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## Research Article

# Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes

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**Abstract.** Tolerance against oxidative stress generated by high light intensities or the catalase inhibitor aminotriazole (AT) was induced in intact tobacco plants by spraying them with hydrogen peroxide ($H_2O_2$). Stress tolerance was concomitant with an enhanced antioxidant status as reflected by higher activity and/or protein levels of catalase, ascorbate peroxidase, guaiacol peroxidases, and glutathione peroxidase, as well as an increased glutathione pool. The induced stress tolerance was dependent on the dose of $H_2O_2$ applied. Moderate doses of $H_2O_2$ enhanced the antioxidant status and induced stress tolerance, while higher concentrations caused oxidative stress and symptoms resembling a hypersensitive response. In stress-tolerant plants, induction of catalase was 1.5-fold, that of ascorbate peroxidase and glutathione peroxidase was 2-fold, and that of guaiacol peroxidases was approximately 3-fold. Stress resistance was monitored by measuring levels of malondialdehyde, an indicator of lipid peroxidation. The levels of malondialdehyde in all $H_2O_2$-treated plants exposed to subsequent high light or AT stress were similar to those of unstressed plants, whereas lipid peroxidation in $H_2O_2$-untreated plants stressed with either high light or AT was 1.5- or 2-fold higher, respectively. Although all stress factors caused increases in the levels of reduced glutathione, its levels were much higher in all $H_2O_2$-pretreated plants. Moreover, significant accumulation of oxidized glutathione was observed only in plants that were not pretreated with $H_2O_2$. Extending the AT stress period from 1 to 7 days resulted in death of tobacco plants that were not pretreated with $H_2O_2$, while all $H_2O_2$-pretreated plants remained little affected by the prolonged treatment. Thus, activation of the plant antioxidant system by $H_2O_2$ plays an important role in the induced tolerance against oxidative stress.

**Key words.** Hydrogen peroxide; oxidative stress; antioxidant enzyme; glutathione.

Throughout life, plants are exposed to many unfavorable environmental conditions such as extreme temperatures, excessive light, pollution, drought, and salinity. These adverse abiotic factors can often lead to the increased production and accumulation to damaging concentrations of reactive oxygen species (ROS), including $H_2O_2$, superoxide anion, and hydroxyl radicals [1, 2], a process referred to as oxidative stress.

One of the mechanisms actively employed by plants to survive this stress is activation of the cell antioxidant system. Stress-resistant plants often possess elevated activities of antioxidant enzymes, including superoxide dismutase, catalases, peroxidases, and glutathione reductase [3, 4]. Studies with transgenic plants that overexpress antioxidant enzymes also substantiate the importance of the antioxidant system for stress tolerance [5].

Although the exact mechanisms determining how the stress signals are perceived and transduced, and the an-

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tioxidant cell machinery is activated are not yet clear, there is evidence that $H_2O_2$ takes an active part in these processes. $H_2O_2$ is the most stable of the ROS and it can rapidly diffuse across cell membranes. At high concentrations it is toxic and can trigger programmed cell death [6, 7]. At non-toxic concentrations, $H_2O_2$ can be a signaling molecule that mediates plant responses to a variety of biotic and adverse abiotic stress factors [8, 9]. For example, a transient increase in the endogenous levels of $H_2O_2$ obtained by exogenous application of salicylic acid or heat can lead to subsequent thermotolerance in mustard seedlings [10] and potato [11]. In maize, protection against chilling injury can be achieved by a transient increase in endogenous $H_2O_2$ levels during low-temperature acclimation [12]. Alternatively, pretreatment with $H_2O_2$ or abscisic acid (ABA) has been shown to protect maize seedling from chilling injury by induction of peroxidases and mitochondrial catalase [13, 14]. $H_2O_2$ is also implicated as part of a systemic signal that sets up an acclimatory response to high light stress in *Arabidopsis* [15]. Taking all these facts together, there is an intriguing possibility that moderately elevated levels of $H_2O_2$ can protect plants from different stress factors. Such a view is also supported by the involvement of ROS in cross-tolerance [16].

