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[1][W][OA]

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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 (H_2O_2) 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, we generated a double mutant lacking both cytosolic APX1 and tylAPX (*apx1/tylapx*). Our analysis of the double and single mutants for *tylapx* and *apx1* reveals a complex mode of integration of ROS signals generated in the cytosol (i.e. mutants lacking APX1) or chloroplast (i.e. mutants lacking tylAPX) of Arabidopsis. In addition, we found that a ROS-related signal, generated in the chloroplast of plants, can enhance basal thermotolerance and that this signal might require an intact retrograde signaling pathway.

RESULTS

Generation of Double Mutants Deficient in Cytosolic APX1 and tylAPX

To generate double mutants deficient in cytosolic APX1 and tylAPX (*apx1/tyapx*), 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 (*tylapx*; 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/tylapx* 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/tylapx* and 10 different homozygous crossing events of the wild-type control WS/Col. The WS/Col and *apx1/tylapx* pools were used for all subsequent analysis in which *apx1* plants were compared to WS, *tylapx* plants were compared to Col, and *apx1/tylapx* plants were compared with WS/Col. Protein-blot analysis of the different mutants grown under controlled

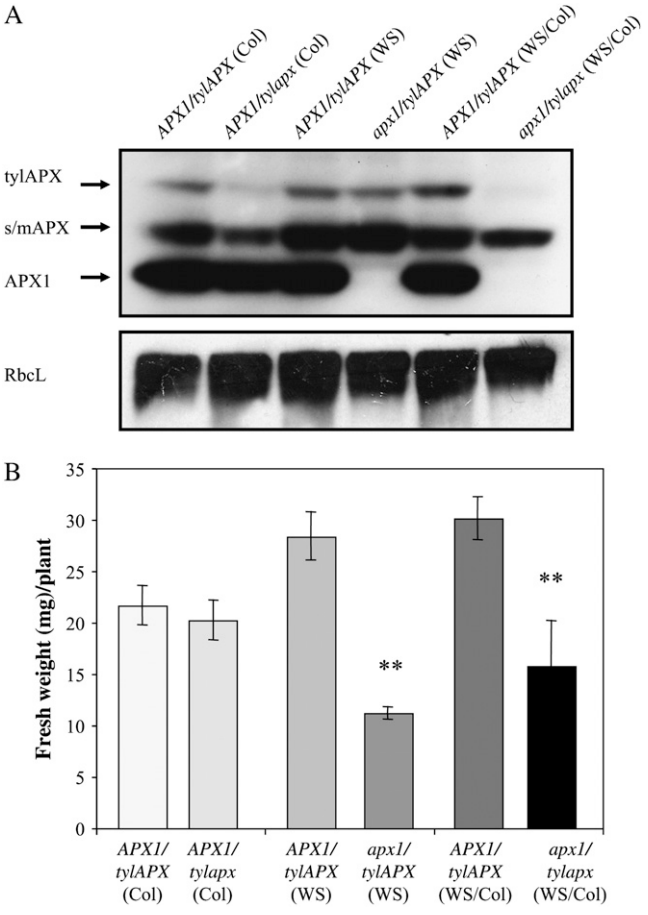

Figure 1. Growth suppression of double mutant *apx1/tylapx*. A, Protein blots showing the absence of cytosolic APX1 and the decreased expression of tylAPX in the different mutants and the double mutant grown under controlled growth conditions. B, Fresh weights of 17-d-old plants grown under controlled conditions, presented as an average of 10 individual plants per line. **, $P = 0.01$ in t test. The single mutants *APX1/tylapx*, *apx1/tylAPX*, and the double mutant *apx1/tylapx* are presented next to their respective wild-type controls, and the genetic background of each line is given in parentheses.

growth conditions confirmed the absence of APX1 in *apx1* and *apx1/tylapx* plants and the reduced expression of tylAPX in *tylapx* and *apx1/tylapx* plants (Fig. 1A). Our results therefore demonstrate that single and double mutants for *APX1* and *tylAPX* are viable, at least when grown under controlled growth conditions.

