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Hydrogen peroxide, nitric oxide and cytosolic ascorbate peroxidase at the crossroad between defence and cell death

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Summary

An increase in the production of reactive oxygen species (ROS) is a typical event occurring during different stress conditions and activating conflicting responses in plants. In order to investigate the relevance of different timing and amounts of ROS production, tobacco (*Nicotiana tabacum*) Bright Yellow-2 (TBY-2) cells were incubated with different amounts of glucose plus glucose oxidase, for generating H_2O_2 during time, or directly with known amounts of H_2O_2. Data presented here indicate that, in TBY-2 cells, a difference in H_2O_2 level is a critical point for shifting metabolic responses towards strengthening of antioxidant defences, or their depletion with consequent cell death. Timing of ROS production is also critical because it can determine programmed cell death (PCD) or necrosis. Depending on the different kinds of activated cell death, ascorbate (ASC) and glutathione (GSH) pools are altered differently. Moreover, an H_2O_2-dependent activation of nitric oxide synthesis is triggered only in the conditions inducing PCD. Ascorbate peroxidase (APX) has been analysed under different conditions of H_2O_2 generation. Under a threshold value of H_2O_2 overproduction, a transient increase in APX occurs, whereas under conditions inducing cell necrosis, the activity of APX decreases in proportion to cell death without any evident alteration in APX gene expression. Under conditions triggering PCD, the suppression of APX involves both gene expression and alteration of the kinetic characteristics of the enzyme. The changes in ASC, GSH and APX are involved in the signalling pathway leading to PCD, probably contributing to guaranteeing the cellular redox conditions required for successful PCD.

Keywords: ascorbate peroxidase, programmed cell death, necrosis, hydrogen peroxide, nitric oxide, tobacco Bright Yellow-2 cells.

Introduction

Reactive oxygen species (ROS) are produced by all aerobic organisms as inevitable by-products of several metabolic pathways, including electron flows in mitochondria and chloroplasts, lipid catabolism and photorespiration in glyoxysomes and peroxisomes, as well as enzymatic oxygenase reactions having a different cellular localization. In order to avoid ROS toxicity, aerobic cells are provided with a flexible set of enzymes and metabolites involved in ROS catabolism, which often acts at the site of ROS production (Foyer, 2004; Mittler *et al.*, 2004; Shigeoka *et al.*, 2002). Despite much metabolic energy being spent on ROS removal by plant cells, ROS are also actively produced by cell metabolism under optimal growth conditions. In particular, hydrogen peroxide is required for biosynthesis and developmentally related modifications of the structural components of cell walls (Ros Barcelò *et al.*, 2004). An increasing body of data suggests that ROS also behave as second messengers. In bacteria, yeasts and animals, alterations in ROS production modify redox-sensitive transcription factors, which activate metabolic pathways involved in ROS-scavenging (Catani *et al.*, 2001; Costa and Moradas-Ferreira, 2001; Georgiou, 2002). Redox-sensitive regulation of gene expression has also been described in plants (Mou *et al.*, 2003), as well as the presence of MAPK pathways and

some phosphatases, similar to those involved in animal ROS-dependent signalling (Apel and Hirt, 2004). Eukaryotic organisms also exploit ROS toxicity for their defence: specific enzymatic systems producing ROS are activated by opportune stimuli in both plant and animal cells (Desikan *et al.*, 1996; Diebold and Bokoch, 2005; Hu *et al.*, 2003; Walters, 2003).

In plant cells, ROS overproduction is induced by biotic and abiotic stresses. Survival under these conditions depends on the capability of plants to increase specific pathways involved in ROS removal (Asada, 1999; Noctor and Foyer, 1998). Recent studies utilizing knockout and antisense plant cell lines or organisms further underline the strong link existing between ROS-removal enzymes and defence responses against stresses (Mittler *et al.*, 2004). ROS overproduction is also an early event in plant programmed cell death (PCD; Dat *et al.*, 2003; Levine *et al.*, 1994).

During the activation of PCD, cytosolic ascorbate peroxidase (APX), a pivotal enzyme for ROS removal, strongly decreases as do ascorbate (ASC) and glutathione (GSH), metabolites of crucial relevance for cellular redox balance (de Pinto *et al.*, 2002; Vacca *et al.*, 2004). These changes in cellular antioxidant capability probably contribute to the ROS increase that generates the oxidative burst characterizing PCD. However, the expression/activity of APX is also regulated downstream by ROS (de Pinto *et al.*, 2002), suggesting that complicated cause–effect relations occur between APX–ASC and ROS levels.

It is known that changes in ROS production may activate contrasting responses: an increase or a decrease in ROS-scavenging systems, which will guarantee cell survival or induce cell death, respectively, according to the situation faced by the plant (Mittler, 2002). Recently, it has been underlined that metabolic changes triggered in order to improve cell fitness under specific conditions are the result of a network of different stimuli, rather than a response to a unique stimulus (Trewavas, 2005). The signal transduction pathways are themselves a network of reactions in which thousands of second messengers, protein kinases and other molecules generate an information flow that diverges, adapts, synergizes and integrates in order to optimize the final response (Taylor and McAinsh, 2004).

It has been reported consistently that PCD requires the simultaneous presence of hydrogen peroxide and nitric oxide, both *in planta* and in cultured cells (Delledonne *et al.*, 1998; de Pinto *et al.*, 2002). Nevertheless, contrasting data on the simultaneous requirements of the different reactive species are also present in the literature. Both in tobacco and Arabidopsis cells, PCD has been activated by single treatments of hydrogen peroxide or nitric oxide (Clarke *et al.*, 2000; Houot *et al.*, 2001).

Despite several papers underlining the pleiotropic effects of ROS overproduction, no study has attempted to evaluate whether ROS generation in different amounts, or with different rates and times, might provoke different effects not merely due to a dose-dependent result, but as a consequence of the activation of diverging metabolic pathways.

To obtain further information on the relevance of timing and intensity of oxidative stress for PCD induction, tobacco cultured cells were subjected to treatments giving rise to an increase in hydrogen peroxide during different times and in different amounts. The results obtained demonstrate that the metabolic responses activated in cells differ greatly depending on both the intensity of the oxidative stress generated and the different timing of ROS production.

Results

Hydrogen peroxide and cell death

To study the effects of different amounts and timing of H_2O_2 production in the responses activated by plant cells, glucose (G) plus different concentrations of glucose oxidase (GOX) or different amounts of H_2O_2 were added to the culture medium of TBY-2 cells.

In the culture medium without cells, the GGOX system generated H_2O_2 at a constant rate for the first 2–3 h. H_2O_2 remained at a constant concentration in the culture medium for the following 2 h, after which it decreased progressively (data not shown). When GGOX was added to the TBY-2 cell suspension, the level of H_2O_2 in the culture medium was always lower than that observed when the enzymatic system was added to the medium without cells, probably because a certain amount of ROS was scavenged by the cells. In cell suspension, the H_2O_2 reached its maximum level 15 min after the beginning of the GGOX treatment and then decreased (Figure 1a,c). After direct addition of H_2O_2 for 15 min, the reactive species were present in the culture medium with concentrations proportional to those added (Figure 1b). At this stage of treatment, the addition of 50 mM H_2O_2 determined H_2O_2 concentration in the cell suspension medium comparable with those obtained by the highest GGOX treatment. However, under direct H_2O_2 addition, the decrease in H_2O_2 over time was much faster than that observed under GGOX treatment (Figure 1d).

