

# Different involvement of the mitochondrial, plastidial and cytosolic ascorbate–glutathione redox enzymes in heat shock responses

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Plant survival under heat stress requires the activation of proper defence mechanisms to avoid the impairment of metabolic functions. Heat stress leads to the overproduction of reactive oxygen species (ROS) in the cell. In plants, the ascorbate (ASC)-GSH cycle plays a pivotal role in controlling ROS levels and cellular redox homeostasis. Ascorbate peroxidase (APX) is the enzyme of this cycle mainly involved in ROS detoxification. In this study, the ASC-GSH cycle enzymes were analysed in the cytosol, mitochondria and plastids of tobacco Bright Yellow-2 cultured cells. The cells were also subjected to two different heat shocks (HSs; 35 or 55°C for 10 min) and the cell compartments were isolated in both conditions. The results reported here indicate that moderate HS (35°C) does not affect cell viability, whereas cell exposure to 55°C HS induces programmed cell death (PCD). In relation to ASC-GSH cycle, the three analysed compartments have specific enzymatic profiles that are diversely altered by the HS treatments. The cytosol contains the highest activity of all ASC-GSH cycle enzymes and the data reported here suggest that it acts as a redox buffer for the whole cells. In particular, the cytosolic APX seems to be the most versatile enzyme, being its activity enhanced after moderate HS and reduced during PCD induction, whereas the other APX isoenzymes are only affected in the cells undergoing PCD. The relevance of the changes in the different ASC-GSH cycle isoenzymes in allowing cell survival or promoting PCD is discussed.

## Introduction

Life in aerobic conditions requires the acquisition of efficient strategies allowing cells to cope with the unavoidable presence of reactive oxygen species (ROS). This led to the evolution of flexible and redundant antioxidant networks controlling cellular ROS levels in all aerobic organisms. The alterations in the components of the antioxidant networks, because of their interaction with

ROS, as well as ROS themselves, constitute sensor systems able to perceive modifications in the cellular redox balance and to consequently activate the opportune metabolic responses. In green tissues, ROS are largely produced in chloroplasts during photosynthesis, whereas in non-green tissues or dark-growing plants, mitochondrial respiration represents the principal ROS source, such as in all the others aerobic organisms (Foyer and Noctor 2003, Puntarulo et al.

*Abbreviations* – ASC, ascorbate; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GOGAT, glutamine oxoglutarate aminotransferase; GR, glutathione reductase; HS, heat shock, MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; PCD, programmed cell death; ROS, reactive oxygen species; TBY-2, tobacco Bright Yellow-2.

1988). Plants also produce significant amounts of ROS in cell walls, especially under adverse environmental conditions and in the microbodies, in particular during photorespiration or germination of oil seeds.

Among the different ROS produced within cells,  $H_2O_2$  is the most stable form (half-life of 1 ms). Several reports suggest that  $H_2O_2$  can react with other molecules far from the production sites because in plants its capability to cross biological membranes is highly facilitated by aquaporins (Dynowski et al. 2008). Catalase (CAT) is the  $H_2O_2$  scavenger enzyme present in all aerobic organisms. It dismutates hydrogen peroxide to water and oxygen (Noctor and Foyer 1998). In plants, CAT is often considered a peroxisomal marker enzyme because its presence is suggested to be limited to these organelles (Corpas et al. 1999, Jimenez et al. 1997); however, its localization in other cellular compartments is still under debate (Blackman and Hardham 2008). Another enzyme dedicated to the removal of hydrogen peroxide is ascorbate peroxidase (APX). It reduces hydrogen peroxide to water by using ascorbate (ASC) as an electron donor and has a much higher affinity for  $H_2O_2$  than CAT. Its strong affinity for  $H_2O_2$  makes APX a very versatile enzyme for a fine modulation of the levels of  $H_2O_2$  in cells and/or within specific cellular compartments. APX is an ubiquitous enzyme in plants. It is present in both higher plants and algae (Grodén and Beck 1979, Nakano and Asada 1981) and different isoenzymes have been characterized in different tissues and cellular compartments (De Gara 2004, Shigeoka et al. 2002), whereas its presence in animal kingdom has only been reported in bovine ocular tissues (Wada et al. 1998). The utilization of ASC as electron donor links APX to a set of reactions that contributes to the cellular ROS scavenging: the ASC–GSH cycle. In this cycle, ASC is converted to monodehydroascorbate (MDHA), a radical that spontaneously undergoes dismutation giving ASC and dehydroascorbate (DHA), the final ASC oxidation product. MDHA can be reduced back to ASC by a NAD(P)H-dependent reductase [MDHA reductase (MDHAR)]. DHA is also reconverted to ASC by another reductase [DHA reductase (DHAR)] that uses GSH as electron donor. The cycle is completed when the oxidized GSH is reduced back by a NADPH-dependent reductase [GSH reductase (GR)]. The presence of a complete ASC–GSH cycle has been identified in chloroplasts (Asada 1987, 1999), mitochondria (Creissen et al. 1995, Jimenez et al. 1997, 1998), peroxisomes (Jimenez et al. 1997, 1998) and cytosol (Mittler et al. 2004). The whole cycle contributes to ROS detoxification, not only by ASC regeneration, which allows APX to continuously remove hydrogen peroxide, but also by modulating the cellular redox balance through the regulation of ASC and GSH pools.