Growth and Development of Double Mutants Deficient in Cytosolic APX1 and tylAPX

As shown in Figure 1B, fresh weight and plant size of mutants with suppressed expression of 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 *apx1/tylapx* mutants appeared to be suppressed in their growth similar to *apx1* plants, suggesting that *APX1* is the more important of the two genes in controlling growth. As shown in Figure 2, bolting of *tylapx* or *apx1* plants was early compared to their corresponding backgrounds when plants were grown under controlled growth conditions ($P = 0.01$). In contrast, bolting of *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 *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 APX1 and tylAPX to Light Stress

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

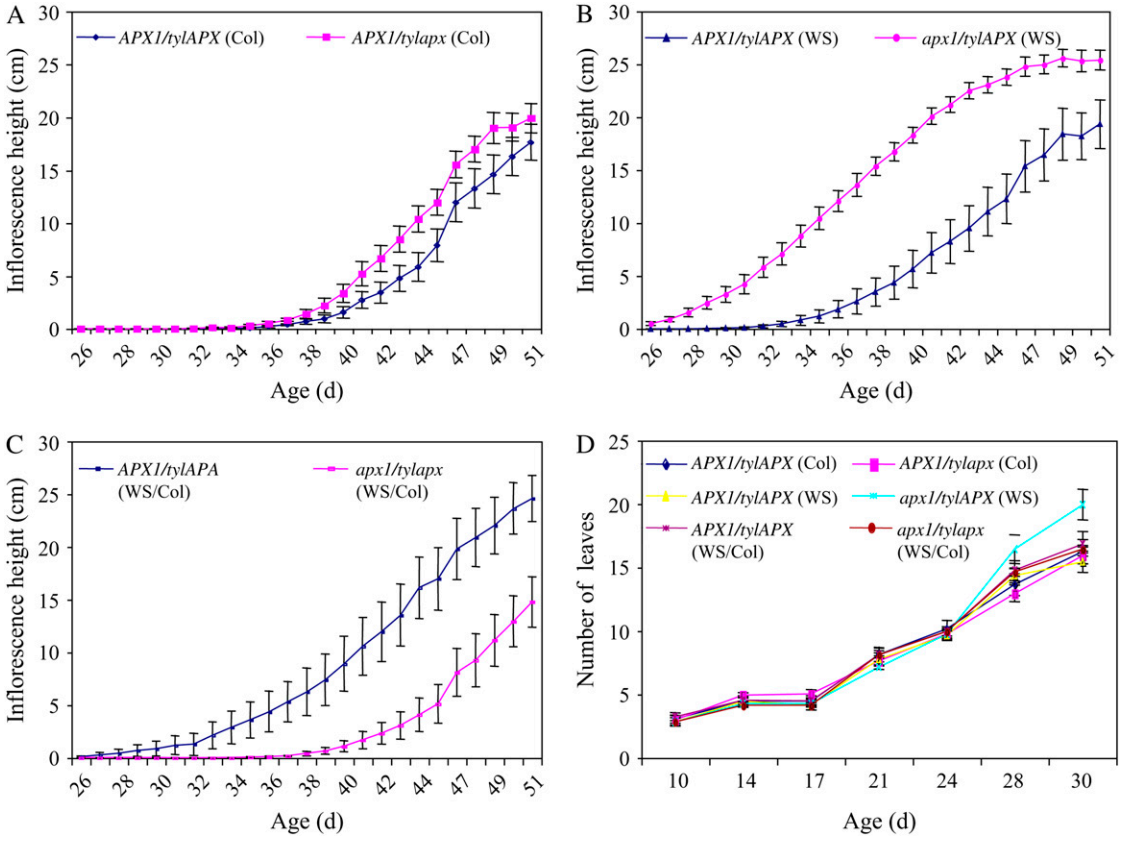

Figure 2. Differences in time of bolting between the different APX mutants and the double mutant. A to C, Inflorescence length was measured for each mutant during growth under controlled conditions. The different mutant lines are presented with respect to their corresponding controls. Genetic backgrounds are shown in parentheses. D, Number of leaves for all lines measured during growth under controlled conditions is given as a control. SEs are given for 40 replicates of each line. Controlled growth conditions: 22°C to 23°C, continuous light, 40 μmol m^{-2} s^{-1}, and a relative humidity of 70%.

