https://doi.org/10.1093/plphys/kiad109 PLANT PHYSIOLOGY 2023: 192: 4–6

Plant Physiology®

News and Views

Four is better than one: Structure and function of a unique ascorbate peroxidase with four binding sites

Dyoni M. Oliveira [1,2,*] **and Igor Cesarino** [3,4,*]

1 VIB Center for Plant Systems Biology, Ghent 9052, Belgium
2 Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent 9052, Belgium
3 Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, 277, São Paulo 05508-090, Brazil
4 Synthetic and Systems Biology Center, InovaUSP, Avenida Professor Lucio Martins Rodrigues, 370, São Paulo 05508-020, Brazil

*Author for correspondence: dyoni.oliveira@psb.vib-ugent.be (D.M.O.), icesarino@usp.br (I.C.)

Ascorbate peroxidase (APX) is a key enzyme involved in scavenging cytotoxic H_2O_2, a type of reactive oxygen species (ROS). ROS are reactive molecules and free radicals derived from molecular oxygen (O_2) that cause oxidative stress unless regulated. The major forms of ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), the hydroxyl radical ($HO^{\cdot}$), superoxide ($O_2^{\cdot-}$), and other organic and inorganic peroxides whose properties and chemical reactivity vary greatly. ROS can donate an electron or transfer an excited-state energy to an acceptor molecule, causing the oxidation of lipids, proteins, nucleic acids, and other molecules in the cell (Mittler et al. 2022).

ROS, including H_2O_2, are produced at high concentrations and normally in a controlled manner during several essential biological processes, including respiration, photosynthesis, and photorespiration. They also accumulate upon biotic and abiotic stresses, playing a key role in the integration of different stress-response signaling pathways and the activation of plant defense mechanisms (Savelli et al. 2019; Mittler et al. 2022; Peláez-Vico et al. 2022). Given, however, that the accumulation of ROS can be highly toxic for the cell, their levels are kept under control to prevent unintended damage. The control of H_2O_2 levels is particularly important, due to its capacity to pass through biological membranes and invade other subcellular compartments (Pandey et al. 2017). Removal of H_2O_2 is achieved via the activity of enzymes from the peroxidase-catalase superfamily, a large group of evolutionarily related but rather divergent enzymes (Lazzarotto et al. 2021). Among them, APX catalyzes the removal of H_2O_2 using ascorbate as an electron donor (Fig. 1A). In plants, APXs are encoded by small gene families and are located in the cytosol, peroxisome, mitochondria, and chloroplast, playing a critical role in removing H_2O_2 produced by photosynthesis and photorespiration to maintain cellular homeostasis (Pandey et al. 2017; Lazzarotto et al. 2021).

In addition to ascorbate, APXs can catalyze the H_2O_2-dependent oxidation of a variety of aromatic compounds, a feature that allowed the recent characterization of biological functions of APXs beyond ROS scavenging. For instance, a mitochondrial APX is relocated to cell walls of xylem cells undergoing programmed cell death where it oxidizes lignin monomers, thus catalyzing lignin polymerization during the early stages of xylem development in Chinese white poplar (*Populus tomentosa*; Zhang et al. 2022). Additionally, a bifunctional cytosolic APX catalyzing the 3-hydroxylation of *p*-coumarate to caffeate was identified in Arabidopsis (*Arabidopsis thaliana*) and purple false brome (*Brachypodium distachon*; Barros et al. 2019), which implied the existence of an alternate route toward lignin monomers that bypasses the canonical shikimate shunt using *p*-coumaroyl-CoA (Fig. 1A) (Volpi e Silva et al. 2019). Genetic manipulation of APX supports a role for this enzyme in the early steps of lignin biosynthesis in both species (Barros et al. 2019). However, to what extent the different functions of APX in lignification are conserved across the plant kingdom remains unknown. In addition, little is known about the structural features underlying APX activity in both ROS scavenging and lignification, especially in monocots.

In this issue of *Plant Physiology*, Zhang et al. (2023) report on the structural and biochemical characterization of cytosolic APX from monocot sorghum (*Sorghum bicolor*), including its crystal structure, steady-state kinetics for H_2O_2/ascorbate, and substrate specificity for aromatic compounds