The viability of TBY-2 cells subjected to the different treatments was analysed (Figure 2). The lowest amount of GOX (0.75 U ml^{-1}) did not induce cell death, whereas when higher GOX units were used, dose-dependent cell death was clearly evident. The highest effect was observed after 6 h treatment, independently from the amount of GOX used (Figure 2a). As the GGOX system generates D-gluconolactone as well as H_2O_2, the dependence of cell death on H_2O_2 was verified by the addition of catalase (CAT) to the cell suspension a few minutes before starting the GGOX treat-

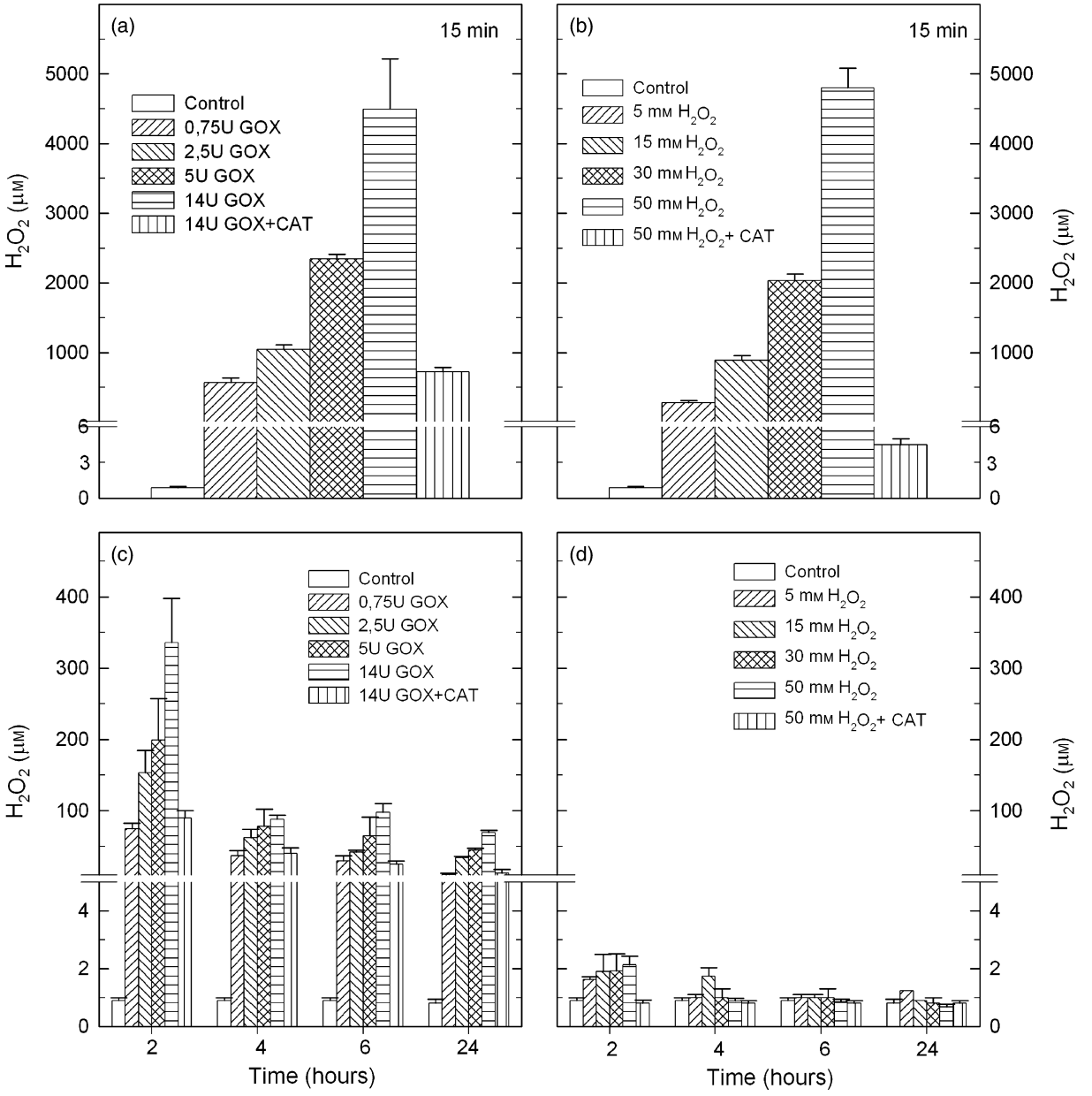


Figure 1. Presence of H_2O_2 in the culture medium of tobacco BY-2 cells.
H_2O_2 concentration was measured over 24 h in the cell suspension in the absence (control) or after the addition of 14 mM glucose (G) plus 0.75, 2.5, 5, 14 U ml^{-1} glucose oxidase (GOX) or 5–50 mM H_2O_2. At the times indicated, 1 ml cell suspension was taken and the concentration of H_2O_2 in the medium determined as described in Experimental procedures. Values represent mean (±SE) of five experiments.
(a, b) Concentrations of H_2O_2 after 15 min treatment with G + GOX or H_2O_2, respectively; (c, d) concentrations of H_2O_2 in the following 24 h treatment with G + GOX or H_2O_2, respectively.

ment. The addition of up to 30 U ml^{-1} CAT was not able to remove completely the H_2O_2 produced by GGOX, as a certain amount of the H_2O_2 produced remained in the cell suspension (Figure 1a,b). In the presence of CAT, GGOX-dependent cell death was reduced to about 80% (Figure 2a), in agreement with the inability of CAT pre-treatment to avoid H_2O_2 generation completely. The direct addition of H_2O_2 to the cell suspension also induced cell death with a dose-dependent behaviour (Figure 2b). During the first 6 h of treatment, 50 mM H_2O_2 induced a decrease of cell viability comparable with that caused by the highest GOX addition (14 U ml^{-1}). However, under direct H_2O_2 addition, the intensity of cell death increased progressively throughout the period analysed (Figure 2b). The viability of the H_2O_2-treated cells was completely preserved by CAT pre-treatment (Figure 2b), consistent with the very rapid and complete removal of the added H_2O_2 by CAT pre-treatment (Figure 1b,d). The addition of glucose to the cell suspension