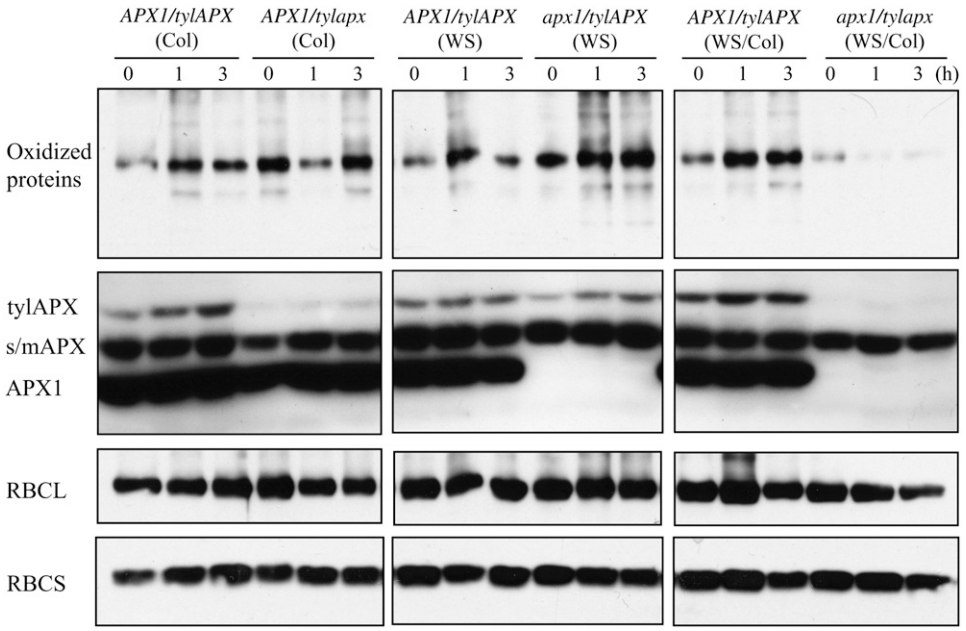

Figure 3. Suppressed level of protein oxidation in the double mutant (*apx1/tylapx*) in response to light stress. Plants were grown under continuous low light conditions (25 μmol m^{-2} s^{-1}) and transferred to moderate light (300 μmol m^{-2} s^{-1}). Detection of proteins containing carbonyl groups (indicative of protein oxidation) was performed by a protein-blot assay (top). The expression level of APX proteins in the different mutants was determined by a protein blot (second segment from the top). Rubisco protein level was also determined by protein blots (rbcL and rbcS) and is used to demonstrate equal loading (bottom). Measurements were performed as described in "Materials and Methods."

