# Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate

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## ABSTRACT

**Exposure to adverse temperature conditions is a common stress factor for plants. In order to cope with heat stress, plants activate several defence mechanisms responsible for the control of reactive oxygen species (ROS) and redox homeostasis. Specific heat shocks (HSs) are also able to activate programmed cell death (PCD). In this paper, the alteration of several oxidative markers and ROS scavenging enzymes were studied after subjecting cells to two different HSs. Our results suggest that, under moderate HS, the redox homeostasis is mainly guaranteed by an increase in glutathione (GSH) content and in the ascorbate peroxidase (APX) and catalase (CAT) activities. These two enzymes undergo different regulatory mechanisms. On the other hand, the HS-induced PCD determines an increase in the activity of the enzymes recycling the ascorbate- and GSH-oxidized forms and a reduction of APX; whereas, CAT decreases only after a transient rise of its activity, which occurs in spite of the decrease of its gene expression. These results suggest that the enzyme-dependent ROS scavenging is enhanced under moderate HS and suppressed under HS-induced PCD. Moreover, the APX suppression occurring very early during PCD, could represent a hallmark of cells that have activated a suicide programme.**

*Key-words*: ascorbate; glutathione; nitric oxide; oxidative stress; programmed cell death; reactive oxygen species; tobacco Bright Yellow-2 cells.

## INTRODUCTION

Adverse thermic conditions play a major role in the yield reduction for crops. Heat stress is particularly common and dangerous for plants as they lack metabolic mechanisms for thermic homeostasis and need light energy, which easily causes temperature increases in the exposed tissues. Plant survival under heat stress requires the activation of proper defence mechanisms in order to avoid the damage of

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metabolic machineries. The defence responses against heat stress consist in the induction of heat-shock (HS) proteins, through the activation of specific transcription factors, known as HS factors (Hsf), which bind specific DNA sequences, called HS elements [reviewed in plants by Baniwal *et al.* (2004), in animals by Burdon (1986) and in procaryotes by Arsene, Tomoyasu & Bukau (2000)]. The presence of HS proteins in all living organisms and their ability to act as defence mechanisms against different kinds of abiotic stress, suggest an ancient origin for this system (Wang *et al.* 2004).

Heat stress, as other stress conditions, leads to the over-production of reactive oxygen species (ROS) within the cells. Several results suggested that ROS can act as signalling molecules involved in triggering defence responses against potentially damaging temperatures. In human, *Drosophila* and yeast Hsf can sense hydrogen peroxide ($H_2O_2$), which plays a pivotal role in regulating their activation, nuclear translocation and DNA binding activity (Zhong, Orosz & Wu 1998; Lee *et al.* 2000; Ahn & Thiele 2003). In rice and tomato, $H_2O_2$ induces the expression of HS proteins (Banzet *et al.* 1998; Lee *et al.* 2000). A recent study also demonstrated that the formation of Hsf–DNA binding complex, occurring in the early phase of HS response, requires $H_2O_2$ (Volkov *et al.* 2006). Consistently, treatment with ROS scavengers, as ascorbate (ASC), inhibits the bond between HS elements and Hsf occurring after HS or $H_2O_2$ treatment (Volkov *et al.* 2006). These results are also coherent with the evidence that many compounds inducing oxidative burst also increase thermotolerance (Dat *et al.* 1998).

A possible relationship between ascorbate peroxidase (APX), one of the key player in $H_2O_2$ catabolism (Kubo *et al.* 1995; Karpinski *et al.* 1997; Storozhenko *et al.* 1998), and heat stress has also been investigated. The expression of APX1, an APX localized in the cytosol of *Arabidopsis thaliana*, is enhanced during heat stress (Karpinski *et al.* 1997). This is consistent with the presence of HS element in the promoter region of the *APX1* gene (Mittler & Zilinskas 1992; Storozhenko *et al.* 1998).

In *Arabidopsis*, HS has also been reported to induce a thermostable APX (Panchuk, Volkov & Schöffl 2002). This enzyme is constitutively expressed in transgenic plants

overexpressing Hsf3, which has been recently recognized as a regulator of stress gene expression in *A. thaliana* (Lohmann *et al.* 2004). Another evidence suggested that the cytosolic APX is suppressed after HS condition, leading to programmed cell death (PCD) in tobacco cells (Vacca *et al.* 2004).

A major challenge in studying stress responses in plants is to understand how different signalling pathways are activated by similar stimuli. It has been suggested that, under stress conditions, cellular fate depends on several factors, among which ROS play a pivotal role. Timing, kinds and amounts of ROS production, as well as possible interactions occurring between different kinds of ROS or between ROS and other chemical species [e.g. nitric oxide (NO), redox molecules], are all important factors in determining cellular response. Moreover, the endogenous cellular antioxidant capability is also crucial in determining the ability of a plant to cope with stress conditions.

Cell cultures represent an optimal system for studying relationships between heat stress and defence response as they are a homogeneous system in which cells are all in the same growth phase and have comparable antioxidant properties. In this study, the alteration of several redox parameters and the changes in the activity and/or expression of the enzymes involved in ROS scavenging have been studied after cell exposure to two different heat stress conditions: an HS inducing PCD and a more moderate HS determining a redox impairment without affecting cell viability.

## MATERIALS AND METHODS

### Cell culture and HS treatments

The tobacco Bright Yellow-2 (TBY-2) cell (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspensions were routinely propagated and cultured according to Nagata, Nemoto & Hasezawa (1992). For the experiments, 4 mL of stationary culture (7 d) was diluted in 100 mL of fresh culture medium into a 250 mL flask and cultured for 4 d. HSs were induced by transferring the flasks containing cell suspensions into a water bath at 35 or 55 °C. The cells were subjected to the HSs for 10 min in the dark. After HS, the cells were returned to the normal growth temperature (27 °C). The temperature was monitored in the cell suspensions during the HS treatments and after, until it reached 27 °C again. At the indicated times, aliquots of cells were collected by vacuum filtration on Whatman 3MM paper (Whatman International LTD, Kent, UK) and used for the analyses.

### Cell viability and measurements of $H_2O_2$ and NO

Cell viability was measured by trypan blue staining as previously described (de Pinto, Francis & De Gara 1999).

The extracellular release of $H_2O_2$ was determined by measuring the absorbance at 560 nm of the $Fe^{3+}$–xylenol orange complex according to de Pinto *et al.* (2006).

Intracellular $H_2O_2$ production was measured by using dihydrorhodamine (DHR) 123 (Sigma-Aldrich Italia,

Milan, Italy) as a fluorescent probe (Royall & Ischiropoulos 1993). The cells were treated with 5 $\mu$M DHR123 for 30 min and then rinsed and re-suspended in a fresh medium. The cells were then exposed to the HS and their fluorescence was monitored at regular intervals by a Shimadzu RF-1501 luminescence spectrophotometer (Shimadzu Italia s.r.l., Milan, Italy) at 490 nm excitation and 525 nm emission.

The intracellular production of $H_2O_2$ was also observed in the DHR123-treated cells by a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 450–490 nm and a barrier filter of 510 nm.

For NO measurements, cell cultures were treated with 20 $\mu$M 4,5-diaminofluorescein diacetate (DAF-2DA) for 1 h and then rinsed and re-suspended in a fresh medium. The cells were then subjected to the heat treatments and their fluorescence was monitored at regular intervals. The fluorescence intensity was measured using a Shimadzu RF-1501 luminescence spectrophotometer at 495 nm excitation and 515 nm emission.

NO production in the cells was observed on a Leica DMLS fluorescence microscope, with an excitation filter of 450–490 nm and a barrier filter of 510 nm.

The specificity of DHR123 and DAF-2DA for $H_2O_2$ and NO, respectively, was checked by pretreating cells with appropriate scavengers before fluorescent probe loading. This analysis was performed 1 h after the HS to avoid the temperature-dependent degradation of the scavengers. In particular, the cells were incubated with 1 mM ASC ($H_2O_2$ scavenger) or 0.5 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, Na (cPTIO) (NO scavenger) for 10 min. The cells were then treated with the fluorescent probes for 15 min, rinsed, re-suspended in a fresh medium and observed through a fluorescence microscope.

### Determination of oxidative stress markers

Lipid peroxidation was measured as malondialdehyde (MDA) content determined by thiobarbituric acid (TBA) reaction as reported by Guidi *et al.* (1999). The amount of MDA–TBA complex was calculated using an extinction coefficient of 155 $mM^{-1} cm^{-1}$.

