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RESEARCH PAPER

Compartment-specific role of the ascorbate–glutathione cycle in the response of tomato leaf cells to *Botrytis cinerea* infection

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Abstract

Changes in AA-GSH cycle activity following *Botrytis cinerea* infection were studied in tomato whole-leaf extracts as well as in chloroplasts, mitochondria, and peroxisomes. The oxidative effect of infection affected all cellular compartments although mitochondria and peroxisomes underwent the most pronounced changes. Apart from organelle-specific variations, a general shift of the cellular redox balance towards the oxidative state was found. It was manifested by the significant decline in concentrations and redox ratios of the ascorbate and glutathione pools as well as by the insufficient activity of MDHAR, DHAR, and GR needed for antioxidant regeneration. There was no compatibility between the ascorbate- and glutathione-mediated changes in different compartments. It was concluded that *B. cinerea* was able to break down the protective antioxidant barrier of the AA-GSH cycle at both the cellular and organellar levels. The changes in the AA-GSH cycle activity could partly be related to the *B. cinerea*-induced promotion of senescence that favoured disease progress.

Key words: Ascorbate–glutathione cycle, *Botrytis cinerea*, chloroplasts, mitochondria, peroxisomes, tomato.

Introduction

Under optimal conditions the constitutive antioxidants in plant cells control the basal production of ROS maintaining their concentrations at levels compatible with the specific metabolic requirements of different cellular compartments. However, in natural environments plants are exposed to many adverse abiotic and biotic factors that cause an overproduction of ROS leading to the imbalance between ROS production and degradation, a process referred to as oxidative stress (Dat *et al.*, 2000; Bolwell *et al.*, 2002). This phenomenon being a common trait of all environmental stresses is regarded as the major cause of the oxidative damage found in cells under stressful conditions. Plant resistance to stresses is closely associated with the capacity of the antioxidant system (Mittler, 2002). Much evidence points to a pivotal role for the AA-GSH cycle that scavenges ROS, and particularly H_2O_2. Its activity relies on the sequential oxidation and re-reduction of AA and GSH in reactions catalysed by the enzymes constituting the cycle, namely APX, MDHAR, DHAR, and GR (Noctor and Foyer, 1998). In plant cells this cycle has been shown to operate in all organelles in which ROS detoxification is needed, i.e. in chloroplasts, mitochondria, and peroxisomes (Jiménez *et al.*, 1997; Asada, 1999). Apart from its function directly to detoxify ROS and thereby to combat the potentially harmful effects of environmental stresses, the AA-GSH cycle is also involved in redox sensing and signalling (Pastori and Foyer, 2002). Hence, the specific interplay between ROS and the AA-GSH cycle constituents could generate compartment-specific changes in both the absolute concentrations of ROS and the antioxidant compounds, and in ascorbate and glutathione redox ratios. Under stress conditions these redox signals could interfere with signalling networks complementary to the antioxidant system and regulate defence gene expression (Grant and Loake, 2000; Vranová *et al.*, 2002; Kiddle *et al.*, 2003), thus co-ordinating the necessary readjustments in the redox-regulated plant defence to overcome the oxidative stress. Although the implication of the oxidative stress in

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Abbreviations: AA, reduced ascorbate; AA–GSH cycle, ascorbate–glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione, GSSG, oxidized glutathione; MDHAR, monodehydroascorbate reductase; ROS, reactive oxygen species

plant–pathogen interactions is well documented (Baker and Orlandi, 1995; De Gara *et al.*, 2003), to date very little information is available on the involvement of the AA-GSH cycle in plant defence against biotic stress.

ROS overproduction constitutes one of the first responses of plant cells to infection (Wojtaszek, 1997*a*). Following pathogen attack, the generation of ROS occurs mainly extracellularly via membrane NADPH-dependent oxidase, cell wall peroxidase or amine, diamine and polyamine oxidase-type enzymes (Wojtaszek, 1997*b*; Bolwell *et al.*, 2002). The intracellular ROS sources include chloroplasts, mitochondria, and peroxisomes. ROS generated upon pathogen attack have been proposed to play two different roles: exacerbating the harmful oxidative effect of infection or participating in the defence response by being toxic to the invading pathogen, contributing to the programmed cell death during the hypersensitive response, driving the cell wall reinforcement processes as well as serving as signal molecules for the activation of local and systemic resistance (Levine *et al.*, 1994; Mehdy *et al.*, 1996; Alvarez *et al.*, 1998; Grant and Loake, 2000; Corpas *et al.*, 2001). This picture is further complicated by the fact that the ROS-mediated plant response is variable and depends on the pathogen life style (biotrophy versus necrotrophy), the type of plant–pathogen interaction (compatibility versus incompatibility) and the stage of plant development (Somssich and Hahlbrock, 1998; Govrin and Levine, 2000; Barnes and Shaw, 2002; Hückelhoven and Kogel, 2003). Thus, the tightly regulated balance between ROS production and removal at both the cellular and subcellular levels seems to be of primary importance for fulfilling the multiple functions of ROS and controlling redox homeostasis.

At the subcellular level, chloroplasts, mitochondria, and peroxisomes that have been considered to be the most powerful sources of ROS under optimal conditions are simultaneously equipped with highly effective antioxidant mechanisms involving the AA-GSH cycle (Jiménez *et al.*, 1997; Noctor and Foyer, 1998; Asada, 1999; del Río *et al.*, 2002). Balancing the production of ROS is therefore important for many different aspects of their normal metabolism as well as under stress conditions. Although the pathogen-induced oxidative stress is generated mainly extracellularly, chloroplasts, mitochondria, and peroxisomes and their antioxidant machinery could be also targeted during pathogenesis. There is a growing body of evidence that either ROS or changes in the redox balance generated at sites of primary action of stressors could activate a co-ordinated response in other compartments to ensure a successful defence strategy (Foyer and Noctor, 2003). Over the past few years progress has been made in the functional characterization of plant peroxisomes and in dissecting their potential role in plant cell defence against oxidative stress (Corpas *et al.*, 2001). While the contribution of antioxidant systems in different organelles to abiotic stress defence has been well documented, the data on their compartment-specific role during plant–pathogen interaction is still scarce and equivocal. In the present work the compartment-specific role of the antioxidant system under biotic stress conditions was assessed by following changes in the AA-GSH cycle activity in chloroplasts, mitochondria, and peroxisomes isolated from *B. cinerea*-infected tomato leaves. This comparative study contributes to knowledge of the molecular mechanisms of plant response to infection-induced oxidative stress and of the possible functional differences between the cellular compartments in the protecting mechanism.

