Double Mutants Deficient in Cytosolic and Thylakoid Ascorbate Peroxidase Reveal a Complex Mode of Interaction between Reactive Oxygen Species, Plant Development, and Response to Abiotic Stresses

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Reactive oxygen species (ROS) play a key signaling role in plants and are controlled in cells by a complex network of ROS metabolizing enzymes found in several different cellular compartments. To study how different ROS signals, generated in different cellular compartments, are integrated in cells, we generated a double mutant lacking thylakoid ascorbate peroxidase (tylapx) and cytosolic ascorbate peroxidase1 (apx1). Our analysis suggests that two different signals are generated in plants lacking cytosolic APX1 or tylAPX. The lack of a chloroplastic hydrogen peroxide removal enzyme triggers a specific signal in cells that results in enhanced tolerance to heat stress, whereas the lack of a cytosolic hydrogen peroxide removal enzyme triggers a different signal, which results in stunted growth and enhanced sensitivity to oxidative stress. When the two signals are coactivated in cells (i.e. tylapx/apx1), a new response is detected, suggesting that the integration of the two different signals results in a new signal that manifests in late flowering, low protein oxidation during light stress, and enhanced accumulation of anthocyanins. Our results demonstrate a high degree of plasticity in ROS signaling in Arabidopsis (Arabidopsis thaliana) and suggest the existence of redundant pathways for ROS protection that compensate for the lack of classical ROS removal enzymes such as cytosolic and chloroplastic APXs. Further investigation of the enhanced heat tolerance in plants lacking tylAPX, using mutants deficient in chloroplast-to-nuclei retrograde signaling, suggests the existence of a chloroplast-generated stress signal that enhances basal thermotolerance in plants.

Reactive oxygen species (ROS) control in-plant processes such as growth, development, stomata signaling, and biotic and abiotic stress responses (Torres and Dangl, 2005; Asada, 2006; Gapper and Dolan, 2006; Halliwell, 2006; Kwak et al., 2006; Mullineaux et al., 2006; Pitzschke and Hirt, 2006; Torres et al., 2006; Van Breusegem and Dat, 2006; Zaninotto et al., 2006). The steady-state level of ROS in cells, as well as the duration, subcellular localization, and intensity of ROS signals, are thought to be controlled in cells by the ROS gene network (Mittler et al., 2004; Bailey-Serres and Mittler, 2006). This network is composed of over 150 genes in Arabidopsis (Arabidopsis thaliana) encoding ROS-generating proteins, such as NADPH oxidases, and ROS-scavenging enzymes, including superoxide dismutases, catalases (CATs), thio- and glutaredoxins, and ascorbate peroxidases (APXs; Mittler et al., 2004).

Although the ROS gene network of plants is thought to play a key role in the regulation of different biological processes (Bailey-Serres and Mittler, 2006), the function of fewer than 5% of the genes that compose the ROS network of Arabidopsis has been determined (Mittler et al., 2004). Moreover, the mode of coordination and the degree of redundancy and cross talk between different branches of the ROS network, as well as the way in which the network senses and transduces ROS signals, are virtually unknown.

The different scavenging and producing enzymes encoded by the ROS gene network are found in many different cellular compartments. In addition, usually more than one enzymatic scavenging activity per a specific ROS can be found in each of the different compartments (Mittler, 2002; Mittler et al., 2004). Because ROS such as hydrogen peroxide (H2O2) can diffuse between different cellular compartments (Henzler and Steudle, 2000; Bienert et al., 2007), ROS metabolism in a particular compartment can effect or alter the

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ROS homeostasis/signaling of a neighboring compartment. Recent studies in Arabidopsis suggested that the mode of coordination between the different cellular compartments of plants is complex (Mittler et al., 2004; Mullineaux et al., 2006). For example, the application of light stress to Arabidopsis resulted in the induction of cytosolic and not chloroplastic ROS removal enzymes (Karpinski et al., 1997, 1999; Pnueli et al., 2003; Davletova et al., 2005a), even though most ROS produced during light stress are thought to be generated in chloroplasts and/or peroxisomes (Mittler, 2002). In addition, a cytosolic APX (APX1) and not a chloroplastic APX (stromal/mitochondrial APX [s/mAPX]), was shown to protect the chloroplast from oxidative damage during light stress (Davletova et al., 2005a). Cytosolic APX1 is also essential for the proper function of chloroplastic APXs, and in its absence, both thylakoid APX (tylAPX) and s/mAPX are degraded during light stress (Davletova et al., 2005a).

