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Oxidative Signals in Tobacco Increase Cytosolic Calcium

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Tobacco (*Nicotiana plumbaginifolia*) seedlings genetically transformed to express apoaequorin were incubated in *h*-coelenterazine to reconstitute the calcium-sensitive luminescent protein aequorin. Treatment of these seedlings with hydrogen peroxide resulted in a transient burst of calcium-dependent luminescence lasting several minutes. Even though the hydrogen peroxide stimulus was persistent, the change in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) was transient, suggesting the presence of a refractory period. When seedlings were pretreated with hydrogen peroxide, there was no increase in $[Ca^{2+}]_{cyt}$ upon a second application, which confirmed the refractory character of the response. Only when the two treatments were separated by 4 to 8 hr was full responsiveness recovered. However, treatment with hydrogen peroxide did not inhibit mobilization of $[Ca^{2+}]_{cyt}$ induced by either cold shock or touching, suggesting that these three signals mobilize different pools of intracellular calcium. To examine whether $[Ca^{2+}]_{cyt}$ is regulated by the redox state of the cytoplasm, we pretreated seedlings with buthionine sulfoximine (to modify cellular glutathione levels) and inhibitors of ascorbate peroxidase. These inhibitors modify the hydrogen peroxide–induced transients in $[Ca^{2+}]_{cyt}$, which is consistent with their effects on the cellular prooxidant/antioxidant ratio. Treatment with hydrogen peroxide that elicited $[Ca^{2+}]_{cyt}$ increases also brought about a reduction in superoxide dismutase enzyme activity. This reduction could be reversed by treatment with the calcium channel blocker lanthanum. This indicates that there is a role for calcium in plant responses to oxidative stress.

INTRODUCTION

Chemical activation of molecular oxygen as a result of partial reduction or photosensitization has shaped the biochemistry of life since oxygen evolution began. The involvement of oxygen in normal respiratory processes and the production of oxygen during photosynthesis results in the generation of superoxides, hydrogen peroxide, hydroxyl radicals, and other free radicals that react with and damage DNA, proteins, and lipids (Bowler et al., 1992). This phenomenon is described as oxidative stress, and complex protective mechanisms have been evolved by plants (and other organisms) to mitigate and repair the damage initiated by free radicals. The primary constituents of these protective mechanisms include enzymes such as superoxide dismutase (SOD), catalases and peroxidases, and free radical scavengers, such as carotenoids, ascorbate, tocopherols, and oxidized and reduced glutathione (GSSG and GSH, respectively). Enzymes of scavenger metabolism, such as ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase that together form the Halliwell-Asada pathway, are shown in Figure 1 and also contribute (Halliwell and Gutteridge, 1985).

In green plants, these protective mechanisms play important roles in resistance to environmental stress. This is because many conditions that limit productivity result in the enhanced production of activated oxygen species either directly (for example, from herbicide treatment, UV radiation, ozone treatment, and transition metal toxicity) or indirectly (for example, drought, chilling, and senescence) (Elstner, 1982; Bowler et al., 1992; Fryer, 1992). An important feature of these protective mechanisms is that their activity is enhanced when plant cells are exposed to conditions increasing free radical production. Cellular activities of SOD, for example, can increase by up to an order of magnitude during chilling, anaerobiosis, fungal invasion, and ozone or SO_2 treatments. Glutathione reductase and the levels of low molecular weight protectants may also increase (Bowler et al., 1992). During senescence, SOD levels may decline allowing enhanced free radical production and thus accelerating the death of plant tissues.

The mechanism whereby plant cells are able to perceive increased free radical production and compensate by increasing the level of oxidative protection (or the converse) is not understood. Special significance, however, is placed on the so-called prooxidant/antioxidant ratio. The primary prooxidant constituents are dehydroascorbic acid, quinones, and GSSG, and the antioxidants are the equivalent reduced forms. The prooxidant/antioxidant ratio is an indicator of the redox state of the cytoplasm. In animal cells, the ratio of GSH to GSSG

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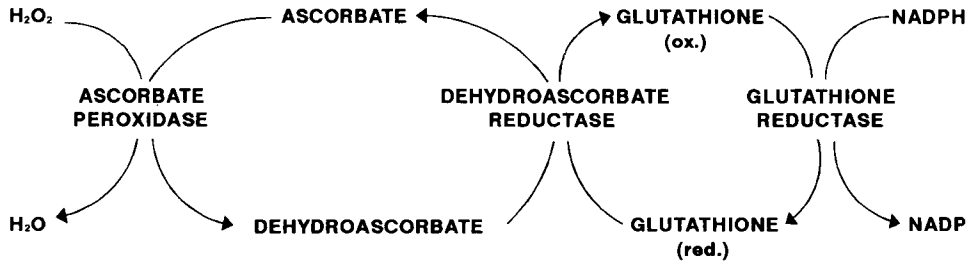

Figure 1. The Halliwell-Asada Enzyme Pathway.

Pathway involving enzymes of scavenger metabolism, such as ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase, that together form the Halliwell-Asada pathway (adapted from Bowler et al., 1992). Ox., oxidation; red., reduction.

is believed to be pivotal to cellular responses (Nicotera et al., 1991, 1992). The GSH/GSSG ratio determines the levels of protein thiols and can modify cytosolic calcium homeostasis via oxidative changes in sensitive thiols of Ca^{2+}-ATPases. During severe oxidative stress, the disruption of cytosolic calcium homeostasis is probably a critical step in the injury of animal cells leading to the activation of numerous calcium-dependent degradative enzymes (Nicotera et al., 1985; Tsokos-Kuhn et al., 1988; Orrenius et al., 1989). A more moderate rise in oxidative stress, however, potentiates the protective mechanisms, and it is suggested that changes in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) resulting from enhanced oxygen activation help to mediate the necessary biochemical changes (Nicotera et al., 1991).

There is currently no direct evidence in plant cells that relates oxidative stress or exogenous hydrogen peroxide treatment to $[Ca^{2+}]_{cyt}$, although indirect evidence (Price, 1990) suggests the possibility. Hydrogen peroxide has been speculated to be a second messenger in its own right, and exogenous hydrogen peroxide can initiate plant defense responses, such as phytoalexin production (Apostol et al., 1989). The addition of exogenous hydrogen peroxide can also mimic the chilling-induced oxidative stress response in maize and stimulate the expression of catalase and peroxidase (Prasad et al., 1994). We have recently described the production of genetically transformed plants expressing apoaequorin (Knight et al., 1991, 1992, 1993). When these plants were incubated in the luminophore coelenterazine, the calcium-dependent photoprotein aequorin was reconstituted. The resultant luminous plants have luminosity directly reporting $[Ca^{2+}]_{cyt}$. Use of this simple method greatly eases the measurement of $[Ca^{2+}]_{cyt}$ and has enabled us to investigate the effects of hydrogen peroxide treatment of whole tobacco seedlings.