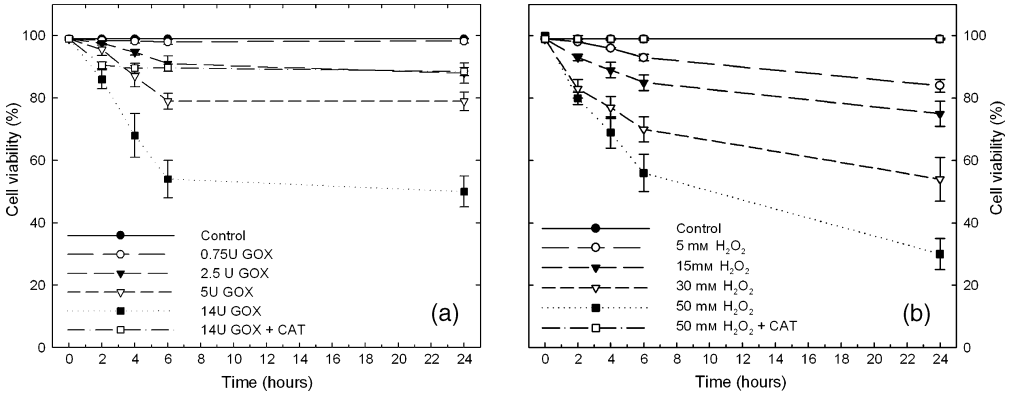


Figure 2. Time-dependent effect of H_2O_2 production on cell viability.
Viability of control and treated cells was measured as reported in Experimental procedures.
(a) Glucose plus 0.75, 2.5, 5, 14 U ml^{-1} glucose oxidase; (b) 5–50 mM H_2O_2. Where indicated, catalase (30 U ml^{-1}) was added to the culture medium 10 min before treatment. The percentage (±SE) of viable cells was counted in a population of at least 1000 cells in five independent experiments.

did not alter the intensity of cell death induced by direct H_2O_2 addition (data not shown).

Some cytological markers were analysed to obtain information on which kind of cell death (necrosis or PCD) was induced by the different treatments. Figure 3 shows that only the direct addition of H_2O_2 induced cytoplasm shrinkage, a typical marker of plant PCD, in almost 80–90% of the Trypan blue-dyed cells (Figure 3c). No cellular shrinkage was evident in control cells or in those treated with G plus 14 U ml^{-1} GOX (Figure 3a,b). DAPI-staining cells also showed the presence of chromatin condensation and apoptotic-like nuclei (Houot *et al.*, 2001) only in the cells directly treated with H_2O_2 (Figure 3f); whereas, nuclei from control and GGOX-treated cells had normal morphology (Figure 3d,e). Moreover, DNA laddering was evident in the cells directly treated with H_2O_2 but not in those subjected to GGOX treatment (Figure 4).

Nitric oxide production

As the simultaneous production of H_2O_2 and NO appears to be required for the activation of PCD in TBY-2 cells (de Pinto *et al.*, 2002), the capability of the two different treatments (H_2O_2 generation by G plus 14 U ml^{-1} GOX versus direct 50 mM H_2O_2 addition) to induce NO production was investigated. The analysis of nitric oxide synthase (NOS) activity indicated that the enzyme was active only in cells treated with H_2O_2 and not in those treated with GGOX (Figure 5a). Production of NO was also quantified spectrofluorimetrically by means of the NO-specific probe DAF-2DA (Foissner *et al.*, 2000). The results obtained clearly indicate that this reactive species was produced under H_2O_2 treatment but not under GGOX (Figure 5b). The increase in fluorescence occurring in the H_2O_2-treated cells was specifically due to NO, as it was strongly reduced by the NO scavenger cPTIO

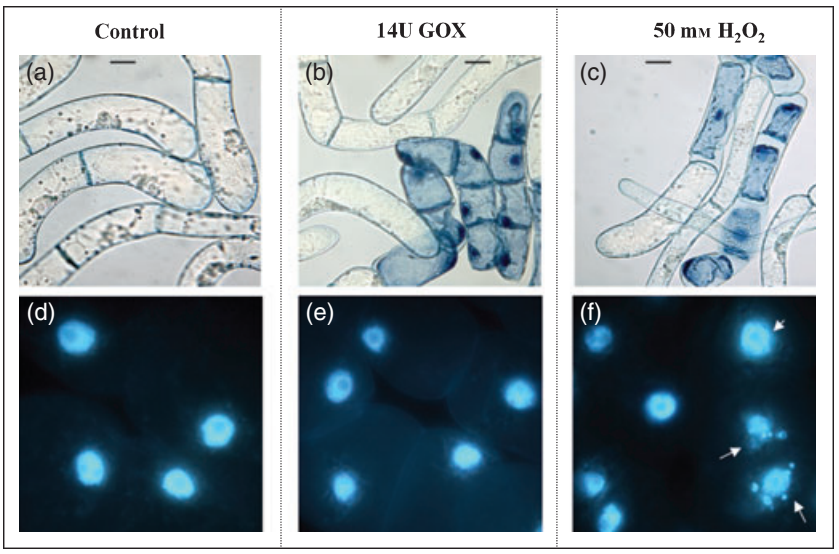


Figure 3. Cytological analysis of GGOX- or H_2O_2-treated cells.
Cell suspensions were treated with 14 mM glucose (G) plus 14 U ml^{-1} glucose oxidase (GOX) or 50 mM H_2O_2. Aliquots of control and treated cells were collected, stained with trypan blue or DAPI dyes, and visualized under phase-contrast microscopy (a–c) or fluorescence microscopy (d–f). Pictures represent typical examples after 6 h treatment: (a, d) control cells; (b, e) G + GOX-treated cells; (c, f) H_2O_2-treated cells. Bar, 20 μm. Arrows and arrowheads indicate apoptotic-like nuclei and chromatin condensation, respectively.

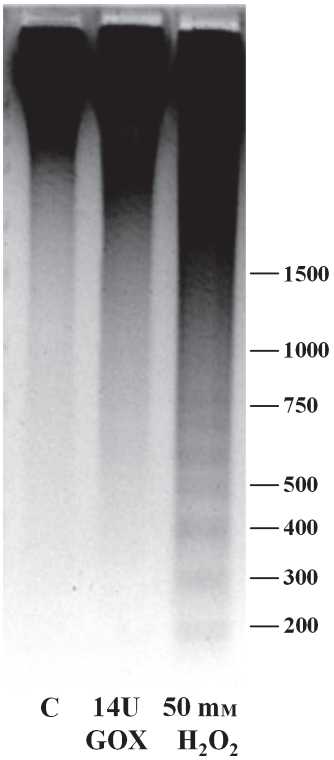


Figure 4. DNA analysis.
DNA was extracted from cells collected 24 h after treatment with 14 mM glucose plus 14 U glucose oxidase or 50 mM H_2O_2. A representative electrophoretic run in which 100 μg DNA was loaded in each line.

(Figure 5b). Interestingly, the addition of cPTIO to the H_2O_2-treated cells was also able to prevent PCD (Figure 5c). The capability of H_2O_2 to induce NO production was also evident when treated cells were observed under a florescent light microscope. At the beginning of H_2O_2 treatment all cells produced NO, whereas after 2 h only the cells that were still alive showed a clear NO-dependent green fluorescence (Figure 6).