Like other stress conditions, heat shock (HS) leads to ROS overproduction in the cell; on this basis, many studies focused on the search of a putative interlink between oxidative signalling and HS response mechanism. Many researches suggest that HS proteins and HS transcription factors are induced by  $H_2O_2$  (Banzet et al. 1998, Desikan et al. 2001, Hihara et al. 2001, Lee et al. 2000). Moreover, it has been demonstrated that, in the early phase of HS response, the binding of HS transcription factors with HS elements requires  $H_2O_2$  and is inhibited by the ROS scavenging action of ASC (Volkov et al. 2006). These results are consistent with the evidence that many compounds inducing oxidative burst also increase thermotolerance (Dat et al. 1998).

A possible relationship between cytosolic APX and HS proteins/HS transcription factors has also been investigated. It has been reported that transgenic tobacco cells under-expressing a cytosolic APX (APX1) are more resistant to adverse environmental situations, including heat stress (Ishikawa et al. 2005), probably because in the APX1-depleted cells alternative defence systems are already activated when the stress is imposed. Consistently, in the APX1 under-expressing plants, the expression of HS proteins is enhanced, and this reinforcement of the HS defence system has been proposed to act as an alternative strategy controlling ROS accumulation (Pnueli et al. 2003). In *Arabidopsis thaliana* a thermostable APX is constitutively expressed in transgenic plants over-expressing HS transcription factor 3 (Panchuk et al. 2002). This transcription factor has been recently characterized as a regulator of stress genes in *A. thaliana* (Lohmann et al. 2004). Moreover, this novel APX is also inducible in wild-type plants, as a consequence of moderate HS (Karpinski et al. 1999). The involvement of cytosolic APX has been reported in the programmed cell death (PCD) induced by HS. In this case, HS determines a decrease in the activity and expression of cytosolic APX, probably as part of the strategy aimed at generating the typical oxidative burst occurring during PCD (Locato et al. 2008, Vacca et al. 2004).

As different compartments differ in their capability to generate and to scavenge ROS, the efficiency of the ASC–GSH cycle working in each compartment could have a pivotal role in maintaining cellular redox homeostasis. Very few studies have made comparative analysis of the enzyme activities of the ASC–GSH cycle present in the different cell compartments and of their eventual modulation under stress conditions. With the aim of better understanding the role of the ASC–GSH metabolism in controlling the redox homeostasis of different cell compartments, the localization of ASC–GSH cycle enzymes has been studied in etiolated tobacco Bright Yellow-2 (TB2) cell culture. In particular, the ASC–GSH

cycle isoenzymes have been studied in cytosol, mitochondria and plastids. Two different heat stress conditions were imposed on the basis of their different effects on cell viability: a HS-inducing PCD (10 min at 55°C) and a more moderate HS (10 min at 35°C) having no effect on cell viability (Locato et al. 2008). In both conditions, the regulation of the ASC-GSH cycle enzymes has been examined in the purified cellular compartments.

## Materials and methods

### Cell culture and HS treatments

The TBY-2 (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell suspensions were routinely propagated and cultured according to Nagata et al. (1992). For the experiments, 4 ml of stationary culture (7 days) were diluted in 100 ml of fresh culture medium into 250-ml flask and cultured for 4 days. HSs were induced by transferring the flasks containing cell suspensions into a water bath at 35 or 55°C. Cells were subjected to the HSs for 10 min in the dark and then returned to their normal growth temperature (27°C). The temperature was monitored in the cell suspensions during the HS treatments. Four minutes was required to reach the HS temperatures (35 and 55°C) within cell suspensions (Locato et al. 2008). At the indicated times, aliquots of cells were collected by vacuum filtration on Whatman 3MM paper and used for the analyses.

### Cellular fractioning

After both HSs, protoplast preparation was performed to isolate cytosol, plastids and mitochondria from TBY-2 cells. Approximately 30-g cells were resuspended in 100 ml protoplast extraction buffer [0.4 M mannitol, 25 mM Tris-MES, pH 5.5 containing 0.25% (w/v) cellulase, 0.05% (w/v) pectolyase, 0.1% (v/v) pectinase]. The cells were incubated at 27°C in the dark with gentle agitation for about 2 h to obtain protoplasts. Progress was monitored by observing subsamples of the cells under the light microscope. Protoplasts were recovered by centrifugation at 100 g for 5 min at room temperature without brake. The pellet was washed twice with the protoplast washing buffer (0.4 M mannitol and 25 mM Tris-MES, pH 6.5). After the second washing step, protoplasts were resuspended in the organelle isolation buffer (20 mM Tris-HCl pH 7.6, 0.4 M mannitol, 0.5 mM EDTA, 4 mM cysteine and 0.1% BSA). The lysis of the protoplasts was accomplished by homogenizing them at 4°C in a Potter, the pestle of which was connected to a motor-driven grinder and by passing the cells through a 20- $\mu$ m mesh placed in a vacuum apparatus that gave a constant

pressure of 0.5 kg cm<sup>-2</sup>. The homogenates were centrifuged at 1500 g for 5 min at 4°C and the pellets containing nuclei were eliminated. The supernatants were added with 15% Percoll and centrifuged at 14 000 g for 30 min at 4°C. In Percoll gradient, plastids were recovered as a yellow pellet on the bottom and mitochondria as a darker layer above them. Plastids and mitochondria were rinsed in their washing buffer (20 mM Tris-HCl pH 7.6, 0.4 M mannitol, 0.5 mM EDTA, 4 mM cysteine, 1 mM ASC) and then resuspended in the same medium without mannitol to induce osmotic shock. Cytosol was recovered using an aliquot of the homogenates without nuclei and submitted to an ultracentrifugation at 100 000 g for 1 h at 4°C. The obtained fraction was concentrated in Amicon centricon concentrator tube (Millipore, Billerica, MA) for 1 h. Protein measurement was performed according to Bradford (1976) using BSA as a standard.

