Nitric Oxide Acts as an Antioxidant and Delays Programmed Cell Death in Barley Aleurone Layers

Maria Veronica Beligni, Angelika Fath, Paul C. Bethke*, Lorenzo Lamattina, and Russell L. Jones

Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, 7600, Mar del Plata, Argentina (M.V.B., L.L.); and Department of Plant and Microbial Biology, University of California, Berkeley, California 94720–3102 (A.F., P.C.B., R.L.J.)

Nitric oxide (NO) is a freely diffusible, gaseous free radical and an important signaling molecule in animals. In plants, NO influences aspects of growth and development, and can affect plant responses to stress. In some cases, the effects of NO are the result of its interaction with reactive oxygen species (ROS). These interactions can be cytotoxic or protective. Because gibberellin (GA)-induced programmed cell death (PCD) in barley (Hordeum vulgare cv Himalaya) aleurone layers is mediated by ROS, we examined the effects of NO donors on PCD and ROS-metabolizing enzymes in this system. NO donors delay PCD in layers treated with GA, but do not inhibit metabolism in general, or the GA-induced synthesis and secretion of 1-amylase. 1-Amylase secretion is stimulated slightly by NO donors. The effects of NO donors are specific for NO, because they can be blocked completely by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. The antioxidant butylated hydroxy toluene also slowed PCD, and these data support our hypothesis that NO is a protective antioxidant in aleurone cells. The amounts of CAT and SOD, two enzymes that metabolize ROS, are greatly reduced in aleurone layers treated with GA. Treatment with GA in the presence of NO donors delays the loss of CAT and SOD. We speculate that NO may be an endogenous modulator of PCD in barley aleurone cells.

1 Nitric oxide (NO) is an important second messenger in animal cells (Brunori et al., 1999; Chung et al., 2001) and accumulating evidence suggests it is important in plant cells as well (Beligni and Lamattina, 2001; Wendehenne et al., 2001). Exogenous NO affects several aspects of plant growth and development (Ribeiro et al., 1999), and can affect the responses of plants to pathogens (Delledonne et al., 1998; Durner et al., 1998), light (Giba et al., 1998; Beligni and Lamattina, 2000), gravity (Pedroso and Durzan, 2000), and oxidative stress (Beligni and Lamattina, 1999). NO may also be an endogenous maturation and senescence factor in higher plants (Lesch et al., 1998). As a short-lived, readily diffusible free radical, NO can react with a variety of intracellular and extracellular targets. In some cases, these interactions are cytotoxic and result in cell death. In macrophages, thymocytes, and tumor cells, for example, NO has been shown to hasten apoptosis (Cui et al., 1994; Fehsel et al., 1995; Messmer and Bruene, 1996). NO can also paradoxically act as an antioxidant and an antiapoptotic modulator that prevents cell death (Chung et al., 2001). These cytotoxic and protective effects of NO are often concentration dependent (Wink and Mitchell, 1998). When suspension-cultured soybean (Glycine max) cells were infected with the bacterial pathogen Pseudomonas syringae, for example, NO increased in parallel with other reactive oxygen species (ROS) and promoted the hypersensitive response and programmed cell death (PCD; Delledonne et al., 1998). In potato (Solanum tuberosum) leaves infected by the pathogen Phytophthora infestans or treated with ROS-producing herbicides, on the other hand, NO acted as an antioxidant and prevented cell death (Laxalt et al., 1997; Beligni and Lamattina, 1999). More recently, NO was shown to protect wheat (Triticum aestivum) seedlings against oxidative stress resulting from drought (Garcia-Mata and Lamattina, 2001). This protective role of NO may result, in part, from its interaction with lipid hydroperoxyl radicals or highly reactive superoxide, both of which promote lipid peroxidation (Wink and Mitchell, 1998). An additional role in promoting stomatal closure may be equally important (Garcia-Mata and Lamattina, 2001).

