The Plant Journal (2002) **32**, 329–342

Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase

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Received 22 May 2002; revised 2 July 2002; accepted 7 July 2002.
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Summary

The plant genome is a highly redundant and dynamic genome. Here, we show that double antisense plants lacking the two major hydrogen peroxide-detoxifying enzymes, ascorbate peroxidase (APX) and catalase (CAT), activate an alternative/redundant defense mechanism that compensates for the lack of APX and CAT. A similar mechanism was not activated in single antisense plants that lacked APX or CAT, paradoxically rendering these plants more sensitive to oxidative stress compared to double antisense plants. The reduced susceptibility of double antisense plants to oxidative stress correlated with suppressed photosynthetic activity, the induction of metabolic genes belonging to the pentose phosphate pathway, the induction of monodehydroascorbate reductase, and the induction of IMMUTANS, a chloroplastic homologue of mitochondrial alternative oxidase. Our results suggest that a co-ordinated induction of metabolic and defense genes, coupled with the suppression of photosynthetic activity, can compensate for the lack of APX and CAT. In addition, our findings demonstrate that the plant genome has a high degree of plasticity and will respond differently to different stressful conditions, namely, lack of APX, lack of CAT, or lack of both APX and CAT.

Keywords: alternative oxidase, ascorbate peroxidase, catalase, oxidative stress, pentose phosphate pathway, photosynthesis.

Introduction

While molecular oxygen has a relatively low reactivity towards most cellular components, partially reduced forms of atmospheric oxygen, globally named 'reactive oxygen intermediates' (ROI), may react with many cellular substances potentially leading to the oxidative destruction of cells (Asada and Takahashi, 1987). ROI are formed in plant cells as by-products of many metabolic reactions. For example, hydrogen peroxide (H_2O_2) is produced through the catalytic activity of glycolate oxidase in peroxisomes during photorespiration, and superoxide (O_2^-) is generated by leakage of electrons from chloroplastic or mitochondrial electron transport systems. While under normal growth conditions the formation of ROI occurs at a low rate, many stresses that disrupt the biochemical and physiological homeostasis of cells cause a dramatic increase in the rate of ROI production (Allen, 1995; Asada and Takahashi, 1987; Dat *et al.*, 2000; Mittler, 2002). Recent studies suggested that ROI may play an important role in plant cells as signalling molecules involved in the regulation of gene expression during stress or pathogen infection (Grant *et al.*, 2000; Hirt, 2000; Karpinski *et al.*, 1999; Knight and Knight, 2001; Kovtun *et al.*, 2000; Pei *et al.*, 2000).

Plants, as well as most aerobic organisms, contain complex enzymatic and non-enzymatic mechanisms capable

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of detoxifying O_2^- and H_2O_2. Superoxide radicals are scavenged through the catalytic activity of superoxide dismutase (SOD; Asada, 1999), whereas H_2O_2 is removed through the catalytic action of ascorbate peroxidase (APX; Mittler *et al.*, 1999), glutathione peroxidase (GPX; Roxas *et al.*, 1997), and catalase (CAT; Willekens *et al.*, 1997). Non-enzymatic scavenging of ROI involves ascorbic acid, glutathione, α-tocopherol, and carotenoids (Asada and Takahashi, 1987; Foyer and Halliwell, 1976). The importance of ROI scavenging for the growth and defense of plants was recently demonstrated using transgenic tobacco plants with reduced CAT or APX expression. These were found to be hypersensitive to treatments that involved the accumulation of ROI, including high light, ozone, application of the O_2^- generating herbicide paraquat, salt stress, pathogen infection, and low CO_2 combined with high light (Mittler *et al.*, 1999; Orvar and Ellis, 1997; Willekens *et al.*, 1997). In addition, mutants deficient in the biosynthesis of the antioxidant ascorbic acid were shown to be hypersensitive to environmental stresses (Conklin *et al.*, 1996).

ROI appear to play a central role in the defense of plants against pathogens. During many plant-pathogen interactions ROI are produced by plant cells at a very high rate and are thought to activate plant defenses, including programmed cell death (Dangl *et al.*, 1996; Delledonne *et al.*, 2001; Hammond-Kosack AND Jones, 1996). In contrast, the activity and expression of APX and CAT is suppressed during this response (Clark *et al.*, 2000; Durner and Klessig, 1995; Mittler *et al.*, 1998). Thus, the plant simultaneously produces more ROI and diminishes its own capability to scavenge H_2O_2.

The studies described above point to a complex mode of regulation controlling the steady state level of ROI in cells. Depending upon the physiological condition encountered by plants, i.e. biotic or abiotic stress, plants may alter the balance between ROI production and ROI removal to enhance (biotic), or suppress (abiotic), the cellular level of ROI. Balancing ROI production and ROI scavenging is therefore crucial for many different aspects of plant metabolism. In addition, due to the potential toxicity of ROI, a high degree of redundancy is expected to occur between different ROI scavenging mechanisms.

We previously reported that tobacco plants with suppressed cytosolic APX or CAT expression are limited in their capability to balance their intracellular level of ROI (Mittler *et al.*, 1999). However, APX and CAT might be functionally redundant and compensate for the lack of each other. We therefore generated double antisense tobacco plants lacking both CAT and cytosolic APX. To our surprise, at least under a defined set of environmental conditions, these appeared to be less sensitive to oxidative stress than single antisense plants lacking APX or CAT.

Results

Characterization of double antisense plants (Nicotiana tabacum) *with suppressed expression of APX and CAT*

All crosses between antisense cytosolic APX (*apx I*; AS-APX; Orvar and Ellis, 1997), antisense CAT (*cat 1*; AS-CAT; Willekens *et al.* 1997), and wild-type (WT) plants, as well as the isolation of double antisense (dAS) plants are described in Experimental procedures. We compared the growth of WT, AS-APX, AS-CAT, and dAS plants under three different light intensities: low (75 μmol photons m^{-2} sec^{-1}), moderate (150–200 μmol photons m^{-2} sec^{-1}), and high (600–800 μmol photons m^{-2} sec^{-1}), using a continuous light source, at 22–24°C. At low light there was no apparent difference between the different plants (not shown). At moderate light AS-CAT plants developed lesions on their leaves while WT, AS-APX, and dAS plants did not (Figure 1a). At high light all antisense plants, including dAS, developed lesions, while WT plants did not (not shown). We therefore focused our studies on plants that grow at moderate or low light. RNA blot analysis was performed on leaf samples obtained from the different plants grown at moderate light. As shown in Figure 1b, dAS plants that had suppressed expression of APX and CAT did not appear to induce stress response genes such as cytosolic CuZn-SOD (SOD) or cytosolic glutathione reductase (GR). In contrast, AS-APX plants induced CAT, GR, and SOD in an apparent attempt to compensate for the reduced APX expression, and AS-CAT plants induced APX and PR-1 (PR-1a), developed lesions, and had a reduced expression of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS), and glucose-6-phosphate dehydrogenase (G6PDH; Figure 1a,b). The response of AS-APX and AS-CAT plants indicated that they were subjected to oxidative stress. However, at least under moderate light, dAS plants appeared not to be subjected to a similar level of stress.