Materials and methods

Plant material and pathogen infection

Tomato plants (*Lycopersicon esculentum* Mill.) were grown as described earlier (Kuźniak and Skłodowska, 2001). At the age of 6 weeks plants were inoculated with spore solution containing *Botrytis cinerea* Pers:Fr conidia (2×10^6 spores ml^{-1}), 5 mM glucose, and 2.5 mM KH_2PO_4. The inoculation solution was sprayed uniformly on all leaves. Thereafter plants were kept at 100% relative humidity to ensure conidia germination and to facilitate infection. The activity of the AA-GSH cycle was studied in whole-leaf extracts as well as in chloroplasts, mitochondria, and peroxisomes obtained from the first, second, and third leaves harvested in the middle of the 16 h light period. Analyses were performed at time 0 (t_0) and 1, 2, 3, 4, and 5 d post-inoculation (dpi).

Isolation and characterization of organelles

Isolation of chloroplasts was as described earlier (Kuźniak and Skłodowska, 2001). The activity of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9), used as a stromal marker, was assayed as described by Bradbeer (1969). The activities of CAT (EC 1.11.1.6) being a peroxisomal marker enzyme and fumarase (EC 4.2.1.2) as a specific mitochondrial marker were assayed using the UV method described by Dhindsa *et al.* (1981), and according to Hatch (1978), respectively. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was considered an auxiliary marker for cytosol and was assayed using a Sigma diagnostic kit. Chlorophyll was extracted with 80% acetone and measured according to Porra *et al.* (1989). Tomato leaf mitochondria were isolated as described by Kuźniak and Skłodowska (2004) and purified by a Percoll density-gradient containing sucrose according to Struglics *et al.* (1993). The mitochondrial fraction located close to the top was identified on the basis of the activity of specific marker enzymes: succinate:cytochrome *c* oxidoreductase (EC 1.6.99.3) and NADH:cytochrome *c* oxidoreductase activity, both assayed as described by Douce *et al.* (1972), and fumarase. To determine the purity of the mitochondrial fraction, chlorophyll content and the following marker enzyme activities were assayed: glucose-6-phosphate dehydrogenase for cytosol, $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase for the chloroplast stroma, and CAT for peroxisomes. For the isolation of peroxisomes leaves (70 g) without the main midribs were cut into pieces, chilled in an ice bath for 20 min, and homogenized in a blender for 2×15 s in an ice-cold isolation medium (1:5 w/v). The medium (pH 7.4) contained 20 mM TRIS-HCl, 0.3 M mannitol, 1 mM EDTA, 20 mM sodium ascorbate, 0.1% bovine serum albumin, and 2 mM $MgCl_2$. The homogenates were filtered through six layers of gauze and centrifuged at 200 *g* for 20 min to remove chloroplasts. The supernatants were centrifuged again at 15 000 *g* for 10 min to recover peroxisomes. The pellet was gently resuspended in a medium containing 0.3 M mannitol, 0.05% bovine serum albumin, 1 mM EDTA, 10 mM

Table 1. *Marker enzyme activities and chlorophyll concentration in purified organelle fractions isolated from tomato leaves*

Marker enzyme	Organelle fractions		
	Chloroplasts	Mitochondria	Peroxisomes
Glucose-6-phosphate dehydrogenase (nmol min^{-1} mg^{-1} protein)	ND[a]	ND	7.30
$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (μmol min^{-1} mg^{-1} protein)	4.47	ND	ND
Chlorophyll (μg mg^{-1} protein)	126.46	1.50	2.46
Acid phosphatase (μmol min^{-1} mg^{-1} protein)	1.77	ND	ND
Fumarase (nmol min^{-1} mg^{-1} protein)	ND	190.80	24.10
NADH:cytochrome *c* oxidoreductase (nmol min^{-1} mg^{-1} protein)	9.20	88.50	8.10
Catalase (mmol min^{-1} mg^{-1} protein)	ND	0.03	1.51
Hydroxypyruvate reductase (μmol min^{-1} mg^{-1} protein)	ND	0.17	10.07

[a] ND, not detected.

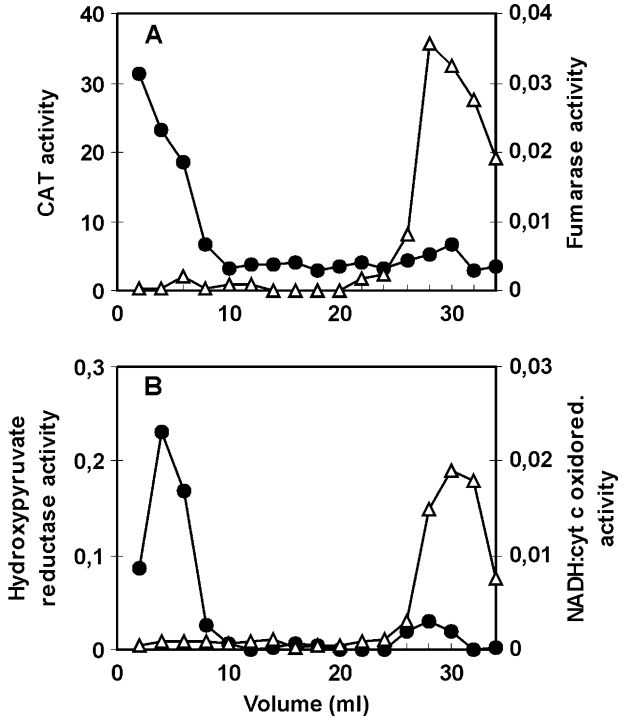

Fig. 1. Analysis of the Percoll density-gradient used for the isolation of mitochondria and peroxisomes from non-infected tomato leaves. (A) Distribution of CAT (closed circles) and fumarase (open triangles). (B) Distribution of hydroxypyruvate reductase (closed circles) and NADH: cytochrome *c* oxidoreductase (open triangles). Fumarase and NADH;cytochrome *c* oxidoreductase were used as markers for mitochondria. CAT and hydroxypyruvate reductase were used as peroxisomal markers. The enzyme activities were expressed as μmol min^{-1} ml^{-1}.

TRIS-HCl, 1 mM sodium ascorbate, and 0.3 M sucrose (pH 7.2) and separated on a continuous Percoll gradient containing sucrose as the osmoticum as described by Struglics *et al.* (1993). The peroxisomal band located at the bottom was identified on the basis of activity of specific marker enzymes: hydroxypyruvate reductase (EC 1.1.1.29) (Schwitzguebel and Siegenthaler, 1984) and CAT. The collected fraction was diluted 10 times with the buffer containing 10 mM TRIS-HCl (pH 7.2), 0.3 M mannitol, 1 mM EDTA, 1 mM sodium ascorbate, and pelleted at 15 000 *g* for 10 min. In order to remove Percoll the washing procedure was carried out three times. All operations were carried out at 0–4 °C. The buffers lacking sodium ascorbate were used when ascorbate content was determined. The quality of the peroxisomal fraction was monitored by measurements of chlorophyll content and marker enzyme activities. The activities of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase, fumarase, and acid phosphatase (EC 3.1.3.2), being specific marker enzymes for chloroplast stroma, mitochondria, and vacuoles, respectively were measured. Acid phosphatase was assayed using Sigma diagnostic kit.

