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Regulation and function of ascorbate peroxidase isoenzymes

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Abstract

Even under optimal conditions, many metabolic processes, including the chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems of higher plants, produce active oxygen species (AOS). Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess concentrations of AOS, resulting in oxidative damage at the cellular level. Therefore, antioxidants and antioxidant enzymes function to interrupt the cascades of uncontrolled oxidation in each organelle. Ascorbate peroxidase (APX) exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. APX is also found in eukaryotic algae. The characterization of APX isoenzymes and the sequence analysis of their clones have led to a number of investigations that have yielded interesting and novel information on these enzymes. Interestingly, APX isoenzymes of chloroplasts in higher plants are encoded by only one gene, and their mRNAs are generated by alternative splicing of the gene's two 3′-terminal exons. Manipulation of the expression of the enzymes involved in the AOS-scavenging systems by gene-transfer technology has provided a powerful tool for increasing the present understanding of the potential of the defence network against oxidative damage caused by environmental stresses. Transgenic plants expressing *E. coli* catalase to chloroplasts with increased tolerance to oxidative stress indicate that AOS-scavenging enzymes, especially chloroplastic APX isoenzymes are sensitive under oxidative stress conditions. It is clear that a high level of endogenous ascorbate is essential effectively to maintain the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses.

Key words: Ascorbate peroxidase, gene regulation, oxidative stress.

Introduction

In agreement with the fact that ascorbate (AsA) is a reductant of H_2O_2 in chloroplasts (Foyer and Halliwell, 1977), the occurrence of APX in plants (Groden and Beck, 1979; Nakano and Asada, 1981) and *Euglena* (Shigeoka *et al*., 1980*a*) shows that AsA is a very important reducing substrate for H_2O_2 detoxification in photosynthetic organisms. The code number (EC 1.11.1.11) for APX was first given on the basis of detailed information that was available about the enzymological properties of the *Euglena* enzyme (Shigeoka *et al*., 1980*a*, *b*).

APX utilizes AsA as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDAsA), a univalent oxidant of AsA (Fig. 1). MDAsA is spontaneously disproportionated to AsA and dehydroascorbate (DAsA). MDAsA is also directly reduced to AsA by the action of NAD(P)H-dependent MDAsA reductase. DAsA reductase utilizes glutathione (GSH) to reduce DAsA and thereby regenerate AsA. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD(P)H. Thus, APX in combination with the effective AsA–GSH cycle functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Asada, 1992, 1997). In the chloroplasts of higher plants,

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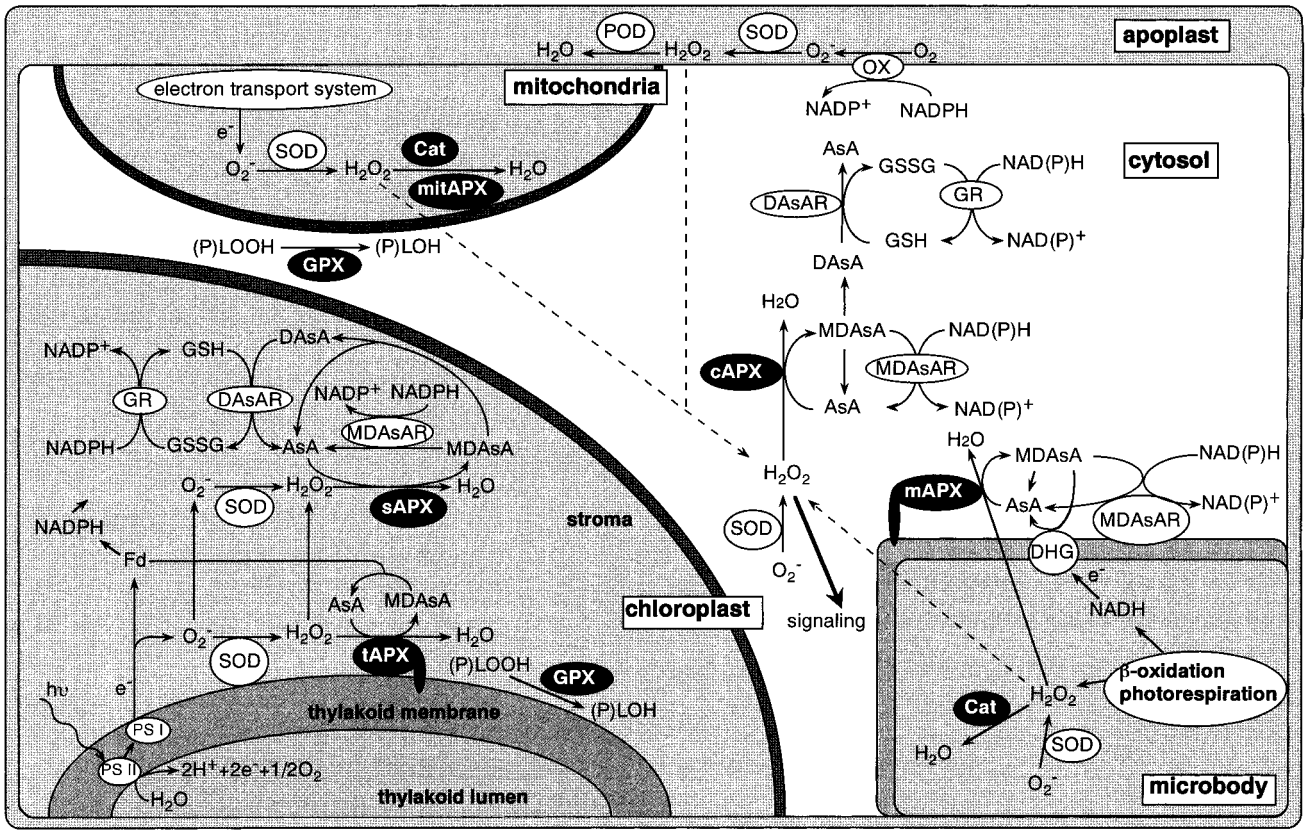

Fig. 1. Scavenging system of the active oxygen species in higher plants.

in addition to the AsA–GSH cycle located in the stroma the water–water cycle, which is the photoreduction of oxygen to water in PSI by the electrons derived from water in PSII, participates in the detoxification of AOS and the dissipation of the energy of excess photons (Asada, 1999).

To date, many reports on the purification, molecular cloning, and physiological function of APX isoenzymes based on enzymological and molecular approaches have been published; these studies indicate that APX isoenzymes are critical components that prevent oxidative stress in photosynthetic organisms. Additionally, recent studies on the response of APX expression to some stress conditions and pathogen attack indicate the importance of APX activity in controlling the H_2O_2 concentration in intracellular signalling.