RESULTS

Effects of Hydrogen Peroxide and Lanthanum Chloride on SOD Enzyme Activity

The effects on SOD enzyme activity resulting from treatment of tobacco seedlings with 10 mM hydrogen peroxide are shown in Figure 2A. Treatment with hydrogen peroxide brought about a 45 and 67% reduction in SOD activity after 1 and 4 hr, respectively. Treatment with hydrogen peroxide for 10 min did not cause a significant inhibition in SOD activity after 1 hr, but 37% inhibition was detected after 4 hr. The fact that no inhibition was seen after 1 hr indicates that it is unlikely that inhibition of SOD activity seen is a result of direct inhibition of SOD by hydrogen peroxide in the reaction assay itself. Also, if this were the case, no increased inhibition would be expected after 4 hr, but this does indeed occur. To see whether intracellular calcium might have a role in inhibiting SOD enzyme activity, we incubated plants with the calcium channel blocker lanthanum (III) chloride along with hydrogen peroxide. Figure 2B shows that treatment with lanthanum reverses the hydrogen peroxide–mediated inhibition of SOD activity. The lower response to hydrogen peroxide alone in this experiment compared to Figure 2A illustrates the differences in response that we observed between different preparations of plants that presumably received different levels of stress during growth. Certainly, plants grown at higher densities showed a lower $[Ca^{2+}]_{cyt}$ response to hydrogen peroxide (S.J. Ripley, A. Taylor, M.R. Knight, and A.J. Trewavas, unpublished results). Lanthanum on its own stimulated SOD activity almost twofold over untreated controls. Because these experiments suggested a role for calcium in mediating cellular responses to hydrogen peroxide, we decided to measure $[Ca^{2+}]_{cyt}$ in plants challenged with hydrogen peroxide.

Hydrogen Peroxide Increases $[Ca^{2+}]_{cyt}$ in Tobacco Seedlings

Seeds of the transformed tobacco line MAQ2.4 expressing apoaequorin (Knight et al., 1991) were germinated on half-strength MS medium (Murashige and Skoog, 1962) in white light (16-hr photoperiod) for 1 to 2 weeks. Reconstitution of active aequorin was accomplished by incubating the seedlings in *h*-coelenterazine (Knight et al., 1993). When single seedlings were placed in a luminometer and immersed in 10 mM hydrogen peroxide at room temperature as shown in Figure 3 (Nicotera et al., 1991), a single spike of calcium-dependent luminescence was observed. This spike followed a definite lag

period, which was observed to vary from 20 to 40 sec in different seedlings. The increase in $[Ca^{2+}]_{cyt}$ was transient, lasting between 1 and 2 min, even though the seedlings remained immersed in the solution of hydrogen peroxide: a persistent stimulus induced a transient response.

The mechanism of calcium-induced light emission from aequorin involves oxidation of the luminophore coelenterazine. To establish that hydrogen peroxide does not directly react with aequorin, thus initiating spurious light emission, the controls shown in Figure 4 and Table 1 were performed. Hydrogen peroxide treatments were performed on MAQ2.4 seedlings incubated in the absence of coelenterazine (Figure 4, trace b)

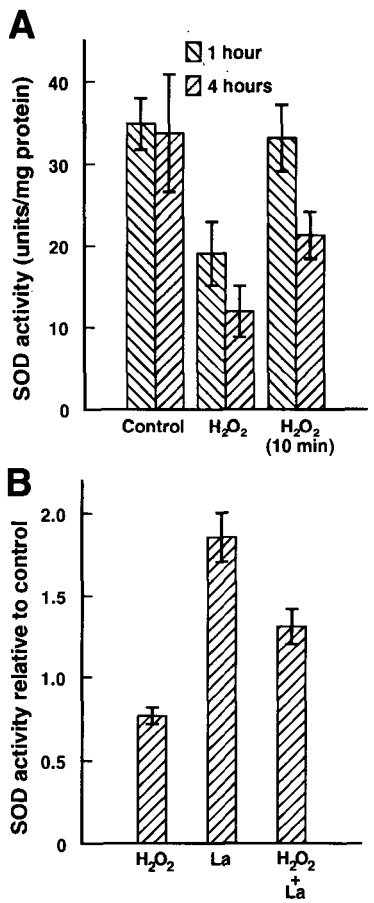

Figure 2. Effect of Hydrogen Peroxide and Lanthanum Chloride on SOD Enzyme Activity.

(A) Tobacco seedlings were treated for 10 min with hydrogen peroxide or continuously with hydrogen peroxide. Seedlings on water were used as a control. Total protein was extracted after 1 and 4 hr. SOD activity was measured as described in Methods and is expressed as units of enzyme per milligram of total protein. For each treatment, $n = 6$. **(B)** Seedlings were treated as given in **(A)** but incubated for 1 hr in either 10 mM hydrogen peroxide, 10 mM lanthanum (III) chloride, or a combination of the two. SOD measurements are as given in **(A)**. SOD activity is expressed as relative to a water control (control = 1). For each treatment, $n = 6$. La, lanthanum. Bars = ± standard error (SE).

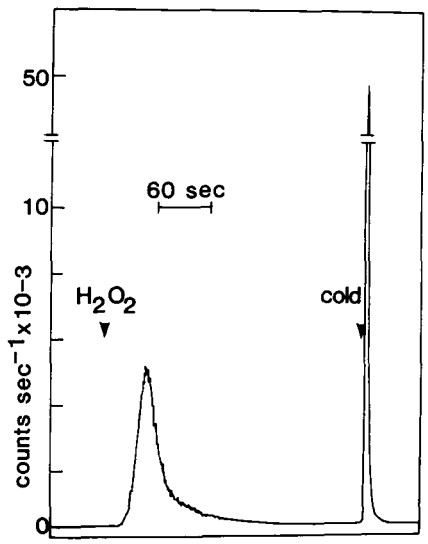

Figure 3. The Hydrogen Peroxide–Induced $[Ca^{2+}]_{cyt}$ Response.

A single transformed (MAQ2.4) tobacco seedling was incubated in 2 μM *h*-coelenterazine for 4 hr. The seedling was then challenged sequentially with 10 mM hydrogen peroxide followed by the addition of ice-cold water as indicated by the arrowheads. Luminescence was recorded graphically as described in Methods.

and on untransformed wild-type tobacco seedlings that were incubated for the requisite period in *h*-coelenterazine (Figure 4, trace c). These traces also show cold shock treatments (Knight et al., 1991), and again no response was elicited. Two-week-old seedlings of MAQ2.4 were also homogenized and aequorin reconstituted in the homogenate in vitro using *h*-coelenterazine (Table 1). The addition of 25 mM $CaCl_2$ to the reconstituted extract gave luminescence values of over 100,000 counts in 10 sec. The addition of 10 mM hydrogen peroxide to aliquots of this extract, in place of $CaCl_2$, produced an average of 253 counts over 10 sec, whereas the addition of hydrogen peroxide to aliquots of the reconstitution mixture minus the plant homogenate gave 242. These results indicate that hydrogen peroxide does not detectably induce light emission from isolated aequorin or *h*-coelenterazine, and the induced light emission from reconstituted aequorin in MAQ2.4 (Figure 3) truly represents an increase in $[Ca^{2+}]_{cyt}$.