### Marker enzyme assays

Cytochrome *c* oxidase [EC 1.9.3.1] activity was performed following the decrease in absorbance at 550 nm because of the oxidation of reduced cytochrome *c*. The reaction mixture contained 0.1 M phosphate buffer pH 7 and the sample pretreated with 0.1% Triton-X-100 in ice for 1 min. The reaction started with the addition of 50  $\mu$ M reduced cytochrome *c*. An extinction coefficient of 21 mM<sup>-1</sup> cm<sup>-1</sup> was used (de Pinto et al. 2000).

NADH-dependent glutamine:2-oxoglutarate aminotransferase (NADH-GOGAT) [EC 1.4.1.13] activity was measured following the decrease in absorbance at 340 nm because of the NADH oxidation. The reaction mixture contained 0.05 M phosphate buffer pH 7.5, 10 mM  $\alpha$ -ketoglutarate, 10 mM glutamine. The reaction started adding 0.15 mM NADH and was followed for 20 min. An extinction coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup> was used (Lea and Mifflin 1980).

Alcohol dehydrogenase [EC 1.1.1.1] activity was tested measuring the increase of absorbance at 340 nm because of NAD<sup>+</sup> reduction. The reaction mixture contained 50 mM Tris-HCl pH 9, 0.867 mM NAD<sup>+</sup>. The reaction started adding 20% ethanol. An extinction coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup> was used (Chung and Robert 1999).

### ASC-GSH cycle enzyme assays

The activity of APX (L-ascorbate: hydrogen peroxide oxidoreductase) [EC 1.11.1.11], MDHAR (NADH: ascorbate free radical oxidoreductase) [EC 1.6.5.4], DHAR (glutathione: dehydroascorbate oxidoreductase) [EC 1.8.5.1] and GR (NADPH: glutathione disulphide

oxidoreductase) [EC 1.6.4.2] were tested according to de Pinto et al. (2000).

Native PAGE for cAPX and DHAR were performed according to de Pinto et al. (2000) When cAPX isoenzymes were analysed by native PAGE, ASC was omitted from the extraction buffer (de Pinto et al. 2000). Native PAGE for GR was performed according to Madamanchi et al. (1992).

## Statistics

The reported values are the average of five independent experiments  $\pm$  SE. The differences between treatments were analysed by one-way ANOVA.

## Results

### Localization of ASC-GSH cycle enzymes in TBY-2 cells

Cellular fractioning was performed to isolate cytosol, mitochondria, and plastids from TBY-2 cells. The purity of the obtained compartments was tested by measuring the activity of marker enzymes: cytochrome *c* oxidase for mitochondria (Wikström 1977); NADH-GOGAT for plastids (Lea and Miflin 1980) and alcohol dehydrogenase for cytosol (Shimomura and Beevers 1983). The percentage of the recovered specific activity of each marker enzyme represents the purity of the correspondent compartment (Table 1) (Lackey et al. 2003).

Once the quality of the purification procedure was established, the activities of the ASC-GSH cycle enzymes were measured in all the obtained compartments (Fig. 1). Most of APX activity was recovered in the cytosolic fraction. However, considerable activity of this enzyme, much higher than the contamination levels, was also found in plastids and mitochondria (19 and 12%, respectively; Table 2). The activities of the enzymes responsible for the recycling of the ASC oxidized forms (MDHAR; DHAR) showed similar profiles in the three analysed compart-

ments, having the highest activity in cytosol and the lowest in mitochondria. On the other hand, GR activity was more equally distributed in the investigated compartments: no difference in the GR activity of cytosol and plastids was detected, whereas in mitochondria this activity was only about 40% lower than in the other cellular compartments. The analysis of the percentages of the enzyme activities supported the presence of specific isoenzymes of ASC-GSH cycle in each cell compartments (Table 2).

Native PAGE analyses were also performed for APX, DHAR and GR. Two isoenzymes of APX were present in cytosol, three in plastids and two in mitochondria. The isoenzymes were different in migration rate and specific for each compartment (Fig. 2). Consistently with data of marker enzymes, a slight contamination of mitochondria with plastid isoenzymes, and vice versa, was evident in the native PAGE, whereas no cytosolic contamination was present in the two organelle fractions.

DHAR had two isoenzymes in cytosol, two in mitochondria and one in plastids, which showed the same electrophoretic mobility of one mitochondrial isoenzyme (Fig. 3).

