ASCORBATE PEROXIDASES

Peroxidases, and among them APXs, are proteins containing the heme prosthetic group in which iron plays an important role in the catalytic site. This was clearly shown by applying iron deficiency conditions in sugar beet, which doubled the ascorbate concentration while approximately halving the activity of cytosolic APX in whole roots, probably reflecting depletion of the available iron pool in the root cell (Zaharieva and Abadia, 2003).
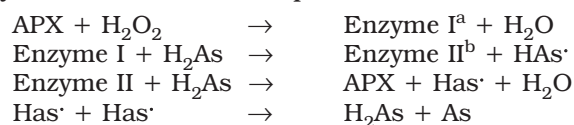
Ascorbate, the electron donor to which the enzymes show high affinity, is the substrate utilized in reduction of hydrogen peroxide. APXs are extremely sensitive to the ascorbate concentration. If it is too low (lower than 20 μM) the enzymes lose stability and their activity declines (Shigeoka et al., 2002). This explains the difficulties encountered during attempts to isolate the enzymes from plants. Mittler and Zielinskas (1991b) were the first to isolate and purify homogenous cytosolic APX from pea. Ascorbate binds to the active site of the proteins by four hydrogen bonds with lysine and arginine residues, and the heme, the site of substrate binding (Cys-32, Arg-172, Lys-30, Fig. 1) is highly conserved (Sharp and Raven, 2004).

Ascorbate peroxidases occur mainly in higher plants, algae and some cyanobacteria (Shigeoka et al., 1980a,b; Miyake et al., 1991; Takeda et al., 1992, 1998, 2000; Sano et al., 2001; Sharma and Dubey, 2004), but they have also been found in insects (Mathews et al., 1997), *Trypanosoma cruzi* (Boveris et al., 1980) and in the choroid and iris epithelium of the bovine eye, tissues rich in ascorbate (Boveris et al., 1980; Wada et al., 1998).

Plant ascorbate peroxidase isoforms differ in molecular weight (Tab. 1), optimal pH, stability and substrate specificity. Jespersen et al. (1997) suggested that tryptophan found at position 175 of chloroplast APX increases their substrate specificity.

The reaction scheme catalyzed by these enzymes is as follows (Sharp and Raven, 2004):

APX + H_2O_2	$\rightarrow$	Enzyme I[a] + H_2O
Enzyme I + H_2As	$\rightarrow$	Enzyme II[b] + HAs·
Enzyme II + H_2As	$\rightarrow$	APX + Has· + H_2O
Has· + Has·	$\rightarrow$	H_2As + As

[a,b] Enzyme I and II – forms with different oxidation levels (I – with 2 free electrons; II – with 1 free electron)
· Reactive form of ascorbate

In the case of APXs, two molecules of ascorbate (H_2As) are used as the preferred substrate. After their oxidation, monodehydroascorbate (HAs·) and dehydroascorbate (As·) are formed, which are subsequently reduced by monodehydroascorbate reductase and dehydroascorbate reductase, respectively, in order to maintain the ascorbate pool (Badyal et al., 2006).
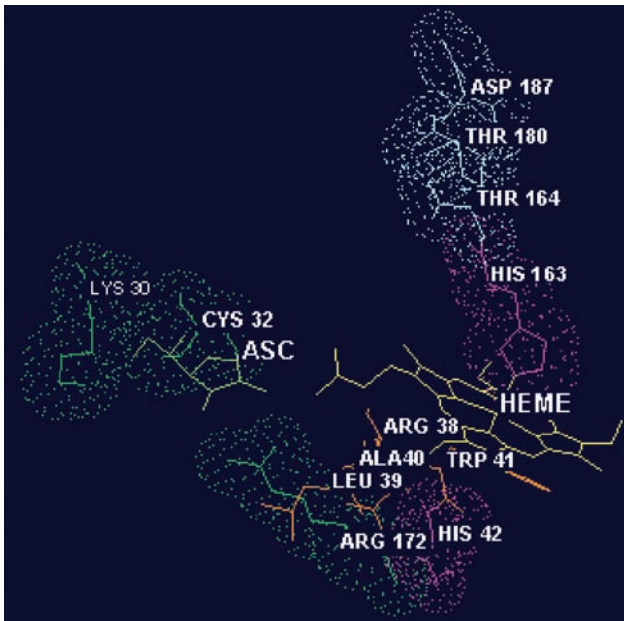
Fig. 1. Structure of the active site of APX with substrate (ascorbate). Amino acid residues forming hydrogen bonds with the substrate – green; residues responsible for binding of K^+ – blue; proximal and distal histidine – violet; residues near the distal histidine – orange; heme – yellow; ascorbate (ASC) – light green. Modified from Protein Data Base (PDB).

In animals, a similar role to APX is played by glutathione peroxidase; interestingly, it possesses selenium instead of sulphur in cysteine in the active site (Shigeoka et al., 2002).

APX ISOFORMS AND THEIR SUBCELLULAR LOCALIZATION

Plant APXs are found in several cellular compartments. In *Arabidopsis thaliana*, for example, the presence of eight isozymes has been confirmed: soluble cytosolic (APX1, APX2, APX6), bound to the microsome membrane (APX3, APX4, APX5), and chloroplast sAPX and tAPX (Jespersen et al., 1997; Panchuk et al., 2002). APX4 contains the SKL motif (Ser-Lys-Leu) at the C-terminal end (Tab. 1), which suggests targeting of this protein to peroxisome. APX has also been found in mitochondria in pea and potato (Jimenez et al., 1997; Leonardis et al., 2000).

Cytosolic APX

The cytosolic isoforms of ascorbate peroxidases are homodimers (Tab. 1). Amino acid residues participating in electrostatic interactions between subunits (Lys-18, Arg-21, Lys-22, Arg-24, Asp-112 or Glu, Glu-228, Asp-229) are highly conserved (Jespersen et al., 1997).