The intactness of all organelles was determined on the basis of enzyme latency calculated for the activities of specific marker enzymes in the isotonic medium and in the organellar extract according to the formula of Burgess *et al.* (1985). The integrity of chloroplasts was estimated by the ferricyanide method according to Heber and Santarius (1970). The integrity of the outer mitochondrial membrane was determined based on NADH:cytochrome *c* oxidoreductase. The intactness of peroxisomes was determined on the basis of enzyme latency calculated for hydroxypyruvate reductase. Moreover, for all organelles, latency of APX (EC 1.11.1.11) and GR (EC 1.6.4.2) was also calculated.

Preparation of extracts for analyses

For preparing the whole-leaf extracts, leaves were homogenized (1:10 v/w) using 50 mM potassium phosphate buffer pH 7.0 containing 1 M NaCl, 1% polyvinylpyrrolidone, and 1 mM EDTA. For assays of APX, extracts were prepared in the same medium containing 1 mM sodium ascorbate. After centrifugation (20 000 *g*, 15 min) the supernatant was used for determination of APX, MDHAR, DHAR, and GR activities as well as glutathione content. For preparing the organellar extracts the chloroplastic, mitochondrial, and peroxisomal pellets obtained from the respective isolation procedures were homogenized as described above. For the determination of ascorbate, extracts were prepared in 6% ice-cold trichloroacetic acid.

Antioxidant enzyme assays and determination of ascorbate and glutathione

APX activity was assayed following the oxidation of ascorbate at 265 nm (ε=13.7 mM^{-1} cm^{-1}) by a modified method of Nakano and Asada (1981) and DHAR (EC 1.8.5.1) was determined by following the formation of ascorbate at 265 nm (ε=14.6 mM^{-1} cm^{-1}) according to Hossain and Asada (1984). The activity of MDHAR (EC 1.6.5.4) was determined following the oxidation rate of NADH at 340 nm (ε=6.22 mM^{-1} cm^{-1}) as described by Kuźniak and Skłodowska (2004). The activity of GR was assayed by the determination of GSSG-dependent oxidation of NADPH at 340 nm (ε=6.22 mM^{-1} cm^{-1}) as described by Beutler (1975). The activities of APX, MDHAR, and DHAR were expressed in μmol min^{-1} mg^{-1} protein and GR activity was given in nmol min^{-1} mg^{-1} protein. For the determination of ascorbate, leaves were homogenized in 6% trichloroacetic acid. Ascorbate was determined according to the

Table 2. *Latency of marker and antioxidant enzymes in the purified organelle fractions isolated from tomato leaves before infection (time 0) and 5 dpi or 4 dpi*

Enzyme	Latency					
	Chloroplasts		Mitochondria		Peroxisomes	
	t_0	5 dpi	t_0	5 dpi	t_0	4 dpi
$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase	64	73	–	–	–	–
Ferricyanide reduction	83	80	–	–	–	–
Fumarase	–	–	98	93	–	–
NADH:cytochrome *c* oxidoreductase	–	–	71	69	–	–
Hydroxypyruvate reductase	–	–	–	–	80	71
APX	73	76	85	80	77	70
GR	67	69	98	97	95	81

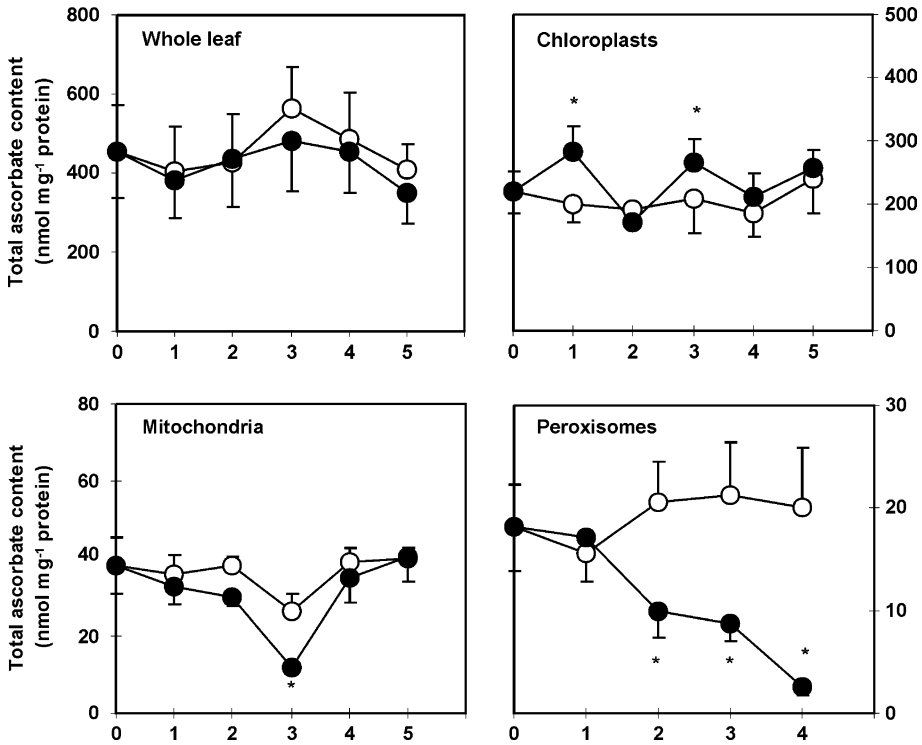

Fig. 2. The effect of *B. cinerea* infection on ascorbate content in organelles isolated from tomato leaves. * Indicates means that differ significantly from control. Open symbols, control; closed symbols, infected. Data are means ±SD.

colorimetric bipyridyl method as described by Knörzer *et al.* (1996). Total ascorbate was assayed by adding dithiothreitol to the samples for reducing DHA to AA. DHA concentration was calculated by subtracting the AA value from the total ascorbate. For the determination of glutathione, the sample obtained as described for enzyme assays was deproteinized immediately using 30% $HClO_4$. The levels of non-protein sulphydryl groups (-SH) were determined colorimetrically with 5,5′-dithio*bis*-2-nitrobenzoic acid according to Brehe and Burch (1976). For specific assay of GSSG the GSH could be masked by derivatization with 2-vinylpyridine in the presence of triethanolamine for 60 min at 20 °C. The concentrations of non-enzymatic antioxidants were expressed in nmol mg^{-1} protein.

Other determinations

The protein content was determined by the Bradford method (1976).

Statistical analysis

Each value is the mean from at least four individual replicates ±SD. Values that differ significantly from the control at $P < 0.05$ were indicated by asterisks.