After the hydrogen peroxide (10 mM) response had been recorded for 4 min, the seedlings were then immersed in several volumes of ice-cold water to induce the cold shock $[Ca^{2+}]_{cyt}$ response (Knight et al., 1991, 1992). Figure 3 shows that as previously observed, the cold shock response was very rapid and was generally completed within 5 to 10 sec of immersion. Most importantly, the inability of $[Ca^{2+}]_{cyt}$ to respond to further stimulation by hydrogen peroxide did not prevent a normal $[Ca^{2+}]_{cyt}$ response to cold shock. Table 2 contains a summary of the effects of various hydrogen peroxide treatments on subsequent cold shock–induced luminescence. Pretreatment of seedlings with 1 or 10 mM hydrogen peroxide

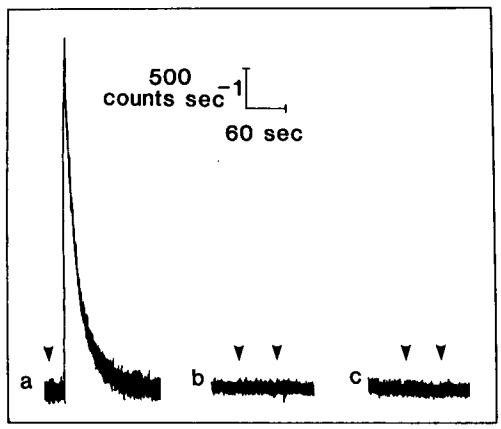

Figure 4. Effect of Hydrogen Peroxide on Aequorin Luminescence in Tobacco Seedlings.

Transformed (MAQ2.4) or untransformed tobacco seedlings were incubated in 2 μM *h*-coelenterazine or in water for 4 hr. The seedlings were then challenged with 10 mM hydrogen peroxide and/or ice-cold water as indicated by the arrowheads: luminescence was recorded graphically as described in Methods. Trace a represents MAQ2.4 seedlings incubated in *h*-coelenterazine and challenged only with hydrogen peroxide. Trace b denotes MAQ2.4 seedlings not incubated in *h*-coelenterazine; hydrogen peroxide is indicated by the first arrowhead and ice-cold water by the second arrowhead. Trace c shows the response of untransformed seedlings incubated for 4 hr in *h*-coelenterazine. This untransformed seedling was treated as given for seedlings represented by trace b.

slightly increased the luminescent light released in response to cold shock, although the difference was small. Because the cold shock response is much more rapid than that of hydrogen peroxide, responses in most experiments were measured numerically rather than estimated from chart recordings. A hydrogen peroxide treatment consumes only ~5% of the reconstituted aequorin in the seedling.

The effect of hydrogen peroxide on $[Ca^{2+}]_{cyt}$ is concentration dependent. Figure 5 illustrates a dose response of hydrogen peroxide on luminescent light emission and shows that treatments with concentrations of hydrogen peroxide as low as 0.05 mM produced detectable responses. Ten experiments were performed to construct these dose–response data, and the traces shown best represent the average size of response for each concentration. The Figure 5 inset shows the average emitted light (plus standard errors) summed over the first minute for these 10 experiments. At the lower concentrations of hydrogen peroxide (0.1 and 0.05 mM), the lag period before a luminescence response commences was increased to 45 to 60 sec.

Seedlings that had been immersed in hydrogen peroxide for periods of 10 min were removed, washed, and placed back on agar containing half-strength MS medium in light or darkness as described previously (Knight et al., 1991). At hydrogen peroxide concentrations of 20 mM or below, there were no discernible effects either on the tissues or on the growth or tropic sensitivity of the seedling in light or dark. With concentrations of 100 mM (a concentration we have not used elsewhere), slight browning of the cotyledons was observed after 24 to 48 hr in the light. Leaving seedlings in solutions of 100 mM hydrogen peroxide in darkness for periods of several days produced no oxidation or bleaching effects.

To identify the source of calcium giving rise to the hydrogen peroxide–induced increases shown in Figures 3 through 5, we pretreated seedlings with a variety of inhibitors. Substantive effects were only obtained with the plasma membrane channel blocker lanthanum chloride (Tester, 1990) and the putative mitochondrial calcium channel blocker ruthenium red (Campbell, 1983). Data were obtained in 10 separate experiments, and the traces shown in Figure 6 best represent the average result. Lanthanum chloride at 1 mM reduced the increase in $[Ca^{2+}]_{cyt}$ to approximately one-half, whereas ruthenium red at 20 μM resulted in almost total abolition of the response. Lanthanum chloride concentrations had to be increased to 10 mM to obtain an equivalent reduction in response to that of ruthenium red (data not shown). From what is understood concerning the sites of action of these two inhibitors, hydrogen peroxide is mobilizing $[Ca^{2+}]_{cyt}$ from both external and internal pools, although the greater sensitivity to ruthenium red might argue for an emphasis on internal sources. However, the sites of action of these two inhibitors in plant cells are not yet well characterized, and conclusions can only be very tentative.

All seedlings subjected to treatments with lanthanum chloride and ruthenium red were given a cold shock treatment, and luminescence was recorded. The data (not shown) merely confirmed observations previously recorded (Knight et al., 1992) concerning these two inhibitors. Lanthanum chloride at 1 mM very substantially inhibited cold shock–induced luminescence, whereas ruthenium red at 20 μM had no detectable effect. This contrast seems to suggest that cold shock and hydrogen peroxide mobilize different pools of $[Ca^{2+}]_{cyt}$.

Table 1. Effect of Hydrogen Peroxide on the Luminescence of Reconstituted Aequorin

Treatment	Counts over 10 Sec
25 mM $CaCl_2$	118,802
10 mM H_2O_2	253
10 mM H_2O_2 with no homogenate	242
Water	106

Five MAQ2.4 seedlings were homogenized, and aequorin was reconstituted as described in Methods. Aliquots (10 μL) of the reconstituted aequorin were treated with $CaCl_2$, hydrogen peroxide, or water. In one case, hydrogen peroxide was added to the reconstitution mixture from which plant homogenate was omitted.