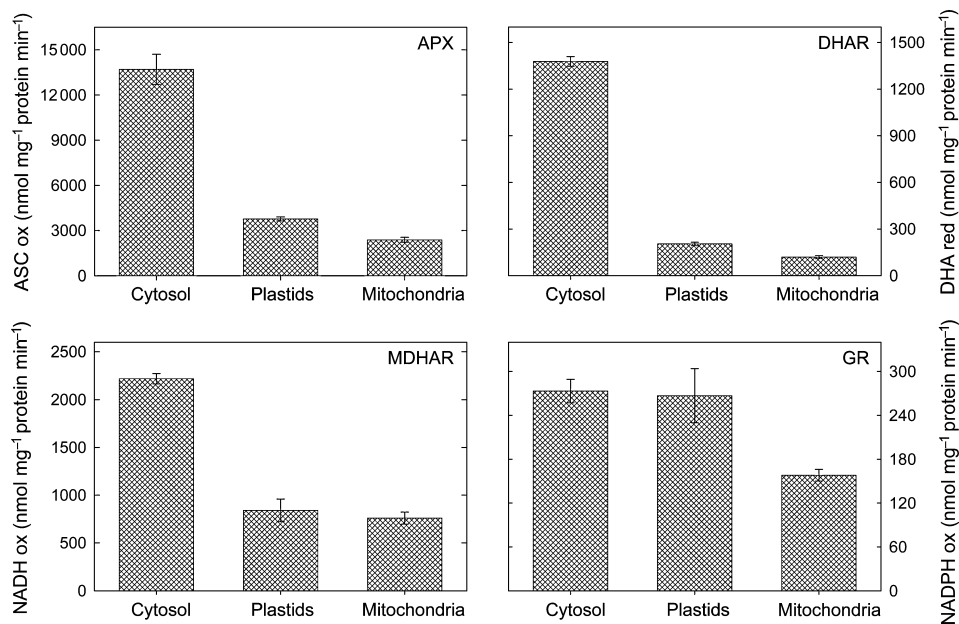
The native PAGE of GR showed the presence of several different isoenzymes, most of them characteristic for each compartment. Four isoenzymes were evident in cytosol, two in plastids and four in mitochondria. Some of them appeared to be specific of a certain compartment, others could be common in more than one compartment, at least according to their electrophoretic mobility (Fig. 4).

### Effects of HS on cell viability and ASC-GSH cycle enzymes

Once the subcellular localization of the ASC-GSH cycle enzymes in TBY-2 cells was known, the involvement of this cycle in the HS responses was analysed. At this purpose, TBY-2 cell cultures were exposed to two different HS conditions by transferring the cell suspension into a water bath at 35 and 55°C for 10 min. Both temperatures represent stressful conditions for TBY-2

**Table 1.** Cell fraction purities according to the enrichment of the marker enzyme activities. After cell fractioning, the measurement of the marker enzyme activity has been performed in all isolated compartments. The activity of alcohol dehydrogenase (cytosol marker enzyme), NADH-GOGAT (plastid marker enzyme) and cytochrome *c* oxidase (mitochondrion marker enzyme) has been measured as reported in Materials and methods. Values represent the means ( $\pm$ SE) of five experiments. 1 unit = 1 nmol substrate oxidized or reduced  $\text{min}^{-1} \text{mg}^{-1}$  prot.

Cell compartments	Cytochrome <i>c</i> oxidase		NADH-GOGAT		Alcohol dehydrogenase	
	Specific activity (units)	Activity distribution (%)	Specific activity (units)	Activity distribution (%)	Specific activity (units)	Activity distribution (%)
Cytosol	43 $\pm$ 5	1.5 $\pm$ 0.2	6 $\pm$ 2	3.6 $\pm$ 1.2	741 $\pm$ 200	92.9 $\pm$ 25.1
Plastids	141 $\pm$ 29	4.8 $\pm$ 1.0	140 $\pm$ 15	83.8 $\pm$ 9.0	6.4 $\pm$ 0.5	0.8 $\pm$ 0.1
Mitochondria	2746 $\pm$ 184	93.7 $\pm$ 6.3	21 $\pm$ 6	12.6 $\pm$ 3.6	50 $\pm$ 20	6.3 $\pm$ 2.5



**Fig. 1.** Distribution of the activity of ASC-GSH cycle enzymes in cytosol, plastids and mitochondria of TBY-2 cells. The activities of APX, DHAR, MDHAR and GR have been measured in each isolated cell fraction as reported in Materials and methods. Values represent the means ( $\pm$ SE) of five experiments.

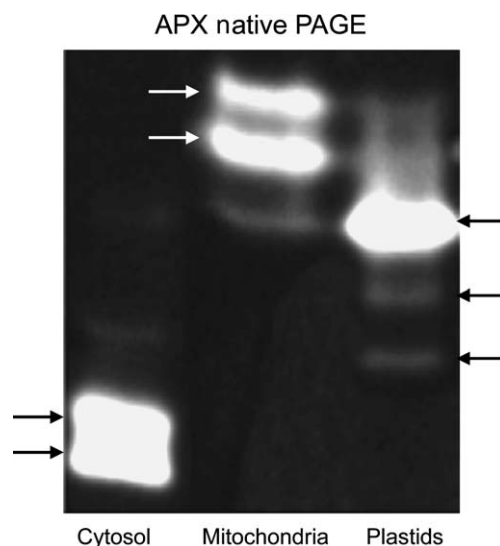
cells, which normally grow at 27°C. After each treatment, cells were brought back to their standard growth temperature. Cell viability was monitored during the period following the HSs. Consistently with results previously reported (Locato et al. 2008), the exposure at 35°C HS did not affect cell viability. On the contrary, 55°C HS caused a strong and immediate reduction of cell viability. Such reduction started to be visible 2 h after the HS exposition, when cell viability decreases of  $7 \pm 4\%$ . In the first 4 h following the 55°C HS, cell viability decreased by  $18 \pm 2\%$ ; after 24 and 48 h following the treatment, it reached values of  $50 \pm 6\%$  and  $20 \pm 3\%$  of the control, respectively. The cell death induced by 55°C HS was a PCD because it was accompanied by DNA laddering and cytoplasmic shrinkage (data not shown) as well as cytochrome *c* release from mitochondria and