To confirm that the suppression of CAT and APX expression, evident by a reduction in the steady state level of transcripts encoding APX and CAT (Figure 1b), resulted in a reduction in APX and CAT activities, we measured these activities in the different plants grown under moderate light. As shown in Figure 2a, the activity of CAT was suppressed in AS-CAT plants and the activity of APX was suppressed in AS-APX plants. In contrast, the activity of APX was enhanced in AS-CAT plants and the activity of CAT was enhanced in AS-APX plants. These results are in agreement with the steady state level of transcripts encoding APX and CAT in the different plants (Figure 1b). In contrast to AS-APX and AS-CAT plants, both CAT and APX activities were suppressed in dAS plants. To expand our analysis of plants grown under moderate light we measured the level of the antioxidants ascorbic acid (ASC) and glutathione (GSH) in dAS plants. The overall level of these antioxidants

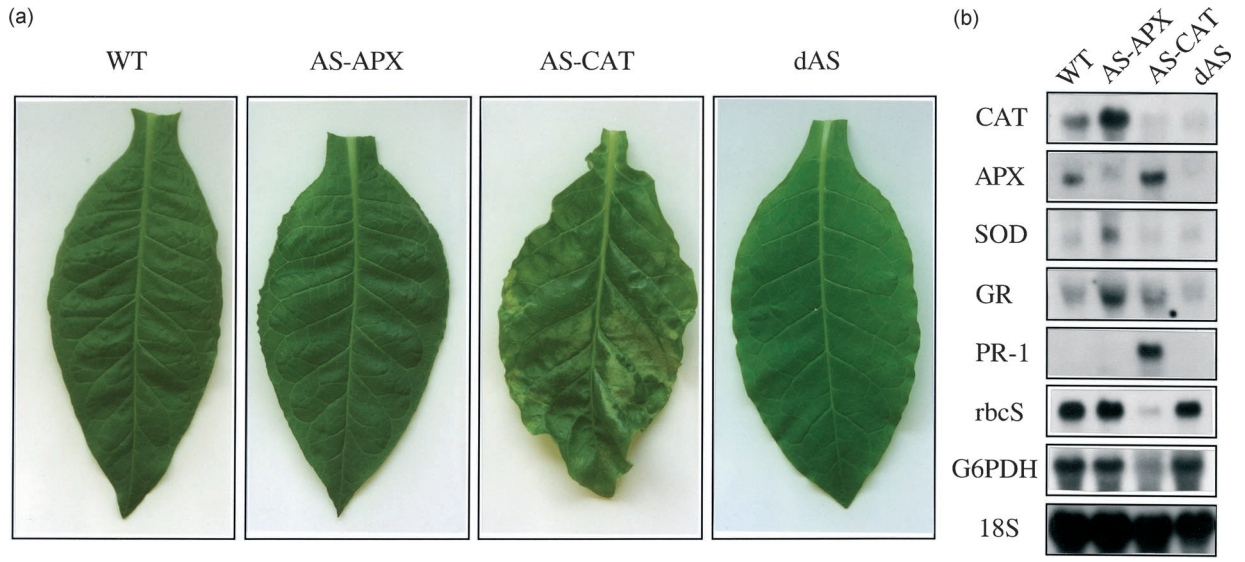

Figure 1. Molecular characterization of double antisense plants.
(a) Photographs of leaves from wild-type (WT), APX antisense (AS-APX), CAT antisense (AS-CAT), and APX and CAT antisense (dAS) plants, showing the formation of lesions on leaves of AS-CAT plants but not on leaves of dAS plants that lack both APX and CAT. Leaves of similar developmental age and size were photographed from 5- to 6-week-old plants grown under moderate light intensity (150 μmol photons m^{-2} sec^{-1}).
(b) RNA gel blot analysis performed with RNA obtained from the leaves shown in (a). The expression of the stress response genes SOD, GR and/or PR-1 is shown to be induced in AS-APX and AS-CAT plants but not in dAS plants.

as well as the size of their reduced pool can provide an indication to the degree of oxidative stress encountered by plants (Herouart *et al.*, 1993; Noctor and Foyer, 1998). As shown in Figure 2b, the levels of reduced ASC and GSH in dAS plants were similar to those of WT plants. In contrast, AS-CAT and AS-APX plants had high levels of ASC and GSH, suggesting that these plants were exposed to oxidative stress. The levels of oxidized ASC (DHA) and GSH (GSSG) were maintained at 15–20% of their corresponding pools in all plants (not shown). This result is in accordance with the presence of ASC and GSH regenerating enzymes in the different plants, postulated to be sufficient for maintaining the antioxidant pools at a reduced level (Polle, 2001). It should however, be noted that on a molar basis the pool of oxidized ASC was higher in AS-APX and AS-CAT plants compared to WT or dAS plants (not shown).

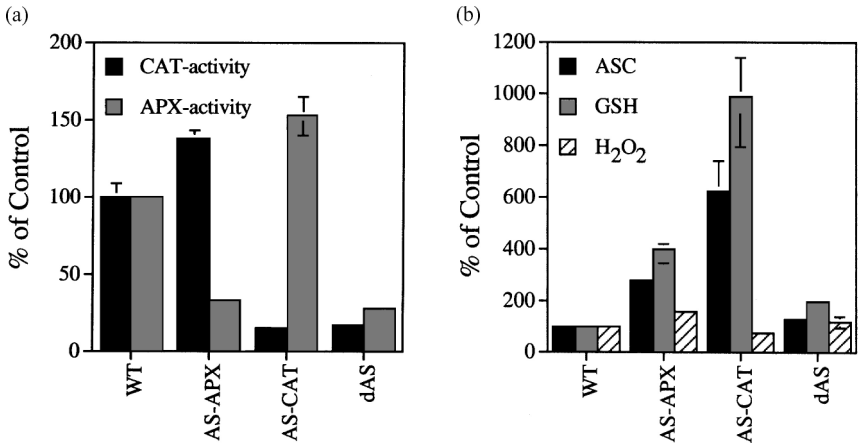

Figure 2. Biochemical characterization of double antisense plants.
(a) Measurements of CAT and APX activities in WT, AS-APX, AS-CAT, and dAS plants. The measurements of APX and CAT activities are in good agreement with the level of transcripts encoding CAT and APX in the different plants shown in Figure 1b. Results are mean and standard deviation of 3 independent measurements.
(b) Ascorbate (ASC), glutathione (GSH), and H_2O_2 levels in WT, AS-APX, AS-CAT, and dAS plants. Results shown are mean and standard deviation of 3 independent measurements.
Leaves of similar developmental age and size were sampled and analysed from 4-week-old plants grown under moderate light intensity (150 μmol photons m^{-2} sec^{-1}).

Measurements of overall H_2O_2 levels in leaf tissues of the different plants, grown under moderate light, did not reveal dramatic differences between dAS plants and the other plants (Figure 2b). However, the concentrations of H_2O_2 at the different cellular compartments of the different plants that may be critical for the activation of different signal transduction pathways, are not known. It is possible that grinding of plant tissue to measure H_2O_2 results in measurements that do not reflect the true *in vivo* level of this compound (Mittler, 2002).

To further characterize the phenotype of dAS plants, and examine their resistance to oxidative stress, we subjected 4- to 5-week-old plants to stress imposed by treatment with the superoxide generating agent paraquat, at the three different light conditions, and assayed plants for cell death by measuring ion leakage. Because paraquat's mode of action in chloroplasts is to accept electrons from PSI and to donate them to oxygen to form superoxide radicals, the effectiveness of paraquat as an ROI generator in the chloroplast is dependent to a large extant on the intensity of light applied to plants during the treatment. At high and moderate light there was no apparent difference between the different antisense plants, including dAS (not shown), however, at low light AS-CAT plants were more sensitive to paraquat treatment compared to AS-APX or dAS plants (Figure 3). Thus, although the expression of CAT and APX was suppressed in dAS plants, they appeared to be less sensitive than AS-CAT plants to this treatment.