In this review, the focus will be on recent molecular and physiological findings about APX isoenzymes in photosynthetic organisms such as higher plants and eukaryotic and prokaryotic algal cells. In fact, elucidation of the regulation mechanisms of APX isoenzymes in response to environmental stress is a subject of great interest with respect to APX research and will help to clarify the oxidative stress-tolerance responses of these organisms.

H_2O_2/hydroperoxide-scavenging enzymes in higher plants

APX has been identified in many higher plants and comprises a family of isoenzymes with different characteristics. Many isoenzymes of guaiacol peroxidase (GP) in plant tissues are localized in vacuoles, the cell wall, and the cytosol, but not in organelles (Asada, 1992). By contrast, APX isoenzymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), and cytosolic APX (cAPX) (Chen and Asada, 1989; Miyake *et al.*, 1993; Yamaguchi *et al.*, 1995*a*, *b*; Bunkelmann and Trelease, 1996; Ishikawa *et al.*, 1996*a*, *c*, 1998). A fifth APX isoenzyme (mitAPX) occurs in a mitochondrial membrane-bound form (Jiménez *et al.*, 1997; Leonardis *et al.*, 2000). Recently, the cDNA encoding a putative APX in the chloroplast thylakoid lumen of *Arabidopsis* was identified (Kieselbach *et al.*, 2000). APX has also been found in the protozoan *Trypanosoma cruzi* (Boveris *et al.*, 1980) and the bovine eye (Wada *et al.*, 1998). The finding of APX in the bovine eye suggests that APX may also

contribute, together with glutathione peroxidase (GPX: EC 1.11.1.9), to the detoxification of AOS in AsA-rich tissues of animals.

To date, cDNAs homologous to those of mammalian GPXs, especially phospholipid hydroperoxide GPX (PHGPX), have been found for higher plants, including *Citrus sinensis* (Holland *et al*., 1993), *Nicotiana sylvestris* (Criqui *et al*., 1992), and yeast (Tran *et al*., 1993). However, the corresponding genes carry a codon for a Cys residue at the putative catalytic site instead of the TGA codon for the selenocysteine (Sec) of mammalian GPXs. Furthermore, *Arabidopsis* has a homologue of thioredoxin peroxidase, which is known to be an anti-oxidative enzyme in animals and bacteria (Baier and Dietz, 1999).

Enzymatic properties of APX isoenzymes

The molecular and enzymatic properties of APX isoenzymes are different from those of other haem-peroxidases such as GP. APX isoenzymes have high specificity for AsA as the electron donor, which is especially the case for the chloroplastic APX (chlAPX) and mitAPX isoenzymes (Yoshimura *et al*., 1998; Asada, 1999; Leonardis *et al*., 2000). In addition to oxidizing AsA, cAPX and mAPX of higher plants and algal APXs can also oxidize artificial electron donors such as pyrogallol or guaiacol at appreciable rates (Chen and Asada, 1989; Ishikawa *et al*., 1995, 1996*b*; Yoshimura *et al*., 1998; Asada, 1999). Plant APX isoenzymes cannot reduce lipid hydroperoxides. APX, a haem-containing enzyme whose prosthetic group is protoporphyrin, is inhibited by cyanide and azide (Shigeoka *et al*., 1980*b*; Chen and Asada, 1989). APX is also inhibited by thiol-modifying reagents such as *p*-chloromercuribenzoate and suicide inhibitors such as hydroxylamine, *p*-aminophenol, and hydroxyurea (Chen and Asada, 1990). Since these suicide inhibitors do not affect GP, these reagents are useful for assays that differentiate between APX and GP (Amako *et al*., 1994). One of the most characteristic properties of APX is its instability in the absence of AsA. Under conditions where the concentration of AsA is lower than 20 μM, APX activity is rapidly lost. The half-inactivation times of chlAPX and mitAPX are less than 30 s, while those of cAPX and mAPX are about 1 h or more (Chen and Asada, 1989; Miyake *et al*., 1993; Ishikawa *et al*., 1998; Yoshimura *et al*., 1998; Leonardis *et al*., 2000). The instability of APX seems to be one reason that APX was not found for a long time in photosynthetic organisms. ChlAPX isoenzymes exist in a monomeric form, but cAPX is a dimer consisting of identical subunits with a molecular mass of 28 kDa (Mittler and Zilinskas, 1991*a*; Miyake *et al*., 1993). As for chlAPX isoenzymes, the molecular mass of tAPX is about 4.5 kDa larger than that of sAPX (33.2 kDa); the difference in molecular mass between tAPX and sAPX is related to the requirement for membrane binding (Chen and Asada, 1989; Miyake *et al*., 1993; Ishikawa *et al*., 1996*a*). The molecular masses (31 kDa) of mAPX and mitAPX are similar (Yamaguchi *et al*., 1995*a*; Ishikawa *et al*., 1998; Leonardis *et al*., 2000).

H_2O_2/hydroperoxide-scavenging systems in algae

Both eukaryotic and prokaryotic algae produce H_2O_2 via O_2^-, as do higher plants (Patterson and Myers, 1973; Miyake *et al*., 1991; Ishikawa *et al*., 1993; Collén *et al*., 1995). Algal APX isoenzymes have enzymological and immunological properties similar to those of APX isoenzymes from higher plants except for the amino acid sequence of the N-terminus, the affinity for substrates, and/or their stability. *Euglena gracilis* lacks catalase and contains the enzymes involved in the AsA–GSH cycle, including APX, solely in the cytosol (Shigeoka *et al*., 1980*a*; Ishikawa *et al*., 1996*b*). *Chlorella vulgaris* contains APX as well as catalase (Takeda *et al*., 1998). *Galdieria partita*, a unicellular red alga, has at least two APX isoenzymes (Sano *et al*., 2001). Fresh water *Chlamydomonas reinhardtii* C9 (*C*. C9) contains catalase, APX, and selenium-induced GPX (Yokota *et al*., 1988; Takeda *et al*., 1992). Halotolerant *Chlamydomonas* sp. W80 (*C*. W80) contains catalase, APX, and a selenium-independent GPX-like protein (Takeda *et al*., 2000).

In *C*. C9 cells grown in the absence of selenium, APX functions mainly to scavenge H_2O_2 in chloroplasts (Shigeoka *et al*., 1991; Takeda *et al*., 1992). When the *C*. C9 cells are transferred to medium containing sodium selenite, APX is completely lost after 6 h, and the GPX activity appears and gradually increases until 24 h (Takeda *et al*., 1993). Recently, a cDNA encoding a GPX-like protein was isolated from *C. reinhardtii* $cw_{15}arg_7mt$-(CC-325) (Leisinger *et al*., 1999). The GPX-like protein contained a normal Cys residue, instead of a selenocysteine, which is critical for the activity of the reactive centre in cytosolic GPX from mammals. Accordingly, it seems likely that *Chlamydomonas* cells contain both a selenium-dependent GPX and a selenium-independent GPX-like protein.

