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Award Review

Recent Advances in Ascorbate Biosynthesis and the Physiological Significance of Ascorbate Peroxidase in Photosynthesizing Organisms

Takahiro ISHIKAWA[1,†] and Shigeru SHIGEOKA[2]

[1]*Department of Life Sciences and Biotechnology, Faculty of Life and Environmental Sciences, Shimane University, 1060 Nishikawatsu, Matsue, Shimane 690-8504, Japan*
[2]*Department of Advanced Bioscience, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan*

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Ascorbate (AsA), the most abundant water-soluble redox compound in plants and eukaryotic algae, has multiple functions. There is compelling genetic evidence that the biosynthesis of AsA proceeds *via* a D-mannose/L-galactose pathway and is the most significant source of AsA in plants. AsA plays important roles in antioxidative defense, particularly *via* the AsA/glutathione cycle. AsA peroxidase (APX) plays a central role in the cycle and is emerging as a key enzyme in cellular H_2O_2 metabolism. Plants possess diverse APX isoenzymes in cellular compartments, including the chloroplast, cytosol, and microbody. In algae, however, the number and distribution of APX proteins are quite limited. Recent progress in molecular biological analysis of APX isoenzymes has revealed elaborate mechanisms for the tissue-dependent regulation of two chloroplastic APX isoenzymes by alternative splicing, and for redox regulation of cytosolic APX gene expression in response to light stress. Furthermore, transgenic plants overexpressing a chloroplastic APX isoenzyme enable us to evaluate the behavior of the enzyme under conditions of photo-oxidative stress. Molecular physiological analysis has revealed that cytosolic APX is part of the system modulating the cellular H_2O_2 level in redox signaling.

Key words: ascorbate biosynthesis; ascorbate peroxidase; reactive oxygen species; gene expression; environmental stress

Reactive oxygen species (ROS), such as the superoxide radical (O_2^-) and H_2O_2, are generated by all aerobic organisms, and excessive production results in a toxic state called oxidative stress. On the other hand, the physiological use of ROS by cells is now being demonstrated in areas such as redox signaling *via* the regulation of protein and gene expression. In plants and eukaryotic algae, chloroplasts are potentially the major site for the generation of ROS due to an abundance of O_2 produced by the primary photochemical reactions of photosynthesis.[1)] Additionally, other metabolic processes, including the mitochondrial and plasma membrane-linked electron transport systems, also produce ROS even under optimal conditions. The imposition of biotic or abiotic stress can give rise to excess amounts of ROS, leading to critical damage to cells.

In order to maintain an adequate cellular concentration of ROS, photosynthesizing organisms have developed many enzymatic and non-enzymatic antioxidative defense systems in various cellular compartments. Among these and of particular importance is ascorbate (AsA), the most abundant water-soluble antioxidant in plants and eukaryotic algae.[1,2)] AsA is present at high-millimolar levels in all subcellular compartments; in particular, it is found in chloroplasts, at concentrations of 20 mM or more. It has also become apparent that AsA has multiple roles in metabolism, electron transport, control of the cell cycle, and expansion, and in the responses of plants to pathogens and abiotic stress.[2,3)]

AsA is quantitatively the most predominant antioxidant and much more effective at regulating the cellular H_2O_2 level when coupled with its redox system, termed the AsA/glutathione (GSH) cycle or Asada-Halliwell pathway.[4,5)] AsA peroxidase (APX) utilizes AsA as its electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDA), a univalent oxidant of AsA. MDA is either

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† To whom correspondence should be addressed. Tel: +81-852-32-6580; Fax: +81-852-32-6092; E-mail: ishikawa@life.shimane-u.ac.jp

Abbreviations: APX, ascorbate peroxidase; cAPX, cytosolic APX; mAPX, microbody APX; sAPX, stromal APX; tAPX, thylakoid membrane-bound APX; AsA, ascorbate; DHA, dehydroascorbate; FBPase, fructose 1,6-bisphosphatase; L-Gal, L-galactose; L-GalL, L-galactono-1,4-lactone; D-GalUA, D-galacturonic acid; GAPDH, NADP+ glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; L-GulL, L-gulono-1,4-lactone; HSF, heat shock transcription factor; D-Man, D-mannose; MDA, monodehydroascorbate; PET, photosynthetic electron transport; PRK, phosphoriblokinase; ROS, reactive oxygen species; SRE, splicing regulatory element