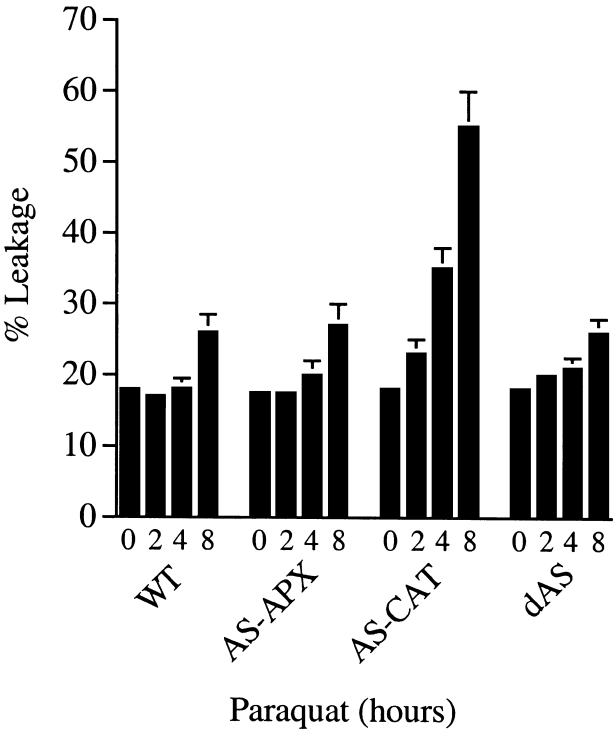

Figure 3. Induction of cell death by paraquat in AS-CAT plants.
Wild-type (WT), APX antisense (AS-APX), CAT antisense (AS-CAT), and APX and CAT antisense (dAS) plants were treated with 10^{-6} M paraquat at a low light intensity (75 μmol photons m^{-2} sec^{-1}) and assayed for cell death at different times following paraquat application by measuring ion leakage from leaf discs. Data shown is the mean and standard deviation of 3 independent measurements.

Suppression of photosynthetic activity in double antisense plants

Measurements of photosynthetic activity and dark respiration were performed on the different plants grown at low light intensity using a saturating light experimental design. Plants were clamped with a Li-Cor LI-6400 apparatus and the light intensity within the measuring chamber was changed to 2000 μmol photons m^{-2} sec^{-1}. The rate of CO_2 assimilation was then measured every minute over the course of 15 min. As shown in Figure 4a, the maximal photosynthetic activity of dAS plants was lower than that of WT or AS-CAT and AS-APX plants. No significant differences in stomatal conductance were detected between the different plants during the course of the experiment (not shown).

Additional measurements were performed on WT and dAS plants grown at moderate light intensity, using this light intensity as a light source for the measurements. These measurements confirmed that dAS plants had about 50% lower photosynthetic activity compared to WT plants (WT, 8.2, SE = 1.4, $n = 5$; dAS, 4.7, SE = 0.9, $n = 5$; μmol CO_2 cm^{-2} sec^{-1}). The decreased photosynthetic activity of dAS plants did not appear to be a result of a lower number of stomata, because stomata counting indicated that dAS plants had a high number of stomata (80 mm^{-2}, SE = 9.6, $n = 10$) compared to WT (53 mm^{-2}, SE = 4.2, $n = 8$) or AS-APX (55 mm^{-2}, SE = 4.8, $n = 10$) plants, and an equivalent number of stomata to AS-CAT plants (76 mm^{-2}, SE = 7.2, $n = 10$), all measured on leaf number 4 of the different plants.

The suppression of photosynthetic activity in dAS plants may result from the inability of these plants to use ambient CO_2 levels for photosynthesis. To examine this possibility we compared the degree of increase in photosynthetic activity between the different plants upon changing the CO_2 concentrations from ambient (350 ppm) to near saturating (2200 ppm). This analysis was performed at saturating light as described for Figure 4a. As shown in Figure 4b, there was no difference between the fold increase in photosynthetic activity of dAS plants and WT plants. In contrast, AS-CAT plants appeared to significantly benefit from the increase in CO_2 levels. Because the activity of the photorespiration pathway in AS-CAT plants is damaging to plants (Willekens *et al.*, 1997), it is possible that the suppression of this pathway in AS-CAT plants at high CO_2 levels resulted in an increase in photosynthetic activity. In contrast, a similar improvement was not observed in dAS plants. The results

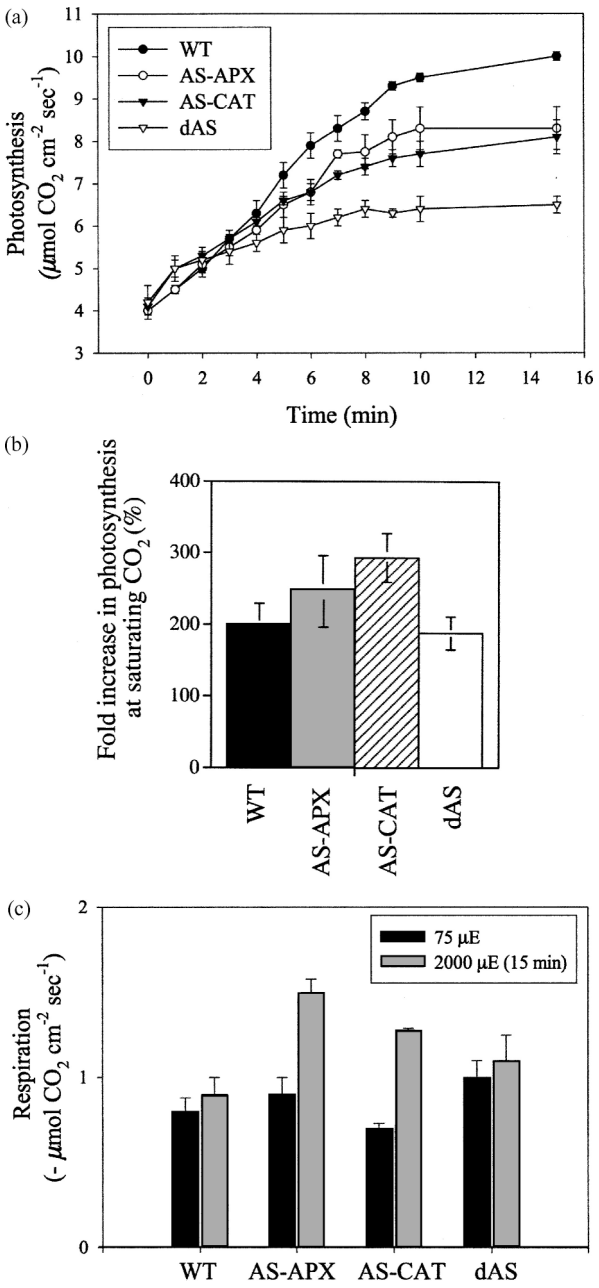

Figure 4. Measurements of photosynthetic activity and dark respiration in the different antisense plants.

(a) Measurements of photosynthetic activity in 3- to 4-week-old control and antisense plants grown at low light (75 μmol photons m^{-2} sec^{-1}) and subjected to saturating light for 15 min. Leaves of similar developmental age and size were clamped with a LI-6400 photosynthetic measuring apparatus, subjected to saturating light (2000 μmol photons m^{-2} sec^{-1}), and assayed for their rate of CO_2 assimilation. Double antisense plants (dAS) are shown to have a low photosynthetic activity compared to WT or AS-APX and AS-CAT plants.

(b) Fold-increase in photosynthetic activity upon changing the CO_2 concentration from 350 ppm to 2200 ppm. Plants were clamped with a LI-6400 apparatus as described for (a). When they reached maximal photosynthetic activity at 350 ppm CO_2 the concentration of CO_2 was changed to 2200 ppm and the new maximal photosynthetic activity was measured. The graph shows the fold-increase in activity calculated from the two photosynthetic values, i.e. at 350 and at 2200 ppm.

presented in Figure 4b suggest that the mode of suppression of photosynthetic activity in dAS plants is different from that observed in AS-CAT plants (Figure 4a). Thus, at least some of the suppression in photosynthetic activity, observed in AS-CAT plants (Figure 4a), could be prevented by increasing the level of CO_2, whereas the photosynthetic activity of dAS plants appeared not to be affected in a similar manner by a similar increase (Figure 4b).

Dark respiration, i.e. CO_2 emission in the dark, measured at the start and at the end of the experiment illustrated in Figure 4a is shown in Figure 4c. Interestingly, compared to AS-CAT and AS-APX plants, dAS plants did not have a high rate of dark respiration following the high light treatment. The behaviour of dAS plants was therefore comparable to that of WT plants, suggesting that they were not affected in a similar manner by the high light treatment as AS-CAT or AS-APX plants. However, the nature of the enhanced dark respiration and its relation to oxidative stress is unknown.