Cyanobacteria contain catalase-peroxidase (CPX) and thioredoxin peroxidase (Mutsuda *et al*., 1996; Baier and Dietz, 1999; Yamamoto *et al*., 1999). APX activity has been detected in some prokaryotic algae (Miyake *et al*., 1991). However, the corresponding protein and gene for APX have not yet been identified. The absence or low level of AsA may account for the lack of APX in these organisms. *Synechocystis* PCC 6803 cells have two *gpx* (GPX-1 and GPX-2) homologue genes (slr1171 and slr1992 according to CyanoBase) with significant similarity to the PHGPX gene in mammals. Interestingly, both recombinant proteins in *E. coli* are able to utilize

NADPH, but not GSH, as an electron donor and alkyl hydroperoxide or unsaturated fatty acid hydroperoxides like α-linolenic acid hydroperoxide as an acceptor (Gaber *et al*., 2001). It seems accurate to refer to GPX-1 and GPX-2 as NADPH-dependent GPX-like proteins that serve as a novel defence system for the reduction of unsaturated fatty acid hydroperoxides.

The photosynthesis of *Euglena*, *Chlamydomonas*, and some cyanobacteria, including *Synechococcus* PCC 7942 and *S*. PCC 6803, is not susceptible to H_2O_2 up to 1 mM, as a result of the resistance of fructose-1,6-/sedoheptulose-1, 7-bisphosphatase, $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase, and phosphoribulokinase in the Calvin cycle to H_2O_2 (Takeda *et al*., 1995; Tamoi *et al*., 1996*a*, *b*, 1998*a*, *b*, 2001). H_2O_2 formed in chloroplasts and mitochondria from algal cells diffuses from each organelle to the cytosol, where it is decomposed by H_2O_2-scavenging enzymes (Ishikawa *et al*., 1993). A similar finding has also been obtained in several cyanobacteria as well as *Ulva rigida* C. Ag (Collén *et al*., 1995). It is concluded that the H_2O_2-scavenging, H_2O_2-diffusion, and H_2O_2-tolerance systems in algal cells function co-operatively to protect cellular components against oxidative stress caused by AOS.

Characterization of cDNAs encoding APX isoenzymes

The cDNAs encoding APX isoenzymes from plant species have been isolated and characterized by several research groups (Mittler and Zilinskas, 1991*b*; Ishikawa *et al*., 1995, 1996*a*; Bunkelmann and Trelease, 1996). The cDNA encoding mitAPX has not been isolated yet. Higher plants contain two or more putative cAPXs, indicating that cAPX isoenzymes are encoded by a multigene family (Santos *et al*., 1996; Jespersen *et al*., 1997). In spinach, cAPX2 (SAP1), which appears to belong to a second family of cAPX-related enzymes, has been cloned and characterized (Ishikawa *et al*., 1995); however, neither the protein nor the activity of cAPX2 has been detected in crude homogenates of spinach leaves, although its mRNA is constitutively expressed (Yoshimura *et al*., 2000). Thus, the expression of cAPX2 seems to be suppressed by translational regulation. In *Mesembryanthemum crystalinum*, cDNAs encoding three types of putative cAPXs have been isolated; however, their corresponding proteins have not been identified yet. cDNA clones encoding APXs localized in the stroma of *C*. C9 and *C*. W80 were recently isolated (Takeda *et al*., 2000).

Interestingly, the N-terminal 364 amino acids of spinach sAPX are completely identical with those of tAPX, whereas the C-terminal 50 amino acids differ (Ishikawa *et al*., 1996*a*). A similar finding was also made in pumpkin (Mano *et al*., 1997), *M*. *crystalinum*, and tobacco. These chlAPX isoenzymes are encoded by only one gene (*APXII*), and their mRNAs are generated by alternative splicing of the gene's two 3′-terminal exons as described below. By contrast, in *Arabidopsis thaliana*, the nucleotide sequences of the cDNAs for sAPX and tAPX share only 66.1% identity and these two isoenzymes are encoded by different genes (Jespersen *et al*., 1997).

chlAPX isoenzymes have a transit peptide consisting of approximately 70 residues in their N-terminus. This peptide contains few acidic residues and is rich in Ser and Thr, thus resulting in a net positive charge (Ishikawa *et al*., 1996*a*; Yamaguchi *et al*., 1996; Jespersen *et al*., 1997). In all sequences of transit peptides for import across the chloroplast envelopes, position–2 relative to the cleavage site is occupied by a Lys residue; however the functional significance of this is unknown (Jespersen *et al*., 1997). tAPX has one major hydrophobic domain which is responsible for spanning to the stroma-exposed thylakoid membranes in chloroplasts. A similar membrane-spanning region in the C-terminal region is present in mAPX and is bound to the external side of the membrane of glyoxysomes (Yamaguchi *et al*., 1995*b*; Bunkelmann and Trelease, 1996; Ishikawa *et al*., 1998). This fact indicates that the physiological function of mAPX is to scavenge H_2O_2 generated both in microbodies and the cytosol in combination with catalase and cAPX (Yamaguchi *et al*., 1995*b*; del Río *et al*., 1998; Ishikawa *et al*., 1998). The transmembrane domain within mAPX functions as an overlapping peroxisomal endoplasmic reticulum-sorting signal and a peroxisomal membrane-targeting signal type 2 (Mullen and Trelease, 2000).

Evolution of APX isoenzymes

On the basis of the amino acid sequences, plant peroxidases can be classified into three well-separated classes (Welinder, 1992). Class I includes the intracellular peroxidases of prokaryotic origin, Class II the fungal peroxidases, and Class III the classical plant peroxidases. APX, yeast cytochrome *c* peroxidase (CCP) and cyanobacterium CPX belong to the Class I family (Welinder, 1992; Mutsuda *et al*., 1996; Zámock'y *et al*., 2000). On the other hand, GP belongs to the Class III family, which constitutes a separate lineage from APX. A novel Class III peroxidase in tea leaves showed high specificity for AsA as an electron donor (Kvaratskhelia *et al*., 1997).