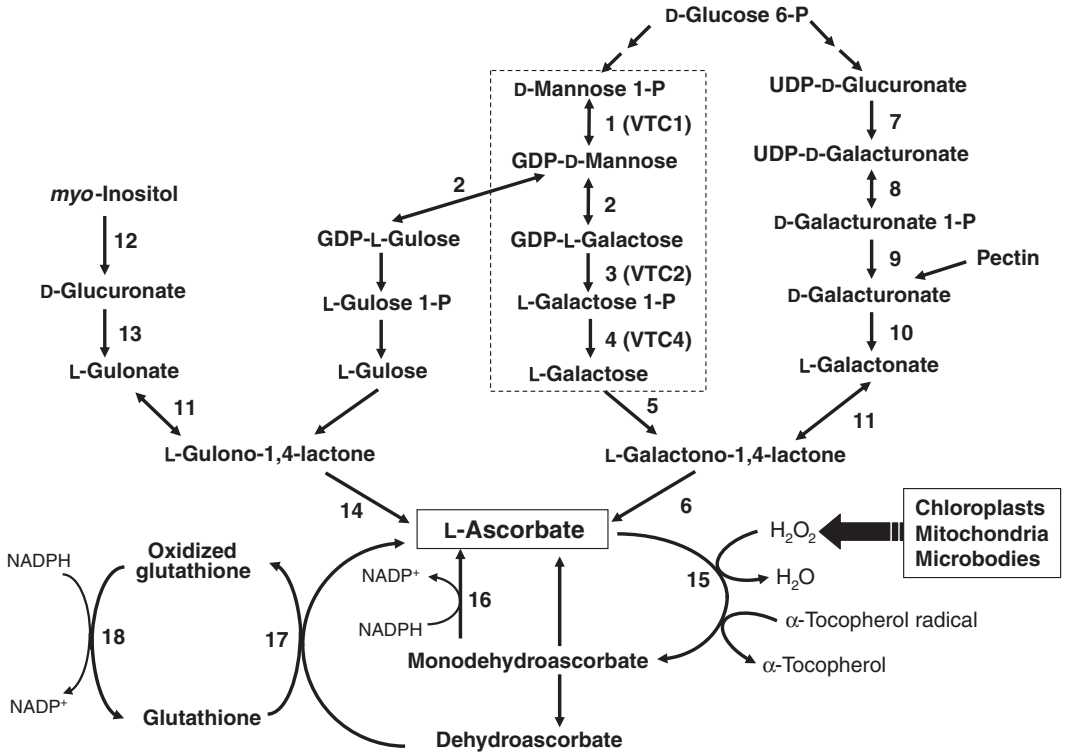


Fig. 1. Proposed Ascorbate Biosynthetic Pathways and Ascorbate/Glutathione Cycle in Photosynthesizing Organisms.

The broken line box shows D-Man/L-Gal pathway. Parentheses indicate the names of ascorbate-deficient (VTC) *Arabidopsis* mutants. Enzymes: 1, GDP-Man pyrophosphorylase; 2, GDP-Man-3′,5′-epimerase; 3, GDP-L-Gal phosphorylase/L-Gal guanylyltransferase; 4, L-Gal 1-phosphate phosphatase; 5, L-Gal dehydrogenase; 6, L-GalL dehydrogenase; 7, UDP-glucuronate 4-epimerase; 8, UDP-galacturonate pyrophosphatase or phosphorylase or uridylyltransferase; 9, D-galacturonate 1-phosphate phosphatase; 10, D-galacturonate reductase; 11, aldonolactonase; 12, *myo*-inositol oxygenase; 13, D-glucuronate reductase; 14, L-GulL dehydrogenase or oxidase; 15, ascorbate peroxidase; 16, monodehydroascorbate reductase; 17, dehydroascorbate reductase; 18, glutathione reductase.

disproportionated to AsA and dehydroascorbate (DHA) or reduced to AsA by MDA reductase. DHA is also reduced back to AsA by the actions of GSH-dependent DHA reductase, and the resulting oxidized GSH is then regenerated by GSH reductase. The enzymes and processes involved in the AsA/GSH are shown in Fig. 1. It is apparent that APX has numerous isoenzymes in various plant cell compartments and that it plays a central role in scavenging H_2O_2 in plants and eukaryotic algae.[5] APX also helps to dissipate excess photonenergy through the water-to-water cycle in chloroplasts.[6] Meanwhile, convincing evidence of the involvement of APX in the regulation of redox signal transduction is also accumulating.[7,8]

This review focuses on recent advances concerning the physiological function and molecular properties of APX isoenzymes in photosynthesizing organisms. In addition, the pathway of AsA biosynthesis is highlighted, given the recent elucidation of the entire D-mannose (D-Man)/L-galactose (L-Gal) pathway in plants.

I. Ascorbate Biosynthesis in Photosynthesizing Organisms

AsA biosynthesis occurs in all plant tissues and eukaryotic algal cells. In spite of the nutritional importance of AsA to humans, the major AsA biosynthetic pathway in higher plants has only recently been properly elucidated.[9–11] This pathway, designated the D-Man/L-Gal pathway, proceeds *via* GDP-D-Man and L-Gal, and the final aldonolactone precursor of AsA is L-galactono-1,4-lactone (L-GalL) (Fig. 1): no carbon skeleton inversion can be seen. L-GalL dehydrogenase, the enzyme catalyzing the last step of the pathway, is now well characterized. It is located in mitochondria and uses cytochrome *c* as its electron acceptor.[12–14] Other important enzymes and genes related to the pathway, such as L-Gal dehydrogenase,[15] GDP-Man pyrophosphorylase,[16] GDP-Man 3′,5′-epimerase,[17] and L-Gal-1-phosphate phosphatase,[18,19] have also been identified during the last decade. More recently, complete picture of the D-Man/L-Gal pathway has finally emerged with the finding that the *VTC2* gene encodes a newly identified enzyme, GDP-L-Gal phosphorylase/L-Gal guanylyltransferase.[20,21]

