

Production of Reactive Oxygen Species, Alteration of Cytosolic Ascorbate Peroxidase, and Impairment of Mitochondrial Metabolism Are Early Events in Heat Shock-Induced Programmed Cell Death in Tobacco Bright-Yellow 2 Cells¹

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To gain some insight into the mechanisms by which plant cells die as a result of abiotic stress, we exposed tobacco (*Nicotiana tabacum*) Bright-Yellow 2 cells to heat shock and investigated cell survival as a function of time after heat shock induction. Heat treatment at 55°C triggered processes leading to programmed cell death (PCD) that was complete after 72 h. In the early phase, cells undergoing PCD showed an immediate burst in hydrogen peroxide (H₂O₂) and superoxide (O₂^{•-}) anion production. Consistently, death was prevented by the antioxidants ascorbate (ASC) and superoxide dismutase (SOD). Actinomycin D and cycloheximide, inhibitors of transcription and translation, respectively, also prevented cell death, but with a lower efficiency. Induction of PCD resulted in gradual oxidation of endogenous ASC; this was accompanied by a decrease in both the amount and the specific activity of the cytosolic ASC peroxidase (cAPX). A reduction in cAPX gene expression was also found in the late PCD phase. Moreover, changes of cAPX kinetic properties were found in PCD cells. Production of ROS in PCD cells was accompanied by early inhibition of glucose (Glc) oxidation, with a strong impairment of mitochondrial function as shown by an increase in cellular NAD(P)H fluorescence, and by failure of mitochondria isolated from cells undergoing PCD to generate membrane potential and to oxidize succinate in a manner controlled by ADP. Thus, we propose that in the early phase of tobacco Bright-Yellow 2 cell PCD, ROS production occurs, perhaps because of damage of the cell antioxidant system, with impairment of the mitochondrial oxidative phosphorylation.

In plants, programmed cell death (PCD) is responsible for removal of redundant, misplaced, or damaged cells, and, thus, contributes significantly to both development and maintenance of these multicellular organisms. Activation of PCD in plants takes place during a variety of processes including differentiation of tracheary elements (Fukuda, 2000; Yu et al., 2002) and female gametophytes (Wu and Cheun, 2000), the development of cereal endosperm and aleurone cells (Young and Gallie, 2000; Fath et al., 2002), responses to external stimuli such as the hy-

persensitive reaction against pathogen attacks (Lamb and Dixon, 1997; Beers and McDowell, 2001) and severe abiotic stresses (Koukalová et al., 1997; Weaver et al., 1998; Rao and Davis, 2001). At present, however, how signaling pathways lead plant cells toward death via apoptosis and how death occurs are far from being elucidated. Mammalian and plant PCD processes share several morphological and biochemical features, including cytoplasm shrinkage, nuclear condensation, DNA laddering, expression of caspase-like proteolytic activity, and release of cytochrome c from mitochondria (Balk et al., 1999; Sun et al., 1999; Kim et al., 2003). Nonetheless, in distinction from mammals (for instance, see Atlante et al., 2003a, 2003b), how plant PCD takes place remains somewhat obscure. In this context, the role of the reactive oxygen species (ROS) in plants merits special attention because ROS can exert two opposite effects: They can activate pathways aimed at saving the cell from demise (Pastore et al., 1999, 2000; Di Cagno et al., 2001; Mittler, 2002; Vranova et al., 2002) but can also impair the cellular redox balance, thus triggering PCD, perhaps with mitochondria playing a pivotal

¹ This work was supported by the Italian Ministry of Instruction, University, and Research (MIVR) and by the Programmi di Ricerca di Rilevante Interesse Nazionale ("Bioenergetica: Aspetti Genetici, Biochimici e Fisiologici" to S.P. and "Specie Reattive Dell'Ossigeno e Sistemi Antiossidanti Nella Morte Cellulare Programmata e Nell'Interazione Pianta-Patogeno" to L.D.G.).

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Article, publication date, and citation information can be found at <http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.035956>.

role in integrating cellular stress and regulating PCD (Jones, 2000; Lam et al., 2001; Tiwari et al., 2002). These ROS-dependent responses could be dependent on localization, timing, and level of ROS production in response to different stimuli (Mittler, 2002; De Gara et al., 2003) and on the occurrence of cell scavenger systems. Among the latter, ascorbate (ASC) plays a crucial role (Noctor and Foyer, 1998; Smirnov, 2000; De Gara, 2003), it being both a direct scavenger and electron donor of ASC peroxidase, a key enzyme for scavenging hydrogen peroxide (H_2O_2) in plant cells (Asada, 1992; Shigeoka et al., 2002).

In this work, we first developed a plant model system consisting of heat-shocked tobacco (*Nicotiana tabacum*) Bright-Yellow 2 (TBY-2) cells, in which PCD occurs in the absence of added PCD inducers. Then, we investigated events occurring in the early phase of PCD including ROS production, changes in the expression and activity of cytosolic ASC peroxidase (cAPX), and the alterations in cell oxidative metabolism that depends on mitochondrial function. We found that both cAPX function and mitochondrial energy metabolism are already impaired in the early stages of PCD.

RESULTS

Heat Shock-Induced PCD in Cultured TBY-2 Cells

To ascertain whether and how heat shock can induce PCD in cultured cells of TBY-2, cells were kept for 10 min at temperatures ranging between 27°C (control cells) and 65°C, and cell viability was analyzed as a function of time after treatment. Cell viability was essentially unaffected after heating up to 45°C, whereas incubation at 55°C resulted in a progressive decrease in cell viability. The effect started after 2 h (cells in these conditions will be referred to as 2-h PCD cells). Viability of cells was 50% after 24 h (24-h PCD cells) and negligible after 72 h (Fig. 1). Incubation at 60°C and 65°C resulted in cell death in 24 h (data not shown).

To distinguish whether cell death occurred by PCD or by necrosis, we investigated the occurrence of indicators of PCD including cytoplasm shrinkage, chromatin condensation, and DNA laddering. Cytoplasm shrinkage was found in most 24-h PCD cells ($72\% \pm 7\%$; Fig. 2B) but only in $12\% \pm 2\%$ and $2\% \pm 2\%$ of dead cells heated at 60°C and 65°C respectively. As expected, control cells exhibited normal morphology (Fig. 2A). In parallel experiments, chromatin morphology was observed by DAPI staining. Nuclei of control cells exhibited a large central nucleolus surrounded by uniformly stained chromatin (Fig. 2C), whereas the chromatin had a granular appearance with lobated nuclei in cells shocked at 55°C (Fig. 2D). Further confirmation that 55°C heat shock does induce PCD was obtained by assaying DNA cleavage in oligo-nucleosomal units by cell death-specific endonucleases. DNA laddering was found in

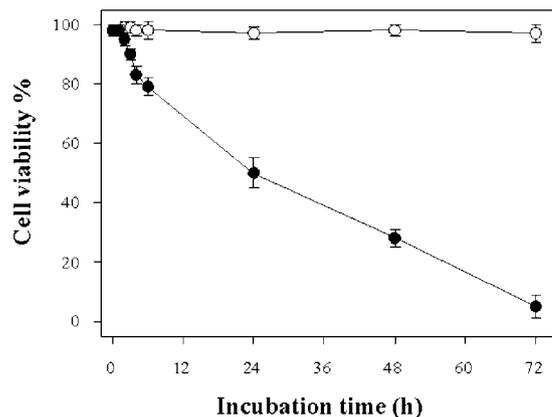


Figure 1. Time-dependent effect of heat shock on cell viability. One hundred milliliters of TBY-2 cell cultures was treated for 10 min at 55°C and returned to 27°C for a 72-h period. At indicated time intervals after heat treatment, viability of both control (○) and heat-shocked (●) cells was measured using trypan blue staining and examination by phase contrast microscopy. In both cases, the percentage (\pm SE) of viable cells was counted in a population of at least 1,000 cells in six separate experiments.

