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Catalase is a sink for H_2O_2 and is indispensable for stress defence in C_3 plants

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Hydrogen peroxide (H_2O_2) has been implicated in many stress conditions. Control of H_2O_2 levels is complex and dissection of mechanisms generating and relieving H_2O_2 stress is difficult, particularly in intact plants. We have used transgenic tobacco with ~10% wild-type catalase activity to study the role of catalase and effects of H_2O_2 stress in plants. Catalase-deficient plants showed no visible disorders at low light, but in elevated light rapidly developed white necrotic lesions on the leaves. Lesion formation required photorespiratory activity since damage was prevented under elevated CO_2. Accumulation of H_2O_2 was not detected during leaf necrosis. Alternative H_2O_2-scavenging mechanisms may have compensated for reduced catalase activity, as shown by increased ascorbate peroxidase and glutathione peroxidase levels. Leaf necrosis correlated with accumulation of oxidized glutathione and a 4-fold decrease in ascorbate, indicating that catalase is critical for maintaining the redox balance during oxidative stress. Such control may not be limited to peroxisomal H_2O_2 production. Catalase functions as a cellular sink for H_2O_2, as evidenced by complementation of catalase deficiency by exogenous catalase, and comparison of catalase-deficient and control leaf discs in removing external H_2O_2. Stress analysis revealed increased susceptibility of catalase-deficient plants to paraquat, salt and ozone, but not to chilling.
Keywords: ascorbate–glutathione cycle/ozone stress/ photorespiration/redox control/salt stress

Introduction

Active oxygen species (AOS), including hydrogen peroxide (H_2O_2), have been implicated in irradiation, heat-shock and metal stress, in degenerative diseases such as arthritis, cataract, cancer and Lou Gehrig's disease, in developmental degenerative processes such as ageing and apoptosis, in macrophage-mediated destruction of pathogens and in the activation of stress responses (Halliwell and Gutteridge, 1989; Deng *et al.*, 1993; McConkey and Orrenius, 1994; Khan and Wilson, 1995; Spector, 1995; Sundaresan *et al.*, 1995; Sohal and Weindruch, 1996). In plants, the production of AOS has been evidenced during pathogen infection (Baker and Orlandi, 1995) and in photooxidative processes induced during abiotic stress conditions such as chilling, drought, salt and ozone stress (Foyer and Mullineaux, 1994). Recent evidence suggests that AOS formation in plant–pathogen interactions is mechanistically similar to the oxidative burst in macrophages (Groom *et al.*, 1996). In contrast, processes leading to photooxidative damage during abiotic stresses are mostly unique to plants and cyanobacteria. There is currently great interest in elucidating the nature of these photooxidative processes and in understanding how photooxidation can lead to cellular destruction. Biochemical studies have identified over-reduction of photosystem II (PSII), the Mehler reaction and photorespiration as potentially important sources of AOS in photosynthetic cells (Foyer, 1996). Over-reduction of PSII occurs when carbon assimilation is repressed, e.g. due to environmental stress and when light influx is high. In such a state of photon excess, PSII becomes progressively reduced, and this can lead to oxidative stress through the production of singlet oxygen and possibly superoxide. To prevent over-reduction of PSII, reduction of oxygen through photorespiration and the Mehler reaction may function as alternate routes for de-energizing the photosystems (Krause, 1994; Osmond and Grace, 1995). In the Mehler reaction, oxygen is reduced monovalently first to superoxide ($O_2^{\bullet -}$) and then to H_2O_2. This H_2O_2 is subsequently converted to water by ascorbate peroxidase, thus generating a pseudocyclic electron flow, in which electrons from the oxygen-splitting complex pass through the photosynthetic electron carriers back to oxygen. Photorespiration recycles carbon that is used by oxygenation of ribulose-1,5-bisphosphate and produces H_2O_2 in the peroxisomes through the enzyme glycolate oxidase. The relative importance of these different AOS-forming pathways towards photooxidation in natural stress conditions remains an issue of debate, due mainly to a lack of good experimental procedures to dissect mechanistically these photooxidative processes in intact plants.

Characterization of the antioxidant response may give insight into the nature of the photooxidative processes induced by a specific stress. Likewise, analysis of transgenic plants with modified levels of antioxidant enzymes should identify the critical components of this complex defence system and may provide a basis for designing strategies for oxidative stress tolerance. For example,

transgenic plants with increased levels of superoxide dismutase have demonstrated the importance of this enzyme in the cytosol and chloroplasts during stress defence (Allen, 1995; Inzé and Van Montagu, 1995). A similar analysis of H_2O_2-scavenging enzymes has thus far not been performed, with the exception of a H_2O_2-consuming extracellular peroxidase, possibly involved in lignin biosynthesis (Lagrimini *et al.*, 1990). The principal H_2O_2-scavenging enzyme in plants is catalase, which is located in peroxisomes/glyoxysomes and—at least in maize—in mitochondria and ascorbate peroxidase (APx), which is primarily found in the cytosol and chloroplasts (Asada, 1992; Willekens *et al.*, 1995). The subcellular distribution of these enzymes suggests that chloroplastic APx removes H_2O_2 produced during the Mehler reaction and other chloroplastic processes, whereas catalase scavenges photorespiratory H_2O_2. However, the situation is likely to be far more complex, firstly because H_2O_2 is not strictly compartmentalized, being able to diffuse freely through membranes, and secondly, because catalase and APx have distinct catalytic properties. Catalase ($2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$) does not consume reducing power and has a very high reaction rate, but only poor affinity for H_2O_2. APx (H_2O_2 + ascorbate $\rightarrow 2\ H_2O$ + dehydroascorbate) requires a source of reductant, ascorbate, and has a higher affinity for H_2O_2 than catalase.

We have adopted a transgenic approach to unravelling the role of catalase in tobacco, which—in terms of oxidative stress defence—is probably the best-characterized C_3 plant to date. *Nicotiana plumbaginifolia* contains three active genes encoding catalase (*Cat1*, *Cat2*, *Cat3*), two of which are expressed in mature leaves (Willekens *et al.*, 1994). Cat1 represents ~80% of leaf catalase activity and is located in palisade parenchyma cells. Cat2 accounts for ~20% and is found in the phloem. Using sense and antisense technology, transgenic *Nicotiana tabacum* lines were generated that are deficient in Cat1 (Cat1AS), Cat2 (Cat2AS), or both (Cat-suppressed CatGH lines; Chamnongpol *et al.*, 1996). Cat2-deficient plants did not show any phenotype, under either low- and high-light conditions. Cat1-deficiency had no effect on tobacco grown at low light [<100 μmol/m^2/s photosynthetic photon fluence rates (PPFR 400–700 nm)], but caused white necrotic lesions on the leaves when plants were exposed to higher light intensities (300–1000 μmol/m^2/s PPFR). This phenotype is similar to that observed in a catalase-deficient mutant of barley (Kendall *et al.*, 1983), but it contrasts with catalase deficiency in maize, which is not associated with any visible disorders (Scandalios, 1994). The research presented here was undertaken to elucidate the critical determinants responsible for lesion formation in Cat1-deficient tobacco under high light and to define the function of catalase in tobacco under stress conditions. We report that photorespiration is required for the development of leaf necrosis in Cat1-deficient plants. Biochemical data are presented which show that necrosis is not associated with a discernible increase in H_2O_2 within the leaf, but concurs simultaneously with the inception of a prooxidant state in the cell. Stress experiments with Cat1-deficient tobacco demonstrate that catalase, apart from its housekeeping function during normal photosynthetic growth, is also an indispensable component of the antioxidant defence against environmental stress. Finally, we present evidence that Cat1 deficiency in tobacco provides a highly versatile system for studying the biochemical and molecular effects of intracellular oxidative stress.

