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FULL RESEARCH PAPER

Antioxidant, ethylene and membrane leakage responses to powdery mildew infection of near-isogenic barley lines with various types of resistance

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Abstract Leaves of powdery mildew-susceptible barley (*Hordeum vulgare* cv. Ingrid) and related near-isogenic lines bearing various resistance genes (*Mla12*, *Mlg* or *mlo5*) were inoculated with *Blumeria graminis* f. sp. *hordei* race A6. Fungal attack induced several-fold increases in ethylene emission and electrolyte leakage in leaves of susceptible Ingrid beginning 3 days after inoculation. Activities of peroxidase, superoxide dismutase, glutathione *S*-transferase, ascorbate peroxidase and glutathione reductase enzymes were induced markedly in susceptible leaves 5–7 days after inoculation. Similar, but less pronounced pathogen-induced changes were detected in inoculated leaves of Mla-type resistant plants that show hypersensitive cell death upon inoculation, and, to an even lesser extent, in the Mlg and mlo lines, where no visible symptoms accompanied the incompatible interaction. Glutathione content increased only in susceptible barley 7 days after inoculation. Catalase activity, total ascorbate content and redox state were not influenced by inoculation in any of the genotypes. The activity of dehydroascorbate reductase was significantly reduced 3–5 days after inoculation in the susceptible parental plants and after 5 days in Mla and Mlg lines, while it was stable in the mlo barley. Slightly elevated levels of H_2O_2 were observed in the inoculated resistant plants. In contrast, H_2O_2 content decreased in the susceptible line 7 days after pathogen attack. These data indicate that high levels of antioxidants are involved in the compatible interaction of susceptible barley and powdery mildew by protecting the pathogen from oxidative damage.

Keywords Ascorbate–glutathione cycle · *Blumeria graminis* f. sp. *hordei* · *Hordeum vulgare* · Hydrogen peroxide · Reactive oxygen species · Susceptibility

Abbreviations

APX	ascorbate peroxidase
Bgh	*Blumeria graminis* f. sp. *hordei*
CDNB	1-chloro-2,4-dinitrobenzene,
dai	day(s) after inoculation
DCF	2′,7′-dichlorofluorescein
DCFH-DA	2′,7′-dichlorofluorescin diacetate

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DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
GR	glutathione reductase
GST	glutathione *S*-transferase
hai	hour(s) after inoculation
HR	hypersensitive reaction
MDA	monodehydroascorbate
POX	guaiacol peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase

Introduction

Powdery mildew disease of barley (*Hordeum vulgare*) is caused by a biotrophic parasitic fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*) that keeps host plant cells alive during infection. It is important to understand the various mechanisms that protect plants resistant to this dangerous pathogen. Here we studied the interaction of *Bgh* with appropriate susceptible barley (cv. Ingrid) and related near-isogenic lines with genes *Mla12*, *Mlg* or *mlo5* for resistance to powdery mildew. The genes *Mla12* and *Mlg* mediate race-specific resistance to *Bgh* race A6, while the recessive *mlo5* allele of the *Mlo* locus confers race-nonspecific resistance to the plant against various barley powdery mildew isolates (Heitefuss 2001). These genes interfere with different stages of fungal development, thereby resulting in distinctive interaction phenotypes. Functional alleles of the *Mla* locus mediate fungal arrest by inducing a hypersensitive reaction (HR) in the underlying mesophyll cells after the fungal penetration of the target cell (Hückelhoven and Kogel 1998). The gene *Mlg* mediates resistance via single-cell HR of the attacked epidermal cells, while the gene *mlo5* controls penetration resistance through an effective papilla formation, leaving the attacked cell alive (Hückelhoven and Kogel 1998).

One of the earliest responses of plant cells to pathogen attack is the accumulation of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Specific staining and microscopic analysis have indicated that powdery mildew infection can induce a strong, early accumulation of H_2O_2 in attacked leaves of Mla-, Mlg- and mlo-type resistant, but not in susceptible barley lines (Hückelhoven et al. 1999). Here we addressed the question whether these differences in accumulation of H_2O_2 can be confirmed by quantitative methods.

Bgh-induced changes in the antioxidant status of barley have been studied extensively in the past decades, especially in susceptible and Mla-type resistant plants (Kerby and Somerville 1989; El-Zahaby et al. 1995; Vanacker et al. 1998; Kristensen et al. 1999; Burhenne and Gregersen 2000). Here we compared antioxidant responses to powdery mildew attack in susceptible Ingrid and in three distinct types of incompatible interactions: not only in Mla, but also in the poorly studied Mlg and mlo backcross barley lines. Therefore we investigated the activity of several antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1), glutathione reductase (GR, EC 1.6.4.2) and glutathione *S*-transferase (GST, EC 2.5.1.18). To gain more information on the ascorbate–glutathione cycle in barley defence reactions against powdery mildew we measured ascorbate and glutathione content, as well as the ratio of reduced and oxidized forms of ascorbate and glutathione, which reveals the cellular redox state in inoculated leaves. In addition, we were interested in whether accumulation of H_2O_2 correlates with membrane damage and with the accumulation of ethylene, a secondary messenger plant hormone. The potential mechanisms that mediate the termination of host colonisation are discussed.

Materials and methods

Plant materials, pathogen and inoculation

The barley (*H. vulgare*) cv. Ingrid, and the backcross lines Ingrid Mla12, Ingrid Mlg and Ingrid mlo5 were kindly supplied by Lisa Munk (University of Copenhagen, Denmark). Their generation was described previously (Kølster et al. 1986). Plants were grown in a growth chamber at 17°C with 60% RH, and a photoperiod of 16 h (100 μE m^{-2} s^{-1}). Barley powdery mildew fungus (*Bgh*, race A6) was inoculated onto primary leaves of 7 day-old barley plants to give an inoculation density of approximately 50 conidia mm^{-2}. Powdery mildew was maintained on barley cv. Ingrid under the same conditions.