Protein oxidation was determined by measuring the content of carbonyl groups assayed using the dinitrophenyl-hydrazine according to Levine *et al.* (1994). Carbonyl content was calculated using an extinction coefficient of 22 $mM^{-1} cm^{-1}$.

Identification of proteic sulphydrylic (–SH) groups was performed by their labelling with monobromobimane (mBBr) according to Kobrehel *et al.* (1992). The cells (0.5–2 g) were ground in liquid $N_2$ with a mortar and pestle. Four volumes of 2 mM mBBr (dissolved in acetonitrile) in 100 mM Tris–HCl, pH 7.5 buffer were added just as the last trace of liquid $N_2$ disappeared. The thawed mixture was then ground for 1 min, transferred to a microfuge tube and centrifuged at 30 000 *g* for 15 min at 4 °C. Ten microlitres of 10% sodium dodecyl sulphate (SDS) and 10 $\mu$L of 100 mM 2-mercaptoethanol were added to 80 $\mu$L of the mBBr-labelled extracts to stop the reaction and derive excess

mBBr. The samples were then applied to gels for electro-phoretic analysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the mBBr-labelled extracts was performed as described in De Gara *et al.* (2003b). The intensity of the –SH groups was measured by the Quantity One (Bio-Rad, Hercules, CA, USA) software.

Proteins were determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

## Total antioxidant activity and ASC, glutathione assays

Total antioxidant activity (TAA) was measured according to Arnao, Cano & Acosta (2001) by the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS)/horseradish peroxidase decolouration method. This method enables lipophilic antioxidant activity (LAA) and hydrophilic anti-oxidant activity (HAA) to be measured separately in the same sample. Therefore, the relative contribution of both LAA and HAA antioxidant systems to the TAA can be evaluated. Briefly, cells (1 g) were ground in a mortar in liquid nitrogen with 2 mL of 50 mM sodium phosphate buffer (pH 7.5) and 5 mL of ethyl acetate. The homogenate was centrifuged at 4000 $g$ for 10 min in order to separate the aqueous phase from the organic phase. The capability of the aqueous and organic phases to scavenge the ABTS radical cations was compared with a standard dose–response curve obtained using ascorbic acid or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), respec-tively, and was expressed as micromoles of ascorbic acid or Trolox equivalent per gram fresh weight. Trolox and ascorbic acid have the same antioxidant activity (Arnao *et al.* 2001).

In order to obtain deproteinized extracts for ASC and glutathione (GSH) determinations, 0.3 g of cells was homogenized with cold 5% metaphosphoric acid at 4 °C in 1:4 ratio (w/v). After centrifugation at 20 000 $g$ for 15 min, the supernatants were collected and used for the analysis of ASC and GSH levels and redox state according to de Pinto *et al.* (1999).

## Enzyme assays

The cells were ground in liquid nitrogen 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 and 1 mM ASC]. Homogenates were centrifuged at 20 000 $g$ for 15 min. The supernatants were used for both spectrophotometric and electrophoretic analyses. The activities of APX (L-ASC: $H_2O_2$ oxidoreductase, EC 1.11.1.11), monodehy-droascorbate reductase (MDAR) (NADH: ASC-free radical oxidoreductase, EC 1.6.5.4), dehydroascorbate reductase (DHAR) (GSH: dehydroascorbate oxidoreduc-tase, EC 1.8.5.1) and GSH reductase (GR) (NADPH: GSH disulphide oxidoreductase, EC 1.6.4.2) were tested accord-ing to de Pinto, Tommasi & De Gara (2000).

Catalase (CAT–$H_2O_2$: $H_2O_2$ oxidoreductase, EC 1.11.1.6) activity assay was performed according to Beaumont *et al.*

(1990) with minor modification, by following the $H_2O_2$ dis-mutation at 240 nm in a reaction mixture, which consisted of 0.1 M phosphate buffer, pH 7.0, 50–100 $\mu$g protein and 18 mM $H_2O_2$ (extinction coefficient 23.5 mM$^{-1}$ cm$^{-1}$).

Native PAGE for cytosolic APX (cAPX) and CAT were performed according to de Pinto *et al.* (2000) and Chandlee & Scandalios (1983), respectively. When cAPX isoenzymes were analysed by Native PAGE, ASC was omitted from the extraction buffer (de Pinto *et al.* 2000).

## Total RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from TBY-2 cells using the RNeasy plant minikit (QIAGEN S.p.A., Milan Italy) according to the supplier's recommendation. Residual DNA was removed from the RNA samples using a DNA-*free* kit (Ambion, Inc. Austin, TX, USA). Synthesis of cDNA was performed from 2 $\mu$g total RNA with 10 $\mu$M random primers (Amersham Biosciences Europe GMBH, Milan, Italy), utilizing an Omniscript Reverse Transcriptase kit (QIAGEN S.p.A.) according to the supplier's recommen-dation. PCR reactions were performed with specific primers for cAPX (cAPX, D85912, 5′-CACTGTAAGCGAGG AGTACC-3′ and 5′-TGAGCCTCAGCATAGTCAGC-3′), CAT (CAT1, U93244, 5′-CCA TAC AAG TAC CGT CCG TC-3′ and 5′ GAA TGT GTG GAC ACC AGA AC 3′) and 18S rRNA (18S, AJ236016, 5′-CATGATAACTCGAC GGATCG-3′ and 5′-GAAGGCCAACGTAATAGGAC-3′). The 18S rRNA was used as an internal control in order to normalize each sample for variations in the amount of initial RNA. PCR reactions were performed as described in Vacca *et al.* (2004). The products of PCR amplification pro-duced a single band at the predicted sizes of 699 bp, 624 bp and 594 bp for cAPX, CAT and 18S, respectively. These were analysed on 1.5 % agarose gel containing 0.5 $\mu$g mL$^{-1}$ ethidium bromide.

## RESULTS

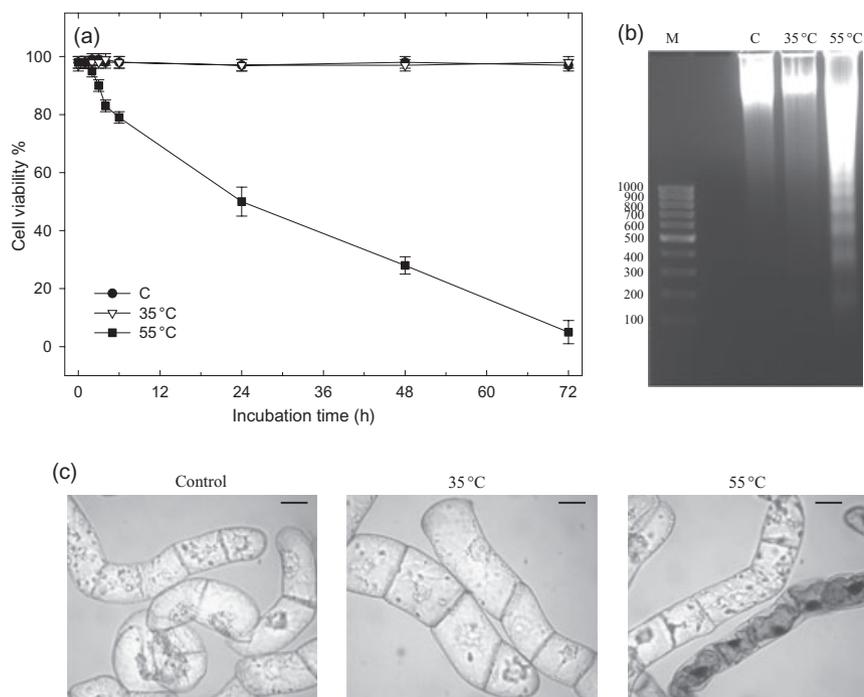### Cell viability and production of $H_2O_2$ and NO

In order to study the defence responses activated by plant cells after HS exposition, cell suspensions of *N. tabacum* Bright Yellow-2 (TBY-2) were subjected to HS at 35 and 55 °C for 10 min and then reported at their normal growth temperature (see Materials and Methods for details). Temperature changes within cell cultures were monitored during the HS treatments and in the recovery phase (Fig. 1). Four minutes were required to reach the HS temperatures (35 and 55 °C) within cell suspensions; while after the HS exposure, the temperature slowly decreased requiring 75 min to reach the normal growth temperature (27 °C) again.