Results

Light-induced leaf necrosis in Cat1-deficient plants is dependent on photorespiration

We have previously reported on the induction of pathogenesis-related responses in Cat1-deficient tobacco plants (Chamnongpol *et al.*, 1996). This induction was only observed when plants were transferred from low (<100 μmol/m^2/s PPFR) to moderate or high light intensities (300–1000 μmol/m^2/s PPFR). Under these circumstances, Cat1-deficient plants developed severe necrosis on the leaves. As a first step to understanding the mechanisms leading to this phenotype, we have performed a more detailed symptomatic analysis of this light-dependent leaf necrosis. Cat1-deficient plants obtained by antisense inhibition (Cat1AS) or co-suppression (CatGH) and control plants were precultivated under low light (80 μmol/m^2/s PPFR) for 2 months and subsequently exposed to 1000 μmol/m^2/s PPFR (14 h light/10 h dark) for 4 days. The first signs of leaf deterioration on Cat1-deficient plants appeared after 1 day at high light and consisted of a slight bleaching of the leaves. This bleaching then evolved further leading to the formation of necrotic areas. The development of leaf necrosis was dependent on the leaf age and the cell type. This is illustrated in Figure 1A for leaves of a Cat1AS plant. Necrosis occurred first on the older leaves and generally started at the leaf tip. Not fully expanded leaves remained symptomless within the time course of the experiment (4 days). At the end of the high-light treatment, the entire leaf surface of the oldest leaf had become necrotic with the exception of the veins and some surrounding mesophyll cells, which remained green (Figure 1A). Catalase-suppressed CatGH lines developed a phenotype similar to that of Cat1AS plants, but with a delay of ~1 day. Control plants showed no damage at high light.

High light had no direct effect on catalase activity (data not shown), suggesting that necrosis at high light is due to increased H_2O_2 production rather than to a reduced scavenging capacity. The spatial expression of Cat1 is indicative for a role of Cat1 in H_2O_2-scavenging during photorespiration (Willekens *et al.*, 1994) and the lethal effect of Cat deficiency in barley could be avoided by growth under non-photorespiratory conditions (Kendall *et al.*, 1983). To assess the role of photorespiration towards leaf necrosis in Cat1-deficient tobacco, a second set of plants was exposed to 1000 μmol/m^2/s PPFR in a CO_2-enriched atmosphere (3000 p.p.m. CO_2, 21% O_2). The latter condition strongly inhibits photorespiration of C_3 plants, because it favours carboxylation of ribulose-1,5-bisphosphate carboxylase/oxygenase against oxygenation. If photorespiration is the major cause of leaf necrosis in Cat1-deficient tobacco, then damage should be attenuated or impeded under elevated CO_2. In accord with this hypothesis, we found that the light-induced damage in Cat1-deficient lines was completely prevented under non-photorespiratory conditions (Figure 1B).

Undamaged leaves of Cat1-deficient plants are physiologically indistinguishable from control plants

We subsequently investigated whether the upper leaves of Cat1-deficient plants, in which necrosis had not yet initiated, could be distinguished from controls based on physiological parameters. Leaf gas exchange was measured daily on the youngest, fully expanded leaf of Cat1-deficient and control lines (corresponding to the right-most leaf in Figure 1A). This was the only fully expanded leaf which, in all lines, showed no macroscopic damage after 4 days at 1000 μmol/m^2/s PPFR. Transpiration, conductance and photosynthesis rates of the youngest fully expanded leaf were inferred from the gas exchange values and were very similar in Cat1-deficient and control lines during the entire exposure period. This is illustrated in Table I for day 3. Also chlorophyll fluorescence values (F_v/F_m), which are commonly used to estimate the integrity of PSII (Krause, 1988), were very similar in young leaves of Cat1-deficient and control plants (data not shown). Elevated CO_2 levels did not affect PSII efficiency, compared with ambient air (data not shown), but clearly permitted much higher levels of net CO_2 exchange (Table I).

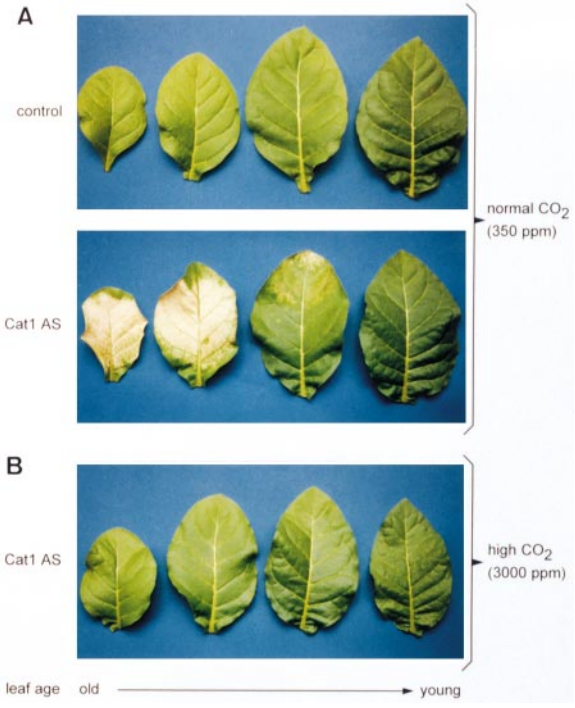


Fig. 1. Light-induced necrosis in Cat1AS plants and protection by elevated CO_2. (**A**) Effect of light stress on leaf integrity in Cat1AS and wild-type tobacco. Plants precultivated for 2 months at 80 μmol/m^2/s PPFR photon flux were exposed to 1000 μmol/m^2/s PPFR for 4 days in normal air (380 p.p.m. CO_2, 21% O_2). Leaves were detached for making photographs. (**B**) Plants were exposed to light stress as in (A), in an atmosphere enriched for CO_2 (3000 p.p.m. CO_2, 21% O_2).

We then addressed the question of whether a loss in physiological activity could be detected before the onset of visible necrosis. When PSII efficiency and photosynthesis were followed in leaves of different age, a clear correlation was found between leaf damage and repression of PSII efficiency (data not shown) and photosynthetic activity. Photosynthesis was reduced by 15–30% in old versus young leaves of control plants (Table II). In old leaves of Cat1-deficient lines, photosynthetic activity decreased by >50% in catalase-suppressed CatGH plants and by 75% in Cat1AS plants. In young leaves of Cat1-deficient plants, however, photosynthetic activity was similar to that in controls, whereas in middle-aged leaves only a small reduction was observed compared with controls. Taken together, these results show that light-induced leaf necrosis in Cat1-deficient plants is associated with a loss of PSII efficiency and photosynthetic activity, and that until the appearance of visible damage, Cat1-deficient leaves are physiologically indistinguishable from controls. The fact that no decrease in photosynthesis was observed in non-necrotic leaves suggests that, once initiated, the loss of physiological competence associated with Cat1 deficiency rapidly leads to visible tissue damage.