We previously reported that antisense tobacco (Nicotiana tabacum) plants with suppressed expression of cytosolic APX1 and peroxisomal CAT1 are more tolerant to light stress compared to plants with suppressed expression of APX1 or CAT1 (Rizhsky et al., 2002). This result suggested that different signaling pathways are activated in plants with reduced expression of APX1 or CAT1 and that suppression of both APX1 and CAT1 results in the activation of a new type of response, possibly resulting from the integration of the two different pathways activated in plants lacking APX1 or CAT1 (Rizhsky et al., 2002). Interestingly, plants with reduced activity of CAT1 activated cell death in response to light stress, whereas plants with reduced APX1 and CAT1 did not. This result supported the hypothesis that different signals are generated in cells lacking different ROS-scavenging enzymes localized to different cellular compartments.

To further study how different ROS signals, generated in different subcellular compartments, are integrated in cells lacking different ROS-scavenging enzymes localized to different cellular compartments.

**RESULTS**

**Generation of Double Mutants Deficient in Cytosolic APX1 and tylAPX**

To generate double mutants deficient in cytosolic APX1 and tylAPX (apx1/tyltpx), we crossed knockout plants deficient in APX1 (apx1; At1g07890; Pnueli et al., 2003; Davletova et al., 2005a; Wassilewskija [WS] background) with knockdown plants with suppressed expression of tylAPX (tyltpx; At1g77490; SALK_072948; Columbia [Col] background, harboring a T-DNA insert in the promoter of tylAPX). Progenies of crosses were then selfed, tested for a homozygous genotype of the double mutant, and bulked up. As controls for the apx1/tyltpx double mutants, we crossed wild-type WS plants with wild-type Col plants. To overcome genetic variability that potentially results from crossing two different genetic backgrounds, seeds were pooled from six different homozygous crossing events of apx1/tyltpx and 10 different homozygous crossing events of the wild-type control WS/Col. The WS/Col and apx1/tyltpx pools were used for all subsequent analysis in which apx1 plants were compared to WS, tyltpx plants were compared to Col, and apx1/tyltpx plants were compared with WS/Col. Protein-blot analysis of the different mutants grown under controlled growth conditions.
growth conditions confirmed the absence of APX1 in \textit{apx1} and \textit{apx1/tylapx} plants and the reduced expression of \textit{tylAPX} in \textit{tylapx} and \textit{apx1/tylapx} plants (Fig. 1A). Our results therefore demonstrate that single and double mutants for \textit{APX1} and \textit{tylAPX} are viable, at least when grown under controlled growth conditions.

**Growth and Development of Double Mutants Deficient in Cytosolic \textit{APX1} and \textit{tylAPX}**

As shown in Figure 1B, fresh weight and plant size of mutants with suppressed expression of \textit{tylAPX} were similar to that of wild type. In contrast, mutants lacking APX1 had stunted growth (Pnueli et al., 2003; Davletova et al., 2005a). Interestingly, double \textit{apx1/tylapx} mutants appeared to be suppressed in their growth similar to \textit{apx1} plants, suggesting that \textit{APX1} is the more important of the two genes in controlling growth. As shown in Figure 2, bolting of \textit{tylapx} or \textit{apx1} plants was early compared to their corresponding backgrounds when plants were grown under controlled growth conditions \((P = 0.01)\). In contrast, bolting of \textit{apx1/tylapx} plants was significantly delayed compared to the WS/Col controls \((P = 0.01)\), suggesting a complex mode of interaction between the two genes in controlling bolting and flowering time. In contrast to the significant differences in time of bolting between each of the mutants and their corresponding controls (Fig. 2, A–C), leaf number of the different lines was not significantly different during plant growth (Fig. 2D). The low variability observed between the different plants, assayed for time of bolting and leaf number in the \textit{apx1/tylapx} and WS/Col lines, obtained from the pools of different crossing events could be viewed as an indication that the pools represented a population of plants with low genetic variability (compare the se bars in Fig. 2, A and B, with the se bars in Fig. 2, C and D). Our results suggest a complex mode of interaction between ROS scavenging in the cytosol and/or chloroplast and plant growth and development.