Results

The first clearly visible symptoms of *B. cinerea* infection in the form of dark necrotic lesions appeared 2–3 dpi. Later on, starting from the lowermost leaves, a subset of lesions gradually developed into typical spreading lesions. The youngest leaves remained symptomless over all 5 d of the experiment.

Characterization of the organelle fractions prepared from tomato leaves

The purity of the chloroplasts, mitochondria, and peroxisomes was monitored by determination of the chlorophyll content and of specific marker enzyme activities (Table 1). On the Percoll gradient the intact mitochondrial band was located close to the top (28–32 ml) and the peroxisomal band was almost at the bottom (2–6 ml) as judged on the basis of high activities and high latency of marker enzymes (Fig. 1). The cross-contamination of organelles was in the range routinely found for subcellular fractionation of leaves, reflecting the relatively clean separation of chloroplasts, mitochondria, and peroxisomes (Fig. 1; Table 1). The integrity of chloroplasts, mitochondria, and peroxisomes was estimated by measuring the latency of marker enzymes as well as two antioxidant enzymes: APX and GR (Table 2). The comparative analysis of enzyme latency at t_0 and 5(4) dpi revealed that *B. cinerea* infection did not significantly modify the integrity of isolated organelles (Table 2).

Infection-dependent changes in ascorbate and glutathione pools

There was no effect of *B. cinerea* infection on the total ascorbate concentration in whole-leaf extracts (Fig. 2). Similarly, AA content did not change, except for a 17% (P <0.05) decrease found 3 dpi, which was accompanied by a rise in DHA content up to 157% of the control (P <0.05). Conversely, 5 dpi DHA concentration fell to the level that was 64% (P <0.05) lower than in the control. The *B. cinerea*-induced perturbations in the DHA pool drove concomitant changes in the AA:DHA ratio. After a transient decrease found 3 dpi, 5 d after challenge, the AA:DHA ratio was about 2-fold higher than in the control (Fig. 3).

In chloroplasts, the total ascorbate content was significantly increased (P <0.05) 1 and 3 dpi (Fig. 2). This was mainly the effect of the strong DHA concentration increase ranging from 144% to 320% of the control (P <0.05) found over all 5 d of the experiment and bringing about a significant decline in the chloroplastic AA:DHA ratio (Fig. 3). Contrary to the changes observed for chloroplasts, a transient and long-lasting visible decrease was found in the ascorbate pools in mitochondria and peroxisomes, respectively. In mitochondria, the AA content decreased down to 23% of the control (P <0.05) at 3 dpi while DHA increased from day 1 onward, except for the 3rd day after challenge (68% of the control, P <0.05). The peroxisomal total ascorbate content decrease from the 2nd dpi onward was the effect of a significant concentration decrease observed for both AA and DHA. *B. cinerea*-induced changes in the concentrations of the mitochondrial and peroxisomal ascorbate pools provoked a significant decrease in the AA:DHA ratios (Fig. 3).

The glutathione pool underwent similar pathogen-induced changes in all cellular compartments, although the rate and timing were organelle-specific. Following *B. cinerea* infection total glutathione was significantly

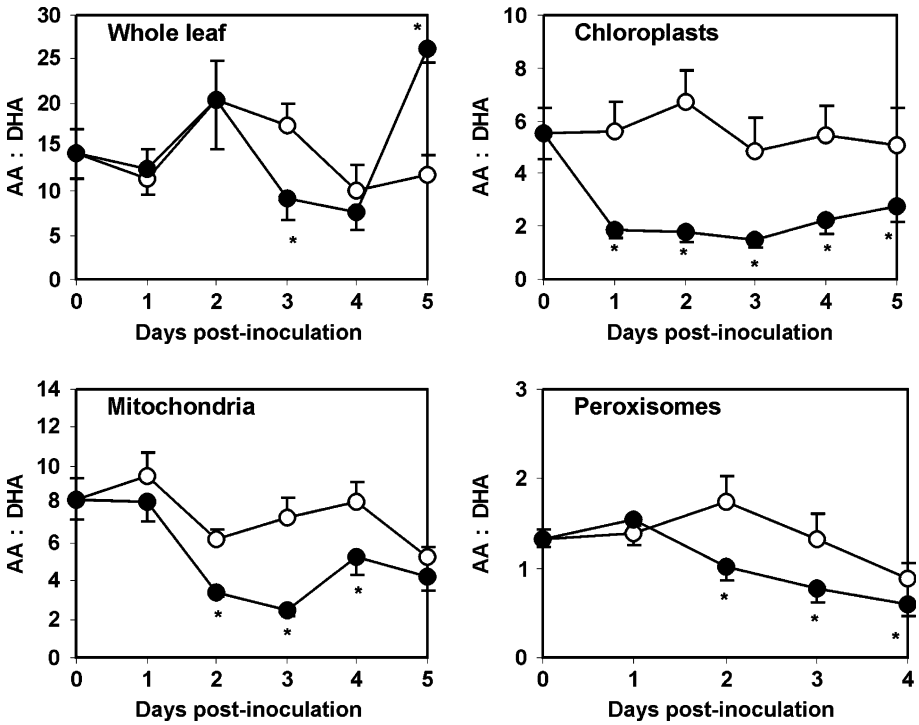

Fig. 3. Ascorbate redox ratios in organelles from *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. * Indicates means that differ significantly from control.

diminished over all 5 d of the experiment, except for chloroplasts where the decline in the glutathione pool started only 2 dpi (Fig. 4). This resulted mainly from a GSH concentration decline. The much pronounced decreases in GSH concentration, to the levels about 60% (P <0.05) lower than in the control, were evident 3 dpi and 4 dpi in peroxisomes and 5 dpi in mitochondria. As to *B. cinerea*-induced GSSG concentration changes, its accumulation was detected in peroxisomes and mitochondria, while in chloroplasts and whole-leaf extracts its content did not change significantly. However, in all compartments studied infection induced a marked and prolonged GSH:GSSG redox ratio decrease (Fig. 5). The strongest glutathione redox state decline, down to a level about 5-fold lower than in the control (P <0.05), was observed in chloroplasts isolated from the inoculated leaves. The redox state decreases found in mitochondria and peroxisomes, although ultimately proved to be less pronounced as compared with chloroplasts, appeared earlier and were stronger at the initial stage of infection development, i.e. on the 1st and 2nd dpi, respectively. It is worth noting that the levels at which the ascorbate and glutathione redox state finally operated in a given cellular compartment depended not only on the ratio of infection-provoked changes in the antioxidant pools, but also on their unique basal redox ratios, for example, peroxisomes were maintained at significantly lower ascorbate and glutathione redox ratios than chloroplasts (Figs 3, 5).