Regarding location, transcripts of APX1 in sweetpotato (*Ipomoea batatas*) were detected clearly in leaves, weakly in stems, and not in non-storage and storage roots. The expression level appeared to be higher in mature leaves than in immature leaves, suggesting that it is regulated by growth stage (Park et al., 2004). Expression of APX2, another cytosolic isoform, is limited to bundle sheath cells in leaves exposed to excess light (Fryer et al., 2003).

Peroxisomal APX

Reactive oxygen species are also byproducts of processes taking place in peroxisomes (i.e., photorespiration, fatty acid β-oxidation and ureide metabolism), which require effective protection. Peroxisomal ascorbate peroxidase plays such a role and scavenges hydrogen peroxide escaping the peroxisomal matrix by passive diffusion. The N-terminal active domain of the enzyme faces the cytosol, and its C-terminal domain is anchored (APXs are single-pass peroxisomal membrane proteins), which facilitates the protein's functioning (Lisenbee et al., 2003). Its cytosolic orientation has also been studied within glyoxysomes of oilseed species (Jimenez et al., 1997). Peroxisomal APX preferentially accumulates in spongy parenchyma rather than palisade parenchyma, and can also be found in large amounts near the central vascular bundles (Pereira et al., 2005).

Peroxisomes possess the capacity to generate and release into the cytosol signalling molecules such as O_2^-, H_2O_2 and nitric oxide, which are involved in signal transduction pathways and play a key role in redox-regulated responses to pathogen attack and abiotic stresses. In tomato, the activity of peroxisomal APX decreased markedly following inoculation of a pathogen; this may have resulted from diminution of the peroxisomal ascorbate pool in response to infection, and from inhibition by NO, generated by peroxisomal NADPH-dependent NO synthase (Kuźniak and Skłodowska, 2005).

Chloroplastic APX

tAPX and sAPX are both involved in the water-water cycle, where O_2^- is reduced to water in a two-step reaction catalyzed by superoxide dismutase (SOD) and APX. O_2^- is produced at the PSI site by reduction of O_2 by electrons donated from PSI – the Mehler reaction, which is one of several mechanisms for dissipating excess excitation energy. The water-water cycle contributes to maintaining a proper ATP/NADPH ratio and to alleviating the over-reduction of photosystems when plants are exposed to photoinhibitory conditions (Asada, 1999).

tAPX transcript levels are weakly responsive to external stimuli, exposing the plant to oxidative

stress (Yoshimura et al., 2000; Panchuk et al., 2002; Shigeoka et al., 2002). This suggests that tAPX is involved in direct detoxification of H_2O_2 but not in protection from excess ROS produced under environmental stress (Shigeoka et al., 2002). Overexpression of cAPX in chloroplasts of transgenic tobacco produced enhanced tolerance to oxidative stress generated by paraquat and salinity, confirming the function of tAPX as an immediate scavenger of ROS (Badawi et al., 2004).

Nitric oxide (NO), which, with H_2O_2 as partner molecule, regulates the hypersensitive response against pathogen attack, inhibits tAPX transcript accumulation and reduces tAPX enzymatic activity (Murgia et al., 2004; Tarantino et al., 2005). It has been suggested that the higher levels of tAPX enzyme in tAPX-overexpressing lines are responsible for the reduced symptoms of damage seen upon treatment with the NO-donor sodium nitroprusside (Murgia et al., 2004); this would accord with the increased susceptibility of tAPX-antisensed plants to NO-induced damage and cell death (Tarantino et al., 2005).

Subcellular localization of APX

Subcellular localization of APX is determined by the presence of targeting peptides and transmembrane domains in the N- and COOH-terminal regions of the proteins (Shigeoka et al., 2002; Teixiera et al. 2004). The peroxisomal targeting signal comprises a COOH-terminal transmembrane domain rich in valine and alanine, followed by a positively charged domain containing five amino acid residues (Mullen and Trelease, 2000). These isoforms face the cytosol and are indirectly sorted to the peroxisomes via a subdomain of the rough endoplasmic reticulum (Lisenbee et al., 2003). In contrast, all chloroplastic isoforms possess a hydroxylated peptide at the N-terminus that is processed in mature proteins (Madhusudhan et al. 2003). Thylacoid-bound isoforms present a transmembrane hydrophobic domain at their COOH-terminus (Ishikawa et al. 1996). Chew et al. (2003) showed that the *Arabidopsis* stromal APX is dual-targeted to plastids and mitochondria, due to the ambiguity of the targeting peptide at the N-terminus of the proteins, but Mittova et al. (2004b) suggested that specific genes generate the mitochondrial and stromal isoform in tomato plants, despite the fact that no corresponding gene, cDNA, or protein sequences for the specific mitochondrial isoform have been described so far.

STRUCTURE OF APX GENES

Many experiments have been carried out on various APX genes, but the gene encoding APX1 is the one whose structure is best known. APX1 is encoded by a single gene. The promoter has typical structure: it possesses a TATA box at -28 bp, a CCAAT box at -176 bp, and several regulatory elements (Mittler and Zilinskas, 1992). The heat-shock element (HSE), binding the heat-shock factor (HSF) characteristic for heat-shock genes, is a regulatory element (Storozhenko et al., 1998). It is also present in Apx2 promoter (Panchuk et al., 2002). Apx3 and Apx5 possess a sequence similar to HSE (Tab. 1), but it does not bind HSF. However, microsomal APXs are involved in the response to heat stress (Shi et al., 2001). The next characteristic element is the antiperoxidative element (ARE), a sequence responsible for H_2O_2-dependent response. Other regulatory elements found in the Apx1 gene are the CACGCA sequence, probably responsible for the xenobiotic response, and the CACGTG sequence which constitutes part of the G box (Mittler and Zilinskas, 1992). In the first intron at position 97 the TGATTCAG sequence has been reported; probably it is part of the GPE I enhancer (a regulatory element for glutathione transferase P) which regulates gene transcription through the interaction with transcription factors and RNA II polymerase (Okuda et al., 1989).