Table 2. Effect of Pretreatment with Hydrogen Peroxide, HU, HA, and BSO on the Cold Shock–Induced Luminescence from Transformed Tobacco Seedlings

Pretreatment	Cold Shock–Induced Luminescence
None	203,679 ± 8,982 (n = 17)
10 mM H_2O_2	252,346 ± 10,106 (n = 23)
1 mM H_2O_2	247,848 ± 19,608 (n = 12)
10 mM HU followed by 10 mM H_2O_2	301,727 ± 30,720 (n = 11)
1 mM HA followed by 10 mM H_2O_2	254,597 ± 53,623 (n = 6)
10 mM BSO followed by 10 mM H_2O_2	241,711 ± 16,186 (n = 8)

One- or two-week-old tobacco seedlings were incubated in 2 μM *h*-coelenterazine to re-form aequorin. Cold shock was induced by irrigating the seedlings with water at 0°C, and luminescence was recorded numerically for 20 sec. Values are given as ± standard error (SE). Seedlings were either pretreated with water or with hydrogen peroxide for 4 min, or they were incubated for 4 hr in 10 mM HU, 1 mM HA, or 10 mM BSO, followed by 10 mM hydrogen peroxide for 4 min before cold shock.

Recovery of $[Ca^{2+}]_{cyt}$ Responsiveness from Hydrogen Peroxide Stimulation

The data in Figures 3 through 6 show that transients in $[Ca^{2+}]_{cyt}$ occur when seedlings are immersed in hydrogen peroxide: a transient response is induced by a persistent stimulus. This implies that a refractory period follows hydrogen peroxide treatment in which no further hydrogen peroxide stimulation of $[Ca^{2+}]_{cyt}$ can be elicited. However, the $[Ca^{2+}]_{cyt}$ homeostasis system is evidently not globally modified by hydrogen peroxide because cold shock–induced luminescence can be elicited a few minutes after treatment with hydrogen peroxide (Figure 3 and Table 2).

The presence of a refractory period was confirmed in experiments summarized in Figure 7. Seedlings were challenged with an initial stimulus of 10 mM hydrogen peroxide, and the luminescence response was recorded. The seedlings were then removed from the luminometer and incubated on distilled water (to wash out cell wall hydrogen peroxide) for periods up to 8 hr before being rechallenged with 10 mM hydrogen peroxide. In both cases, luminescence was recorded numerically over a 4-min period, and chart traces were used to assess $[Ca^{2+}]_{cyt}$ kinetics. Figure 7 shows traces of repetitive treatment with hydrogen peroxide within 0.5 and 4 hr of the original stimulus; treatment was then followed by cold shock. After a 0.5-hr recovery from hydrogen peroxide, the touch response was not abated. The remaining data are summarized in the inset to Figure 7, and recovery is expressed as a percentage of the original response using numerical estimation of the luminescence over a 4-min period. The data indicate that complete recovery of responsiveness required 4 to 8 hr of incubation and that recovery starts within half an hour. At the end of each assay, seedlings were cold shocked (Figure 7), and luminescence was recorded, but equivalent changes or recovery in cold shock response was not observed.

Effects of Inhibitors of Glutathione Synthesis and Ascorbate Peroxidase on the Hydrogen Peroxide–Induced Increase in $[Ca^{2+}]_{cyt}$

Is the hydrogen peroxide–induced increase in $[Ca^{2+}]_{cyt}$ the result of a direct cellular perception of hydrogen peroxide concentrations or the result of changes in the prooxidant/antioxidant ratio of the cytoplasm? To distinguish between these two possibilities, we conducted experiments in which seedlings were pretreated with (1) buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis that can reduce cellular glutathione contents by up to 80% (Griffith and Meister, 1979; Hell and Bergmann, 1990), and (2) inhibitors of ascorbate peroxidase (hydroxyurea [HU], hydroxylamine [HA], and 4-aminophenol [AP]; Chen and Asada, 1989). Ascorbate peroxidase is the first member of the Halliwell-Asada pathway (Figure 1); it degrades hydrogen peroxide using NADPH (Bowler et al., 1992). The two remaining enzymes in this pathway, dehydroascorbate reductase and glutathione reductase, involve glutathione directly as a redox couple.

Both groups of inhibitors should similarly retard the rate at which hydrogen peroxide is metabolized through the Halliwell-Asada pathway, ensuring that cellular hydrogen peroxide concentrations are maintained for longer after the plants are challenged with external hydrogen peroxide. But both groups of inhibitors should affect the prooxidant/antioxidant ratio in opposite ways. A lower cellular glutathione content, induced

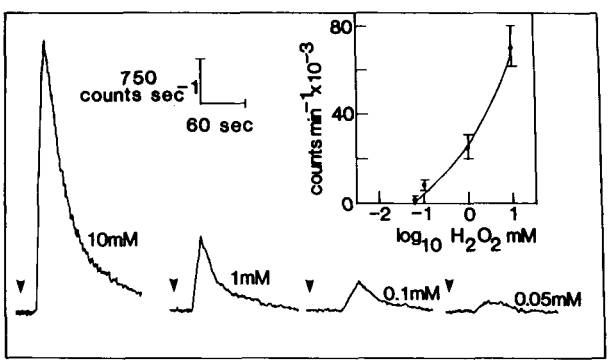

Figure 5. Effect of Different Hydrogen Peroxide Concentrations on the Luminescence of Reconstituted Aequorin in Transformed Tobacco Seedlings.

Hydrogen peroxide (10, 1, 0.1, and 0.05 mM) was added to tobacco seedlings containing reconstituted aequorin as indicated by the arrowheads, and calcium-dependent luminescence was recorded. The inset shows the accumulated luminescence counts (means plus standard error from 10 experiments) for the first minute in which hydrogen peroxide stimulation was observed.

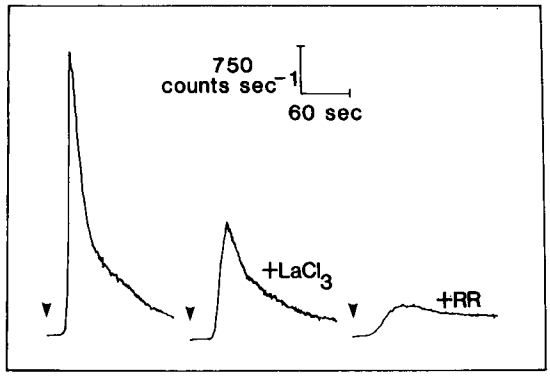

Figure 6. Effect of Lanthanum Chloride and Ruthenium Red on Hydrogen Peroxide–Induced Luminescence of Transformed Tobacco Seedlings Containing Reconstituted Aequorin.