activation of caspase-like proteins (Vacca et al. 2004, 2006).

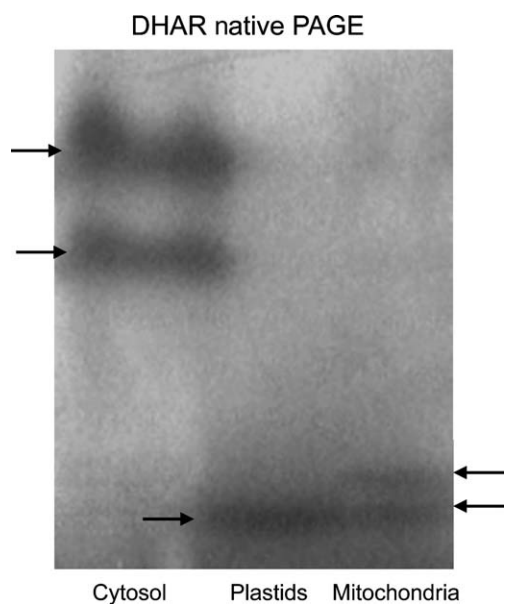
The changes induced by the two different HS conditions on the activities of the ASC-GSH cycle enzymes were measured after 2 h from cells exposition to HS. This

**Table 2.** Percentages of the activities of ASC-GSH cycle enzymes in different cellular compartments. The specific activities of the ASC-GSH cycle enzymes of each cellular compartment have been quantified as percentage of the total activity according to Lackey et al. 2003. Values represent the means ( $\pm$ SE) of three independent experiments.

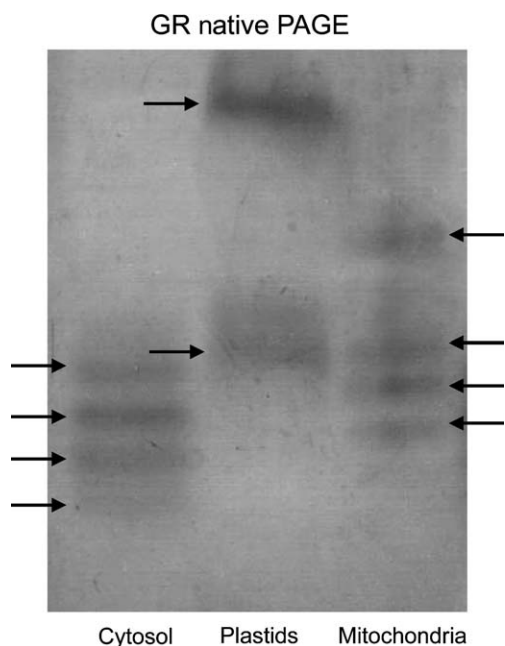
Cell compartments	Activity distribution (%)			
	APX	DHAR	MDHAR	GR
Cytosol	$69 \pm 5.0$	$81 \pm 1.8$	$58 \pm 1.4$	$39 \pm 2.3$
Plastids	$19 \pm 0.7$	$12 \pm 0.6$	$22 \pm 3.0$	$38 \pm 5.3$
Mitochondria	$12 \pm 0.8$	$7 \pm 0.6$	$20 \pm 1.6$	$23 \pm 1.1$



**Fig. 2.** Native PAGE of APX isoenzymes in cytosol, plastids and mitochondria of TBY-2 cells. Cytosol, plastids and mitochondria of TBY-2 cells have been submitted to the native PAGE for identification of proteins with APX activity as described in Materials and methods; 200  $\mu$ g of proteins were loaded in each lane. The shown result has been confirmed in three independent experiments.



**Fig. 3.** Native PAGE of DHAR isoenzymes in cytosol, plastids and mitochondria of TBY-2 cells. Cytosol, plastids and mitochondria of TBY-2 cells have been submitted to the native PAGE for identification of proteins with DHAR activity as described in Materials and methods; 200  $\mu$ g of proteins were loaded in each line. The shown result has been confirmed in three independent experiments.



**Fig. 4.** Native PAGE of GR isoenzymes in cytosol, plastids and mitochondria of TBY-2 cells. Cytosol, plastids and mitochondria of TBY-2 cells have been submitted to the native PAGE for identification of proteins with GR activity as described in Materials and methods; 200  $\mu$ g of proteins were loaded in each line. The shown result has been confirmed in three independent experiments.