Detection of hydrogen peroxide

To elucidate the possible relationships between antioxidant activities and inhibition of ROS accumulation in powdery mildew-infected barley, H_2O_2 levels of inoculated leaves were determined quantitatively. We measured the accumulation of H_2O_2 in whole, detached leaves of powdery mildew-inoculated barley plants by a cell-permeable fluorescent dye as described by Lu and Higgins (1998). The fluorescent compound, 2′,7′-dichlorofluorescein (DCF) is produced from the oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma) by H_2O_2 in the presence of cellular peroxidases.

Uninfected and powdery mildew-infected barley leaves were treated with 0.4 mM DCFH-DA 4 dai. DCFH-DA stock solution (20 mM) was prepared in 100% ethanol and diluted to 1:50 with distilled water immediately before use. To measure the relative generation of H_2O_2, DCFH-DA was introduced into intercellular spaces of barley leaves by vacuum infiltration. The infiltrated leaves (0.1 g) were frozen in liquid nitrogen 8 min after infiltration and homogenized in 2 ml of 2 mM KCN in a mortar with pestle. The homogenate was centrifuged (12,000 *g*) for 20 min at 4°C. Supernatants were diluted to 1:1,000 with distilled water and the relative fluorescence was measured immediately. Conversion of DCFH-DA to 2′,7′-dichlorofluorescein was measured by a spectrofluorometer (FluoroMax-3, Jobin Yvon Horiba, France) with the excitation and emission wavelengths set to 488 and 525 nm, respectively.

Enzyme assays

For the detection of antioxidant enzyme activities, 0.5 g leaf tissue was homogenised at 0–4°C in 3 ml of TRIS–HCl buffer (50 mM, pH 7.8) containing 1 mM EDTA-Na_2 and 7.5% (w/v) soluble polyvinylpyrrolidone. Homogenates were centrifuged (12,000 *g*, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All measurements were carried out at 25°C, using a model UV-160A spectrophotometer (Shimadzu, Japan).

Activity of guaiacol-dependent peroxidases (POX) was assayed by measuring the formation of the conjugate product of guaiacol at 436 nm (Rathmell and Sequeira 1974). The reaction mixture contained 2.2 ml of 100 mM sodium phosphate buffer (pH 6.0), 100 µl guaiacol (62 mg dissolved in 10 ml distilled water, final concentration was 2 mM), 100 µl of 12 mM H_2O_2 and 10 µl of leaf extract. The increase in A_{436} was measured as the conjugate was formed using an extinction coefficient of 26.6 mM^{-1} cm^{-1} for the conjugate. Control reaction was not necessary.

Ascorbate peroxidase (APX) activity was determined in 2.25 ml total volume containing 2 ml TRIS–HCl buffer (50 mM, pH 7.8), 100 µl ascorbic acid (10 mg in 10 ml distilled water, final concentration was 0.25 mM), 100 µl of 11.25 mM H_2O_2 (final concentration was 0.5 mM) and 50 µl supernatant. The oxidation of ascorbic acid was followed in a quartz cuvette at 290 nm (extinction coefficient of ascorbic acid was 2.8 mM^{-1} cm^{-1}) according to the method of Nakano and Asada (1981). The respective control reaction mixtures contained buffer instead of 100 µl H_2O_2 solution.

Catalase (CAT) activity was determined as described by Aebi (1984). The decomposition of hydrogen peroxide was followed at 240 nm in a quartz cuvette, (extinction coefficient of H_2O_2 was 0.040 $mM^{-1}cm^{-1}$). The reaction mix consisted of 2 ml 0.1 M Na-phosphate buffer (pH 6.5), 100 µl of 269 mM hydrogen peroxide solution (12.5 mM H_2O_2 was the final concentration) and 50 µl plant extract. Control reaction was not necessary.

Activity of dehydroascorbate reductase (DHAR) was measured by following the reduction of dehydroascorbate (DHA) at 265 nm (extinction coefficient of ascorbic acid was 14 mM^{-1} cm^{-1}) in a quartz cuvette (Klapheck et al. 1990). The assay mix consisted of 2 ml buffer (50 mM Na-phosphate, pH 6.5, 1 mM EDTA-Na_2), 100 µl reduced glutathione (7 mg GSH dissolved in 1 ml of the above buffer, the final concentration was 1 mM), 100 µl dehydroascorbate (2 mg DHA dissolved in 1 ml of the above buffer, the final concentration was 0.5 mM) and 100 µl supernatant. The control reaction mix contained buffer instead of supernatant.

To measure glutathione reductase (GR) activity, the oxidation of NADPH was detected at 340 nm (extinction coefficient of NADPH was 6.2 mM^{-1} cm^{-1}) (Klapheck et al. 1990). The assay mix consisted of 2 ml TRIS–HCl buffer (50 mM, pH 7.8), 100 µl NADPH (2 mg dissolved in 1 ml buffer, the final

concentration was 0.1 mM), 300 µl oxidized glutathione (3 mg GSSG dissolved in 1 ml buffer, the final concentration was 5.8 mM) and 100 µl supernatant. The respective control reaction mix contained buffer instead of 300 µl GSSG solution.

Glutathione S-transferase (GST) activities were determined by measuring the formation of the conjugate reaction product at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (extinction coefficient of the conjugate was 9.6 mM^{-1} cm^{-1}) (Mauch and Dudler 1993). The reaction mixture contained 2 ml buffer (0.1 M Na-phosphate, 1 mM EDTA-Na_2, pH 6.5), 150 µl CDNB (37 mg dissolved in 10 ml 100% ethanol, final concentration was 1 mM), 500 µl GSH (60.8 mg dissolved in 10 ml of the above buffer, final concentration was 3.6 mM), and 100 µl supernatant. The control reaction mix contained buffer instead of supernatant.

The activity of superoxide dismutase (SOD) was also determined spectrophotometrically according to the method of Paoletti and Mocali (1990). Each set of assays included its own control that consisted of 800 µl triethanolamine–diethanolamine buffer (100 mM each, pH 7.4, adjusted with cc. HCl), 4 µl NADH (7.5 mM), 25 µl EDTA-$MnCl_2$ solution (100 mM EDTA and 50 mM $MnCl_2$) and 100 µl of sample. The absorbance was recorded for 5 min at 340 nm and then 100 µl of 10 mM mercaptoethanol solution was added into the cuvettes. The decrease of absorbance was monitored for another 20 min. Calculations were carried out using a calibration curve prepared by SOD purchased from Sigma.