The effect of 35 and 55 °C HS on cell viability was analy-sed by trypan blue staining at different time intervals up to 72 h following the HS induction (Fig. 2a). HS at 35 °C did not alter the viability of the treated cells, which remained
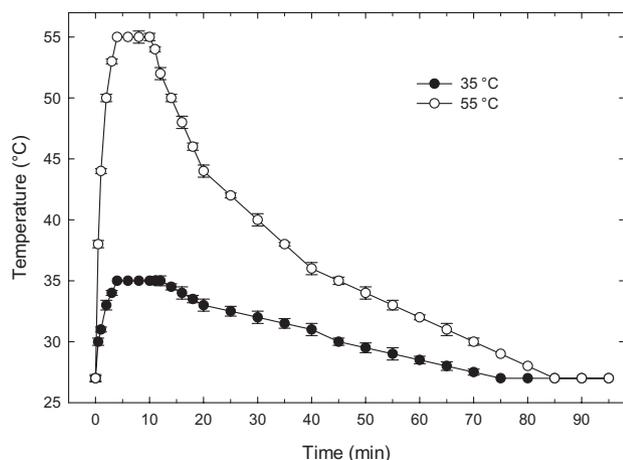
**Figure 1.** Temperature profile within cell cultures during the heat-shock (HS) treatments and in the recovery phase. Temperature changes were monitored within heat-shocked cell suspensions during 35 and 55 °C treatments and in the time following the HSs until the normal growth temperature (27 °C) was reached. The reported values represent the mean (±SE) of two independent experiments.
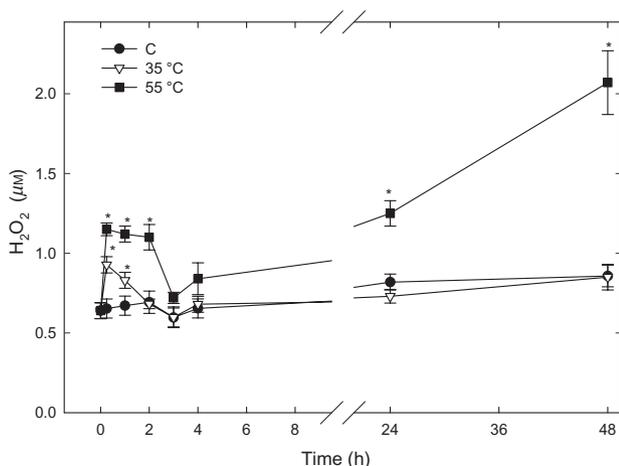
similar to that of the control ones (98%). On the other hand, 55 °C HS induced a strong decrease in cell viability, which was already evident 2 h after the treatment; cell viability further decreased over time (Fig. 2a). The cell death induced by 55 °C HS was PCD, as indicated by cytoplasm shrinkage and DNA laddering (Fig. 2b,c). These hallmarks of PCD were undetectable in the 35 °C heat-shocked cells and, as expected, in the control ones.

The levels of $H_2O_2$ and NO were determined in the cells exposed to the two different HS temperatures. The amount of the extracellular $H_2O_2$, released after 35 °C HS, immediately increased; but 2 h after the treatment, its value returned comparable to that of the control cells (Fig. 3). On the other hand, cell suspensions exposed to 55 °C HS showed an $H_2O_2$ increase higher than that induced by the 35 °C HS. The production of $H_2O_2$ after 55 °C HS had a biphasic behaviour, with a first peak during the first 3 h after the HS and a second, more relevant increase, starting at about 5 h after the treatment and further rising during the following hours (Fig. 3). $H_2O_2$ production was also analysed by using the permeable fluorescent probe DHR123 and quantified by fluorimetric assay. The control and 35 °C-treated cells had a comparable production of $H_2O_2$ over time; whereas cell exposure to 55 °C HS caused a raise in the $H_2O_2$ content that was already significant 30 min after the heat treatment and progressively increased until 24 h from the HS (Fig. 4a). Microscopic fluorescence analysis performed on cells exposed to 55 °C HS showed that 30 min after 55 °C HS, all the cells produced $H_2O_2$. The number of the $H_2O_2$-producing cells decreased during the time and after 4 h, only the cells that were negative to trypan blue staining (the still viable cells) showed a high fluorescence (Fig. 4b).

The production of NO induced by the two different HSs was quantified by fluorimetric assay. As it is shown in Fig. 5a, the control and the 35 °C heat-shocked cells had a low and comparable production of NO. On the other hand, the cells exposed to 55 °C HS showed a fast-increasing production of NO, reaching its highest value at 24 h from HS induction. A fall in NO production was observed after 48 h, when the NO level in the cells exposed to 55 °C HS was



**Figure 2.** Time-dependent effect of heat shock (HS) on cell viability and DNA integrity. (a) Viability of control and heat-shocked cells was measured after trypan blue staining as reported in the Materials and Methods. The percentage (±SE) of the viable cells was counted in a population of at least 1000 cells in five independent experiments. (b) DNA was extracted from cells collected 24 h after the beginning of HS. The picture shows a representative electrophoretic run in which 10 $\mu$g of DNA was loaded in each line. (c) Typical examples of trypan blue-stained cells after 24 h from the beginning of HS in the control 35 and 55 °C heat-shocked cells. Bar = 20 $\mu$m.

**Figure 3.**  Release of H$_2$O$_2$ into the culture medium by tobacco Bright Yellow-2 cells exposed to heat shocks. H$_2$O$_2$ concentration was measured during 48 h in the control and heat-shocked cell suspensions. At the indicated times, 1 mL of cell suspension was taken, and the H$_2$O$_2$ concentration was determined in the medium as described in the Materials and Methods. The reported values represent the mean ($\pm$SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.05$).

very similar to that of the control cells. Fluorescence microscopy has also been used in order to verify the NO production within 55 °C heat-shocked cells. This analysis showed that only the cells that were still viable, but in

which PCD had been induced, were responsible for NO production (Fig. 5b).

The specificity of fluorescent probes DHR123 and DAF-2DA for H$_2$O$_2$ and NO, respectively, was verified by the addition of compounds that lower the accumulation of these reactive species in the cells. Figure 6a shows that the addition of 1 mM ASC lowered the fluorescence intensity of DHR123 observed in the 55 °C exposed cells, thus confirming the specificity of the fluorescent probe for H$_2$O$_2$. Similarly, the increase in fluorescence occurring in the DAF-2DA-loaded cells, after the 55 °C HS, was specifically because of NO, as it was strongly reduced by the addition of the NO scavenger cPTIO (Fig. 6).

## Oxidative markers

In order to verify the level of oxidative stress induced in the TBY-2 cells by HS exposure, lipid peroxidation and protein oxidation were assayed. The amount of MDA–TBA complex was measured as an indicator of lipid peroxidation. In the control and the 35 °C-treated cells, the lipid peroxidation, measured on fresh weight basis, decreased at 24 h and, even more, at 48 h. Such decrease might be explained by cellular elongation, as such process is mainly because of the vacuole enlargement occurring during the analysed period (data not shown). The HS at 35 °C had no effect on lipid peroxidation, while 55 °C HS induced a significant increase of this oxidative marker, which was already evident shortly after the treatment (Fig. 7). The oxidative

(a)