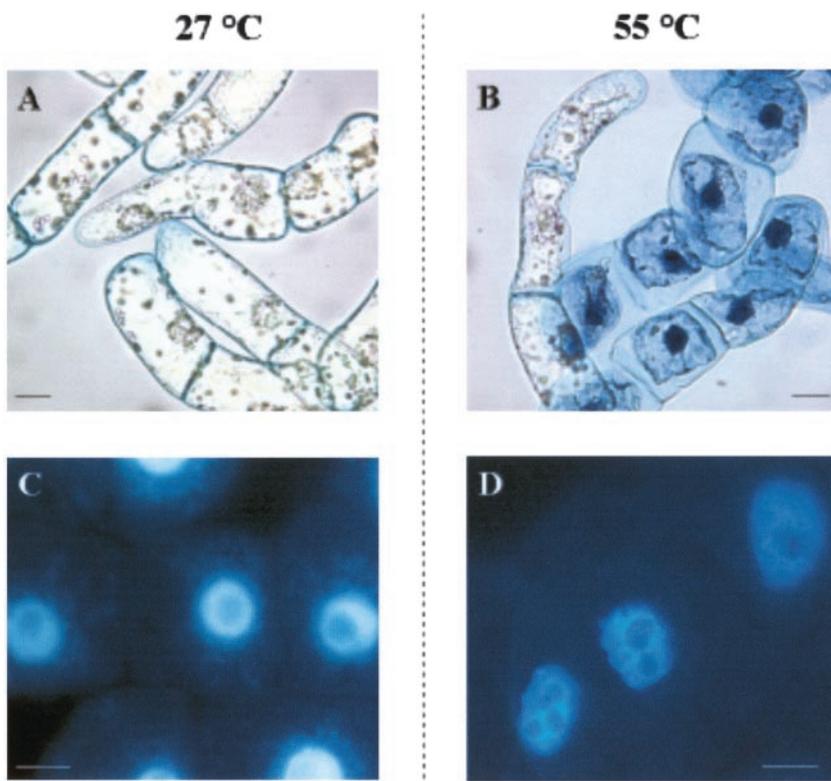
48- and 72-h PCD cells, at which times 75% to 98% cells were already dead (Fig. 3). In cells treated at 60°C and 65°C, neither chromatin condensation nor DNA laddering were found (not shown), thus showing that cell death occurred by necrosis.

ROS Production in TBY-2 Cells Undergoing PCD

In the light of the crucial role played by ROS in PCD (Dat et al., 2003), we investigated production of ROS in heat-shocked TBY-2 cells by monitoring both H_2O_2 and superoxide anion ($O_2^{\cdot-}$) production. Generation of H_2O_2 , measured by following the fluorescence of the dye rhodamine produced from hydrorhodamine 123 (DHR123) in the presence of H_2O_2 , occurred in the majority of cells immediately after heat shock, (0-h PCD cells) as shown by the bright fluorescence resulting from staining with DHR123 (Fig. 4), whereas production of ROS in control cells was negligible. Production of H_2O_2 was also investigated as a function of time (Fig. 5). As a result of heat shock, the level of H_2O_2 increased by about 200% with respect to the control cells and remained constant over the investigated time period. Pre-incubation with ASC failed to prevent the increase in H_2O_2 in 0-h PCD cells, but the H_2O_2 was gradually removed over a 3-h period. It should be noted that an immediate and very large (500% over control levels) production of H_2O_2 was found in cells heat shocked at 65°C (not shown).

In another set of experiments (Fig. 6), generation of $O_2^{\cdot-}$ was measured in the culture medium as a function of time, both in control and in heat-shocked TBY-2 cells, and in the absence or presence of superoxide dismutase (SOD). The level of $O_2^{\cdot-}$ was found to increase enormously (up to 1,000%) over the first

Figure 2. Chromatin condensation and cytoplasm shrinkage induced by heat shock in TBY-2 cells. Cell suspension was subjected to heat shock and returned to growth temperature for 24 h. Aliquots of both control (27°C) and heat-shocked (55°C) cells were collected, stained with trypan blue, and visualized under phase contrast microscopy (A and B) or stained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy (C and D). Pictures represent typical examples. Bar = 20 μm .



30 min in 55°C shocked cells and then to drop to control levels by 1 h. The levels of $\text{O}_2^{\cdot -}$ superoxide production both in control cells and in cells pre-incubated with SOD were low and remained constant at all times.

Effects of Scavengers of ROS and Transcription/Translation Inhibitors on Heat-Induced PCD

In the light of the results quoted above and given that protein synthesis is needed for plant PCD to occur (Solomon et al., 1999; Clarke et al., 2000), the abilities of two ROS scavengers (ASC and SOD) and of inhibitors of transcription (actinomycin D [Act D]) and translation (cycloheximide [Chex]) to prevent PCD were compared. Both the scavengers and the inhibitors were added to the culture medium 30 min before heat shock, and cell viability was measured in 24-h PCD cells. In the absence of either scavengers or inhibitors, cell survival was about 50% compared with control cells (Fig. 7). About 90% and 80% survival was found for cells pre-incubated with ASC and SOD, respectively. A small but statistically significant ($P < 0.01$) prevention of cell death (65% cell survival) resulted from addition of either Act D or Chex used at concentrations that totally inhibit protein synthesis both in tobacco (Elbaz et al., 2002) and soybean (*Glycine max*; Solomon et al., 1999). In experiments not reported in detail, it was confirmed that

none of the compounds used had any effect on viability of control cells.

ASC Pool and cAPX in Heat Shock-Induced PCD

Because steady-state levels of ROS depend on the balance between ROS-producing and -scavenging reactions, we measured levels of both ASC and dehydroascorbate (DHA) and the activity of cAPX; the latter plays a major role in scavenging H_2O_2 in plants (Asada, 1992; Noctor and Foyer, 1998; Smirnov, 2000; Shigeoka et al., 2002).

Results of measurements of the ASC and DHA levels are given in Table I. Interestingly, although the total ASC pool (ASC + DHA) remained constant at the control level during the first 6 h after induction of PCD, a 50% increase in concentration of DHA was found already at 0-h PCD. In 24-h PCD cells, the total ASC pool was decreased by 35% with respect to the control, with a further increase (up to 80%) of the percentage of oxidized ASC (DHA). No significant change in either the ASC+DHA pool or in its redox state was found in the control cells over the 24-h period.

The role of cAPX during PCD was investigated by monitoring the expression of its gene and the amount of cAPX protein present in the cells and by measuring the rate of the enzyme reaction (Fig. 8). Gene expression was analyzed by semiquantitative RT-PCR using a specific primer for cAPX. In 0-h PCD cells, gene

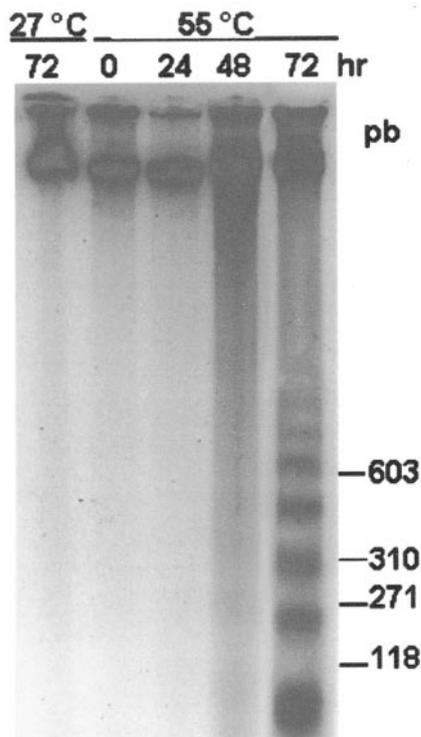


Figure 3. Fragmentation DNA induced by heat shock in TBY-2 cells. Cell cultures were treated at 55°C and returned to 27°C for a 72-h period. At the indicated time intervals after heat shock, cells were collected, frozen in liquid nitrogen, and genomic DNA was extracted as described in "Materials and Methods." Five hundred nanograms of genomic DNA was separated on a 1.8% (w/v) agarose gel and detected by staining the gel with ethidium bromide.

expression was found to only have a weak decrease; however, its expression clearly decreased after 6 h and was negligible in 24-h PCD cells (Fig. 8A).

Levels of cAPX protein were measured using immunoblotting with a specific monoclonal antibody (Fig. 8B). In 0-h PCD cells, the amount of cAPX protein was about 50% of that in control cells and decreased by about 80% in 24-h PCD cells. These decreases were largely prevented by addition of either ASC or SOD.

Because changes in gene expression do not necessarily reflect changes in enzyme catalysis, the activity of cAPX was also measured in both control and PCD cells. A reduction of more than 40% was found in the cAPX activity in 0-h PCD cells (Fig. 8C). At 6 h, the reduction reached 80%, after which the cAPX activity remained constant. On the other hand, when TBY-2 cells were pretreated with either ASC or SOD, no significant decrease in the enzyme activity was found as a result of heat shock (Fig. 8C).