Cellular antioxidants and cytosolic ascorbate peroxidase

To analyse the cellular redox state determined by different treatments, the ASC and GSH pools (reduced plus oxidized forms) were determined during the first 6 h of treatment. The ASC pool decreased significantly both under GGOX treatment, inducing necrosis, and under H_2O_2 addition, inducing PCD. However, the decrease was much more severe in cells undergoing PCD (direct addition of H_2O_2), with an 80% decrease in the first 2 h versus a 25% decrease under GGOX treatment (Figure 7a). The two treatments caused still more different effects on GSH. The continuous H_2O_2 production (GGOX treatments) induced an increase in the GSH pool, whereas direct addition of H_2O_2 caused a strong decrease in the GSH pool (about 75% during the first 4 h; Figure 7b). Nevertheless, direct addition of H_2O_2 and GGOX treatment induced similar decreases in the ASC and GSH redox state (ratio between reduced and reduced plus oxidized forms). During the first 2 h of treatment the ASC redox state decreased from 0.87 to 0.62 and 0.56 in GGOX- and H_2O_2-treated cells, respectively, and the GSH redox state from 0.98 to 0.83 and 0.81 in GGOX- and H_2O_2-treated cells, respectively.

The behaviour of cytosolic APX, one of the main ROS-scavenging enzymes in plant cells, was analysed during the different treatments. APX activity was increased transiently when GOX was added at the lowest concentration (0.75 U ml^{-1}; Figure 8a). This increase was small but statis-

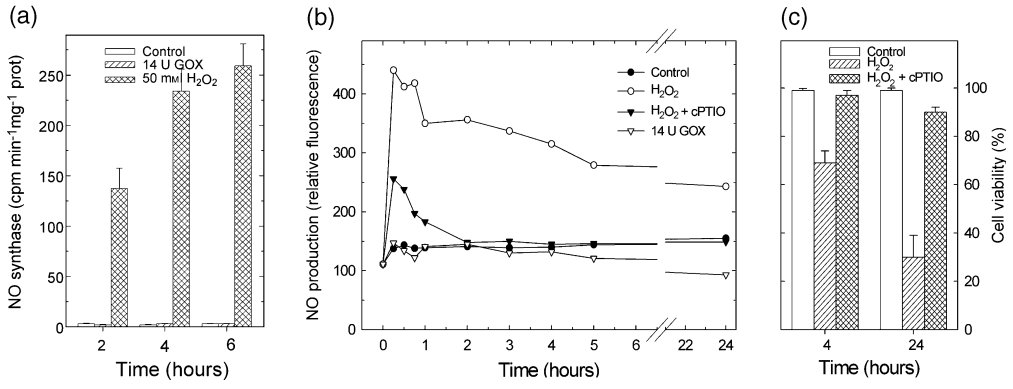


Figure 5. Nitric oxide production by tobacco BY-2 cells in response to glucose + glucose oxidase (G + GOX) or H_2O_2 treatments and its effect on cell viability.
(a) Nitric oxide synthase: enzyme activity was measured in control cells and in cells treated with 50 mM H_2O_2 or 14 mM G plus 14 U ml^{-1} GOX 2, 4 and 6 h after treatments. Values represent mean ± SE of five experiments.
(b) Nitric oxide production: fluorescence emission of TBY-2 cells was measured at the times indicated in cells loaded with DAF-2DA then treated with 14 mM G plus 14 U ml^{-1} GOX or 50 mM H_2O_2, and control cells (loaded with DAF-2DA without any subsequent treatments). Where indicated, cell suspensions were pre-incubated for 10 min with 500 μM carboxy-PTIO, an NO-scavenger, before treatment. Results are the mean of three independent experiments; variation in values obtained for the same treatments was in the range 5–10%.
(c) Cell viability of H_2O_2-treated cells in the absence or presence of 500 μM carboxy-PTIO. Percentage (±SE) of viable cells was counted in a population of at least 1000 cells in three independent experiments.

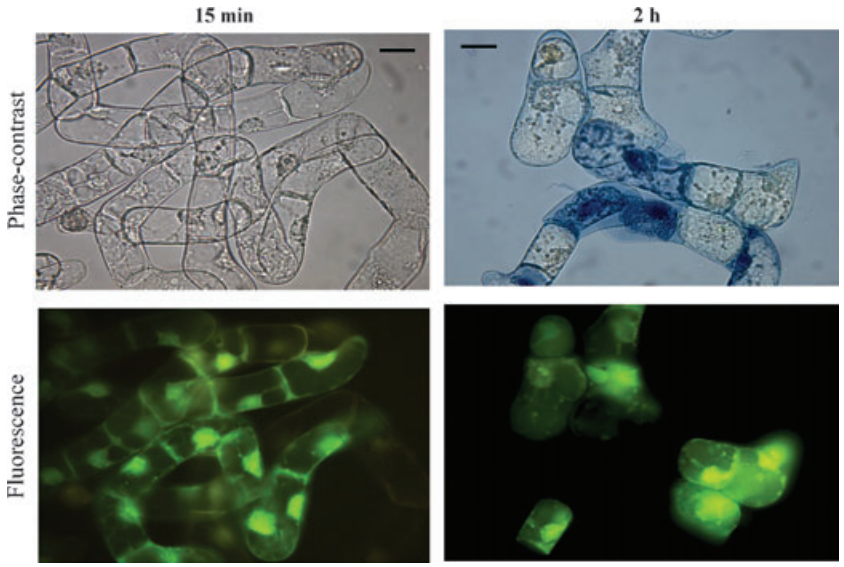


Figure 6. NO accumulation induced by H_2O_2 in tobacco BY-2 cells.
Cells were pre-loaded with DAF-2DA and treated with 50 mM H_2O_2. At the times indicated, 1 ml cell suspension was treated with 0.4% trypan blue and examined by microscope. Representative phase-contrast and fluorescence images of H_2O_2-treated cells are reported in upper and lower panels, respectively. Bar, 20 μm.

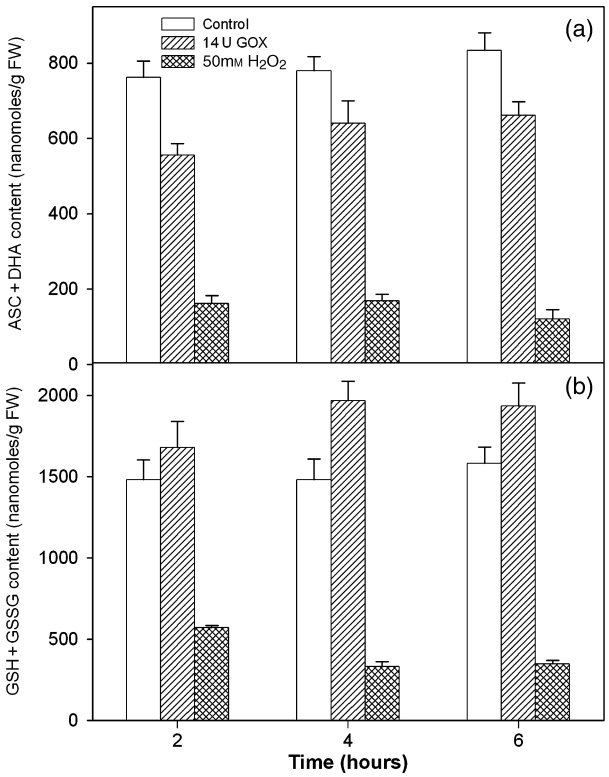


Figure 7. Changes in ascorbate (ASC) and glutathione (GSH) content induced by glucose + glucose oxidase (G + GOX) and H_2O_2 treatments.
Control and treated cells (14 mM G plus 14 U ml^{-1} GOX or 50 mM H_2O_2) were collected at the times indicated and used for the determination of the total ASC (a) or GSH (b) pools (reduced plus oxidized forms) as reported in Experimental procedures. Values represent means (±SE) of three experiments.