Ascorbate and glutathione assays

Besides enzymes, the low-molecular-weight antioxidants, particularly ascorbic acid and reduced glutathione (GSH), play an important role in antioxidative defence. The levels of reduced and oxidised forms of ascorbic acid and glutathione were detected in whole-leaf extracts. We focused on the late stages of the barley-powdery mildew interaction, 4 and 7 dai.

Leaf material (0.5 g) was homogenised in 2.5 ml of 5% (w/v) metaphosphoric acid at 0–4°C. After centrifugation (19,000 *g*, 30 min, 4°C) the supernatant was used for the antioxidant assays. Ascorbate content was determined using ascorbate oxidase (Foyer et al. 1983). For the determination of reduced ascorbate, a 125 µl sample of the metaphosphoric acid extract was neutralised with 25 µl 1.5 M triethanolamine. After thorough mixing, 150 µl sodium phosphate buffer (150 mM, pH 7.4) and 75 µl H_2O were added and mixed again. The reaction mixture contained 2 ml sodium phosphate buffer (100 mM, pH 5.6) and 200 µl of the above mix in a quartz cuvette. The extinction of this solution was measured immediately at 265 nm and then the decrease of absorbance at 265 nm was followed upon the addition of 1 unit of ascorbate oxidase from Cucurbita (Sigma).

To determine DHA we performed non-enzymatic reduction of this metabolite to ascorbate using dithiothreitol, then measured total ascorbate content in the samples. Leaf extracts (125 µl) were neutralised as above, mixed with 150 µl sodium phosphate buffer and 75 µl of 10 mM dithiothreitol, and incubated for 15 min at room temperature to reduce DHA. Then total ascorbate levels were measured as above. The decrease of extinction at 265 nm was followed spectrophotometrically until the minimum was reached and the minimal extinction value was recorded. The difference between the extinction at the beginning and the minimal extinction was proportional to the total ascorbate content. The concentration of DHA was then calculated by subtracting the ascorbate content from the total ascorbate level. Standard curve for ascorbate determination was prepared with stock solutions of ascorbate and DHA prepared in 5% metaphosphoric acid.

Oxidised and reduced glutathione were determined by a spectrophotometric enzymatic recycling assay using glutathione reductase by the method of Law et al. (1983). Samples (100 µl) of the metaphosphoric acid extracts were neutralised with 36 µl of 1 M triethanolamine. Total glutathione was estimated from the increase of absorbance at 412 nm following the addition of GR and NADPH. The assay mix contained 50 mM potassium phosphate buffer (pH 7.5), 2.5 mM EDTA, 1 mM 5,5′-dithio-bis(2-nitrobenzoic acid), 1 U of GR (from Baker's yeast, Type III, Sigma), 0.2 mM NADPH and 20 µl of neutralised sample in a total volume of 1 ml. To determine GSSG, GSH was derivatized by adding 8 µl of 2-vinylpyridine to the neutralised samples, which were then incubated at 25°C for 1 h. GSSG was measured as described above, adding 50 µl sample to the assay mix of a total volume of 1 ml. Calibration curves were produced with stock solutions of GSH and

GSSG prepared in 5% metaphosphoric acid. Fine chemicals were all purchased from Sigma-Aldrich Co.

Electrolyte leakage assay

In order to gain information on membrane damage in powdery mildew-attacked barley leaves, conductivity tests were carried out. Ten leaf segments (each 1 cm long) were cut from the middle parts of healthy and infected primary barley leaves 24 hai and floated on distilled water (40 ml) in Petri dishes as described by Ádám et al. (2000). Changes in conductivity of the bathing solutions were measured by a conductivity bridge (Oakton Benchtop Meter, type pH/Con 510).

Analysis of ethylene by gas chromatography

Primary leaves of powdery mildew-inoculated and healthy barley plants were detached and incubated in 25-ml glass test tubes containing 2 ml distilled water and sealed with gas tight rubber stoppers, which enabled gas sampling. The tubes were kept in a growth chamber at constant temperature and light (20°C, 250 μE m^{-2} s^{-1}). One milliliter gas samples were withdrawn by syringe from the headspace for ethylene determination daily for four consecutive days following inoculation. Ethylene content of the samples was quantitatively analysed by gas chromatography using a Packard 427 GC, which was equipped with an aluminium oxide column (1/8 in.×1 m) and a flame ionisation detector. The injector, column and detector temperatures were 80, 80 and 220°C, respectively (Heiser et al. 1998).

Statistical analysis

At least three independent biological experiments were conducted in each case. Statistical analysis was performed using Student's *t*-test. Differences were considered to be significant at $P<0.05$.

Results

Levels of hydrogen peroxide in whole-leaf extracts

Here we investigated the changes of H_2O_2 levels in barley 4 and 7 dai with powdery mildew to reveal the long-term consequences of this plant-pathogen interaction. Our results showed a slight (20–25%) increase of H_2O_2 content in all near-isogenic resistant barley lines 4 dai (Fig. 1a). At this time-point, H_2O_2 levels did not change notably in the interaction of susceptible barley with powdery mildew. However, 7 dai the concentration of H_2O_2 was markedly reduced (to about 50% of the uninoculated control) in the inoculated susceptible leaves (Fig. 1b). On the other hand, H_2O_2 levels increased in the leaves of powdery mildew-infected resistant (Mla, Mlg, mlo) barley plants compared to their respective uninfected controls.

Antioxidant enzyme activities

Activities of antioxidant enzymes were assayed in whole-leaf extracts of susceptible barley cv. Ingrid and related backcross lines Mla, Mlg and mlo after powdery mildew attack. Following inoculation, samples were taken at 'early' stages of the interaction: after 15 h (before fungal penetration of epidermal cells), 30 h (after penetration attempt), 60 h (after establishment of pathogenesis or HR), and at 'late' stages: after 4 days (before fungal sporulation), 5 and 7 days (after sporulation).