To check whether the kinetics of cAPX were modified as a result of heat shock, the dependence of the reaction rate on ASC (Fig. 9A) or H₂O₂ (Fig. 9B) concentrations was investigated at a fixed concentration of the second substrate. At a fixed concentration of H₂O₂ (Fig. 9A), a sigmoidal dependence of rate on

ASC concentration was found for control cells showing the occurrence of cooperativity. The Hill coefficient was found to be between 1.4 and 1.6 in four different experiments. On the other hand, in PCD cells, the dependence of rate on ASC concentration was hyperbolic. As expected in light of the results in Figure 8, a very large reduction in the V_{\max} was found for the enzyme in PCD cells (about 5-fold). Similarly, K_m for ASC was reduced 5-fold compared with $K_{0.5}$ (i.e. the substrate concentration that gives one-half maximum rate). A hyperbolic dependence of rate on H₂O₂ concentration at a fixed level of ASC was found for the enzyme from both control and heat-shocked cells (Fig. 9B). The enzyme from shocked cells showed a decrease in the V_{\max} (again about 5-fold) compared with that from control cells, but the K_m values were essentially the same.

Energy Metabolism in PCD Cells

The protection of cells against PCD by antioxidants posed the question as to whether impairment of cell oxidative metabolism, involving mitochondria, occurs together with induction of PCD. Thus, oxygen consumption by the cell suspension arising from addition of glucose (Glc) (10 mM) was measured (Fig. 10). In a typical experiment, in which control and 4-h PCD cells were used, the oxygen content of the cell suspensions incubated in the absence of Glc remained constant, as expected because endogenous respiratory substrates had been oxidized previously (see "Materials and Methods"). When Glc was added, the oxygen content decreased at rates of 22 and 11 natoms min⁻¹ mg⁻¹ cell protein in control and 4-h PCD cells, respectively. In both cases, O₂ consumption was largely inhibited by 1 mM cyanide (KCN), a powerful inhibitor of mitochondrial cytochrome c oxidase. Residual oxygen consumption (6 and 3 natoms min⁻¹ mg⁻¹ cell protein in control and 4-h PCD cells, respectively) was observed after addition of KCN, probably because of the cyanide-insensitive alternative oxidase activity (Vanlerberghe et al., 1995). The rate of oxygen consumption measured as a function of the time after heat shock is shown in Figure 10B. In distinction to the control cells, a continuous decrease in this rate was observed over 0 to 6 h of PCD. In heat-shocked cells, viability decreased more slowly than did oxidative capacity, and at 2 h, viability was still about 100%, whereas 30% inhibition of Glc oxidation had already occurred (Fig. 10B). In a parallel experiment, we found no impairment of oxidative metabolism when TBY-2 cells were incubated with the antioxidants ASC and SOD, whereas partial prevention (60%) occurred in the presence of ActD or Chex (Fig. 10C). None of the compounds used had any effect on the rate of Glc oxidation in control cells (not shown).

To confirm that impairment of Glc oxidation involved intracellular metabolism, a further experiment was carried out under the same experimental

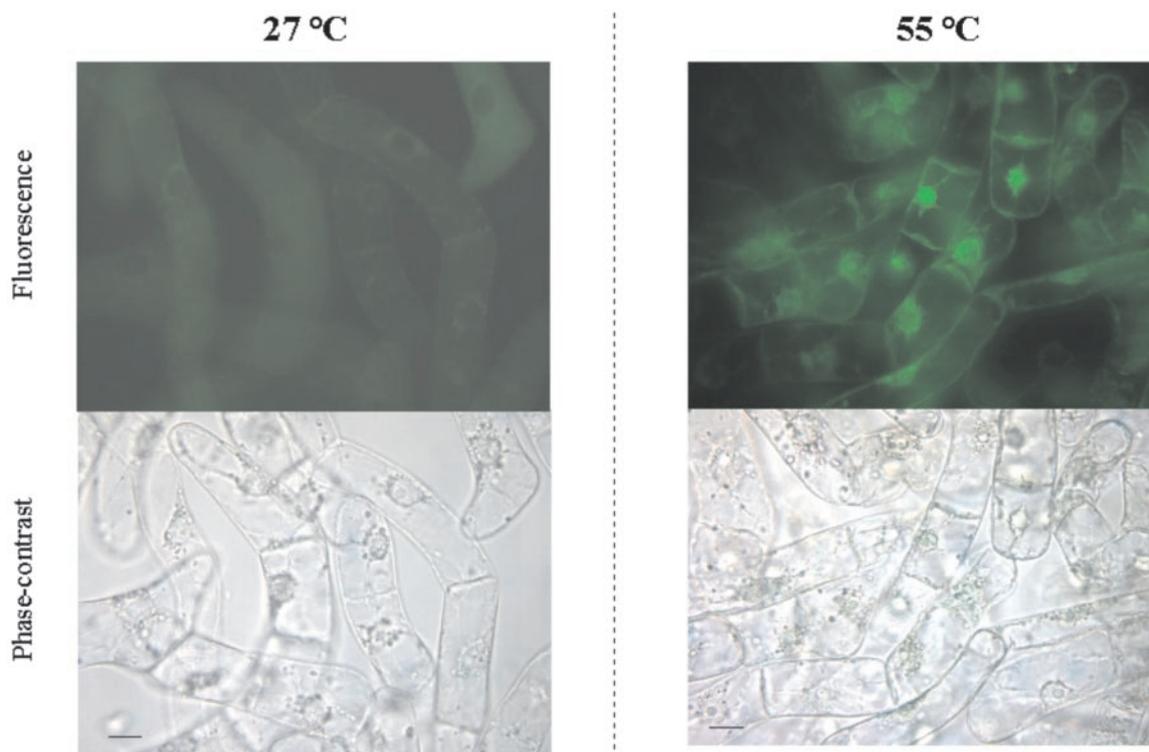


Figure 4. Production of H₂O₂ in PCD-induced cells. TBY-2 cells were kept either at 27°C or taken immediately after heat shock at 55°C. The cells were stained with DHR123, and H₂O₂ production was visualized by fluorescent microscopy as described in “Materials and Methods.” Cells were photographed under both fluorescent field and phase contrast. Pictures represent typical examples. Bar = 20 μm.

conditions, in which the fluorescence emission spectra of both control and PCD cells were measured using a wavelength pair designed to monitor the intracellular NAD(P)/NAD(P)H ox/redox state as described by Atlante et al., 1999 (Fig. 11). PCD, but not control cells, showed an increase in fluorescence emission close to a wavelength of 456 nm, arising from an increase in intracellular NAD(P)H concentration. The effect increased with time after heat shock. Pretreatment with ASC and SOD was found to prevent the increase in intracellular NAD(P)H fluorescence (data not shown).

Irrespective of the mechanism by which cytosolic NADH oxidation takes place, i.e. via the malate/oxaloacetate shuttle as in durum wheat (*Triticum durum*) mitochondria (Pastore et al., 2003) or via other shuttles and/or an external NADH dehydrogenase, the increase of fluorescence in Figure 11 suggests that the mitochondria were somehow impaired. As a consequence, mitochondria were isolated from both control and 4-h PCD cells and assessed with respect to their ability to generate a membrane potential ($\Delta\Psi$); this was monitored by changes in absorbance of safranin (Fig. 12A). In control mitochondria, $\Delta\Psi$ generation after addition of succinate (5 mM) was revealed by the decrease in absorbance of safranin. Generation of $\Delta\Psi$ was found to be prevented by antimycin and was abolished by the uncoupler car-

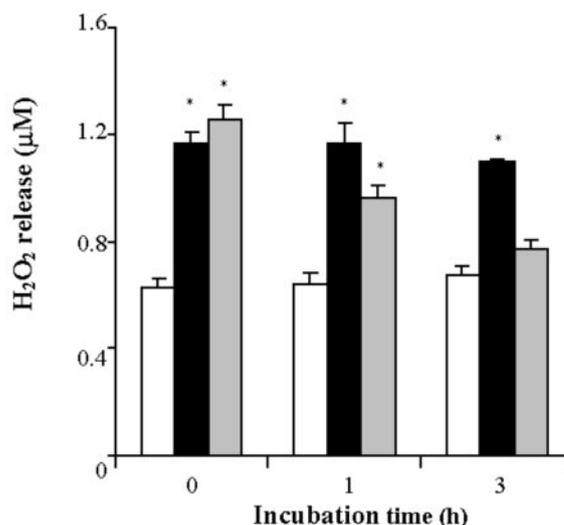


Figure 5. Release of H₂O₂ into the culture medium by TBY-2 cells. The concentration of H₂O₂ was measured in the absence (black columns) or in the presence of 0.4 mM ASC (gray columns) added to the culture medium before heat shock. The concentration of H₂O₂ in control cells (white columns) was also measured. At the indicated times, 1 mL of cell suspension (0.12 g of cells) was taken, and the concentration of H₂O₂ was determined in the medium as described in “Materials and Methods.” Values represent mean (\pm SE) of five experiments. An asterisk indicates the values that are significantly different from the control ($P < 0.01$).