Cat1 deficiency compromises the ascorbate–glutathione cycle

Cat1 deficiency reduces the H_2O_2-removing capacity of plant cells and, consequently, may lead to higher steady-

Table II. Effect of leaf age on net CO_2 exchange in Cat1-deficient and wild-type tobacco lines exposed to high light (1000 μmol/m^2/s PPFR) and ambient CO_2 (380 p.p.m.) for 4 days

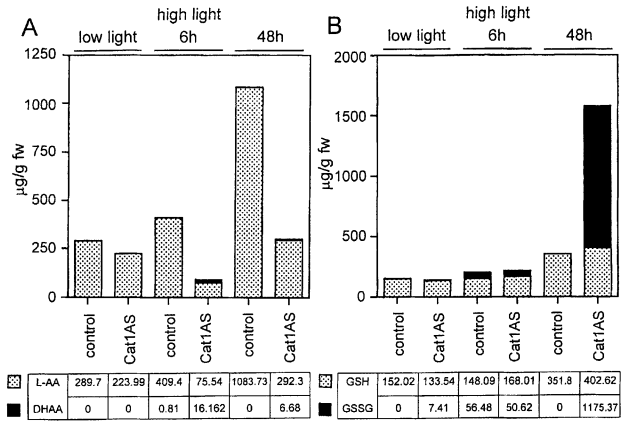
Line	Net CO_2 exchange (μmol/m^2/s)		
	Young leaves	Middle-aged leaves	Old leaves
Cat1AS	14.79 ± 0.27	10.53 ± 0.80	3.41 ± 0.74
Co-sup. CatGH1	12.59 ± 0.6	10.32 ± 0.18	5.88 ± 0.44
Co-sup. CatGH2	12.50 ± 0.65	10.68 ± 0.91	5.80 ± 1.26
Control 1	14.77 ± 0.99	14.53 ± 1.06	12.57 ± 0.51
Control 2	14.33 ± 0.08	13.79 ± 0.25	10.52 ± 0.61

Means ± SE (n = 4). Co-sup., co-suppressed.

Table I. Photosynthetic gas exchange parameters of tobacco leaves under ambient and elevated CO_2 conditions

Line	Net CO_2 exchange (μmol/m^2/s)		Transpiration (mmol/m^2/s) Ambient CO_2	Conductance (mmol/m^2/s) Ambient CO_2
	Ambient CO_2 (380 p.p.m.)	Elevated CO_2 (3000 p.p.m.)		
Cat1AS	9.48 ± 1.10	19.9 ± 1.4	1.75 ± 0.06	319 ± 30
Co-sup. CatGH1	10.38 ± 0.21	17.9 ± 1.3	1.85 ± 0.39	379 ± 18
Co-sup. CatGH2	9.22 ± 0.33	15.4 ± 0.6	1.88 ± 0.27	379 ± 7
Control 1	10.03 ± 0.33	21.4 ± 0.7	1.75 ± 0.55	332 ± 25
Control 2	10.24 ± 0.36	18.8 ± 1.0	1.85 ± 0.38	363 ± 26

Values are from the youngest fully expanded leaf on the third day after transfer from low (80 μmol/m^2/s PPFR) to high light (1000 μmol/m^2/s PPFR) and are means ± SE (n = 4). Co-sup., co-suppressed.



L-AA	289.7	223.99	409.4	75.54	1083.73	292.3
DHAA	0	0	0.81	16.162	0	6.68

GSH	152.02	133.54	148.09	168.01	351.8	402.62
GSSG	0	7.41	56.48	50.62	0	1175.37

Fig. 2. Changes in ascorbate and glutathione contents in leaves of Cat1AS and wild-type tobacco during light stress. (**A**) Effect of a shift from low light (80 µmol/m^2/s PPFR) to high light (300 µmol/m^2/s PPFR) on the levels of reduced (L-AA) and oxidized (DHAA) ascorbate. Samples were taken before, after 6 h and after 48 h exposure to high light. Ascorbate and glutathione levels in these samples were determined by HPLC analyses with simultaneous UV and electrochemical detection. Values are expressed in µg/g fresh weight of leaf material. A value of zero indicates that the signal was below the detection limit. (**B**) Effect on reduced (GSH) and oxidized (GSSG) glutathione.

state levels of H_2O_2 inside leaves. If all the photorespiratory H_2O_2 would accumulate, then concentrations of H_2O_2 would exceed 100 mM within 1 day [assuming 10 µmol CO_2/m^2/s for rate of CO_2 fixation (see Table I), 20% as the ratio of oxygenation versus carboxylation of ribulose-1,5-bisphosphate carboxylase and 1 mm leaf thickness]. We observed considerable variation in H_2O_2 levels among different leaf samples of Cat1-deficient and wild-type tobacco (100–500 µmol H_2O_2/g fresh weight), but we found no evidence for higher H_2O_2 levels in leaf tissue of Cat1-deficient versus control plants, either at low or at high light. Recovery rates of added H_2O_2 indicated that differential losses of H_2O_2 during extraction could at most account for 10% variation. It is concluded that the increase in H_2O_2 levels in Cat1AS plants, if any, is minor compared with the intrinsic variation in H_2O_2 levels in tobacco leaves, implying that most of the H_2O_2 in Cat1-deficient leaves was removed by cellular mechanisms other than catalase.

The ascorbate–glutathione cycle, located both in the cytosol and the chloroplasts of plant cells, is likely to be the first H_2O_2-scavenging mechanism to be challenged, when H_2O_2 removal in the peroxisomes falls short (Asada, 1994; Creissen *et al.*, 1994). Ascorbate removes H_2O_2 non-enzymatically, as well as enzymatically via APx. The oxidation products of ascorbate are recycled by different reductive mechanisms involving either ferredoxin, glutathione and/or NAD(P)H (Asada, 1994). Levels of ascorbate and glutathione have been shown to increase upon stress, indicating that the capacity of the ascorbate–glutathione cycle can be modulated according to the demand for antioxidant potential (reviewed in Alscher, 1989; Foyer, 1993). Along the same line, we found a 3- to 4-fold increase in reduced ascorbate and a 2-fold increase in reduced glutathione 48 h after tobacco SR1 was transferred from 100 to 300 µmol/m^2/s PPFR (Figure 2). The oxidized forms of ascorbate and glutathione were below the detection limit in tobacco SR1 at 100 µmol/m^2/s PPFR and after adaptation to 300 µmol/m^2/s PPFR (48 h sample). However, a transient increase in oxidized ascorbate and glutathione, indicative of oxidative stress, was observed after 6 h at 300 µmol/m^2/s PPFR, which is before the strong increment in levels of reduced ascorbate and glutathione. This suggests that tobacco SR1 underwent a transitory condition of oxidative stress during adaptation to the elevated light regime.

At 100 µmol/m^2/s PPFR, Cat1AS plants showed levels of reduced ascorbate and glutathione similar to that of controls, and undetectable levels of dehydroascorbate. The presence of small amounts of oxidized glutathione was the only indication of stress in Cat1AS plants exposed to low light. However, exposure to 300 µmol/m^2/s PPFR had a dramatic effect on the total ascorbate pool of Cat1AS plants and controls. In Cat1AS plants, the total ascorbate pool decreased >2-fold within 6 h at 300 µmol/m^2/s PPFR, against a slight increase in controls; the amount of dehydroascorbate accounted for 20% of the total ascorbate pool in Cat1AS, but for only 0.5% in the controls. After 48 h, the levels of reduced ascorbate in Cat1AS plants had recovered to the initial level, but were still 3- to 4-fold lower than in the controls. At this time, levels of dehydroascorbate had not increased further, which indicates that, to some extent, Cat1AS cells were able to control dehydroascorbate accumulation, presumably via the ascorbate–glutathione cycle. Yet, the control of dehydroascorbate levels in Cat1AS plants occurred at great cost, since it was paralleled by a massive accumulation of oxidized glutathione. After 48 h, oxidized glutathione was at least 1000-fold higher in Cat1AS plants than in controls, whereas levels of reduced glutathione were similar. No differences in oxidized or reduced glutathione levels were observed after 6 h at 300 µmol/m^2/s PPFR. Taken together, these results suggest that Cat1AS plants are unable to generate sufficient reducing power for the ascorbate–glutathione cycle to allow continuous recycling of the substrates.