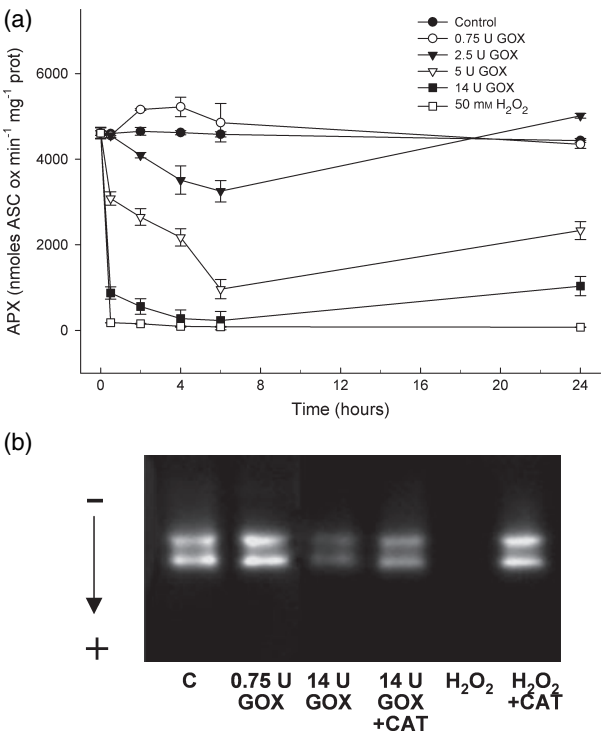


Figure 8. Effects of glucose + glucose oxidase (G + GOX) or H_2O_2 treatments on ascorbate peroxidase (APX) activity.
(a) Specific activity of APX was measured in control and treated cells (14 mM G plus 0.75, 2.5, 5, 14 U ml^{-1} GOX or 50 mM H_2O_2) during time. Values represent means (±SE) of five experiments.
(b) Representative native PAGE of APX from tobacco BY-2 cells treated for 2 h with 14 mM G plus 0.75 U ml^{-1} GOX, 14 U ml^{-1} GOX or 50 mM H_2O_2. Where indicated, cell suspensions were pre-treated with catalase (30 U ml^{-1}). For each treatment, 300 μg total protein was loaded.

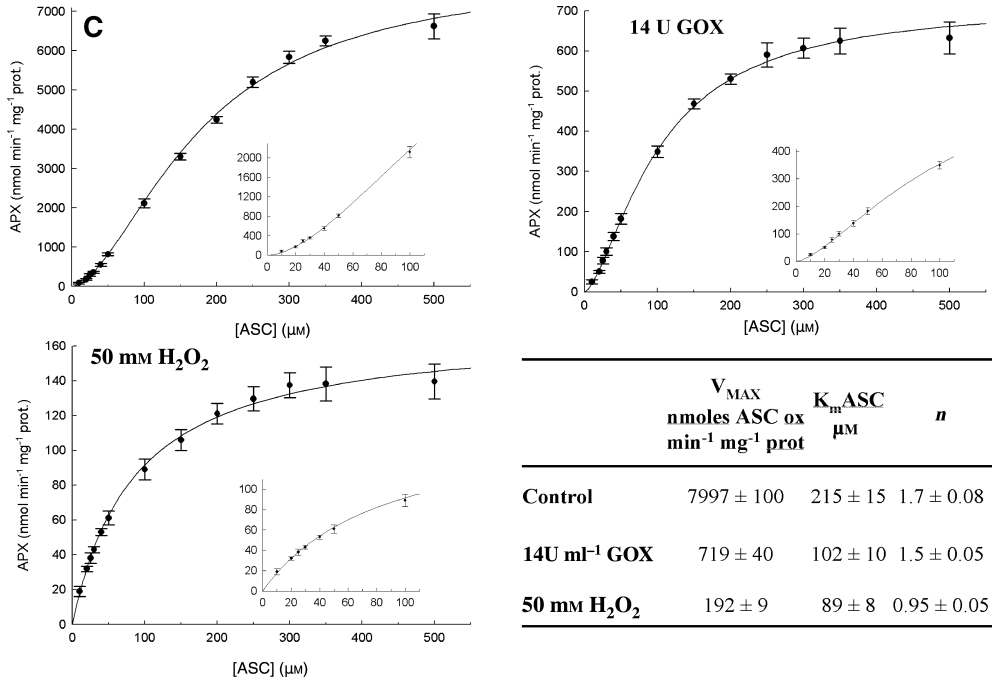
tically significant after 2 and 4 h treatment (Student's *t*-test, $P < 0.01$). On the other hand, higher amounts of GOX determined a dose-dependent inhibition of APX activity. Such APX inhibition induced by GGOX was attenuated over time, as the values of APX activity after 24 h of GGOX treatment were higher than those observed at 6 h (Figure 8a).

The direct H_2O_2 addition induced a dose-dependent APX inhibition (with an APX activity of 1800 ± 66, 660 ± 25, 280 ± 33, 155 ± 18 at 2 h treatment with 5, 15, 30 and 50 mM H_2O_2, respectively). As shown in Figure 8a, the addition of 50 mM H_2O_2 to the culture medium induced a prompt decrease in enzyme activity. However, under this treatment no recovery in APX activity was observed with time (Figure 8a). These results were also confirmed by electrophoretic analyses. Figure 8b shows a representative native PAGE of APX activity after 2 h treatment. Both cytosolic isoforms of APX were increased slightly under treatment with 0.75 U GOX. In contrast, the two bands were attenuated under 14 U GOX treatment and disappeared completely under treatment with 50 mM H_2O_2. CAT pre-treatment made H_2O_2 treatment completely inefficient at inhibiting APX. It also remarkably reduced the inhibition of APX due to GGOX (Figure 8b).

To verify whether the decrease in enzyme activity was also due to changes in the kinetic characteristics of APX, the dependence of its reaction rate on increasing ascorbate concentration, in the presence of a fixed concentration of hydrogen peroxide, was analysed. The kinetic parameters of APX were investigated in both control cells and cells treated for 15 min with GGOX (14 U ml^{-1} GOX) or H_2O_2 (50 mM). As shown in Figure 9, the enzyme V_{max} of cells treated with H_2O_2 and GOX was lower than that of control cells. However, APX from both GGOX-treated and control cells showed a sigmoidal dependence of rate on ASC concentration. This means that the enzyme from control and GGOX-treated cells showed a co-operative pattern, having a Hill coefficient of 1.7 and 1.5, in control and GGOX-treated cells, respectively. This is consistent with the presence of two binding sites for ASC (Lad *et al.*, 2002). On the other hand, in the H_2O_2-treated cells, APX lost this co-operative pattern, showing a hyperbolic dependence of rate on ASC concentration with a Hill coefficient close to 1 (Figure 9).