The aims of this work were (i) to examine whether hydrogen peroxide can induce tolerance against oxidative stress generated by either high light or aminotriazole (AT) in intact tobacco plants and (ii) to study the role of the cell antioxidant system in that tolerance. Stress tolerance was induced by pretreatment with $H_2O_2$ and assessed by determining lipid peroxidation, because levels of lipid peroxides rapidly increase during oxidative stress-induced cell damage [17, 18]. The role of the cell antioxidant system was assessed by following changes in the activities of the antioxidant enzymes catalase, ascorbate peroxidase (APx), and guaiacol peroxidases, as well as the glutathione peroxidase (GPx) protein and glutathione levels.

## Materials and methods

### Plant material, growth conditions, and application of stress

Tobacco plants *Nicotiana tabacum* cv. Petit Havana SR1 were germinated on soil in a greenhouse with 14 h light/10 h dark, at 25 °C, photosynthetic photon flux density (PPFD) 100 µmol $m^{-2}$ $s^{-1}$, and relative humidity 70%. Four-week-old tobacco plants in the rosette stage were selected for the experiments. All plants were divided into two groups: a control group and an $H_2O_2$-treated group. Each of the two groups was divided into three subgroups: unstressed control plants, plants subjected to high light stress, and plants subjected to oxidative stress generated by AT. For induction of stress tolerance, $H_2O_2$ was applied by spraying an aqueous solution directly on the plant leaves. As a control treatment, water alone was applied. Each plant was sprayed with a 5 mM $H_2O_2$ solution corresponding to approximately 1.7 mg $H_2O_2$ per single plant. The high light treatment was conducted for 24 h at 25 °C in a plant growth chamber with 14 h light/10 h dark, PPFD 500 µmol $m^{-2}$ $s^{-1}$, and relative humidity 70%. AT treatment was applied by spraying a 10 mM aqueous solution on the plant leaves. The duration of the AT stress was either 1 or 7 days. The approximate amount of AT applied on each plant was 2 mg per day. High light or AT stress were applied 1 day after the $H_2O_2$ pretreatment. Samples were taken immediately after the stress treatments and kept frozen until analyzed.

### Protein extraction and enzyme assays

The extraction buffer for all enzymatic assays contained 50 mM potassium phosphate, 1 mM EDTA, 0.1% Triton X-100, 2% (w/v) PVPP, and Complete protease inhibitors (Roche). Ascorbate was added to the buffer at a concentration of 30 mM for APx. For the analysis of catalase, guaiacol peroxidases, and GPx, ascorbate was omitted from the extraction buffer. Antioxidant enzymes were determined according to Slooten et al. [19] and enzyme activity was expressed as metabolized substrate per minute per milligram protein. Protein concentration was determined by the method of Bradford [20] with a kit supplied by Bio-Rad.

### Total peroxidase

For peroxidase determination, protein samples were separated by 10% native PAGE. After electrophoresis, the gel was preincubated in 50 mM potassium phosphate buffer (pH 7.0) and then stained with 0.5 mg $ml^{-1}$ diaminobenzidine and 5 mM $H_2O_2$ in 50 mM potassium phosphate buffer (pH 7.0) for 15 min.

### Lipid peroxidation

Determination of malondialdehyde levels was performed with thiobarbituric acid essentially as described by Dhindsa et al. [21].

### Western blot analysis

Proteins were separated on 15% SDS-PAGE and transferred onto Hybond ECL membrane (APBiotech). The membrane was blocked with 5% skimmed milk in phosphate-buffered saline (PBS) for 3 h and washed for 10 min three times with PBS + 0.05% Tween-20 (PBST). The membrane was probed for 3 h with a polyclonal antibody raised against GPx [22], washed again three times and probed with a second anti-rabbit IgG antibody conjugated with horseradish peroxidase for 1 h. After the final washes, the antibody was detected by the enhanced chemiluminiscence method according to the instructions of the manufacturer (APBiotech) and the signal was

recorded on X-ray film. The intensity of the signals from the Western blot and the peroxidase staining were quantified using Image Master software (APBiotech).