Peroxidase induction in conditions of Cat1 shortage

The accumulation of oxidized ascorbate and glutathione in Cat1AS plants shows that the reducing part of the ascorbate–glutathione cycle could not keep pace with the increased rate of oxidation. Oxidation of ascorbate and glutathione was presumably accelerated because of a greater availability of H_2O_2 but, in addition, it could result from increased activity of peroxidases that consume ascorbate or glutathione. These include APx, as outlined above, and glutathione peroxidase (GPx). GPx from plants show highest homology to a class of mammalian GPx which, besides removing H_2O_2, can also repair other peroxides such as fatty acid and phospholipid peroxides (Criqui *et al.*, 1992; Beeor-Tzahar *et al.*, 1995). Induction of peroxidase activity has been observed in mutants of *Saccharomyces cerevisiae* and *Hansenula polymorpha* that were deficient in peroxisomal catalase (Verduyn *et al.*, 1988). At low light, we found no difference between the amounts of GPx in Cat1AS and control plants, whereas the level of APx protein was only slightly higher in Cat1AS plants (Figure 3). After transfer to high light, a transient induction of GPx and a sustained increase of APx

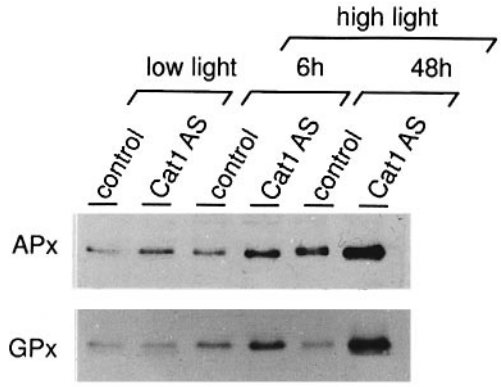


Fig. 3. Effect of Cat1 deficiency on the expression of APx and GPx at low light (80 μmol/m^2/s PPFR) and after 6 and 24 h at high light (300 μmol/m^2/s PPFR). Protein extracts of Cat1AS and wild-type tobacco leaves were separated by denaturing polyacrylamide gel electrophoresis and assayed by immunoblot analysis for APx (using anti-cytAPx serum, upper panel) and GPx levels (using anti-GPx serum, lower panel).

protein levels was observed in control plants (Figure 3). Cat1AS plants showed a persistent increase in both GPx and APx after transfer to high light, and the level of induction was much higher in Cat1AS plants than in the controls. This high induction of APx in Cat1AS plants was also manifested at the level of total APx activity. After 6 h of high light, APx activity was 2.5-fold higher in Cat1AS plants than in controls (data not shown). These data demonstrate that tobacco activates alternate enzymatic mechanisms for H_2O_2 scavenging to compensate for the shortage in catalase.

Exogenous catalase can attenuate leaf necrosis in Cat1-deficient plants

An *in planta* leaf assay was developed that allowed us to test whether exogenously added catalase or peroxidase could compensate for Cat1 deficiency at elevated light. Using a syringe without a needle, components were injected into the intercellular space of leaves and scored for their ability to delay leaf necrosis. The injected solution spread easily through the leaf mesophyll but was arrested at primary and secondary veins. Soaked areas were initially dark-green, but this coloration disappeared within 1 h, indicating that most of the liquid was rapidly absorbed by the cells. Injection of bovine catalase (~500 μl of a 2000 U/ml solution) gave protection against leaf necrosis (Figure 4). The amount of injected catalase corresponds to ~250 U/cm^2 leaf surface, which is within the range found in tobacco (Zelitch *et al.*, 1991). By contrast, injection of distilled water, an osmotically adjusted solution, heat-inactivated catalase or horseradish peroxidase (25–2500 U/ml) had no discernible effect on the development of leaf necrosis (Figure 4; data not shown).

Catalase functions as a sink for cellular H_2O_2

The observation that catalase, injected into the intercellular space of the leaf, can compensate for peroxisomal catalase deficiency suggests that catalase functionally protects cells against H_2O_2 that is produced at a distant location. This would imply that catalase could be involved in the removal of H_2O_2 from subcellular compartments other than the peroxisomes. The validity of this model was assessed by

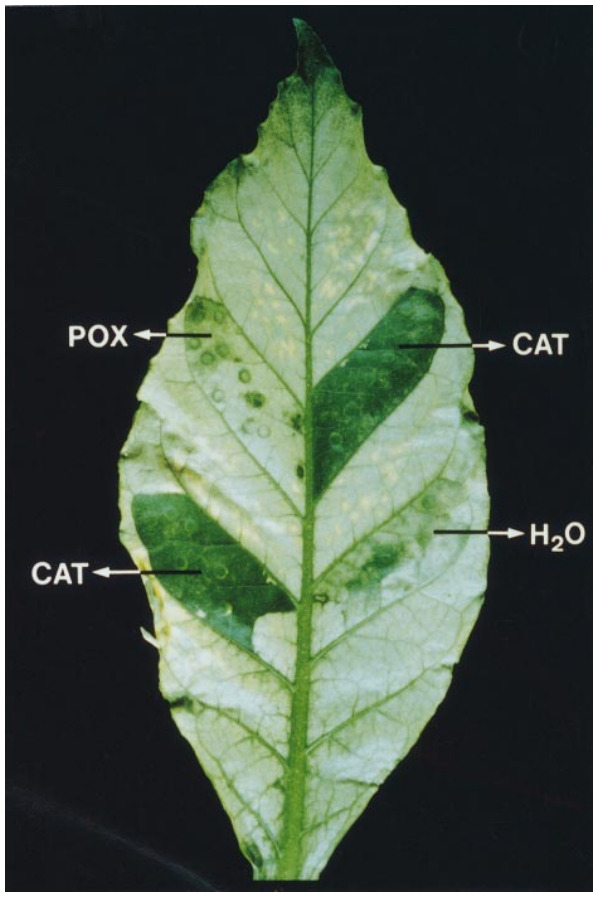


Fig. 4. Complementation of Cat1 deficiency with exogenous catalase. Commercial bovine catalase (1000 U), horseradish peroxidase (1250 U) and water were injected with a syringe without a needle into Cat1-deficient leaves. After injection, plants were exposed to high light (300 μmol/m^2/s PPFR) for 48 h. The zone of the leaf infiltrated with catalase (CAT) remained green during this period, whereas zones injected with peroxidase (POX) and water (H_2O) were bleached. This bleaching was comparable in time and severity with that in non-infiltrated parts.

the reverse experiment, i.e. determination of the contribution of peroxisomal catalase to the removal of extracellular H_2O_2. Plant cells are relatively tolerant to exogenous H_2O_2 in comparison with mammalian cells (e.g. Levine *et al.*, 1994; Guyton *et al.*, 1996) and this is generally thought to be due to the presence of extracellular peroxidases in the plant cell wall. Yet, experimental data on the relative contribution of peroxidases and catalases to the removal of exogenous H_2O_2 have so far not been presented.

Leaf discs of control and Cat1-deficient plants were floated on a solution of H_2O_2 and assayed for their H_2O_2-scavenging capacity. Under low light (100 μmol/m^2/s PPFR), four leaf discs of 8 mm diameter from control plants scavenged ~60% of the H_2O_2 from 10 ml of 10 mM H_2O_2 solution in 24 h, which corresponds to 50 μmol H_2O_2/g fresh weight/h (Figure 5A). Leaf discs from Cat1-deficient plants removed only one-quarter to one-third of this amount. As outlined above, Cat1-deficient plants developed normally under low-light conditions and contained similar levels of reduced ascorbate and glutathione as control plants (see Figure 2). Nevertheless, it is likely that even under low light, part of the peroxidase activity in Cat1-deficient plants is engaged in the removal of

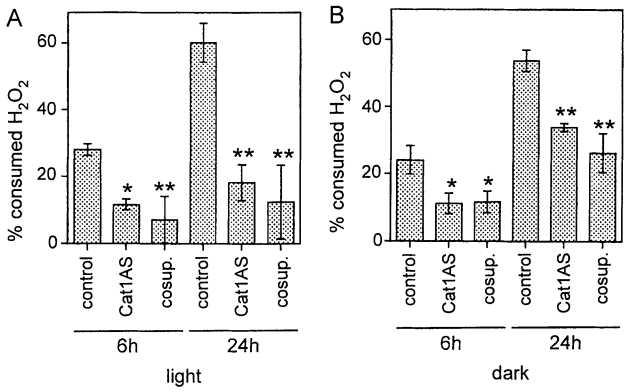


Fig. 5. Elimination of extracellular H_2O_2 by Cat1-deficient and wild-type SR1 leaf discs. (**A**) Four leaf discs of 8 mm diameter were floated on 10 ml of 10 mM H_2O_2 solution under low light (100 μmol/m^2/s PPFR). The amount of H_2O_2 consumed by the leaf discs after 6 and 24 h was determined by measuring the absorbency of the medium at 240 nm. Means with an asterisk are significant (* = P <0.05, ** = P <0.01) compared with the control (SR1). (**B**) The same experiment as in (A), but in dark conditions.