Levels of APX protein were measured by immunoblotting using a specific monoclonal antibody (Figure 10). In cells treated with the lowest concentration of GOX (0.75 U ml^{-1}) the amount of APX protein was always higher than in control cells. A significant decrease in the level of APX protein was evident only after 6 h cell treatment with the highest concentration of GOX (14 U ml^{-1}). On the other hand, direct addition of H_2O_2 induced an immediate decrease in the amount of APX protein that was completely prevented by addition of CAT to the culture medium.

Finally, the expression of cytosolic APX (cAPX) was investigated in cells treated with H_2O_2 and GGOX. Gene expression was analysed by semi-quantitative RT-PCR using specific primers for tobacco APX. As shown in Figure 11, addition of H_2O_2 to the culture medium determined a remarkable decrease in APX gene expression, which was progressively more evident during the treatment.



	V_{MAX} nmoles ASC ox min^{-1} mg^{-1} prot	K_mASC μM	*n*
Control	7997 ± 100	215 ± 15	1.7 ± 0.08
14U ml^{-1} GOX	719 ± 40	102 ± 10	1.5 ± 0.05
50 mM H_2O_2	192 ± 9	89 ± 8	0.95 ± 0.05

Figure 9. Kinetic analysis of cytosolic ascorbate peroxidase (cAPX) in cells treated with glucose + glucose oxidase (G + GOX) or H_2O_2.
The analysis was performed in extracts from control cells and cells treated for 15 min with 14 mM G plus 14 U ml^{-1} GOX or 50 mM H_2O_2. Concentration of H_2O_2 was 170 μM; concentration of ascorbate (ASC) varied over the range 10–700 μM. *n* = Hill coefficient. V_{max} and K_m values for ASC in control and treated cells represent the mean (±SE) of five experiments.

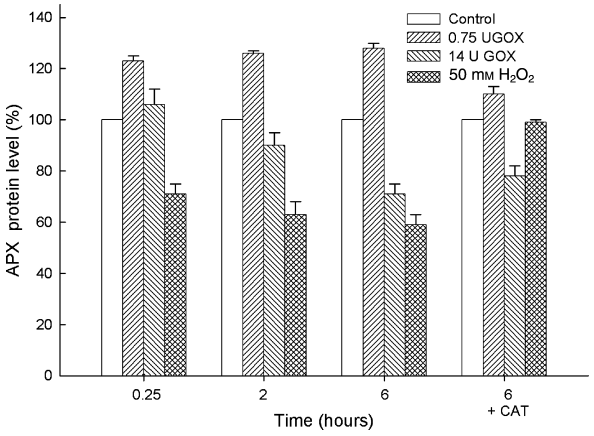


Figure 10. Changes in the level of ascorbate peroxidase (APX) protein under glucose + glucose oxidase (G + GOX) or H_2O_2 treatment.
Immunoblotting analysis was performed using a specific APX antibody (see Experimental procedures) in control cells and in cells treated with 14 mM G plus 0.75 and 14 U ml^{-1} GOX or 50 mM H_2O_2. Where indicated, catalase (30 U ml^{-1}) was added to the culture medium 10 min before treatment; 5 μg total protein was loaded in each line. After densitometry, levels of APX protein in treated cells were compared with the control and calculated as a percentage of alteration. Values represent mean (±SE) of three independent experiments.

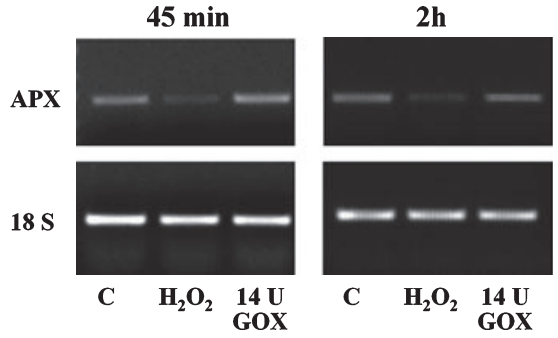


Figure 11. Levels of ascorbate peroxidase (APX) mRNA in cells treated with glucose + glucose oxidase (G + GOX) or H_2O_2.
Total RNA was extracted at the times indicated from control cells and cells treated with 14 mM G plus 14 U ml^{-1} GOX or 50 mM H_2O_2. Semi-quantitative RT-PCR for APX was performed as described in Experimental procedures. Levels of 18S rRNAs were also determined so that results could be normalized for recovery of RNA from the initial samples.

Discussion

Cell death and nitric oxide requirement for programmed cell death

The results reported here indicate that production of H_2O_2 over a threshold value induces cell death in TBY2 cells (Figure 2). However, the kind of death (necrotic versus PCD) depends strongly on the timing of H_2O_2 production. The prolonged production of this ROS for several hours induces a necrotic process as, under these conditions, no cytological PCD hallmarks are evident. On the other hand, an amount of H_2O_2 comparable with that inducing cell necrosis, but given as a single pulse, triggers PCD (Figures 3 and 4). Direct H_2O_2 addition probably determines a situation similar to the oxidative burst induced by several biotic or abiotic stresses against which PCD is frequently activated (Overmyer *et al.*, 2003). However, not all the signalling pathways leading to PCD require the generation of an oxidative burst and, under certain situations, ROS production even seems to suppress cell death (Torres *et al.*, 2005). It has been reported that the mesophyll cells of *Zinnia elegans*, when *in vitro trans*-differentiating into tracheids, require a constant oxidative state for PCD induction (Gomez-Ros *et al.*, 2006). In this process of tracheid differentiation, NO production is another crucial event during PCD (Gabaldon *et al.*, 2005). One noteworthy result reported here is that only the direct addition of H_2O_2 activates NO synthesis, whereas the constant production of H_2O_2 over time has no effect on NO synthesis, when either NOS activity or NO production is analysed (Figure 5). The capability of the NO-scavenger cPTIO in restoring cell viability under direct addition of H_2O_2 confirms the requirement of the two reactive species in the signalling pathway leading to PCD. The activation of NO biosynthesis by H_2O_2, probably by a NOS-like enzyme, has also been reported in mung bean leaves (Lum *et al.*, 2002). Moreover, it is known that treatment with NO generators is able to make ROS effects vanish or strengthen (Delledonne *et al.*, 2001; Orozco-Cárdenas and Ryan, 2002), further supporting the view that signalling pathways, based on the same chemical messengers, interplay differently according to the different environmental or cellular contexts. Increasing evidence suggests that NO and ROS play an active role in regulating metabolic changes occurring both during plant development and in responses against stress (Foyer and Noctor, 2005; Lamattina *et al.*, 2003); our results support the hypothesis that, at least in TBY-2 cells, H_2O_2 and NO are team players in the PCD-signalling pathway when produced in a precise amount and with precise timing.