A phylogenetic tree constructed using the deduced amino acid sequences of the catalytic active domains of APX isoenzymes, not including the transit peptide and the membrane-spanning sequences, shows that APX isoenzymes in higher plants and algae can be divided into four groups (cAPX I, cAPX II, chlAPX, and mAPX) (Fig. 2). The earliest event in APX evolution resulted in the appearance of separate groups: a cAPX I group, a

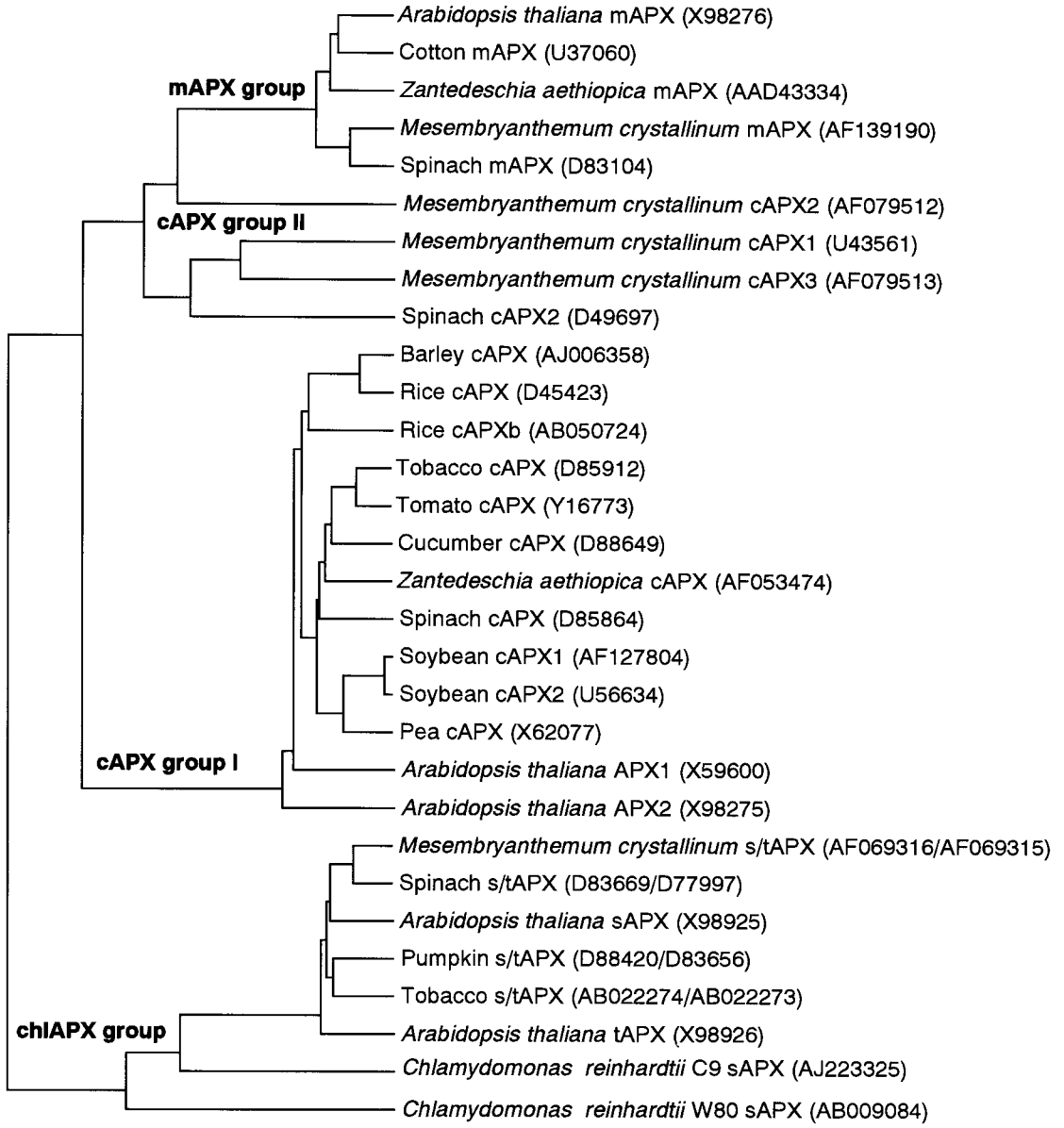

Fig. 2. Phylogenic tree for APX isoenzymes.

chlAPX group, and mAPX and cAPX II groups. It is likely that cAPX I, chlAPX, and mAPX share common features conserved among plant species, whereas the cAPX II group may have evolved from cAPX in a species-specific manner. chlAPX isoenzymes are divided into two types that arose by alternative splicing from a single gene and by different genes at a very recent stage.

APX isoenzymes in higher plants show high homology (70–90%) within each group. Furthermore, the four groups of APX isoenzymes show 50–70% homology with each other. The Arg-38, Asn-71, Glu-65, and Asp-208 residues around the distal His-42 and proximal His-163 residues of pea cAPX are conserved in all APX groups as well as the entire plant peroxidase family. These residues are essential for binding of the ligand haem (Welinder, 1992). Trp-179 is conserved in most APX groups and is the third participant in a hydrogen-bonding network together with the proximal His-163 and Asp-208 residues; however, the residue in the cAPX II group is changed to Phe, which is the predominant residue at this position in the Class II and Class III peroxidases of higher plants (Jespersen *et al.*, 1997). The change from Trp-41 to Phe is also found in the cAPX II group as well as in Class II and III peroxidases. Phe-175 is conserved in all APX groups except for a Trp in the chlAPX group, suggesting that the Trp residue may be the major determinant of the greater specificity toward AsA of chlAPX isoenzymes. Four additional regions in the internal

sequences and seven residues in the C-terminal extension of the catalytic domain are observed in chlAPX isoenzymes, but not in the other APX isoenzymes. Therefore, chlAPX isoenzymes generally have larger molecular weights than cAPX and mAPX, suggesting that these additional sequences may be associated with the greater instability of chlAPX isoenzymes.

Structural investigations of the catalytic domain

The instability of APX isoenzymes seems to be one of the reasons for the difficulty in obtaining large amounts of highly purified APX isoenzymes. Therefore, advances in the expression of the recombinant APX isoenzymes utilizing cDNA clones have provided new approaches for characterizing the structure and function of each APX isoenzyme (Raven, 2000). Some functional amino acid residues which are related to the binding of haem and AsA have been identified by mutation analysis using recombinant pea cAPX. Arg-172 of pea cAPX has an important role in AsA utilization to form Compound II (Bursey and Poulos, 2000). Glu-112 is located at the dimer interface, and is related to an alteration in solvent structure (Mandelman *et al*., 1998*a*). Arg-38 has a functional role in the control of substrate binding and orientation (Celik *et al*., 2001). The active site structures, including the hydrogen-bonding interactions between the proximal His-163, buried Asp-208, and Trp-179, are nearly identical to those of CCP. On the other hand, there are many structural differences between CCP and APX, including the cation binding site and orientations of the vinyl substituents on the haem chromophore (Patterson and Poulos, 1994, 1995; Nissum *et al*., 1998). AsA oxidation does not occur at the exposed haem edge but at an alternate binding site in the vicinity of Cys-32 and the haem propionates (Mandelman *et al*., 1998*b*).