**Response of Double Mutants Deficient in Cytosolic \textit{APX1} and \textit{tylAPX} to Light Stress**

To determine how \textit{apx1/tylapx} responds to a light stress treatment, we subjected WS, \textit{apx1}, Col, \textit{tylapx}, and WS/Col and \textit{apx1/tylapx} plants to a light stress treatment \((900 \mu\text{mol m}^{-2}\text{s}^{-1} \text{for } 0, 1, 3, 6, 24, \text{and } 48\text{ h})\).

![Figure 2](image-url)

**Figure 2.** Differences in time of bolting between the different \textit{APX} mutants and the double mutant. A to C, Inflorescence length was measured for each mutant during growth under controlled conditions. The different mutant lines are presented with respect to their corresponding controls. Genetic backgrounds are shown in parentheses. D, Number of leaves for all lines measured during growth under controlled conditions is given as a control. sxs are given for 40 replicates of each line. Controlled growth conditions: 22°C to 23°C, continuous light, 40 \mu\text{mol m}^{-2}\text{s}^{-1}, and a relative humidity of 70%.
As shown in Figure 3, light stress resulted in the accumulation of oxidized proteins in the different wild-type and control lines. In contrast, no significant changes were observed in the level of Rubisco protein (large subunit of Rubisco [rbcL] and small subunit of Rubisco [rbcS]; Fig. 3). In agreement with Davletova et al. (2005a), a high level of protein oxidation was detected in apx1 plants in response to light stress. In contrast, the basal level of protein oxidation under controlled growth conditions was high in tylapx plants. These results suggest that plants deficient in APX1 or tylAPX are subjected to oxidative stress when grown under controlled growth conditions (tylapx) or when subjected to light stress (tylapx or apx1; Davletova et al., 2005a). In contrast to tylapx or apx1 mutants, oxidized proteins did not accumulate in the apx1/tylapx double mutant grown under controlled growth conditions or subjected to light stress (Fig. 3). Interestingly, in the tylapx mutant, a transient decrease in protein oxidation was observed during light stress. This decrease could be the result of a transient increase in the expression level of different ROS defense mechanisms during light stress, as described by Davletova et al. (2005a). Thus, in response to light stress, additional defense mechanisms might be activated in tylapx mutants to fend off the increasing levels of ROS. However, these
are insufficient, and protein oxidation is therefore only transiently decreased.

RNA blots performed on the different lines subjected to light stress revealed that light stress in apx1 plants resulted in enhanced expression of the ROS response zinc-finger protein Zat12 (Rizhsky et al., 2004; Davletova et al., 2005b) and the Respiratory burst oxidase homolog-D (RbohD; Fig. 4). This result was in accordance with our previous analysis of apx1 plants (Davletova et al., 2005a). We previously reported that in wild-type plants, Zat12 is expressed in two different phases: an early phase (phase I) that is not dependent on retrograde signaling, and a late phase (phase II) that is dependent on retrograde signaling (Koussevitzky et al., 2007). In apx1 plants, the two phases cannot be distinguished, because Zat12 expression is highly elevated (Fig. 4). In contrast, in tylapx plants and in apx1/tylapx plants, phase I of Zat12 expression could not be detected. The enhanced expression of phase II of Zat12 expression and RbohD expression observed in apx1 plants could also not be detected in apx1/tylapx plants (Fig. 4).

Anthocyanin accumulation was previously linked to ROS accumulation and light stress (Vanderauwera et al., 2005). Interestingly, while anthocyanin accumulation was only slightly elevated in tylapx or apx1 plants, anthocyanin accumulated to high levels in apx1/tylapx plants during light stress (Fig. 5). Our results suggest that, at least in the apx1/tylapx double mutant, a redundant mechanism(s) for ROS removal is activated to provide plants with enhanced tolerance to oxidative stress.