In addition to the "non-inversion" D-Man/L-Gal pathway, evidence of alternative AsA biosynthetic pathways has become apparent with the cloning and characterization of other enzymes. Biochemical analysis of GDP-Man 3′,5′-epimerase indicated that the enzyme catalyzes the conversion of GDP-D-Man not only to GDP-L-Gal but also to GDP-L-gulose, which result from

the 3′- and 5′-epimerization and the 5′-epimerization of GDP-D-Man respectively.[17] GDP-L-gulose represents approximately 25% of the products of the reaction at equilibrium.[22] Therefore, it is quite possible that an alternative L-gulose pathway exists for AsA biosynthesis. Moreover, overexpression of novel genes encoding D-galacturonic acid (D-GalUA) reductase and *myo*-inositol oxigenase (MIOX4) resulted in a several-fold increase in the total amount of AsA in transgenic *Arabidopsis*, suggesting the participation of these enzymes in alternative uronic acid pathways.[23,24] D-GalUA reductase catalyzes the reduction of D-GalUA to L-galactonic acid, which is subsequently converted to L-GalL. This pathway actually operates in the unicellular alga *Euglena gracilis*.[25,26] On the other hand, the product of the *myo*-inositol oxygenase reaction is presumably a D-glucuronate, assuming the occurrence of the AsA biosynthetic pathway in higher plants, similarly to animals. In the cases of both alternative L-gulose and D-glucuronate pathways, to generate AsA, the presence of L-gulono-1,4-lactone (L-GulL) oxidase or dehydrogenase is essential to complete them. Although there is still an incomplete understanding of the pathways in plants, Wolucka and Van Montagu[27] have reported L-GulL dehydrogenase activity in a cytosolic fraction from potato tubers, and the *Arabidopsis* genome contains several genes homologous to rat L-GulL oxidase. It is of interest to clarify whether the homologous genes are functional.

Genetic evidence that the D-Man/L-Gal pathway is the major AsA biosynthetic pathway in plants was provided by an analysis of a series of *Arabidopsis* AsA-deficient (*vtc*) mutants.[28] Among several *vtc* lines, *vtc2-1*, with a splice site mutation of the GDP-L-Gal phosphorylase gene, showed the lowest AsA level (approximately 20% of the wild-type AsA level). More importantly, a double knockout *Arabidopsis* mutant for *VTC2* and its paralogous gene, *VTC5*, showed growth arrest after germination and resumed normal growth on supplementation with AsA or L-Gal.[20] This result clearly supports the evidence that the D-Man/L-Gal pathway is the only significant source of AsA in *Arabidopsis* seedlings. The possibility remains that alternative uronic acid pathways contribute to AsA levels in other species or in tissues, especially in fruits, although recent metabolite profiling in tomato does not support this idea.[29]

The AsA content of plants is influenced by light/dark conditions,[20,30] photosynthesis electron transport (PET),[31] senescence,[32,33] various forms of environmental stress,[34] germination,[35] and fruit ripening,[23,36] suggesting the presence of a control mechanism for the biosynthesis. There is a suggestion that AsA biosynthesis is regulated by feedback inhibition.[37,38] Control by respiration and plant hormones such as jasmonic acid has also been proposed.[39–41] However, at present, information to explain the crucial molecular mechanism involved is quite limited.

II. Enzymatic and Molecular Properties of APX Isoenzymes

APX is widely distributed in plants and eukaryotic algae that have acquired the ability to produce AsA. APX as well as cytochrome *c* peroxidase from yeast and catalase-peroxidase from cyanobacteria belong to the Class I category of plant-type heam peroxidases,[42] indicating that these enzymes developed from the same ancestral gene. Recent genome-wide analysis in higher plants indicated that APX belongs to a multigenic family, since there are nine genes in *Arabidopsis*, eight in rice, and seven in tomato.[43–45] These APX families are further classified into three subfamilies according to subcellular localization, *viz.*, chloroplastic, cytosolic, and microbody isoenzymes.[5] Chloroplastic isoenzymes have stroma-soluble (sAPX) and thylakoid membrane-bound (tAPX) forms. The tAPX sequence exhibits a C-terminal extension with a hydrophobic anchor region for binding the thylakoid membrane. Another chloroplastic APX isoenzyme (At4g09010) was found in the *Arabidopsis* thylakoid lumen by a proteomic approach,[46,47] although its function remains unknown. Two or three isoenzymes are usually found in plant cytosol. Microbody APX (mAPX) isoenzymes have a primary structure very similar to the cytosolic form, except that the C-terminal extension for membrane sorting is similar to that of tAPX and the catalytic domain faces the cytosol fraction.[48] In spite of recent study on mAPX based by a molecular genetics approach, its compelling physiological function is still largely unknown.[49] Although no gene encoding APX located in mitochondria has been identified to date, Chew *et al.*[43] demonstrated the possibility that sAPX is located in mitochondria, based on the dual targeting function of its transit signal. Further investigation is needed to conclude that dual targeting is a common mechanism locating APX in mitochondria and chloroplast stroma, because no APX activity or protein has been found in tobacco mitochondria.[50]

In contrast to higher plants, in algae the number and distribution of APX proteins are quite limited. *Euglena gracilis* lacks catalase and contains APX and enzymes related to the ascorbate redox cycle solely in the cytosol.[51,52] Two cytosolic APX (cAPX) isoenzymes have been identified in the unicellular red algae *Galdieria partita* and *G. sulphuraria*.[53,54] Halotolerant *Chlamydomonas* ap. W80 contains one APX gene, the translated product of which is predicted to be located in the chloroplast stroma.[55] The limited number and subcellular localization of APX isoenzymes in these eukaryotic algae suggest a cellular metabolism of H_2O_2 different from that in higher plants. It is apparent that some parastic protoza, such as *Trypanosome cruze* and *Leishmania major*, also have a functional APX, but the location in the endoplasmic reticulum is very different from that of plant APXs.[56,57] Protozoa APXs, including *Euglena* APX, form a novel category, because the