Antibodies raised against APX isoenzymes

Monoclonal antibodies have been developed against spinach sAPX (chl-mAb6) and cAPX (cyt-mAb1) isoenzymes and *Euglena* cAPX (EAP1 and EAP2) (Ishikawa *et al*., 1996*b*; Yoshimura *et al*., 2001). Each spinach APX antibody reacts specifically with the respective isoenzyme from higher plants, but none cross-react with the others. On the other hand, EAP1 reacts with both chlAPX isoenzymes and cAPX from higher plants, although EAP2 shows much higher cross-reactivity with cAPX than with sAPX (Ishikawa *et al*., 1996*b*). EAP1 recognizes the common epitope at the site around the proximal His residue in APX isoenzymes, while EAP2 recognizes a relatively homologous region downstream of the distal His residue in the APX isoenzymes. Cyt-mAb1 and chl-mAb6 recognize the distal His region of cAPX and the inherent region of chlAPX isoenzymes, respectively.

The potential of the defence system involving APX isoenzymes to oxidative stress

The rate of O_2^- and H_2O_2 formation within intact chloroplasts under optimal conditions is estimated to be 240 and 120 $\mu M\ s^{-1}$, respectively (Asada and Takahashi, 1987). SOD isoenzymes and H_2O_2-sacavenging enzymes are preferentially located near the site of O_2^- production (Miyake and Asada, 1992; Ogawa *et al*., 1995). As a consequence, it is estimated that the H_2O_2 concentration is less than 8×10^{-7} M under normal conditions (Asada, 1999). Under photo-oxidative conditions, the actual level of H_2O_2 must increase several orders of magnitude with respect to that estimated for non-stressed chloroplasts, especially within the sites of localization of antioxidant enzymes. When the AOS production far exceeds the endogenous AOS-scavenging capacity, the regulated balance between the generation system and the scavenging system of AOS may be upset, leading to the inactivation of defence enzymes. Thus, O_2^- and H_2O_2 must be completely scavenged to preserve photosynthetic activity.

A characteristic property of APX isoforms is their lability in the absence of their electron donor, AsA (Nakano and Asada, 1981; Chen and Asada, 1989). In the presence of AsA, the reaction intermediate of APX Compound I successively oxidizes AsA to produce two molecules of MDAsA in the catalytic cycle. However, unless Compound I of APX is reduced by AsA, it is oxidized and degraded to the inactivated form by 10 nM levels of H_2O_2 within 2 min, levels which are generated via auto-oxidation of AsA at micromolar levels. Cu/Zn-SOD is inactivated by H_2O_2 with an apparent rate constant of 0.8 $M^{-1}\ s^{-1}$; the half-time for the inactivation is about 30 min in 0.4 mM H_2O_2 at neutral pH (Asada *et al*., 1975; Casano *et al*., 1997). Stromal Fe-SOD is also inactivated by H_2O_2 at a similar rate of 0.6 $M^{-1}\ s^{-1}$. H_2O_2 reduces the Cu(II) and then reacts with the Cu(I) to give a $\cdot OH$ at the reaction centre of the enzyme. The $\cdot OH$ oxidizes His-118 of the active site of the enzyme to 2-oxo-His, resulting in the inactivation and fragmentation of the radical (Casano *et al*., 1997). DAsA reductase is labile in the absence of thiols and inactivated by 0.5 mM H_2O_2 (Hossain and Asada, 1984). GSH reductase is inactivated by NADPH in the absence of GSSG (Lascano *et al*., 1998). Thus, based on the data reported so far, it seems likely that the AOS-scavenging enzymes themselves show sensitivity to oxidative stress.

Manipulation of the expression of enzymes involved in the AOS-scavenging systems has focused on SOD, APX,

and GR isoenzymes that are targeted into the cytosol or plastids (Sen Gupta *et al*., 1993; Pitcher *et al*., 1994; Aono *et al*., 1995; Foyer *et al*., 1995; Slooten *et al*., 1995; Webb and Allen, 1996). The modification of AOS-scavenging systems can lead to considerable changes in oxidative stress tolerance (Allen, 1997). To evaluate the potential of the AOS-scavenging system of chloroplasts to respond to photo-oxidative stress imposed by drought at high light intensity or paraquat treatment under illumination, *E. coli* catalase encoded by *katE* was introduced into tobacco chloroplasts (Shikanai *et al*., 1998; Miyagawa *et al*., 2000). Under high light and drought conditions (1600 μmol m^{-2} s^{-1} without watering), the degradation of chlorophyll in the leaves of wild-type plants was detectable after 24 h, and severe chlorosis occurred at 72 h. However, transgenic plants did not show any chlorosis for at least 96 h under the stress conditions. Furthermore, when sprayed with 50 μM paraquat and exposed to high light intensity (300 μmol m^{-2} s^{-1} or 1600 μmol m^{-2} s^{-1}), wild-type plants developed visible severe leaf injury after 24 h, while the transgenic plants did not exhibit any signs of chlorosis. These results demonstrate that transgenic tobacco plants have increased tolerance to photo-oxidative damage imposed by AOS. Interestingly, the chlAPX isoenzymes in the wild-type and transgenic plants were completely inactivated under the stress conditions, while PRK remained active. The total SOD activity in the control plants dramatically decreased, while, in the transgenic plants, the initial activity of SOD was retained. These data suggest that AOS-scavenging enzymes, especially chlAPX isoenzymes, are much more strongly inactivated by oxidative stress than PRK, which is believed to be one of the thiol-modulated enzymes that is most sensitive to H_2O_2. chlAPX isoenzymes have been found to be the primary targets among the H_2O_2-sensitive enzymes under paraquat-induced photo-oxidative stress in spinach leaves (Mano *et al*., 2001).

Why are chlAPX isoenzymes sensitive to photo-oxidative stress in both plant lines? Concentrations of H_2O_2 as low as 2 μM inactivate chlAPX isoenzymes within several seconds when the level of AsA is too low for the catalytic cycle of the APX isoenzymes to function (Miyake and Asada, 1996). A significant decrease in the total AsA level is in agreement with the decreases in the activities of chlAPX isoenzymes in the wild-type and transgenic plants with stress treatments (Shikanai *et al*., 1998; Miyagawa *et al*., 2000). When tobacco leaves suffer from photo-oxidative stress, the level of AsA in the vicinity of chlAPX isoenzymes may decrease due to the excess of AOS generated by paraquat treatment, which causes irreversible damage of chlAPX isoenzymes. Total APX activity and AsA content show identical reductions in pea plants under stress conditions induced by severe water deficit or paraquat (Iturbe-Ormaetxe *et al*., 1998). The *Arabidopsis* mutant *vtc1* is deficient in AsA, accumulating approximately 30% of wild-type levels, and is more sensitive than the corresponding wild-type to stress conditions such as ozone, UV-B light, and SO_2, which generate AOS (Conklin *et al*., 1997). In spinach leaves, the activities of the chlAPX isoenzymes decrease as the intensity of light increases, despite the fact that the transcript and protein levels of these isoforms are not altered (Yoshimura *et al*., 2000). A reduction in APX activity is observed in *Lupinus arbustus* seedlings, in which AsA deficiency is induced by lycorine treatment (Arrigoni *et al*., 1997). Application of the AsA precursor L-galactono-γ-lactone increases the AsA level of water-stressed leaves of wheat, enhances photochemical and non-photochemical quenching of chlorophyll fluorescence, and thus reduces oxidative damage to their photosynthetic apparatus (Tambussi *et al*., 2000). Based on the data accumulated thus far, it is clear that a high level of endogenous AsA is essential for effectively maintaining the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses.

