Plant Cytosolic Ascorbate Peroxidase with Dual Catalytic Activity Modulates Abiotic Stress Tolerances

HIGHLIGHTS
- Cytosolic ascorbate peroxidase in several plants possess two substrate oxidation activities.
- Amino acid residues Pro63, Asp75, and Tyr97 were identified as GSH oxidation sites.
- OgcytAPX1-catalyzing GSH is an independent biochemical step in AsA-GSH cycle.
- CytAPXs with two substrate-binding activities enhance stress tolerance and flowering.

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Plant Cytosolic Ascorbate Peroxidase with Dual Catalytic Activity Modulates Abiotic Stress Tolerances

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SUMMARY
Ascorbic acid-glutathione (AsA-GSH) cycle represents important antioxidant defense system in planta. Here we utilized Oncidium cytosolic ascorbate peroxidase (OgCytAPX) as a model to demonstrate that CytAPX of several plants possess dual catalytic activity of both AsA and GSH, compared with the monocatalytic activity of Arabidopsis APX (AtCytAPX). Structural modeling and site-directed mutagenesis identified that three amino acid residues, Pro63, Asp75, and Tyr97, are required for oxidation of GSH in dual substrate catalytic type. Enzyme kinetic study suggested that AsA and GSH active sites are distinctly located in cytosolic APX structure. Isothermal titration calorimetric and UV-visible analysis confirmed that cytosolic APX is a heme-containing protein, which catalyzes glutathione in addition to ascorbate. Biochemical and physiological evidences of transgenic Arabidopsis overexpressing OgCytAPX1 exhibits efficient reactive oxygen species-scavenging activity, salt and heat tolerances, and early flowering, compared with Arabidopsis overexpressing AtCytAPX. Thus results on dual activity CytAPX impose significant advantage on evolutionary adaptive mechanism in planta.

INTRODUCTION
Plants generate reactive oxygen species (ROS) continuously as by-products of various metabolic pathways and stresses in different cell compartments. Several antioxidants are usually employed by plants to eliminate the oxidative damage from ROS under various growth and stress conditions (Suzuki et al., 2012). The ascorbate (AsA)-glutathione (GSH) cycle is an essential metabolic pathway for the detoxification of ROS and regulation of the cellular level of H2O2 (Foyer and Shigeoka, 2011). The pathway contains ascorbate peroxidase (APX) together with dehydroascorbate reductase (DHAR) and glutathione reductase (GR), in addition to antioxidant metabolites AsA, GSH, and NADPH. AsA, GSH, and NADPH form redox couples with different redox potential and concentration and play the important role of maintaining redox homeostasis in plants to protect them from oxidation damage (Foyer and Noctor, 2016). APX enzymes (EC1.11.1.11) are class I heme peroxidases and catalyze the electron transfer from AsA to scavenge H2O2. In plant cells, AsA is the most important reducing substrate for H2O2 detoxification, with the oxidized product being dehydroascorbate (DHA). DHA is reduced to AsA by the action of DHAR, which uses GSH as the reducing substrate, and subsequently generates glutathione disulfide (GSSG). GSSG is in turn reduced to GSH by the catalysis of GR using NADPH as electron donor (Sharma et al., 2012). GSH is a non-protein thiol metabolite with a tripeptide (γ-glu-cys-gly) structure. The fundamental function of GSH is in thiol-disulfide interactions, in which reduced GSH is interchangeable with the oxidized form, GSSG (Frendo et al., 2013). Both AsA and GSH are ubiquitous in eukaryotic organisms, but only AsA is specific and highly abundant in plants, where it is essential for growth and development (Foyer and Noctor, 2016).

The AsA level and redox state have been reported to play a role in cell proliferation and elongation (Gest et al., 2013; Pignocchi and Foyer, 2003) and flowering (Chin et al., 2014; Kotchoni et al., 2009). The role of APX isoforms in overcoming various environmental stresses has been reviewed recently (Pandey et al., 2017). Knockout APX-1 mutants in Arabidopsis are sensitive to both drought and heat stress, resulting in increased Calvin cycle enzymes without changing the amount of glyceral-3-phosphate and ribulose-5-phosphate (Koussevitzky et al., 2008). Likewise, APX is more sensitive to heavy metals in double-silenced APX1 and APX2 transgenic rice plants, which displayed normal growth and enhanced tolerance (Rosa et al., 2010). In comparison, the GSH redox system has been implicated in the regulation of cell death...
Figure 1. Polyacrylamide Gel Electrophoresis Analysis of Recombinant Cytosolic Ascorbate Peroxidase 1 (CytAPX1) Proteins of Oncidium and Arabidopsis

(A) Total protein extracts of Oncidium OgCytAPX1 (left panel) and Arabidopsis AtCytAPX1 (right panel) expressed in E. coli; lane 1, non-isopropyl β-thiogalactopyranoside (IPTG) induction (40 μg); lane 2, 0.1 mM IPTG induction (40 μg); lane 3, purified fusion protein, MBP-CytAPX1 (10 μg).

(B) Purified recombinant MBP-CytAPX (30 μg) after factor Xa digestion; fusion protein (74 kDa), MBP tag (47 kDa), free CytAPX1 (27 kDa); lane 1 for Oncidium and lane 2 for Arabidopsis.

(C) In-gel staining for assaying recombinant APX activity. Digested recombinant protein (50 μg) was assayed on native polyacrylamide gel electrophoresis (10%); lane 1 for Oncidium and lane 2 for Arabidopsis.
OgCytAPX activity. Ultraviolet-visible (UV-vis) analysis and isothermal titration calorimetry confirmed that two distinct active sites are present for binding AsA and GSH, respectively, of Pro63, Asp75, and Tyr97, in addition to AsA-binding site, was identified in flowering initiation and environmental adaption.

Of several plants possessing APX/glutathione peroxidase (GPX) activities with two substrate recognition sites of oxidizing AsA and GSH in plants being functional to enhance stress tolerance and modulate the compounds shift toward a more oxidized state in response to increases in intracellular ROS, suggesting that the changes in AsA/GSH redox status under oxidative stress have the ability to trigger several processes in development and growth, including phase transition and flowering initiation.