Infection-dependent changes in the activities of AA-GSH cycle enzymes

B. *cinerea* infection differentially affected APX isoforms located in the respective cellular compartments. The activity increase found in the whole-leaf extracts and chloroplasts starting from the 2nd and 3rd dpi, respectively, contrasted with an APX activity decrease in mitochondria and peroxisomes (Fig. 6). The strongest APX activity induction in whole-leaf extracts found at 2 dpi (165% of the control, P <0.05) preceded that detected for chloroplasts (179% of the control, P <0.05, at 3 dpi). This enzyme activity in mitochondria and peroxisomes isolated from the inoculated leaves was constantly decreased compared with the control. The maximum inhibitory effect for APX located in mitochondria and peroxisomes was observed at the advanced stage of infection development, i.e. on the 3rd and 4th dpi, respectively (Fig. 6).

In the inoculated leaves MDHAR activity in whole-leaf extracts was about the same as in control ones, except for the 38% (P <0.05) increase found on the 2nd day after challenge (Fig. 7). In response to *B. cinerea* infection MDHAR activity in chloroplasts, mitochondria, and peroxisomes generally decreased, except for a peroxisomal activity increase (127% of the control, P >0.05) detected at 1 dpi. In all cellular compartments the strongest MDHAR activity reduction, to the levels ranging from 55% to 74% of the respective controls (P <0.05), was evident at 3 and 4 dpi.

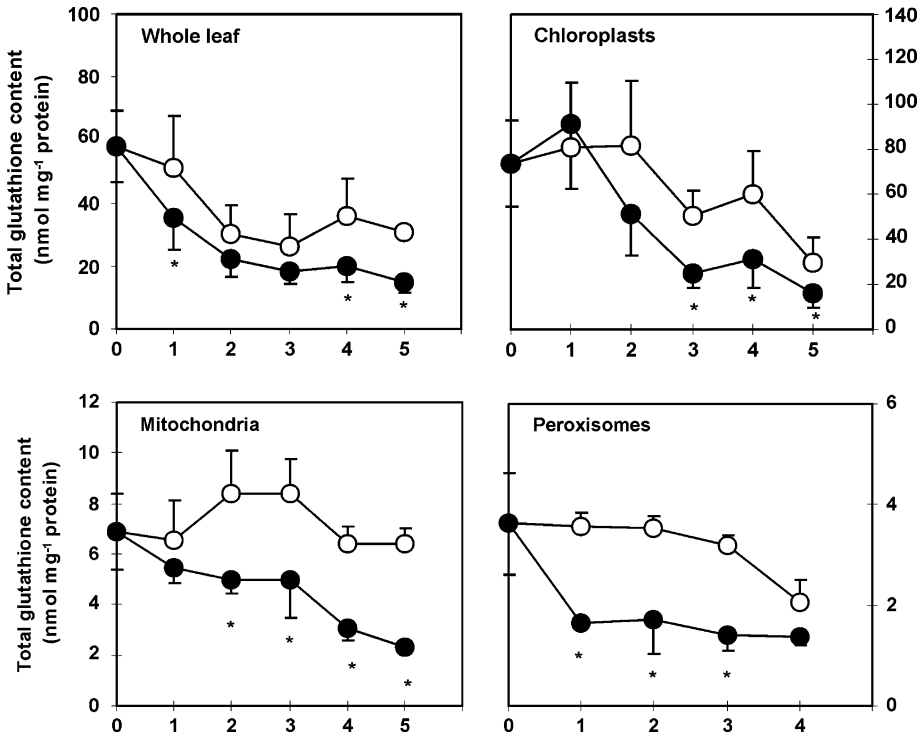

Fig. 4. The effect of *B. cinerea* infection on glutathione content in organelles isolated from tomato leaves. * Indicates means that differ significantly from control. Open symbols, control; closed symbols, infected. Data are means ±SD.

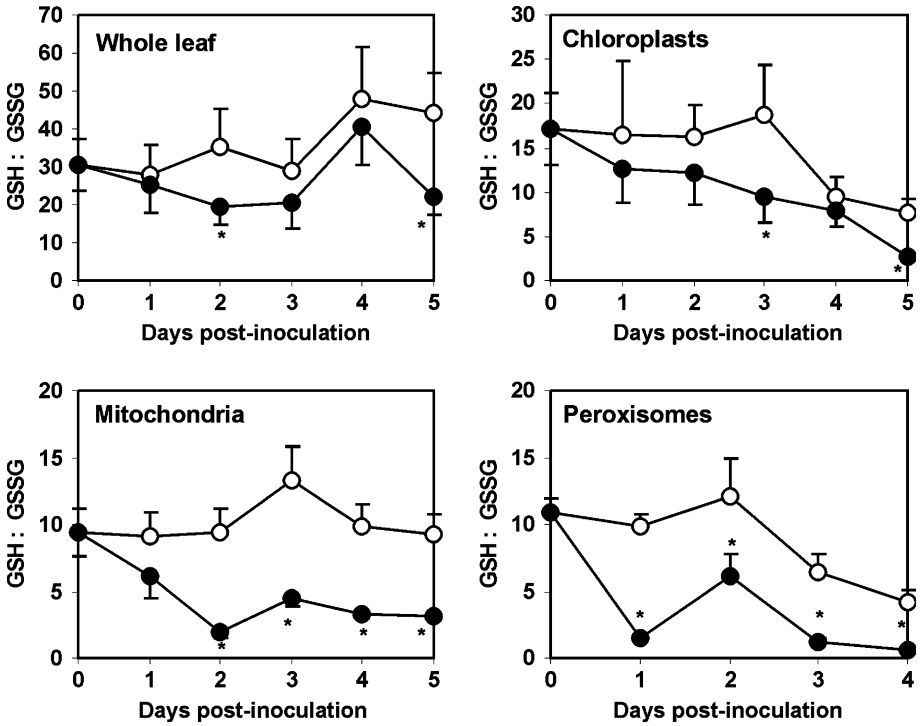

Fig. 5. Glutathione redox ratios in organelles from *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. * Indicates means that differ significantly from control.

B. cinerea infection induced a significant (P <0.05) decrease in whole-leaf extract DHAR activity 2, 3, and 4 dpi (Fig. 8). The chloroplastic DHAR activity was not affected except for a marked increase found at 3 dpi (156% of the control, P <0.05) whereas the mitochondrial and peroxisomal activities were distinctly decreased starting from the 3rd day after challenge. The strong inhibitory effect found for DHAR activity at 3 and 4 dpi matched that described for MDHAR.

The initial GR activity increase found on the 1st day in all compartments was maintained during the following 4 d only for GR in the whole-leaf extract and chloroplasts (Fig. 9). However, the whole-leaf extract GR activity enhancement was less profound compared with the marked effect observed for the chloroplast fraction where GR activity was induced up to the level of 219% of the control (P <0.05, 4 dpi). Conversely, the peroxisomal and mitochondrial GR activities were sequentially inhibited in response to infection (Fig. 9).