Regulation of APX expression

Change in APX activity

Recent studies have focused on the changes in activities of APX isoenzymes in higher plants subjected to several environmental stresses such as ozone, high light, extremes of temperature, salt, and paraquat (Tanaka *et al*, 1985; Mittler and Zilinskas, 1994; Prasad *et al*., 1994; Conklin and Last, 1995; Rao *et al*., 1996; Dat *et al*., 1998; López *et al*., 1996; Donahue *et al*., 1997; Yoshimura *et al*., 2000). Interestingly, APX activities generally increase along with activities of other antioxidant enzymes like catalase, SOD, and GSH reductase in response to various environmental stress factors, suggesting that the components of AOS-scavenging systems are co-regulated. Drought-resistant maize shows greater induction of APX activity than sensitive plants, in addition to a significant increase in GSH reductase activity (Pastori and Trippi, 1992). An *Arabidopsis* mutant (*pst1*) isolated based on its ability to grow photoautotrophically on a NaCl-containing medium showed significantly enhanced APX and SOD activities (Tsugane *et al*., 1999). The *pst1* also showed cross-resistance to other stress conditions, suggesting that enhanced enzyme activities might be conferred by the mutation of a recessive nuclear gene and that regulation of these enzyme levels provides plants with additional stress-defence capabilities. Transgenic tobacco plants expressing antisense RNA for cAPX show 45–55% less activity than the non-transgenic plants, and increased susceptibility to ozone (Övar and Ellis, 1997).

Change in APX transcript level

Arabidopsis fumigated by ozone shows a large increase in the steady-state transcript level of cAPX (Conklin and Last, 1995; Övar *et al*., 1997). The transcript level of pea cAPX also increases 4-fold in response to drought stress, but is even more dramatically enhanced (15-fold) following recovery from stress (Mittler and Zilinskas, 1994). The analysis of the protein level and activity of APX indicates that, during recovery from drought stress, cAPX expression in pea is regulated at the post-transcriptional level, at least in part at the level of protein synthesis. Furthermore, many environmental factors such as high light, salt, wounding, pathogen infection, fruit ripening, and paraquat affect the steady-state transcription level of cAPX (Mittler and Zilinskas, 1992; Pastori and Trippi, 1992; Schantz *et al*, 1995; Karpinski *et al*., 1997, 1999; Övar *et al*., 1997; Biemelt *et al*., 1998; Mittler *et al*., 1998, 1999; Morita *et al*., 1999; Yoshimura *et al*., 2000). A simultaneous analysis of the response of each APX isoenzyme in spinach leaves to several stress conditions has been carried out (Yoshimura *et al*., 2000). Among the APX isoenzymes (chlAPX, two cAPXs, and mAPX) tested, the steady-state transcript level of the typical cAPX isoenzyme markedly increased in response to high light stress and paraquat treatment, but not in response to other stress treatments such as drought and salt. The transcript levels of the total chlAPX and mAPX did not change in response to any of the stress treatments. These results indicate that the genes for chlAPX and mAPX are constitutively expressed for the immediate and efficient detoxification of H_2O_2 under normal and stress conditions, while the gene expression for cAPX is responsive to environmental changes, resulting in the protection of important cellular compartments from oxidative stress and in strict control of the level of H_2O_2 in intracellular signalling.

Signal for APX expression

Recently, many researchers have focused on other functional aspects of H_2O_2 generation. The increases in H_2O_2 levels are rapid and transient and are thought to constitute a general signal indicating cellular stress (Foyer *et al*., 1997). H_2O_2 levels increase in plant cells after exposure to various environmental stress conditions (Okuda *et al*., 1991; O'Kane *et al*., 1996; Foyer *et al*., 1997; Karpinski *et al*., 1997; Dat *et al*., 1998; Yoshimura *et al*., 2000). Plants treated with low concentrations of H_2O_2 can develop stress tolerance (Prasad *et al*., 1994; Matsuda *et al*., 1994). More recent reports demonstrated a link between H_2O_2 and a MAP kinase pathway in plants, supporting the idea that H_2O_2 acts as a signal during stress (Desikan *et al*., 1999; Kovtun *et al*., 2000; Samuel *et al*., 2000).

Interesting observations regarding regulation of APX gene expression by H_2O_2 have been reported in the last few years. Treatment of cultured soybean cells with exogenous H_2O_2 resulted in the alteration of cAPX transcription levels (Lee *et al*., 1999). Furthermore, the treatment of cultured rice cells with hydroxyurea, a suicide inhibitor of APX, or aminotriazole, an effective inhibitor of catalase, led to increased cellular H_2O_2 content and a large increase in the cAPX transcript level (Morita *et al*., 1999). These results support the idea that cAPX gene expression is up-regulated in response to cellular H_2O_2 levels. The accumulation of H_2O_2 via redox signal transduction may play an important role in cAPX gene expression regulation under some stress conditions. In addition to H_2O_2 accumulation, during high light stress, redox changes in electron transport through plastoquinone may be essential for the induction of *APX2*, a member of the cAPX gene family, in *Arabidopsis* leaves (Karpinski *et al*., 1999; Karpinska *et al*., 2000).

Upon pathogen attack, AOS including O_2^-, H_2O_2, and nitric oxide can help to induce cell death in infected cells or serve as a signal to activate defence responses in distant uninfected cells. cAPX expression in TMV-infected tobacco leaves is suppressed by inhibition of protein synthesis in the polysomes (Mittler *et al*., 1998). Transgenic antisense tobacco with reduced APX suggests that the suppression of APX plays a key role in elevating cellular H_2O_2 levels and results in enhanced cell death in response to pathogen attack (Mittler *et al*., 1999). A recent report that nitric oxide and peroxynitrite derived from nitric oxide inhibit APX and catalase activities strongly supports the possibility that these enzymes contribute to controlling cellular H_2O_2 levels (Clark *et al*., 2000). Prolonged accumulation of toxic amounts of H_2O_2 within plant cells is eventually lethal. It is likely that the regulation of APX expression is part of a system for regulating the balance between the detrimental and beneficial roles of H_2O_2 in plant cells.