Apx1 contains 9 introns having high content of A and T nucleotides. The first intron is in an unusual position in the 5'UTR region in mRNA. In species in which sAPX and tAPX are formed by alternative splicing of one gene (e.g., in spinach), chloroplast Apx contains 12 introns (Yoshimura et al., 1999). Such genes have a conserved SRE sequence (*splicing regulatory cis-element*), which controls alternative splicing. This element is not present in plants that possess separate genes for chloroplast forms (Teixeira et al., 2004).

It has been discovered that the zinc finger protein Zat12 is required for cAPX1 expression during oxidative stress caused by hydrogen peroxide, heat, paraquat and wounding in *Arabidopsis*. Moreover, Zat12 enhances the expression of an NADPH oxidase, which suggests that NADPH oxidases, via enhanced production of ROS, are also involved in regulating the response of plants to oxidative stress (Rizhsky et al., 2004).

There are recent findings on hormone activation of APX. Abscisic acid treatment was shown to strongly increase transcription of APX1 in sweetpotato (Park et al., 2004; Hu et al., 2005), while ethylene treatment had no effect on ascorbate content and gene expression involved in ascorbate metabolism (Nishikawa et al., 2003b).

THE ASCORBATE PEROXIDASE FAMILY AND EVOLUTION OF PLANT APX

Ascorbate peroxidases, which are of the prokaryotic origin, belong to class I of the superfamily of bacter-
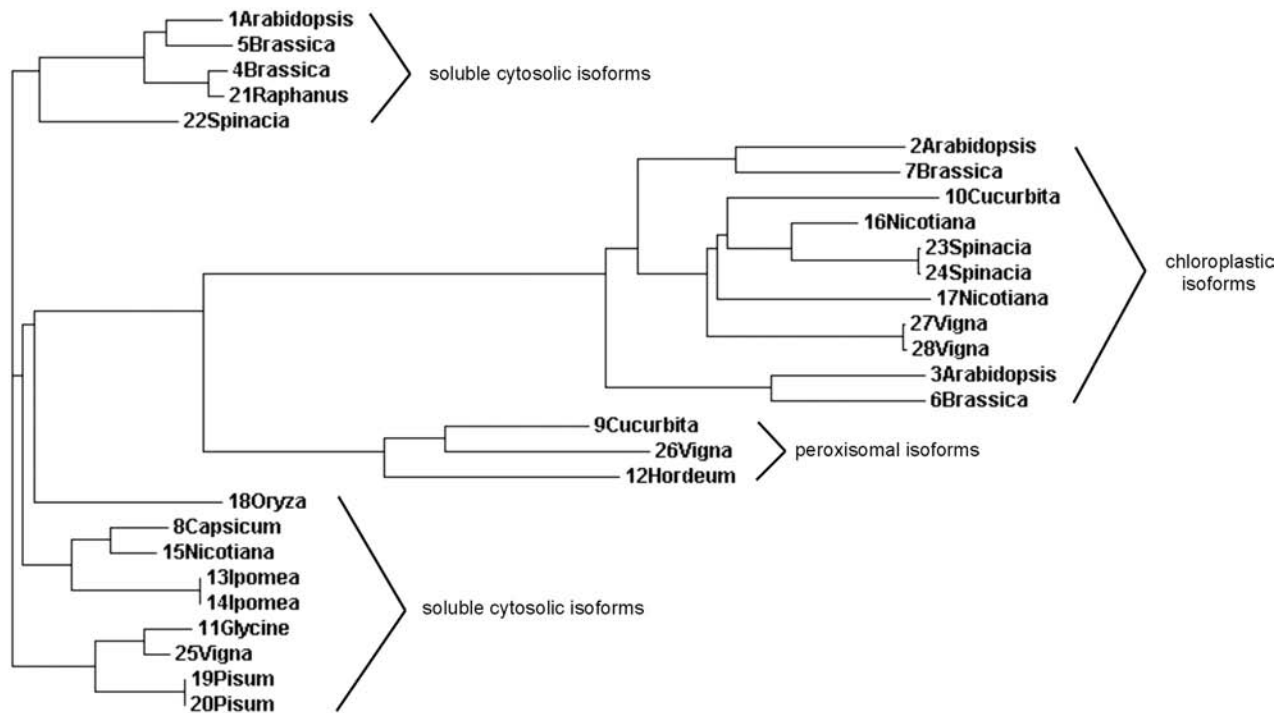
Fig. 2. Phylogenetic tree of plant ascorbate peroxidases (Table 2) based on the complete NCBI (National Center for Biotechnology Information) amino acid sequences. Phylogram constructed using ClustalW software.

ial, fungal and plant peroxidases. APXs have several characteristic structural features:

(1) In known ascorbate peroxidases, two histidine residues, His-42 and His-163, have been found (Fig. 1). Both residues are essential for the catalytic activity of the enzyme. His-42 is located on the distal side of the heme cavity; on its proximal side, His-163 forms the axial heme ligand connected to the heme iron. Near the distal histidine there are residues, Arg-38, Leu-39, Ala-40 and Trp-41 (Fig. 1), which are highly conserved in this class (Jespersen et al., 1997).

(2) The enzymes bind single K^+ ion to the proximal domain. This ion is essential for APX activity. The site of K^+ binding is unchanged (Thr-164, Thr-180, Asp-187, Fig. 1). Moreover, APXs have a putative metal-binding site in the distal domain occupied by a monovalent metal ion (Jespersen et al., 1997).