## Results

### Tobacco leaves sprayed with 5 mM $H_2O_2$ are protected against a subsequent oxidative stress

To evaluate the potential stress-protective effect of $H_2O_2$, we sprayed an aqueous solution of $H_2O_2$ on 4-week-old tobacco plants. In a pilot experiment to determine the optimal $H_2O_2$ concentration that exerts biological effect, we sprayed 0, 0.5, 5, 50, and 500 mM $H_2O_2$ on the plants. Initially, we measured catalase and guaiacol peroxidase activities and the degree of lipid peroxidation to quantify the induction of cellular defense and damage, respectively. Leaves were harvested the day following spraying. Catalase activity was induced by 5 and 50 mM $H_2O_2$ (fig. 1 A). Guaiacol peroxidase activity was most prominently induced by 50 mM $H_2O_2$; 0.5 and 50 mM $H_2O_2$ also induced it, while 500 mM had the opposite effect (fig. 1 B). All tested $H_2O_2$ concentrations had no significant effect on lipid peroxidation (fig. 1 C). Up to 50 mM, no visible effects were detected on the sprayed leaves. Spraying 500 mM $H_2O_2$ led to the induction of necrotic lesions resembling those occurring during a hypersensitive response (data not shown). In all further experiments, 5 mM was used.

To test whether plants pretreated with $H_2O_2$ were also more protected against a subsequent oxidative stress, we measured lipid peroxidation in $H_2O_2$-pretreated or non-treated plants exposed to high light conditions or spraying with AT. Figure 2 A shows that in non-treated plants, lipid peroxidation was increased up to 1.5- to 2-fold by high light and AT, respectively. This increase could be prevented by $H_2O_2$ pretreatment.

Extending the AT stress up to 7 days resulted in the appearance of necrotic lesions on the plant leaves and subsequent death of the stressed plants (fig. 2 B). This damaging effect of prolonged AT stress was completely prevented by $H_2O_2$ pretreatment.

### $H_2O_2$-inducible stress tolerance correlates with increased antioxidant levels

Changes in the activities of antioxidant enzymes, GPx protein, and glutathione levels were measured both in $H_2O_2$-pretreated and non-treated plants after 1 day high light or AT stress treatment in order to assess the role of the cell antioxidant system in $H_2O_2$-induced stress tolerance.

High light caused a slight increase in catalase activity in the $H_2O_2$-untreated plants whereas AT caused a severe, about 3-fold reduction in catalase activity (fig. 3 A). The same trend was observed in the $H_2O_2$-pretreated plants; however, catalase activities, compared with those of the untreated plants, were higher in all variants. Thus, $H_2O_2$-pretreated controls and $H_2O_2$-pretreated plants exposed to subsequent light stress possessed higher catalase activities, and catalase activity in $H_2O_2$-pretreated plants subsequently exposed to AT stress was less inhibited (fig. 3 A).