(b)
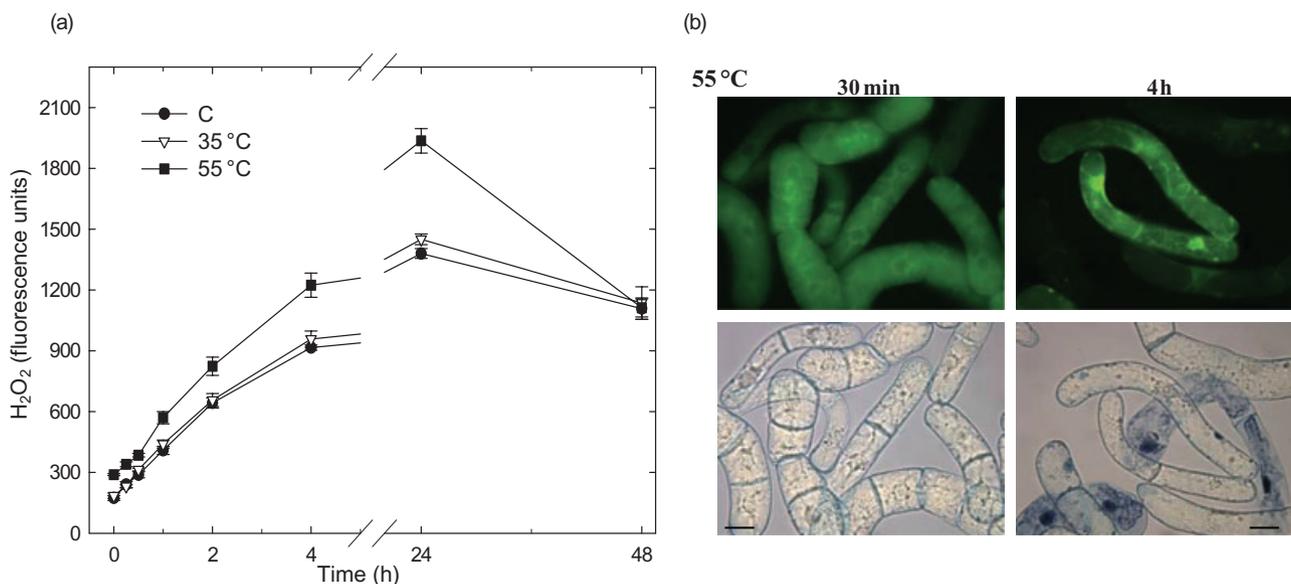


**Figure 4.**  Production of H$_2$O$_2$ in the control and heat-shocked tobacco Bright Yellow-2 (TBY-2) cells. (a) H$_2$O$_2$-dependent fluorescence emission of TBY-2 cells was measured at the indicated times in the cells loaded with dihydrorhodamine (DHR) 123 and then exposed to 35 and 55 °C heat shock (HS) and in the control cells (cells loaded with DHR123 without any subsequent treatments). The reported results are the mean ($\pm$SE) of three independent experiments. All the values of 55 °C HS until 24 h significantly differ from the control and 35 °C HS values (Student's *t*-test with $P < 0.05$). (b) Microscope visualization of the H$_2$O$_2$ accumulation induced by 55 °C HS in TBY-2 cells. The cells were pre-loaded with DHR123 and exposed at 55 °C HS. At the indicated times after the HS, 1 mL of cell suspension was treated with 0.4% trypan blue and examined using a microscope. Representative phase-contrast and fluorescence images of 55 °C heat-shocked cells are reported in the upper and lower panels, respectively. Bar = 20 $\mu$m.

(a)



(b)
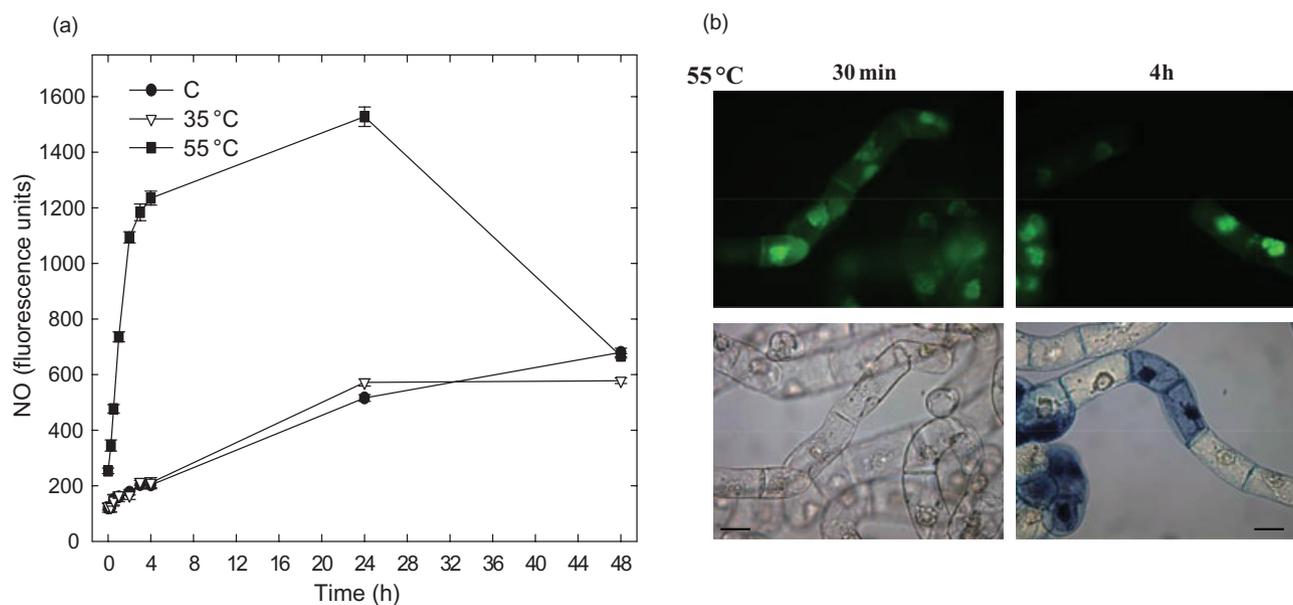


**Figure 5.** Nitric oxide production by tobacco Bright Yellow-2 (TBY-2) cells exposed to heat shocks (HSs). (a) NO-dependent fluorescence emission of TBY-2 cells was measured at the indicated times in the cells loaded with 4,5-diaminofluorescein diacetate (DAF-2DA) and then exposed to 35 and 55 °C HS and in the control cells (cells loaded with DAF-2DA without any subsequent treatments). The reported results are the mean (±SE) of three independent experiments. (b) Microscope visualization of the NO accumulation induced by 55 °C HS in TBY-2 cells. The cells were pre-loaded with DAF-2DA and exposed at 55 °C HS. At the indicated times after the HS, 1 mL of cell suspension was treated with 0.4% trypan blue and examined using a microscope. Representative phase-contrast and fluorescence images of 55 °C heat-shocked cells are reported in the upper and lower panels, respectively. Bar = 20 $\mu$m.
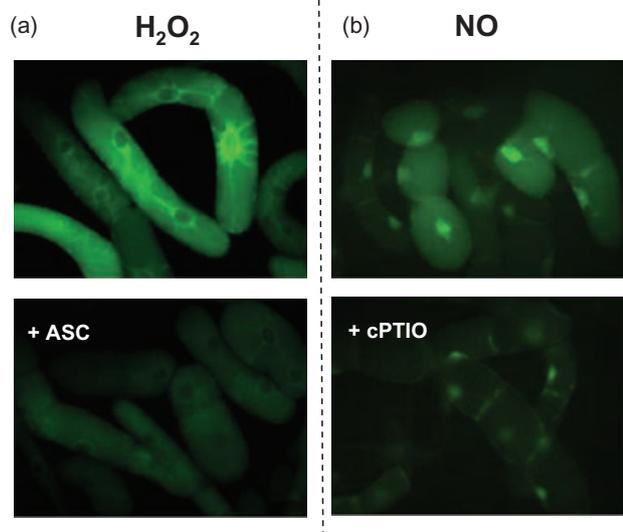


**Figure 6.** Fluorescent probes' specificity for hydrogen peroxide and nitric oxide. One hour after 55 °C HS treatment, the heat-shocked cells were incubated with 1 mM ascorbate (ASC) (a) and 0.5 mM 2-(4-carboxypenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, Na (cPTIO) (b) for 15 min. The cPTIO- or ASC-loaded cells were treated with 20 $\mu$M dihydrorhodamine 123 (a) or 20 $\mu$M 4,5-diaminofluorescein diacetate (b), respectively, and after 10 min, were observed under a fluorescence microscope.

damage of proteins was evaluated by quantifying the increase in carbonyl groups and the decrease in –SH groups in the total protein fraction. In cells exposed to 35 °C HS, the amount of carbonyl groups was similar to that of the control cells (Fig. 8a). On the other hand, cell exposition to 55 °C HS induced a significant and immediate increase in protein oxidation, which further rose over time (Fig. 8a). The increase in protein oxidation following 55 °C HS was still more significant if the reduction in the amount of total proteins occurring after this treatment is considered. On the other hand, 35 °C HS did not alter cellular protein content (Fig. 8b). The changes in the redox state of the protein –SH groups induced by the two different HSs were analysed by labelling the proteic –SH groups with mBBr, which stoichiometrically reacts with the –SH groups of cysteine residues but not with the oxidized form of the amino acid fixed in disulphide bonds (Crawford *et al.* 1989). The electrophoretic analysis of the labelled proteins showed an increase in the protein –SH groups during the first 24 h after the 35 °C HS (Fig. 9a); whereas a clear decrease in the protein –SH groups was evident starting at 4 h after 55 °C HS (Fig. 9b).