The gene encoding APX isoenzymes in higher plants

The cAPX genes from pea, *Arabidopsis*, tomato, and strawberry have been isolated and characterized (Mittler and Zilinskas, 1992; Kubo *et al*., 1993; Santos *et al*., 1996; Kim and Chung, 1998; Gadea *et al*., 1999). The pea cAPX gene (*APX1*) contains nine introns, the first of which is located within the 5′-untranslated region. Similar observations have also been made in those of other plant species, except for the *Arabidopsis APX1b* gene encoding a second family of cAPX. The expression of cAPX is regulated in response to several oxidative stress factors, indicating the presence of some functional *cis*-regulatory elements in the promoter. A putative

heat-shock *cis*-element is present in the cAPX genes from all plant species and is associated with the *in vivo* induction of the gene (*APX1*) from *Arabidopsis* (Storozhenko *et al*., 1998). The expression of the cAPX gene in rice is mediated by high temperature; furthermore, rice seedlings previously subjected to high temperature show increased tolerance to chilling stress (Sato *et al*., 2001). A functional G/C-rich element, which is essential for ethylene induction, is found in the APX1 promoter (Storozhenko *et al*., 1998). Furthermore, the leader intron in the 5′-untranslated region of the tomato cAPX gene is required to confer the constitutive gene expression (Gadea *et al*., 1999).

The *APXII* gene, which encodes spinach chlAPX isoenzymes and is present in only one copy, consists of 13 exons split by 12 introns. The intron–exon splice junctions conform to the consensus sequences GT at the donor site and AG at the acceptor site (Brown, 1986). Exons 1–11 encode the common amino acid sequence for sAPX and tAPX isoenzymes. The important point to note regards the two 3′-terminal exons. Two splice acceptor sites occur in exons 12 (penultimate) and 13 (final) separated by 14 bp nucleotides. The penultimate exon 12 consists of one codon for Asp-365 before the TAA termination codon and the entire 3′-untranslated region including a potential polyadenylation signal (AATAAA) of the sAPX mRNA. The final exon 13 contains the corresponding coding sequence of the hydrophobic C-terminal region, the TGA termination codon, and the entire 3′-untranslated region, including a potential polyadenylation signal (AATATA) of the tAPX mRNA. These facts show that the mRNAs for the chlAPX isoenzymes arise from only one gene by alternative use of the two final exons. The same mechanism has also been observed in the cases of pumpkin, *M. crystallinum*, and tobacco chlAPX isoenzymes, indicating that the alternative splicing of chlAPX isoenzymes is a common regulation mechanism in higher plants (Mano *et al*., 1997; K Yoshimura and S Shigeoka, unpublished data).

Generation of chloroplastic APX isoenzymes by alternative mRNA splicing

Alternative splicing pattern

By alternative processing of the 3′-terminal region as a result of alternative polyadenylation and splicing, four types of mature mRNA variants, one form encoding tAPX (tAPX-I) and three forms (sAPX-I, sAPX-II, sAPX-III) encoding sAPX, are produced as mature and functional forms, transported from the nucleus to the cytoplasm, and then incorporated into polysomes (Yoshimura *et al*., 1999). When the AAUAAA signal in exon 12 is selected, the only resulting product after the splicing of intron 11 should be sAPX-I mRNA. In contrast, the selection of the AAUAUA signal in exon 13 causes more complexity and, as a result, three mRNA variants (sAPX-II, -III, and tAPX-I) are produced by the alternative excision of intron 11 or intron 12 (Fig. 3).

Alternative splicing is a common mechanism of gene regulation at the post-transcriptional stage in eukaryotic organisms (McKeown, 1992; Simpson and Filipowicz,

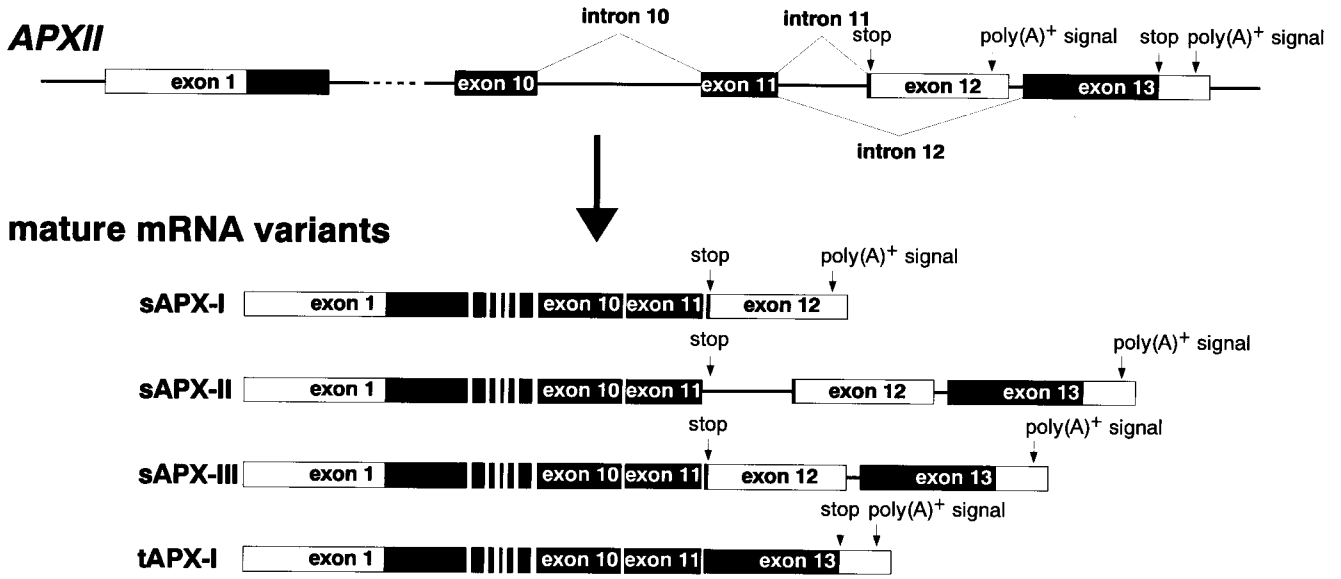

Fig. 3. Alternative splicing patterns producing spinach chlAPX mRNA variants. Exon regions are shown as boxes and introns as lines. The open reading frame and untranslational regions are indicated by black and white boxes, respectively. Functional stop codons and polyadenylation signals are indicated.

1996). The production of heterogeneity in the amino acid sequences by various regulation systems such as alternative splicing, 3′-end cleavage, and polyadenylation of mRNA precursors is well documented in animal systems. In higher plants, production of heterogeneous C-terminals by alternative splicing which induces an in-frame shift of the open reading frame has been shown for spinach ribulose bisphosphate carboxylase/oxygenase (Rubisco) activase and pumpkin hydroxypyruvate reductase (Werneke *et al*., 1989; Hayashi *et al*., 1996).