Discussion

Emerging evidence points to the importance of the subcellular compartmentation in the antioxidant defence to a successful defeat of the oxidative stress (Foyer and Noctor, 2003). However, the response of the AA-GSH cycle to pathogen-induced stress has been mainly studied in apoplast and whole-leaf extracts without discriminating between intracellular compartments (El-Zahaby *et al.*, 1995; Vanacker *et al.*, 1998). These data did not adequately reflect the role of the AA-GSH cycle in the protective mechanism. It was found that *B. cinerea* infection differentially upset ascorbate- and glutathione-mediated redox homeostasis at the subcellular level. A general down-regulation of glutathione content was found for both the whole-leaf extract and the organelles studied, although mitochondria and peroxisomes were mostly affected. This inhibitory effect was an early event that, except for chloroplasts, was already visible at 1 dpi. Significant GSSG accumulation accompanying GSH decrease was only found in mitochondria and peroxisomes. As GSH synthesis in photosynthetic cells was shown to take place in the cytosol and chloroplasts (Noctor *et al.*, 2002), the GSH depletion found in whole-leaf extracts and chloroplasts could either reflect an impaired biosynthetic capacity or an enhanced GSH engagement in other processes, for example, glutathione nitrosylation or glutathione transferase-mediated conjugation with endogenous compounds generated by ROS (Eshdat *et al.*, 1997; Foyer *et al.*, 1997; Dixon *et al.*, 2002). Accordingly, previous studies from this laboratory indicated that glutathione transferase could contribute to an enhanced GSH pool decrease, in particular, in chloroplasts from *B. cinerea*-infected tomato leaves (Kuźniak and Skłodowska, 1999, 2001). It could also be attributable to GSH transport from its biosynthetic sites to other compartments. By contrast, the heavily decreased GSH concentration in mitochondria and peroxisomes could be the effect of up-regulated oxidative processes as well as perturbations of

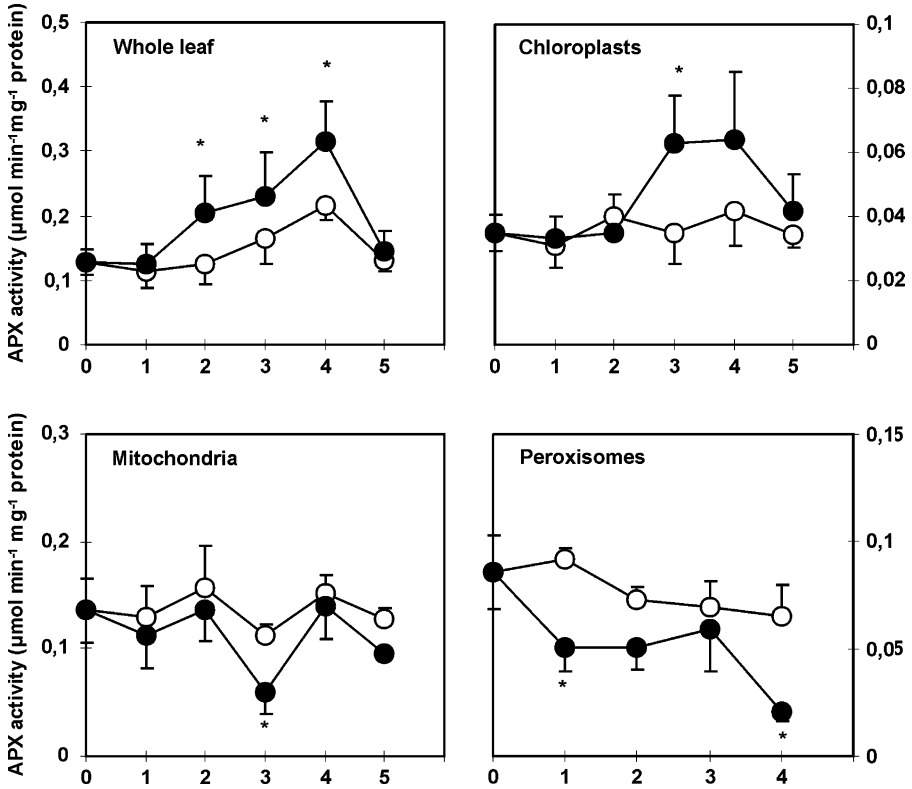

Fig. 6. APX activity in organelles isolated from control and *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. Data are means ±SD. * Indicates means that differ significantly from control.

glutathione transport (Foyer *et al.*, 2001). Although the physiological significance of the latter under stress conditions is unclear, an effectively operating glutathione transport across membranes could be assumed to counteract perturbations of glutathione homeostasis resulting from differential demands in individual compartments (Foyer *et al.*, 2001; Noctor *et al.*, 2002). Under the experimental conditions tested here, the integrity of leaf organelles from diseased plants did not significantly decrease when compared with the control ones, as shown by enzyme latency. Nevertheless, based on these data it cannot be excluded that *B. cinerea* infection impaired the active membrane transport systems and the decreases in non-enzymatic antioxidant contents were partly due to their leakage from the organelles. If that was the case, the data on the absolute concentrations of ascorbate and glutathione could be, to some extent, underestimated, particularly for peroxisomes surrounded by a single membrane. The evaluation of the contribution of each of the explanations could be a fascinating long-term project.

Changes in the ascorbate pool in mesophyll cells experiencing *B. cinerea*-induced stress were different from those observed for glutathione. Although the AA content also tended to decrease the changes, when compared with those in the glutathione pool, the changes were less pronounced and transient, with the strongest effect being limited to the phase of visible disease symptoms appearance. AA concentration decline to a level comparable with that for glutathione was evident in peroxisomes only. Similarly to the compartment-specific differences in GSH pool changes, the AA content decrease did not occur proportionally in all cellular compartments. The strongest effect was again found for the mitochondrial and peroxisomal pools. However, the AA decrease in peroxisomes was long-lasting, while that in mitochondria was no longer observed at the advanced stage of infection development. This reversion of the decreasing tendency was tightly related to AA biosynthesis, as it was shown that mitochondrial AA concentration correlated with the activity of L-galactono-γ-lactone dehydrogenase catalysing the final step of AA synthesis in mitochondria (Kuźniak and Skłodowska, 2004). Given that, in the whole-leaf extracts, comparable proportions of AA and GSH come from the cytosol and chloroplasts (Noctor and Foyer, 1998), the infection-induced changes in cytosolic and chloroplastic non-enzymatic antioxidants were proportional, apart from minor changes. This could be viewed as a homeostatic attempt to maintain the balance between the cytosol and chloroplasts with respect to the absolute AA and GSH concentrations. However, chloroplasts were the only organelles where the ascorbate pool underwent extensive oxidative processes, as judged on the basis of the strongly enhanced DHA content. The factors that may allow DHA accumulation in the presence of different chloroplastic