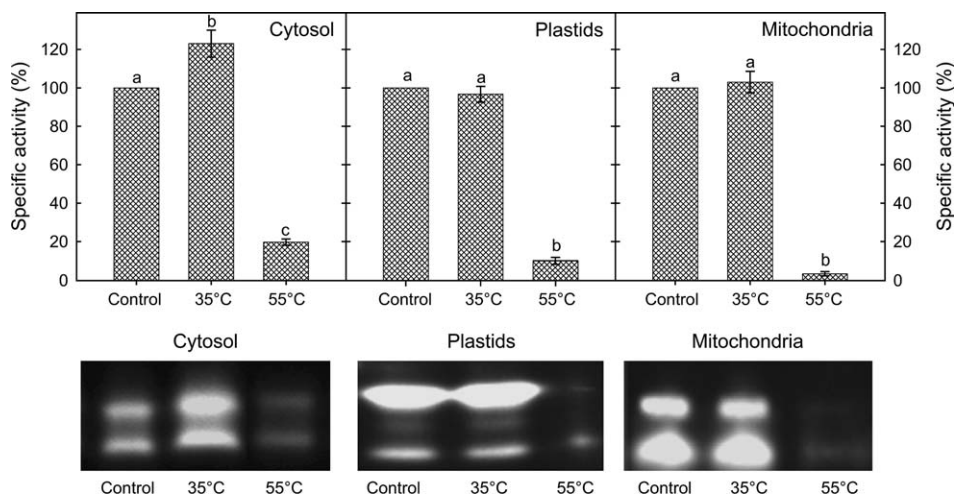
was the necessary time for protoplast isolation, which is the preliminary step required by cell fractioning. Furthermore, in the 55°C heat shocked cells, the 2 h were sufficient for triggering the pathways leading to PCD, even if a very modest percentage of cells had already died.

The effect of the two HSs on the activity of APX in cytosol, plastids and mitochondria is shown in Fig. 5. The cytosolic fraction seems to be the most involved compartment in stress responses. In fact, after treatment at 35°C, increase of APX activity was only observed in the cytosolic fraction, because the APX activity of both mitochondria and plastids almost remained unchanged. On the contrary, exposure at 55°C HS induced a strong decrease in the activities of the APX isoenzymes of all the analysed cellular compartments. Fig. 5 also reports a representative native PAGE of the different APX isoenzymes from cells exposed to the two HSs. The HSs did not induce any change in the number of the isoenzymes present in the various compartments, whereas the intensity of all the bands was altered according to the changes observed in the specific activities of the enzyme, even if it must be underlined that this technique is more appropriate for giving qualitative information rather than quantitative ones.

The enzymes responsible for the reduction of the oxidized forms of ASC and GSH showed different behaviours from APX. After 35°C HS exposition, neither MDHAR nor DHAR nor GR significantly altered their subcellular activities with the exception of the plastidial MDHAR, whose activity significantly increased (Fig. 6). On the other hand, after 55°C HS, the activities of the three enzymes increased in the cytosol, whereas they showed specific changes in the other cellular compartments. MDHAR and GR decreased in both plastids and mitochondria, the former enzyme being almost undetectable in mitochondria. DHAR activity decreased in plastids but it rose in mitochondria. Similar to what occurred for APX, native PAGE of DHAR and GR did not induce HS-dependent appearance or disappearance of specific bands (Fig. 7).

## Discussion

Data reported in this study show that ASC-GSH cycle enzymes are present in the cytosol, plastids and mitochondria isolated from TBY-2 cells. This result is consistent with the literature, which suggests that ASC-GSH cycle plays a pivotal role in maintaining the redox homeostasis of mostly cellular compartments (De Gara 2004, Pastori and Foyer 2002, Pavet et al. 2005). The low contamination level of each compartment by the others, as verified by marker enzymes (Table 1) and native PAGE analysis (Figs 2, 3 and 4), clearly indicates that the

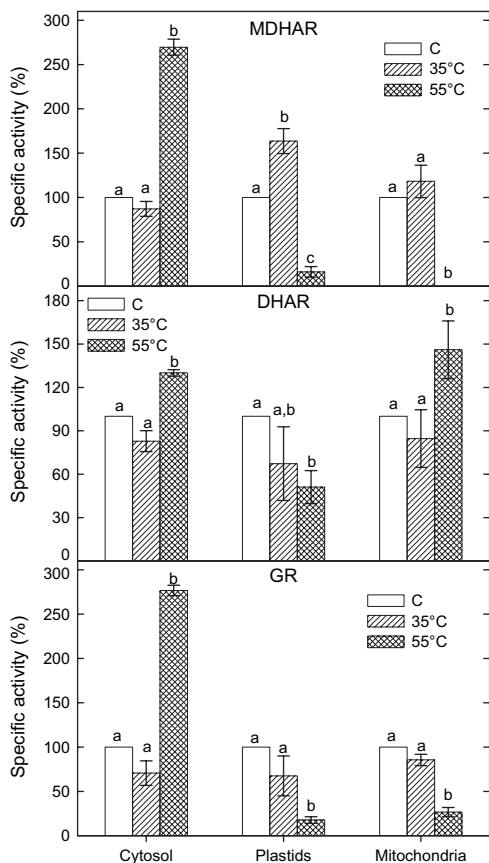


**Fig. 5.** Changes in APX activity induced by HSs in tobacco BY-2 subcellular fractions. (A) APX-specific activity. Cell fractioning has been performed after HS and the APX activity was measured in each isolated compartment as reported in Materials and methods. Values represent the means ( $\pm$ SE) of five experiments. Different letters represent values that are statistically different (ANOVA test;  $P \leq 0.05$ ). (B) Representative native PAGE of APX from cells exposed to HS; 200  $\mu$ g of proteins were loaded in each line. The shown result has been confirmed in two independent experiments.