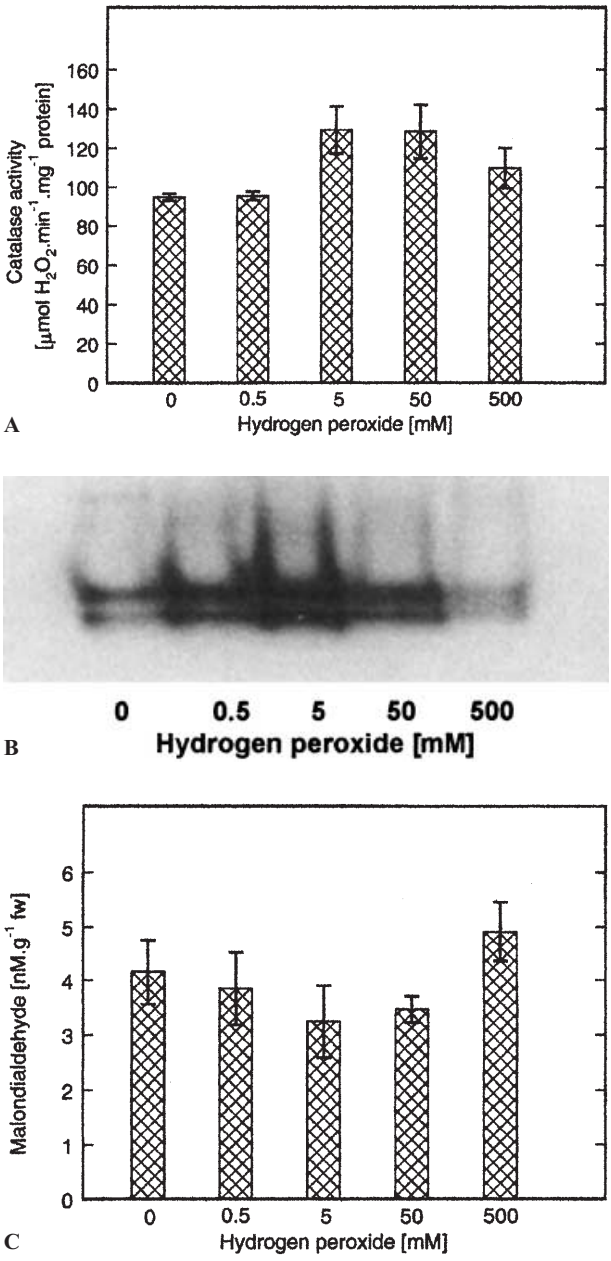


Figure 1. Dose-dependent effect of $H_2O_2$ on the activity of the antioxidant enzymes catalase (*A*) and guaiacol peroxidases (*B*) and on lipid peroxidation (*C*) in tobacco. Intact 4-week-old plants were sprayed with increasing concentrations of $H_2O_2$ (0–500 mM). Catalase activity was determined photometrically, while guaiacol peroxidases were separated by native 10 % PAGE and stained with guaiacol. Lipid peroxidation was assessed by determining malondialdehyde levels. Data are means ± SE; n = 3.

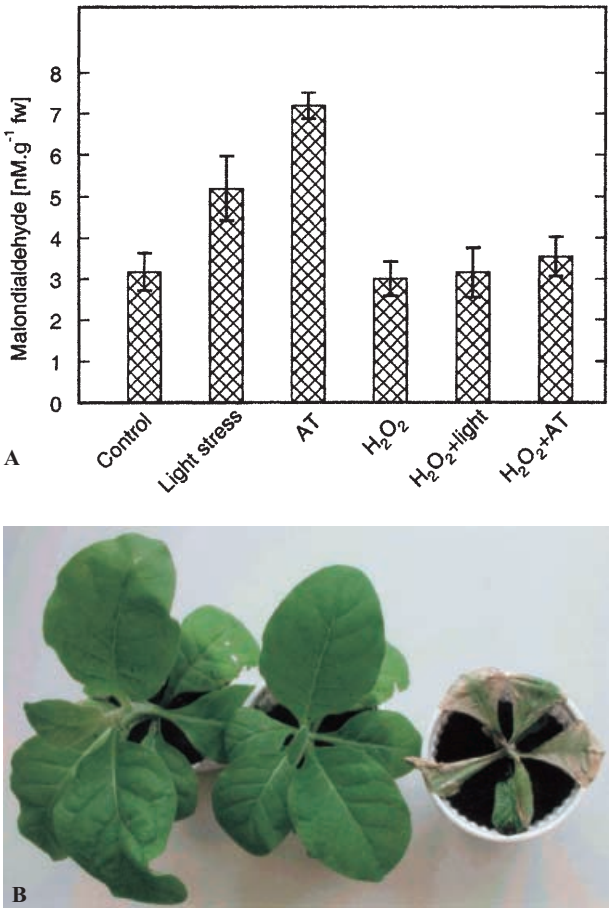


Figure 2. $H_2O_2$ protects tobacco against oxidative stress. (*A*) $H_2O_2$ prevents a stress-associated increase in lipid peroxidation. 4-week-old tobacco plants were not treated (columns 1–3) or were pretreated with 5 mM $H_2O_2$ for 1 day (columns 4–6). On the following day, plants from the two groups were subjected to 1 day of oxidative stress generated by either high light intensities or aminotriazole (AT) and the tolerance against oxidative stress was assessed by comparing lipid peroxidation (malondialdehyde levels) of the stressed plants with that of unstressed controls. Data are means ± SE; n = 3. (*B*) $H_2O_2$ protects tobacco from AT-generated oxidative stress. On the left, unstressed control; in the middle, plant pretreated with $H_2O_2$ for 1 day and then exposed to 7 days AT stress; on the right, plant exposed to 7 days AT stress.