Transformed tobacco seedlings containing reconstituted aequorin were incubated in the presence (+) or absence of 1 mM lanthanum (III) chloride ($LaCl_3$) or 20 μM ruthenium red (RR) for 2 hr before being challenged with 10 mM hydrogen peroxide as shown by the arrowheads. The traces were selected as being best representative of the means from 10 different experiments.

by BSO, should diminish the apparent activities of the last two enzymes in the Halliwell-Asada pathway. The rate of accumulation of ascorbic acid should be decreased, and the prooxidant/antioxidant ratio achieved should be higher than uninhibited controls. In contrast, inhibition of ascorbate peroxidase should increase the rate at which dehydroascorbic acid and GSH accumulate, and the prooxidant/antioxidant ratio achieved should be lower than uninhibited controls. The data on $[Ca^{2+}]_{cyt}$ obtained with these inhibitors are summarized in Figures 8 and 9, and appropriate cold shock data are presented in Table 2.

Seedlings that had been incubated in 10 mM BSO were challenged with 10 mM hydrogen peroxide, and calcium-dependent luminescence was recorded. Figure 8 summarizes the data from 11 separate experiments, and the traces were selected as being representative of the mean change. The luminescence response was higher (mean total increase in luminescence is ~60%) in seedlings incubated in BSO, but the kinetics of the hydrogen peroxide–induced transient, including the lag period, were largely unaltered. In contrast, the cold shock response was unchanged by preincubation in BSO and hydrogen peroxide (Table 2).

Figure 9A shows that the preincubation of seedlings in 1 mM HU and 0.1 mM HA altered the kinetics of $[Ca^{2+}]_{cyt}$ response to hydrogen peroxide; the increase in $[Ca^{2+}]_{cyt}$ was slower, the peak was lower, and the increased luminescence continued for a much longer period. Figure 9B shows data for higher concentrations of HU (10 mM) and HA (1 mM). The peak is lower again and the response further broadened, with increased luminescence continuing for well over 15 min. Only the first part of the trace is shown in Figure 9B. The lag period was also increased by these higher concentrations of inhibitors. Pretreatment with 1 mM AP completely abolished the response to hydrogen peroxide, but such seedlings retained responses to both touch and cold shock (Figure 9B). We have also observed that seedlings incubated in 10 mM HU and 1 mM HA exhibit a touch response with kinetics similar to the response recorded in Figure 9B. Use of 0.1 mM AP instead of 1 mM produced luminescence changes very similar to those shown with 1 mM HA in Figure 9B (data not shown).

Pretreatment with HU increased subsequent cold shock responses slightly, but HA pretreatment left this response unchanged (Table 2). In three experiments with 1 mM AP, the cold shock luminescence was estimated to be 177,900 counts over the first 20 sec, which is slightly lower than untreated controls (Table 2).

With both groups of inhibitors, the response of $[Ca^{2+}]_{cyt}$ to hydrogen peroxide was similar to the predicted changes in the prooxidant/antioxidant ratio. $[Ca^{2+}]_{cyt}$ was increased by prior reduction in glutathione content and decreased by prior ascorbate peroxidase inhibition.

DISCUSSION

The results described in this study suggest that oxidative stress in the form of hydrogen peroxide treatment increases

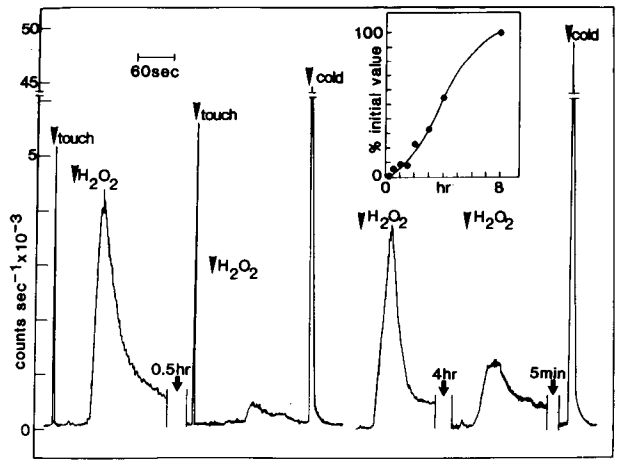

Figure 7. Effect of a Second Challenge with Hydrogen Peroxide on the Luminescence of Transformed Tobacco Seedlings Containing Reconstituted Aequorin.

Tobacco seedlings containing reconstituted aequorin were challenged with 10 mM hydrogen peroxide. Luminescence was chart recorded for 4 min. The seedlings were then removed and incubated on water for the times indicated before being rechallenged with 10 mM hydrogen peroxide. Luminescence response to a second challenge with hydrogen peroxide was recorded numerically and expressed as a percentage of the first challenge (shown as inset).

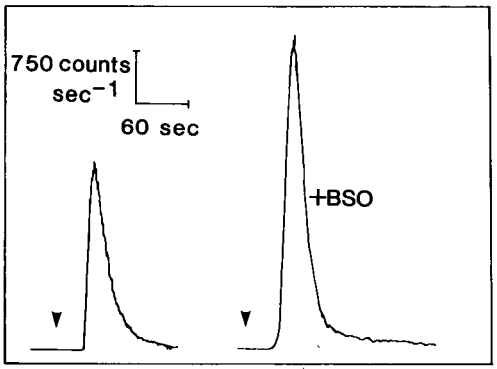

Figure 8. Effect of BSO on Hydrogen Peroxide–Induced Luminescence from Transformed Tobacco Seedlings Containing Reconstituted Aequorin.

Seedlings containing reconstituted aequorin were incubated in the presence (+) or absence of 10 mM BSO for 4 hr before being challenged with 10 mM hydrogen peroxide as indicated by the arrowheads.

$[Ca^{2+}]_{cyt}$. As far as we are aware, the linking of this type of signal directly to changes in $[Ca^{2+}]_{cyt}$ in plant cells is a novel observation. Preliminary imaging data from whole tobacco seedlings (M.R. Knight, N.J. Watkins, and A.K. Campbell, unpublished data) indicate that the hydrogen peroxide–induced $[Ca^{2+}]_{cyt}$ elevations seen are relatively global and occur in most tissues in a manner similar to cold shock (Knight et al., 1993). Although hydrogen peroxide is a chemically reactive compound and oxidative stress causes increases in ultraweak chemiluminescence in plants (Abeles, 1986, 1987), we were unable to obtain any evidence that direct chemical oxidation caused luminescent light emission under our experimental conditions (Figure 4 and Table 1). The effects of known inhibitors of calcium homeostasis (Figure 6) on the hydrogen peroxide–induced luminescence transients certainly support the hypothesis that oxidative stress increases $[Ca^{2+}]_{cyt}$. Hydrogen peroxide–mediated $[Ca^{2+}]_{cyt}$ elevation occurs after a lag period. The size of this lag period seems to be dependent on hydrogen peroxide concentration (Figure 5). This suggests that the lag period is a property of the accessibility of hydrogen peroxide to cells. However, the fact that inhibitors also alter the length of the lag period (Figure 9) suggests that the mechanism involved is perhaps not that simple.