## Antioxidant metabolites

In order to have a first evaluation of the cellular antioxidant properties, the antioxidant activity of the water- and lipid-soluble extracts was separately analysed in the control and HS-exposed cells. As it is shown in Fig. 10a,b,
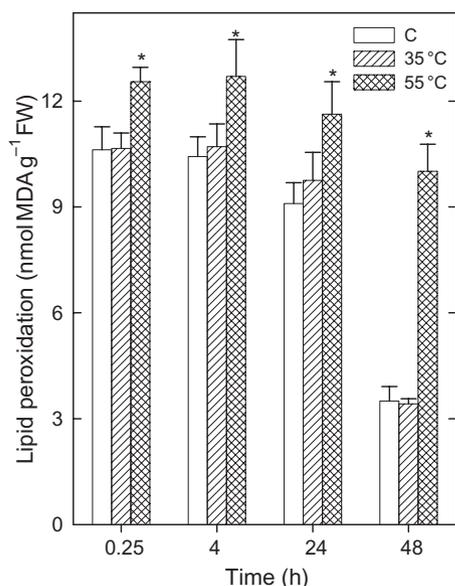
**Figure 7.** Heat-shock (HS) effect on lipid peroxidation in tobacco Bright Yellow-2 (TBY-2) cells. Changes over time in lipid peroxidation of TBY-2 cells exposed to HSs were measured as the malondialdehyde (MDA) content. The results are the means (±SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.01$). FW, fresh weight.

the HAA of TBY-2 cells was much higher than the lipophilic one (LAA). In cells exposed to 35 °C HS, LAA values were comparable to those of the control cells; whereas cells exposed to 55 °C HS showed a progressive decrease of LAA, which was already statistically different from the control value after 15 min from the HS induction (Fig. 10a). A moderate increase of HAA was observed in the cells subjected to 35 °C HS; whereas a progressive decrease, similar to that observed for LAA, occurred in the cells exposed to 55 °C HS (Fig. 10b). Among the hydrophilic antioxidants, attention was focused on the changes in the level and redox state of ASC and GSH. The total ASC content (reduced plus oxidized forms) and redox state (reduced form/total content) did not change significantly in the 35 °C heat-shocked cells. Cell exposition at 55 °C HS determined a significant decrease in the ASC content particularly evident 24 h after the HS induction; ASC redox state immediately decreased after the 55 °C HS (Fig. 11a,b). GSH showed a different behaviour, as in the cells exposed at 35 °C HS, its total content was higher than that of the control cells during the first 24 h, after which it returned to control values. In the 55 °C heat-shocked cells, GSH content decreased more rapidly than ASC. Indeed, a 34% drop of total GSH occurred very soon after 55 °C HS induction and a further decrease occurred over time (Fig. 11c). On the other hand, GSH redox state was less altered than ASC redox state, as it did not change in the 35 °C heat-shocked cells, and it significantly decreased only 24 h after the exposure at 55 °C (Fig. 11d).

## The ROS removal enzymes

In order to have more information on the global $H_2O_2$ detoxification capability of the cells, the changes induced by the HSs in the activities of APX and CAT were analysed. The exposure of cells to 35 °C HS induced an increase in APX and CAT activities (Fig. 12). In the case of CAT, such increase was transient and significant for the first 4 h after the treatment (Fig. 12b). The behaviour of APX and CAT under the 55 °C HS greatly differed: CAT activity increased in the first hours following the treatment, in correspondence to the first peak of $H_2O_2$ production, and then decreased (Fig. 12b). On the other hand, APX activity strongly decreased immediately after the treatment (almost 70% in the first 15 min after the 55 °C exposure) and its further progressive decrease occurred over time (Fig. 12a). The activity of these $H_2O_2$ scavenging enzymes was also
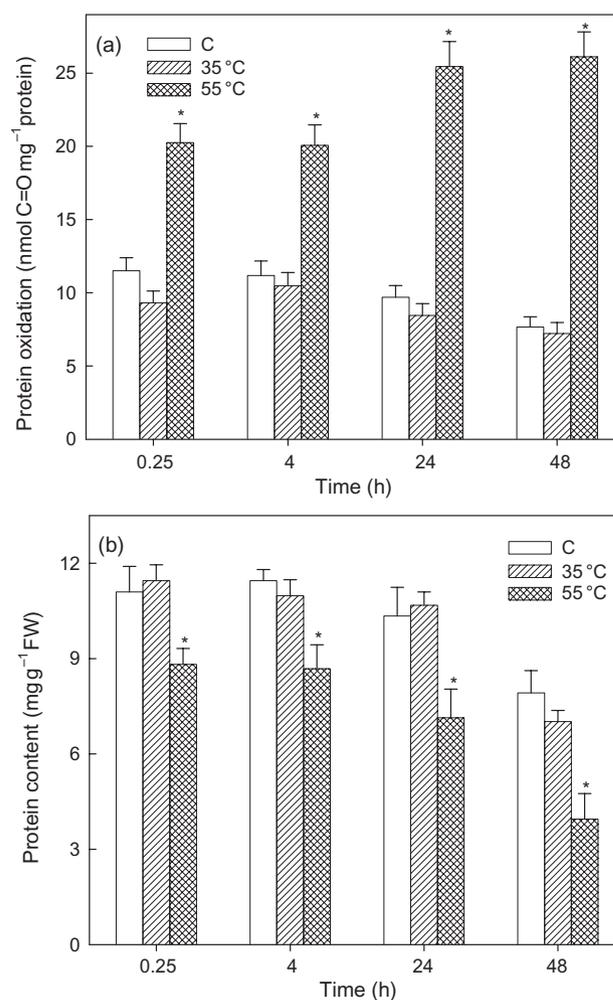


**Figure 8.** Heat-shock effects on level and oxidation of proteins in tobacco Bright Yellow-2 cells. (a) Level of protein content expressed as milligram protein per gram fresh weight (FW); (b) level of protein oxidation measured as total protein carbonyl group content. Data represent the means (±SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.01$).
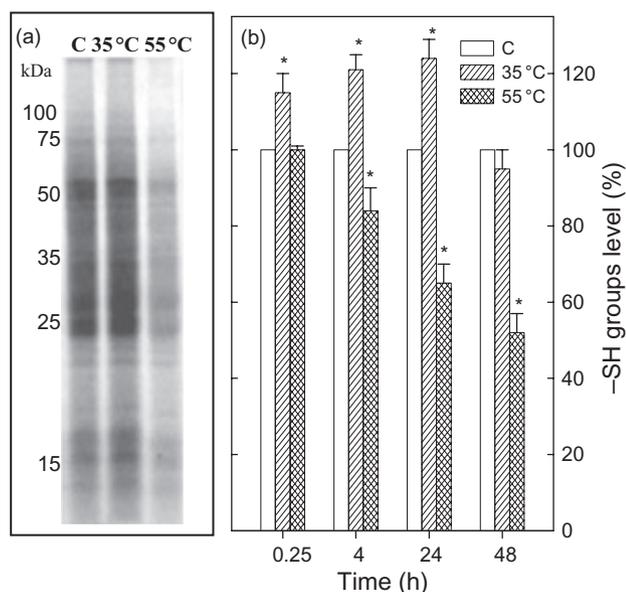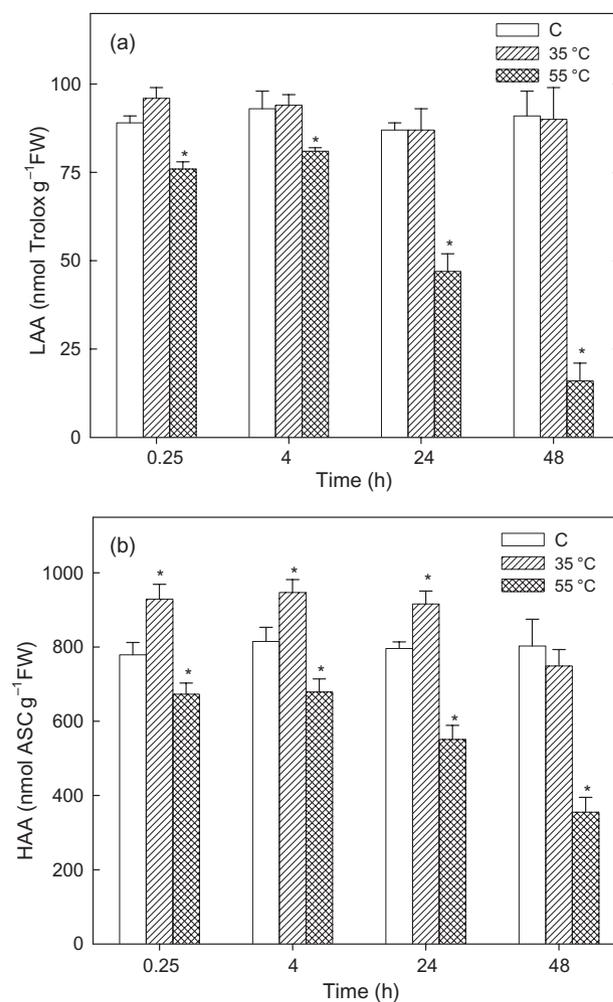
**Figure 9.** Level of sulphydrylic (–SH) proteic groups in the control and heat-shocked cells. (a) Representative sodium dodecyl sulphate–polyacrylamide gel electrophoresis of proteins extracted from the control and treated cells after 4 h from heat-shock induction. –SH groups were labelled with monobromobimane (mBBr) as described in the Materials and Methods. Eighty micrograms of proteins was loaded in each lane. (b) Densitometric analysis of mBBr-labelled proteins. –SH proteic levels in heat-shocked cells were expressed as percentage of that in the control cells. Values represent the mean (±SE) of three independent experiments. The '*' indicates the values that are significantly different from the control ($P < 0.05$).