Received January 17, 2023. Accepted February 7, 2023. Advance access publication February 22, 2023
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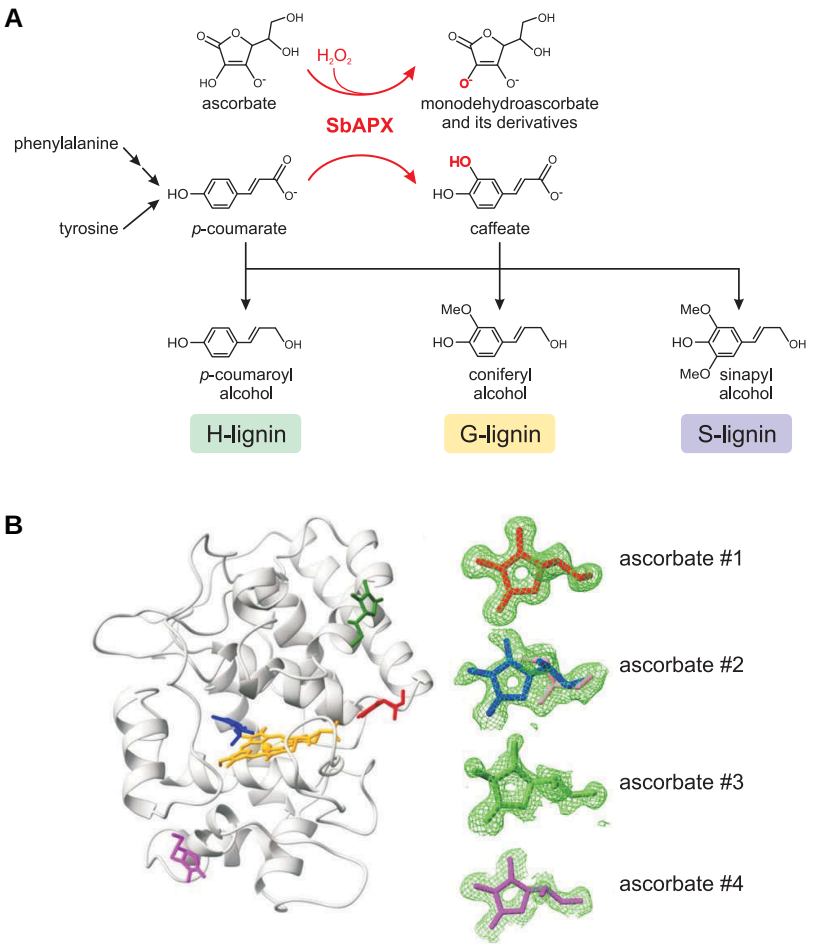

Figure 1. SbAPX catalytic reactions. **A)** Schematic representation of both H_2O_2-dependent oxidation of ascorbate (upper) and hydroxylation of *p*-coumaric acid to produce caffeic acid in the lignin biosynthetic pathway. **B)** SbAPX structure showing the four ascorbate binding sites, which are shown in the right. The heme group is marked in the center of the protein structure. Figure **B)** was modified from Figure 9 of Zhang et al (2023).

of the lignin biosynthetic pathway. The recombinant SbAPX enzyme was expressed and purified from *Escherichia coli* and used for both structural and enzymatic characterization. In addition to ascorbate, enzyme specificity was evaluated using a broad set of lignin-related aromatics. In the presence of H_2O_2, SbAPX catalyzed the oxidation and consequent polymerization not only of *p*-coumarate but also caffeate, ferulate, sinapate, coniferyl alcohol, and coniferyl aldehyde with substantial affinity. No oxidation was observed for cinnamate and tyrosine, indicating that this H_2O_2-dependent reaction is substrate-specific. Although no protein localization was performed, these results led the authors to suggest that SbAPX may be translocated to the cell wall, where it may use H_2O_2 to incorporate the phenolics into lignin polymer via oxidative polymerization in response to stress.

Based on the previous observation that caffeate can be produced via the ascorbate-dependent hydroxylation of *p*-coumarate catalyzed by APX from *B. distachon* and Arabidopsis (Barros et al. 2019), the authors demonstrated that SbAPX catalyzes the same reaction. However, given that similar levels of caffeate were produced in the control reaction without SbAPX from a nonenzymatic activity, the authors questioned whether this *p*-coumarate hydroxylase activity by SbAPX might be relevant for caffeate production in vivo and, thus, whether SbAPX is part of the lignin biosynthetic pathway in sorghum.

The crystal structure at high resolution showed that SbAPX uniquely has 4 ascorbate-binding pockets (Fig. 1B). Consistent with the presence of multiple binding sites, the steady-state kinetics for the H_2O_2-dependent oxidation of ascorbate indicated positive cooperativity. The authors also provided evidence of ascorbate oxidation into bicyclic hemiketal dehydroascorbic acid. Although the physiological role of this oxidized form of ascorbate is largely unknown, dehydroascorbic acid is proposed to be involved in the balance of NAD(P)/$NAD(P)^+$ and glutathione (GSH/GSSG) for redox homeostasis (Carroll et al. 2016). To address the biological importance of these 2 additional ascorbate-binding pockets, the authors combined the structural characterization with enzyme kinetics and site-directed mutagenesis of the conserved amino acid residues. They observed that these additional binding pockets in SbAPX not only allowed the association of 2

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more ascorbate molecules, but also that these additional molecules were connected to the other 2 through a hydrogen-bond network. Furthermore, the substitution of amino acids involved in the substrate binding of SbAPX may result in the diversification of its catalytic function (Pandey et al. 2017; Lazzarotto et al. 2021). These results suggest that the surface-bound ascorbate molecules might synergistically contribute to the catalytic dismutation of H_2O_2, potentially allowing an enhanced activity of SbAPX in maintaining cellular homeostasis.

The work of Zhang et al. (2023) not only demonstrates that SbAPX uniquely displays 4 binding pockets that positively cooperate for the H_2O_2-dependent oxidation of ascorbate, but also raises some questions about the involvement of SbAPX in the lignification process in sorghum. Does SbAPX play a role in caffeate production in vivo during developmental lignification? Alternatively, is this pathway associated with stress-induced lignin deposition, connecting the biosynthesis of free phenolic acids and lignin upon different stress responses (Marchiosi et al. 2020; Oliveira et al. 2020)? Is SbAPX translocated to the cell wall to catalyze the oxidation of lignin monomers and, thus, function in lignin polymerization? Future studies will help answer these questions and contribute to a better understanding of the biological functions of SbAPX and its homologs during stress responses.

Conflict of interest statement. None declared.

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