Calibration of $[Ca^{2+}]_{cyt}$ using recombinant aequorin requires knowledge of how much aequorin has been expressed, reconstituted, and consumed in cells (Allen and Blinks, 1978; Blinks et al., 1978). There is currently no reliable way to make these estimates. One solution to this problem is the use of a ratiometric aequorin method (Knight et al., 1993). However, this method relies on dual-wavelength luminometry using narrow bandwidth selective light filters, and the hydrogen peroxide $[Ca^{2+}]_{cyt}$ response produces too low a luminescence signal to make these measurements technically possible. However, we know that the response of $[Ca^{2+}]_{cyt}$ to hydrogen peroxide is approximately an order of magnitude lower than that of cold shock. Because imaging indicates that the luminescence in response to both these stimuli is occurring globally in most of the plant, we can estimate that hydrogen peroxide–induced $[Ca^{2+}]_{cyt}$ elevations are of the order of a few hundred nanomolar concentrations and certainly no higher than 1 μM.

To obtain easily measurable effects of hydrogen peroxide, we had to immerse seedlings in solutions of 1 or 10 mM. These

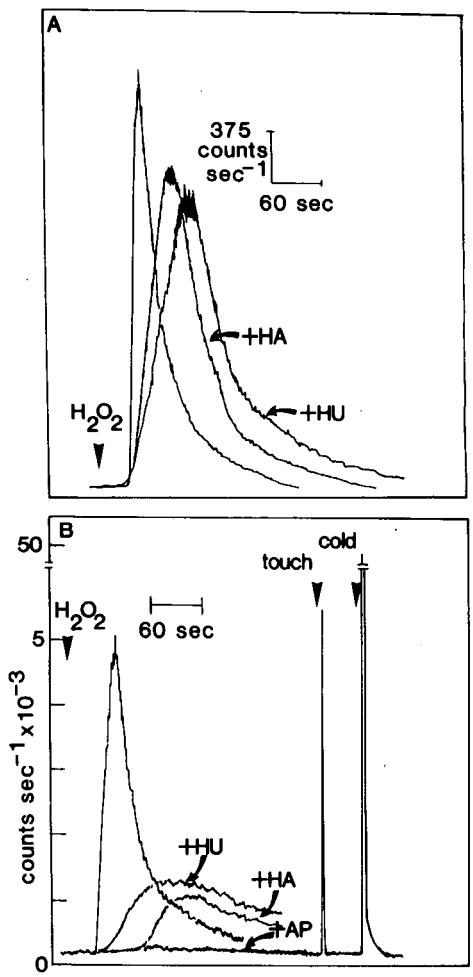

Figure 9. Effect of HU, HA, and AP on the Hydrogen Peroxide–Induced Luminescence from Transformed Tobacco Seedlings Containing Reconstituted Aequorin.

(A) Effects of HU and HA on $[Ca^{2+}]_{cyt}$. Seedlings containing reconstituted aequorin were incubated in the presence (+) or absence of 1 mM HU or 0.1 mM HA for 4 hr before being challenged with 10 mM hydrogen peroxide as indicated by the arrows.

(B) Effects of HU, HA, and AP on $[Ca^{2+}]_{cyt}$. Seedlings containing reconstituted aequorin were incubated in the presence (+) or absence of 10 mM HU, 1 mM HA, or 1 mM AP before being challenged with 10 mM hydrogen peroxide as indicated by the arrows. The AP-treated seedlings were subsequently touch stimulated and finally cold shock treated.

concentrations do not seem to be permanently damaging because seedlings could be left for several days in darkness in 10 mM hydrogen peroxide with no obvious effects on growth or tropic sensitivity. The cuticle and numerous peroxidases in the cell wall may account for some of the resistance to hydrogen peroxide treatments. There is some indication that secretion into or synthesis of hydrogen peroxide in the cell wall may be part of the normal mechanism of resistance to fungal invasion (Bradley et al., 1992). Hydrogen peroxide is produced endogenously in response to chilling, and some of the gene expression changes associated with chilling can be mimicked by adding exogenous hydrogen peroxide (Prasad et al., 1994). Mechanisms for dealing with extracellular hydrogen peroxide would seem, therefore, to be present in plant cells.

Currently, there is little information on the mechanism by which plant cells perceive oxidative stress. Conditions that cause oxidative stress and increase the synthesis of hydrogen peroxide do initiate a counter program of enzyme synthesis designed to sequester free radicals (Bowler et al., 1992). An intracellular perception of hydrogen peroxide in this case is most likely and would be in agreement with our observations on $[Ca^{2+}]_{cyt}$ described here. This suggests that a certain amount of exogenous hydrogen peroxide must penetrate into cells to initiate mobilization of $[Ca^{2+}]_{cyt}$. However, we do not know whether it is hydrogen peroxide or some other component of the redox system that is responsible for some or all of the effects induced by our treatments with hydrogen peroxide. A mobilization of $[Ca^{2+}]_{cyt}$, the ubiquitous second message, could well directly contribute to or help to coordinate the cellular response.

Our data do suggest that a redox control of $[Ca^{2+}]_{cyt}$ exists in plant cells. We used two groups of inhibitors of enzymes concerned with the metabolism of hydrogen peroxide. Seedlings treated with BSO, an inhibitor of glutathione synthesis, were predicted to exhibit an acceleration of the rate of increase of the prooxidant/antioxidant ratio when challenged with hydrogen peroxide. The change in $[Ca^{2+}]_{cyt}$ (Figure 8) paralleled this prediction. Inhibitors of ascorbate peroxidase (HU, HA, and AP) were predicted to produce seedlings with a reduced rate of prooxidant/antioxidant increase when treated with hydrogen peroxide. Again, the changes in $[Ca^{2+}]_{cyt}$ conformed with this prediction (Figure 9). Both groups of inhibitors, it should be noted, should similarly slow the rate of metabolism of hydrogen peroxide. Our data therefore support a redox control of $[Ca^{2+}]_{cyt}$ but do not conform to a hypothesis of direct hydrogen peroxide perception. With seedlings incubated in both BSO and the ascorbate peroxidase inhibitors, a cold shock response on $[Ca^{2+}]_{cyt}$ of approximately normal magnitude could be elicited. HU even increased the total cold shock luminescence slightly. This effect of HU could result from the sensitivity of the thiols of the Ca^{2+}-ATPase to prolonged changes in the redox ratio as described in animal cells (Nicotera et al., 1991) but would not explain the different results obtained with HA and AP.