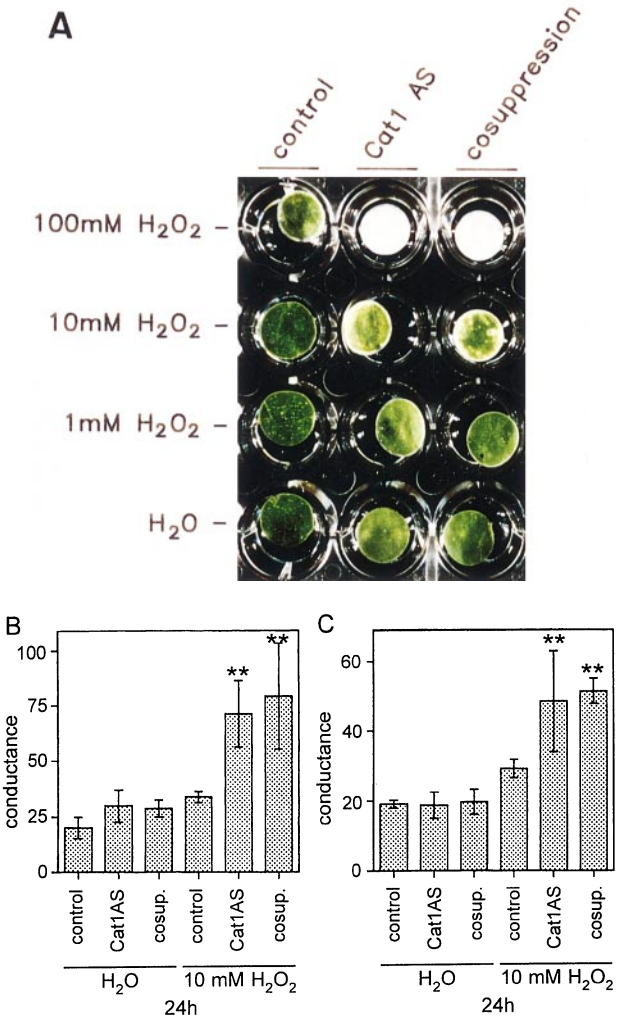


Fig. 6. Sensitivity of Cat1-deficient and wild-type SR1 leaf discs to extracellular H_2O_2. (**A**) Chlorophyll bleaching of Cat1-deficient and wild-type SR1 leaf discs floated for 24 h on water and increasing concentrations of H_2O_2 (1, 10 and 100 mM). (**B**) Ion leakage, measured as conductance of the medium, of Cat1-deficient and wild-type SR1 leaf discs floated on 10 mM H_2O_2 in the light. Experimental conditions are as described in Figure 5. Conductance values for ion leakage (expressed as μSiemens/cm) were corrected for the amount of H_2O_2 present in the medium (see Figure 5). Means denoted with ** are significantly (P <0.01) different from the control (SR1, 10 mM H_2O_2). (**C**) The same experiment as in (B), but in dark conditions.

photorespiratory H_2O_2 and hence is not available for scavenging exogenous H_2O_2. Experiments were therefore repeated in the dark and under non-photorespiratory light conditions (3000 p.p.m. CO_2, 2% O_2). In agreement with our assumption, removal of exogenous H_2O_2 in Cat1-deficient leaf discs was almost twice as efficient in the dark as in the light (Figure 5B). Yet, both in the dark as in non-photorespiratory light conditions (data not shown) less H_2O_2 was disposed of by Cat1-deficient leaf discs than by controls. Considering that none of the Cat1-deficient lines is entirely devoid of catalase activity, it is tentatively estimated that catalase accounted for ~50% of the H_2O_2 consumption from the medium in control leaf discs. It is concluded therefore that Cat1 functions as a cellular sink for H_2O_2.

Sensitivity to oxidative stress

The reduced ability of Cat1-deficient leaf discs to remove exogenous H_2O_2 correlated with an increased sensitivity to H_2O_2. Cat1-deficient leaf discs showed severe bleaching when floated on 10 and 100 mM H_2O_2 solutions under low light (Figure 6A). This bleaching was due to photodestruction of pigments, because it did not occur in dark conditions. A second parameter used for assessing cellular injury in leaf discs exposed to exogenous H_2O_2 was the conductance of the floating solution. Conductance reflects the leakage of ion solutes out of the cell and hence gives an indication of membrane damage. Lipid peroxidation resulting from oxidative stress is likely to initiate this membrane deterioration. Solute conductance was almost 3-fold higher in Cat1-deficient leaf discs than in controls, when challenged with 10 mM H_2O_2 (Figure 6B). A minor increase in solute conductance was also observed in Cat1-deficient leaf discs that were floated on water, suggesting that intracellular H_2O_2 production slightly affects the integrity of the plasma membrane of Cat1-deficient leaf discs. Cat1 deficiency also increased the sensitivity of leaf discs to exogenous H_2O_2 in dark conditions, but the ion leakage was not as pronounced as in the light (Figure 6C).

The redox-cycling herbicide paraquat intercepts electrons from various electron transport chains, thereby reducing oxygen to superoxide (Dodge, 1994). Most of this superoxide subsequently dismutates to oxygen and H_2O_2, either spontaneously or enzymatically via superoxide dismutase (Bowler *et al.*, 1992). In photosynthetic plant cells, electron transport through the photosystems is the primary site of electron capture by paraquat (Halliwell, 1984). The sensitivity of tobacco leaf discs was markedly enhanced by Cat1 deficiency (Figure 7). Leaf discs of Cat1-deficient plants showed increased ion leakage after 3 h of paraquat treatment (Figure 7A). After 6 h of paraquat treatment, ion leakage from Cat1-deficient leaf discs was twice the level of controls (Figure 7B). Taken together, these data demonstrate that Cat1 deficiency leads to enhanced sensitivity to oxidative stress generated at the plasma membrane or in the chloroplasts.

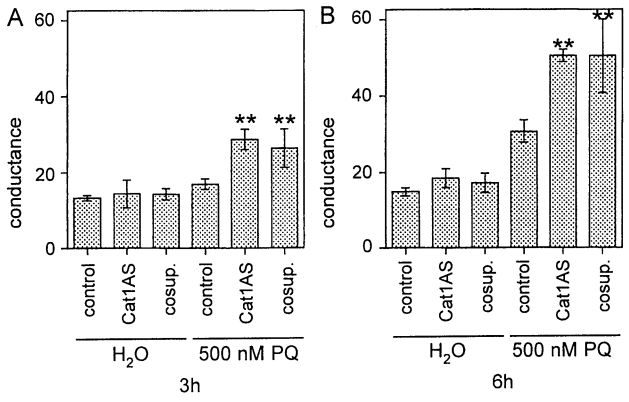


Fig. 7. Ion leakage of Cat1-deficient and wild-type SR1 leaf discs challenged with paraquat. Four leaf discs of 8 mm diameter were floated on 10 ml of 500 nM paraquat solution under low light (100 μmol/m^2/s PPFR). Ion leakage was measured as conductance of the medium after (**A**) 3 h and (**B**) 6 h incubation. Means denoted with ** are significantly (P <0.01) different from the control (SR1, 500 nM paraquat).