Our recent work has indicated that Oncidium orchid grown under prolonged high ambient temperature exposure (at 30°C lasting for 14 days) is induced to early flowering (Chin et al., 2014). Investigation of the flowering mechanism revealed that CytAPX1 gene expression and enzymatic activity are increased by prolonged high-temperature exposure, which leads to decreased AsA level or AsA redox ratio, as well as decrease of GSH level or GSH redox ratio (Chin et al., 2016). Moreover, transgenic Arabidopsis ectopically overexpressing Oncidium CytAPX1 displayed an early-flowering phenotype, accompanied by low level of H2O2 and low AsA redox ratio under 30°C growth condition. This suggests that CytAPX1-mediated AsA and GSH redox homeostasis is a critical factor for the mechanism of flowering induction against prolonged high ambient temperature. This observation prompted us to characterize the physiological, biochemical, and molecular functions of Oncidium CytAPX1. In the current study, we found that Oncidium cytosolic APX1 (OgCytAPX1) and those in some plant species can catalyze not only AsA but also GSH as electron donors to scavenge H2O2. Enzyme kinetic analysis suggested that two distinct active sites are present for binding AsA and GSH, respectively, in OgCytAPX1. Data from structural modeling revealed that a possible GSH-binding site composed of Pro63, Asp75, and Tyr97, in addition to AsA-binding site, was identified in OgCytAPX1, whereas in Arabidopsis the corresponding site is composed of Asp63, His75, and His97 without GSH-binding activity. Ultraviolet-visible (UV-vis) analysis and isothermal titration calorimetry confirmed that OgCytAPX uses heme group to catalyze GSH. When OgCytAPX1 and AtCytAPX1 were overexpressed in Arabidopsis Col-0, only OgCytAPX1-OE plants showed a significant reduction in H2O2 level and GSH redox ratio, thus resulting in earlier flowering. Therefore our finding validates that the CytAPXs of several plants possessing APX/glutathione peroxidase (GPX) activities with two substrate recognition sites of oxidizing AsA and GSH in plants being functional to enhance stress tolerance and modulate flowering initiation and environmental adaption.

RESULTS

In-Gel Assays Illustrate OgCytAPX Possesses Dual Substrate-Binding Activities for Ascorbate and Glutathione

OgCytAPX1 was cloned from mRNA transcripts of pseudobulb tissues using RT-PCR. Sequence analysis predicted 250 amino acid residues with approximate molecular mass 27 kDa. Recombinant protein was produced in E. coli BL21 (Codon Plus) by cloning the OgCytAPX1 gene in pMAL-c5x vector, as described in methods. AtCytAPX1 from Arabidopsis was also produced for comparing experiment. Both fusion proteins linked with an MBP tag (maltose-binding protein) were expressed with approximate molecular weight 74 kDa (Figure 1A). After purification and digestion by protease factor Xa to remove the MBP tag, an...
Figure 3. Sequence Alignment of OgCytAPX1 with Some Related Plant APXs, and the Sequence Homology-Based Predicted Three-Dimensional Structures

(A) Sequence alignment was performed with the program ClustalW (Larkin et al., 2007). The final figure was prepared using Alscript (Barton, 1993). The secondary structure elements on the top of the alignment are based on the crystal structure of soybean CytAPX1 (PDB: 1OAF) (Sharp et al., 2003). The blue bars represent the secondary structure, cylinders are α-helices, bars with arrows are β-sheets, and lines are coil/loop; 22 amino acid residues marked by red background indicate the difference between Oncidium (or Oryza sativa) and Arabidopsis. The conserved residues corresponding to heme binding and AsA...
binding in the crystal structure of soybean CytAPX1 are marked by orange and black diamonds, respectively. Cyan diamonds represent Proline (Pro) 63, Aspartate (Asp) 75, and Tyrosine (Tyr) 97, required for GSH oxidation activity in Oncidium.

(B) C-alpha atom trace form or mode of OgCytAPX1. The homology modeling structure of OgCytAPX1 using the 3D coordination of soybean CytAPX1 (PDB: 1OAF) as the template. The modeling process was carried out using Modeler/Discovery Studio (Accelrys Inc., San Diego, CA, USA). Figures were generated by PyMol (http://pymol.sourceforge.net/). Ligand sites for heme and AsA were adopted from the template. Heme-binding residues were marked in orange, and AsA-binding residues were marked in black. Residues differing between Oncidium (or Oryza sativa) and Arabidopsis were in red. The three residues Pro63, Asp75, and Tyr97 that were mutated in this study were marked by cyan carbon atoms in stick mode.

(C) C-alpha atom ribbon form or mode of OgCytAPX1. The three residues Pro63, Asp75, and Tyr97 required for GSH oxidation are marked in cyan. The structure shows the location of GSH, AsA, and heme-binding sites.

The OgCytAPX1 and AtCytAPX1 recombinant proteins were further assayed in vitro to determine substrate-oxidizing activity. OgCytAPX1 displayed oxidizing activities for both substrates, indicating possession of both APX and GPX activities, whereas AtCytAPX1 displayed only APX activity (Figures 2A, 2B, and 2D–2G). Further confirmation of substrate-oxidizing activity was obtained by overexpressing OgCytAPX1 and AtCytAPX1 in Arabidopsis. Crude proteins extracted from transgenic Arabidopsis lines were used for in-gel activity assay. As shown in Figure 2C, both Arabidopsis lines overexpressed CytAPX1 as demonstrated by the high intensity of the band in the APX in-gel activity assay. Moreover, crude proteins extracted from OgCytAPX1-OE plants displayed a band of GPX activity in addition to APX activity. In contrast, protein samples extracted from AtCytAPX1-OE and empty vector plants showed no signals in GPX activity. Enzyme kinetic assay to determine the Lineweaver-Burk plot was performed (Figures 2H and 2I). The reaction rate of OgCytAPX1 against AsA substrate was Km 0.616 (mM) and Vmax 4.266 (mM AsA mg CytAPX1⁻¹ min⁻¹), whereas against GSH was Km 0.126 (mM) and Vmax 1.936 (mM GSH mg CytAPX1⁻¹ min⁻¹). The result suggested that the binding affinity of OgCytAPX1 to GSH is higher than to AsA. To clarify whether the active sites on OgCytAPX1 for binding AsA and GSH are distinct, the substrate-binding competition assay, by adding AsA and GSH together to react with OgCytAPX1, was performed. As shown in Figure 2J, the varied GSH concentration from 0.02 to 0.12 mM did not interfere with the APX activity on AsA (with 0.1 mM constant concentration). However, at the same constant AsA concentration (0.1 mM), GPX activity of OgCytAPX1 linearly increased with increasing GSH concentration from 0.02 to 0.12 mM (Figure 2K). Likewise, GPX activity of OgCytAPX1 on GSH was not interfered by AsA (Figure 2L). Moreover, APX activity of OgCytAPX1 was identical in Michaelis-Menten behavior at varied concentration of AsA, even at 0.1 mM GSH (Figure 2M). The data ruled out the possibility of substrate-binding competition between AsA and GSH toward OgCytAPX1. It suggested that OgCytAPX1 possessed two distinct active sites for AsA and GSH. Therefore it exerts APX and GPX activities independently.