Antioxidant involvement in cell death induced by H_2O_2

The metabolism of ASC and GSH plays a pivotal role in ROS removal in plants (Mittler, 2002; Noctor and Foyer, 1998; Shigeoka *et al.*, 2002). It has also been reported that APX and ASC decrease remarkably as early events of the PCD process (de Pinto *et al.*, 2002; Vacca *et al.*, 2004; Figures 7 and 8). The decrease in this antioxidant defence line has been considered part of the strategy contributing to generating the oxidative burst that characterizes the PCD triggered by several stimuli (De Gara, 2004; De Gara *et al.*, 2003; Mittler *et al.*, 1998). Data reported here suggest that ASC and GSH levels, more than their redox state, are altered as a specific signal in cells undergoing PCD (Figure 7), as the alterations in ASC and GSH redox state occurring under treatments with GGOX or H_2O_2 direct addition are similar. Consistent with this,

Arabidopsis thaliana mutants, having a 10–25% wild-type ascorbate content but a normal ASC redox state, spontaneously activate processes that typically occur during hypersensitive PCD, such as localized cell death and the expression of pathogenesis-related proteins (Pavet *et al.*, 2005).

Our results also indicate that the level and timing of H_2O_2 production are critical points for APX behaviour. The constant production of low amounts of this reactive species, which was ineffective in inducing cell death (0.75 U ml^{-1} GGOX), determines a transient, modest rise in APX (Figures 8 and 10). Such a rise is aimed at restoring redox impairment due to ROS overproduction, occurring under moderate GGOX treatment. The strengthening of antioxidant metabolism has been reported widely in the literature as a first line of defence against moderate oxidative stress (Asada, 1999; Mittler, 2002; Noctor and Foyer, 1998; Shigeoka *et al.*, 2002).

On the other hand, when the continuous production of H_2O_2 increases, a dose-dependent decrease in APX occurs at the level of enzyme activity and, over longer times, at the level of the amount of protein (Figures 8 and 10). Even in this case, APX inhibition is transient: 6 h after GGOX addition its activity progressively increases again, probably because the cells that are still alive become able to activate their strategies for counteracting the residual oxidative stress. The H_2O_2-dependent decrease in APX activity is not a surprise. Although H_2O_2 is one of the physiological substrates of the enzyme, its presence over a threshold value directly inhibits its activity by causing protein oxidation (Hiner *et al.*, 2000). No information is available, at least to our knowledge, on the possibility that protein oxidation accelerates APX turnover, which might explain the decrease in the amount of protein, evident from Western blotting analysis, in the absence of a decrease in its gene expression (Figures 10 and 11). However, the site-specific oxidation of proteins has been reported to act as a ubiquitination signal triggering protein degradation (Iwai *et al.*, 1998).

Ascorbate peroxidase behaves quite differently in cells undergoing PCD due to direct addition of H_2O_2. In this case, the decrease in enzyme activity appears to involve mechanisms different from simple protein oxidation. In fact, a change in the kinetic behaviour of the enzyme (from sigmoidal to hyperbolic) occurs only under these conditions (Figure 9). A stimulating hypothesis is that the different APX behaviour is a consequence of NO generation. In animal cells the formation of *S*-nitrosothiol groups, ferrous nitrosyl–haem complexes and the nitration of specific tyrosine residues are all well-characterized parts of the signalling pathways triggered by NO (Hofmann *et al.*, 2000). A guanylyl cyclase isoenzyme of smooth muscle is activated by the NO binding to the haeme group located in its regulatory domain, and the consequent increase in cGMP concentration activates a cGMP-dependent protein kinase (Hofmann *et al.*, 2000; Lucas *et al.*, 2000). Moreover, NO *trans*-nitrosylation and oxidation of specific cysteine residues has been reported to induce conformational changes in *trans*-membrane calcium channel proteins in neuronal cells (Nelson *et al.*, 2003). *S*-nitrosylation has also been suggested to play a central role in the signalling pathways leading to disease resistance in plants (Feechan *et al.*, 2005). The fact that APX is a haemoprotein and has a cysteine near its catalytic site (Lad *et al.*, 2002) makes it a putative NO target. An *in vitro* inhibition of APX by NO generators, probably due to the formation of a nitrosoferrous [Fe(II)NO^+] complex (Ferrer and Ros Barceló, 1999), has been reported previously (Clark *et al.*, 2000); even if the *in vivo* effect of NO seems to be more complex and strongly dependent on its concentration as well as the NO donor used and/or the presence of other reactive species (Murgia *et al.*, 2004a; de Pinto *et al.*, 2002; M.C.P. and L.D.G., unpublished data). It is worth noting that, in the presence of NO, GSH can be transformed into GSNO, a powerful nitrosylation agent (Ji *et al.*, 1999). GSNO formation might contribute to the decrease in the GSH pool (Figure 7), also promoting APX nitrosylation, even if further analysis is required in order to confirm this hypothesis.

Nitric oxide or, more properly, the cellular environment determined by the simultaneous presence of NO and H_2O_2, also appears to affect APX expression (Figure 11). Generation of NO in Arabidopsis plants induces a decrease in the thylakoidal APX transcript accumulation; consistently, Arabidopsis plants over- or underexpressing one thylakoidal APX gene show increased or decreased sensitivity, respectively, to both NO-induced cell death and paraquat-induced oxidative stress (Murgia *et al.*, 2004b; Tarantino *et al.*, 2005).

Changes in APX expression could also be correlated to the observed decrease in ASC content: in several experimental systems, a tight correlation has been reported between changes in ASC and APX (Arrigoni, 1994; De Gara *et al.*, 1997). The strong decrease in ASC could be, at least in part, due to an impairment of L-galactone-γ-lactone dehydrogenase, the last enzyme of ASC biosynthesis (Smirnoff *et al.*, 2001). Accordingly, a significant decrease in the activity of this enzyme has been observed in TBY-2 cells undergoing heat shock-induced PCD (M.C.P. and L.D.G., unpublished data), even if no information is yet available on a putative role for NO in such an alteration. The different behaviour of ASC and GSH under necrotic or PCD conditions further underlines that, despite oxidative stress occurring in both situations, the molecules and enzymes involved in the fine regulation of the cellular redox balance are modulated diversely, according to the different fates for which the cells are programmed.

In conclusion, the data reported here suggest that not only the different kinds of reactive species generated under environmental or developmental stimuli, but also the amount and timing of their generation can give reasons for the different responses triggered in plant cells. In this

picture, the interplay between nitric and oxygen reactive species plays a pivotal role in plant fitness.

Experimental procedures

Cell culture and treatments

The tobacco BY-2 cell suspension (*Nicotiana tabacum* L. cv. Bright Yellow-2) was routinely propagated and cultured according to Nagata *et al.* (1992). For the experiments, a stationary culture (7 days) was diluted 5/100 (v/v). At the third day of culture, 14 mM glucose plus glucose oxidase (Calbiochem, La Jolla, CA, USA) 0.75–14 U ml^{-1} or 5–50 mM H_2O_2 was added to 30 ml cell suspension. Where indicated, catalase (30 U ml^{-1}) was added to the culture medium 10 min before treatment. At the times indicated, aliquots of cells were collected by filtration on Whatman 3MM paper for analysis.