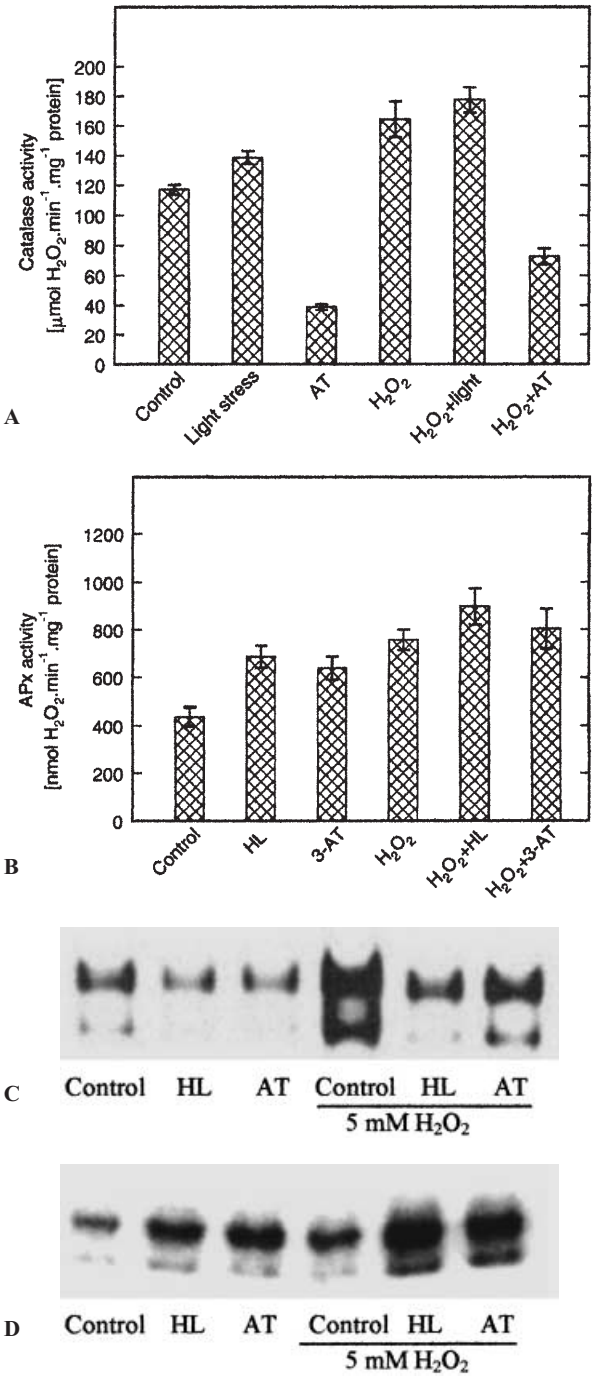


Figure 3. $H_2O_2$ induces a set of antioxidant enzymes. Antioxidant enzymes catalase (*A*), APx (*B*), guaiacol peroxidases (*C*), and GPx (*D*) were studied in 4-week-old tobacco plants that were not treated or were pretreated with 5 mM $H_2O_2$ for 1 day and then subjected to 1 day oxidative stress generated by either high light intensities (HL) or aminotriazole (AT). Catalase and APx activities were determined photometrically, guaiacol peroxidases were separated on 10% native PAGE and then stained with guaiacol, and GPx was determined immunologically by Western blotting with a polyclonal antibody raised against GPx. For catalase and APx, data are means ± SE; n = 3.

Stress treatment either by high light or AT increased APx activity between 1.5- and 2-fold (fig. 3B). A 2-fold increase in APx activity was observed when plants were treated with $H_2O_2$. Subsequent exposure of the $H_2O_2$-treated plants to high light or AT stress elevated APx activities even further. Thus, plants pretreated with $H_2O_2$ and exposed to the two stresses possessed higher APx activities compared with the $H_2O_2$-untreated plants exposed to the same stress factors.