Identification of the GSH-Binding Site by Structural Modeling and Site-Directed Mutagenesis

To dissect the structural site for GSH binding, alignment of amino acid sequence among Oncidium, Arabidopsis, Oryza sativa, Glycine max, and Pisum sativum was carried out. Among them, O. sativa shows high identity (82.1%) to Oncidium and contains GSH oxidation activity as Oncidium (data not shown). As shown in Figure 3A, the AsA-binding site is conserved in all CytAPX1 proteins at Lys30Asn31-Cys32Pro34Ile35His169Arg172 (black diamonds) (Sharp et al., 2003). After comparative analysis of the primary structure, 22 amino acid residues were found to vary between Arabidopsis and Oncidium, and also between Arabidopsis and O. sativa (as indicated by boxes and red background in Figure 3A). The conformation structural models of G. max and P. sativum were used as reference for further analysis of the three-dimensional conformation. The homology-derived structural model revealed that three amino acid residues in OgCytAPX1 (Pro63, Asp75, and Tyr97) had different properties from the corresponding amino acids in AtCytAPX1 (Asp63, His75, and His97). In the structural model, these three amino acids are located at the surface, relatively close to heme and the AsA-binding site among the 22 amino acid residues (Figure 3B, colored red), and are proposed to be the key residues forming the GSH oxidizing activity (Figures 3B and 3C, colored cyan). Therefore these three amino acid residues in OgCytAPX1 and corresponding residues in AtCytAPX1 were chosen for site-directed mutagenesis assay to validate GPX activity. The following derived mutants in OgCytAPX1 were thus generated: (1) single-residue mutation: Pro63Asp, Asp75His and Tyr97His, (2) double-residue mutation: Pro63Asp-Asp75His, Pro63Asp-Tyr97His and Asp75His-Tyr97His, and (3) triple-residues mutation: Pro63Asp-Asp75His-Tyr97His.
Figure 4. APX and GPX Activity Assay of Various Mutant Proteins of OgCytAPX1 and AtCytAPX1

(A) Purified free recombinant proteins of OgCytAPX1 (~27 kDa) with single-residue mutant: Pro63Asp (lane 1), Asp75His (lane 2), and Tyr97His (lane 3); double-residue mutants: Pro63Asp-Asp75His (lane 4), Pro63Asp-Tyr97His (lane 5), and Asp75His-Tyr97His (lane 6); and triple-residue mutant: Pro63Asp-Asp75His-Tyr97His (lane 7) were resolved on 10% native gel and then assayed for APX activity (upper gel) and GPX activity (middle gel).

(B) Purified free recombinant proteins AtCytAPX1 (~27 kDa) and its derived mutants, including with single-residue mutation: Asp63Pro (lane 1), His75Asp (lane 2), and His97Tyr (lane 3); double-residue mutation: Asp63Pro-Asp75His (lane 4), Asp63Pro-His75Asp (lane 5), and His75Asp-His97Tyr (lane 6), and triple-residue mutation: Asp63Pro-His75Asp-His97Tyr (lane 7), were resolved on 10% native gel and then assayed for APX activity (upper gel) and GPX activity (middle gel). Coomassie blue staining was used as internal control (bottom gel).
Each mutant protein was expressed in E. coli BL21 cell, isolated, and purified for biochemical assay. Similar far-UV circular dichroism spectra for wild-type protein and mutants indicated their identical folds, which are not altered by amino acid replacement (Figure S1). Enzymatic activity assays demonstrated that all the mutated OgCytAPX1 recombinant proteins, except the triple-residue mutation, retained both AsA and GSH oxidation activities (Figures 4A–4E). In a similar manner, the corresponding amino acid residues Asp63, His75, and His97 in AtCytAPX1 were mutated to Pro63-Asp75-Tyr97 (as present in OgCytAPX1) and assays of AsA/GSH-oxidizing activity were carried out. Only the triple-residues mutation Asp63Pro-His75Asp-His97Tyr exhibited GPX activity (Figures 4B–4F). All the mutated CytAPX1 showed equal enzymatic activity of APX (Figures 4A–4D). These results demonstrated that Pro63Asp75Tyr97 are required for GSH oxidation activity of OgCytAPX1.

**Phylogeny of CytAPX1 Antioxidant Substrate Recognition Sites in Plants**

To understand the universality of CytAPX1 with dual substrate-binding specificity in planta, CytAPX1 from seven plant species (Brassica juncea, Brassica oleracea, O. sativa, Zea mays, G. max, Solanum lycopersicum, and Nicotiana tabacum) were cloned and the recombinant proteins were produced from E. coli to assay for GPX activity in gel. Recombinant proteins from G. max, O. sativa, and Z. mays displayed both AsA and GSH oxidation activities, as OgCytAPX1 did (Figures 5A–5C), whereas proteins from the other species did not. Analysis of the full amino acid sequences revealed that plant species are hypothetically classified into three groups based on amino acid composition of GSH-binding site. Group I, including Oncidium, G. max, O. sativa, and Z. mays, contains the typical residues Pro63Asp75Tyr97 referred to as the Oncidium type (Figure 5D). Group II, with one to two conserved amino acids to Group I, including S. lycopersicum and N. tabacum, contains residues Lys63Asp75His97 or Lys63Asp75Tyr97. Group III, with no conserved amino acids to Group I, including Arabidopsis, B. juncea, and B. oleracea, contains residues Asp63His75His97, referred to as the Arabidopsis type (Figure 5D). Only Group I plants, possessing the GSH oxidation activity conferred by Pro63Asp75Tyr97, exhibit dual substrate recognition for oxidizing both AsA and GSH, whereas plants of groups II and III do not (Figures 5A–5C). A phylogenetic relationship based on the full amino acid sequence of CytAPX1 was constructed (Figure 5E). A total 27 plant species were grouped into three separate clades based on Maximum-Likelihood method. Group I comprises mainly eudicot Populus trichocapa, N. tabacum, G. max etc., but sequence similarities are more closely related to monocot Oncidium CytAPX1. On the other hand, 10 plant species of group II, including S. lycopersicum and Nicotiana attenuata, and 7 plant species of group III, including Arabidopsis thaliana, Arabidopsis lyrata, and B. oleracea, contain the atypical residues of Asp63His75His97 and do not exhibit GSH oxidation activity (Figures 5D and 5E). It is interesting to note that group II species have the transition-type residues between group I and group III. The clade marked with different colors denotes grouping classification of CytAPX1s. The result corresponds to group classification by three key amino acid residues (Figure 5E).