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

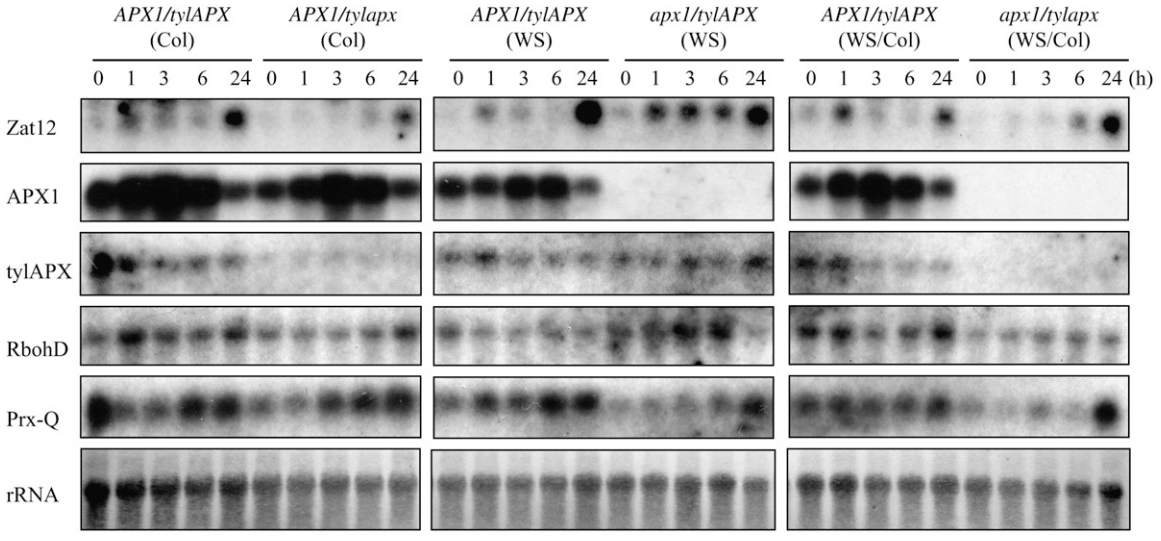

Figure 4. Suppressed expression of the zinc-finger protein Zat12 in *apx1/tylapx* in response to light stress. Plants were grown under continuous light (40 μmol m^{-2} s^{-1}) and exposed to high light intensity (900 μmol m^{-2} s^{-1}). At different times, plant tissue was collected from the different mutants and processed for RNA blots. Blots were probed with different cDNAs encoding proteins involved in ROS production (RbohD) or scavenging (APXs and peroxiredoxin Q [Prx-Q]).

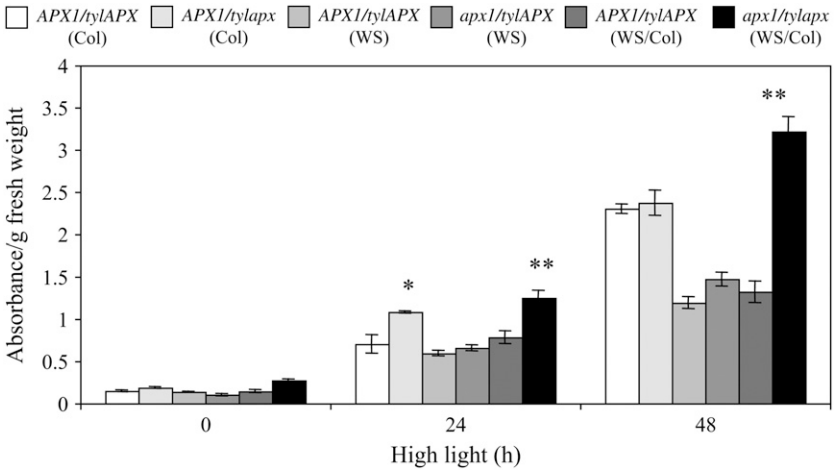

Figure 5. Enhanced accumulation of anthocyanin in *apx1/tylapx* in response to light stress. Anthocyanin levels were measured in shoots of 21-d-old wild-type and mutant plants exposed to light stress (900 μmol m^{-2} s^{-1}) for 24 and 48 h. *, $P = 0.05$; **, $P = 0.01$ in *t* test.