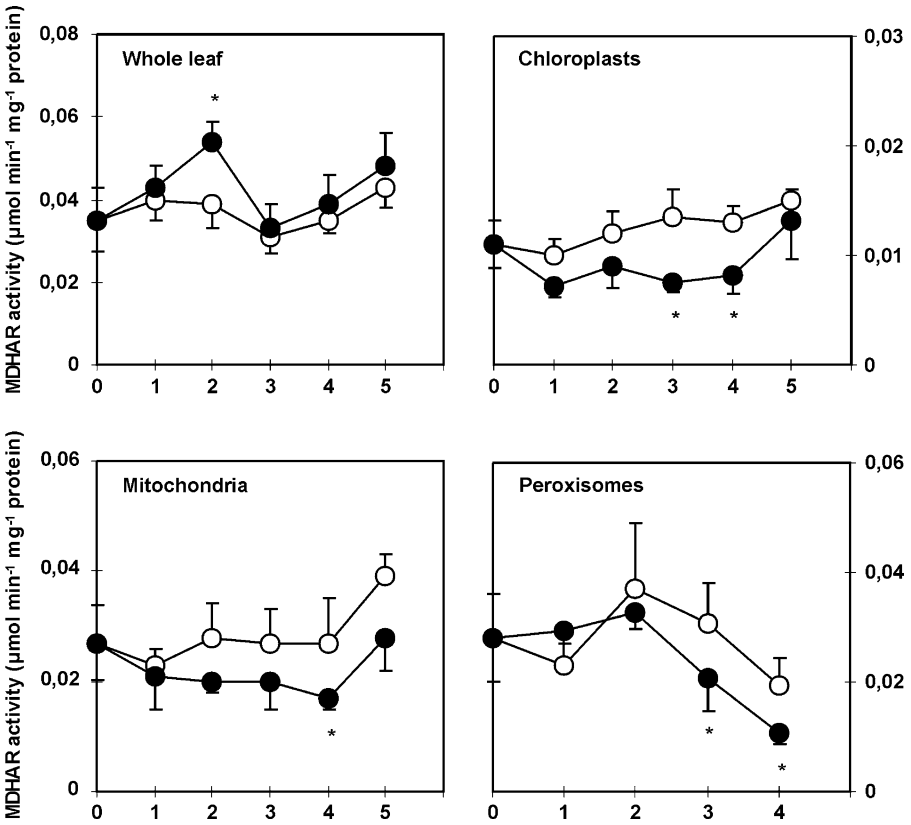

Fig. 7. MDHAR activity in organelles isolated from control and *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. Data are means ±SD. * Indicates means that differ significantly from control.

reduction mechanisms for this compound (Asada, 1999) remain unclear. This could have implications for both ascorbate-mediated signalling (Kiddle *et al.*, 2003; Pastori *et al.*, 2003) and photosynthetic capacity, as inhibition of the Calvin–Benson cycle enzymes by DHA, present even at low concentration, was shown (Wilson, 2002).

B. cinerea affected not only the absolute glutathione and ascorbate contents, but also their redox ratios that were generally decreased following infection. However, redox state changes in total-leaf extract did not adequately reflect the compartment-specific perturbations. Assuming that the redox ratio in the whole-leaf extract is mainly dependent on the cytosolic one, in both control and infected leaves the redox state of the cytoplasm was maintained on a significantly higher level than in the remaining cellular compartments. This could be linked to the necessity of the cytosol to withstand a continuing influx of DHA and GSSG from the apoplastic space (Horemans *et al.*, 2000; Foyer *et al.*, 2001), in particular, after pathogen attack when a massive extracellular ROS generation took place. Moreover, the redox states of individual organelles were maintained at different levels that seemed to correspond to their unique metabolic requirements. The peroxisomal redox ratios of both redox buffers were the lowest under physiological conditions and underwent the most profound reduction following infection. In recent years, the role of peroxisomes as a source of ROS and NO signal molecules integrating the response of different cellular compartments under stress conditions has been proposed (Corpas *et al.*, 2001). Apart from the response specificity of a particular compartment, the ascorbate pool in this study was generally more oxidized than the glutathione one. As it is now widely accepted that plants can sense shifts in the amounts and redox states of antioxidants and redox signals play a key role in plant defence (Foyer and Noctor, 2003), the disturbances in the ascorbate and glutathione pools found in this study, besides reflecting the oxidative nature of *B. cinerea* attack, are likely to be involved in readjustments of the cellular metabolism under stress to meet the demand for a defence strategy. However, the latter was apparently insufficient to arrest the fungus.

As a result of pathogen-induced changes, the ratio of specific APX activity in chloroplasts and cytosol was enhanced relative to that in other organelles, taking into account that the main proportion of the AA-GSH cycle components resides in chloroplasts. According to the studies that link the increase in APX activity to enhanced stress tolerance (Allen *et al.*, 1997) these results could indicate that the protection of cytosol and chloroplasts was favoured under biotic stress. However, APX activity increase found in whole-leaf extracts and chloroplasts also contributed to DHA accumulation and to ascorbate redox

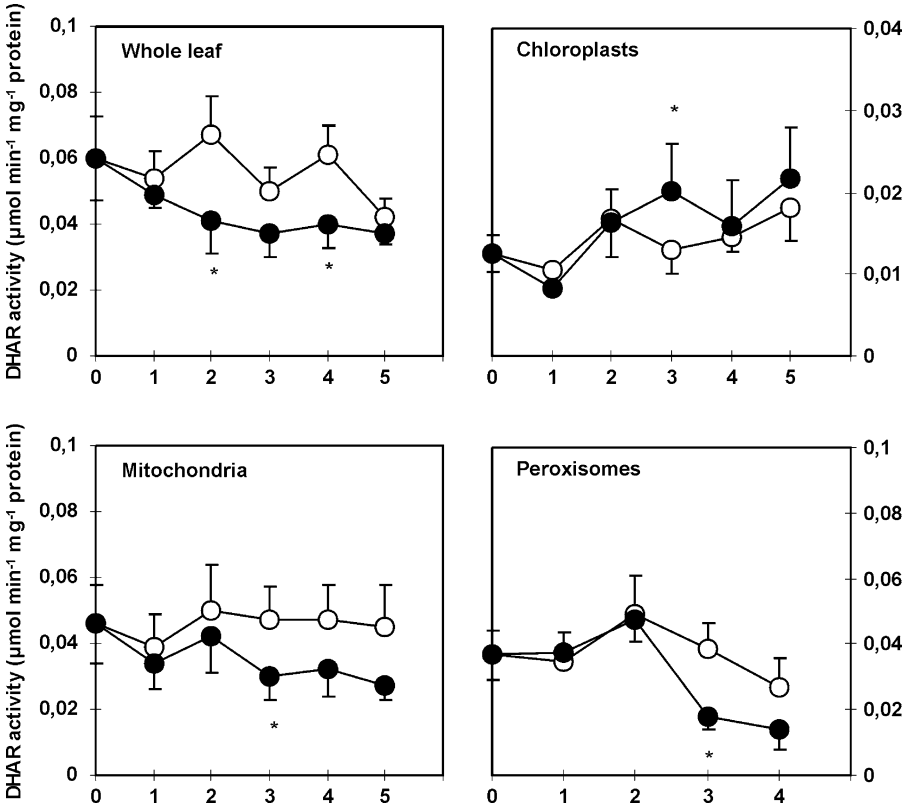

Fig. 8. DHAR activity in organelles isolated from control and *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. Data are means ±SD. * Indicates means that differ significantly from control.