In contrast with APx, guaiacol peroxidases were slightly inhibited by high light and AT (fig. 3C). $H_2O_2$ treatment elevated guaiacol peroxidase activities more than 3-fold. Although to a lesser extent, guaiacol peroxidase activity

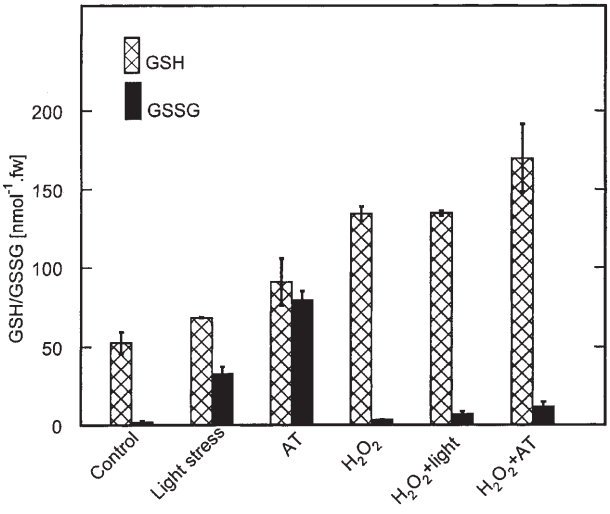


Figure 4. Stimulation of glutathione synthesis by $H_2O_2$ and oxidative stress. Glutathione content was determined in tobacco plants that were either pretreated or not treated with $H_2O_2$ and then exposed to oxidative stress generated by high light or AT. Hatched bars, reduced glutathione (GSH); black bars, oxidized glutathione (GSSG). Data are means ± SE; n = 2.

was also elevated in $H_2O_2$-treated plants subjected to high light or AT stress.

Both stress treatments and $H_2O_2$ application elevated GPx protein levels (fig. 3D). The induction of GPx was particularly strong in the $H_2O_2$-pretreated plants subjected to high light or AT stress.

Stimulation of glutathione synthesis was observed both as a response to the stress treatments and $H_2O_2$ application (fig. 4). However, the accumulation of GSH was much more pronounced in plants pretreated with $H_2O_2$. Moreover, high light and AT treatment resulted in the accumulation of oxidized glutathione (GSSG), while plants pretreated with $H_2O_2$ and exposed to the same stress factors did not accumulate any significant amounts of GSSG.

## Discussion

Testing the effects of different $H_2O_2$ concentrations clearly demonstrated that a relatively moderate concentration of 5 mM is the most effective in inducing the antioxidant enzymes in our system, while higher concentrations may have the opposite effect. This is in agreement with the general belief that $H_2O_2$ is non-toxic or even beneficial at moderate concentrations but toxic at high concentrations [23, 24]. At the same time, we showed that the concentrations that can cause oxidative stress in tobacco are much higher than those causing oxidative stress in *Arabidopsis* [25] or cell death in soybean [26]. This is most likely to be a consequence of the application method rather than the plant species. The advantage of exogenous application by spraying is that plants stay intact and are not mechanically wounded, thus eliminating the difficulties interpreting results obtained from wounded plants [27, 28].

Our first aim was to investigate whether sublethal doses of $H_2O_2$ can exert a protective effect against different oxidative stress-generating factors in this tobacco system. Such an idea is supported by recent evidence that $H_2O_2$ as a signal molecule plays a key role in mediating the plant response to many abiotic and biotic stress factors [8, 9, 29]. The choice of high light was based on the fact that it is one of the most common natural sources of oxidative stress and is therefore of significant biological importance. Not only can high light intensities cause oxidative stress on their own [15] but they are especially damaging in combination with other stress factors like extreme temperatures and an excess or deficiency of mineral elements [30–32]. Our second stress factor of choice was the catalase-specific inhibitor AT, a useful model system to study the effects of oxidative stress. Under illumination, the increased production of $H_2O_2$ in peroxisomes cannot be eliminated due to the low catalase activity and the excess $H_2O_2$ migrates into other cell compartments causing oxidative stress [21].

Accumulation of malondialdehyde, a decomposition product of the lipid hydroperoxides, is a good indicator for the severity of cell injury during oxidative stress [18] and has been successfully used to follow both high light- and AT-generated oxidative stress damage [17]. Although both high light and AT stress increased lipid peroxidation in our experiments, the more significant increase caused by AT treatment suggests a severer oxidative stress in these plants. Indeed, extending the stress period from 1 to 7 days evoked necrotic lesions on the leaves of AT-stressed plants and eventual death of those plants while no major damage or death was observed among the plants exposed to high light. $H_2O_2$ pretreatment not only prevented any increase in lipid peroxidation but AT-dependent leaf damage was also avoided, thus demonstrating fully the protective role of $H_2O_2$.