after HS exposition (Fig. 13a,c), as no significant change in the expression of the genes occurred in previous times (data not shown). This decrease became even more evident 24 h after the HS exposure (Fig. 13b,d).

The activities of the enzymes of ASC–GSH recycling, MDAR, DHAR and GR were also analysed (Fig. 14). The 35 °C HS had no effect on the activity of DHAR (Fig. 14b). On the other hand, in the hours following the HS, a slight and transient increase of MDAR and GR activities was evident (Fig. 14a,b). Under 55 °C HS, all the activities of the ASC–GSH recycling enzymes were affected: the activity of DHAR transiently increased over the first 4 h after the HS

analysed by Native PAGE, 4 h after HS induction. The attention of APX was focused on the cytosolic isoenzymes (cAPX), as it has been reported that these isoenzymes are the most versatile in the heat stress responses of TBY-2 cells (Locato 2008). As it is shown in Fig. 12c, both the cAPX isoforms detectable by Native PAGE (de Pinto *et al.* 2000) were more active after the 35 °C HS; whereas their activities strongly decreased after 55 °C HS even if it must be taken into account that Native PAGE mainly gives qualitative, rather than quantitative, information. A representative Native PAGE of the CAT activity of the cells collected 4 h after HS exposure is reported in Fig. 12d. The control cells showed a single band with CAT activity. The intensity of CAT increased following 35 °C HS. Consistently with the measurement of its specific activity, such increase was even more evident in the cells exposed to 55 °C HS. The expression of cAPX and CAT was also investigated in cells exposed to HSs by semi-quantitative RT-PCR regarding their tobacco isoenzymes whose sequences were available (see Materials and Methods for details; Fig. 13). The exposition of cells to 35 °C HS determined a significant increase in cAPX transcript only 24 h after the HS (Fig. 13b,d), whereas it did not induce any change in CAT expression. On the other hand, cell exposition to 55 °C HS induced a decrease in both CAT and cAPX transcripts starting at 4 h



**Figure 10.** Total antioxidant activity in the control and heat-shocked tobacco Bright Yellow-2 cells. Control and heat-shocked cells were collected at the indicated times and used for the determination of lipophilic antioxidant activity (LAA) (a) or of hydrophilic antioxidant activity (HAA) (b) as reported in the Materials and Methods. One nanomole of ascorbate and 1 nmol of Trolox have the same reducing capability (Arnao *et al.* 2001). Values represent the means (±SE) of three independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.05$). FW, fresh weight.
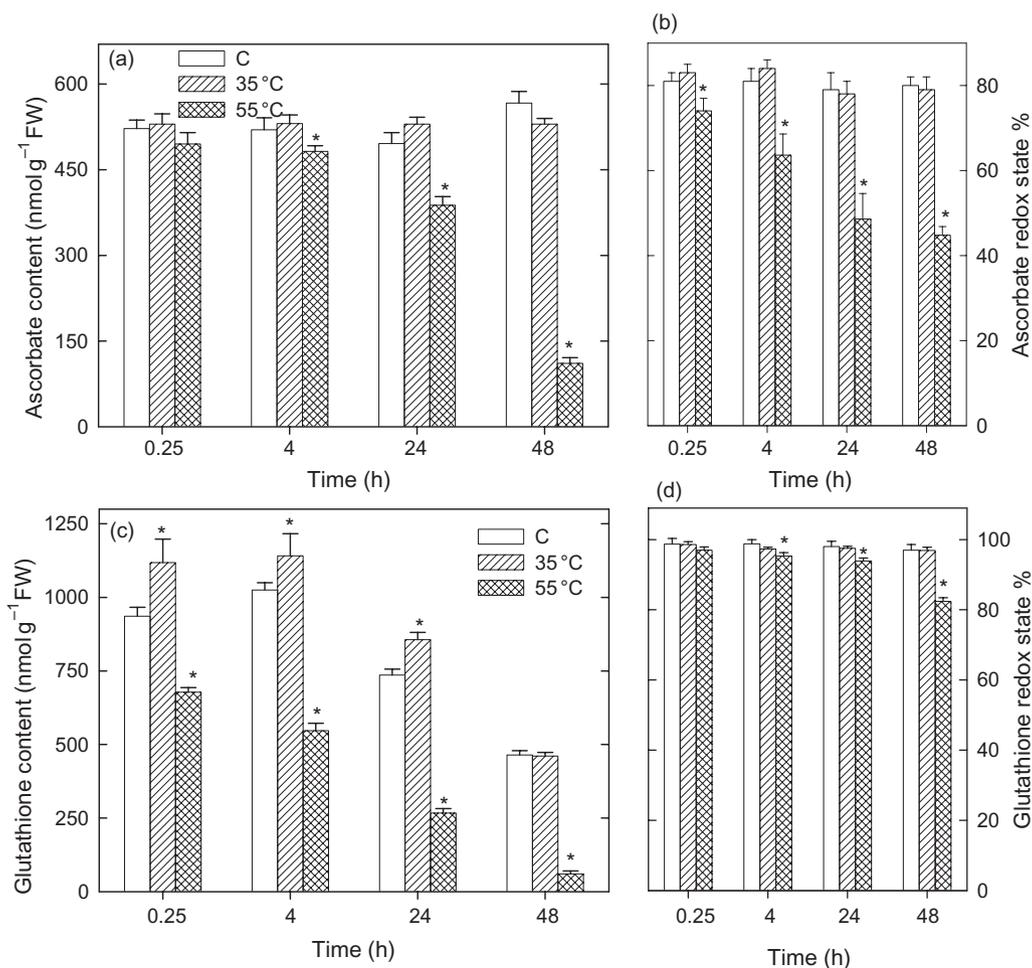
**Figure 11.** Changes in ascorbate (ASC) and glutathione (GSH) content and redox state induced by heat shocks in tobacco Bright Yellow-2 cells. Control and heat-shocked cells were collected at the indicated times and used for the determination of the total ASC (a) or GSH (c) pools (reduced plus oxidized forms) and their redox states, respectively (b,d) as reported in the Materials and Methods. Values represent the means ($\pm$SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.01$).

(Fig. 14b); whereas the activities of MDAR and GR strongly increased during all the analysed periods (Fig. 14a,c).