(3) Unlike plant peroxidases belonging to class III, ascorbate peroxidases have a tryptophan residue at position 41 instead of phenylalanine (Mittler and Zilinskas, 1991a; Jespersen et al., 1997).

Two models of the evolution of multigene peroxidase families in class I have been proposed. The first model represents a concerted mode of evolution in which polymorphism (genetic diversity) has been generated by interlocus recombination or gene conversion followed by natural selection. The other model proposes that multigene families can exist in genomes as a consequence of evolution by the birth-and-death process. It assumes that new genes are created by repeated duplication of existing ones, some of which stay in the genome for a long time, while others are removed or cease to function. Zamocky (2004) suggests that repeated gene duplication has led to the emergence of separate APX families, cytochrome C peroxidase and catalase-peroxidases, and their further evolution. The assumption that ascorbate peroxidases have originated from one common ancestor is supported by the finding that all isoforms possess highly conserved regions in the area of ion binding. Yeast cytochrome c peroxidases, however, do not possess such a region.

The phylogenetic tree shows that the first step in the evolution of the ascorbate peroxidase family was diversification of the cytosolic and chloroplast forms (Tab. 2, Fig. 2). It is worth mentioning that representatives of the same evolutionary branches existing in the same cell compartments of different plant species are more closely related than are representatives of different evolutionary branches existing in the same species. For example, sequences of stromal and thylakoid-bound APX from broccoli (*Brassica oleracea*) show high similarity to those of

TABLE 2. Classification of the complete amino acid sequences used for the phylogram. sAPX – stromal form; pAPX – peroxisomal form; tAPX – thylacoid-bound form; cAPX – cytosolic form; NCBI – National Center for Biotechnology Information

No.	Species	No. of NCBI sequences	Kind of APX
1	*Arabidopsis thaliana*	gi21554322	cAPX
2	*Arabidopsis thaliana*	X98926	tAPX
3	*Arabidopsis thaliana*	AY056319	sAPX
4	*Brassica oleracea*	AB078599	cAPX
5	*Brassica oleracea*	AB078600	cAPX
6	*Brassica oleracea*	BAD14932	sAPX
7	*Brassica oleracea*	BAD14931	tAPX
8	*Capsicum annum*	CAA57140	cAPX
9	*Cucurbita* sp. cv. *Kurokawa Amakuri*	AB070626	pAPX
10	*Cucurbita* sp. cv. *Kurokawa Amakuri*	D83656	tAPX
11	*Glycine max*	BAC92739	cAPX
12	*Hordeum vulgare*	AB063117	pAPX
13	*Ipomoea batatas*	AAP42501	cAPX
14	*Ipomoea batatas*	AY206407	cAPX
15	*Nicotiana tabacum*	gi559005	cAPX
16	*Nicotiana tabacum*	BAC10691	sAPX
17	*Nicotiana tabacum*	BAA78552	tAPX
18	*Oryza sativa*	gi12082341	cAPX
19	*Pisum sativum*	P48534	cAPX
20	*Pisum sativum*	X62077	cAPX
21	*Raphanus sativus*	X78452	cAPX
22	*Spinacia oleracea*	D85864	cAPX
23	*Spinacia oleracea*	D83669	sAPX
24	*Spinacia oleracea*	D77997	tAPX
25	*Vigna unguiculata*	U61379	cAPX
26	*Vigna unguiculata*	AY466858	pAPX
27	*Vigna unguiculata*	AAS55853	sAPX
28	*Vigna unguiculata*	AY484492	tAPX

chloroplastic APX from *Arabidopsis* (Nishikawa et al., 2003b). In cowpea (*Vigna unguiculata*), tAPX (38.2 kDa) was found to share 77.5% and 79.3% sequence identity with spinach and pumpkin tAPX, respectively; tAPX and sAPX were determined to be identical except for the presence of a 49 amino acid region at the C-terminal end of the thylakoidal sequence, with a very hydrophobic profile that could correspond to a membrane-spanning region (Shigeoka et al., 2002). It was suggested that tAPX and sAPX in cowpea are coded by a single gene alternatively spliced (D'arcy-Lameta et al., 2006).

Cytosolic APXs from broccoli gave values of about 90% identity to those of *Arabidopsis thaliana* (both have molecular mass of 28 kDa), and more than 80% identity to cytosolic APX in spinach and cucumber (Nishikawa et al., 2003a). The amino acid sequence of cytosolic APX1 in sweetpotato displayed 80% homology to cAPX from *Arabidopsis thaliana* and 90% to tobacco cAPX (Park et al., 2004). Cytosolic APX from cowpea (molecular mass 27 kDa) gave values of 92% and 80.8% identity to that of pea and radish, respectively, whereas peroxisomal APX (31.7 kDa) shared 84.7% and 75.7% sequence identity with *Cucurbita* and *Hordeum* pAPX, respectively (D'arcy-Lameta et al., 2006).

This clearly means that ascorbate peroxidases localized in the same cell compartment originated from one common gene. In some species (e.g., *Arabidopsis*) the evolutionary path of chloroplast isoforms probably separated fairly recently into stromal- and thylakoid-bound ones. In other species, the isozymes sAPX and tAPX resulted from alternative splicing of one gene, as in spinach (Jespersen et al., 1997; Yoshimura et al., 1999) or in a climbing plant of the genus *Vigna* (Fig. 2).

THE ROLE OF APX

Response to different stresses

APX isoforms play a protective role against ROS produced in excess under environmental stress. Therefore their expression level often reflects the occurrence of stress conditions.

The same stress can differentially change the expression of cytosolic and chloroplastic APX genes (Nishikawa et al., 2003b). In broccoli the expression of two cytosolic APX isoforms was markedly induced just after harvest in different parts of the plant. The transcript level of both chloroplastic isoforms remained unchanged until 6 h after harvest and then diminished rapidly.