An increased capacity of the antioxidant system is one of the possible mechanisms responsible for oxidative stress tolerance as demonstrated by the existence of stress-resistant lines with naturally enhanced antioxidant systems [4] or the properties of transgenic plants overexpressing particular antioxidant enzymes [5, 33]. Direct evidence has recently been provided for the role of $H_2O_2$ in inducing stress-related promoters and genes [15, 34, 35].

Our second aim was to study the components of the plant antioxidant system that are possibly involved in the induced tolerance against high light and AT stress. A number of experiments with transgenic plants demonstrated that APx and catalase, two of the major antioxidant enzymes, play a crucial role in plant defense against the two stress factors [33, 36]. In our experiments, high light in-

creased the activities of both enzymes slightly, in agreement with the induction of APx by light observed by other authors [15, 37]. These increases are, however, not sufficient to prevent the light-dependent lipid peroxidation. Only when these two enzymes were induced to much higher levels by $H_2O_2$ pretreatment was subsequent light-dependent lipid peroxidation prevented.

Catalase seems to play a more general role in stress tolerance because it can function as a cellular sink for $H_2O_2$ [38]. This can explain the fact that a catalase deficiency, caused by the AT treatment, leads to severe oxidative stress despite the fact that APx activity in these plants was elevated. Elevation of APx activity in AT-treated plants may be a consequence of a rise in endogenous $H_2O_2$ levels [39]. The severity of the AT-generated oxidative stress was apparent from the significant lipid peroxidation and leaf necrosis after prolonged stress application. Again, pretreatment with $H_2O_2$ elevated APx activity further and minimized AT-dependent catalase inhibition, thus playing an important role in preventing the AT-induced damage.

$H_2O_2$-elevated levels of guaiacol peroxidases are also likely to contribute significantly to the $H_2O_2$-induced stress tolerance. Guaiacol peroxidases are a heterogeneous group and participate in different physiological processes, such as biosynthesis of lignin, plant development and organogenesis, senescence, and responses to wounding and pathogens [40, 41]. They also consume $H_2O_2$, thus minimizing its accumulation in the plant cell. Moreover, some isoforms of guaiacol peroxidases are directly implicated in plant defense against ROS [12].

Our observations that GPx protein levels increase both under high light and AT stress are in agreement with the responsiveness of GPx to various oxidative stress-generating factors [39, 42]. We also demonstrated that pretreatment with $H_2O_2$ further elevates GPx levels. As elevated levels of GPx have been shown to protect tobacco from oxidative stress generated by salt or chilling [43], the elevation of GPx levels by $H_2O_2$ may also contribute to the stress tolerance against light and AT in our plants.

As a general antioxidant and substrate for glutathione reductase and GPx, glutathione is a crucial part of the cell antioxidant system. Its depletion therefore leads to severe oxidative stress, while elevated levels are thought to contribute to stress tolerance [41]. The elevation of the glutathione pool in our experiments is in accordance with the consensus that an increase in total glutathione is a general response to various abiotic stress factors, including light and AT stress [44, 45]. Such glutathione elevation is executed mainly by posttranscriptional activation of the key enzyme in glutathione synthesis, $\gamma$-glutamylcysteine synthase [46]. However, the increased lipid peroxidation and accumulation of GSSG in the $H_2O_2$-untreated plants subjected to high light or AT stress demonstrates that elevated glutathione levels per se are not sufficient to provide protection against the two stresses. On the other hand, glutathione levels in the $H_2O_2$-pretreated oxidative stress-tolerant plants were elevated even higher, which suggests that plants with larger glutathione pools are better equipped to subsequently encounter oxidative stress.

The results presented here show how a brief application of moderate doses of $H_2O_2$ can significantly enhance oxidative stress tolerance by elevating the antioxidant status of the plant cell. Such a modulation of the plant antioxidant system may be useful in protecting plants against adverse factors that cause oxidative stress.


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