Although NO can have many, often disparate, effects on plants, there is little evidence demonstrating that endogenous NO acts as a regulator of plant growth and development or plant responses to stress. It is clear that plants synthesize NO, and the production of NO from NO$_3^-$ in a reaction catalyzed by nitrate reductase (NR) is well characterized (Yamasaki, 2000). Other sources of NO are likely, though controversy exists regarding the synthesis of NO in plants by NO synthase (NOS). Genes encoding NOS have not been identified in the sequenced genomes of plants (Yamasaki, 2000) or their ancestors (Mallick et
Nitric Oxide Delays Aleurone Cell Death

RESULTS

NO Donors Delay PCD in Barley Aleurone Layers

PCD in intact barley aleurone layers is prevented by ABA and stimulated by GA (Bethke et al., 1999; Fath et al., 2001). ABA can also delay death of aleurone cells that have been treated with GA. In this report, we extend our observations on PCD to show that NO can selectively delay the death of GA-treated aleurone cells. To monitor death and viability of barley aleurone cells, aleurone layers were incubated in 5 μM GA or 5 μM ABA for up to 48 h and were then stained with the fluorescent dyes fluorescein diacetate (FDA) and N-(3-tritylammoniumpropyl)-4-(6-[4-(diethylamino) phenyl]hexatrienyl) pyridinium dibromide (FM 4-64) to detect living (green fluorescence) and dead (red fluorescence) cells, respectively (Fig. 1). PCD in GA-treated layers had already begun 24 h after incubation in GA, when approximately 20% of the cells were dead, and was well under way after 36 h in GA, when about 60% of the cells were dead (Fig. 1A). Two days after hormone treatment, 87% of cells in GA-treated aleurone layers were dead (Fig. 1A), but virtually no cells were dead in ABA-treated aleurone layers (Fig. 1D).

We used net O2 uptake as an additional measure of cell viability in barley aleurone layers and as an indicator of overall metabolic activity (Fath et al., 2001). O2 uptake data for layers from the experiment described above (Fig. 1) are presented in Figure 2. O2 consumption changed in parallel with cell viability as measured by FDA and FM 4-64 staining (Fig. 1). Whereas O2 consumption by ABA-treated layers was relatively constant for 48 h, O2 consumption by GA-treated layers decreased to almost zero between 24 and 48 h of incubation (Fig. 2). The rapid decline in O2 consumption by GA-treated layers between 24 and 48 h of incubation occurs at the time when cells are undergoing PCD (Fig. 1A).

We also measured O2 consumption by barley aleurone layers to gauge the effect of the NO donors sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) on cell death. ABA- or GA-treated aleurone layers were incubated for 48 h with NO donors at various concentrations, and relative rates of O2 consumption were determined (Fig. 3). There was virtually no net O2 uptake by aleurone layers incubated in GA for 48 h, but addition of SNP (100 μM) or SNAP (300 μM to 2 mM) resulted in O2 consumption at rates comparable with those for ABA-treated layers (Fig. 3). SNP and SNAP were most effective at maintaining O2 consumption at 100 μM and 1 mM respectively. O2 consumption declined more slowly between 24 and 48 h when layers were incubated in GA plus 100 μM SNP (Fig. 2A) or GA plus 300 μM SNAP (Fig. 2B) compared with layers incubated in GA alone. This indicates that these NO donors delayed PCD in GA-treated aleurone layers. The O2 consumption data also establish that the delay in PCD resulting from incubation with NO donors does not result from a general inhibition of metabolism. O2 consumption by layers treated with GA and NO donors is not lower than that for layers treated with GA alone (Fig. 2).

Incubation of GA-treated aleurone layers in SNAP or SNP also dramatically reduced the rate of PCD as measured by fluorescence microscopy (Fig. 1, B and C). Virtually no cells were dead 24 h after incubation in GA plus 100 μM SNP (Fig. 1B) or 300 μM SNAP (Fig. 1C), and only 28% (SNP) or 35% (SNAP) of cells were dead at 36 h compared with 60% of the cells in layers incubated in GA alone. After 48 h with NO donors, 61% of cells in GA + SNAP and 52% of the cells in GA + SNAP remained alive. We added the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) to aleurone layers incubated with GA and SNAP to confirm that the effect of SNAP on PCD was mediated specifically by NO (Fig. 1E). Virtually all cells were dead after 48 h with cPTIO and SNAP, and cell death at 24 h in the presence of SNAP and the NO scavenger was higher than in cells incu-
bated in GA alone (compare Fig. 1, A with E). When cPTIO was added to cells incubated in GA alone, cell death was also accelerated (data not shown). This latter result suggests that endogenous sources of NO may play a role in delaying PCD in aleurone layers. cPTIO added to non-hormone-treated aleurone layers had no effect on cell death, and this makes it unlikely that the NO scavenger exerts a toxic effect on its own (data not shown).