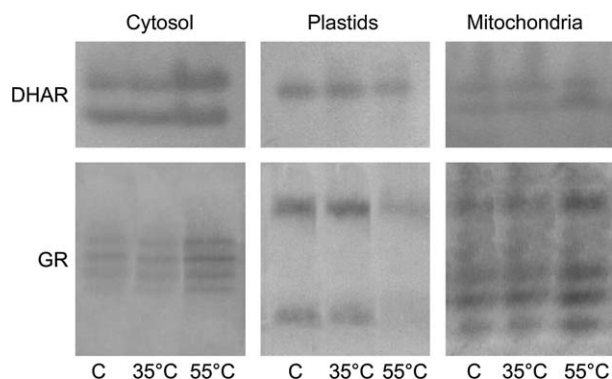
different compartments have specific isoenzymes. In the non-photosynthesizing cells, mitochondria probably are the main producers of ROS (Noctor and Foyer 1998). Despite this, in TBY-2 cells, the ASC-GSH cycle enzymes have the highest activities in the cytosol (Fig. 1). This suggests that cytosol plays a redox-buffering role for the whole cellular metabolism. A substantial part of ROS, in particular  $H_2O_2$ , produced in different organelles, easily permeates into the cytosol by passing across biological membranes, most probably through aquaporins (Dynowski et al. 2008). Consistently, the cytosolic activities of APX, MDHAR and DHAR, the three isoenzymes directly involved in the ASC oxidoreduction, are many folds higher than those of the correspondent isoenzymes localized in mitochondria and plastids. When each compartment is taken into account, a correlation between the activities of APX, MDHAR and DHAR seems to be evident, being that of APX activity 3–5 and 10–15 times higher than those of MDHAR and DHAR of the same compartment, respectively. This consistency supports the hypothesis that the three enzymes coordinately act in each compartment to optimize the ASC availability for ROS detoxification as well as for other metabolic pathways in which this redox molecule is involved. On the other hand, the GR, the enzyme responsible for reducing GSH disulphide into GSH, has very similar activity in cytosol and plastids and only about 40% lower in mitochondria (Fig. 1). It is interesting to notice that GSH is not only involved in ASC recycling, acting as the electron donor of DHAR, but it is also responsible for other metabolic pathways, such as the oxidoreduction of proteic sulphidric groups, or metal and

xenobiotics conjugation (Brazier-Hicks et al. 2008, De Gara et al. 2003, Foyer and Noctor 2005, Paradiso et al. 2008). Moreover, GSH also acts as electron donor for reducing DHA in reactions not catalysed by DHAR, such as that catalysed by a protein disulphide isomerase (Bánhegyi et al. 2003). Because of the redox potential of the GSH disulphide–GSH pair being more negative than that of DHA–ASC ( $-0.23$  and  $0.08$ , respectively), GSH might undergo non-enzymatic oxidation more than ASC. Therefore, in the different cellular compartments, GR might not only be involved in the metabolite flux through ASC-GSH cycle but also in GSH regeneration in other metabolic pathways. This seems to be particularly supported by the enzymatic profile of plastids and mitochondria, where the GR activity is much higher than that of DHAR, thus suggesting that restabilizing a correct GSH redox state requires a GR activity higher than that necessary for ASC regeneration.

In the second part of this study, the effects of two different HSs on the ASC-GSH cycle enzymes were analysed in the isolated compartments of TBY-2 cells. The two HS conditions were chosen to obtain the activation of different cellular responses: 35°C HS determines a moderate oxidative stress that allows cells to promptly recover their redox homeostasis (Locato et al. 2008), whereas 55°C HS induces a much more drastic oxidative stress against which PCD is activated. Consistently with previous results (Locato et al. 2008), 35°C HS has no effect on cell viability, while 55°C HS induces an active programme of cell death that progressively occurs over time and that is prevented by exogenous addition of ROS scavengers (Vacca et al. 2004).



**Fig. 6.** Changes in the activity of the ASC-GSH recycling enzymes induced by HSs in tobacco BY-2 subcellular fractions. Cell fractioning has been performed after HSs and the activity of ASC-GSH recycling enzyme has been measured as reported in Materials and methods. Values represent the means ( $\pm$ SE) of five experiments. Different letters represent values that are statistically different (ANOVA test;  $P \leq 0.05$ ).



**Fig. 7.** Changes in the native PAGE profiles of the ASC and GSH recycling enzymes induced by HS in tobacco BY-2 subcellular fractions. Representative native PAGE of DHAR and GR of cytosol, plastids and mitochondria from cells exposed to HS; 200  $\mu$ g of proteins were loaded in each line. The shown result has been confirmed in two independent experiments.