## DISCUSSION

In this study, the effects of two HS conditions on cell viability, production of reactive species and changes in metabolites and enzymes involved in ROS scavenging, have been analysed, in order to assess the defence response activated by the different heat stress conditions. The two HS conditions, 10 min at 35 or at 55 °C, represent a mild stress, not inducing cellular death, and a strong stress, triggering PCD, respectively (Fig. 2). The results reported here underline that the two HS temperatures cause the production of reactive species, different as for the amount and kind. Indeed, 35 °C HS determines only a transient increase in $H_2O_2$, but no detectable production of NO, while 55 °C HS induces a biphasic production of the former reactive species and a

remarkable and rapid increase in the latter (Figs 3 & 5). These data are in agreement with the literature, as it has been reported that in the early stages of a moderate environmental stress, a transient increase of $H_2O_2$ occurs (Foyer *et al.* 1997), whereas a biphasic production of ROS is a typical feature of several kinds of PCD (Bolwell *et al.* 2002; De Gara, de Pinto & Tommasi 2003a). The different production of NO and $H_2O_2$ and the different fates determined by the two HSs on the cell cultures support the hypothesis that simultaneous and balanced presence of $H_2O_2$ and NO is required for inducing PCD in plant cells (Delledonne *et al.* 1998, 2001; de Pinto, Tommasi & De Gara 2002). It is interesting to notice that, under 35 °C HS, the cellular redox homeostasis is maintained, as no alteration occurs in the analysed markers of oxidative stress: levels of lipid peroxidation and proteic carbonyl groups do not change, and the proteic –SH groups even increase after this treatment (Figs 7–9). Consistently, several players involved in redox homeostasis, GSH, APX, CAT, MDAR and GR, all
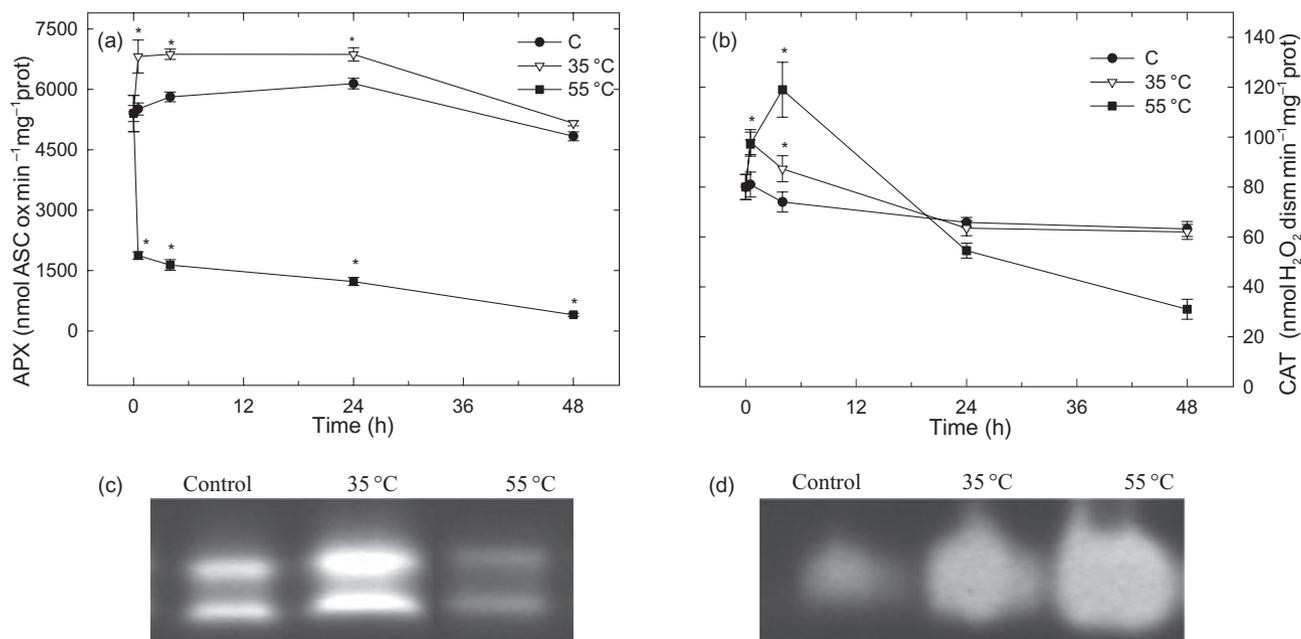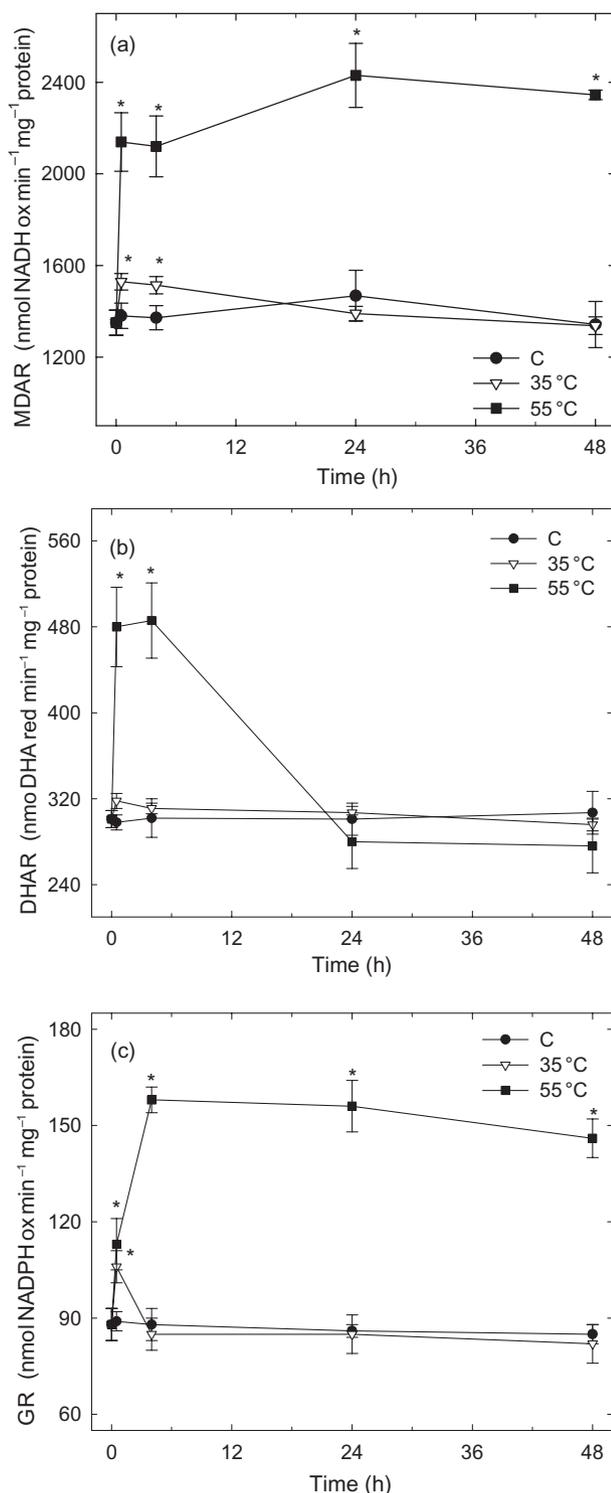
**Figure 12.** Effects of heat shocks (HSs) on ascorbate peroxidase and catalase activities. Specific activities of ascorbate peroxidase (APX) (a) and catalase (CAT) (b) were measured in the control and heat-shocked cells over time. Values represent the means ($\pm$SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.05$). Representative cytosolic APX (c) and CAT (d) Native polyacrylamide gel electrophoresis from tobacco Bright Yellow-2 cells after 4 h from HSs induction. For each treatment, 300 and 50 $\mu$g total proteins were loaded for APX and CAT, respectively.