NO Donors Do Not Inhibit GA-Stimulated α-Amylase Synthesis

NO donors delayed PCD in barley aleurone layers, but did not inhibit overall metabolism as measured by net O2 consumption. To determine if delayed PCD resulted from a global inhibition of GA-induced responses, we carried out experiments to establish whether the GA-stimulated synthesis and secretion of α-amylase was affected by these treatments. To do this, we measured the activity of α-amylase secreted to the incubation medium and the steady-state amounts of α-amylase mRNA in aleurone layers that were incubated in the presence and absence of ABA, GA, and NO donors. As has been reported frequently, GA stimulates and ABA inhibits the synthesis and secretion of α-amylase (Fig. 4). When GA-treated aleurone layers were incubated with NO donors, the rate of α-amylase secretion and the total amount of α-amylase produced were not less than that from layers treated with GA alone. α-Amylase accumulation in the medium was enhanced slightly by NO donors (Fig. 4). Whereas the accumulation of α-amylase activity in the incubation medium ceased 24 h after incubation in GA alone, incubation of layers in 100 μM SNP or 300 μM SNAP prolonged the period of α-amylase synthesis and secretion. This resulted in a significantly greater amount of α-amylase activity in the medium surrounding layers treated with SNAP or SNP (Fig. 4).

RNA blots probed with an α-amylase cDNA are consistent with the observation that NO donors do not inhibit the synthesis of α-amylase (Fig. 5). GA stimulates the accumulation of α-amylase mRNA in

![Figure 1.](#)
barley aleurone layers, and in these experiments, \( \alpha \)-amylase mRNA abundance peaked between 9 and 18 h of incubation. By 24 h of incubation in GA, the amount of \( \alpha \)-amylase mRNA remaining was a small fraction of that present at 9 to 18 h (Fig. 5). When layers were treated with SNP or SNAP, on the other hand, the amount of \( \alpha \)-amylase mRNA remained high through 24 h of incubation in GA (Fig. 5). This effect of GA and NO donors contrasts with that of ABA, which inhibits the accumulation of \( \alpha \)-amylase mRNA in aleurone layers (Fig. 5).

### NO Donors and Enzymes of ROS Metabolism

We showed previously that steady-state amounts of mRNAs encoding the ROS metabolizing enzymes catalase (CAT) and superoxide dismutase (SOD), as well as CAT and SOD protein and enzyme activity, are strongly correlated with GA-induced PCD (Fath et al., 2001). GA treatment reduced the amounts of these mRNAs and proteins and promoted cell death, whereas ABA brought about an increase in their abundance and prevented cell death (Fath et al., 2001). We confirmed and extended these observations in this study to determine if NO donors prolonged the life of cells in GA-treated layers by increasing their capacity to metabolize ROS (Figs. 6 and 7). RNA blotting shows that the steady-state amount of barley \( \text{Cat2} \) mRNA was reduced within 3 h of exposure to GA, and by 6 h in GA, \( \text{Cat2} \) mRNA was not detectable. Incubation in GA plus SNAP slowed the loss of \( \text{Cat2} \) mRNA by up to 6 h (Fig. 6A), but did not result in a dramatic, sustained increase as is seen for layers incubated in ABA (Fig. 6A). Protein blotting shows that GA treatment also caused a rapid decline in the amount of CAT protein, and this decline was delayed by 3 h in GA-treated cells by the presence of SNAP (Fig. 6B). The amount of CAT protein did not decline in ABA-treated tissue (Fig. 6B).