We found enhanced the activities of POX, SOD, GST, APX and GR enzymes in susceptible Ingrid leaves at late stages of the pathogenesis (5 and 7 dai). Fungal attack increased POX activity five-fold, SOD and GST activities three-fold, APX and GR activities about two-fold in infected susceptible leaves by 7 dai as compared to healthy controls (Figs. 2 and 3). While induction of these enzymes in the susceptible leaves could only be observed at the later stages of the infection, POX was induced as early as 15 hai (Fig. 3a). Activity of DHAR transiently decreased until the fourth day of infection in susceptible Ingrid leaves and then recovered to control level by the seventh dai (Fig. 2). We could not detect considerable changes of catalase activity, only a minor, transient increase peaking 4 dai in susceptible Ingrid leaves (Fig. 2).

In Mla barley leaves, activity of POX increased up to four-fold and SOD, GST, APX activities by about 50% at 5 and 7 dai with *Bgh*. However, the induction of these antioxidants observed at the late stages of the interaction was significantly weaker in attacked Mla leaves than in leaves of infected susceptible plants; thus Mla barley seemed to show an intermediate phenotype between parental susceptible plants and the

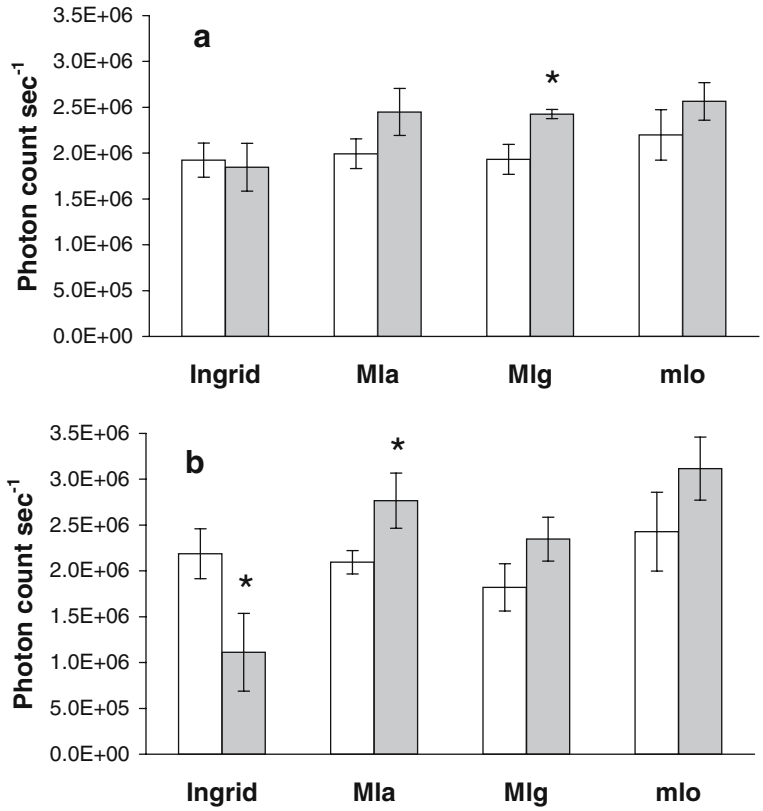

Fig. 1 Hydrogen peroxide content in powdery mildew-inoculated (*dark bars*) and uninoculated control (*white bars*) leaves of susceptible (Ingrid) and resistant (Mla, Mlg, mlo) barley. **a** and **b**: 4 and 7 dai, respectively. Data are presented as mean±S.D., calculated from at least three independent experiments. *Differences are significant at $P<0.05$

other resistant lines. Similarly to susceptible Ingrid, DHAR activity also showed a decrease in the powdery mildew-attacked Mla leaves, but somewhat later than in the susceptible line (Fig. 2).

In contrast to susceptible Ingrid, little or no increase in activities of SOD, GST, APX and GR and were observed in Mlg and mlo lines after *Bgh*-inoculation (Fig. 2), except for GR activity that was induced by 50% in Mlg barley 7 dai. POX activity, however, significantly increased in inoculated leaves of Mlg (up to four-fold) and mlo (more than two-fold) barley (Fig. 3a). Similarly to what was observed in Mla, it peaked at 5 dai in the Mlg line, and in the mlo line at around 3 dai, then started to decrease in all of the investigated barley lines by 7 dai.

It is noteworthy that POX activity also increased significantly over time in the uninoculated mutant mlo barley plants (Fig. 3b) as compared to plants bearing the wild-type *Mlo* gene (susceptible, Mla and Mlg). In 7 day-old seedlings (0 hai in our experiments) only a slight, non-significant difference in POX activity could be observed between the *mlo* and the *Mlo* genotypes. However, when comparing uninoculated plants 1 week later, we could detect a significant, two-fold increase of POX activity in mlo barley as compared to Mla, Mlg or susceptible lines, indicating an early senescence process in Mlo-deficient plants.

Ascorbate and glutathione content

The levels of reduced and oxidised forms of ascorbate and glutathione were investigated in near-isogenic lines 4 and 7 dai with *Bgh*. Neither concentrations of ascorbate and glutathione, nor their redox state changed significantly in any of the barley lines 4 dai (Figs. 4a and 5a). However, we demonstrated a significant increase (about 50%) of glutathione levels in susceptible plants only 7 days after fungal attack, with no change in the ratio of GSH to GSSG (Fig. 5b).