The response of $[Ca^{2+}]_{cyt}$ to a single persistent challenge of hydrogen peroxide was transient and completed in 1 to 2 min (Figure 3), suggesting that a refractory period follows stimulation. Furthermore, seedlings that were challenged with hydrogen peroxide failed to respond fully to a further stimulation until 4 to 8 hr had passed (Figure 7), confirming the existence of this refractory state. No effect of hydrogen peroxide was observed on cold shock– or touch-induced changes in $[Ca^{2+}]_{cyt}$ homeostasis, suggesting possibly that these two signals mobilize different pools of calcium.

One possible explanation for this refractory period is that there is a strong homeostatic regulation of the prooxidant/antioxidant ratio. Upon oxidative challenge, a rapid compensating increase in the activity of protective enzymes could occur. From the kinetics of the $[Ca^{2+}]_{cyt}$ change described here, any putative change in the activity of these enzymes would have to take place within a few seconds. It is known that the redox state controls chloroplast protein kinase activity (Bennett, 1991), and redox-sensitive protein kinases in the cytoplasm might be responsible for phosphorylating protective enzymes. A prominent feature of the effects of the higher concentrations of the ascorbate peroxidase inhibitors HU and HA (Figure 9B) was not only the much slower rate in the rise of $[Ca^{2+}]_{cyt}$ but a much slower rate of $[Ca^{2+}]_{cyt}$ decline. If those variations directly describe equivalent changes in the prooxidant/antioxidant ratio, then it can be hypothesized that the homeostatic regulation of the prooxidant/antioxidant ratio is determined directly by the ratio itself or the rate of change of the ratio.

We have used two channel blockers to try and deduce the possible intracellular or extracellular sources of $[Ca^{2+}]_{cyt}$ that are mobilized by hydrogen peroxide (Figure 6). Ruthenium red is highly effective at inhibiting hydrogen peroxide–induced changes in $[Ca^{2+}]_{cyt}$, suggesting that most of the mobilized calcium has an intracellular origin perhaps commensurate with sites at which hydrogen peroxide may be sensed. However, lanthanum chloride, a putative plasma membrane channel blocker, also reduces oxidatively induced $[Ca^{2+}]_{cyt}$, although complete inhibition required concentrations as high as 10 mM. It is possible that at a concentration of 10 mM, lanthanides may penetrate the plasma membrane (Quiquampoix et al., 1990), resulting in ambiguity in any interpretation. We must again emphasize that uncertainties in identifying the cellular site of action of these two inhibitors mean that conclusions must be equally circumspect.

As well as elevations in $[Ca^{2+}]_{cyt}$, we also observed inhibition of SOD activity in plants treated with hydrogen peroxide (Figure 2A). A 10-min treatment with 10 mM hydrogen peroxide was sufficient to result in a significant reduction in SOD activity after 4 hr. The fact that this hydrogen peroxide–induced inhibition of SOD activity was reversed by treatment with lanthanum (Figure 2B) suggests that the rise in $[Ca^{2+}]_{cyt}$ mediated by hydrogen peroxide is the causal factor for this inhibition. Indeed, lanthanum on its own causes an increase in SOD activity. Assuming that lanthanum is interfering with the constant cycling of calcium through the plasma membrane, which occurs even when cells are at rest, then the elevations in SOD activity seen could be a result of the prevention of the calcium-

mediated control of SOD, which might act to maintain the enzyme activity at normal levels in nonstressed plants. The fact that plants need to and can sense how much hydrogen peroxide is present in cells is indicated by the fact that they use hydrogen peroxide for defense (Apostol et al., 1989) and respond rapidly at the molecular level to exogenous hydrogen peroxide (Prasad et al., 1994). The data presented in this study regarding SOD inhibition are another of such changes in response to exogenously applied hydrogen peroxide. If hydrogen peroxide levels are to be maintained at regulated levels, then SOD activity needs to be tightly coupled to cellular hydrogen peroxide levels because this enzyme is responsible for its synthesis. The simplest scheme of events would involve a system of product inhibition of the enzyme working through the prooxidant/antioxidant ratio and $[Ca^{2+}]_{cyt}$ (Figure 10).

We do not as yet know how $[Ca^{2+}]_{cyt}$ mediates the inhibition of SOD activity and whether it does so directly or indirectly, but either phosphorylation by calcium-dependent protein kinases or the action of specific calcium-dependent proteases are possibilities. Work is currently in progress to help us to understand the exact mechanism(s) involved.

METHODS

Plant Materials and Growth Conditions

Genetically transformed tobacco (*Nicotiana plumbaginifolia* line MAQ2.4) plants expressing apoaequorin (Knight et al., 1991) were used. Plants were grown on half-strength MS medium (Murashige and Skoog, 1962), 0.8% agar at 25°C, with a 16-hr photoperiod and were generally used when 2 weeks old, after which time the first two primary leaves had just emerged.

Determination of Superoxide Dismutase Activity

Seedlings were grown as described above until the primary leaves had fully emerged. They were then floated on distilled water and maintained at 25°C in the dark. Hydrogen peroxide and lanthanum (III) chloride were added to 10 mM, and incubations continued in the dark. After 1 or 4 hr, seedlings were washed in distilled water prior to protein extractions (see below). In the case of the 10-min incubation with hydrogen peroxide, seedlings were washed in distilled water and incubated in water until extractions were performed at 1 and 4 hr. For each replicate experiment, seedlings were homogenized in 0.1 mM EDTA, 20% polyvinylpolypyrrolidone, 50 mM Tris-HCl, pH 7.6. The soluble fraction was obtained as the supernatant after centrifugation at 13,000*g* for 10 min. Protein concentration was determined colorimetrically (Bramhall et al., 1969). Superoxide dismutase (SOD) activity was calculated from the inhibition of the reduction of 0.1 mM nitro blue tetrazolium (monitored at 560 nm) by superoxide, which was generated by 0.05 units of xanthine oxidase in the presence of 0.2 mM xanthine in 50 mM potassium phosphate, pH 7.8.

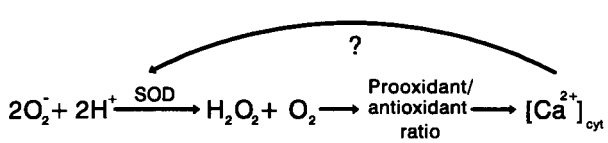

Figure 10. Model for Product Inhibition of SOD Mediated by $[Ca^{2+}]_{cyt}$.

The question mark denotes the unknown mechanism by which $[Ca^{2+}]_{cyt}$ regulates SOD activity.

Reconstitution of Aequorin

Reconstitution of the calcium-sensitive photoprotein aequorin was performed in vivo by floating seedlings (or the excised first leaves) on water containing 2 μM *h*-coelenterazine (Knight et al., 1993) at 16°C for 4 or 6 hr. The use of the more sensitive *h*-coelenterazine enabled lower hydrogen peroxide concentrations to be used for experimental treatments.