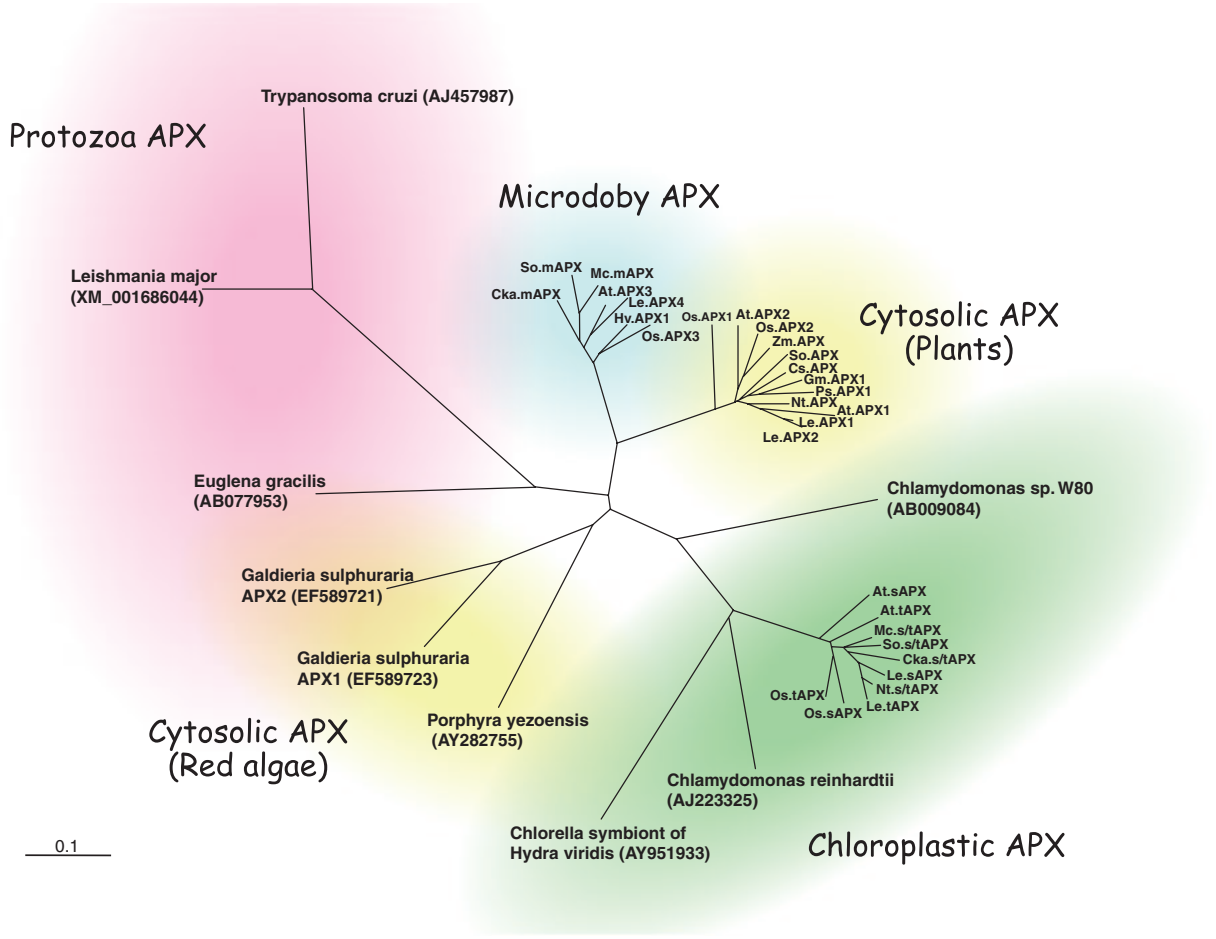


Fig. 2. Classification of Ascorbate Peroxidase Protein Families.

This phylogenic tree was constructed using ClustalW and visualized using TreeView. The abbreviations and accession numbers for plant APXs are as follows: At, *Arabidopsis thaliana* (At.APX1, X59600; At.APX2, NM_001035587; At.APX3, U69138; At.sAPX, X98925; At.tAPX, X98926); Cka, Cucurbita cv. Kurokawa Amakuri (Cka.mAPX, AB070626; Cka.sAPX, D88420; Cka.tAPX, D83656); Cs, *Cucumis sativus* (Cs.APX, D88649); Gm, *Glycine max* (Gm.APX1, L10292); Hv, Hordeum vulgare (Hv.APX1, AB063117); Le, *Lycopersicon esculentum* (Le.APX1, DQ099420; Le.APX2, DQ099421; Le.APX4, DQ131130; Le.sAPX, DQ131133; Le.tAPX, DQ131132); Mc, *Mesembryanthemum crystallinum* (Mc.mAPX, AF139190; Mc.sAPX, AF069316; Mc.tAPX, AF069315); Nt, *Nicotiana tabacum* (Nt.APX, D85912; Nt.sAPX, AB022274; Nt.tAPX, AB022273); Os, *Oryza sativa* (Os.APX1, D45423; Os.APX2, AB053297; Os.APX3, AY382617; Os.sAPX, AB114855; Os.tAPX, AB114856); So, *Spinacia oleracea* (So.APX, D85864; So.mAPX, D84104; So.sAPX, D83669; So.tAPX, D77997); Zm, *Zea mays* (Zm.APX, Z34934).

phylogenetic tree reveals a clear divergence between plants and protozoa (Fig. 2).