Profiling gene expression in double antisense plants

To understand the basis of dAS resistance (Figures 1 and 3), as well as the reason for the suppression in photosynthetic activity observed in dAS plants (Figure 4), we performed a filter array analysis of gene expression in plants grown under moderate light. We screened about 500 different stress response and metabolic genes in an attempt to identify a gene(s)/pathway(s) that may be specifically expressed in dAS plants and provide a possible explanation for the enhanced tolerance of these plants. An example of our filter array hybridization is shown in Figure 5a. Based on this analysis the only pathway that appeared to be specifically affected in dAS plants was the pentose phosphate pathway. Thus, as shown in Table 1, at least three transcripts encoding enzymes belonging to this pathway, i.e. transaldolase, transketolase, and ribulose-5-phosphate isomerase, were induced in dAS plants (2- to 3-fold induction in pentose phosphate pathway genes was also observed by RNA blots; not shown). The pentose phosphate pathway was previously linked to the defense of animal cells against oxidative stress (Pandolfi *et al.*, 1995). It is therefore possible that it plays a similar role in dAS plants enhancing the supply of NADPH for ROI removal. A number of other genes were also induced in dAS plants but their induction did not appear to be specific to dAS plants. The expression level of these genes, as well as that of other ROI-related genes, not shown in Figure 1b, is shown in Table 1. These results suggest that other defense enzymes with an H_2O_2

Figure 4. continued

(c) Rates of dark respiration, i.e. CO_2 emission, measured at the beginning (75 μE) and at the end (2000 μE) of the experiments shown in (a). Dark respiration was measured with a LI-6400 photosynthetic measuring apparatus.

Data shown is mean and standard deviation of 3–5 independent measurements. Abbreviations: μE, μmol photons m^{-2} sec^{-1}.

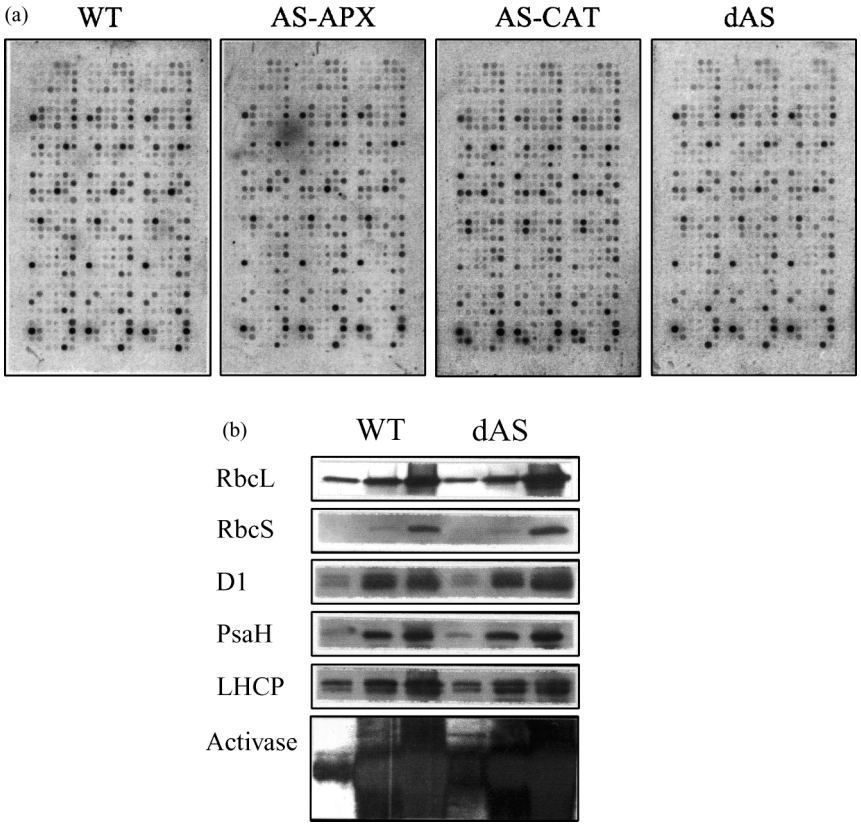

Figure 5. DNA filter arrays and protein blot analysis of gene expression in the different antisense plants.
(a) Filter array hybridization comparing the expression pattern of 200 stress response cDNAs between wild-type (WT), APX antisense (AS-APX), CAT antisense (AS-CAT), and APX and CAT antisense (dAS) plants. Overall about 500 genes were tested using this approach. A short summary of the results shown in (a) is presented in Table 1.
(b) Protein blot analysis comparing the level of different proteins involved in photosynthesis between WT and dAS plants. Samples were loaded in 3 different dilutions (1 : 1, 1 : 5, 1 : 20) based on equal protein or chlorophyll content with similar results. No differences were detected between dAS and WT plants. Abbreviations used: Activase, Ribulose-1,5-bisphosphate carboxylase/oxygenase activase; D1, Photosystem II D1 subunit; LHCP, light harvesting chlorophyll binding protein; PsaH, subunit H of photosystem I; RbcS/l, ribulose-1,5-bisphosphate carboxylase/oxygenase small (S) or large (L) subunit.

detoxification activity such as thylakoid/stromal APX, GPX, or thioredoxin peroxidase were not induced in dAS plants. Interestingly, the level of transcripts encoding cytosolic monodehydroascorbate (MDA) reductase was induced in dAS plants (Table 1). Although MDA reductase was also induced in AS-CAT plants, the combined induction of MDA reductase and transcripts belonging to the pentose phosphate pathway may indicate that in dAS plants these two mechanisms co-operate to enhance the non-enzymatic scavenging of H_2O_2 by ascorbic acid. Although a number of cDNAs encoding proteins involved in photosynthesis were included on the filters, we could not detect changes that could explain the reduction of photosynthetic activity in dAS plants in the expression of their corresponding transcripts (not shown).

Measuring the level of different proteins involved in photosynthesis in dAS plants

In an additional attempt to determine the cause of suppression in photosynthetic activity in dAS plants (Figure 4) we performed protein gel blots comparing the level of different proteins involved in photosynthesis between WT and dAS plants. This analysis was performed on plants growing under low or moderate light. As shown in Figure 5b, we could not detect a decrease in these proteins in dAS plants. Similar results were obtained with AS-CAT and AS-APX plants grown under low light (not shown). However, in AS-CAT plants grown under moderate light there was a decrease in the level of certain photosynthetic proteins including RbcS (not shown; see Figure 1b for a decrease in rbcS transcripts in AS-CAT plants grown under moderate light). Because many of the different components of the photosynthetic apparatus are linked, a reduction in the level of any one of these components may lead to an overall suppression of photosynthetic activity. For example, it was found that even a moderate decrease in the activity of chloroplastic aldolase resulted in the inhibition of photosynthesis in potato (Haake *et al.*, 1998). A comprehensive proteomic and metabolomic analysis may therefore be required to identify a specific protein(s) or metabolite(s) that may account for the suppression in photosynthetic activity in dAS plants.