**Confirmation of GSH-Binding Activity in OgCytAPX by Isothermal Titration Calorimetry and UV-Vis Analysis**

The GSH binding activity of OgCytAPX1 was further validated by ITC analysis. As shown in Figure 6, GSH reacting with wild-type OgCytAPX1 releases corrected heat rate (Figure 6A), whereas it does not happen in mutated OgCytAPX1 (Figure 6B). The optical property assayed by UV-vis analysis revealed that Soret absorption maximal of OgCytAPX1 and AtCytAPX1 are around 410 nm (Figure 7A), and AtCytAPX1 PM (mutation from Asp63His75His97 to Pro63 Asp75Tyr97) shows absorption spectra close to OgCytAPX1 (Figure 7B). This demonstrated that OgCytAPX1 is a heme-containing protein, same as AtCytAPX1.

The UV-vis analysis also provides strong evidence that Soret absorption maxima of OgCytAPX1 is shifted from 410 to 411.5 nm (by +1.5 nm) in parallel with the titration concentration of GSH binding (Figure 7C). This suggests that heme group is oxidized during the GSH redox reaction. In contrast, absorption of AtCytAPX1 is not affected by GSH titration (Figure 7D). However, the mutant of AtCytAPX1 (PM) is shifted
Biochemical and Physiological Assays of Transgenic Arabidopsis Overexpressing OgCytAPX1 and AtCytAPX1, Respectively

OgCytAPX1 exhibits substrate-oxidizing affinities for both AsA and GSH in H$_2$O$_2$ reduction, whereas AtCytAPX1 exhibits only AsA-binding activity. To further examine their difference in function, Arabidopsis lines overexpressing either one of OgCytAPX1 or AtCytAPX1 were generated and APX activities in these lines were determined. Five independent lines for each gene transformation that had similar APX activity were selected for further study (Figure 8A). Transgenic Arabidopsis were grown for 6 weeks and transferred to high ambient temperature (30°C) for 14 days; then their GPX activity, AsA level and AsA redox ratio, GSH level, GSSG level and GSH redox ratio, as well as endogenous H$_2$O$_2$ content were measured. Higher total GPX activity was measured from OgCytAPX1-OE Arabidopsis, compared with Col-0 WT and AtCytAPX1-OE Arabidopsis, suggesting that OgCytAPX1 potentially confers GPX activity in Arabidopsis (Figure 8B). Notably, while the transgenic Arabidopsis plants overexpressing either OgCytAPX1 or AtCytAPX1 were grown at ambient temperature (22°C), no significant differences in AsA and DHA level or in AsA redox ratio were observed (Figures 8C and 8D). This implied that OgCytAPX1 and AtCytAPX1 have equal abilities to oxidize AsA at ambient temperature. However, while they were grown at high ambient temperature (30°C), the endogenous GSH level (Figure 8E) and GSH redox ratio (Figure 8F) in OgCytAPX1-overexpressing Arabidopsis were significantly lower than those in AtCytAPX1-overexpressing Arabidopsis. This is in contrast to that of no difference growing in ambient temperature (22°C) (Figure S2). Moreover, the endogenous H$_2$O$_2$ content of transgenic OgCytAPX1-OE Arabidopsis plants was one-third lower than that of transgenic AtCytAPX1-OE Arabidopsis plants (Figure 8G), suggesting that the potential GPX activity in OgCytAPX1 is effective to scavenge H$_2$O$_2$ and maintain the redox homeostasis at a lower H$_2$O$_2$ level, while plants stay at thermal stress condition, such as at 30°C condition.

Furthermore, observation of the flowering time revealed that all the transgenic Arabidopsis lines showed earlier flowering than wild-type (Col-0), once they were grown in high ambient temperature (30°C) for 14 days. Moreover, OgCytAPX1-OE plants showed much earlier bolting than AtCytAPX1-OE plants (Figure 9A). The number of rosette leaves in OgCytAPX1-OE plants was approximately 20, compared with 25 leaves in AtCytAPX1-OE plants and 37 leaves in wild-type (Figure 9B). This implied that OgCytAPX1 with dual antioxidant is beneficial to plants against environmental stress.

OgcytAPX1 Overexpression in vtc1 Mutant Mitigates ROS Damage through the Direct Utilization of GSH and Enhances Tolerance to Heat and Salt Stress

Arabidopsis vtc1 mutant, an ascorbate biosynthesis-deficient mutant, displays high sensitivity to environmental stresses. To examine the functional role of OgcytAPX1 in enhancing stress tolerance under AsA starvation, vtc1 mutants ectopically overexpressing OgcytAPX1 and AtcytAPX1 were produced. As shown in Figure 10, OgcytAPX1-OE-vtc1 displayed higher tolerance of 42°C heat stress for 2 h (Figures 10A–10C); the percentage of plants surviving stress treatment was 60% for OgcytAPX1-OE-vtc1, compared with 20% for AtcytAPX1-OE-vtc1, 10% for vtc1, and 20% for wild-type Arabidopsis. The salt stress assay on 150 mM NaCl medium for 2 weeks exhibited that OgcytAPX1 conferred elevated salt tolerance (Figure 10D). Root growth
activity is much more vigorous in OgcytAPX1-OE-vtc1 lines than in AtcytAPX1-OE-vtc1 lines, vtc1, and Col-0 WT (Figure 10E). The total chlorophyll content also showed higher level than others (Figure 10F). Taken together, results clearly demonstrated that the function of the dual antioxidant-binding activities of OgcytAPX1 is to enable plants to achieve a much higher tolerance of environmental stresses.