Since APX activity was first described by Shigeoka *et al.* in 1980,[51] its enzymatic properties have been well characterized. The details have been reviewed by Asada[4] and Shigeoka *et al.*[5] The catalytic mechanism of APX has been well characterized based on the three-dimensional structure of the substrate complex of cAPX and sAPX isoenzymes.[58–60] One of the peculiarities of APX is its instability in the absence of AsA. APX activity is rapidly lost under conditions where the AsA concentration is lower than 20 μM *in vitro*.[61] Chloroplastic APX isoenzymes are especially sensitive to inactivation; the half inactivation time is less than 30 s, while that of cAPX and of mAPX is 1 h or more. The crystal structure of tobacco sAPX reveals that it, but not cAPX, has a unique loop in the vicinity of the heme,[59] and a chimera of *Galdieria* cAPX and spinach sAPX, in which the corresponding loop structure was removed from sAPX, showed increased stability under AsA-depleted conditions,[62] indicating a causal relationship between the loop structure and stability of chloroplastic APX. The instability of chloroplastic APX was observed in tobacco chloroplasts exposed to photo-oxidative stress, as described in the section below.

III. Molecular Mechanisms That Regulate APX Expression

1. Signaling and regulation of cytosolic APX expression

Numerous studies of ROS-scavenging enzymes in plants have demonstrated that APX activity generally increases along with activities of other enzymes, such as catalases, superoxide dismutase, and GSH reductase, in response to environmental stress.[5] Among APX iso-

enzymes, cAPX is known to be highly responsive to environmental conditions. The steady-state level of the cAPX transcript in pea and spinach leaves significantly increased in response to high-light illumination, drought, heat, and methyl viologen treatment.[63,64] A recent transcriptome-based analysis also confirms the susceptibility of cAPX to environmental factors.[65]

The signaling mechanisms inducing cAPX expression and its regulation have been examined mostly by analyzing cAPX isoenzymes in *Arabidopsis*. The expression of both *APX1* and *APX2*, paralogous genes encoding cAPX in *Arabidopsis*, was found to be rapidly induced by H_2O_2 accumulation and redox changes in PET system through a plastoquinone pool at the early stages of high-light exposure.[66] The same phenomenon has been observed for cAPX in tobacco leaves.[67] A study using transgenic tobacco overexpressing tAPX or catalase in chloroplasts that show a high degree of tolerance to photo-oxidative stress through a significant reduction in excess accumulation of H_2O_2[68,69] demonstrated cessation of transcription after rapid induction of cAPX expression within 1 h after exposure to excess light. This result indicates that induction of cAPX expression is caused by a PET-derived signal at an early stage in the response to stress from excess light, and thereafter by an accumulation of H_2O_2 in chloroplasts.

Although the H_2O_2 signaling pathway for cAPX expression is still largely unknown, the signaling events are explained, at least in part, by the regulation of heat shock transcription factors (HSFs). Transgenic *Arabidopsis* plants expressing a dominant-negative form of Hsf21 (encoded by *HSFA4a*), which lacks an activation domain, showed inhibition of APX1 mRNA accumulation under H_2O_2-producible light stress conditions ($250\,\mu mol\,m^{-2}\,s^{-1}$).[8] Nishizawa *et al.*[70] reported that HsfA2-overexpressing *Arabidopsis* showed *APX2* gene expression, while *HSFA2* gene-knockout plants almost completely lacked expression, following a combination of high-light ($800\,\mu mol\,m^{-2}\,s^{-1}$) and heat-shock (40 °C) treatments. On the other hand, HsfA2-overexpressing and -knockout plants did not exhibit any significant difference in accumulation of the *APX1* transcript. Overexpression of *Hsf3* (encoded by *HSFA1a*) in *Arabidopsis* resulted in high expression levels of both *APX1* and *APX2* following heat-shock treatment (37 °C).[71] These results indicate a complicated interlinking or coordination of H_2O_2-signaling *via* HSFs in cAPX expression. It might explain why plants use different HSFs at different sites of H_2O_2 generation during applied stress, because possible sources of ROS include mitochondria, chloroplasts, and the plasma membrane during heat stress.[72] A putative HSF-binding *cis*-element can be seen in the cAPX promoters, but there is still no direct evidence of interaction between those elements and known HSFs.

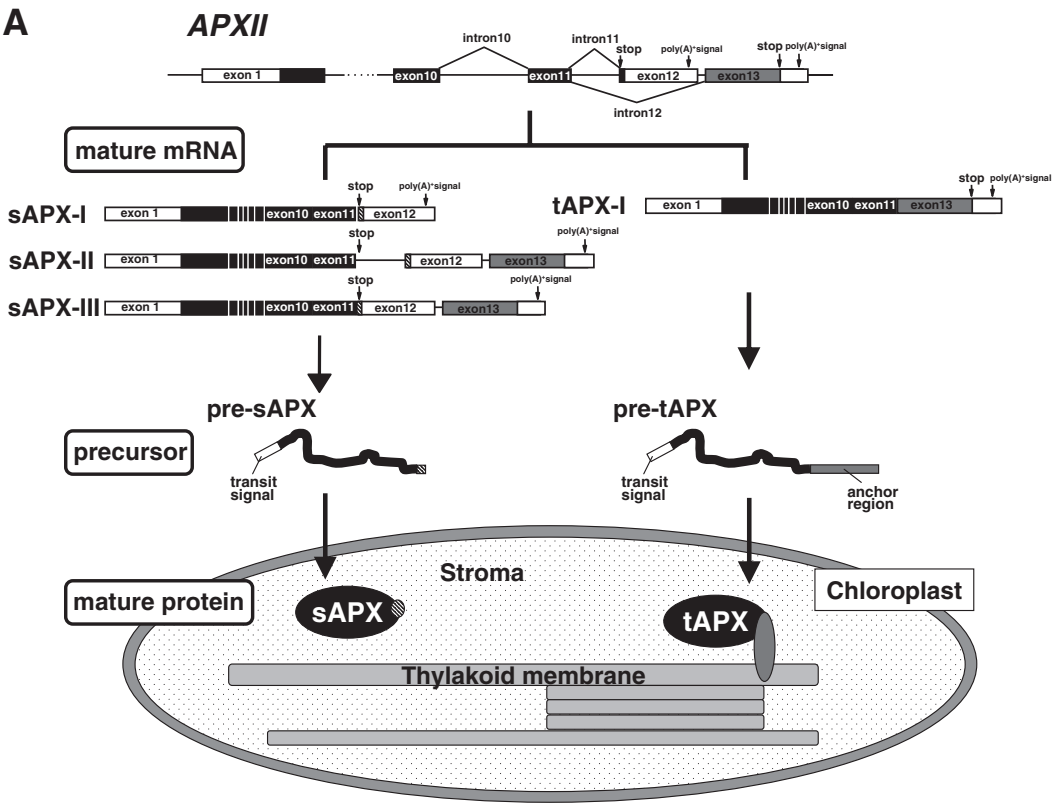
Recently, a mutant (*alx8*) that shows a constitutively higher *APX2* gene expression level even under low light conditions was screened by chemical mutation of *Arabidopsis* seeds transformed with the *APX2*-promoter::luciferase gene.[73] The gain-of-function is thought to be specific to *APX2* gene expression, because the *APX1* gene expression level in the mutant did not change under normal conditions. Identification of the causal gene for *alx8* should help to reveal the signaling specific to *APX2* gene expression.