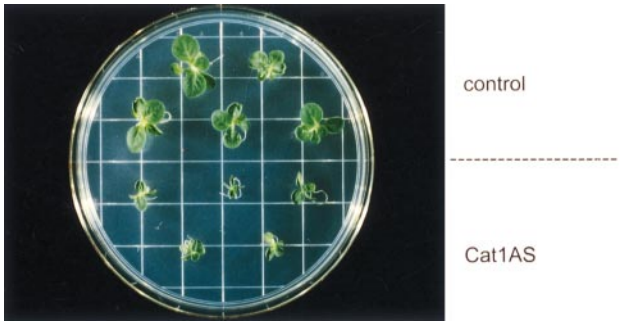


Fig. 8. Differential effect of salinity on the growth of Cat1AS and wild-type SR1 seedlings. Cat1AS seedlings manifested a pronounced growth retardation compared with wild-type, when germinated and grown for 8 weeks on MS medium containing 100 mM NaCl under low light (100 μmol/m^2/s PPFR).

Sensitivity to abiotic stress conditions

Several environmental stress conditions are thought to generate oxidative stress in plants (Foyer and Mullineaux, 1994). Hence, we addressed the question of whether the increased sensitivity of Cat1-deficient plants to photorespiration and oxidative stress is also mirrored in an increased susceptibility to abiotic stress conditions, in particular to salt, ozone and chilling stress. These stresses were applied under low light to distinguish effects due to the stress condition from light-induced damage.

Salt tolerance was examined during early growth. Seeds of Cat1-deficient and control lines were germinated on Murashige and Skoog (MS) medium in the presence and absence of 100 mM NaCl. Growth was assessed after 8 weeks of cultivation on salt. Whereas equal growth was observed on normal MS medium (data not shown), Cat1-deficient plantlets were clearly retarded in growth compared with controls when grown on medium containing 100 mM NaCl (Figure 8).

Ozone induces small necrotic spots on the leaves of tobacco. The kinetics of this process depend on the dosage, plant age and light intensity (Guderian, 1985). Mature preflowering plants of Cat1-deficient and control lines were exposed to ozone (mean value 130 nl/l) during the light period (16 h light, 80 μmol/m^2/s PPFR) and to ozone-free air during the night. After 2 days, leaves of Cat1-deficient plants were covered with glossy necrotic spots (Figure 9). Lesion formation was observed first on the middle leaves, as is usual with ozone stress in tobacco. Control plants remained symptomless during the course of the experiment (4 days).

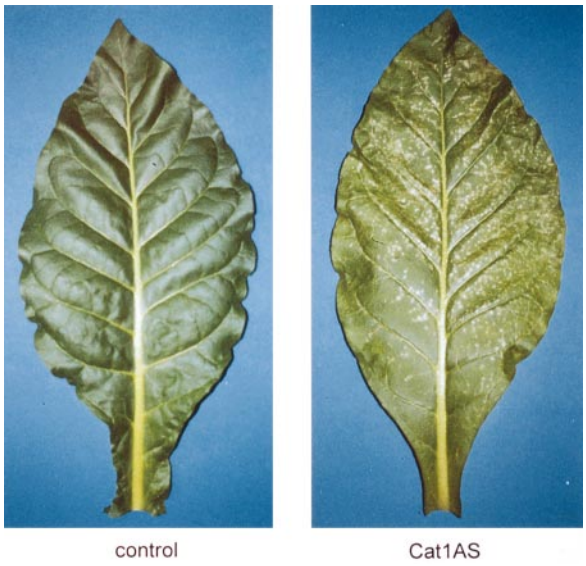


Fig. 9. Ozone sensitivity of Cat1AS versus wild-type tobacco SR1. Representative leaf of a Cat1AS and wild-type plant after 2 days of exposure with ozone (mean value 130 p.p.b.) during the light period (16 h, 80 μmol/m^2/s PPFR) and with ozone-free air during the dark period (8 h). Ozone-induced injury in the Cat1AS line is characterized by the appearance of glossy necrotic spots on the upper parts of the leaf, whereas the wild-type shows no discernible damage under the same conditions.

Chilling stress was applied by exposing mature preflowering tobacco plants to 4°C at 80 μmol/m^2/s PPFR for 4 days. This treatment did not cause any visible sign of injury in either Cat1-deficient or control lines, indicating that the damage observed during salt and ozone stress is not merely the result of combining the low light treatment to a second stress factor. Taken together, these results demonstrate that Cat1 deficiency renders tobacco SR1 markedly more vulnerable to salt and ozone stress, whereas it has no effect on chilling tolerance under the conditions used.

Discussion

Cat1 deficiency greatly enhances the sensitivity of tobacco to light stress

We have studied the effects of shifts in light intensities on the ascorbate and glutathione contents in Cat1-deficient and control tobacco. Plants were shifted from 100 to 300 μmol/m^2/s PPFR, which is a moderate increase compared with what is generally used for inducing photoinhibition in plants. The oxidative siege resulting from such mild stress conditions was nevertheless considerable. Although phenotypically uninjured, wild-type tobacco was unable to maintain its redox status, as illustrated by the transient accumulation of oxidized glutathione (Figure 2). This perturbation of the redox balance was transitory in wild-type plants, possibly because the protective potential of the ascorbate–glutathione cycle (i.e. the amount of

reduced antioxidants) remained unaffected in the first hour after the light shift and showed a strong adaptive response thereafter. The same treatment, however, strongly compromised the ascorbate–glutathione cycle and caused severe necrosis in Cat1-deficient tobacco. Young leaves of Cat1-deficient plants were markedly less sensitive to high light exposure than older leaves. The reason for this is not clear, as we found no large differences in rates of photosynthesis, in APx activity, or in ascorbate and glutathione pools between young and old leaves. A possible explanation is the degree of catalase deficiency, which was less pronounced in young leaves than in older leaves (15% versus 5% of wild-type activity, respectively).

Cat1 is essential for maintaining the redox balance during light stress

Induction of APx and GPx did not prevent necrosis in Cat1-deficient tobacco (Figure 3), suggesting that catalases and peroxidases have fundamentally different H_2O_2-scavenging properties. In contrast to catalase, peroxidase activity requires a source of reductant. The substrates oxidized by peroxidases during H_2O_2 removal are primarily low molecular weight antioxidants, such as ascorbate and glutathione. These are not only directly involved in the non-enzymatic scavenging of AOS, but are also essential for other antioxidant systems, e.g. for the recycling of α-tocopherol (Fryer, 1992) and for the xanthophyll cycle (Demmig-Adams and Adams, 1994). Hence, perturbing ascorbate and glutathione pools can have far-reaching consequences on the antioxidant defence system. Plants have, therefore, developed powerful and versatile systems for maintaining ascorbate and glutathione pools in the reduced state (Asada, 1994).