increased after the 35 °C HS, even though in different amounts and with different timing. This is in agreement with the data suggesting that the strengthening of antioxidant network is a first line of defence against oxidative stress and a mechanism allowing plants to acclimatize and respond more effectively to stress (Noctor & Foyer 1998; Asada 1999; Mittler 2002; Shigeoka *et al.* 2002). Among the analysed metabolites, GSH seems to play a pivotal role in avoiding the onset of oxidative stress, as its content significantly increases in cells exposed to 35 °C HS. Interestingly, GSH is involved in several pathways responsible for oxidative stress protection. It takes part in ROS removal, both being an electron shuttle in the ASC–GSH cycle and being able, for its redox potential, to directly reduce $H_2O_2$ to water
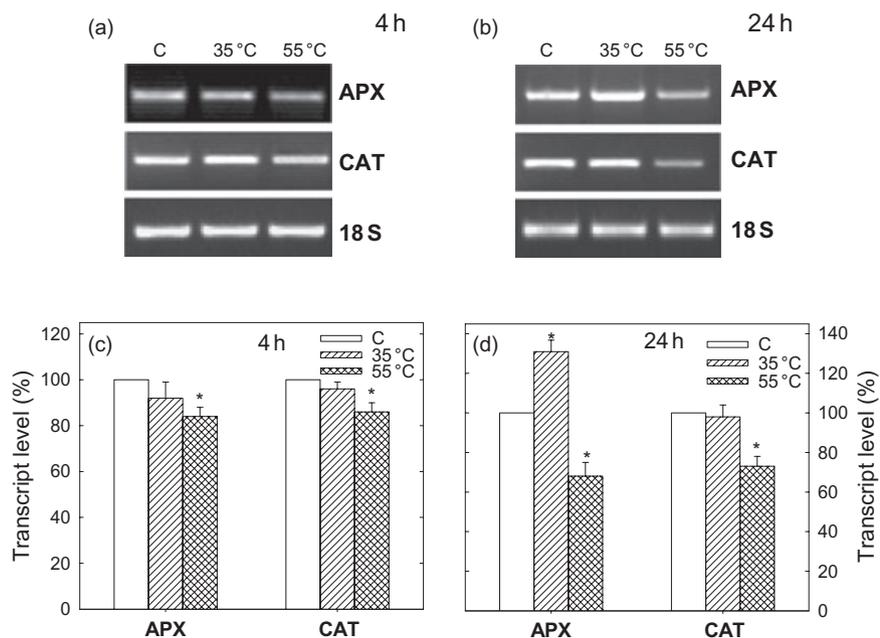


**Figure 13.** Effects of heat shocks (HSs) on ascorbate peroxidase (APX) and catalase (CAT) expression. Representative semi-quantitative RT-PCR results for APX and CAT of the control and heat-shocked cells after 4 h (a) and 24 h (b) from HSs induction. Levels of 18S rRNAs were also determined to normalize the results. Transcript levels for APX and CAT obtained after densitometric analysis and normalization, in cells after 4 h (c) and 24 h (d) from the beginning of HSs. Transcript levels in heat-shocked cells were expressed as percentage of that in the control cells. Values represent the mean ($\pm$SE) of four independent experiments. The '*' indicates the values that are significantly different from the control ($P < 0.05$).

**Figure 14.** Effects of heat shocks on ascorbate (ASC)–glutathione (GSH) recycling enzymes. Specific activity of (a) monodehydroascorbate reductase (MDAR), (b) dehydroascorbate reductase (DHAR) and (c) GSH reductase (GR) over time. The values represent the mean (±SE) of five independent experiments. The '*' indicates the values that are significantly different from the controls (Student's *t*-test with $P < 0.05$).

(Finley, Wheeler & Witt 1981). Moreover, GSH is involved in maintaining the proteic thiols in the reduced state (Foyer & Noctor 2005). It has been recently reported that a complex interplay between GSH and thioredoxin pathways occurs during plant development as well as in responses to oxidative stress (Reichheld *et al.* 2007). This interplay might also explain the increase in the –SH groups observed in the proteins of the cells exposed to 35 °C HS, even if more accurate studies are required in order to support the GSH involvement in the observed increase of protein –SH groups. An increase in GSH biosynthesis has been also reported to be involved in the signalling pathways inducing stress defence gene expression (Ball *et al.* 2004). The comparison of several kinds of stress responses involving GSH suggests that initial responses are often related to a reduction in GSH redox state, with an increase in its oxidized form. Long-term responses or acclimation conditions, on the contrary, are correlated with the increase of the GSH pool, without an evident decrease of its redox state (Tausz, Sircelj & Grill 2004). In our experimental condition, the 35 °C HS does not induce any significant change in the GSH redox state, thus suggesting that TBY-2 cells are in a situation comparable to acclimation. The transient increase in GR, occurring after the 35 °C HS exposition, may also be able to compensate a putative increase in GSH oxidation (Fig. 14).

The $H_2O_2$ scavenger enzymes, APX and CAT, also contribute to the redox adjustment occurring after the 35 °C HS: both their activities are increased, even though APX enhancement is more persistent during that time, while the increase of CAT is only transient and strictly mirrors the transient increase of $H_2O_2$ occurring during this HS (Figs 3 & 12). The different behaviour of these two enzymes could be the consequence of their different kinetic characteristics. It is known that the affinity of CAT for $H_2O_2$ is much lower than that of APX (Willekens *et al.* 1997). This makes CAT less sensitive to small changes in the amount of $H_2O_2$ and, probably, less suitable for a precise control of the cellular $H_2O_2$ levels. Moreover, the cellular localization of the two ROS scavenging enzymes is quite different, being CAT located surely in the peroxisomes and, probably, in the mitochondria (Scandalios, Tong & Roupakias 1980; Mittler *et al.* 2004); whereas APX is widely distributed, with different isoenzymes, in almost all cellular compartments (Shigeoka *et al.* 2002; De Gara 2004). Therefore, it is probable that APX plays a role as a redox buffer enzyme wider than that of CAT. It is also necessary to underline that, during the first hours following the moderate HS, the specific activities of both CAT and cAPX increased, in spite of the evidence showing it had no effect on gene expression (Figs 12 & 13). This suggests that, at least in our condition of moderate oxidative stress, the regulation of these enzymes occurs with a mechanism acting downstream gene expression. As a long-term response, only the expression of the *cAPX* gene is enhanced, and this probably sustains its activity at levels higher than that of the control cells, even when $H_2O_2$ production returns to control value (Figs 12 & 13). The changes in the *cAPX* expression are coherent with the presence of

an HS element in its promoter (Mittler & Zilinskas 1992; Storozhenko *et al.* 1998) and with previous evidence showing that an increase in the activity and expression of *cAPX* but not of CAT occurs in *Arabidopsis* plants subjected to HS challenge (Sato *et al.* 2001); even if the evidence of the increase in CAT activity after HS treatment was also reported to occur in *A. thaliana* leaves (Orendi *et al.* 2001).

Following 55 °C HS, the situation is very different, as the PCD is accompanied by the oxidation of lipids and proteins (Figs 7 & 8), as well as with an impairment of the metabolites and enzymes directly involved in ROS scavenging (Figs 11 & 12). The enzymes responsible for the recycling of the oxidized forms of ASC and GSH increase in the cells undergoing PCD (Fig. 14). This behaviour, coherent with other PCD situations (de Pinto *et al.* 2002), is probably a homeostatic attempt to block the decrease of cellular reducing power under a strong oxidant environment. ASC and GSH also respond differently in 55 °C heat-shocked cells. Our data suggest that the oxidative pressure is higher for ASC than for GSH, as the decrease in the redox state of ASC is stronger and more rapid than that of GSH. On the other hand, the decrease in the content of GSH is more precocious and evident than that of ASC (Fig. 11). It is known that the presence of NO, which occurs in 55 °C heat-shocked cells, causes GSH utilization to form nitrosoglutathione, a nytrosilating agent (Tsikas *et al.* 2001; Huang & Huang 2002). This could contribute to the decrease of GSH levels induced by 55 °C HS. The different behaviours of the two $H_2O_2$ scavenging enzymes, CAT and cAPX, following 55 °C HS, are of particular interest. The increase in CAT activity, occurring during the first hours, in spite of its gene expression decrease, is probably a consequence of the strong rise of its substrate $H_2O_2$, which might induce an activation of the enzyme. About 24 h are necessary before CAT activity mirrors the decrease in its gene expression. In contrast, *cAPX* activity strongly decreases immediately after the 55 °C HS induction, much earlier than the decrease in its gene expression (Figs 12 & 13). The precocious and strong reduction of APX in cells undergoing different kinds of PCD (de Pinto *et al.* 2002; Vacca *et al.* 2004; de Pinto *et al.* 2006) indicates that this enzyme is subjected to different regulatory mechanisms, according to the different fates that cells must pursue: it is more active when cells must survive and suppressed when cells activate a suicide program. The versatility of this enzyme supports its role in controlling ROS levels, allowing ROS to act as signal molecules or to induce the oxidative burst that is typical of several forms of PCD. Consistently, knockout cAPX *Arabidopsis* plants present increased ROS levels and have alteration of several metabolic and developmental processes (Pnueli *et al.* 2003) The immediate change in APX activity might also be considered a PCD signature, identifying those cells destined to death because of their inability to counteract specific stress conditions.

Further studies are in progress in order to better understand the regulatory mechanisms aimed to control cAPX and the other enzymes involved in the homeostasis of cellular redox state.

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