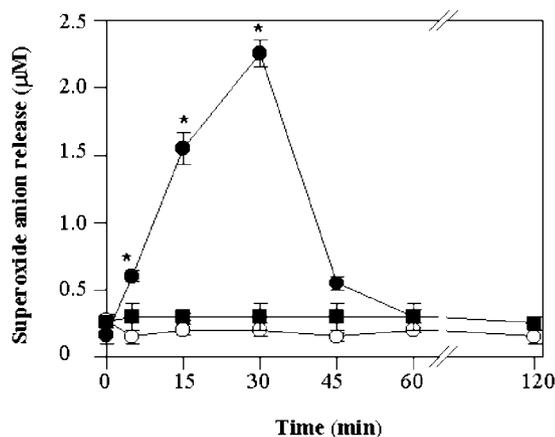


Figure 6. Release of $O_2^{\cdot-}$ into the culture medium by TBV-2 cells. The concentration of $O_2^{\cdot-}$ was measured in the absence (●) or in the presence of 75 units mL^{-1} superoxide dismutase (SOD; ■) added to the culture medium before heat shock. The concentration of $O_2^{\cdot-}$ in control cells (○) was also measured. At the times indicated, 1 mL of cell suspension (0.12 g cells) was taken, and $O_2^{\cdot-}$ concentration was determined in the medium as described in "Materials and Methods." Values represent mean (\pm SE) of four experiments. An asterisk indicates the values that are significantly different from the control ($P < 0.01$).

bonyl cyanide *p*-trifluoromethoxyphenyl-hydrazine (FCCP). In mitochondria from 4-h PCD cells, addition of succinate failed to generate a membrane potential and showed low uncoupling by FCCP (Fig. 12A). In a parallel experiment (Fig. 12B), mitochondria isolated from control and 4-h PCD cells were compared with respect to their capability to oxidize succinate in a manner controlled by ADP. In control mitochondria, measurements of oxygen uptake made in both states 4 and 3 in the presence of 0.5 mM ADP exhibited a control respiratory index (the ratio between the rate of oxygen uptake in the presence or absence of ADP) equal to about 2. Oligomycin inhibited oxygen uptake completely. The uptake was restored by addition of the uncoupler FCCP but with a further strong inhibition arising from addition of antimycin. In contrast, mitochondria isolated from PCD cells showed a decrease in state 4 respiration; more importantly, they were insensitive to addition of ADP but not to FCCP, which caused oxygen uptake at a rate equal to 12 $nmol\ min^{-1}mg^{-1}$ protein. Oxygen uptake

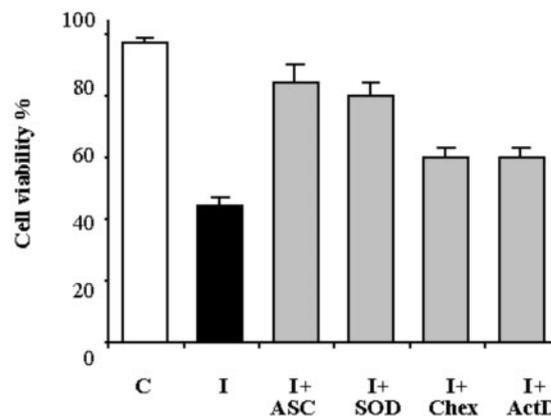


Figure 7. Effect of antioxidants and inhibitors of transcription and translation on PCD. Cell viability was measured 24 h after heat shock either in the absence (I) or presence of 0.4 mM ASC (I+ASC), 75 units mL^{-1} SOD (I+SOD), 50 $\mu g\ mL^{-1}$ Chex (I+Chex), or 10 $\mu g\ mL^{-1}$ ActD (I+ActD). Cell viability in control cells (C) was also measured. The percentage cell viability was calculated after counting a population of at least 1,000 cells; values represent mean (\pm SE) of three experiments. The effects of the added compounds were statistically significant ($P < 0.01$) in all cases.

was inhibited by antimycin (Fig. 12B). The experiments reported in Figure 12 were repeated three times, with similar results in each case.

DISCUSSION

Heat Shock Can Induce PCD in TBV-2 Cells

Plant cells respond to a variety of externally added inducers by initiating PCD (McCabe and Leaver, 2000). However, study of the role played by cell organelles in the PCD pathway is complicated by the fact that interactions between the inducer and cell organelles could occur, resulting in effects not related to PCD. We tried to overcome this complication by inducing PCD in TBV-2 cells using heat shock, as already done with other plant cells including those from carrot (*Daucus carota*), Arabidopsis (McCabe and Leaver, 2000), and cucumber (*Cucumis sativus*; Balk et al., 1999). Here, we show that TBV-2 cells, when heat shocked at 55°C, undergo PCD as judged by the common criteria of cytoplasm shrinkage, chromatin condensation, and DNA laddering (Figs. 2 and 3). This

Table 1. Time-dependent effect of heat-induced PCD on the ascorbate pool

Levels of ASC and DHA were measured in control cells (C) and in cells collected immediately (I_0) and 6 (I_6) and 24 (I_{24}) h after heat shock. Values represent mean \pm SE of five experiments. The redox state was calculated as the ratio ASC/(ASC + DHA), using mean values of the concentrations of the individual substances.

Sample	ASC	DHA	ASC + DHA	Redox State
	<i>nmoles g⁻¹ fresh wt</i>			
C	411 \pm 26	102 \pm 11	513 \pm 37	0.8
I_0	319 ^a \pm 22	157 ^a \pm 16	476 \pm 38	0.67 ^a
I_6	284 ^a \pm 25	219 ^a \pm 19	503 \pm 44	0.56 ^a
I_{24}	145 ^a \pm 20	187 ^a \pm 16	332 ^a \pm 33	0.44 ^a

^a Indicates the values that are significantly different from control ($P < 0.01$).