Tolerance of Mutants Deficient in Cytosolic APX1 and/or tylAPX to Abiotic Stresses

Mutants with altered capability to scavenge ROS show altered tolerance to abiotic stresses (Davletova et al., 2005b; Ciftci-Yilmaz et al., 2007). To evaluate the tolerance of the apx1, tylapx, and apx1/tylapx mutants to abiotic stresses, we subjected 5-d-old seedlings of the different lines to oxidative stress (paraquat), osmotic stress (sorbitol), salinity, and heat and cold stresses using the assays described in Mittler et al. (2007), Davletova et al. (2005b), and Ciftci-Yilmaz et al. (2007). All lines germinated simultaneously, and no differences were observed in the growth and germination rate of the different lines (data not shown). As shown in Figure 6A, apx1 mutants, but not tylapx or apx1/tylapx, appeared to be more sensitive to oxidative stress. Compared to their corresponding controls, all lines appeared to be more tolerant to salinity stress (Fig. 6B). While apx1 and tylapx plants appeared to also be more tolerant to osmotic stress, apx1/tylapx was not (Fig. 6C). Plants lacking tylAPX and apx1/tylapx plants appeared to be more tolerant to heat stress, while apx1 plants were also more tolerant to this stress but to a lesser degree (Fig. 6D). With respect to cold stress, tylapx plants were similar to wild type, apx1 appeared to be more tolerant, but apx1/tylapx plants appeared to be more sensitive (Supplemental Fig. S1).

To further test the tolerance of the different lines to heat stress, we performed basal and acquired thermotolerance survival assays as described by Suzuki et al. (2005). We could not detect differences between the lines in acquired thermotolerance (data not shown). In contrast, tylapx and apx1/tylapx plants were found to be significantly more tolerant to heat stress than their corresponding controls (Fig. 7). In contrast, apx1 and WS plants had a similar level of basal thermotolerance (Fig. 7).

The enhanced basal thermotolerance of tylapx or apx1/tylapx mutants (Fig. 7) suggested that a signal that might be related to ROS metabolism, generated in the chloroplast, enhances the basal thermotolerance of plants. It was recently suggested that all retrograde signaling pathways in Arabidopsis are channeled through a single pathway involving genome-uncoupled gun1 and abscisic acid-insensitive abi4 (Koussevitzky et al., 2007). To test whether this pathway is also involved in enhancing basal thermotolerance in Arabidopsis, we subjected different mutants deficient in retrograde signaling (gun1, gun5, and abi4; Nott et al., 2006) to heat stress. As shown in Figure 8, all retrograde signaling pathways in阿拉伯祖斯 (Arabidopsis) are channeled through a single pathway involving genome-uncoupled gun1 and abscisic acid-insensitive abi4 (Koussevitzky et al., 2007). To test whether this pathway is also involved in enhancing basal thermotolerance in Arabidopsis, we subjected different mutants deficient in retrograde signaling (gun1, gun5, and abi4; Nott et al., 2006) to heat stress. As shown in Figure 8, all retrograde signaling...
mutants had reduced basal thermotolerance. In contrast, gun1, gun5, and abi4 mutants did not appear to be impaired in acquired thermotolerance (Fig. 8). Our results suggest a complex mode of interaction between ROS scavenging in the cytosol and/or chloroplast and plant response to environmental stress. In addition, we identified a new signaling pathway that enhances basal thermotolerance in plants in response to the absence of a chloroplastic ROS-scavenging enzyme (tylAPX).

**DISCUSSION**

Recent studies of knockout, antisense, or RNAi lines for CAT2, APX1, tylAPX, mitochondrial and chloroplastic alternative oxidase, superoxide dismutase, peroxiredoxins, thio- and glutaredoxins, as well as different NADPH oxidases, revealed a strong link between ROS and processes such as growth, development, stomatal responses, and biotic and abiotic stress responses (Danna et al., 2003; Mittler et al., 2004; Torres and Dangl, 2005; Gapper and Dolan, 2006; Kwak et al., 2006; Mullineaux et al., 2006; Pitzschke and Hirt, 2006; Torres et al., 2006; Van Breusegem and Dat, 2006; Zaninotto et al., 2006). These findings demonstrated the complex nature of the ROS gene network of plants and its modulation of key biological processes. Although all the mutants reported above are viable, demonstrating the redundancy of the ROS gene network, a phenotype was associated with each of the different mutants, suggesting that they represent genes that play important roles in the ROS signaling network of plants. In contrast to the studies described above, an analysis of mutants lacking more than one ROS metabolizing enzyme was, to the best of our knowledge, only reported in tobacco for double antisense plants with suppressed expression of CAT1 and APX1 (Rizhsky et al., 2002). The analysis of these plants revealed a high degree of redundancy in the ROS gene network of plants. Thus, plants lacking APX1 and CAT1 appeared less sensitive to oxidative stress compared to plants lacking either APX1 or CAT1 (Rizhsky et al., 2002). Our current analysis of plants lacking tylAPX and APX1 revealed an even higher degree of...
complexity in the ROS gene network that was evident in growth and developmental phenotypes, as well as in responses to abiotic stresses and susceptibility to oxidative stress (Figs. 1–7).