DISCUSSION
Dual Antioxidant-Binding Specificities for AsA and GSH by CytAPX1 Is an Evolutionary Event
APX is a heme peroxidase, found in all kingdoms of life, and typically catalyzes the one- and two-electron oxidation of a number of organic and inorganic substrates. Peroxidases of distinct families generally display representative sequence signatures and essential amino acids in heme cavity, and each family possesses a peculiar fold of the heme peroxidase domain (Zamocky et al., 2008). In the past decade, intensive analysis reveals that distinct families show pronounced catalase, cyclooxygenase, chlorite dismutase, or peroxygenase activities, in addition to the common peroxidatic activity (Zamocky et al., 2015). However, only ascorbate and cytochrome c peroxidases are typical monofunctional peroxidases with either ascorbate or cytochrome c as one-electron donor. The main function seems to be scavenging excess H$_2$O$_2$. Our present findings demonstrate that OgCytAPX1 in Oncidium and some plants possesses an additional activity (or pathway) of GPX, besides APX activity, for H$_2$O$_2$ reduction. Enzyme kinetic analysis showed that the binding affinity of OgCytAPX1 toward GSH is higher than toward AsA (Figures 2H and 2I). Maybe it is due to the lower concentration of GSH present in plant cell, about one-tenth of AsA (Noctor, 2006) (Table S1). The property of OgCytAPX1 is distinct from that of most APXs in plants, such as Arabidopsis (Figure 2), N. tabacum, and S. lycopersicum (Figure 5). It suggests that a new phylogenetic clade of CytAPX1, which can use both AsA and GSH as electron donors, has been evolving.

Pro63, Asp75, and Tyr97 of OgCytAPX1 are required for GSH oxidation, and three groups of CytAPX in planta are classified based on the amino acid residues of GSH-binding site. Based on the structural conformation model and serial site-directed mutations, we concluded that the three residues, Pro63, Asp75, and Tyr97 are required for GSH oxidation activity (Figures 3 and 4). Whether there are any other amino
acid residues involved in the formation of GSH-binding site, further investigation on structural function by using crystallographic approach is required. Plant species containing these three residues, Pro63, Asp75, and Tyr97, are designated as group I. In contrast, plants containing residues of Asp63His75His97 in the corresponding location and without GSH oxidation activity, such as in Arabidopsis and other plants of Brassica spp., are designated group III. The residue composition with either Lys63Asp75Tyr97 or Lys63Asp75His97 in group II is a transition type, such as N. attenuata and S. tuberosum, which have no GSH oxidation affinity either (Figure 5). Interestingly, it seems that more monocot plants belong to group I than eudicot plants (Figure 5). However, many active sites contain conserved substitution, which is structurally related to APX (Lazzarotto et al., 2011). Thus duplication event conserved in the chromosome region reflects that the active site varies for intragenomic duplication in Oncidium.

The Function of CytAPX Is Associated with Redox Homeostasis and Flowering Induction under Environmental Stress

APX is one of the key enzymes involved in the regulation of ROS homeostasis during plant growth or development and under adverse stress conditions (Correa-Aragunde et al., 2013; Maruta et al., 2012; Suzuki et al., 2013). Our previous study has shown that OgCytAPX1 is markedly upregulated to scavenge H$_2$O$_2$ by catalyzing AsA into DHA and causes a drastically reduced level of AsA and AsA redox ratio under high ambient temperature (30°C) (Chin et al., 2014). Also, high expression level with strong enzymatic activity of CytAPX1 is associated with low GSH/high GSSG content and low GSH redox ratio under light, drought, salt stress (Faize et al., 2011; Hernández et al., 2000; Karpinski et al., 1997). In addition, the redox
Figure 8. Effect of Oncidium CytAPX1 and Arabidopsis CytAPX1 on AsA and GSH Level/Redox Ratio after Overexpressing in Arabidopsis

CytAPX1 from Oncidium and Arabidopsis was overexpressed in Arabidopsis; five independent lines of each transformant plant were selected for monitoring the alternation of antioxidant levels and redox state.

(A–G) (A) Similar APX activity shown in five selected independent lines of each gene transformant plant. Comparison of (B) GPX activity, (C) AsA (black bar) and dehydroascorbate (DHA) level (white bar), (D) AsA redox ratio, (E) GSH (black bar) and GSSG level (white bar), (F) GSH redox ratio, and (G) H₂O₂ levels, among OgCytAPX1-OE, AtCytAPX1-OE, and WT (Col-0).
change of AsA, coupled with GSH redox state, plays a crucial role in protecting photosynthetic system from oxidative stress (Foyer and Noctor, 2011; Miller et al., 2010) and in floral induction in Oncidium (Chin et al., 2016). These reports support that CytAPX1 together with DHAR is the main enzyme regulating the redox homeostasis in AsA-GSH cycle (Gallie, 2013; Le Martret et al., 2011). The present study demonstrated that OgCytaPX1 possesses two substrate oxidation specificities for AsA and GSH, and has additional GPX activity (Figure 2). OgCytaPX1 causing lower H2O2 level and lower GSH redox ratio than AtCytAPX1 was demonstrated in overexpressing Arabidopsis (Figure 8). Our work strongly supports that GSH consumption by OgCytaPX1 is an independent biochemical step of AsA-GSH cycle.

Alternation of GSH level and GSH redox ratio affecting flowering time in Arabidopsis, wheat, and Eustoma grandiflorum have been reported (Yanagida et al., 2004) (Gulyas et al., 2014; Hatano-Iwasaki and Ogawa, 2012). Also, GSH level or redox status mediating GSH redox potential changes and glutathionylation from oxidative stress was known to associate with redox signal transduction to affect growth and development in plants (Noctor et al., 2012; Shigeoka and Maruta, 2014). The earlier flowering in association with lower GSH redox ratio in OgCytaPX1-OE Arabidopsis strongly suggests that the dual substrate recognition function of OgCytaPX1 is more efficient to scavenge H2O2, regulate redox homeostasis, and trigger signal transduction to affect development under environmental changes (Figure 8).

CytAPX1 Uses GSH to Enhance the Capability in Scavenging ROS and Confers High Tolerance on Plants in Oxidative Stress

Furthermore, understanding the truly physiological function of dual antioxidant recognition in OgCytaPX1 is the issue of most concern in this work. We used Arabidopsis vtc1 mutant, which is an ascorbate biosynthesis-deficient mutant, lacking ascorbate for ROS detoxification (Smirnoff, 2000), and displays high sensitivity to environmental stresses, such as high temperature and salinity (Wang et al., 2003; Larkindale et al., 2005). Obviously, OgCytaPX1 conferred greater tolerance and survival in transgenic vtc1 plants compared with AtcytaPX1 under heat and salt stress (Figure 10). The similar pattern of AsA level and redox ratio compared with AtcytaPX1-OE-vtc1 Arabidopsis suggests that the greater tolerance of OgCytaPX1-OE vtc1 Arabidopsis is not due to the effect of AsA oxidation to scavenge H2O2, but rather critical is the outcome of GSH consumption. The data demonstrate that the physiological function of OgCytaPX1 is able to compensate for AsA deficiency to mitigate ROS oxidative damage in AsA-deficient vtc1 system. While plants are under environmental stress, AsA is enormously employed for ROS scavenging and is associated with several phytohormones and signaling compound biosynthesis, such as abscisic acid, nitric oxide (NO), ethylene, and salicylic acid (Bethke et al., 2004; Khan et al., 2011; Kerchev et al., 2011). Consequently, AsA pool size decreases markedly in light, drought, heat stress, and high ambient temperature (Bartoli et al., 2005; Song et al., 2005; Chin et al., 2014). Thus it suggests that the capability of OgCytaPX1 to use GSH under AsA starvation condition confers high tolerance on plants in unfavorable environmental conditions.