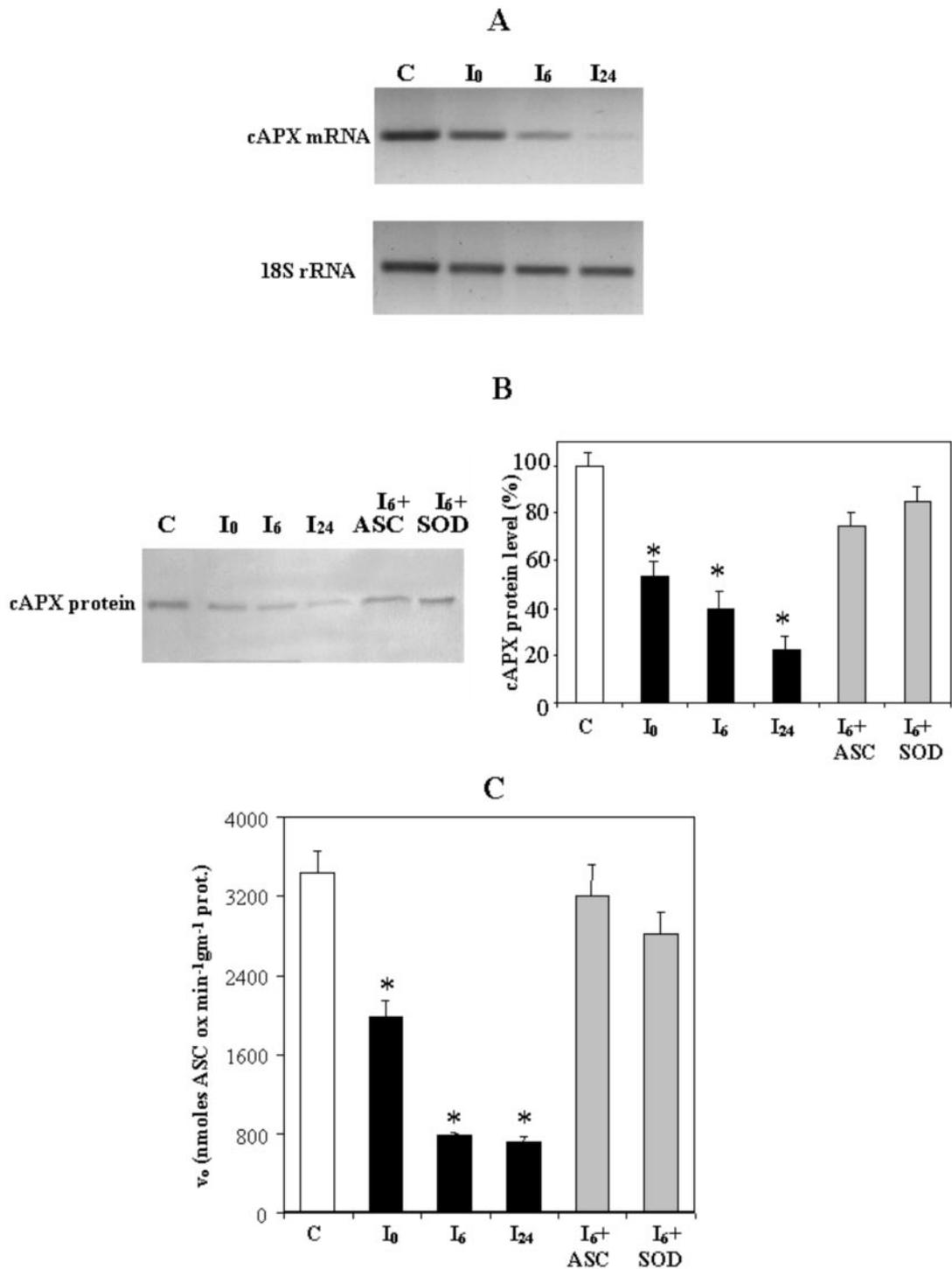


Figure 8. Levels of cAPX mRNA and protein in PCD cells and the effects of heat-dependent PCD induction on specific activity of cAPX. Cells were collected immediately (I₀), 6 h (I₆), and 24 h (I₂₄) after induction of PCD. C, Control cells. A, Total RNA was extracted, and semiquantitative reverse transcriptase (RT)-PCR for cAPX was performed as described in "Materials and Methods." Levels of 18S rRNA were also determined so that results could be normalized for recovery of RNA from the initial samples. B, Immunoblotting analysis performed using a specific cAPX antibody (see "Materials and Methods"). Protein extracts (15 μ g) were loaded in each line. Where indicated, 0.4 mM ASC and 75 units mL⁻¹ SOD were added to the cells before heat shock. After densitometry, levels of cAPX protein were calculated as a percentage of that in control cells. Values represent the mean (\pm SE) of three experiments. C, Specific activity of cAPX. Where indicated, 0.4 mM ASC and 75 units mL⁻¹ SOD were added to the cells before heat shock. Values represent the mean (\pm SE) of five experiments. An asterisk indicates the values that are significantly different from control ($P < 0.01$).

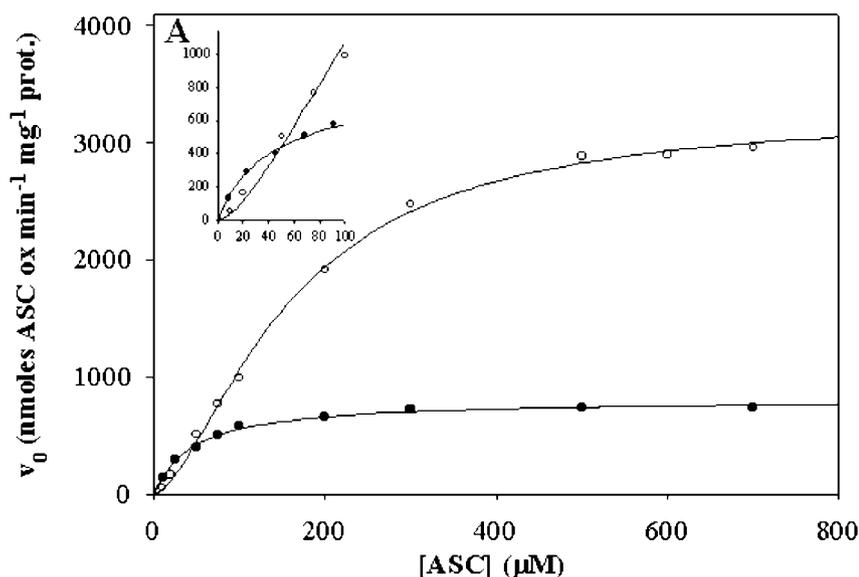
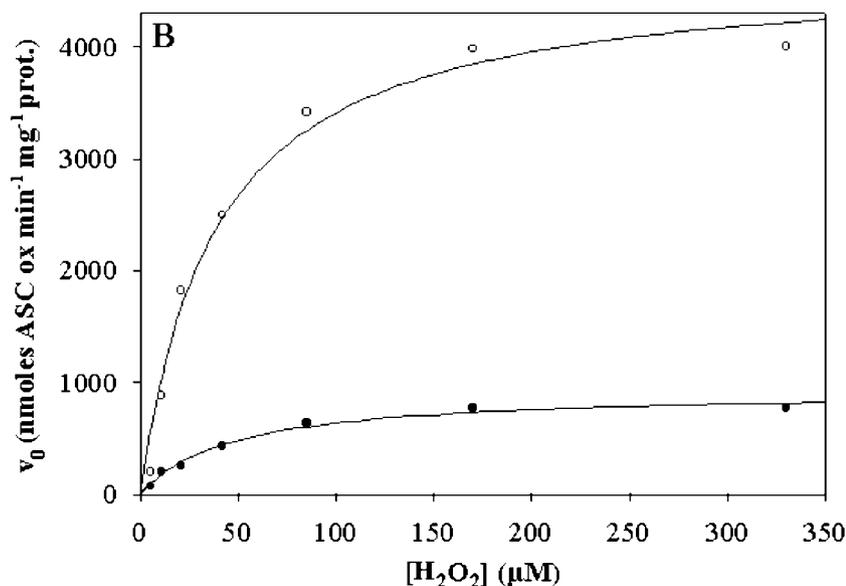


Figure 9. Kinetic behavior of cAPX in control and PCD cells. The analysis was performed in extracts from control cells (○) and from cells 6 h after the heat shock (●). A, Concentration of H_2O_2 was $170 \mu M$, and the concentration of ASC was varied over the range 10 to $700 \mu M$. B, Concentration of ASC was $500 \mu M$, and the concentration of H_2O_2 was varied over the range 5.5 to $330 \mu M$. C, V_{max} and K_m values for ASC and H_2O_2 in control and heat-shocked cells. Values represent the mean ($\pm SE$) of five experiments.



C

	V_{max}^{ASC}	$V_{max}^{H_2O_2}$	K_m^{ASC}	$K_m^{H_2O_2}$
	nmol ASC ox min ⁻¹ mg ⁻¹ prot		μM	
C	4255 ± 298	4711 ± 129	229 ± 30	38 ± 3
I ₆	809 ± 29	905 ± 39	41 ± 5	47 ± 4

provides a model for investigation of PCD-related processes and, in particular, of the possible roles played by cellular constituents and organelles in the early phase of PCD, i.e. when the viability of the cells undergoing PCD was almost unaffected.