We previously reported that plants lacking APX1 are more sensitive to light and oxidative stress (Pnueli et al., 2003; Davletova et al., 2005a). Plants with altered expression of tylAPX were extensively characterized; suppression of tylAPX resulted in enhanced sensitivity to oxidative stress, whereas enhanced expression of tylAPX resulted in increased tolerance to stress (Yabuta et al., 2002; Danna et al., 2003; Murgia et al., 2004; Tarantino et al., 2005). It is therefore possible that anthocyanins could play a protective role during light stress (Vanderauwera et al., 2005). It is therefore possible that anthocyanin accumulation contributes to the survival of tylapx/apx1 plants during light stress. Further studies are ROS and can protect cells under extreme conditions of oxidative stress.

We previously reported that compared to control plants or plants lacking either APX1 or CAT1, tobacco plants with suppressed expression of APX1 and CAT1 have a lower level of photosynthetic activity (Rizhsky et al., 2002). A lower level of photosynthetic activity could result in a low rate of ROS production. Our current analysis of tylapx/apx1 Arabidopsis plants failed, however, to show a similar reduction in photosynthetic activity (data not shown). In contrast, our results showing enhanced tolerance to oxidative stress, salinity, and heat stress in tylapx/apx1 plants (Figs. 3–7) suggest that active defense and/or ROS removal pathways are activated in the tylapx/apx1 double mutants. Double mutants lacking tylapx/apx1 could therefore be activating redundant ROS removal pathways, as opposed to suppressing ROS production via suppressed photosynthesis (Rizhsky et al., 2002), to cope with their reduced capability to scavenge H$_2$O$_2$ in the chloroplast and cytosol. The redundant mechanisms contributing to the survival of tylapx/apx1 plants are largely unknown at present. At least one defense pathway involving Zat12 and RbohD, activated in apx1 plants (Rizhsky et al., 2004; Davletova et al., 2005a, 2005b), appears not to be activated in tylapx/apx1 plants (Fig. 4). In contrast, tylapx/apx1 plants accumulate high levels of anthocyanins in response to light stress (Fig. 5). Anthocyanins could function as antioxidants, and a light-sensitive Arabidopsis mutant lacking CAT2 was shown to be suppressed in anthocyanin accumulation, supporting the hypothesis that anthocyanins could play a protective role during light stress (Vanderauwera et al., 2005). It is therefore possible that anthocyanin accumulation contributes to the survival of tylapx/apx1 during light stress. Further studies are...
Our findings that reproductive phase in Arabidopsis was associated with and Dolan, 2006), and the transition from vegetative to processes in plants (Torres and Dangl, 2005; Gapper response to stress. (Fig. 9). Our results therefore demonstrate a high degree of plasticity in ROS metabolism in Arabidopsis. The only phenotype that appears to be common to apx1, tylapx, and tylapx/apx1 plants is salinity tolerance. It is not clear yet what the common denominator is for this phenotype. It is possible, however, that this response is a result of activating a pathway involving Zat7, WRKY70, and HASTY in all mutants (Ciftci-Yilmaz et al., 2007).

Our results suggest that a complex mode of regulation exists within the ROS gene network of plants and that ROS such as H2O2 could be responsible for communication between different cellular compartments during abiotic stresses. An interesting outcome of our study is the identification of a signal that is possibly generated in tylapx and in tylapx/apx1 plants in chloroplasts and enhances the basal thermotolerance of Arabidopsis. This signal could require an intact retrograde signaling pathway (Fig. 8), demonstrating a link between chloroplast metabolism and sensing or activation of an abiotic stress signal that leads to enhanced stress tolerance. In future studies, this pathway could be further studied as a possible link between chloroplast metabolism and sensing of abiotic stresses.

Figure 9. Venn diagram summarizing the different pathways affected in apx1, tylapx, and apx1/tylapx mutants. The diagram illustrates the complex interactions between the pathways affected in plants by the absence of APX1, tylAPX, and/or APX1 and tylAPX and includes developmental effects on growth and flowering, differences in expression of defense pathways, and tolerance to different abiotic stresses.

required to identify the exact mechanism(s) activated in tylapx/apx1 under nonstressful conditions and in response to stress.