In response to wounding, expression of the cytosolic APX gene from sweetpotato increased only slightly, attaining its highest level at 40 h after treatment (Park et al., 2004). Not only cytosolic isoforms respond to accumulation of ROS. In *Arabidopsis*, the mRNA of APX4 and of tAPX showed strong leaf-age-dependant and plant-age-dependant reduction of mRNA levels at an early stage of leaf senescence, whereas the mRNA of APX5 showed down-regulation in older leaves of each rosette (Panchuk et al., 2005). Interestingly, chloroplastic APXs in pea behaved differently in thylakoids and stroma during long-term NaCl stress: sAPX increased considerably while tAPX activity progressively diminished (Gomez et al., 2004).

In tobacco it was reported that cytosolic (but not chloroplastic) APX was up-regulated after exposure to 6 h of light; the possibility of changes in the cAPX transcript due to circadian rhythmicity was ruled out (Pignocchi et al., 2003).

Response to changes in photosynthetic electron transport (PET) and to programmed cell death

Detoxification of H_2O_2 by APX is followed by a set of reactions catalyzed by monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Together these reactions comprise one of the most important antioxidant systems in plants – the ascorbate-glutathione or Halliwell-Asada cycle. In this cycle, ascorbate and glutathione act as reducing substrates for scavenging of H_2O_2, and are ultimately recycled at the expense of ATP and NAD(P)H (Noctor and Foyer, 1998). The ascorbate-glutathione cycle plays a critical role in chloroplast redox protection. These organelles are devoid of catalases and are a major source of superoxide and H_2O_2 as a consequence of the highly energetic reactions that take place there during photosynthetic activity. A series of reports have convincingly demonstrated the activity of ascorbate-glutathione cycle enzymes in the mito-

chondria of plant cells, confirming that this cycle also plays an important role in protecting this organelle against the harmful effects of the ROS that are regularly produced in respiratory chain reactions (Mittova et al., 2004a).

The signals that initiate APX2 expression in light-stressed leaves are redox changes in PET, probably through the plastoquinone pool at an early stage of stress (Yabuta et al., 2004), H_2O_2 accumulation derived from the Mehler reaction, and transient changes in leaf water status (Karpinski et al., 1999; Fryer et al., 2003). The relationship between induction of APX2 expression and leaf water status has been suggested to be mediated by abscisic acid (Fryer et al., 2003), which is not required for wound-induced APX2 induction. It is also suggested that wound-signalling pathways in *Arabidopsis* might share common mediators with excess light-signalling pathways. Thus, in wounded leaves under light there is increased diversion of photosynthetic electron flux to O_2 in the vascular regions, leading to redox changes in PET and increased production of H_2O_2, which in turn triggers induction of APX2 expression. The highest levels of APX2 expression were always observed in leaves subjected to both wounding and excess light. Moreover, wounded leaves treated with the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) showed no induction of APX2 expression (Chang et al., 2004). It has been suggested that the specific expression of APX2 in bundle sheath cells may be part of the systemic signalling system that facilitates acclimation of plants to challenges such as excess light or wounding (Karpinski et al., 1999, Fryer et al., 2003).

Another important APX isozyme alleviating photooxidative damage is thylakoid APX. It was reported that antisense reduction of tAPX in *Arabidopsis* enhances paraquat-induced photooxidative stress; this conforms with the results observed in tobacco and in *Arabidopsis*, where tAPX overexpression increased tolerance to paraquat, an O_2^--generating herbicide (Yabuta et al., 2002; Danna et al., 2003; Murgia et al., 2004). Double antisense transgenic tobacco plants lacking cAPX and catalase are less susceptible to oxidative stress than wild type plants; such improved resistance correlates with reduced photosynthetic activity (Rizhsky et al., 2002).

Antisense reduction of tAPX also enhances nitric oxide-induced cell death, suggesting that tAPX enzyme participates in regulation of H_2O_2 levels involved in cell death triggered by NO. *Arabidopsis* tAPX transgenic lines are phenotypically indistinguishable from wild plants (Tarantino et al., 2005), unlike their tobacco counterparts (Yabuta et al., 2002). In tobacco, a single gene codes for both tAPX and sAPX by alternative splicing (Shigeoka et al., 2002), so it cannot be excluded that tAPX antisensing also antisensed sAPX, drastically reducing over-

all chloroplastic APX content (Tarantino et al., 2005).

ROS are early messenger molecules in signalling pathways under different stress conditions (Pellinen et al., 2002; Vranova et al., 2002; Mori and Schroeder., 2004; Shin and Schachtman, 2004; Hung et al., 2005), leading to initiation of protective mechanisms such as antioxidative systems aimed at saving the cell from demise or otherwise triggering the impairment of the cellular redox balance and acting as a factor in PCD. Vacca et al. (2004) studied the process of PCD initiation and alteration of cell metabolism. They observed that induction of PCD resulted in gradual oxidation of endogenous ascorbate and decreased both the amount and specific activity of cytosolic APX. Cells were exposed to 10 min heat shock (55°C), leading to PCD and complete loss of viability within 72 h. Immediately after heat shock, the amount of cAPX protein in the cells was about half that in control cells. After 6 h, cAPX expression clearly decreased and was negligible after 24 h. It was also shown that changes of the kinetic properties of cAPX in PCD cells are a non-competitive type of inhibition; that is, they do not involve the active site of the enzyme. cAPX activity in the cells dropped more than 40% immediately after heat shock. At 6 h the reduction reached 80%, after which activity remained constant (Vacca et al., 2004).

Besides inhibiting tAPX, PCD induced by simultaneous production of H_2O_2 and NO (Murgia et al., 2004; Tarantino et al., 2005) also down-regulates cAPX (de Pinto et al., 2002).