ROS Production in Heat Shock PCD

We show that PCD cells produce dramatically increased levels of ROS. This phenomenon has been reported already for cells subject both to pathogen

attack or to abiotic stress (Lamb and Dixon, 1997; Mittler, 2002; Yao et al., 2002), but the novelty of our results is that ROS generation was induced without the addition of exogenous compounds that could, per se, alter the cellular redox homeostasis. That an increase in ROS is a prerequisite for heat-induced PCD was shown by the fact that the cells survived if the ROS scavengers ASC and SOD were present during heat shock. The results in Figures 4 to 7 imply that both H_2O_2 and $O_2^{\cdot-}$ are required for triggering the

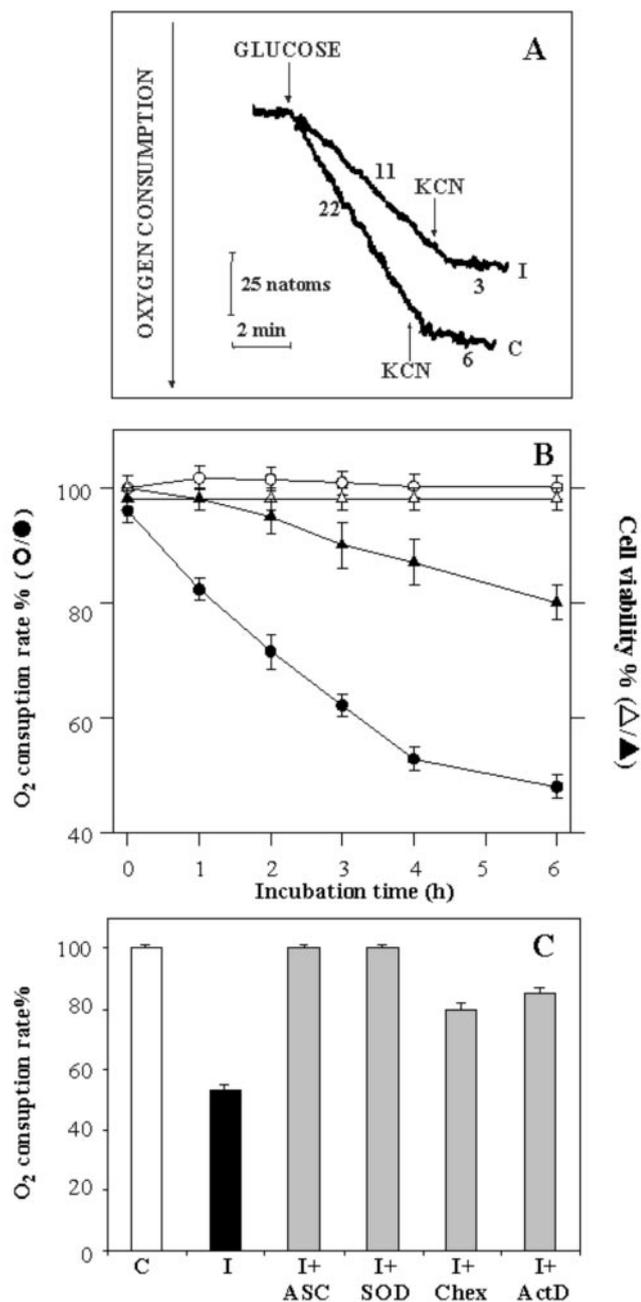


Figure 10. Oxidation of Glc in PCD-induced cells. A, Control and 4-h PCD cells (0.2 mg of protein) were incubated at 25°C in 1.5 mL of the medium reported in "Materials and Methods". At the arrows, Glc (10 mM) and KCN (1 mM) were added. The rate of oxygen uptake is expressed as nanoatoms of oxygen per minute per milligram of cell protein. B, Consumption of O₂ was monitored polarographically in 0- to 6-h PCD cells, as reported in Figure 10A. The rate of Glc-dependent oxygen consumption and the viability of both control (white symbols) and PCD (black symbols) cells were measured as a function of time and expressed as a percentage of the control. C, Glc-dependent rate of oxygen consumption was measured 4 h after heat shock in the absence (I) or in the presence of 0.4 mM ASC (I+ASC), 75 units mL⁻¹ SOD (I+SOD), 50 μg mL⁻¹ Chex (I+Chex), or 10 μg mL⁻¹ ActD (I+ActD). The rate of O₂ consumption measured in control cells (C) was set at 100. For B and C, values represent the mean (±SE) of three experiments.

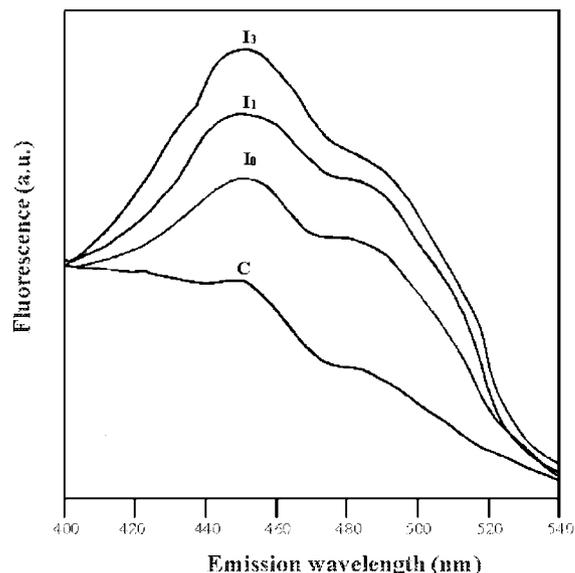


Figure 11. Fluorescence emission spectra of control and PCD cells. Fluorescence emission spectra ($\lambda_{exc}=334$; emission: $400 < \lambda < 540$) of TBV-2 cells (0.2 mg protein) were measured immediately (I₀), 1 h (I₁), and 3 h (I₃) after the heat shock and compared with the control (C). The experiment was repeated four times with different cell preparations, giving values with 5% to 10% variation. Fluorescence intensity is reported in arbitrary units (a.u.).

process of PCD; removal of either of them alone allows the cells to resist the challenge of heat shock. In the case of SOD, protection was exerted presumably by causing efflux of intracellular superoxide.

At present, the temporal connection between ROS production and the qualitative connection between H₂O₂ and superoxide must remain a matter of speculation as the ROS potential source is. Even though mitochondria are considered the main intracellular source of ROS, we cannot suggest them as responsible for the ROS production because of heat shock; this point merits further investigation.

Because partial prevention of PCD was also found when inhibitors of transcription and translation were present, we conclude that PCD in TBV-2 cells triggered by ROS is reinforced by modifications in cellular protein biosynthesis (see also Yao et al., 2002). The finding that cell death and the accompanying impairment of Glc oxidation and cAPX expression were completely prevented by antioxidants, but only partially by ActD or Chex, supports the view that the primary events of PCD are triggered by ROS, with biosynthetic events occurring downstream.

It should be noted that in the cascade of events leading to cell death, the cellular level of ROS is critical. A threshold level of ROS is required to activate the signal transduction pathway that results in PCD, but at high doses, the process is subverted and death occurs rapidly by necrosis (Desikan et al., 1998). Consistently, we show that necrotic cell death occurs as a result of heat shock at temperatures higher than 55°C

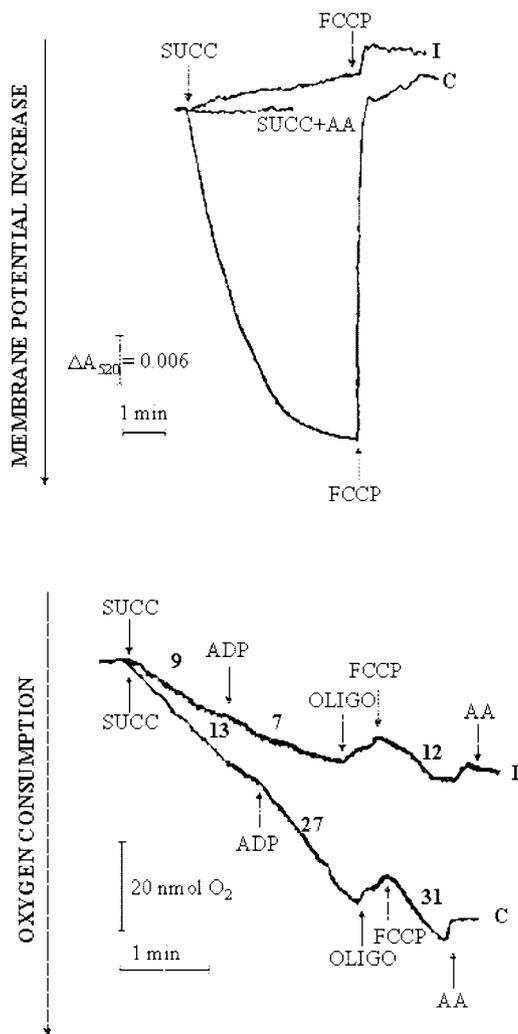


Figure 12. Generation of $\Delta\Psi$ and oxygen uptake by mitochondria isolated from control and PCD cells arising from externally added succinate. A, Mitochondria isolated from control and 4-h PCD cells (0.5 mg of protein) were incubated at 25°C in 2 mL of the medium reported in "Materials and Methods" with continuous measurement of the safranin O A_{520} . At the arrows, succinate (5 mM) and FCCP (1.25 μM) were added. When present, antimycin (AA; 2 μg) was added 1 min before succinate addition. B, Mitochondria from control and 4-h PCD cells (0.5 mg protein) were incubated at 25°C in 1.5 mL of the respiratory medium reported in "Materials and Methods," and oxygen uptake was measured polarographically as a function of time. At the arrows, 5 mM succinate, 0.5 mM ADP, 2.5 μM oligomycin (OLIGO), 1.25 μM FCCP, and 2 μg AA were added. The rate of oxygen consumption is expressed as nanomoles of O₂ per minute per milligram of mitochondrial protein.

and is accompanied by a much higher production of H₂O₂ than that in cells undergoing PCD; this is similar to the situation in mammals (Fleury et al., 2002; Atlante et al., 2003a).