We also determined the steady-state amounts of \( \text{Sod4} \) mRNA and SOD protein in aleurone layers exposed to GA and NO donors (Fig. 7). Using a maize \( \text{Sod4} \) probe, we found that \( \text{Sod4} \) mRNA declined in layers incubated in GA or GA plus SNAP. In both cases, after 9 h of incubation, \( \text{Sod4} \) mRNA was barely detectable. In contrast, the amount of \( \text{Sod4} \) mRNA in ABA-treated layers was greater after 24 h of incubation than in freshly prepared layers (Fig. 7A). Protein blotting shows that the amount of SOD protein declined in aleurone cells exposed to GA, and that this decline was less rapid in layers treated with GA plus SNP or GA plus SNAP (Fig. 7B).
The Antioxidant BHT Mimics the Effects of NO Donors on Aleurone Cell Viability

NO has been shown to act synergistically with ROS in plant and animal cells to promote cell death (Delledonne et al., 1998; Wendehenne et al., 2001), but NO has also been shown to act as an antioxidant and to prevent death (Beligni and Lamattina, 1999; Beligni and Lamattina, 2001; Wendehenne et al., 2001). We tested whether the antioxidant BHT could mimic the effect of NO donors and slow down GA-induced PCD in barley aleurone layers. When aleurone layers were incubated in GA in the presence of 1 mm BHT, the rate of PCD decreased (Fig. 8, A and C). In this experiment, 76% of cells were dead after 36 h in GA alone, and after 48 h in GA, almost all (91%) cells were dead. In aleurone layers treated with GA and 1 mm BHT, only 25% of cells were dead at 36 h, and more than 50% of cells remained alive after 48 h (Fig. 8C). As a positive control for these experiments, cell viability of ABA-treated layers was determined, and all cells were alive 48 h after ABA treatment in the presence or absence of BHT (Fig. 8B). BHT did not reduce the rate or extent of α-amylase secretion by aleurone layers (Fig. 8D).

Detection of NO in Barley Aleurone Protoplasts with Fluorescent Probes

We used NO-sensitive fluorescent probes to establish whether barley aleurone cells produce NO. A method for detecting NO production in live cells was developed by Heiduschka and Thanos (1998). This method uses 1,2-diaminonanthraquinone (DAQ) as an NO-sensitive fluorophore. Aleurone protoplasts accumulate DAQ and become fluorescent when incubated with this probe (Fig. 9A). These observations support the hypothesis that aleurone cells produce NO. We used 1-aminonanthraquinone (1-AQ) as a negative control for DAQ (Fig. 9B). 1-AQ lacks one of the two amino groups that make up the NO-reactive site in DAQ. Therefore, 1-AQ is a useful control to assess the extent that enzymatic activities, independent of NO, might result in the production of a fluorescent compound from DAQ. When aleurone cells were incubated in 1-AQ, fluorescence from these cells was much less intense than that from cells incubated in DAQ (Fig. 9, A and B), though still greater than that from autofluorescence alone (Fig. 9C). These data suggest that the conversion of DAQ from a nonfluorescent to fluorescent molecule was largely dependent on NO. There was no pronounced effect of ABA or GA treatment on the intensity of fluorescence from aleurone protoplasts incubated in DAQ.

4,5-Diaminofluorescein diacetate (DAF-2 DA) is another cell-permeable probe that becomes fluorescent when it reacts with NO (Nakatsubo et al., 1998), and DAF-2 DA has been used previously to demon-
strate NO production by plant cells (Foissner et al., 2000; Pedroso and Durzan, 2000). Figure 9, D and G, shows a field of aleurone protoplasts loaded with DAF-2 DA and viewed by differential interference contrast (Fig. 9G) or fluorescence microscopy (Fig. 9D). Damaged cells (Fig. 9, D and G, arrows) are fluorescent, but undamaged cells are not. To see if DAF-2 DA was reporting NO production by injured cells that was not observed with DAQ, we used 4-aminofluorescein diacetate (4-AF DA) as a negative control. Like 1-AQ, 4-AF DA lacks one of the amino groups that make up the NO-reactive site in DAF-2 DA. In contrast to the low-intensity labeling seen with 1-AQ, all aleurone protoplasts accumulated the negative control 4-AF DA and showed bright fluorescence (Fig. 9E). These data indicate that DAF-2 DA is not a suitable probe for monitoring NO production in barley aleurone cells.