SUMMARY

APX induction is one element of the response to the oxidative stress caused in plants by unfavorable external conditions such as drought, high intensity illumination, chilling, salt stress or heat shock.

Ascorbate peroxidases also play an important role in controlling the concentration of oxygen radicals that participate in signal transduction in many naturally occurring physiological processes in the cell. The function of APXs does not have to be limited to antioxidative protection, but may be more extensive; there may be some connection between APX induction and regulation of metabolism.

Ascorbate peroxidases are enzymes that help to maintain nontoxic levels of hydrogen peroxide in cells or control the signal flux. The large number of APX-encoding genes in plants, differences in the regulation of each member of this family, and the presence of their proteins in different subcellular compartments, also highlight the value of the specificity of response and the complexity of the antioxidant system in plants.

Knowledge of the functional role of changes in APX expression is still insufficient. Future experiments may more precisely clarify the role of APXs in cells.

ACKNOWLEDGEMENTS

REFERENCES

Asada K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 601–639.

Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, and Tanaka K. 2004. Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. *Physiologia Plantarum* 121: 231–238.

Badyal SK, Joyce G, Sharp KH, Seward HE, Mewies M, Basran J, Macdonald IK, Moody PCE, and Lloyd Raven E. 2006. Conformational mobility in the active site of a heme peroxidase. *The Journal of Biological Chemistry* 281: 24512–24520.

Blokhina OB, Chirkova TV, and Fagerstedt KV. 2001. Anoxic stress leads to hydrogen peroxide formation in plant cells. *Journal of Experimental Botany* 52: 1179–1190.

Boveris AH, Sies H, Martino EE, Decampo R, Turreus JF, and Stoppani AOM. 1980. Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi*. *The Biochemical Journal* 188: 643–648.

Cakmak I, and Marschner H. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiology* 98: 1222–1227.

Chang CC, Ball L, Fryer MJ, Baker NR, Karpinski S, and Mullineaux PM. 2004. Induction of ascorbate peroxidase 2 expression in wounded *Arabidopsis* leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis. *The Plant Journal* 38: 499–511.

Chew O, Whelan J, and Millar H. 2003. Molecular definition of the ascorbate-glutathione cycle in *Arabidopsis* mitochondria reveals dual targeting of antioxidant defenses in plants. *Journal of Biological Chemistry* 278: 46869–46877.

D'arcy-Lameta A, Ferrari-Iliou R, Contour-Ansel D, Pham-Thi AT, and Zuily-Fodil Y. 2006. Isolation and characterization of four ascorbate peroxidase cDNAs responsive to water deficit in cowpea leaves. *Annual of Botany* 97: 133–140.

Danna CH, Bartoli CG, Sacco F, Ingala LR, Santa-Maria GE, and Guiamet JJ, Ugalde RA. 2003. Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. *Plant Physiology* 132: 2116–2125.

DE PINTO MC, TOMMASI F, and DE GARA L. 2002. Changes in the antioxidant systems as part of the signalling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright Yellow 2 cells. *Plant Physiology* 130: 698–708.

FRYER MJ, BALL L, OXBOROUGH K, KARPINSKI S, MULLINEAUX PM, and BAKER NR. 2003. Control of ascorbate peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of *Arabidopsis* leaves. *The Plant Journal* 33: 691–705.

GOMEZ JM, JIMENEZ A, OLMOS E, and SEVILLA F. 2004. Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. *Puget*) chloroplasts. *Journal of Experimental Botany* 55: 119–130.

HU X, JIANG M, ZHANG A, and LU J. 2005. Abscisic acid-induced apoplastic H_2O_2 accumulation upregulates the activities of chloroplastic and cytosolic antioxidant enzymes in maize leaves. *Planta* 223: 57–68.

HUNG S, YU C, and LIN C. 2005. Hydrogen peroxide functions as a stress signal in plants. *Botanica Bulletin of Academia Sinica* 46: 1–10.

INZE D, and MONTAGU MV. 1995. Oxidative stress in plants. *Current Opinion in Biotechnology* 6:153–158.

ISHIKAWA T, SAKAI K, YOSHIMURA, TAKEDA T, and SHIGEOKA S. 1996. cDNAs encoding spinach stromal and thylakoid-bound ascorbate peroxidase, differing in the presence or absence of their 3'-coding regions. *FEBS Letters* 384: 289–293.

JESPERSEN HM, KJAERSGARD IVH, OSTERGAARD L, and WELINDER KG. 1997. From sequence analysis of three novel ascorbate peroxidases from *Arabidopsis thaliana* to structure, function and evolution of seven types of ascorbate peroxidase. *The Biochemical Journal* 326: 305–310.

JIMENEZ A, HERNANDEZ JA, DEL RIO LA, and SEVILLA F. 1997. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiology* 114: 275–284.

KARPINSKI S, REYNOLDS H, KARPINSKA B, WINGSLE G, CREISSEN G, and MULLINEAUX P. 1999. Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. *Science* 284: 654–657.

KARPINSKI S, ESCOBAR C, KARPINSKA B, CREISSEN G, and MULINEAUX PM. 1997. Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. *The Plant Cell* 9: 627–640.

KUBO A, SAJI H, TANAKA K, and KONDO N. 1995. Expression of *Arabidopsis* cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. *Plant Molecular Biology* 29: 479–489.

KUŹNIAK E, and SKŁODOWSKA M. 2005. Fungal pathogen-induced changes in the antioxidant systems of leaf peroxisomes from infected tomato plants. *Planta* 222: 192–200.

LEONARDIS SD, DIPIERRO N, and DIPIERRO S. 2000. Purification and characterization of an ascorbate peroxidase from potato tuber mitochondria. *Plant Physiology and Biochemistry* 38: 773–779.