Cell viability and nuclear morphology

Cell viability was measured as described previously using Trypan Blue staining (de Pinto *et al.*, 1999). For the analysis of nuclear morphology, TBY-2 cells were stained with 4,6-diamidino-2-phenylindole (DAPI), as described by de Pinto *et al.* (2002), and visualized using a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 340 nm and a barrier filter of 400 nm.

DNA laddering

Cells were collected from cell suspension and homogenized in liquid nitrogen. DNA was extracted by the cetyl trimethyl ammonium bromide (CTAB) method (Vacca *et al.*, 2004). DNAse-free RNAse was added to a final concentration of 1 mg ml^{-1}; DNA fragments were separated by electrophoresis on 1.8% (w/v) agarose gel, followed by visualization by staining with ethidium bromide.

Measurement of H_2O_2

H_2O_2 was measured according to Bellincampi *et al.* (2000) in the extracellular phase. Briefly, 1 ml cell culture was harvested by centrifugation (10 000 ***g***, 20 sec, 25°C) and the H_2O_2 concentration was measured in the supernatant. An aliquot of supernatant (500 μl) was added to 500 μl assay reagent (500 μM ferrous ammonium sulphate, 50 mM H_2SO_4, 200 μM xylenol orange, 200 mM sorbitol). After 45 min incubation, the peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} was determined by measuring the absorbance at 560 nm of the Fe^{3+}–xylenol orange complex.

Measurements of nitric oxide production

Cell cultures were treated with 20 μM DAF-2DA for 1 h, then rinsed and re-suspended in a fresh medium. Cells were then subjected to the treatments and their fluorescence monitored at regular intervals. To verify whether increases in green epifluorescence could be attributed to NO evolution, DAF-2DA-loaded cells were incubated for 10 min in 500 μM carboxy-PTIO, an NO-scavenger, before treatment. Fluorescence intensity was measured using a Shimadzu RF-1501 luminescence spectrophotometer at 495 nm excitation and 515 nm emission. Production of NO in the cells was observed by fluorescence microscopy on a Leica DMLS fluorescence microscope mounted with an I_3 filter, at 470 nm excitation and 515 nm emission.

Nitric oxide synthase activity

Activity of NO synthase in tobacco cells was measured by monitoring the conversion of L-[U-^{14}C]arginine into L-[^{14}C]citrulline (Rees *et al.*, 1995). In brief, 300 mg cells was ground to a powder under liquid N and homogenized in ice-cold homogenization buffer (250 mM Tris–HCl pH 7.4, 10 mM EDTA, 10 mM EGTA). The homogenates were centrifuged at 10 000 ***g*** for 20 min at 4°C and the supernatant was used for analysis. Activity of NOS was determined using the Nitric Oxide Synthase Assay Kit (Calbiochem) according to the supplier's recommendations. Briefly, 1 μl radioactive arginine (approximately 64 Ci $mmol^{-1}$, 1 μCi μl^{-1}, Amersham Biosciences Europe GMBH, Milan, Italy), was added to 9 μl cellular extracts containing 1 μg μl^{-1} protein and incubated for 30 min at 30°C with 40 μl mixture reaction buffer (50 mM Tris–HCl pH 7.4, 6 μM tetrahydrobiopterin, 2 μm flavin adenin dinucleotide, 2 μM flavin mononucleotide, 10 mM NADPH, 6 mM $CaCl_2$). The reactions were stopped with 400 μl stop buffer (50 mM HEPES pH 5.5 with 5 mM EDTA). Successively, 100 μl equilibrated resin, which binds arginine, was added to the samples and then transferred to the spin cups. The citrulline, being ionically neutral at pH 5.5, passes completely through the column. The NOS activity was then determined by quantifying the radioactivity in the eluate by liquid-scintillation counting. When extracts were boiled for 10 min, no significant conversion into L-[^{14}C]citrulline was detected. For the negative controls, 5 μl of the NOS inhibitor, N^G-nitro-L-arginine methyl ester, HCl were added. Rat cerebellum was used for the positive control.

Ascorbate and glutathione assay

Cells (0.5 g) were collected by filtration on Whatman 3MM paper and homogenized with 2 vol cold 5% (w/v) metaphosphoric acid at 4°C in a porcelain mortar. The homogenate was centrifuged at 20 000 ***g*** for 15 min at 4°C, and the supernatant was collected for analysis. Ascorbate and glutathione contents were measured as described by de Pinto *et al.* (1999).

Activity and kinetics of ascorbate peroxidase

Cells were ground in liquid N and homogenized at 4°C in extraction buffer [50 mM Tris–HCl pH 7.8, 0.05% (w/v) cysteine, 0.1% (w/v) BSA]. The homogenate was centrifuged at 20 000 ***g*** for 15 min. The supernatant was used for both spectrophotometric and electrophoretic analyses.

The reaction catalysed by APX (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11, cytosolic isoenzyme) was determined by following the H_2O_2-dependent oxidation of ASC as decrease in absorbance at 290 nm, as described by Vacca *et al.* (2004). The values for V_{max} and K_m were obtained by either Michaelis–Menten or Hill analysis using SIGMAPLOT software (SPSS Inc., Chicago, IL, USA). Native PAGE of APX was performed according to de Pinto *et al.* (2000).

Protein determination

Assay of proteins was carried out according to Bradford (1976).

Immunoblot analysis

Cells were ground in liquid N and homogenized at 4°C in extraction buffer [50 mM Tris–HCl pH 7.5, 0.05 % (w/v) cysteine, 0.1 % (w/v) BSA]. The homogenate was centrifuged at 20 000 ***g*** for 15 min, and

the supernatant was electrophoresed on 12.5% 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 immunoglobulin G conjugated with alkaline phosphatase (Promega, Madison, WI, USA) as described previously by de Pinto *et al.* (2002). Densitometry values for immunoreactive bands were quantified using a GS-700 imaging densitometer (Bio-Rad, Milan, Italy) as described by Vacca *et al.* (2004).

Total RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from TBY-2 cells using the RNeasy plant minikit (Quiagen, Milan, Italy) according to the supplier's recommendations. Residual DNA was removed from the RNA samples using a DNA-*free* kit (Ambion, Austin, TX, USA). Synthesis of cDNA was performed from 2 μg total RNA with 10 μM random primers (Amersham), utilizing an Omniscript reverse transcriptase kit (Quiagen) according to the supplier's recommendations. PCR reactions were performed with specific primers for cAPX (cAPX, D85912) and 18S rRNA (18S, AJ236016) as described by Vacca *et al.* (2004). 18S rRNA was used as an internal control to normalize each sample for variations in the amount of initial RNA. For semi-quantitative RT-PCR, the cycle number in the linear range was determined empirically. 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 analysed on 1.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

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