**DISCUSSION**

In this paper, we show that NO delays the onset of GA-induced PCD of barley aleurone cells. Whether determined by vital staining with FDA and FM 4-64 (Fig. 1) or by monitoring net O2 consumption (Fig. 2), NO delays PCD by 12 h or more in GA-treated layers when supplied to aleurone cells using the NO donors SNP or SNAP. The effects of SNP and SNAP are specific for NO because the NO scavenger cPTIO reverses the effects of NO donors on cell viability (Fig. 1E) and because aleurone cells incubated with SNP or SNAP remain fully responsive to GA, as shown by the synthesis and secretion of α-amylase (Fig. 4). NO’s effect on cell viability can be mimicked by the antioxidant BHT, indicating that NO may act to protect the cell from oxidative stress. However, unlike NO, BHT does not prolong the period of α-amylase secretion (compare Fig. 4 with Fig. 8D). These data suggest that NO may play an additional role in barley aleurone cells, perhaps as a signaling molecule. Our fluorescence microscopy data indicate that NO is synthesized by aleurone cells.

Cytotoxic and protective effects of NO in animals and plants have been reported (Wink and Mitchell, 1998; Beligni and Lamattina, 2001). The simplest explanation for our data showing that NO donors delay ROS-mediated PCD in barley aleurone layers (Figs. 1 and 2) is that NO acts as an antioxidant in this system. Our results with barley aleurone layers are in line with data showing, for example, that NO can delay senescence (Mallick et al., 2000), prevent ROS-induced cytotoxicity (Beligni and Lamattina, 1999), and slow chlorophyll loss resulting from pathogen infection (Laxalt et al., 1997).

We have shown that ROS play a central role in promoting PCD in barley aleurone cells (Bethke and Jones, 2001; Fath et al., 2001), and ROS are likely targets of NO and BHT in aleurone cells. Aleurone cells store large amounts of triglycerides (approximately 25% of cell volume) and these are converted to sugars through β-oxidation and the glyoxylate cycle. The first step in β-oxidation is catalyzed by fatty acyl coenzyme A, a flavin-containing enzyme that generates superoxide and hydrogen peroxide (Bethke and Jones, 2001; Fath et al., 2001). Hence, glyoxysomes, along with mitochondria, are likely to be significant sites of ROS synthesis. We have proposed that peroxidation of membrane lipids and rupture of the plasma membrane occurs when the rate of ROS production exceeds the cell’s capacity for ROS metabolism. NO has been shown to protect membranes and lipoproteins from oxidation directly, by interacting with lipid peroxyl radicals, or indirectly, by inhibiting lipoxy-
genase activity (Patel et al., 2000). At present, our data do not allow us to specify the exact mechanism by which NO exerts its protective effect.

Aleurone cells contain a suite of enzymes that metabolize ROS. The synthesis of these enzymes is tightly regulated by ABA and GA (Figs. 6 and 7, and Fath et al., 2001). Freshly isolated aleurone layers and those incubated in ABA have high amounts of mRNAs encoding CAT and SOD, and corresponding high amount of these proteins. These mRNAs and proteins decline in GA-treated layers (Figs. 6 and 7, and Fath et al., 2001), and there is a strong correlation between the amounts of CAT and SOD mRNA, protein, and enzyme activity and the effects of GA on PCD (Figs. 1 and 2, and Fath et al., 2001). CAT and SOD proteins decline in amount less rapidly in tissue incubated in GA plus NO donors than in tissue incubated in GA alone (Figs. 6 and 7). For CAT at least, this delayed loss of protein is not a consequence of delayed cell death. CAT loss occurs at a time when all cells in layers treated with GA alone or GA plus NO donors are still alive. An increased ability to metabolize ROS may contribute somewhat to the extended viability of cells treated with NO donors by further reducing the degree of oxidative stress. It is interesting that Yamasaki et al. (2001) reported that NO inhibits cytochrome c oxidase and oxidative phosphorylation in mitochondria isolated from mung bean (Vigna radiata), but that the alternative oxidase pathway is resistant to NO. A greater dependence on the alternative oxidase pathway in the presence of NO may lower the respiration-dependent production of ROS in mitochondria, and thus may be protective by reducing the overall rate of ROS synthesis (Yamasaki et al., 2001).