Induction of chloroplastic alternative oxidase in dAS plants

A defense function against ROI stress was recently suggested for mitochondrial alternative oxidase (mitAOX; Maxwell *et al.*, 1999). However, this gene was mainly induced in AS-CAT plants and not in dAS plants (Table 1; Dat *et al.*, unpublished results). A distantly related homologue of mitAOX was recently reported to exist in chloroplasts (Carol *et al.*, 1999; Wu *et al.*, 1999). It has been

Table 1 Changes in gene expression measured by filter arrays

Gene/Class	Expression Level Compared to WT (% of Control)^{SD}		
	AS-APX	AS-CAT	dAS
ROI-related genes:			
Thylakoid APX	51^{48}	2^{8}	69^{40}
Stromal APX	71^{56}	49^{9}	115^{31}
Glutathione peroxidase	91^{15}	185^{19}	117^{50}
Thioredoxin peroxidase	75^{30}	64^{20}	148^{10}
Thioredoxin H1	48^{40}	298^{16}	149^{21}
MDA reductase	156^{80}	256^{9}	313^{24}
Glutathione-*S*-transferase	87^{38}	320^{18}	185^{60}
Mitochondrial AOX	32^{33}	260^{20}	148^{27}
Induced genes:			
Cytosolic sHSP	40^{27}	327^{24}	81^{38}
Chloroplastic sHSP	141^{83}	1423^{88}	142^{39}
Cytosolic HSP100	335^{22}	182^{64}	31^{5}
PR-1a (See also Figure 1b)	28^{56}	4660^{27}	203^{50}
PR-3	18^{11}	864^{24}	219^{36}
PR-5	150^{39}	7362^{55}	45^{19}
ACC oxidase	58^{14}	376^{52}	179^{31}
Ribulose-5-phosphate isomerase	93^{38}	20^{16}	281^{45}
Transaldolase	33^{17}	164^{39}	455^{49}
Transketolase	12^{8}	46^{6}	514^{17}
Invertase	99^{29}	297^{19}	389^{48}
Histone H3	86^{16}	497^{11}	132^{28}
Housekeeping genes:			
ATP synthase	90^{34}	65^{8}	94^{27}
Actin	65^{39}	67^{21}	115^{18}
Tubulin	66^{28}	60^{53}	67^{12}
RbcS (see also Figure 1B)	64^{30}	8^{12}	87^{18}

Only ROI-related genes, housekeeping genes, and genes with an induction rate of 250% or more are shown. Results, presented as percentage of control compared to WT plants, are an average and standard deviation of three independent experiments each repeated twice. RNA was extracted from WT and antisense plants grown under moderate light conditions, labelled, and hybridized to nylon arrays containing tobacco and tomato cDNAs as described in Experimental procedures. Abbreviations used: AOX, alternative oxidase; APX, ascorbate peroxidase; HSP, heat shock protein; MDA, monodehydroascorbate; PR, pathogenesis-related. RbcS, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; SD, standard deviation.

shown that this enzyme (IMMUTANS) has quinol oxidase activity (Josse *et al.*, 2000), suggesting that it diverts electrons flowing from photosystem II (PSII) to photosystem I (PSI) at the plastoquinone pool and uses these to reduce O_2 into water. Because the majority of ROI production in chloroplasts occurs at PSI using electrons supplied by PSII (Asada, 1999), IMMUTANS might lower the rate of ROI production in chloroplasts by reducing the flow of electrons from PSII to PSI. As shown in Figure 6a, the steady state level of transcripts encoding IMMUTANS was specifically induced in dAS plants growing at low light. At moderate light IMMUTANS was also induced in AS-CAT plants, and at high light it was induced in all plants, including WT plants.

The induction of IMMUTANS in WT plants at high light support a role for this enzyme in preventing the formation of ROI in chloroplasts during high light stress. To test whether IMMUTANS is also induced in other plants in response to high light stress we examined its induction in *Arabidopsis*. As shown in Figure 6b, IMMUTANS was induced following a 5-h high light stress treatment (700 μmol m^{-2} sec^{-1}) in *Arabidopsis*. This induction was observed at the transcript as well as the protein level and coincided with a reduction in the steady state level of transcripts encoding the light harvesting chlorophyll *a*/*b* binding protein LHCP. The findings presented in Figure 6 support a role for IMMUTANS in preventing ROI related damage during high light stress in plants.

Decreased sensitivity of antisense plants with suppressed photosynthetic activity to oxidative stress

Because a decrease in photosynthetic activity was associated with the dAS phenotype (Figure 4a), we tested whether plants with reduced photosynthetic activity are

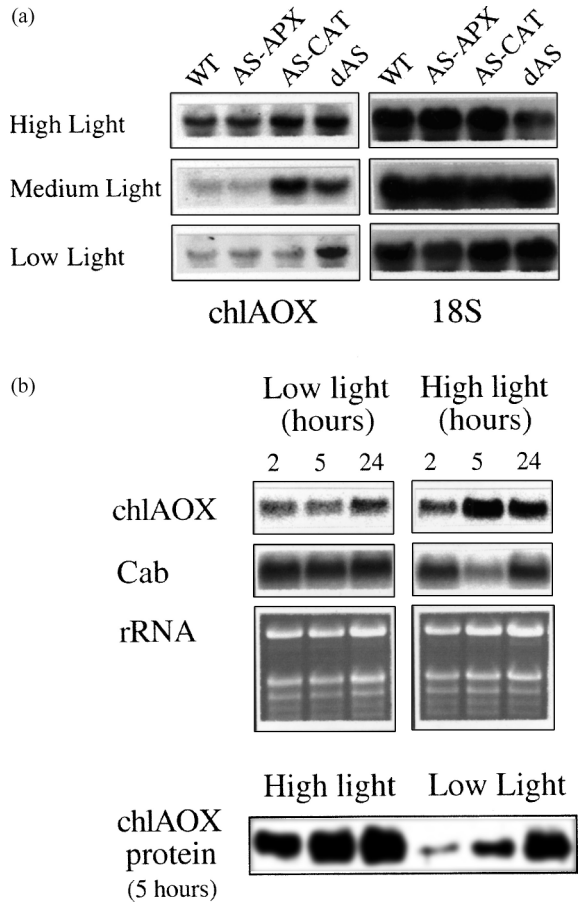

Figure 6. Enhanced expression of chloroplastic alternative oxidase (IMMUTANS) in double antisense plants and in *Arabidopsis* plants subjected to high light stress.
(a) RNA gel blot analysis performed on leaves obtained from wild-type (WT), APX antisense (AS-APX), CAT antisense (AS-CAT), and APX and CAT antisense (dAS) plants grown under low (75 μmol photons m^{-2} sec^{-1}), moderate (150–200 μmol photons m^{-2} sec^{-1}), or high (600–800 μmol photons m^{-2} sec^{-1}) light intensity, showing that IMMUTANS is specifically induced in dAS plants at low light. IMMUTANS is also shown to be induced in AS-CAT plants at moderate light and in all plants, at high light, suggesting that it may be involved in the suppression of ROI production in plants.
(b) RNA (top) and Protein (bottom) blots showing that IMMUTANS is induced in *Arabidopsis* plants subjected to high light stress. IMMUTANS is induced after 5 h of high light stress. This induction is both at the steady state transcript and protein levels. For the analysis of IMMUTANS protein, chloroplast extracts were loaded in 3 different concentrations: 1, 2.5, and 5-μg chlorophyll μ^{-1}l.

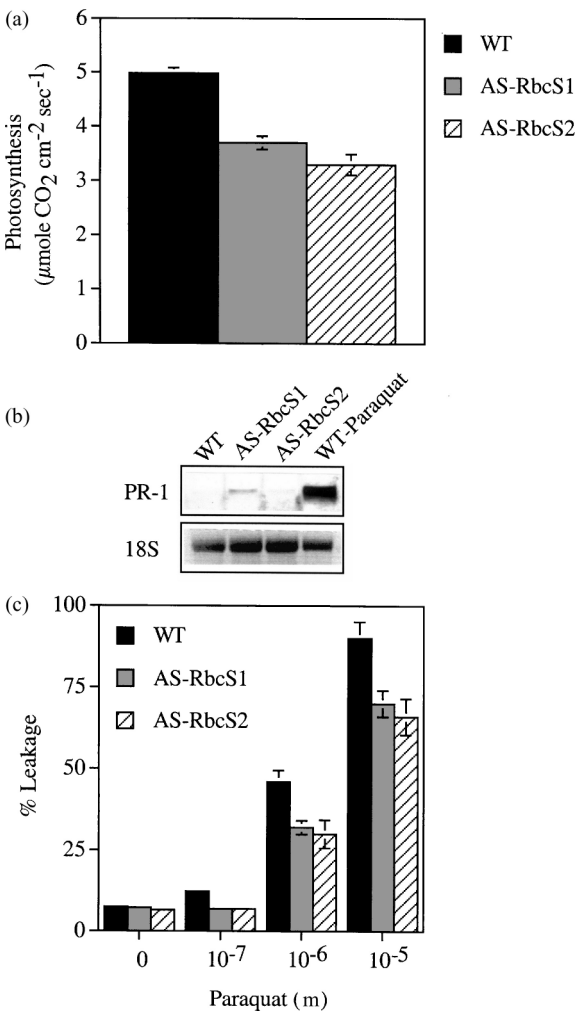

Figure 7. Suppression of paraquat-induced cell death in transgenic plants with decreased photosynthetic activity.
(a) Measurements of photosynthetic activity in wild-type (WT) and two independent antisense plants with reduced expression of *rbcS* (AS-RbcS1 and AS-RbcS2), showing that AS-RbcS plants have reduced photosynthetic activity.
(b) Expression of PR-1a in AS-RbcS plants compared to WT plants or to WT plants treated with paraquat.
(c) Decreased cell death of AS-RbcS plants compared to WT plants in response to a 5-h treatment with paraquat. Cell death was assayed by measuring ion leakage from leaf discs.
Data in (A) and (C) are shown as mean and standard deviation of 3 independent measurements performed on 3-week-old plants grown under low light intensity (75 μmol photons m^{-2} sec^{-1}).