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

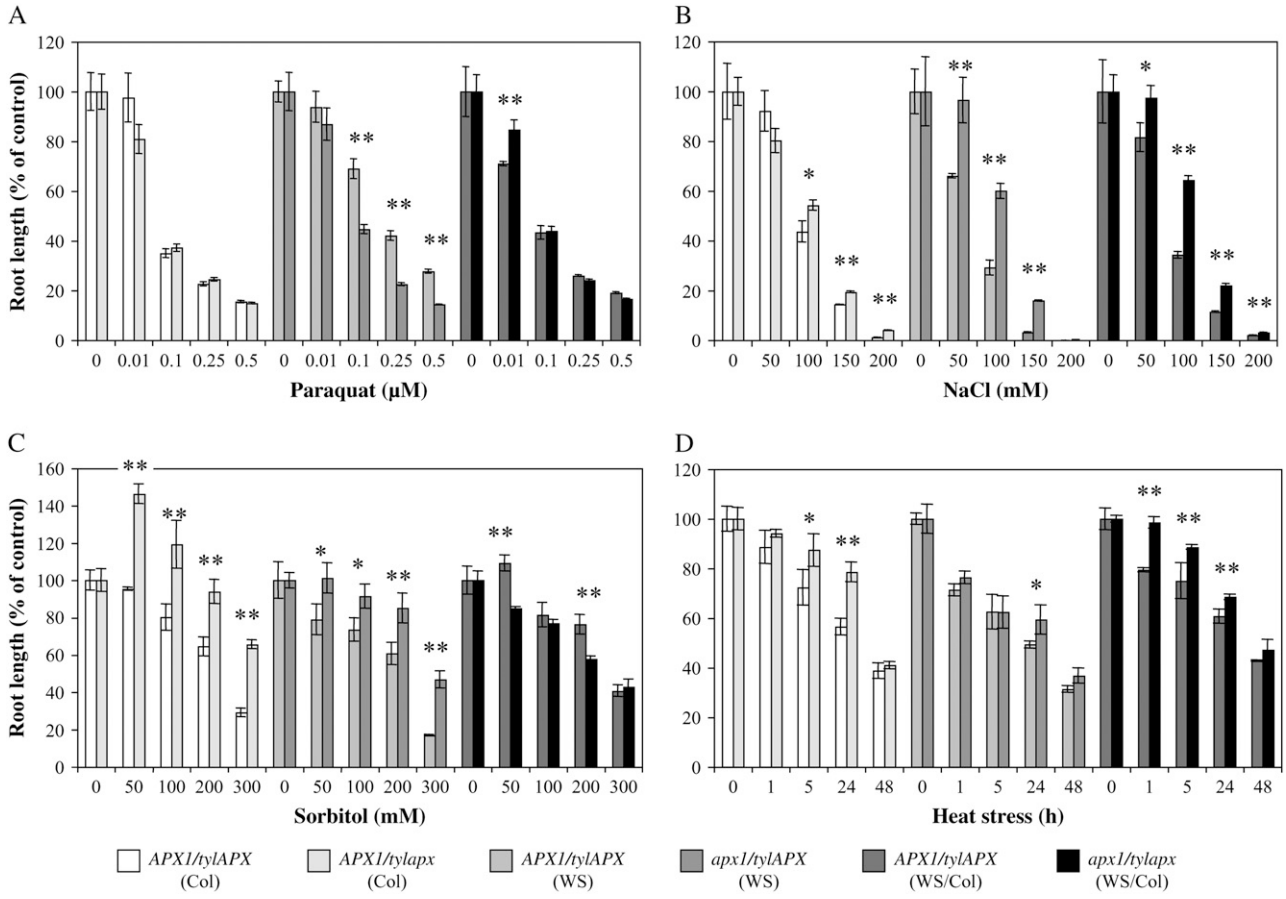

Figure 6. Differences in abiotic stress tolerance among *apx1*, *tylapx*, and *apx1/tylapx* mutants. Effects of abiotic stresses imposed by paraquat (A), salinity (B), osmotic stress (sorbitol; C), and heat stress (38°C; D) on root elongation in 5-d-old seedlings of the different lines and their corresponding controls were measured with seedlings grown on agar plates. All lines germinated simultaneously with a germination rate of 97% to 100%. Measurements were performed as described in "Materials and Methods." *, $P = 0.05$; **, $P = 0.01$ in *t* test.

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

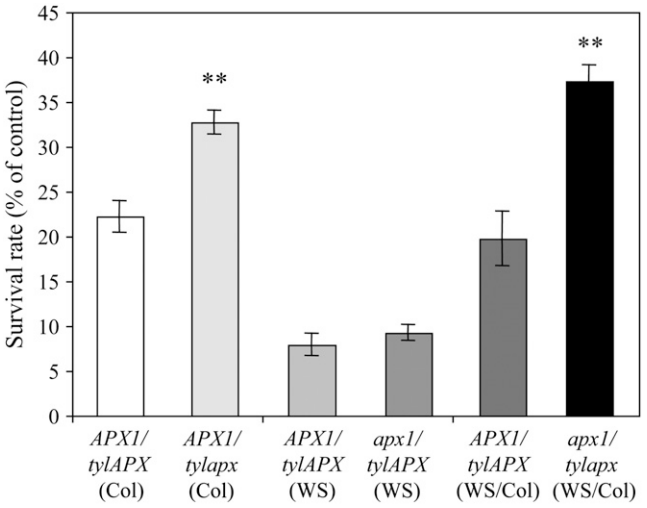

Figure 7. Enhanced basal thermotolerance of *tylapx* and *apx1/tylapx* mutants. Basal thermotolerance was measured by scoring the survival rate of 5-d-old seedlings of the different lines and their corresponding controls subjected to a 2-h heat treatment (45°C). No differences were found among the different lines and their corresponding controls in acquired thermotolerance. All lines germinated simultaneously with a germination rate of 97% to 100%. **, $P = 0.01$ in *t* test.