Electrolyte leakage

Leakage of electrolytes from infected susceptible Ingrid leaf segments was two times higher than that from uninfected leaf segments as early as 40 hai, and by the fifth day following inoculation ion leakage was en-

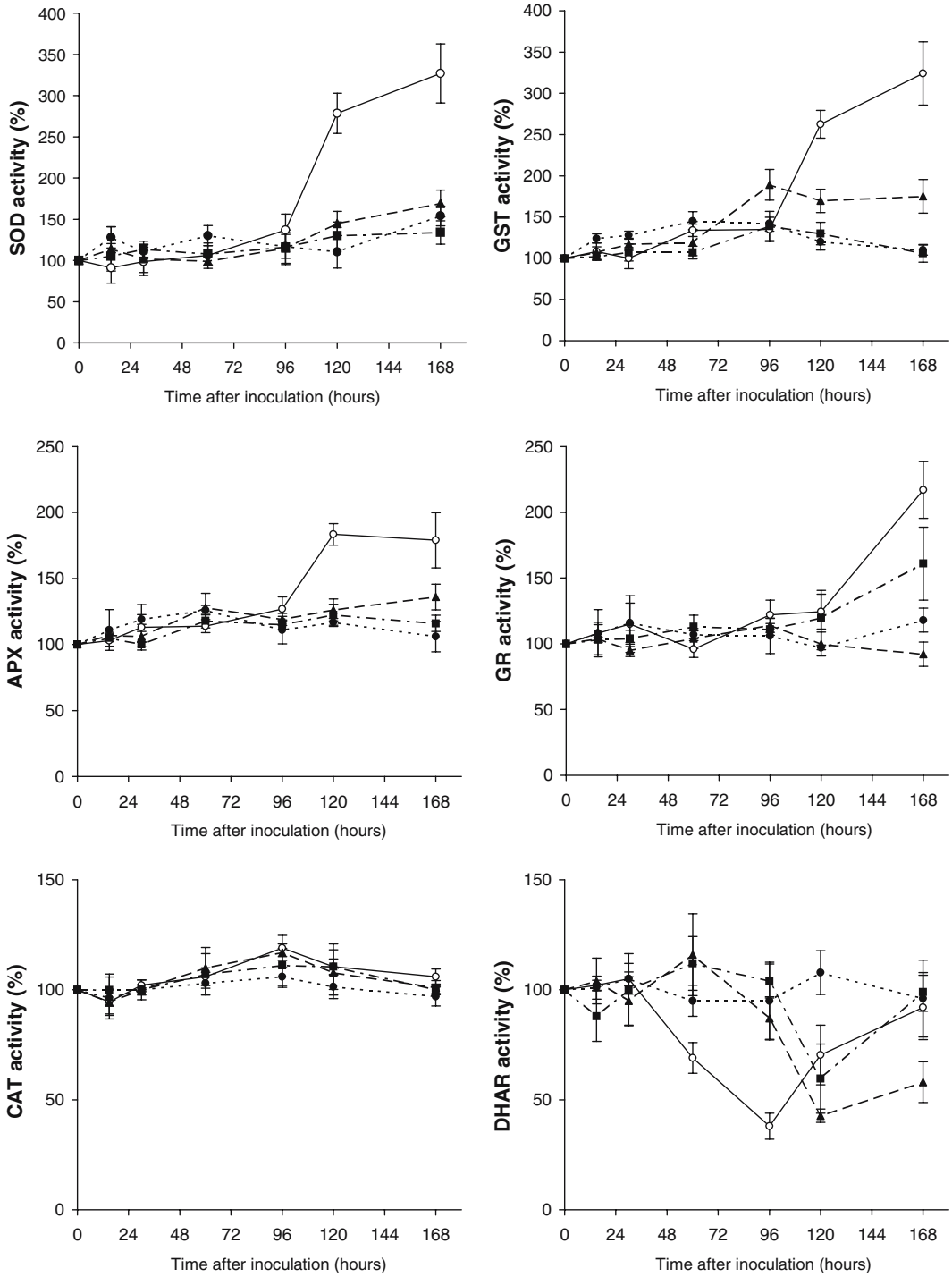

Fig. 2 Effect of powdery mildew inoculation on the activities of SOD, GST, APX, GR, catalase and DHAR enzymes in leaves of susceptible (*empty circle*) and resistant barley lines carrying the gene *Mla* (*filled triangle*), *Mlg* (*filled square*) or *mlo* (*filled circle*), expressed as percentage values. In case of each line the uninoculated control is 100%. Data are presented as mean±S.D., calculated from at least three independent experiments

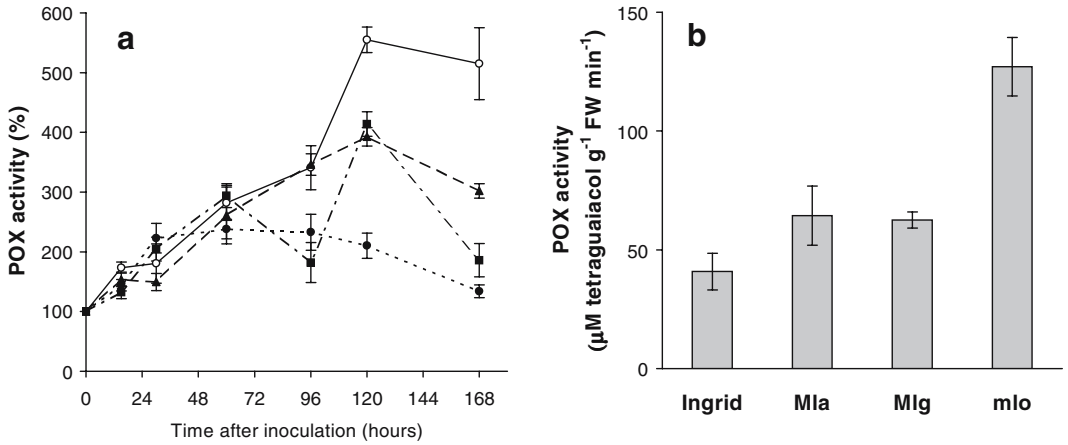

Fig. 3 Changes in activity of POX in leaves of susceptible (*empty circle*) and resistant barley lines carrying the gene *Mla* (*filled triangle*), *Mlg* (*filled square*) or *mlo* (*filled circle*), expressed as percentage values (**a**). In the case of each line the uninoculated control is 100%. POX activities in the leaves of 14 day-old uninfected control plants in susceptible Ingrid and resistant Mla, Mlg, mlo barley (**b**). Data are presented as mean±S.D., calculated from at least three independent experiments

hanced to eight-fold compared to the control (Fig. 6). In infected Mla leaves, only a slight increase in electrolyte leakage was observed and no difference was detected in inoculated Mlg and mlo lines. When comparing healthy leaf segments only, a stronger ion leakage was detected from healthy mlo leaves by the end of the time course, than from the other three genotypes (Fig. 6), indicating again the early senescence of mlo leaves.