ROS were implicated in different developmental processes in plants (Torres and Dangl, 2005; Gapper and Dolan, 2006), and the transition from vegetative to reproductive phase in Arabidopsis was associated with enhanced accumulation of oxidized proteins (Johansson et al., 2004). Our findings that apx1 and tylapx plants have an early bolting phenotype could suggest that these plants are subjected to stress and therefore flower earlier. In contrast, tylapx/apx1 plants have a very late bolting phenotype. It is unknown at present what the cause of this phenotype in tylapx/apx1 plants is. Our results suggest that, at least in the context of plant development, the signal generated in tylapx/apx1 mutants is drastically different from that triggered in apx1 or tylapx plants.

Figure 9 summarizes our phenotypic analysis of the different mutants in the form of a Venn diagram. It suggests that two different signals are activated in plants lacking cytosolic APX1 or tylAPX. Thus, the lack of a chloroplastic H2O2 removal enzyme triggers a specific signal in cells that results, for example, in enhanced tolerance to heat stress, whereas the lack of a cytosolic H2O2 removal enzyme triggers a different signal that results in stunted growth and enhanced sensitivity to oxidative stress (Fig. 9). Very interestingly, when the two signals are coactivated in cells that lack APX1 and tylAPX, a new response is detected, suggesting that the integration of the two different signals results in a new and unique signal that triggers late bolting, low protein oxidation during light stress, and enhanced accumulation of anthocyanins (Fig. 9).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Assays

Arabidopsis (Arabidopsis thaliana) Col-0, WS, and WS/Col plants were grown under controlled conditions: 22°C to 23°C, continuous light, 40 μmol m−2 s−1, and a relative humidity of 70%. Stress treatments were carried out in growth chambers (Percival E-30, AR-66; Percival Scientific). A knockdown Arabidopsis line containing a T-DNA insert in the promoter of the thylakoid-APX gene (SALK, SALK_079484; tylapx obtained through the SIGnAL project, http://signal.salk.edu/tabout.html) was outcrossed and selfed to check for segregation as previously described (Pnueli et al., 2003; Rizhsky et al., 2004). Screening for expression of tylAPX was performed by protein blots using an antibody raised against a conserved domain of cytosolic and chloroplastic APXs (Davletova et al., 2005a). Knockout plants deficient in cytosolic Apx1 (apx1) described by Pnueli et al. (2003) and Davletova et al. (2005a) were crossed with the knockdown-tylAPX and selfed. Screening for double mutants was performed by PCR analysis and protein blots. Light stress treatments were performed on 21-d-old plants by changing the light intensity from 40 to 900 μmol m−2 s−1. All other parameters were maintained constant (Davletova et al., 2005a).

Protein oxidation was measured with 10-d-old seedlings, grown on solid 0.5× Murashige and Skoog media, and subjected to a light stress treatment of 25 to 300 μmol m−2 s−1 as described by Davletova et al. (2005a) and Rizhsky et al. (2004). Analysis of seedlings’ tolerance to oxidative stress imposed by paraquat, heat (38°C), cold (4°C), salinity, and osmotic stresses was performed as described by Davletova et al. (2005b), Ciftci-Yilmaz et al. (2007), and Mittler et al. (2007). Basal and acquired thermotolerance assays were performed as described by Suzuki et al. (2005). Statistical analysis was performed as described by Suzuki et al. (2005) and Davletova et al. (2005a).

Molecular, Physiological, and Biochemical Analyses

Measurements of plant growth and development were performed as described in Pnueli et al. (2003) and Suzuki et al. (2005). Anthocyanin content was determined in 21-d-old plants according to Bariola et al. (1999). RNA and protein were isolated and analyzed by RNA and protein blots as previously described (Pnueli et al., 2003; Rizhsky et al., 2004; Davletova et al., 2005a).
RNA staining or a ribosomal 18S RNA probe were used to control for RNA loading. Antibodies against Rubisco (rbcL and rbcS) were used in western analysis as control for protein loading (Rizhsky et al., 2004). Detection of protein oxidation by protein blots was performed with the OxyBlot protein oxidation kit (Chemicon International) as recommended by the manufacturer (Rizhsky et al., 2004; Davletova et al., 2005a). Antibodies against tylAPX, s/mAPX, and APX1 were obtained as described by Davletova et al. (2005a).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Response of mutants to cold stress.

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