less sensitive to oxidative stress. For this analysis we used antisense tobacco plants with reduced expression of *rbcS* (Jiang and Rodermel, 1995). We used two lines: AS-RbcS1 and AS-RbcS2, that have 60% and 80% reduction in WT Rubisco content, respectively (Jiang and Rodermel, 1995). We subjected 3-week-old antisense and control (WT) plants grown under low light conditions to oxidative stress imposed by paraquat, and compared the degree of cell death induced by this treatment in the different plants. As shown in Figure 7a, AS-RbcS plants had a reduction of about 20–25% in their photosynthetic activity. Prior to paraquat application we tested the expression level of PR-1 by RNA blot analysis to confirm that these plants were not subjected to any additional stress and/or had an induced systemic acquired resistance response. Consistent with this, Figure 7b shows that PR-1 mRNA was not induced in the antisense plants. As illustrated in Figure 7c, compared to WT plants AS-RbcS plants were less sensitive to oxidative stress induced by paraquat and had a decreased level of cell death in response to this treatment.

Response of dAS plants to pathogen infection

Reactive oxygen intermediates play a key role in the defense of plants against pathogen attack (Hammond-Kosack and Jones, 1996). Accordingly, plants with reduced CAT or APX expression are hyper-responsive to pathogen attack and activate the hypersensitive response (HR) upon infection with low titres of bacteria that do not induce the HR in WT plants (Mittler *et al.*, 1999). Our findings that under certain environmental conditions dAS plants are less sensitive to oxidative stress compared to AS-CAT or AS-APX plants, prompted us to examine the response of dAS plants to pathogen infection. Because growth of plants at moderate light resulted in the induction of PR gene expression in AS-CAT plants, we infected plants with bacteria and measured cell death and PR-1 induction in plants grown under low light conditions. For these studies we used a bacterial pathogen that induces the HR in tobacco but cannot propagate within tobacco leaves (Mittler *et al.*, 1999). As shown in Figure 8,

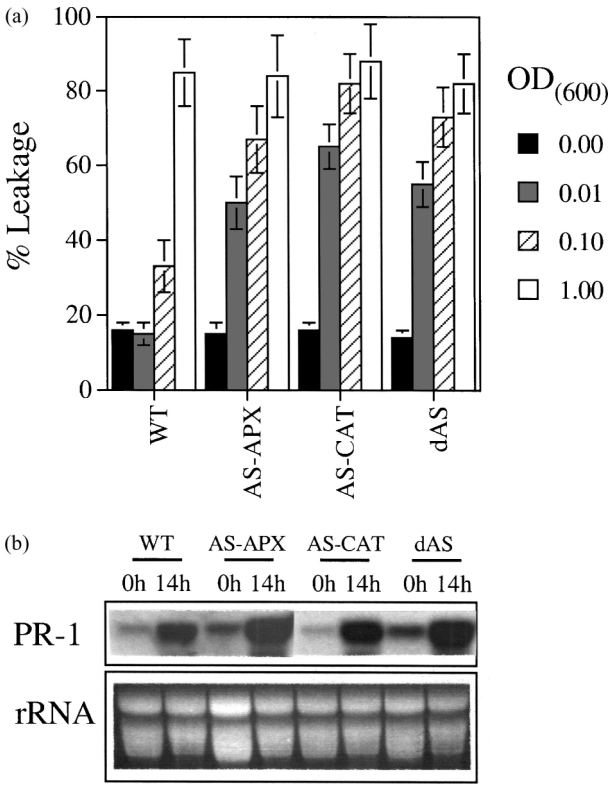

Figure 8. Response of double antisense plants to infection with an HR-inducing bacterium.
(a) Induction of HR-cell death in WT, AS-APX, AS-CAT, and dAS plants 12 h following infection with *Pseudomonas syringae* pv. *phaseolicola* at different concentrations. Cell death was measured by leakage of ions from cells as described in Methods. Results shown are mean and standard deviation of 3 independent measurements performed on 3-week-old plants grown under low light intensity (75 μmol photons m^{-2} sec^{-1}). All antisense plants are shown to be hyper-responsive to bacterial infection compared to WT plants.
(b) RNA gel blots showing the induction of PR-1 transcripts 14 h following bacterial infection ($OD_{600} = 0.1$). PR-1 is shown to be induced in all plants including WT plants. These results are in agreement with Mittler *et al.* (1999).

dAS plants were similar to AS-CAT and AS-APX plants in their response to bacterial infection. Thus, they activated HR-cell death upon infection with a low titre of bacteria similar to AS-CAT and AS-APX plants (Figure 8a), and they induced PR-1 expression in a similar manner to that of WT, AS-CAT, or AS-APX plants (Figure 8b). The differences observed between dAS plants and AS-CAT or AS-APX plants in their response to various abiotic conditions (Figures 1–3) were therefore not reflected in the response of these plants to pathogen infection.

Discussion

At least 3 central players are involved in the removal of H_2O_2 in plants: APX, CAT, and GPX. In a previous study Willekens *et al.* (1997) reported that a deficiency in CAT resulted in the induction of APX and GPX, suggesting that these enzymes were induced to compensate for CAT suppression. Here we show that CAT deficiency is also accompanied by an induction in mitAOX and MDA reductase, two additional enzymes involved in ROI protection (Table 1; Maxwell *et al.*, 1999; Murthy and Zilinskas, 1994). In addition, we show that APX deficiency results in the induction of CAT, SOD, and GR (Figure 1b, Table 1). Taken together these findings suggest a high degree of redundancy in ROI detoxifying mechanisms in plants.

Interestingly, compared to plants with suppressed APX or CAT, a decrease in both APX and CAT did not result in the induction of similar ROI scavenging mechanisms in dAS plants (Figure 1b, Table 1). Moreover, dAS plants, with reduced APX and CAT, should have been more sensitive to ROI stress compared to AS-APX or AS-CAT. However, at least under low or moderate light, dAS plants did not appear to be subjected to the same degree of oxidative stress as AS-CAT or AS-APX plants (Figures 1–3). It is possible that dAS plants induced an unknown ROI scavenging mechanism that protected them from ROI-induced damage. Alternatively, they might have suppressed the rate of ROI production, thereby lowering their intracellular level of ROI. The suppression of photosynthetic activity in dAS plants may account for such a response (Figure 4). The photosynthetic apparatus is one of the major sources of ROI production in photosynthetic tissues, and suppression of photosynthetic activity was shown to accompany the defense response of certain plants to adverse physical conditions (Mittler *et al.*, 2001). It is unlikely that the suppression of photosynthetic activity in dAS plants was a result of an oxidative damage to the photosynthetic apparatus because such damage would have been accompanied by the induction of ROI scavenging mechanisms (Table 1; Figure 1b). Furthermore, measurements of variable fluorescence at low and moderate light failed to detect damage to PSII in dAS plans (not shown), and enhanced CO_2 levels did not result in an increase in photosynthetic

activity that was higher than that observed in WT plants (Figure 4b).