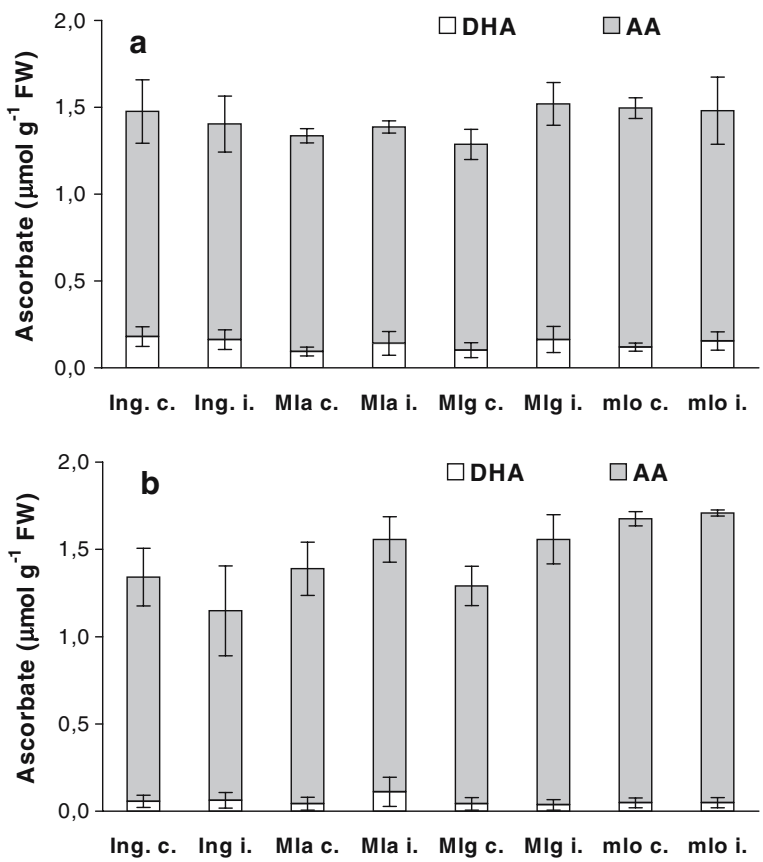

Fig. 4 Ascorbate content in barley leaves 4 days (**a**) and 7 days (**b**) after inoculation with powdery mildew. Ascorbic acid (*dark bars*) and dehydroascorbate (*white bars*) were both determined in uninoculated control (c.) and infected (i.), near-isogenic barley lines. Data are presented as mean±S.D., calculated from three independent experiments

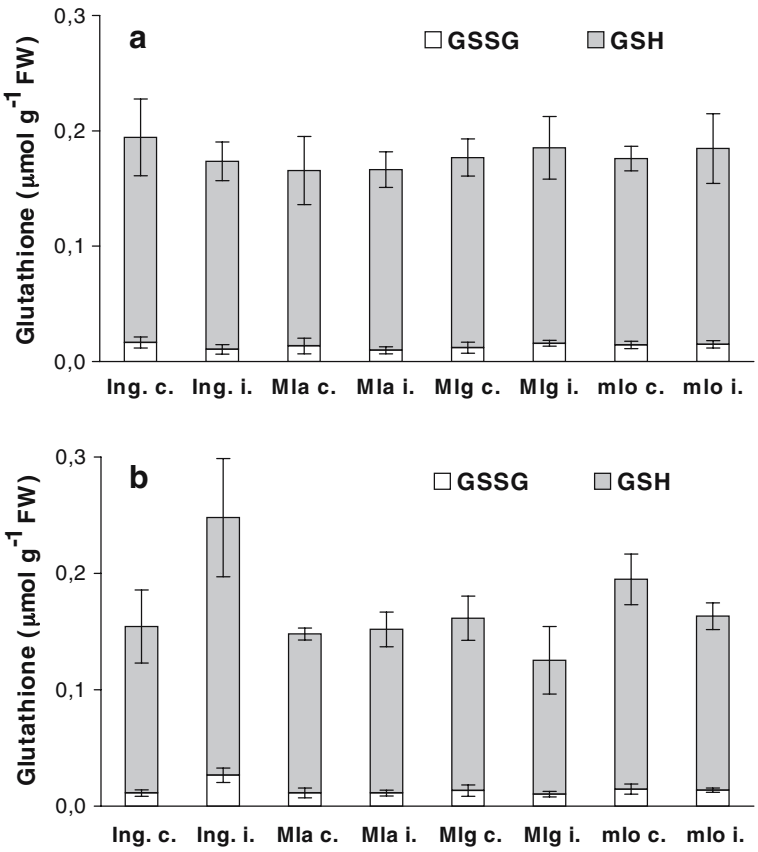

Fig. 5 Glutathione content in barley leaves 4 days (**a**) and 7 days (**b**) after inoculation with powdery mildew. Reduced (*dark bars*) and oxidised (*white bars*) forms of glutathione were both determined in uninoculated control (c.) and infected (i.), near-isogenic barley lines. Data are presented as mean±S.D., calculated from three independent experiments

Ethylene emission

A gradual and exponential growth in ethylene emission was found during the pathogenesis starting 2 dai in the powdery mildew-infected parental susceptible barley and, to a much lesser extent, in Mla-type resistant plants (Fig. 7). Such an increase upon inoculation was not detectable in case of Mlg or mlo barley leaves.