2. Alternative splicing of chloroplastic APX isoenzymes

As for the genes of chloroplastic APX isoenzymes among plants, interestingly, they are divided into two groups. The first group comprises single genes encoding two isoenzymes, and the second, two individual genes for each isoenzyme. In the former group, which includes genes from spinach, tobacco, pumpkin, and ice plant (*M. crystallium*), the expression of two chloroplastic APXs is post-transcriptionally regulated by alternative splicing. On the other hand, the chloroplastic APX genes from *Arabidopsis*, rice, and tomato belong to the second group and are individually regulated. Duplication leads to the evolution of chloroplastic APXs with different preferences among plant species, although why this is so is not clear.

The mechanism of alternative splicing in chloroplastic APX has been studied in spinach in detail (Fig. 3A). The gene, designated *APXII*, consists of 13 exons split by 12 introns, and penultimate exon 12 consists of the terminal amino acid, termination codon, and 3′-untranslated region, including a potential polyadenylation signal of the sAPX mRNA.[74,75] The final exon, exon 13, contains the corresponding sequence of the hydrophobic anchor region and the entire 3′-untranslated region of tAPX mRNA. As a result of alternative polyadenylation and splicing, four types of mature mRNA variants, one form for tAPX (tAPX-I) and three forms for sAPX (sAPX-I, -II, -III), are produced.[76] *APXII* expression is constitutive and can occur even in root tissue. However, the ratio of sAPX mRNA variants to tAPX-I mRNA differs markedly between leaves and roots (Fig. 3B). In leaves, the ratio is almost equal, while in roots, it is extremely elevated as a result of an increase in sAPX-III mRNA and a decrease in tAPX-I mRNA.[77] These results clearly indicate that alternative splicing plays a key role in controlling the expression of sAPX and tAPX in a tissue-dependent manner. The sAPX proteins, not tAPX, are detectable in non-photosynthesizing tissue and cultured cells like tobacco BY-2, although the physiological function in plastids must be clarified.[50,77]

The molecular mechanism of tissue-dependent regulation of sAPX and tAPX by alternative splicing was determined by the identification of a functional *cis*-acting element on the pre-mRNA transcribed from *APXII*. The *cis*-acting element, designated SRE (Splicing Regulatory Element), was identified just upstream of the acceptor site of intron 12 by a homologous search of the conserved sequence region, mutational analysis of



B

	Relative mRNA ratio (%)		
	Leaf	Stem	Root
sAPX-I	21	21	19
sAPX-II	5	5	3
sAPX-III	32	56	73
tAPX-I	42	18	5

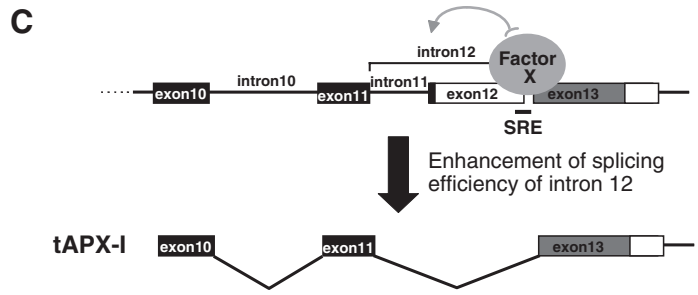


Fig. 3. Regulation of Chloroplastic APX Expression by Alternative Splicing.