Decrease in the ASC Pool and Early Impairment of cAPX in PCD

The level of ROS in a cell derives from a balance between ROS-producing and -scavenging systems.

A

As a consequence, reduced efficiency of ROS scavenging by metabolites and enzymes would be expected to play a major role in induction of PCD. Our results clearly show the importance of ASC in heat-induced PCD. Heat shock resulted in an increase in ASC oxidation, which started immediately after heat-induced PCD and continued over time (Table I). We also show that a change in the activity of cAPX is an early event in the process, with a 50% decrease in activity occurring immediately after induction of PCD. This is consistent with a role for the enzyme in maintaining ROS below threshold levels in non-PCD cells.

B

The regulation of the activity of cAPX is complex. Although there was a decrease in the amount of mRNA for cAPX in PCD cells (Fig. 8A), which was mirrored by a decrease in amount of cAPX protein present (Fig. 8B), the two phenomena are not necessarily related. The loss of protein was very substantially reduced by the presence of ROS scavengers, which suggests that it may result from oxidative damage and subsequent removal of damaged protein. This is consistent with the reported posttranscriptional suppression of cAPX in hypersensitive PCD, where cAPX gene expression is increased over normal levels (Mittler et al., 1998).

In addition to the effect on amount of enzyme present, we also show that its kinetic properties change in response to PCD (Fig. 9). The enzyme from control cells showed a sigmoidal dependence on ASC concentration, as has been reported already for cAPX purified from several sources (Lad et al., 2002, and refs. therein). In contrast, the enzyme from PCD cells showed a hyperbolic dependence on ASC concentration. This strongly suggests that the very large decrease in the V_{max} for the reaction in PCD cells arises not only from a decrease in the amount of enzyme protein but also from modifications involving substrate-enzyme interaction. It is known that cAPX from other sources possesses two ASC-binding sites (Lad et al., 2002); therefore, the loss of cooperativity in the enzyme from PCD cells suggests that the modification results in loss of one of these sites.

As far as H₂O₂ is concerned, cAPX from both control and PCD cells showed saturation characteristics, with the latter having decreased V_{max} but an unchanged K_m ; that is, there is a noncompetitive type of inhibition. In this case, we conclude that the processes resulting in modification of cAPX in PCD cells do not involve the H₂O₂ binding site interaction but cause damage to other parts of the enzyme protein. It should be noted that inactivation of purified cAPX because of H₂O₂ has been reported already, but this was under conditions of complete ASC depletion (Hiner et al., 2000).

Impairment of Energy Metabolism in PCD

We investigated certain aspects of cellular energy metabolism that might be assumed to play a key role in PCD given that the involvement of mitochondria

in oxidative stress (Jones, 2000; Tiwari et al., 2002), cytochrome c release (Balk et al., 1999), and PCD in the hypersensitive response (Lam et al., 2001) has been proposed already. We show here that Glc oxidation is impaired during PCD in a manner completely prevented by ROS scavengers (Fig. 10). It is important to note that oxidation of Glc was already impaired under conditions where cell viability was unaffected; that is, the impairment of energy metabolism is a process occurring in the early phase of PCD, when the cells are still viable. In this regard, plants resemble mammalian cells (Atlante et al., 1996; 1998). Recently, cryptogein has been shown to inhibit Glc transport in tobacco cells (Bourque et al., 2002); whether this occurs in our cells remains to be established. That notwithstanding, the increase in concentration of cellular NAD(P)H in PCD cells (Fig. 11) shows both that the cells can take up Glc and that mitochondrial NAD(P)H oxidation is impaired in PCD. Consistently, we found that mitochondria isolated from 4-h PCD cells were unable to generate a membrane potential or to increase oxygen consumption in state 3 respiration (Fig. 12). Because mitochondrial coupling appeared to be completely impaired when cell viability was almost unaffected, we conclude that mitochondrial damage occurs in the early phase of PCD.

The picture emerging from this work is, then, as follows. After heat shock, as a result of the immediate production of ROS, the death program starts and evokes biosynthetic processes, imbalance in ASC-dependent H₂O₂ scavenging, and impairment of oxidative mitochondrial metabolism as early events. In addition to genetic control, changes in enzyme kinetics are suggested to contribute to regulation of PCD, thus integrating the regulatory mechanisms acting at transcriptional and posttranscriptional levels.

MATERIALS AND METHODS

Cell Culture, Growth Conditions, and Heat Treatments

The suspension of tobacco (*Nicotiana tabacum* L. cv Bright-Yellow 2) cells was routinely propagated and cultured at 27°C according to Nagata et al. (1992). In this work, a stationary culture was diluted 4:100 (v/v) and cultured for 4 d. Heat shock at 55°C was induced by transferring the cell suspension into a water bath at 55°C with a heating time of either 5 or 10 min depending on the cell suspension volume (20 or 100 mL, respectively). After heat shock, the cells were returned to 27°C. Where indicated, ASC (0.4 mM), SOD (75 units mL⁻¹), Chex (50 µg mL⁻¹), or Act D (10 µg mL⁻¹) were added to the culture medium 30 min before heat shock. At the indicated times, aliquots of cells were collected by filtration on Whatman 3MM paper (Whatman, Clifton, NJ) for analysis. Heat treatments at the other temperatures (37°C, 45°C, 60°C, and 65°C) were carried out under the same experimental conditions. Assay of cell protein was done according to Bradford (1976).

Cell and Nuclear Morphology

Cell viability was measured using trypan blue staining as described by de Pinto et al. (1999), and cell morphology was investigated by using a phase contrast light microscope.

For the analysis of nuclear morphology, TBY-2 cells were stained with 4,6-diamidino-2-phenylindole (DAPI) as in de Pinto et al. (2002) and visu-

alized using a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 340 to 380 nm and a barrier filter of 400 nm.

DNA Fragmentation Analysis

At time intervals over a 72-h period after the heat shock, cells were collected from cell suspension and homogenized in liquid nitrogen. DNA was extracted by the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). DNA samples were digested with 100 µg mL⁻¹ DNase-free RNase for 1 h at 37°C, and DNA fragments were separated by electrophoresis on a 1.8% (w/v) agarose gel, followed by visualization by staining with ethidium bromide.

Measurement of H₂O₂

Intracellular H₂O₂ production was measured using DHR123 (Sigma-Aldrich Italia, Milan) as a probe (Royall and Ischiropoulos, 1993). The cells were stained for 5 min with 20 µM DHR123 and then viewed under a fluorescence microscope (DMLS, Leica) with an excitation filter of 450 to 490 nm and a barrier filter of 510 nm.

H₂O₂ in the extracellular phase was measured according to Bellincampi et al. (2000). In brief, 1 mL of cell cultures was harvested by centrifugation (10,000g, 20 s, 25°C), and H₂O₂ concentration was measured in the supernatant. An aliquot of supernatant (500 µL) was added to 500 µL of assay reagent (500 µM ferrous ammonium sulfate, 50 mM H₂SO₄, 200 µM xylenol orange, and 200 mM sorbitol). After 45 min of incubation the peroxide-mediated oxidation of Fe²⁺ to Fe³⁺ was determined by measuring the A₅₆₀ of the Fe³⁺-xylenol orange complex.