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; Laloi et al., 2007). Because suppression of APX1 or tylAPX resulted in enhanced sensitivity to ROS stress, it was expected that plants lacking APX1 and tylAPX will have an even higher degree of sensitivity to oxidative stress compared to plants that lack either tylAPX or APX1. Our results, however, are contrary to this hypothesis. Thus, in contrast to *apx1* or *tylapx* plants, *apx1/tylapx* plants did not accumulate oxidized proteins during light stress, were less sensitive to oxidative stress imposed by paraquat, and did not have elevated expression of the ROS response zinc-finger protein Zat12 (Davletova et al., 2005a, 2005b; Figs. 3–6). These findings, together with our previous report on double antisense plants in tobacco (Rizhsky et al., 2002), provide strong evidence for the existence of a redundant signaling and/or defense pathway(s) that can compensate for the lack of two different ROS metabolizing proteins. The classical ROS removal enzymes in the cytosol and chloroplast of higher plants (i.e. APXs; Asada, 2006) could therefore be supplemented by a yet unknown set of proteins or enzymes that are highly efficient in the removal of 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_2O_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

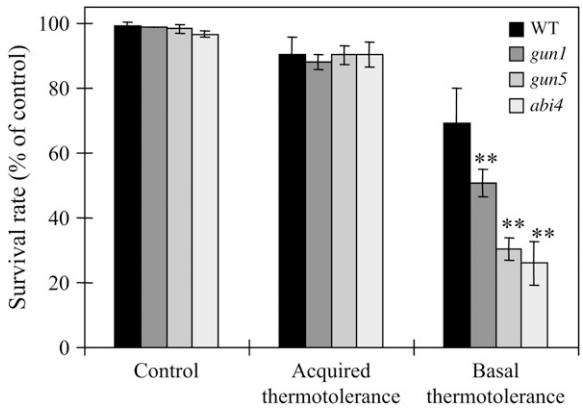

Figure 8. Impaired basal thermotolerance of mutants deficient in retrograde signaling. Survival rates were scored for 5-d-old seedlings of different mutants impaired in chloroplast-to-nuclei retrograde signaling (*gun1*, *gun5*, and *abi4*), subjected to a 2-h heat treatment (45°C; basal thermotolerance), or treated at 38°C for 1.5 h, allowed to recover for 1 h at 21°C, and subjected to 45°C for 2 h (acquired thermotolerance). All lines germinated simultaneously with a germination rate of 97% to 100%. **, $P = 0.01$ in *t* test.

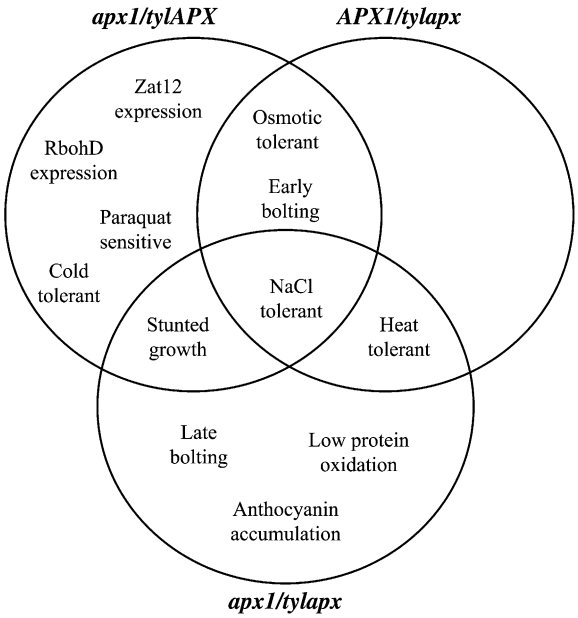

Figure 9. Venn diagram summarizing the different pathways affected in *apx1*, *tylapx*, and *apx1/tylapx* mutants. The diagram illustrates the complex interactions between the pathways affected in plants by the absence of APX1, tylAPX, and/or APX1 and tylAPX and includes developmental effects on growth 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 could 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 H_2O_2 removal enzyme triggers a specific signal in cells that results, for example, in enhanced tolerance to heat stress, whereas the lack of a cytosolic H_2O_2 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). 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 H_2O_2 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.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Assays

Arabidopsis (*Arabidopsis thaliana*) Col, 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_072948; *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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LITERATURE CITED

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol **141:** 391–396

Bailey-Serres J, Mittler R (2006) The roles of reactive oxygen species in plant cells. Plant Physiol **141:** 311