state decrease, as the constitutive or incidentally increased MDHAR and DHAR activities did not prevent DHA accumulation. However, in mitochondria and peroxisomes the diminished activities of the AA regenerative pathway enzymes correlated with decreased DHA content. The most dynamic changes in the AA-recycling capacity generally overlapped the initiation of disease progression, similar to the pattern found for the ascorbate pool changes. An effective protection against GSSG accumulation mediated by increased GR activity was observed for whole-leaf extracts and chloroplasts only. As 80% of the total cellular GR activity resides in chloroplasts (Noctor *et al.*, 2002), GR-related changes could indicate that the maintenance of a relative redox balance of the glutathione pool in chloroplasts and cytosol, being the sites of GSH biosynthesis (Noctor *et al.*, 2002), was favoured under infection stress. However, this attempt seemed to be insufficient to return the glutathione system to the pre-stress balance.

It was found that the changes in AA-GSH cycle resulting from *B. cinerea* infection resembled the senescence-associated oxidative events described for senescencing leaves (del Río *et al.*, 1998). These included the decline in AA-GSH cycle activity in chloroplasts, mitochondria, and peroxisomes that have all been shown to be involved in senescence (Jimenéz *et al.*, 1998; del Río *et al.*, 1998; Munné-Bosch and Alegre, 2004), as well as the decay of chlorophyll (Kuźniak and Skłodowska, 2001) and peroxisomal superoxide dismutase and CAT decreases (data not shown). As senescence is one of the most important factors for tissue susceptibility to *B. cinerea* (Elad and Evensen, 1995) it is suggested that the observed changes could, at least partly, be the effect of a fungus-promoted precocious senescence that favoured disease development, the more so as the degree of AA-GSH cycle response correlated with disease progression.

Conclusions

Under optimal conditions the inherent ascorbate- and glutathione-related redox balance in individual compartments was maintained at different levels compatible with their specific metabolic requirements. The oxidative effects of *B. cinerea* infection have been proved to affect all cellular compartments, although organelle-specific variations were observed. However, these changes were masked when the whole-leaf extract was analysed. Under *B. cinerea*-induced stress the cellular compartments were differentially protected by the AA-GSH cycle so the degree of antioxidant capacity changes, as well as the timing of events, seemed not to be complementary at the subcellular level. The diversification of the AA-GSH cycle response in

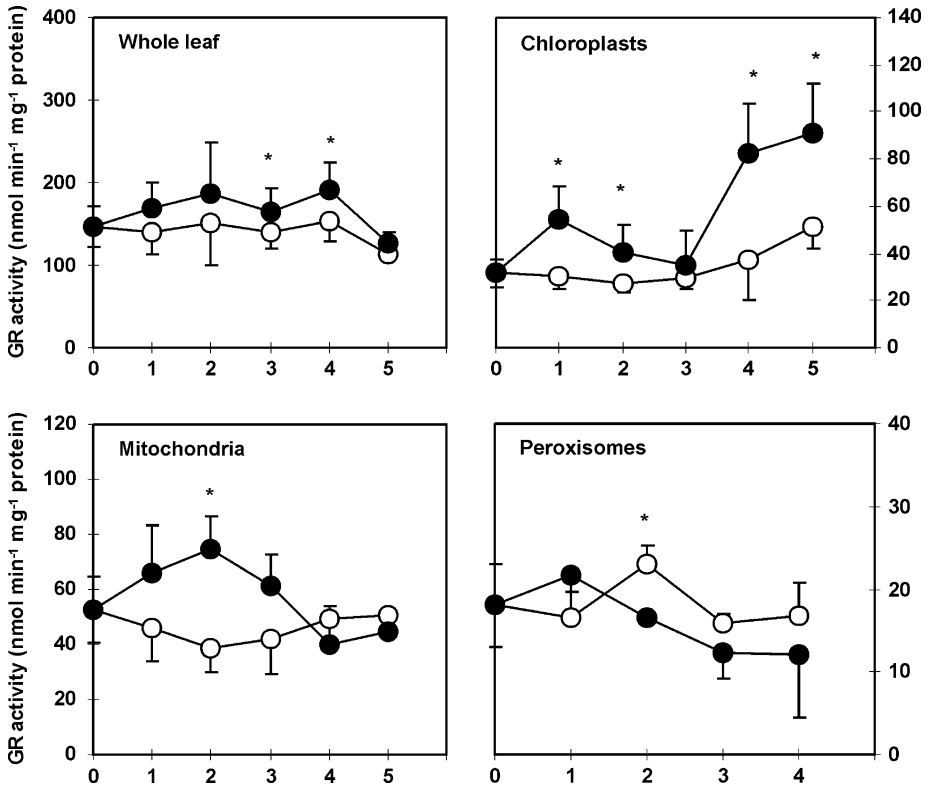

Fig. 9. DHAR activity in organelles isolated from control and *B. cinerea*-infected tomato leaves. Open symbols, control; closed symbols, infected. Data are means ±SD. * Indicates means that differ significantly from control.

different compartments could be the result of either their specific metabolic requirements or of a differential role of individual organelles in maintaining the redox balance and in redox signalling. The roughly stable redox equilibrum found in the whole-leaf extract could, at least partly, be attributable to the buffer capacity of the cytosol that mediates the export and uptake of antioxidants, as well as their recycling, in order to maintain large redox gradients between subcellular compartments. Apart from the response specificity of a particular compartment, there was a general shift of the cellular redox environment towards the oxidative state, expressed as the remarkable decrease in concentrations and redox ratios of the ascorbate and glutathione pools. The latter was partly related to the insufficient activities of enzymes needed for the regeneration of active forms of the antioxidants. At both the cellular and subcellular levels, *B. cinerea* was able to overcome the protective antioxidant barrier related to the antioxidant AA-GSH cycle capacity, although functional differences between organelles were evident. The most dynamic changes in enzyme activities and redox potentials were correlated with the appearance of disease symptoms indicating the breakdown of an active plant defence response. The changes in the AA-GSH cycle activity, in particular those in mitochondria and peroxisomes, could be partly related to the *B. cinerea*-induced promotion of senescence that favoured disease progress.

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