Despite these elaborate mechanisms for ascorbate and glutathione regeneration in plants, our data show that Cat1-deficient tobacco plants were unable to maintain ascorbate and particularly glutathione pools in the reduced state when exposed to elevated light (Figure 2). The GSSG/GSH ratio in Cat1-deficient tobacco increased 60-fold within 48 h after the light shift. A similar build-up of oxidized glutathione, although less pronounced, was also observed in a catalase-deficient barley mutant (Smith *et al.*, 1984). The accumulation of oxidized glutathione suggests that glutathione reductase (GR) activity, which determines the amount of reducing power specifically directed towards glutathione, becomes rate-limiting in Cat1-deficient plants. Overproduction of GR was shown to partially protect the ascorbate pool from oxidation in tobacco (Foyer *et al.*, 1991) and to enhance the stress tolerance in tobacco (Aono *et al.*, 1993) and poplar (Foyer *et al.*, 1995), indicating that recycling of reduced glutathione can indeed be rate-limiting during the antioxidant defence. Another explanation could be that the recycling of glutathione is defective because of a general shortage of reducing power in Cat1-deficient plants. Measurements of the pyridine nucleotide pools, as well as analysis of crosses between Cat1-deficient and GR-overproducing tobacco should allow a clear discrimination to be made between both possibilities.

The ascorbate pool was also compromised in Cat1-deficient plants exposed to elevated light. Levels of reduced ascorbate were 3- to 4-fold lower in Cat1-deficient tobacco plants than in controls after 48 h under elevated light, but the accumulation of the oxidized form was much less pronounced than for glutathione (Figure 2). Possibly, the transfer of reducing equivalents through the ascorbate–glutathione cycle is controlled so that ascorbate is maintained in the reduced state in preference of glutathione. On the other hand, dehydroascorbate might not be found to accumulate because it decomposes further to products such as tartrate and oxalate (Foyer, 1993). It is concluded that Cat1 is essential in tobacco for protecting ascorbate and glutathione pools from oxidation and, since glutathione is the major sulfhydryl component in plant cells, for maintaining the redox balance in light-stressed cells.

Cat1 is a sink for cellular H_2O_2

Our data show that the reductive pathway for H_2O_2 removal in tobacco is not sufficiently powerful to cope with the amounts of H_2O_2 that are produced during a mild light stress. This result suggests that the function of catalase in the cell is to remove the bulk of the H_2O_2, whereas peroxidases (apart from their biosynthetic function) would be mainly involved in scavenging H_2O_2 that is not taken by catalase. The biochemical characteristics of catalases and peroxidases are in accord with these functions. Catalases have a high V_{max} but a lower affinity for H_2O_2 than peroxidases. The catalase/peroxidase system may thus act cooperatively to remove H_2O_2 at a minimal expense of reducing power and yet at a maximal rate. Plant catalases are mostly found in peroxisomes, where many H_2O_2-producing oxidases are also located. The results presented indicate however, that this compartmentalization does not preclude catalase from operating as a general sink for H_2O_2 within the cell. Cat1-deficient leaf discs were strongly impaired in their ability to remove extracellular H_2O_2 (Figure 5), whereas injection of catalase into the intercellular space of the leaf delayed necrosis in Cat1-deficient plants (Figure 4). It follows that catalase could also be essential for the antioxidant defence during biotic and abiotic stresses that generate AOS in cellular compartments other than the peroxisomes.

Sensitivity to stress conditions

Cat1-deficient tobacco was markedly more sensitive to light, paraquat, salt and ozone stress, but not to chilling stress at low light. To our knowledge, this is the first direct evidence that substantiates the importance of AOS during salt stress in plants. Ozone gives rise to superoxide formation and to the production of hydrogen peroxide in the apoplast (Schraudner *et al.*, 1996). The relevance of this AOS formation was demonstrated in studies showing that symptom development during ozone stress was less severe in tobacco plants overproducing superoxide dismutase (Van Camp *et al.*, 1994; Pitcher and Zilinskas, 1996). Moreover, a massive accumulation of oxidized glutathione, similar to that occurring during catalase deficiency at high light, was also observed in ozone-stressed poplar leaves (Sen Gupta *et al.*, 1991).

The sensitivity of Cat1-deficient plants to these various stresses provides proof for H_2O_2 being an important mediator of cellular toxicity during environmental adversity. Identification of the source of this H_2O_2 during these various conditions will be important for identifying common mechanisms between various stress conditions. The primary sources of H_2O_2 in photosynthetic cells are

thought to be the Mehler reaction in the chloroplasts and glycolate oxidase (being part of photorespiration) in the peroxisomes. Other sources are cytosolic and mitochondrial superoxide dismutases and various oxidases, other than glycolate oxidase. We have identified photorespiration as the principal source of H_2O_2 stress in Cat1-deficient tobacco exposed to high light (1000 μmol/m^2/s PPFR), based on experiments with elevated CO_2 (Figure 1B). C_4 plants have much lower levels of photorespiration than C_3 plants, which may explain why catalase deficiency in maize had no apparent detrimental effect (Scandalios, 1994). During light stress, the photoproduction of ATP and NADPH exceeds the consumption in anabolic processes and the photosystems will become increasingly reduced, leading to the formation of AOS in the chloroplasts. It is thought that photorespiration may mitigate these oxidative processes, by functioning as a sink for energy and reducing potential (Wu *et al.*, 1991; Osmond and Grace, 1995). This model was corroborated by a recent study which showed that transgenic tobacco plants with enhanced photorespiratory capacity were more tolerant to photooxidative damage induced by high light (Kozaki and Takeba, 1996). Photorespiration may also be fundamental for maintaining the balance between light and dark reactions of photosynthesis during other conditions of environmental adversity. A reduction in carbon assimilation, either because of limited CO_2 availability or because the enzymes for carbon assimilation are not fully operational, is frequently observed during stress and will also result in a condition of photon excess. In accordance, induction of photorespiratory activity has been observed in salt-treated pea (Fedina *et al.*, 1994), whereas ozone-induced oxidative damage in wheat was diminished by simultaneous exposure to elevated CO_2 (Rao *et al.*, 1995). The latter result suggests that photorespiration actually contributed to ozone damage, yet a direct effect of elevated CO_2 on ozone uptake cannot be excluded. Collectively, these data demonstrate that H_2O_2, probably arising from photorespiration, is an important component of ozone and salt stress, and that catalase is critical for the cellular defence against these stresses.

Cellular responses induced by H_2O_2 stress

We have shown that Cat1 deficiency is a very elegant system for studying the cellular responses to H_2O_2 stress in intact plants. Responses that were identified in Cat1-deficient plants are an increase in total glutathione, enhanced expression of defence-related proteins and activation of cell death. The massive increase in total glutathione levels shows that new synthesis of glutathione is greatly stimulated in Cat1-deficient plants exposed to high light. Glutathione biosynthesis is regulated by feed-back inhibition of one of the biosynthetic enzymes, γ-glutamylcysteine synthetase (Hell and Bergmann, 1990). Our data indicate that this feed-back inhibition on the biosynthetic route is alleviated in Cat1-deficient plants or, more probably, that only the reduced form of glutathione is active in this feed-back control.

Cat1-deficient plants induced oxidative stress-related genes (APx, GPx) and certain components of the pathogenesis response (Chamnongpol *et al.*, 1996; also unpublished results). This induction was found exclusively at high light, suggesting the presence of oxidative stress-sensing pathways for the activation of nuclear gene expression. Similar evidence has previously been presented in soybean suspension cells with respect to the activation of defence related genes by extracellular H_2O_2 during elicitor treatment (Levine *et al.*, 1994). In the same study, by using selectively permeable membranes, H_2O_2 was shown to function as a molecular signal for cell-to-cell communication in suspension-cultured cells. Our data on the sink function of catalase (Figures 4 and 5) demonstrate that H_2O_2 is able to diffuse throughout the cell and between various cellular compartments, thus providing evidence that H_2O_2 has a sufficient life-time within a plant cell to function as a diffusible signal.