To test the possibility that suppression of photosynthetic activity might have enhanced the tolerance of dAS plants to oxidative stress we tested the response of plants with suppressed photosynthetic activity, i.e. AS-RbcS plants (Jiang and Rodermel, 1995), to oxidative stress. The suppression of photosynthetic activity in these plants is a result of a reduction in RbcS protein and is unlikely to involve changes in ROI metabolism. The enhanced tolerance of AS-RbcS plants to oxidative stress induced by paraquat (Figure 7) supported the hypothesis that suppression of photosynthetic activity plays an important role in protecting dAS plants against oxidative stress. Our results may therefore reveal another aspect of the defense response of plants to oxidative stress: the suppression of a cellular pathway that enhances ROI production, i.e. the photosynthetic apparatus, in order to balance the overall level of ROI in cells.

Preventing the formation of ROI in chloroplasts is likely to protect plant tissue from oxidative stress. Our findings that IMMUTANS is induced in dAS plants at low light, and in WT tobacco and *Arabidopsis* plants at high light (Figure 6), may suggest that this enzyme plays an active role in suppressing the production of ROI in these plants. It has been suggested that IMMUTANS act as a terminal oxidase to intercept electrons flowing from PSII to PSI, by reducing oxygen into water at the plastoquinone step (Carol *et al.*, 1999; Cournac *et al.*, 2000; Josse *et al.*, 2000; Wu *et al.*, 1999). Thus, the activity of this enzyme might decrease the overall rate of ROI production in chloroplasts by reducing the flow of electrons to PSI and the reduction of oxygen to ROI by PSI during stress or paraquat application (Asada and Takahashi, 1987). IMMUTANS is essential for chloroplast biogenesis, and leaves of mutants lacking this enzyme bleach when exposed to high light during germination (Wetzel *et al.*, 1994). These findings, as well as the induction of IMMUTANS in wild-type tobacco and *Arabidopsis* plants at high light, may suggest that this enzyme plays a defense role against ROI production, similar to mitAOX (Maxwell *et al.*, 1999). It is thus possible that two different mechanisms, aimed at lowering the rate of ROI production, are specifically activated in dAS plants: suppression of photosynthetic activity (Figure 4), and induction of IMMUTANS (Figure 6). The combined action of these mechanisms may attenuate the rate of ROI production in dAS plants and enable them to survive in the absence of APX and CAT. Although the flux of electrons through the alternative pathway in chloroplasts is estimated at 2% of total electron flux through the thylakoid under normal conditions (Joet *et al.*, 2002), we cannot rule out the possibility that the overexpression of IMMUTANS in dAS plants (Figure 6) may have increased this flux and resulted in the suppression of photosynthetic activity in dAS plants (Figure 4). Further research is required to address this question.

In addition to the mechanisms that suppress ROI production, at least two different mechanisms may co-operate in dAS plants to actively detoxify H_2O_2: the pentose phosphate pathway, and MDA reductase (Table 1). The pentose phosphate pathway was found to be indispensable for the removal of ROI in mammalian cells and yeast (Juhnke *et al.*, 1996; Pandolfi *et al.*, 1995). In addition, the expression of transcripts encoding enzymes of the pentose phosphate pathway was found to be induced in plants in response to stress (Fahrendorf *et al.*, 1995; Moehs *et al.*, 1996). It was suggested that during stress the pentose phosphate pathway serves as a key source for the supply of reduced NADPH for ROI removal (Juhnke *et al.*, 1996; Pandolfi *et al.*, 1995). Because dAS plants had an enhanced expression of MDA reductase (Table 1), it is possible that the enhanced supply of NADPH via the pentose phosphate pathway in these plants is used for enhancing the regeneration of ASC by this enzyme. Ascorbic acid can react non-enzymatically with hydrogen peroxide in a reaction similar to that catalysed by APX, resulting in the oxidation of ASC to monodehydroascorbate (MDA; Foyer and Halliwell, 1976; Asada and Takahashi, 1987; Noctor and Foyer, 1998). The rapid reduction of MDA to ASC by MDA reductase may therefore facilitate the non-enzymatic detoxification of H_2O_2 by ASC in dAS plants, and enhance the detoxification of H_2O_2 in the absence of APX and CAT. Although the steady state level of ASC was not significantly higher in dAS plants compared to control plants (Figure 2b), it is possible that the rate of regeneration of MDA to ASC is higher in dAS plants. Thus, using the same pool size of ASC as WT plants, the non-enzymatic scavenging of H_2O_2 may be higher in dAS plants due to a high turnover rate of ASC oxidation and ASC reduction. Further studies, including a comprehensive metabolomic analysis, are required to address this possibility.

The importance of APX and CAT to the defense of plants against oxidative stress should not be undermined by our results. Under high light conditions, or in response to pathogen infection, when ROI production is significantly enhanced, the redundant mechanism(s) activated in dAS plants appeared not to be sufficient to prevent tissue damage or HR activation. Thus, dAS plants grown under high light conditions developed lesions similar to AS-APX and AS-CAT plants, while wild-type plants did not (not shown), and dAS plants activated the HR in a similar manner to AS-CAT and AS-APX upon challenge with a low titre of bacteria (Figure 8). Nevertheless, identifying redundant mechanisms or alternative strategies for ROI protection may allow, through genetic manipulations, to enhance the tolerance of plants and crops to adverse environmental conditions. For example, it may be beneficial to transiently reduce the photosynthetic activity of crops during periods of environmental stress. This type of manipulation may protect plants from ROI production that is

associated with the photosynthetic apparatus during stress. Alternatively, overexpression of IMMUTANS during stress may protect plant tissues from oxidative stress. Because the abiotic stress conditions tested in our study, i.e. light intensity and paraquat, were both directly linked to the photosynthetic apparatus, it will be interesting to test in future experiments the resistance of dAS plants to stresses such as salt, drought, heat shock, and cold that may affect other cellular pathways and compartments.

The reason for the plasticity observed in the activation of redundant ROI defense mechanisms between the different antisense plants, i.e. AS-CAT, AS-APX, and dAS, is not known. Because APX and/or CAT expression was most likely suppressed in antisense plants immediately upon germination, due to the use of the 35S CaMV promoter it is possible that early events in gene expression were affected in a different manner in plants that lacked APX, CAT, or APX and CAT. An early change in gene expression might affect a number of critical systems in plants, including events associated with chloroplast biogenesis. The constitutive induction of IMMUTANS in dAS plants may provide some evidence for an early effect on gene expression in these plants because this gene was found to be essential for chloroplast biogenesis during the early stages of germination (Wetzel *et al.*, 1994).

The differences observed between the response of AS-CAT and AS-APX plants to moderate light (Figures 1–3; Table 1) suggest that under these conditions at least two different signal transduction pathways are activated in these plants. Thus, AS-CAT plants activate a cell death pathway resulting in the formation of lesions, whereas AS-APX plants do not form lesions (Figure 1a). In addition, the defense mechanisms activated in AS-APX plants appear to be different from those activated in AS-CAT plants (Figure 1b; Table 1). The induction of cell death and mitAOX in AS-CAT plants suggest that in these plants a signal transduction pathway that involves the mitochondria is activated. The mitochondrion was suggested to be involved in controlling the activation of programmed cell death (PCD) in plants (Lam *et al.*, 2001). In addition, peroxisomes, where CAT is localized (Willekens *et al.*, 1997), are thought to be one of the cellular sites of nitric oxide synthesis (NO; Corpas *et al.*, 2001). Because NO is a key regulator of PCD and defense response activation in plants (Delledonne *et al.*, 2001; Klessig *et al.*, 2000), it is possible that the lack of CAT in peroxisomes of AS-CAT plants activated a signal transduction pathway that triggered PCD. In contrast, AS-APX plants that lack the cytosolic isoform of APX but contain CAT, do not activate PCD or mitAOX expression. Interestingly, the response of dAS plants to growth at moderate light is very different from that of AS-APX or AS-CAT plants (Figures 1–3). It is possible that in dAS plants, that lack APX and CAT, the two different pathways activated in the single antisense plants are simultaneously activated. However, their integration results in a completely different outcome. Thus, these plants suppress photosynthesis and activate different defense mechanisms including IMMUTANS. Future studies using microarray or chip technology and knockout or antisense *Arabidopsis* plants lacking cytosolic APX and/or CAT, may identify the specific components of the different signalling pathways activated in these plants. These studies are underway in our laboratories.