In summary, we have discovered that CytAPX of Oncidium orchid and of several plants has an additional GSH oxidation activity to facilitate redox homeostasis under high temperature stress condition (Figure 11). This is coincident with the strong growth potential of Oncidium and several monocot plants in adaption to wild field. The three amino acid residues Pro63, Asp75, and Tyr97, required for GSH oxidation, were identified. Our study pointed out that the GSH oxidation activity in CytAPX distributes in most monocot plants, such as O. sativa, Z. mays, and G. max. Phylogenetic relationship based on the variation of GSH-binding residues of CytAPX showed that the phylogenetis clade is classified into three groups. The significance and evolutionary mechanism in plants are worthy of further investigation.

Limitations of Study

This work first describes that OgCytaPX1 possesses two substrate oxidation specificities for AsA and GSH and has the additional GPX activity to facilitate redox homeostasis under high temperature stress...
conditions. We confirmed that OgcytAPX1 causing lower H$_2$O$_2$ level and lower GSH redox ratio than AtcytAPX1 in Arabidopsis overexpression lines suggested that OgcytAPX1 uses GSH as an independent biochemical step in AsA-GSH cycle. UV-vis analysis further confirmed its heme-containing protein. Furthermore, we discovered three amino acid residues Pro63, Asp75, and Tyr97, required for GSH oxidation. Our investigation pointed out that the GSH oxidization activity in cytAPX1 distributes in many plants, such as O. sativa, Z. mays, and G. max, signifying the evolutionary mechanism in plants.

**METHODS**
All methods can be found in the accompanying Transparent Methods supplemental file.

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**Figure 9. Effect of Oncidium CytAPX1 and Arabidopsis CytAPX1 on Flowering Time after Overexpressing in Arabidopsis**
CytAPX1 from Oncidium and Arabidopsis was overexpressed in Arabidopsis to monitor the flowering time. (A) Photography shows OgCytAPX1-OE plants flowering earlier than AtCytAPX1-OE plants and WT. (B) Rosette leaves number of OgCytAPX1-OE plants, AtCytAPX1-OE plants, and WT while bolting. Experiments were repeated twice using 20 plants in each group. The number of rosette leaves was determined when inflorescences were 1 cm in length. Plants were first grown at 22°C under short day condition (8/16-h photoperiod) for 6 weeks and then transferred to 30°C under short day conditions until bolting. After bolting, plants were placed at 22°C under short day conditions for recovery from stress to determine the number of rosette leaves. Error bar indicates the SD (standard deviation of the mean [n = 40]). Statistical significance was analyzed by ANOVA with post-hoc test. Different letters indicate significant differences between wild-type and transgenic lines according to Fisher’s protected least significant difference test at a significant level of p < 0.05.
Figure 10. Effects of Oncidium CytAPX1 and Arabidopsis CytAPX1 on Heat and Salt Stress Tolerance after Overexpressing in Arabidopsis vtc1-Deficient Mutant

(A and B) Phenotypic survival of Arabidopsis seedlings WT(Col-0), vtc1 mutant and overexpression lines of OgCytAPX1-OE and AtCytAPX1-OE after subjecting to heat stress at temperature 42°C for 2 hours in light/day photoperiod.

(C–F) (C) Survival rate of transgenic Arabidopsis lines, vtc1 mutant, and WT after treatment at 42°C for 2 h. Comparison of (D) salt stress tolerance, (E) the primary root length, (F) leaf chlorophyll content among vtc1 mutant, WT (Col-0), and OgcytAPX1-OE and AtcytAPX1-OE in vtc1 mutant after salt treatment with 150 mM NaCl for 2 weeks. Error bar indicates the SD (standard deviation of the mean [n = 30]). Statistical significance was analyzed by ANOVA with post-hoc test. Different letters indicates significant differences between wild-type and transgenic lines according to Fisher’s protected least significant difference test at a significance level of p < 0.05.
**DATA AND SOFTWARE AVAILABILITY**

The accession number for CytAPX1 reported in this paper is NCBI database: Oncidium [ACJ38537], B. juncea [AAC37695], B. oleracea [HQQ87184], G. max [BAC92739], S. lycopersicum [AAZ77770], N. tabacum [Xp:016432750], O. sativa [Q10N21], Z. mays [ALF39503], A. thaliana [Q05431].

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.05.014.

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**AUTHOR CONTRIBUTIONS**

D.-C.C., C.-S.S., M.-J.H., and K.-W.Y. designed the experiments. D.-C.C. and C.-S.S. performed the experiments. D.-C.C. R.S.K., and C.-S.S assisted and analyzed the data. D.-C.C., R.S.K., M.-J.H., and K.-W.Y. reviewed the manuscript. Z.-X.L supervised the experiment, X.X. performed statistical analysis.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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Supplemental Information

Plant Cytosolic Ascorbate Peroxidase
with Dual Catalytic Activity Modulates
Abiotic Stress Tolerances

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Supplemental information

Transparent Methods

Plant materials and growth condition

Oncidium ‘Grower Ramsey’ plants were grown and maintained in a greenhouse at 20-25°C for the observation of normal growth, following protocol described previously (Chin et al., 2016). For various biochemical assays, orchid plants were shifted and grown in phytotron at 30°C with 16-h-light/8-h-dark cycle photoperiod for the observation of number of inflorescence bud developed (Chin et al., 2016). For all biochemical activity assay, crude soluble proteins were obtained from Oncidium pseudobulb and Arabidopsis tissues. The tissues were ground to fine powder in liquid nitrogen and then homogenized with the extraction buffer containing 50mM sodium phosphate buffer, pH 7.2, 2mM EDTA, 2mM DTT, 20% glycerol and PVPP, following protocol described previously (Chin et al., 2016). Transgenic Arabidopsis from the Col-0 SALK-line vtc1 mutants and ectopically overexpressing Oncidium genes (OgCytAPx1) and Arabidopsis gene (AtCytAPX1), were grown at 22°C under short-day (SD) conditions (8/16 hr photoperiod) for 6 weeks and subsequently maintained at 22°C or transferred to 30°C to conduct various biochemical assays and to determine the number of leaves prior to floral initiation. Oncidium orchid used RNA extraction was grown on peat moss in pot at 22-30°C under a 16-h-light/8-h-dark cycle.