LISENBEE CS, HEINZE M, and TRELEASE RN. 2003. Peroxisomal ascorbate peroxidase resides within a subdomain of rough endoplasmic reticulum in wild-type *Arabidopsis* cells. *Plant Physiology* 132: 870–882.

LOPEZ F, VANSUYT G, CASSE-DELBART F, and FOURCROY P. 1996. Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Plant Physiology* 97: 13–20.

MADHUSUDHAN R, ISHIKAWA T, SAWA Y, SHIGEOKA S, and SHIBATA H. 2003. Characterization of an ascorbate peroxidase in plastids of tobacco BY–2 cells. *Physiologia Plantarum* 117: 550–557.

MATHEWS MC, SUMMERS CB, and FELTON GW. 1997. Ascorbate peroxidase: a novel antioxidant enzyme in insects. *Archives of Insect Biochemistry and Physiology* 34: 57–68.

MEHLHORN H, COTTAM DA. LUCAS PW, and WELLBURN AR. 1987. Induction of ascorbate peroxidase and glutathione reductase activities by interactions of mixtures of air pollutants. *Free Radical Research Communications* 3: 193–197.

MEHLHORN H, LELANDAIS M, KORTH HG, and FOYER CH. 1996. Ascorbate is the natural substrate for plant peroxidases. *FEBS Letters* 378: 203–206.

MITTLER R, and ZILINSKAS B. 1991a. Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase. *FEBS Letters* 289: 257–259.

MITTLER R, and ZILINSKAS BA. 1991b. Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiology* 97: 962–968.

MITTLER R, and ZILINSKAS BA. 1992. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *Journal of Biological Chemistry* 267: 21802–21807.

MITTLER R, and ZILINSKAS BA. 1994. Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. *The Plant Journal* 5: 397–405.

MITTOVA V, GUY M, TAL M, and VOLOKITA M. 2004a. Salinity upregulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. *Journal of Experimental Botany* 55: 1105–1113.

MITTOVA V, THEODOULOU FL, KIDDLE G, VOLOKITA M, TAL M, FOYER CH, and GUY M. 2004b. Comparison of mitochondrial ascorbate peroxidase in the cultivated tomato, *Lycopersicon esculentum*, and its wild, salt-tolerant relative, *L. pennellii* – role for matrix isoforms in protection against oxidative damage. *Plant, Cell and Environment* 27: 237–250.

MIYAKE C, and ASADA K. 1996. Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxidase decomposes compound I of ascorbate peroxidase. *Plant and Cell Physiology* 37: 423–430.

MIYAKE C, MICHIHATA F, and ASADA K. 1991. Scavenging of hydrogen peroxide in procaryotic and eucaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria. *Plant and Cell Physiology* 32: 33–43.

MORI IC, and SCHROEDER JI. 2004. Reactive oxygen species activation of plant Ca^{2+} channels. A signalling mechanism in polar growth, hormone transduction, stress signalling and hypothetically mechanotransduction. *Plant Physiology* 135: 702–708.

Mullen RT, and Trelease RN. 2000. The sorting signals for peroxisomal membrane-bound ascorbate peroxidase are within its C-terminal tail. *Journal of Biological Chemistry* 275: 16337–16344.

Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, and Soave C. 2004. *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and nitric oxide-induced cell death. *The Plant Journal* 38: 940–953.

Nishikawa F, Kato M, Hyodo H, Ikoma Y, Sugiura M, and Yano M. 2003b. Ascorbate metabolism in harvested broccoli. *Journal of Experimental Botany* 54: 2439–2448.

Nishikawa F, Kato M, Wang R, Hyodo H, Ikoma Y, Sugiura M, and Yano M. 2003a. Two ascorbate peroxidases from broccoli: identification, expression and characterization of their recombinant proteins. *Postharvest Biology and Technology* 27: 147–156.

Noctor G, and Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* 49: 249–279.

Okuda A, Imagawa M, Maeda Y, Sakai M, and Muramatsu M. 1989. Structural and functional analysis of an enhancer GPE I having a phorbol 12-0-Tetradecanoate 13-Acetate responsive element-like sequence found in the rat glutathione transferase P gene. *Journal of Biological Chemistry and Molecular Biology* 264: 16919–16926.

Panchuk I, Zentgraf U, and Volkov RA. 2005. Expression of the Apx gene family during leaf senescence of *Arabidopsis thaliana*. *Planta* 222: 926–932.

Panchuk II, Volkov RA, and Schöffl F. 2002. Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis*. *Plant Physiology* 129: 838–853.

Park SY, Ryu SH, Jang IC, Kwon SY, Kim JG, and Kwak SS. 2004. Molecular cloning of a cytosolic ascorbate peroxidase cDNA from cell cultures of sweetpotato and its expression in response to stress. *Molecular Genetics and Genomics* 271: 339–346.

Pellinen RI, Minna-Sisko K, Tauriainen AA, Palva ET, and Kangasjä̈rvi J. 2002. Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiology* 130: 549–560.

Pereira CS, da Costa DS, Teixeira J, and Pereira S. 2005. Organ-specific distribution and subcellular localisation of ascorbate peroxidase isoenzymes in potato (*Solanum tuberosum* L.) plants. *Protoplasma* 226: 223–230.

Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, and Foyer CH. 2003. The function of ascorbate oxidase in tobacco. *Plant Physiology* 132: 1631–1641.

Rao MV, Paliyath G, and Ormrod DP. 1996. Ultraviolet-B- and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiology* 110: 125–136.

Rizhsky L, Davletova S, Liang H, and Mittler R. 2004. The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *Journal of Biological Chemistry* 279: 11736–11743.

Rizhsky L, Hallak-Herr E, Van Breusegem F, Rachmilevitch S, Barr JE, Rodermel S, Inze D, and Mittler R. 2002. Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. *The Plant Journal* 32: 329–342.