Regulatory mechanism of alternative splicing of chlAPX isoenzymes

Some alternative splicing events are constitutive, with similar ratios of mRNA variants in different cells, whereas others are subject to tissue-specific or developmental regulation (McKeown, 1992). In higher plants, a number of cases of alternative splicing have been reported, although the biological significance of the isoforms produced is unknown (Simpson and Filipowicz, 1996). It is likely that a few alternative splicing events are regulated by unique exogenous factors specific to plants. In the case of Rubisco activase, although two isoforms produced by alternative splicing can activate Rubisco, only the larger one is redox-regulated (Rundle and Zielinski, 1991). In the case of pumpkin hydroxypyruvate reductase, which produces the microbody and cytosol isoenzymes by alternative splicing, the production of the cytosol type is greatly enhanced by light (Mano *et al*., 1999).

In the case of chlAPX isoenzymes, it seems likely that the expression ratio of tAPX to sAPX changes according to the plant species (Miyagawa *et al*., 2000; Yoshimura *et al*., 2000). However, known *cis*-elements that might regulate the transcription level have not been found in the promoter of the *APXII* gene (Ishikawa *et al*., 1997), and the amount of the chlAPX transcript is not changed in mature spinach leaves under various stress conditions (Yoshimura *et al*., 2000). Furthermore, even in non-photosynthetic tissues such as root, the chlAPX gene is expressed, and almost equal amounts of chlAPX transcripts are detected in the leaf, stem, and root of mature plants (K Yoshimura and S Shigeoka, unpublished data). However, the expression ratio of sAPX mRNAs to tAPX mRNA variants is extremely elevated in stem and root as a result of an increase in sAPX-III mRNA and a decrease in tAPX-I mRNA (K Yoshimura and S Shigeoka, unpublished data), suggesting that the efficiency of the polyadenylation site selection in the alternative splicing event of the 3′-terminal region in chlAPX pre-mRNA is not modulated in a tissue-specific manner. Therefore, it is likely that the alternative splice-site selection in the alternative splicing event of chlAPX pre-mRNA is a major regulatory mechanism for changing the expression ratio of chlAPX isoenzymes in each tissue, because sAPX-III and tAPX-I mRNAs are produced by the alternative excision of intron 11 or intron 12. In addition to this observation, the patterns of accumulation of sAPX and tAPX isoenzymes in pumpkin cotyledons were different during germination versus subsequent greening, indicating that light regulates the alternative splicing of chloroplastic APX isoenzymes (Mano *et al*., 1997).

What *cis*-elements and *trans*-acting regulators are involved in alternative splicing to determine the level of each mRNA variant for chlAPX isoenzymes? In higher plants, not much is known about the mechanisms underlying alternative splicing events. Judging from the data reported so far, the regulatory factors for alternative splicing in higher plants may also have unique features because the recognition of introns in higher plants differs from that in yeast and vertebrates (Lorkovi'c *et al*., 2000; Reed, 2000). The 3′-terminal regions of the chlAPX gene involved in the alternative splicing event show high homology; in addition, a putative splicing regulatory *cis*-element (SRE) in the region immediately upstream of exon 13 is highly conserved among spinach (Ishikawa *et al*., 1997), pumpkin (Mano *et al*., 1997), and tobacco (K Yoshimura and S Shigeoka, unpublished data). It is likely that the binding of a splicing-enhancing *trans*-acting factor to the SRE region in chlAPX pre-mRNA may induce an increase in the splicing efficiency of intron 12, leading to the enhancement of the production of the tAPX-I mRNA. The identification of a *trans*-acting factor is currently being attempted in the authors' laboratory.

Future prospects

Photosynthetic organisms including higher plants and eukaryotic algae have developed AOS-scavenging systems, including APX isoenzymes. Cloning of cDNAs and genes encoding APX isoenzymes has facilitated a diverse rage of molecular and physiological studies on these isoenzymes. Prokaryotic cyanobacteria have an AOS-scavenging system as well as an H_2O_2-tolerance system of the Calvin cycle and a H_2O_2-diffusion system. APX isoenzymes are expressed by distinct regulatory mechanisms in response to various environmental stresses or cell conditions, and play a co-operative role to protect each organelle and minimize tissue injury. The regulatory mechanism of the expression of chlAPX isoenzymes via alternative splicing will provide new outlooks on plant gene expression because little is known about the mechanisms of post-transcriptional regulation in plant cells (Lorkovi'c *et al*., 2000).

At the present stage, there is no definite information on the signal transduction pathway that regulates the expression of APX isoenzymes, although it is clear that increased reduction-state of quinone B or plastoquinone

in chloroplasts may be essential for the cAPX induction under high light intensity (Karpinski *et al*., 1999).

H_2O_2 functions as a second messenger to regulate the gene expression of some antioxidative enzymes in plant cells, and a transient increase of H_2O_2 is observed during the early stages under oxidative stress conditions (Foyer *et al*., 1997). The balance between the detrimental and beneficial roles of H_2O_2 could be determined by the local concentration of H_2O_2 related to the function of each organelle and the state of the cellular scavengers, including the levels of various antioxidants such as APX, catalase, AsA, and GSH. The cytosol fraction is a very important cellular compartment for communicating the information from each organelle to the nucleus. Taking into account these facts and the occurrence of two or more putative cAPX variants in plants, it is likely that cAPX variants have a critical function in controlling the H_2O_2 concentration in signalling, and that their major subcellular localization in the cytosol has strong relevance to gene regulation and thus to the mechanism of tolerance against various oxidative stress factors.

APX isoenzymes, especially chlAPX, are labile in the absence of AsA as the electron donor; therefore, they are inactivated with decreased AsA content under photo-oxidative stress conditions. It is clear that a high level of endogenous AsA is essential effectively to maintain the antioxidant system that protects plants from oxidative damage. Therefore, the biosynthesis, intracellular translocation, subcellular distribution, and the regeneration system of AsA strongly affect the final activity of each APX isoenzyme. At the present time, a major unanswered question is whether the instability of APX has a physiological role *in vivo*. The fact that the H_2O_2 level is controlled by cAPX suppression during pathogen infection suggests the possibility that the instability of APX might have functions related to stress signalling. Crystallographic studies of APX isoenzymes will provide new information about the inactivation mechanism in relation to the catalytic mechanisms. In addition to recent molecular analyses of APX isoenzymes described in this review, knowledge about AsA metabolism, including the AsA synthetic pathway and the AsA regeneratory cycle in plant cells, has greatly increased within the past few years (Smirnoff, 2000). The combined results of studies of APX isoenzymes together with advances in knowledge of AsA metabolism will provide a fuller understanding of the physiological function of APX in plant stress responses.

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