We measured the production of NO in aleurone cells using two NO-sensitive probes, DAQ (Heiduschka and Thanos, 1998) and DAF-2 DA (Nakatsubo et al., 1998 and Fig. 9). Although others have used DAF-2 DA to monitor NO production in plant cells, DAF-2 DA proved unsuitable for this purpose in barley aleurone cells, even though we could detect a fluorescent product when aleurone protoplasts were incubated with the probe. Increases in fluorescence from DAQ were more specific for NO than those seen with DAF-2 DA because the signal from protoplasts incubated in the NO-insensitive probe 1-AQ was much less intense than that from protoplasts incubated in DAQ. Our data suggest that DAQ is interacting with endogenous NO, and they support the hypothesis that aleurone cells synthesize NO. We did not observe a difference in the intensity of the fluorescent signal between GA- and ABA-treated cells, but these data do not allow us to conclude that NO production is equivalent regardless of hormone

Figure 8. BHT delays GA-induced PCD in barley aleurone layers. Aleurone layers were incubated with GA alone or with GA plus 1 mM BHT, and were then loaded with the fluorescent probes FDA and FM 4-64 at 24, 36, and 48 h. Epifluorescence images (A) of live (green) and dead (red) cells were quantified as shown in C. α-Amylase secretion by layers from the same experiment is quantified in D. Note that cells in layers treated with ABA alone or ABA plus BHT do not die (B).
treatment. Fluorescence from an NO-reactive probe depends on the rate of NO production and on the rate that NO reacts with molecules other than the probe. ABA-treated cells have a much higher capacity for the metabolism of ROS than GA-treated cells. If endogenous molecules that react with NO are also more abundant in ABA-treated cells than in GA-treated cells, their interaction with NO would lessen the intensity of fluorescence from the cell. As a consequence, fluorescence intensity would underrepresent the extent of NO production in ABA-treated cells relative to GA-treated cells.

Although we have not explored the possibility that aleurone cells synthesize NO via a NOS, we hypothesize that NO is likely to be synthesized enzymatically via NR or nonenzymatically in the apoplast (Yamasaki, 2000). NR is known to generate NO in plants, via NR or nonenzymatically in the apoplast (Yamasaki, 2000). Aleurone layers are incubated in FDA (2 μM) in 20 mM CaCl₂ for 30 min, briefly rinsed with 20 mM CaCl₂, and then incubated in FM 4-64 (1 μM) in 20 mM CaCl₂ for 3 min. Layers were briefly washed with 20 mM CaCl₂ and were mounted on microscope slides. Aleurone cells were observed with a fluorescent microscope (Axioskop; Zeiss, Thornwood, NJ) using a 20× objective. Images of the fluorescent signal were captured using a digital camera. Randomly selected fields from at least three different aleurone layers per treatment were counted to determine the rate of O₂ consumption. The data presented are means from at least four aleurone layers per treatment.

**O₂ Consumption**

O₂ consumption was measured using an O₂-sensitive electrode (model 10, Digital Oxygen System; Rank Brothers, Cambridge, UK). Layers were transferred to a measuring chamber containing 5 mL of sterile distilled water at least 20 min prior to determining the rate of O₂ consumption. The number of live and dead cells was determined by double staining with the fluorescent probes FDA and FM 4-64 (Molecular Probes; Bethke and Jones, 2001). Aleurone layers were incubated in FDA (2 μM) in 20 mM CaCl₂ for 30 min, briefly rinsed with 20 mM CaCl₂, and then incubated in FM 4-64 (1 μM) in 20 mM CaCl₂ for 3 min. Layers were briefly washed with 20 mM CaCl₂ and were mounted on microscope slides. Aleurone cells were observed with a fluorescent microscope (Axioskop; Zeiss, Thornwood, NJ) using a 20× objective. Images of the fluorescent signal were captured using a digital camera. Randomly selected fields from at least three different aleurone layers per treatment were counted to determine the percentage of live cells.