Sano S, Ueda M, Kitajima S, Takeda T, Shigeoka S, Kurano N, Miyachi S, Miyake C, and Yokota A. 2001. Characterization of ascorbate peroxidases from unicellular red alga *Galdieria partita*. *Plant and Cell Physiology* 42: 433–440.

Sharma P, and Dubey RS. 2004. Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes. *Plant Science* 167: 541–550.

Sharp KH, and Raven E. 2004. Enzyme – substrate interactions in ascorbate peroxidase. Department of Chemistry and Centre for Chemical Biology, University of Leicester, http://www.le.ac.uk/chemistry/research/chreel10.html#pub

Shi WM, Muramato Y, Ueda A, and Takabe T. 2001. Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. *Gene* 273: 23–27.

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, and Yoshimura K. 2002. Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany* 53: 1305–1319.

Shigeoka S, Nakano Y, and Kitaoka S. 1980a. Metabolism of hydrogen peroxide in *Euglena gracilis* Z by L-ascorbic acid peroxidase. *Biochemical Journal* 186: 377–380.

Shigeoka S, Nakano Y, and Kitaoka S. 1980b. Purification and some properties of L-ascorbic acid-specific peroxidase in *Euglena gracilis* Z. *Archives of Biochemistry and Biophysics* 201: 121–127.

Shin R, and Schachtman DP. 2004. Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proceedings of the National Academy of Sciences* 101: 8827–8832.

Storozhenko S, de Pauw P, van Montagu M, Inze D, and Kushnir S. 1998. The heat-shock element is a functional component of the *Arabidopsis* APX1 gene promoter. *Plant Physiology* 118: 1005–1014.

Takeda T, Shigeoka S, Hirayama O, and Mitsunaga T. 1992. The presence of enzymes related to glutathione metabolism and oxygen metabolism in *Chlamydomonas reihardtii*. *Bioscience, Biotechnology and Biochemistry* 56: 1662–1663.

Takeda T, Yoshimura K, Ishikawa T, and Shigeoka S. 1998. Purification and characterization of ascorbate peroxidase in *Chlorella vulgaris*. *Biochemie* 80: 295–301.

Takeda T, Yoshimura K, Yoshii M, Kanahoshi H, Miyasaka H, and Shigeoka S. 2000. Molecular characterization and physiological role of ascorbate peroxidase from halotolerant *Chlamydomonas* W80 strain. *Archives of Biochemistry and Biophysics* 376: 82–90.

Tanaka K, Suda Y, Kondo N, and Sugahara K. 1985. O_3 tolerance and the ascorbate-dependent hydrogen peroxide decomposing system in chloroplasts. *Plant and Cell Physiology* 26: 1425–1431.

Tang L, Kwon S-Y, Kim S-H, Kim J-S, Choi JS, Cho KY, Sung CHK, Kwak S-S, and Lee H-S. 2006. Enhanced tolerance

of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Reports* 25: 1380–1386.

TARANTINO D, VANNINI C, BRACALE M, CAMPA M, SOAVE C, and MURGIA I. 2005. Antisense reduction of thylakoidal ascorbate peroxidase in *Arabidopsis* enhances paraquat-induced photooxidative stress and nitric oxide-induced cell death. *Planta* 221: 757–765.

TEIXEIRA FK, MENEZES-BENAVENTE L, MARGIS R, and MARGIS-PINHEIRO M. 2004. Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: inferences from the rice genome. *Journal of Molecular Evolution* 59: 761–770.

VACCA RA, PINTO MC, VALENTI D, PASSARELLA S, MARRA E, and GARA L. 2004. Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. *Plant Physiology* 134: 1100–1112.

VANSUYT G, LOPEZ F, INZE D, BRIAT JF, and FOURCROY P. 1997. Iron triggers a rapid induction of ascorbate peroxidase gene expression in *Brassica napus*. *FEBS Letters* 410: 195–200.

VRANOVA E, INZE D, and BREUSEGEM FV. 2002. Signal transduction during oxidative stress. *Journal of Experimental Botany* 53: 1227–1236.

WADA N, KINOSHITA S, MATSUO M, AMAKO K, MIYAKE C, and ASADA K. 1998. Purification and molecular properties of ascorbate peroxidase from bovine eye. *Biochemical and Biophysical Research Communications* 242: 256–261.

YABUTA Y, MARUTA T, YOSHIMURA K, ISHIKAWA T, and SHIGEOKA S. 2004. Two distinct redox signalling pathways for cytosolic APX induction under photooxidative stress. *Plant and Cell Physiology* 45: 1586–1594.

YABUTA Y, MOTOKI T, YOSHIMURA K, TAKEDA T, ISHIKAWA T, and SHIGEOKA S. 2002. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. *The Plant Journal* 32: 915–925.

YOSHIMURA K, YABUTA Y, ISHIKAWA T, and SHIGEOKA S. 2000. Expression of spinach ascorbate peroxidase. *Plant Physiology* 123: 223–233.

YOSHIMURA K, YABUTA Y, ISHIKAWA T, and SHIGEOKA S. 1999. Alternatively spliced mRNA variants of chloroplast ascorbate peroxidase isoenzymes in spinach leaves. *Biochemical Journal* 338: 41–48.

ZAHARIEVA TB, and ABADIA J. 2003. Iron deficiency enhances the levels of ascorbate, glutathione, and related enzymes in sugar beet roots. *Protoplasma* 211: 269–275.

ZAMOCKY M. 2004. Phylogenetic relationships in class I of the superfamily of bacterial, fungal, and plant peroxidases. *European Journal of Biochemistry* 271: 3297–3309.

ZHANG X, ZHANG L, DONG F, GAO J, GALBRAITH DW, and SONG CP. 2001. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiology* 126: 1438–1448.