Gene cloning by RACE

Sequences of the full-length CytAPX1s from various plant species were completed using the rapid amplification of cDNA ends (RACE) method (GeneRacer RLM-RACE kit, Invitrogen™). Sequence was identified, confirmed and deposited in GeneBank under the assigned accession numbers.
Recombinant protein expression and purification

Recombinant protein expression and purification were performed by the pMAL™ Protein Fusion and Purification System (New England BioLabs). cDNAs for the target proteins were cloned into the pMAL-c5X vector and were transformed into *E. coli* (BL21-Codon-Plus). The recombinant proteins expressed in *E. coli* following 37°C incubation for 2 h (OD$_{600}$ ~0.5), and induction with 100 μM isopropyl β-thiogalactopyranoside (IPTG) for 4 h at 28 °C. Harvested cells were sonicated with 10-s pulses separated by 10-s intervals for 10 min, and the insoluble materials were removed by centrifugation at 16,000x g for 15 min at 4 °C. The supernatant was sterile-filtered and the fusion protein was purified on a 2.5 x 10 cm column with amylose resin (New England BioLabs). The purified target protein fused with MBP (maltose binding protein) tag was digested by Factor-Xa (New England BioLabs) to remove the tag, and the digested proteins were monitored by 10% SDS-PAGE.

In-gel assay for ascorbate peroxidase (APX) and glutathione peroxidase (GPX) activity

The *in-gel* assay for APX activity was carried out following the method described previously (Mittler and Zilinskas, 1993). Appropriate amount of homogenates and the purified recombinant proteins were resolved using 10% native gel electrophoresis. Following electrophoresis, the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for a total of 30 min with the equilibration buffer changed every 10 min. The gel was then incubated with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 20 mM H$_2$O$_2$ for 20 min. The gel was subsequently
washed with sodium phosphate buffer (pH 7.8), 28 mM tetramethylethylenediamine (TEMED) and 24 mM Nitro Blue Tetrazolium (NBT) with gentle agitation for approximately 10 min and the reaction stopped by a brief wash with distilled water. After staining, an achromatic band appeared against the dark purple background.

The *in-gel* assay for GPX activity was performed following the method described previously (Lin et al., 2002). Appropriate amount of homogenates and purified recombinant proteins were resolved using 10% native gel electrophoresis. After native PAGE, gel was submerged for 20 min twice in 50 mM Tris-HCl buffer (pH 7.9) before activity staining. Activity staining of GPX was as follows (MP method). Gel was soaked in the substrate solution (50 mM Tris-HCl buffer, pH 7.9, 13 mM GSH, and 0.004% hydrogen peroxide) with gentle shaking for 10–20 min. After a brief rinse, the GPX activity was developed in darkness at room temperature with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) in distilled water for 10 min. The clear zone of GPX activity is present against a purple background.

**APX and GPX activity assay**

Ascorbate peroxidase was assayed by the method described previously (Nakano and Asada, 1981). The reaction mixture to measure APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM AsA, 2 mM H₂O₂ (all the component are from Sigma) and 2mL of crude protein extract in a total volume 5mL. The activity was recorded as decrease in absorbance at OD₂₉₀ for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.6 mM⁻¹ cm⁻¹.

Activity of GPX from the transgenic *Arabidopsis* was assayed by a modified method
of the coupled enzyme system (Drotar et al., 1985). The reaction solution includes 50mM potassium phosphate (pH 7.0), 2mM H$_2$O$_2$, 2mM GSH, 2.5 units of glutathione reductase, 2mM 0.1 mM NADPH (all the component are from Sigma), and 2mL of desalted crude protein extract in a total volume 5mL. The reaction rate was measured by the loss of NADPH at OD$_{340}$ for 2mins. Glutathione peroxidase activity assay in GPX kinetic analysis was measured by DTNB method with modification (Drotar et al., 1985). The reaction solution contains 50mM potassium phosphate (pH 7.0), 2mM H$_2$O$_2$, 0-0.12mM GSH, 0.06% 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) and 2mL of crude protein extract in a total volume 5mL. The absorbance at OD$_{412}$ was performed for measuring GSH concentration. The activity was recorded as decrease of GSH concentration and calculated the values obtained from the standard curve.

**Heat and Salinity stress assay.**

*Arabidopsis* seedlings of WT (Col-0), *vtc1* mutant and overexpression lines of *OgCtyAPX1* and *AtCytAPx1* were grown on MS medium horizontally, and were subjected to heat stress by submerging them in a water bath maintained at temperature 42° C, as described (Mishra et al., 2018). This was performed for 2 hrs in light/day photoperiod. The seedlings were then returned to growth room and were allowed to recover for 7 days. The number of viable seedlings were quantified and the survival rate was determined. For salt stress, seedlings were transferred to plates containing 150mM NaCl for 2 weeks (Mishra et al., 2018). Fresh weight, phenotypic differences of ten seedling, root length and chlorophyll content were recorded after one week. All experiments were repeated three times.

**Enzyme kinetic analysis**
50µg purified recombinant OgCytAPX1 protein was used for kinetic analysis assay. The kinetic parameters were calculated based on Lineweaver–Burk plots of the Michaelis–Menten equation, \( \frac{1}{V} = \frac{K_m}{V_{\text{max}}}\left(\frac{1}{[C]} + \frac{1}{K_m}\right) \). \( V \) is the initial reaction rate, \( V_{\text{max}} \) is the maximum reaction, \([C]\) is the substrate concentration, and \( K_m \) is the Michaelis-Menten constant.

**Estimation of \( \text{H}_2\text{O}_2 \), \( \text{AsA}/\text{DHA} \), and \( \text{GSH}/\text{GSSG} \)**

\( \text{H}_2\text{O}_2 \) content in *Arabidopsis* was measured by DMAB–MBTH–POX method (Queval et al., 2008; Veljovic-Jovanovic et al., 2002). 50 mg *Arabidopsis* leaves were ground in liquid nitrogen and the tissue powder extracted in 2 mL 1 M \( \text{HClO}_4 \) including 5% PVP. Homogenates were centrifuged at 12,000xg for 10 min at 4 °C and the supernatant was neutralized by 5 M \( \text{K}_2\text{CO}_3 \) to pH 5.6. The homogenate then was centrifuged at 12,000xg for 1 min to remove \( \text{KClO}_4 \). The sample was incubated with 1 U ascorbate oxidase (Sigma) for 10 min to oxidize ascorbate. The reaction mixture includes 0.05 M phosphate buffer (pH 6.5), 3.3 mM DMAB, 0.07 mM MBTH, and 0.1U horseradish peroxidase (Sigma). The reaction was initiated by adding 50 µL of sample. The change of absorbance at 590 nm was monitored at 25 °C for 5 min, and this value is the total peroxide in samples.