Experimental procedures

Plant material

The production and characterization of transgenic tobacco plants expressing antisense RNA for APX (*apx I*; AS-APX; Orvar and Ellis, 1997), CAT (*cat 1*; AS-CAT; Willekens *et al.* 1997), or *rbcS* (AS-RbcS; Jiang and Rodermel, 1995) was previously reported. Crosses were performed between AS-APX and AS-CAT plants, between their corresponding controls (Orvar and Ellis, 1997; Willekens *et al.*, 1997; referred herein as WT), and between AS-CAT and AS-APX and wild-types. Progenies of all crosses were selfed and their seedlings were germinated and examined by RNA and protein blots for the expression of APX and CAT. Blots were performed on leaves of similar size and age from the different plants, subjected to the same light intensity. At least 3 different plants from each of the groups studied, i.e. WT, AS-APX, AS-CAT, and dAS were selected and propagated by shoot cuttings and seeds to generate a large number of plants. These were again tested by RNA and protein blots prior to analysis. All experiments were performed in triplicates and repeated at least twice.

Growth conditions and photosynthetic measurements

Growth of plants and experiments were conducted under controlled environmental conditions at 22–24°C. Continuous illumination was provided by cool-white fluorescent lamps (75 or 150–200 μmol m^{-2} sec^{-1}), or cool-white fluorescent lamps plus sodium vapour lamps (600–800 μmol m^{-2} sec^{-1}). Photosynthetic activity, dark respiration, and stomatal conductance of leaves was measured with a Licor LI-6400 apparatus using the following measuring cell (6 cm^2) parameters: 24°C, 0, 75, 150, 600, or 2000 μmol photons m^{-2} sec^{-1}, 350 or 2200 ppm CO_2, and an air flow of 300 μl sec^{-1}, as previously described (Mittler *et al.*, 2001). Stomata were counted with an epifluorescence microscope (CARL Zeiss, Germany).

Arabidopsis (*Arabidopsis thaliana* ecotype Colombia) plants were grown for 3 weeks at 22°C, 70 μmol m^{-2} sec^{-1}. High light stress was imposed by shifting of plants to 700 μmol m^{-2} sec^{-1} at 22°C for 48 h. Control and high light treated plants were sampled at different times and analysed by RNA and protein blots as described below. For the detection of IMMUTANS protein, chloroplasts were isolated from the different plants (Wu *et al.*, 1999) and loaded on gels based on equal levels of chlorophyll or protein.

Bacterial infections

Fully expanded leaves of 3-week-old plants were inoculated with *Pseudomonas syringae* pv. *phaseolicola* (NPS3121) according to Mittler *et al.* (1999). Mock-infected plants were infiltrated with water. Mock and pathogen-infected plants were kept at 22–24°C

under continuous illumination (75 μmol m^{-2} sec^{-1}). At different times after infection leaves were sampled, photographed, and analysed for HR cell death and expression of the PR protein, PR-1, as described below by measurements of ion leakage and RNA blots. Additionally, bacteria were extracted from infected leaves and plated, to assay for viability and *in planta* growth. In accordance with previous reports *P. s.* pv. *phaseolicola* (NPS3121) was unable to grow *in planta* in tobacco (Mittler *et al.*, 1999).

Paraquat treatment and cell death measurements

Fully expanded leaves of 3- to 4-week-old plants were infiltrated with different concentrations of paraquat (methyl viologen, Sigma; Mittler *et al.*, 1999). Plants were incubated at 24°C under continuous illumination (75 μmol m^{-2} sec^{-1}), sampled and assayed for cell death as described below.

Measurement of ion leakage from leaf discs

Cell death was assayed by measuring ion leakage from leaf discs. For each measurement, five leaf discs (9-mm diameter) were floated abaxial side up on 5 ml of double distilled water for 3 h at room temperature. Following incubation, the conductivity of the bathing solution was measured with a conductivity meter (Consort, Turnhout, Belgium); referred to as value A. The leaf discs were then returned to the bathing solution, introduced into sealed tubes and incubated with the bathing solution at 95°C for 25 min. After cooling to room temperature the conductivity of the bathing solution was again measured; referred to as value B. For each measurement ion leakage was expressed as percentage leakage, i.e. (value A/value B) × 100.

RNA and protein blots and biochemical measurements

Immunodetection RbcL, RbcS, D1, PsaH, and LHCP was performed by protein blot analysis of total leaf protein with a chemiluminescence detection system (Mittler *et al.*, 1998). Total RNA was isolated as previously described (Mittler *et al.*, 1998) and subjected to RNA gel blot analysis (Mittler *et al.*, 1999). A probe for 18S rRNA was used to ensure equal loading of RNA. The level of ASC and GSH was determined in frozen plant tissue as previously described (Mittler and Tel-Or, 1991, Mittler *et al.*, 1991). Hydrogen peroxide levels were measured by infiltrating leaves with 50 μM H_2DCF-DA (Sigma) in a 20-mM K_2HPO_4, pH 6.5 buffer. Leaf discs (2 × 6 cm) were prepared from plants 30 min after infiltration and immediately grounded in 400 μl ice-cold 20 mM K_2HPO_4, pH 6.5 buffer. The extracts were cleared by filtration and centrifugation (Karpinski *et al.*, 1999) and assayed using a Hitachi F-2000 fluorescence spectrophotometer at 488 nm excitation and 520 nm emission. Control experiments in which H_2O_2 (0.1–10 mM) was infiltrated into leaves, prior (30 min), or with H_2DCF-DA were also performed for calibration.

Filter array hybridization

Clones for the production of filter arrays were ordered from the tomato EST library at Clemson University, or isolated by a differential display screen from stressed tobacco tissue, following paraquat application (Vranova *et al.*, in preparation). Filter arrays were prepared from the clones by spotting PCR products in triplicates on nylon membranes at the Hadassah Medical School DNA Facility of the Hebrew University, and at the Ghent laboratory. Filters were hybridized with radiolabelled cDNAs prepared from total RNA isolated from the different plants (pooling RNA from 3 to 5 plants per sample) using oligo-dT and Superscript™ reverse transcriptase (Life Technologies, GibcoBRL, Cleveland, USA) as suggested by the manufacturer. Hybridization conditions were as follows: 60°C, 5× SSC, 5× Denhart, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA, overnight. Washing conditions were as follows: 60°C, 2× SSC, 0.1% SDS, for 20 min, followed by 0.2× SSC, 0.1% SDS, 60°C for 20 min. Following hybridization and washes the signals were assayed with a phosphoimager (Fuji BAS1000) and analysed with TINA™ software (Raytest, Pittsburgh, PA, USA). A number of control 'housekeeping' genes, animal-specific genes (as negative controls), and empty spots (for background) were also spotted on the membrane. These were used to normalize the intensity of signals between the different filters and calculate the changes in gene expression presented in Table 1. When pertinent, the expression level of specific genes was verified by RNA blots.

Acknowledgements

We thank Dr Brian E. Ellis for providing seeds of AS-APX plants. We also thank Dr Itzhak Ohad for his help in performing variable fluorescence measurements, and Drs Rachel Nechushtai, Barbara A. Zilinskas, and Gadi Schuster for their gifts of antibodies. This work was supported by the Israeli Academy of Science, The Biotechnology Council Iowa State University, US Department of Energy (grant no DE-FG02–94ER20147 to S.R), and The Fund for the Promotion of Research at the Technion.

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