**Cell Viability and Death**

The number of live and dead cells was determined by double staining with the fluorescent probes FDA and FM 4-64 (Molecular Probes; Bethke and Jones, 2001). Aleurone layers were incubated in FDA (2 μM) in 20 mM CaCl₂ for 30 min, briefly rinsed with 20 mM CaCl₂, and then incubated in FM 4-64 (1 μM) in 20 mM CaCl₂ for 3 min. Layers were briefly washed with 20 mM CaCl₂ and were mounted on microscope slides. Aleurone cells were observed with a fluorescent microscope (Axioskop; Zeiss, Thornwood, NJ) using a 20× objective. Images of the fluorescent signal were captured using a digital camera. Randomly selected fields from at least three different aleurone layers per treatment were counted to determine the percentage of live cells.

**Enzyme Assays**

α-Amylase activity secreted into the medium by aleurone layers was assayed as described by Bush et al. (1986).

**RNA Isolation and Northern Blotting**

Aleurone layers (10–15) were ground to powder with liquid N₂. Total RNA was isolated using the plant RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNAs (5 μg lane⁻¹) were separated on denaturing 1.2% (w/v) agarose gels and then blotted onto a nylon membrane (Hybond-N; Amersham Biosciences, Piscataway, NJ). α-Amylase, Cat2, and Sod4 cDNA probes were labeled with [32P]dCTP (ICN Biomedicals, Costa Mesa, CA) using the Prime-it RmT random prime labeling kit (Stratagene, La Jolla, CA). Membranes were hybridized at 65°C in 7% (w/v) SDS, 0.5 mM Na₂PO₄ pH 7.2, 1 mM EDTA, and 0.1 mg mL⁻¹ sonicated herring sperm DNA. Blots were washed at 65°C in 2× SSC and 1% (w/v) SDS for 15 min, then in 0.5× SSC and 1% (w/v) SDS for 20 min, and finally in 0.1× SSC and 1% (w/v) SDS for 10 min. The blots were reprobed with an rRNA probe to verify equal loading. The amount of [32P]-labeled cDNA probe

**Figure 9.** Fluorescent probes report the presence of NO in barley aleurone protoplasts. Epifluorescence (A–F) and differential interference contrast (G–I) images of barley aleurone protoplasts incubated with DAQ (A), 1-AQ (B), DAF-2 DA (D and G), 1-AF DA (E and H), or no probe (C, F, and I). Arrows point to damaged cells.
hybridizing to specific mRNAs was quantified by laser densitometry using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Detection of NO Using Fluorescent Probes**

Barley aleurone protoplasts were prepared as described previously (Bethke and Jones, 2001). GA- or ABA-treated protoplasts were washed at least twice with fresh incubation medium, incubated with or without probe, and examined using epifluorescence microscopy. The concentration of the probe stock solution (in dimethyl sulfoxide), the dilution used, and the incubation times were: DAF (Calbiochem, San Diego) 10 mm, 1:500, 10 to 60 min; 1-AQ (Aldrich, Milwaukee, WI), 10 mm, 1:500, 10 to 60 min; DAF-2 DA (Calbiochem), 4 mm, 1:500, 1 h; and 4-aminofluorescein diacetate (Calbiochem), 4 mm, 1:500, 1 h.

**ACKNOWLEDGMENTS**

We thank Dr. Ron Skadsen (U.S. Department of Agriculture-Agricultural Research Service, Cereal Crops Research Unit, Madison, WI) for providing the barley Cat2 cDNA and Dr. John Scandalios (North Carolina State University, Raleigh) for the gift of the maize Sod4 cDNA and the anti-maize SOD4 and anti-maize-CAT polyclonal antibodies.

Received December 24, 2001; returned for revision February 19, 2002; accepted April 30, 2002.

**LITERATURE CITED**


Heiduschka P, Thanos S (1998) NO production during neuronal cell death can be directly assessed by a chemical reaction in vivo. Neureport 9: 4051–4057


Leschem Ya, Wills RBH, Ku VV-V (1998) Evidence for the function of the free radical gas, nitric oxide (NO), as an endogenous maturation and senescence regulating factor in higher plants. Plant Physiol Biochem 36: 825–833