Discussion

Elevated antioxidant activities can attenuate the harmful effects of ROS generated during plant-pathogen interactions. This is the first record where activities of important antioxidants involved in detoxification of ROS were compared in compatible and three different incompatible (Mla, Mlg and mlo)

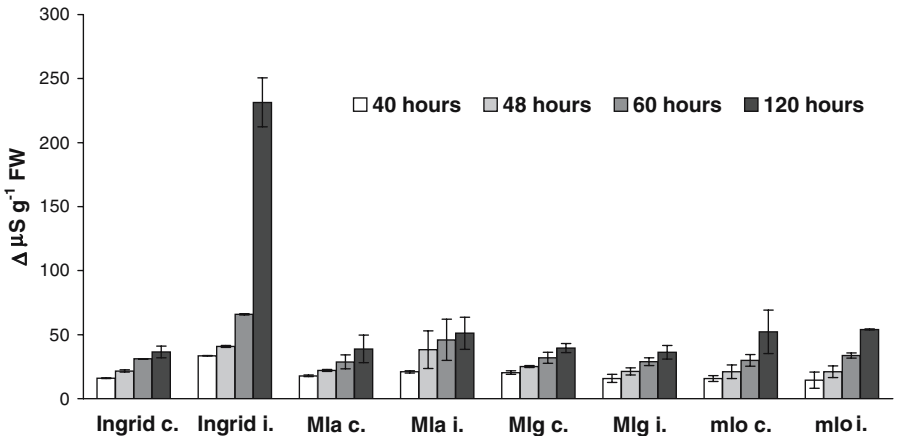

Fig. 6 Electrolyte leakage from uninoculated control (c.) and infected (i.) susceptible (Ingrid) and resistant (Mla, Mlg, mlo) barley leaves following powdery mildew inoculation. Data are presented as mean±S.D., calculated from five independent experiments

interactions between barley and powdery mildew. Our results indicate that up-regulation of the antioxidative system of host cells plays an important role in maintaining the compatible, biotrophic relationship between powdery mildew and barley.

Most of the tested antioxidant enzymes (POX, SOD, APX, GST and GR) excluding catalase and DHAR showed similar activity changes following powdery mildew inoculation (Figs. 2 and 3). After 5–7 days, the activities of POX, SOD, APX, GST and GR were significantly induced in susceptible barley compared to its uninfected control. As compared to susceptible plants, we found very similar but less pronounced changes of enzyme activities in near-isogenic resistant Mla plants. One can suppose that this is due to the similar initial development of *Bgh* in the susceptible and the Mla-type resistant barley plants during the first 30 h of infection. In the Mlg and mlo lines even weaker, or no induction of SOD, GST, APX and GR was detectable upon inoculation. We found a definite induction of POX activity in all of the barley lines after *Bgh*-inoculation. POX activity increased most markedly (to more than five-fold by the later stages of infection) in attacked susceptible barley leaves, in Mla and Mlg leaves it peaked at four-fold activity 5 dai, while in mlo at two-fold of the uninoculated control 3 dai (Fig. 3). Kerby and Somerville (1989) have found enhanced POX activities in both susceptible and Mla-type resistant barley lines between 8 and 16 hai. Kristensen et al. (1999) have shown by activity staining pronounced and continuously growing induction of POX over a time course of 1–5 days following *Bgh*-inoculation in susceptible and in Mla-type resistant barley. No new peroxidase isoforms were found, and no difference was observed in the timing of accumulation of isoperoxidases between the compatible and the incompatible interaction. We may thus conclude that the role of peroxidases in defence responses does not seem to be specific.

DHAR activity, on the other hand, was transiently reduced in susceptible Ingrid, Mla and Mlg plants, reaching its minimum 4 dai in the susceptible and 5 dai in the Mla and Mlg lines. In the mlo line DHAR activity did not change significantly. Along the time course no markedly significant changes in CAT activity could be observed either in any of the tested barley lines. Earlier, a rapid, transient growth in catalase activity has been demonstrated in susceptible barley 24 hai after inoculation and in Mla-type resistant line 16 hai (Vanacker et al. 2000). In wheat, no changes in catalase activity were found 48 hai with powdery mildew (Renard-Merlier et al. 2007).

Ascorbic acid is present in high concentrations in plant tissues and possesses a prominent antioxidative role in barley leaves as the most important reducing agent (Noctor and Foyer 1998). Ascorbate is univalently oxidized by APX to monodehydroascorbate radical (MDA). MDA can be reduced to ascorbate by NADPH-dependent MDA reductase or disproportionates spontaneously to ascorbate and DHA. DHA is regenerated to ascorbate by glutathione-dependent

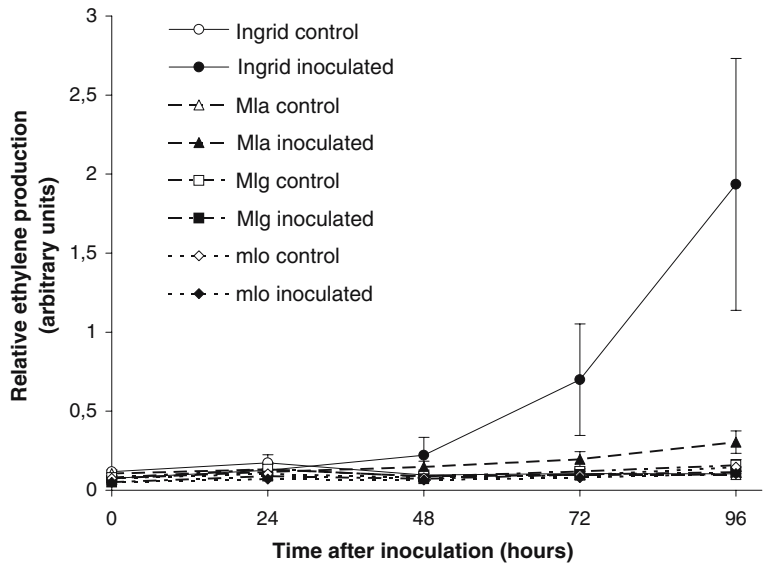

Fig. 7 Ethylene production in leaves of powdery mildew-infected, near-isogenic barley genotypes (susceptible Ingrid, Mla-, Mlg- and mlo-type resistant) and in respective uninoculated controls. Data are presented as mean±S.D., calculated from three independent experiments

DHAR. Activities of APX and MDA reductase have been reported to increase in susceptible but not in Mla-type resistant barley 4 days after powdery mildew attack (Burhenne and Gregersen 2000). However, we observed a decline of DHAR activity in powdery mildew-attacked susceptible Ingrid lines. Our findings, that DHAR activity decreased in the susceptible interaction 4 dai and then recovered, are consistent with the results of El-Zahaby et al. (1995). These data suggest that the regeneration of ascorbate takes place through MDA reductase activity, because MDA reductase may counteract the low DHAR activity, keeping the oxidation of ascorbate under control.