Measurement of O₂⁻

The detection of O₂⁻ in the extracellular phase was performed by using the nitroblue tetrazolium method (Murphy et al., 1998). In brief, 1 mL of cell cultures was harvested by centrifugation (10,000g, 20 s, 25°C), and O₂⁻ concentration was measured in the supernatant by monitoring the reduction of nitroblue tetrazolium (100 µM) at 530 nm. SOD (100 units) was added in a duplicate cuvette to access the specificity of measured changes of absorbance on O₂⁻ concentration. The amount of O₂⁻ was then calculated using ε_{530 nm} = 12.8 mM⁻¹ cm⁻¹ (Murphy et al., 1998).

Total RNA Extraction and Semiquantitative RT-PCR

Total RNA was isolated from TBY-2 cells using the RNeasy plant minikit (QUIAGEN S.p.A., Milan) according to the supplier's recommendation. Residual DNA was removed from the RNA samples using a DNA-free kit (Ambion, Inc., Austin, TX). Synthesis of cDNA was performed from 2 µg of total RNA with 10 µM random primers (Amersham Biosciences Europe GMBH, Milan), utilizing an Omniscript Reverse Transcriptase kit (QUIAGEN S.p.A.) according to the supplier's recommendation. PCR reactions were performed with specific primers for cAPX (cAPX, D85912, 5'-CACTGTAAGCGAGGAGTACC-3' and 3'-TGAGCCTCAGCATAGTC-AGC-5') and 18S rRNA (18S, AJ236016, 5'-CATGATAAAGTCGACGG-ATCG-3' and 3'-GAAGGCCAACGTAATAGGAC-5'). 18S rRNA was used as an internal control to normalize each sample for variations in the amount of initial RNA. The PCR mix contained 2 µL of the template, 0.2 mM dNTPs, 0.5 mM primers (forward and reverse), 1.5 units of *Taq* polymerase (Amersham Biosciences Europe GMBH), and 1× PCR buffer (supplied from Amersham with *Taq* polymerase) in a final volume of 50 µL. PCR was carried out in a programmable Primus Thermal Cycler (MWG-AG Biotech, Ebersberg, Germany) at an annealing temperature of 55°C. For semiquantitative RT-PCR, the cycle number in the linear range was empirically determined. The products of PCR amplification produced a single band at the predicted sizes of 699 and 594 bp for cAPX and 18S, respectively. These were analyzed on 1.5% (w/v) agarose gel containing 0.5 µg mL⁻¹ ethidium bromide.

Immunoblot Analysis

Cells were ground in liquid nitrogen and homogenized at 4°C in extraction buffer (50 mM Tris-HCl [pH 7.5], 0.05% [w/v] Cys, and 0.1% [w/v]

bovine serum albumin). The homogenate was centrifuged at 20,000g for 15 min, and the supernatant was electrophoresed on 12.5% (w/v) SDS-polyacrylamide gel under reduction conditions. Immunoblotting was carried out using anti-cAPX monoclonal antibody (AP6 from Saji et al., 1990) and with goat anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, WI) as previously described by de Pinto et al. (2002). Densitometry values for immunoreactive bands were quantified using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA); multiple expressions of the same immunoblots in a linear range were performed. cAPX protein levels were calculated as a percentage of the control taken as 100 in arbitrary units. The variability of protein levels in the control cells, measured by comparing the percentage of protein level from different control samples on the same filter, was always less than 5%.

Activity and Kinetics of cAPX

Cells were homogenized and centrifuged as reported by de Pinto et al. (2002). The supernatant was used for analysis of cAPX activity. The absence of ASC in the extraction buffer made it possible to determine the cytosolic isoenzyme alone (Shigeoka et al., 2002). The reaction catalyzed by cAPX (L -ASC; H_2O_2 oxidoreductase; EC 1.11.1.11), was determined by following the H_2O_2 -dependent oxidation of ASC as decrease in A_{290} in a reaction mixture containing 0.1 M phosphate buffer (pH 6.4), 500 μ M ASC, 170 μ M H_2O_2 , and 50 μ g of protein. The extinction coefficient (ϵ) was found to be 2.8 $mm^{-1} cm^{-1}$. The rate of the reaction, measured as a tangent to the initial part of the progress curve, was corrected for spontaneous ASC oxidation measured in the absence of H_2O_2 and for nonenzymatic ASC oxidation by H_2O_2 and expressed in nanomoles ASC oxidized per minute per milligram of protein. The values for V_{max} and K_m were obtained by either Michaelis-Menten or Hill analysis using Sigma Plot software (SPSS Inc., Chicago).

ASC Assay

Cells (0.5–1 g) were homogenized in two volumes of cold 5% (w/v) meta-phosphoric acid at 4°C in a porcelain mortar. The homogenate was centrifuged at 20,000g for 15 min at 4°C, and the supernatant was collected for analysis of ASC. ASC content and redox state were measured as described by de Pinto et al. (1999).

Measurement of Oxygen Uptake by TBY-2 Cells

One milliliter of either control or PCD cell suspension was taken at the indicated times and centrifuged at 360g for 5 min at 25°C; the resulting pellet was washed and suspended in 1 mL of plant salt mixture (PSM) (4.3 g L^{-1} Murashige and Skoog plant salt mix [ICN Biomedicals Inc., Aurora, OH], and 0.4 mM KH_2PO_4 [pH 5.8]). The cells (0.2 mg of protein) were then incubated at 25°C in 1.5 mL of 0.5 M Tris-acetate (pH 6.5), and oxygen uptake was started by adding 10 mM Glc to the cell suspension. Oxygen consumption was measured by means of a Gilson 5/6 oxygraph (Gilson Medical Electronics Inc., Middletown, WI) using a Clark electrode according to Atlante et al. (1996). The rate of oxygen consumption was obtained as a tangent to the initial part of the progress curve and expressed as nanoatoms of oxygen per minute per milligram of protein.

Fluorescence Emission Spectra of TBY-2 Cells

Cell suspension (1 mL) was taken at the indicated times, centrifuged (360g, 5 min, 25°C), and the resulting pellets were washed and suspended in 1 mL of PSM. The cells (0.2 mg of protein) were then incubated at 25°C in 1.5 mL of PSM, and fluorescence emission spectra (excitation wavelength 334 nm, emission range 400–540 nm) were recorded using an LS50B Luminescence Spectrophotometer (Perkin-Elmer Applied Biosystems, Foster City, CA).

Isolation of Mitochondria from TBY-2 Cells and Measurement of Their Oxygen Uptake

Mitochondria were isolated by protoplast fractionation and lysis, followed by differential centrifugation essentially as described by de Pinto et al. (2000).

Oxygen uptake measurements were carried out at 25°C using a Gilson 5/6 oxygraph with a Clark electrode. Mitochondria (0.5 mg protein) were added to 1.5 mL of the respiration medium containing 210 mM mannitol, 70 mM sucrose (Suc), 20 mM TRIS-HCl, 5 mM potassium phosphate (pH 7.4), 3 mM $MgCl_2$, and 5 mg mL^{-1} bovine serum albumin in the presence of 2 μ g of rotenone. Succinate (5 mM) was used as a respiratory substrate, and either ADP (0.5 mM) or FCCP (1.25 μ M) were added to induce state 3 respiration.

Assay of the Safranin Response

This was done at 25°C, essentially as in Pastore et al. (1999), by measuring absorbance changes at 520 nm using a Perkin-Elmer Applied Biosystems Lambda-5 spectrophotometer. Measurements were carried out at 25°C with 2 mL of standard medium consisting of 300 mM Suc, 10 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES-Tris (pH 7.2) containing 10 μ M safranin O and 1 mg of protein.

Statistics

The data are reported as the mean \pm SE for the indicated experiments. The statistical significance of differences between groups was determined by one-way ANOVA followed by a Student-Newman-Keuls test. Statistical differences between mean values of control and treated cells were determined with the Student's *t* test. All experiments were repeated at least three times.

ACKNOWLEDGMENTS

The authors wish to thank Prof. Shawn Doonan for his critical reading and Mr. Riccardo S. Merafina for technical assistance. The authors thank Dr. Akihiro Kubo (Environmental Biology Division National Institute for Environmental studies, Onogawa, Japan) for kindly supplying ASC peroxidase antibody.

Received November 7, 2003; returned for revision December 9, 2003; accepted December 16, 2003.

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