Cat1 deficiency caused cell death under certain stress conditions, but it is currently unclear whether active mechanisms play a role in this cell death process. Low doses of H_2O_2 induce apoptosis in animal and plant cells, whereas necrosis occurs when high concentrations are present (McConkey and Orrenius, 1994; Levine *et al.*, 1996). Cell death by low doses of H_2O_2 in soybean cell cultures was triggered only by sustained accumulation above a threshold (Levine *et al.*, 1996). These results suggest that the cell death mechanism operative at low levels of H_2O_2 is distinct from that at high levels and is unlikely to be due to direct cellular killing by H_2O_2. A possible candidate to be involved in this programmed cell death process is oxidized glutathione, because we observed a massive accumulation of oxidized glutathione, but not of H_2O_2, in Cat1-deficient plants that continuously produce H_2O_2 from photorespiration. Being the major sulfhydryl molecule in the cell, glutathione is essential for the antioxidant defence, the redox balance and the regulation of gene expression in plants (reviewed by Foyer *et al.*, 1997); therefore, it is conceivable that oxidation of the glutathione pool could function as a potent initiator of cell death. The validity of this model needs further assessment, but it provides a novel perspective for the analysis of cell death pathways in which oxidative stress could play a role, such as the hypersensitive response and ozone-mediated lesion formation.

Materials and methods

Plant material

Cat1AS and co-suppressed CatGH are transgenic lines of *N.tabacum* cv. Petit Havana SR1 with ~10% of wild-type catalase activity. Cat1AS contains a *Cat1* antisense cassette and is specifically reduced in the activity of the Cat1 isoform. Co-suppressed CatGH lines carry a cassette for sense expression of the cotton catalase SU2, but show reduced catalase activity, presumably due to a co-suppression process. In co-suppressed CatGH plants, both leaf isoforms of catalase (Cat1 and Cat2) are reduced in activity. Construction and characterization of these lines have been described elsewhere (Chamnongpol *et al.*, 1996). F_2 progeny homozygous for the transgene locus were used for analysis. Seedlings were germinated on Murashige and Skoog solid medium under low irradiation [PPFR (400–700 nm) 80 μmol/m^2/s] and, after transfer to soil, grown under low light conditions (100 μmol/m^2/s PPFR, 14 h light/10 h dark cycle, 25°C, 70% relative humidity) unless stated otherwise.

Exposure to elevated CO_2

Wild-type SR1, Cat1AS and co-suppressed CatGH lines were precultivated in controlled environment chambers (Heræus, Balingen, Germany) under low light conditions. Two-month-old plants were transferred into walk-in 'EPOKA' chambers (Thiel *et al.*, 1996). PPFR (400–700 nm) in the EPOKA chambers was ~1020 μmol/m^2/s PPFR at the top of the canopy level. The light system was designed to simulate the solar spectrum and consisted of metal halide, quartz halogen and blue

fluorescent lamps (238 in total) mounted at the ceiling of a separate lamp cabinet on top of the experimental chamber (Thiel *et al.*, 1996). Plants were either exposed to normal air (380 p.p.m. CO_2 concentration) or to CO_2-enriched air (3000 p.p.m. CO_2 concentration). The chamber atmosphere was sampled at the canopy level using Teflon lines at eight locations per chamber and CO_2 concentrations were recorded for 24 h/day.

Stress treatments

Leaf discs (8 mm diameter) were punched using a cork-bore from the youngest fully expanded leaves of 2.5-month-old tobacco plants cultivated at 60–80 μmol/m^2/s PPFR. For H_2O_2 consumption analysis, three leaf discs were floated on a 10-ml aliquot of H_2O_2 solution (various concentrations in deionized H_2O). Consumption of H_2O_2 by the leaf discs was measured as the change in OD_{240} of the medium. Ion leakage of the leaf discs during H_2O_2 stress was measured as conductivity of the medium using a K610 conductivity meter (Consort, Turnhout, Belgium). A correction was made for the conductivity of the H_2O_2 present in the medium as calculated from the OD_{240} values.

Analysis of ion leakage during paraquat (Protex, Deurne, Belgium) stress was performed as described for H_2O_2, except that the conductivity of the paraquat in the medium, which was negligible, was not taken into account in the calculations. Salt stress was applied by germinating seeds on MS solid medium containing 100 mM NaCl. Growth was assessed visually after 2 months. Ozone treatment was performed on mature preflowering plants, cultivated in controlled environment chambers (Heræus). Ozone (mean value 130 ± 10 nl/l) was applied during the light period (16 h, 80 μmol/m^2/s PPFR) and ozone-free air during the dark period. Ozone values in the chambers were registered during the light period with intervals of 5 min. Damage was assessed visually as the appearance of necrotic spots. Chilling stress consisted of exposing mature preflowering plants, precultivated at 25°C, to 4°C for 4 consecutive days. Sensitivity was scored as visual damage. *P* values were obtained by using Dunnett Multiple Comparisons Test.

Molecular and biochemical techniques

H_2O_2 was extracted from leaf tissue according to Okuda *et al.* (1991). Leaf material (0.5 g) was ground in liquid nitrogen and 2 ml of 0.2 M $HClO_4$. The mixture was thawed and transferred to a 10-ml plastic tube. After adding another 2 ml of 0.2 M $HClO_4$, the homogenate was centrifuged at 2700 *g* for 30 min at 4°C. The supernatant was taken, neutralized to pH 6.0 with 4 M KOH and centrifuged at 110 r.c.f. for 1 min at 4°C. The supernatant was loaded on a AG1x8 prepacked column (Bio-Rad, Hercules, CA). H_2O_2 was eluted from the column with 4 ml double-distilled H_2O. Equal recovery from the different samples was checked by analysing duplicate samples to which H_2O_2 was added during grinding at a final concentration of 50 μM. H_2O_2 was determined by a spectrophotometric assay (Shindler *et al.*, 1976). The sample (800 μl) was mixed with 400 μl 4× reaction buffer (1× reaction buffer: 1 mM 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid, 25 mM potassium acetate, pH 4.4), 400 μl deionized water, and 0.25 U horseradish peroxidase. H_2O_2 was measured at OD_{412}.

The simultaneous analysis of ascorbate and glutathione was carried out using a recently developed HPLC method, full details of which will be published elsewhere (M.Davey, G.Bauw and M.Van Montagu, in preparation). Fresh plant material was weighed, snap-frozen in liquid nitrogen and stored at –70°C until analysis. Samples of 100 mg were then ground to a fine powder with a pre-cooled pestle and mortar in liquid nitrogen and 0.4 ml of 3% metaphosphoric acid containing 1 mM EDTA was added. The sample was homogenized with the frozen extraction solvent until the mixture thawed. The crude extract was then transferred to a 2-ml microcentrifuge tube and centrifuged for 7 min at 20 100 *g*. The supernatant was transferred to a fresh microcentrifuge tube and weighed, and the pellet extracted once more. After centrifugation, the supernatants were combined and filtered before HPLC analysis. All manipulations were carried out at 4°C. Isocratic HPLC analyses were carried out using a reverse-phase HPLC column with simultaneous UV and electrochemical detection. Duplicate analyses of 10–20 μl extract were performed and oxidized ascorbate and glutathione calculated by subtraction after reduction of samples with dithiothreitol and repeat HPLC analysis. Values were calculated using standard curves prepared from pure standards in extraction solvent.

Protein extraction and Western analysis were performed as described (Chamnongpol *et al.*, 1996). Rabbit antisera against the cytosolic APx from maize (Koshiba, 1993) and the GPx from *Nicotiana sylvestris* (our unpublished results) were used at a dilution of 1:2000 and 1:5000, respectively.

Photosynthesis measurements

Leaf gas exchange was analysed with a mini-cuvette system (CQP-130, Walz, Effeltrich, Germany) under standardized climatic conditions of the growth chamber (25 ± 0.5°C, 70 ± 5% relative humidity). All gas exchange parameters were calculated according to von Cämmerer and Farquhar (1981) and related to the projected leaf area which was determined with an area meter (Delta-T, Hoddesdon, UK).

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