HS at 35°C only induces an increase in the cytosolic APX and plastidial MDHAR, whereas DHAR and GR are not altered at all (Figs 5 and 6). The increase in the cytosolic APX is aimed to remove  $H_2O_2$  overproduced under this condition (Locato et al. 2008) and to restore the cellular redox homeostasis. The maintenance of ASC and GSH in the correct redox state is also pivotal for cellular redox homeostasis because these two redox pairs are very sensitive to redox unbalances occurring in cell (de Pinto et al. 1999, Noctor and Foyer 1998, Tausz et al. 2004). On the other hand, under 35°C HS, no alteration in the redox state of these two metabolites occurs (Locato et al. 2008), in spite of an increase in the APX-dependent oxidation of ASC being observed (Fig. 5). This suggests that the subcellular activities of the ASC recycling enzymes are probably redundant to better cope with moderate and commonly occurring oxidative stress. The increase of APX activity only in the cytosol supports a special role for this compartment's isoenzymes in controlling cellular  $H_2O_2$  levels. Consistently, several indications support that cytosolic APXs are involved in stress responses more than the organellar isoenzymes (Karpinski et al. 1997, Shigeoka et al. 2002). For example, in *A. thaliana* subjected to photo-oxidative stress, that affects chloroplasts, cytosolic APX is also systemically over-expressed (Karpinski et al. 1999). The major involvement of the cytosolic APX in the oxidative stress responses could also be the result of the ability of this isoenzyme in maintaining its activity under ASC depletion. In fact, it is known that the APX isoenzymes present in the organelles are very sensitive to ASC depletion, an event occurring during oxidative stresses (Shigeoka et al. 2002).

When a strong HS is imposed, the picture obtained for the ASC-GSH cycle enzymes is completely different. Under HS conditions that induce PCD, the activities of ASC-GSH recycling enzymes strongly increase in the cytosolic fraction. As previously reported, under 55°C HS, a strong decrease occurs in ASC and GSH pools (Locato et al. 2008). It is probable that the cytosolic increases in the ASC and GSH recycling enzymes are aimed to compensate for the depletion in these redox metabolites. On the other hand, in the organelles, the activities of the ASC-GSH recycling enzymes decrease, with the exception of that of the mitochondrial DHAR that increases under 55°C HS. At present, we are quite far from explaining the peculiar enzymatic regulation of each organelle. The results presented here underline that the various cellular compartments are diversely affected even if the same stress is imposed to the whole cell. Moreover, the cytosol seems to be particularly sensitive playing a crucial role in controlling redox homeostasis. In relation to the different behaviour of the mitochondrial DHAR under 55°C HS, it is worth noticing that mitochondrion is



the ASC production site because the final enzyme of its biosynthetic pathway, galactono- $\gamma$ -lactone dehydrogenase, is localized in mitochondrial inner membrane and its catalytic activity requires oxidized cytochrome *c* (Bartoli et al. 2000). It is known that an impairment of the electron respiratory transport occurs during oxidative stress and such impairment seems to contribute to ASC depletion through its synthesis inhibition (Millar et al. 2003); moreover, during HS-induced PCD, part of cytochrome *c* is released from the mitochondrion (Vacca et al. 2006). Cytochrome *c* depletion could contribute to ROS overproduction and, consequently, cause respiratory chain damage and ASC biosynthesis reduction. Both events were already reported to occur in TBY-2 cells subjected to 55°C HS (Valenti et al. 2007). It is also interesting to mention that the activation of PCD induces the release from mitochondria of an apoptosis-inducing factor, which seems to be homologue to a MDHAR isoenzyme (Lorenzo et al. 1999). Taking into account this information, the release of the apoptosis-inducing factor, having MDHAR activity, might explain the particularly evident drop of this enzyme activity observed in the mitochondria of cells undergoing PCD. In fact, mitochondrial MDHAR is the only ASC recycling isoenzyme whose activity is no more detectable after 55°C HS. In 55°C heat shocked mitochondria devoid of MDHAR and with reduced ASC biosynthetic capability, the role of DHAR seems to be particularly relevant. In fact, this enzyme might optimize the utilization of the still available ASC in a cellular organelle that is strongly subjected to oxidation by the ROS overproduced here. Therefore, the increase in DHAR activity could be a sort of feedback regulation mechanism occurring to improve ASC regeneration from DHA when ASC depletion occurs at its production site.

The decrease in APX activity occurring after 55°C HS plays a role in promoting PCD occurrence because such decrease probably lets oxidative burst taking place and, as a consequence, induces the activation of all the other metabolic events that orchestrate PCD, as it has been previously discussed in detail (Locato et al. 2008). Here, it is important to underline that the impairment of the isoenzymes working in all the cellular compartments contributes to the oxidative burst occurrence during PCD.

In conclusion, our data suggest that cytosol, mitochondria and plastids are provided with a complete ASC-GSH cycle enzymes, the activities of which have similar ratio among them, but different specific activities in different cellular compartments. The changes in the ASC-GSH cycle of the different compartments also diversely contribute to the redox homeostasis maintenance under moderate stress conditions (35°C HS) or to determine the cellular conditions leading to PCD (55°C HS).

It is probable that the different changes occurring in the ASC-GSH cycle during the two imposed stresses are also the consequences of alterations of other metabolic pathways that are also affected by the HSs in different compartments. Further analyses comprising a more general view of the metabolic networks working in each organelle are needed to better understand the relevance and the interplay of different cell compartments in PCD as well as in defence responses against stresses affecting whole cells and/or organisms.

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