We found that powdery mildew inoculation did not result in significant changes of ascorbate levels, nor in the redox state in either resistant or susceptible Ingrid barley plants at the later stages of the interaction. Vanacker et al. (1998) demonstrated unchanged total foliar ascorbate content 24 h after *Bgh*-infection with a slight shift towards the reduced from in the susceptible line, but not in the Mla-type resistant barley. El-Zahaby et al. (1995) have found a 50–60% decrease of ascorbate levels 4–6 dai in highly susceptible barley cv. Emir attacked by powdery mildew fungus. We could only demonstrate a slight and not significant decrease of ascorbic acid level in susceptible Ingrid 7 days after mildew attack. This inconsistency might not only be caused by the different genetic background of the plants, but also by the differences in growth conditions used. Resistance to powdery mildew has been shown to correlate with increased foliar glutathione content 18–24 hai (Vanacker et al. 2000). In our study, total glutathione content increased significantly 7 dai in the susceptible leaves, whereas El-Zahaby et al. (1995) observed such an increase in glutathione concentration 4 days after powdery mildew attack. One can conclude that with the protection of the active antioxidant systems and with the help of cytokinin and auxin induction biotrophs keep plant cells alive for a longer time in order to develop the necessary long, intimate connection between the pathogen and host cell (Schulze-Lefert and Panstruga 2003). The role of these hormones is to maintain tissue viability and to establish a nutrient sink for the biotrophic pathogen (Jameson, 2000).

The early accumulation of H_2O_2 in barley leaves is well documented in response to powdery mildew (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999; Vanacker et al. 2000), whereas changes in H_2O_2 levels at later stages of interaction was poorly investigated. Upon inoculation with powdery mildew, we found slightly elevated H_2O_2 levels in Mla, Mlg and mlo barley lines; however in susceptible barley leaves H_2O_2 concentration was reduced significantly 7 dai (Fig. 1). Previously it was found microscopically that *Bgh* attack of these resistant barley lines results in an early (1–2 dai) accumulation of H_2O_2 (Hückelhoven et al. 1999). Enhanced H_2O_2 production seems to be closely associated with plant defence against powdery mildew fungus (Thordal-Christensen et al. 1997; Hafez and Király 2003). Hydrogen peroxide has been demonstrated to play an important role in plant resistance to pathogens (Bradley et al. 1992; Olson and Varner 1993; Levine et al. 1994). The external application of ROS sources has been shown to protect susceptible plants from powdery mildew and rust fungi by inhibiting or killing the pathogens (Ouf et al. 1993; Wang and Tzeng 1998; Hafez and Király 2003). Toxic effects of ROS to the pathogen can be prevented by the simultaneous application of antioxidants (Király and El-Zahaby 2000; Hafez and Király 2003). However, any attempt to induce susceptibility to powdery mildew in genetically resistant barley by antioxidant treatments has so far been unsuccessful (Király, personal communication). The hypothesis that H_2O_2 may play a pivotal role in resistance of barley to powdery mildew is further supported by our finding that in contrast to resistant barley lines, H_2O_2 level decreased in leaves of susceptible Ingrid plants after inoculation with powdery mildew fungus.

There was no correlation between accumulation of H_2O_2 and membrane damage measured by leakage of electrolytes. This is probably due to the minimal number of affected cells compared to the whole leaf in the resistant interactions. In the case of the *Mla*-mediated resistance, a slight increase of leakage was detected, but Mlg and mlo lines showed no increase in electrolyte efflux after powdery mildew attack. On the other hand, 2 days growth of the fungus was enough to induce significant electrolyte leakage, and 5 days after infection the leakage was about eight times higher from infected than from control leaf segments of susceptible Ingrid plants. In addition, in the same barley-powdery mildew systems a short-term (2 hai) non-specific, an intermediate (8–12 hai) papilla-specific and a long-

term, HR-specific alkalinisation have been detected in the apoplast with the help of a non-invasive microprobe technique (Felle et al. 2004).

Ethylene production showed a similar tendency as ion leakage in powdery mildew-attacked barley leaves. Probably again due to the limited number of affected cells, no changes of ethylene production were detected in Mlg and mlo plants and only a slight increase was found in Mla leaves even at 4 dai. However, at only 3 dai, significantly more ethylene was produced in powdery mildew-attacked susceptible leaves than in control, uninfected leaves (Fig. 7). In accordance with our results, in powdery mildew-infected wheat varieties exhibiting various levels of quantitative resistance, ethylene evolution was correlated with the number of pustules on the inoculated leaves 5–9 dai with *B. graminis* f. sp. *tritici* (Fauth and Hoffmann 1992).

It is also noteworthy that POX activity and leakage of electrolytes was significantly higher in case of the uninfected mlo barley as compared to other tested barley lines bearing the wild-type *Mlo* gene, strengthening earlier suggestions that mutation of the *Mlo* gene induces an early senescence of barley leaves (Schulze-Lefert and Panstruga 2003; Piffanelli et al. 2002).

In conclusion, powdery mildew inoculation resulted in a significant induction of antioxidant defence in the susceptible barley line. One can suppose that this up-regulation of antioxidants could prevent the accumulation of ROS in powdery mildew-attacked susceptible plants. Indeed, we found decreased H_2O_2 levels in leaves of infected susceptible barley. Based on our observations we can not exclude the possibility that the powdery mildew fungus itself produces antioxidant substances or enzymes to defend itself against ROS. This idea is supported by previous findings of Zhang et al. (2004), who have detected that *Bgh* secreted extracellular catalase from haustoria during infection of barley. We could not find higher catalase activity in powdery mildew-infected susceptible barley; however, we measured catalase activity in total leaf extracts and not locally, at the host-pathogen interface. The question, whether high antioxidant activities in mildewed susceptible barley were generated mainly by the plant cells or by the fungus, awaits further investigations.

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