To avoid the interference of other peroxides, the samples incubated with 1 U catalase (Sigma) for 10 min, and then followed the same procedures described as above. This absorbance change at \( \text{OD}_{590} \) is other peroxide, excluding \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) contents were subtracted other peroxide from total peroxide, and calculated by the reference to an internal standard (1.5 nmol \( \text{H}_2\text{O}_2 \)).

Total AsA, reduced AsA, and total DHA were measured following the method described previously (Gillespie and Ainsworth, 2007). 40 mg plants tissue was extracted
by 6% trichloroacetic acid (TCA) followed by the addition of 10 mM dithiothreitol (DTT) to reduce the pool of oxidized AsA. Total AsA content was measured at OD\textsubscript{525}. Total AsA (DTT added) and reduced AsA levels (DTT not added) were obtained using this method. Total DHA content was obtained by subtracting the reduced AsA from total AsA.

The levels of GSH and GSSG were assayed by the modified method described previously (Rahman et al., 2006). Tissue sample (0.1 g) was homogenized in 0.6% sulfosalicylic acid solution and then centrifuged at 16,000x g for 10 min at 2–4 °C. The clear supernatant was transferred to a new tube and used for the total GSH assay. In a 96-well microtitre plate, 20 μl of 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt pH 7.5 (KPE) was placed in a well, and then 20 μl of the sample was added. Next, equal volumes of freshly prepared 5,5-dithio-bis-2-nitrobenzoic acid (DTNB; 0.06% in KPE) and glutathione reductase (GR; 3 units in KPE) solutions were mixed together and 120 μl of the mixture added to each well. A yellow color was obtained after the final reaction. Tube containing the DTNB:GR mixture was covered by aluminum foil to avoid direct exposure to light. After 30 s to allow for the conversion of GSSG to GSH, 60 μl of β-NADPH was added. The absorbance at OD\textsubscript{412} was performed immediately in a microplate reader, measured every 30 s for 2 min (five readings in total from 0–120 s). The rate of 2-nitro-5-thiobenzoic acid formation (change in absorbance min\textsuperscript{-1}) was calculated. The actual total GSH concentration in the samples was determined by using linear regression to calculate the values obtained from the standard curve. In GSSG assay, 2 μL 2-vinylpyridine (Sigma) was added to 100μl extract and mixed well to derivative GSH. The mixture was reacted for 1 h at room temperature in a hood. Measurement of the derivative samples by the method described as same as total GSH.
The concentration of total GSH and GSSG in samples were determined by using linear regression to calculate values obtained from a standard curve of GSH and GSSG (containing 2 μl 2-vinylpyridine) (Sigma).

**Isothermal Titration Calorimetry (ITC)**

Binding of GSH to wild-type OgCytAPX1 and its mutant were measured by ITC with a Nano Isothermal Titration Calorimeter (TA Instruments). Aliquots of 4 μl of 2.0 mM GSH were titrated by injection into protein (0.1 mM in 0.98 ml) in 25 mM phosphate (pH 7.0) and 100 mM NaCl. Experiments were carried out at 25 °C with 250 rpm stirring.

Background heat from ligand to buffer titrations was subtracted during data processing, and the corrected heat from the binding reaction was used to derive values for the stoichiometry of the binding (n), K_d, apparent enthalpy of binding (ΔH), and entropy change (ΔS). Data were fitted by use of an independent binding model with Launch NanoAnalyze version 2.3.6.

**Circular Dichroism (CD) Spectroscopy**

Far-UV CD spectra were measured over 190-260 nm wavelength with 20 μM protein sample placed into a 1-mm path length cuvette and recorded on a JASCO J-810 spectropolarimeter (JASCO international Co.) equipped with a Peltier temperature control system (JASCO PTC-423S). All samples were centrifuged at 10,000g for 10 min before analysis. Experiments were carried out at 25 °C. Data processes including baseline subtraction and smoothing were done by Origin.

**UV-Visible spectroscopy**

Spectra of 5 μM recombinant proteins were placed into a 1-mm path length cuvette and monitored on a UV–visible spectrophotometer (Hitachi U-3010) between 350 and
500 nm (300 nm min⁻¹) in buffer containing 25 mM phosphate (pH 7.0) and 100 mM NaCl in absence and presence of GSH at different concentration (from 0.1 to 3.0 mM). Experiments were carried out at 25 °C.
Table S1 The reaction rate of *CytAPX1* against AsA substrate in planta (related to Figure 2)

| Organisms          | K_m value          | References                     |
|--------------------|--------------------|--------------------------------|
| *Oncidium*         | 0.626mM            | In this study                  |
| *Theobroma cacao*  | 0.419 µM           | (Camillo et al., 2013)         |
| *Pea*              | 20µM               | (Mittler and Zilinskas, 1991)  |
| *Soybean*          | 11 µM              | (Dalton et al., 1987)          |
| *Pallavicinia*     | 28.7 µM            | (Sajitha Rajan and Murugan, 2010) |
Figure S1 Protein folding comparison of wild-type protein and mutants (related to Figure 4).

Far UV circular dichroism spectra of (A) OgCytAPX1 wild type and mutant protein (at 100 µM concentration in 20mM phosphate, pH 7.0 room temperature). (B) AtCytAPX1 wild-type and mutant protein (at 200 µM concentration in 200mM phosphate, pH 7.0, room temperature), and (C) OgCytAPX1 wild type, AtCytAPX1 wild type and AtCytAPX1 mutant protein (at 200µM concentration in 20mM phosphate, pH 7.0, room temperature) were monitored over 190-260nm wavelength. The experiments were repeated three times and representative as dotted traces.
Figure S2 (A) GSH, GSSG content, and (B) GSH redox ratio in *OgCytAPX1-OE* and *AtCytAPX1-OE* Arabidopsis independent lines grown at 22°C (related to Figure 8). Error bar indicates SD (standard deviation of the mean (n=30). Statistical significance was analysed by Analysis of variance (ANOVA) with post-hoc test. Different letters indicates significant differences between wild type and transgenic lines according to Fisher’s protected LSD test at a significant level of *p*<0.05.