Aequorin was reconstituted in vitro by grinding five seedlings in water and clarifying by centrifugation (13,000*g*, 10 min). Supernatant (50 μL) was added to 50 μL of 0.5 M NaCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 0.1% gelatin (w/v), and 10 mM Tris-HCl, pH 7.4. To this mixture, *h*-coelenterazine was added (final concentration 2 μM), and the mixture was left in darkness for 2 hr.

Luminescence Measurements

Luminescence measurements were made using a digital chemiluminometer with a photomultiplier, model 9757 AM (THORN EMI Electron Tubes Limited, Ruislip, Middlesex, U.K.) at 1 kV with a discriminator (Campbell, 1988). Seedlings were floated on 300 μL of distilled water in a cuvette in the luminometer, and background readings were taken until luminescence had stabilized from touch stimulation (generally 1 to 2 min). Hydrogen peroxide was added from a syringe through a luminometer port, and luminescence counts were recorded every 10 sec for at least 4 min with continuous traces being made on a chart recorder attached to the luminometer. The seedlings were then irrigated with distilled water at 0°C to induce cold shock or touch stimulated as described previously (Knight et al., 1991). For estimation of aequorin reconstituted in vitro, 10 μL of the reconstitution mixture described above was added to 500 μL of 200 mM Tris-HCl, 0.5 mM EDTA, pH 7.0, in a luminometer cuvette. After placing the cuvette in the luminometer, the aequorin was discharged by the addition of 500 μL of 50 mM calcium (II) chloride, and luminescence counts were measured over 10 sec.

Inhibitor Studies

When the inhibitors lanthanum chloride, ruthenium red, hydroxyurea (HU), hydroxylamine (HA), or buthionine sulfoximine (BSO) were used, aequorin reconstitution was first performed as described above, and then the seedlings were floated on solutions of the inhibitor in darkness for 2 hr (lanthanum chloride and ruthenium red) or for 4 hr for the others. After this time, the plants were washed in distilled water before stimulation with hydrogen peroxide.

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REFERENCES

Abeles, F.B. (1986). Plant chemiluminescence. Annu. Rev. Plant Physiol. **37,** 49–72.

Abeles, F.B. (1987). Plant chemiluminescence: An overview. Physiol. Plant. **71,** 127–130.

Allen, D.G., and Blinks, J.R. (1978). Calcium transients in aequorin injected from cardiac muscle. Nature **273,** 509–513.

Apostol, I., Heinstein, P.F., and Low, P.S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol. **90,** 109–116.

Bennett, J. (1991). Protein phosphorylation in green plant chloroplasts. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 281–311.

Blinks, J.R., Mattingly, P.H., Jewell, B.R., Van Leeuwen, M., Harrer, G.C., and Allen, D.G. (1978). Practical aspects of the use of aequorin as a calcium indicator: Assay, preparation, microinjection, and interpretation of signals. Methods Enzymol. **57,** 292–328.

Bowler, C., Van Montagu, M., and Inzé, D. (1992). Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43,** 83–116.

Bradley, D.J., Kjellbohm, P., and Lamb, C.J. (1992). Elicitor-induced and wound-induced oxidative cross-linking of a proline rich plant-cell wall protein; a novel, rapid defense response. Cell **70,** 21–30.

Bramhall, S., Noak, N., Wu, M., and Loewenberg, J.R. (1969). A simple colorimetric method for determination of protein. Anal. Biochem. **31,** 146–148.

Campbell, A.K. (1983). Intracellular Calcium: Its Universal Role as Regulator. (Chicester, UK: Wiley).

Campbell, A.K. (1988). Chemiluminescence: Principles and Applications in Biology and Medicine. (Chicester, UK: Horwood/VCH).

Chen, G.X., and Asada, K. (1989). Ascorbate peroxidase in tea leaves—Occurrence of 2 isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol. **30,** 987–998.

Elstner, E.F. (1982). Oxygen activation and oxygen toxicity. Annu. Rev. Plant Physiol. **33,** 73–96.

Fryer, M.J. (1992). The antioxidant effect of thylakoid vitamin-E (α-tocopherol). Plant Cell Environ. **15,** 381–392.

Griffith, O.W., and Meister, A. (1979). Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). J. Biol. Chem. **254,** 7558–7560.

Halliwell, B., and Gutteridge, J.M.C. (1985). Free Radicals in Biology and Medicine. (Oxford, UK: Oxford University Press).

Hell, R., and Bergmann, L. (1990). γ-Glutamylcysteine synthetase in higher plants—Catalytic properties and subcellular-localisation. Planta **180,** 603–612.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold shock and elicitors on cytoplasmic calcium. Nature **352,** 524–526.

Knight, M.R., Smith, S.M., and Trewavas, A.J. (1992). Wind-induced plant motion immediately increases cytosolic calcium. Proc. Natl. Acad. Sci. USA **89,** 4967–4971.

Knight, M.R., Read, N.D., Campbell, A.K., and Trewavas, A.J. (1993). Imaging calcium dynamics in living plants using semisynthetic recombinant aequorins. J. Cell Biol. **121,** 83–90.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Nicotera, P., Moore, M., Mirabelli, F., Bellomo, G., and Orrenius, S. (1985). Inhibition of hepatocyte plasma membrane Ca^{2+}-ATPase activity by menadione metabolism and its restoration by thiols. FEBS Lett. **181,** 149–153.

Nicotera, P., Kass, G.E.N., Duddy, S.K., and Orrenius, S. (1991). Calcium and signal transduction in oxidative cell damage. In Calcium, Oxygen Radicals and Cellular Damage, C.J. Duncan, ed (Cambridge, UK: Cambridge University Press), pp. 17–33.

Nicotera, P., Bellomo, G., and Orrenius, S. (1992). Calcium mediated mechanisms in chemically-induced cell death. Annu. Rev. Pharmacol. Toxicol. **32,** 449–470.

Orrenius, S., McConkey, D.J., Bellomo, G., and Nicotera, P. (1989). Role of calcium in toxic cell killing. Trends Pharmacol. Sci. **10,** 281–285.

Prasad, T.K., Anderson, M.D., Martin, B.A., and Stewart, C.R. (1994). Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6,** 65–74.

Price, A.H. (1990). A possible role for calcium in oxidative plant stress. Free Radical Research Communications **10,** 345–349.

Quiquampoix, H., Ratcliffe, R.G., Ratkovic, S., and Vucinic, Z. (1990). ^{1}H and ^{31}P NMR investigation of gadolinium uptake in maize roots. J. Inorg. Biochem. **38,** 265–275.

Tester, M. (1990). Plant ion channels—Whole cell and single channel studies. New Phytol. **114,** 305–340.

Tsokos-Kuhn, J.O., Smith, C.V., Hughes, H., and Mitchell, J.R. (1988). Liver membrane calcium-transport in diquat-induced oxidative stress *in vivo*. Mol. Pharmacol. **34,** 209–214.