A, Outline of stromal (sAPX) and thylakoid membrane-bound APX (tAPX) expression by *APXII* gene by alternative splicing.[74,75] Inset table shows relative mRNA expression levels calculated by results of nuclease protection assay.[76] B, Relative expression levels of the splice variants in different tissues of spinach. C, Model of tissue-dependent regulation of chloroplastic APX isoenzymes generated by alternative splicing.[76] Factor X stands for protein factors that interact with the splicing regulatory element (SRE) facilitating the generation of tAPX-I splice variant in leaf.

the SRE, and RNA gel-shift assay with nuclear proteins extracted from various spinach tissues.[77] The SRE region consists of at least eight essential nucleotides that are rich in adenine and uridine. RNA gel-shift analysis showed that protein factors interacting specifically with SRE were present in nuclear extracts from leaves, but not roots, although their identity is not known. Based on the results obtained to date, a possible mechanism that regulates chloroplastic APX in a tissue-dependent manner through alternative splicing is outlined in

Fig. 3C. The finding of SRE represents one of the first examples of a plant *cis*-acting element that controls tissue-specific alternative splicing. Interaction between SRE and protein factors expressed in nuclei in leaves facilitates the splicing of intron 12 and results in the generation of tAPX-I mRNA for tAPX, while in roots the sAPX-III mRNA variant is predominantly produced by the splicing of intron 11 due to suppression of the specific nuclear protein factors.

A recent genome-wide analysis estimated that approximately 22% of all pre-mRNAs undergo alternative splicing in *Arabidopsis* and rice[78] and there is increasing evidence to suggest that alternative splicing plays an important regulatory role in development,[79] in flowering time[80] and in the response to environmental stress.[81] The finding of alternative splicing of chloroplastic APX provides new information on its biological significance in controlling sub-organelle translocation.

3. Regulation of APX expression in algae

Information on the regulation of APX in algae is quite limited, although it has been suggested that algal APX is controlled in a different fashion from that in plants. For instance, in *Clamydomonas reinhardtii*, although APX activity is expressed under ordinary growth conditions, when cells were transferred to a medium supplemented with sodium selenite, activity completely disappeared after 6 h and a selenium-dependent GSH peroxidase was expressed in place of APX.[82,83] In the case of *Euglena*, iron-deficient cells completely lacked APX activity, and activity recovered on supplementation with ferrous ammonium sulfate.[84,85] Activity increased in two phases: a rapid increase right after supplementation and a steady gradual increase. The former was not affected by the addition of cycloheximide or the heme synthesis inhibitor succinyl acetone, indicating activation of APX protein. These findings indicate that nutritional conditions, especially the status of the enzyme cofactor, affect APX expression in algae.

Expression of *Euglena* APX was also observed in dark-adapted cells during light adaptation, and it was completely dependent on chloroplast development.[86] APX mRNA was constitutively expressed, and transcriptional inhibitors did not have any effect on expression. These results suggest that APX expression in *Euglena* is post-transcriptionally regulated in multiplesteps, including ribosome association of the mRNA, translational elongation, and heme-incorporation into apo-APX protein.

IV. Molecular Physiological Analysis of APX Isoenzymes in Plants

1. Evaluation of physiological role of chloroplastic APX under photo-oxidative stress

Chloroplasts are the most important source of ROS in plants. The rate of H_2O_2 formation in chloroplasts under optimal conditions is estimated to be $120\,\mu M\,s^{-1}$.[87] The H_2O_2 level in chloroplasts increases several orders of magnitude under photo-oxidative stress. A low level ($10\,\mu M$) of H_2O_2 inhibits CO_2 fixation by 50% due to the oxidation of thiol-modulated enzymes, *e.g.*, NADP+ glyceraldehyde 3-phosphate dehydrogenase (GAPDH), fructose 1,6-bisphosphatase (FBPase), phosphoriblokinase (PRK), and sedoheptulose 1,7-bisphosphatase, in the Calvin cycle.[88,89] Because the inhibition of photosynthetic efficiency caused by the production of ROS is an important limitation on yield in many useful plants, including crops, it is desirable to create a transgenic plant with a high tolerance to photo-oxidative stress.

In view of the physiological significance of APX against oxidative stress, transgenic plants overexpressing APX isoenzymes have been generated to improve stress tolerance, as in the case of other antioxidative enzymes. Transgenic tobacco and *Arabidopsis* plants overexpressing tAPX in chloroplasts showed remarkable tolerance against photo-oxidative stress following exposure to strong light and paraquat treatment.[69,90] In contrast to the gain of function study, suppression of chloroplast APX isoenzymes in *Arabidopsis* by antisense or T-DNA insertion resulted in increased susceptibility to photo-oxidative stress,[91,92] demonstrating the importance of APX in chloroplasts under such stress. Chloroplastic APX isoenzymes in wild-type tobacco plants were much more strongly inactivated at the early stage of photo-oxidative stress than FBPase, GAPDH, or PRK, which are believed to be most sensitive to H_2O_2 in chloroplasts.[69] The same phenomenon was also observed in transgenic tobacco overexpressing *KatE*, a gene encoding *E. coli* catalase, in stroma, which showed high tolerance to photo-oxidative stress due to compensation for chloroplastic APX inactivation by catalase expression.[68,93] Due to the inherent susceptibility of chloroplastic APX isoenzymes to H_2O_2 in the absence of AsA *in vivo*, chloroplastic APX isoenzymes should be the primary targets under photo-oxidative stress and might be the limiting factor in protecting photosynthesis. The finding that an enzyme that was believed to be the central player as ROS scavenger is extremely labile is somewhat surprising. The physiological significance of this observation is not known, but we cannot exclude the possibility that it plays a regulatory role, including signal transduction, under certain stressful conditions.