Bariola PA, MacIntosh GC, Green PJ (1999) Regulation of S-like ribonuclease levels in Arabidopsis: antisense inhibition of RNS1 or RNS2 elevates anthocyanin accumulation. Plant Physiol **119:** 331–342

Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem **282:** 1183–1192

Ciftci-Yilmaz S, Morsy MR, Song L, Coutu A, Krizek BA, Lewis MW, Warren D, Cushman J, Connolly EL, Mittler R (2007) The ear-motif of the C2H2 zinc-finger protein ZAT7 plays a key role in the defense response of Arabidopsis to salinity stress. J Biol Chem **282:** 9260–9268

Danna CH, Bartoli CG, Sacco F, Ingala LR, Santa-Maria GE, Guiamet JJ, Ugalde RA (2003) Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. Plant Physiol **132:** 2116–2125

Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Shulaev V, Schlauch K, Mittler R (2005a) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. Plant Cell **17:** 268–281

Davletova S, Schlauch K, Coutu J, Mittler R (2005b) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. Plant Physiol **139:** 847–856

Gapper C, Dolan L (2006) Control of plant development by reactive oxygen species. Plant Physiol **141:** 341–345

Halliwell B (2006) Reactive species and antioxidants: redox biology is a fundamental theme of aerobic life. Plant Physiol **141:** 312–322

Henzler T, Steudle E (2000) Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H(2)O(2) across water channels. J Exp Bot **51:** 2053–2066

Johansson E, Olsson O, Nystrom T (2004) Progression and specificity of protein oxidation in the life cycle of Arabidopsis thaliana. J Biol Chem **279:** 22204–22208

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9:** 627–640

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P (1999) Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science **284:** 654–657

Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim J, Mittler R, Chory J (2007) Multiple signals from damaged chloroplasts converge on a common pathway to regulate nuclear gene expression. Science **316:** 715–719

Kwak JM, Nguyen V, Schroeder JI (2006) The role of reactive oxygen species in hormonal responses. Plant Physiol **141:** 323–329

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana. Proc Natl Acad Sci USA **104:** 672–677

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci **7:** 405–410

Mittler R, Kim Y, Song L, Coutu J, Coutu A, Ciftci-Yilmaz S, Lee H, Stevenson B, Zhu JK (2007) Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. FEBS Lett **580:** 6537–6542

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9:** 490–498

Mullineaux PM, Karpinski S, Baker NR (2006) Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. Plant Physiol **141:** 346–350

Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C (2004) Arabidopsis thaliana plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. Plant J **38:** 940–953

Nott A, Jung HS, Koussevitzky S, Chory J (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol **57:** 739–759

Pitzschke A, Hirt H (2006) Mitogen-activated protein kinases and reactive oxygen species signaling in plants. Plant Physiol **141:** 351–356

Pnueli L, Liang H, Rozenberg M, Mittler R (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient Arabidopsis plants. Plant J **34:** 187–203

Rizhsky L, Davletova S, Liang H, Mittler R (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in Arabidopsis. J Biol Chem **279:** 11736–11743

Rizhsky L, Hallak-Herr E, Van Breusegem F, Rachmilevitch S, Barr JE, Rodermel S, Inze D, Mittler R (2002) Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. Plant J **32:** 329–342

Suzuki N, Rizhsky L, Liang H, Shuman J, Shulaev V, Mittler R (2005) Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. Plant Physiol **139:** 1313–1322

Tarantino D, Vannini C, Bracale M, Campa M, Soave C, Murgia I (2005) Antisense reduction of thylakoidal ascorbate peroxidase in Arabidopsis enhances paraquat-induced photooxidative stress and nitric oxide-induced cell death. Planta **221:** 757–765

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8:** 397–403

Torres MA, Jones JD, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. Plant Physiol **141:** 373–378

Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. Plant Physiol **141:** 384–390

Vanderauwera S, Zimmermann P, Rombauts S, Vandenabeele S, Langebartels C, Gruissem W, Inze D, Van Breusegem F (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol **139:** 806–821

Yabuta Y, Motoki T, Yoshimura K, Takeda T, Ishikawa T, Shigeoka S (2002) Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. Plant J **32:** 915–925

Zaninotto F, La Camera S, Polverari A, Delledonne M (2006) Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. Plant Physiol **141:** 379–383