2. Role of cAPX in redox signal modulation

Besides H_2O_2-scavenging reducing plant oxidative stress caused by excess ROS accumulation, cAPX isoenzymes have many more subtle functions in controlling the metabolism of H_2O_2. For instance, upon pathogen attack, accumulation of cellular ROS helps to induce cell death in infected cells, or serves as a signal activating defense responses in distant uninfected cells. cAPX expression in tobacco mosaic virus-infected tobacco leaves is suppressed by inhibition of protein synthesis in the polysomes,[94] indicating that cAPX suppression contributes to endogenous ROS accumula-

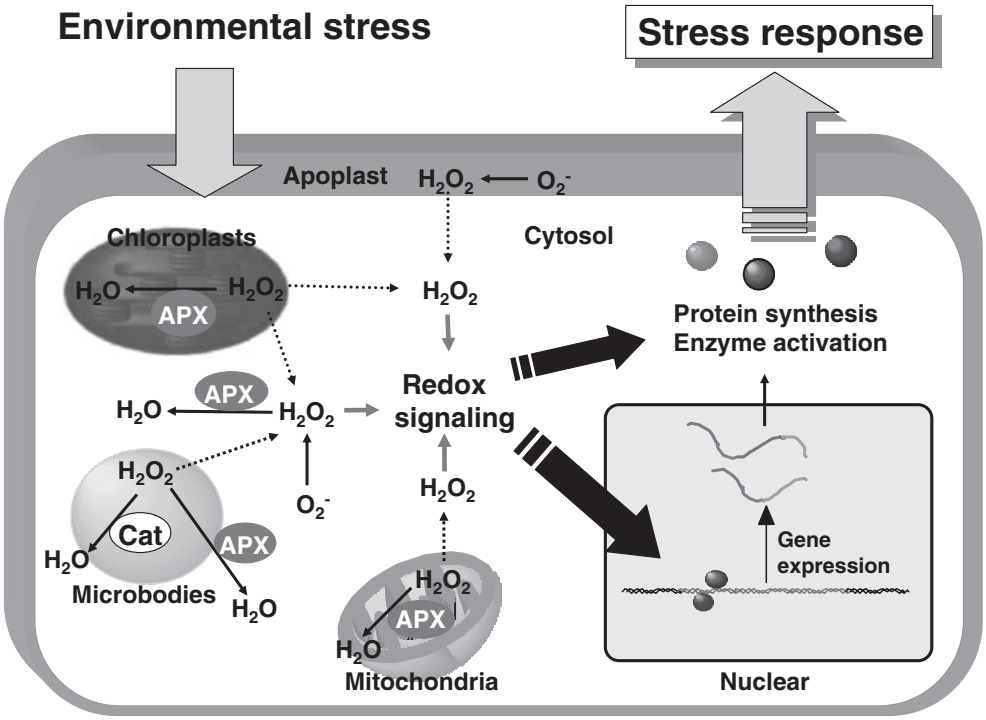


Fig. 4. Outline of the Physiological Function of APX Controlling Cellular Redox Signaling.

tion, which evokes redox-signaling. In addition, the role of cAPX isoenzymes related to cellular redox control was investigated by phenotypic characterization of plants lacking or showing less cAPX activity. Cultured transgenic tobacco cells with lowered cAPX activity due to co-suppression showed greater tolerance to environmental stress, such as heat and salinity, than wild-type cells.[7] This tolerance was accompanied by activation of a specific protein kinase and upregulation of the expression of stress-responsive genes including HSP as well as increased cellular ROS accumulation. Pnueli *et al.*[95] have also demonstrated that cAPX (APX1)-knockout *Arabidopsis* plants showed upregulation of the expression of various genes, including those for stress-related proteins and transcription factors. Moreover, induction of HSP gene expression was much greater in APX1-knockout plants than in wild-type plants under light stress. These observations strongly support the idea that cAPX takes an active role in the regulation of redox gene expression by modulating the concentration of H_2O_2 (cellular redox status) to adjust its activity or expression to a level sufficient for second messenger activity (Fig. 4). On the other hand, because prolonged accumulation of cellular ROS is eventually toxic and lethal, the increase in cAPX expression in response to H_2O_2 accumulation, as described above, is essential to reduce overproduction of ROS to toxic levels.

V. Conclusion and Future Perspectives

Within the past five years our knowledge of the metabolism and physiological roles of AsA and its redox system, including APX, has increased greatly. The pool size and redox status of AsA are also thought to affect signaling, due to AsA's quantitative predominance in the cell. Signaling should closely interlink with the phytohormone network, because AsA is an essential cofactor in the biosynthesis of phytohormones such as abscisic acid and gibberellins.[96,97] Recent elucidation of the entire D-Man/L-Gal pathway as the only physiologically significant route of AsA biosynthesis in plants should provide a clue as to how it is produced and how its pool size is regulated.

Although we limit our discussion to AsA and APX in this review, photosynthesizing organisms contain a diverse array of antioxidants and large number of hydroperoxides and metabolic enzymes, such as catalase, GSH, and thioredoxin peroxidases (peroxiredoxin). Genetic and experimental evidence exists of their cellular functions as both antioxidants and redox signaling modulators.[98,99] The possible simultaneous presence of all these antioxidants and enzymes surely suggests complex cross-talk and raises the question of their specificity and redundancy. It is apparent that AsA acts as a natural electron donor not only for APX, but also for 1-Cys type peroxiredoxin.[100] Recent proteomic analysis also indicates that cAPX is a potential thioredoxin (Trx-*h*) and glutaredoxin-interacting protein.[101,102] Although there are obviously still large gaps to fill to elucidate their precise relationships in redox signaling *in vivo*, it is likely that ROS production in cellular compartments affects redox signaling during stress. Detailed analysis of ROS accumulation, antioxidant levels, and enzyme activities in each compartment coupled with compre-

hensive gene and proteomics assays in plants and algae overexpressing or suppressing a target gene encoding an antioxidative enzyme are necessary to resolve this matter.

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