The White Barley Mutant *Albostrians* Shows Enhanced Resistance to the Biotroph *Blumeria graminis* f. sp. *hordei*

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We performed cytological and molecular analyses of the interaction between the biotrophic barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* and white and green leaves of the barley *albostrians* mutant. The leaves have the same nuclear genotype but differ from each other in respect to plastid differentiation. White leaves showed enhanced penetration resistance to *B. graminis* f. sp. *hordei*, associated with higher epidermal H₂O₂ accumulation beneath the appressorial germ tubes and protein cross-linking in papillae. Very low basal salicylic acid content was found in white leaves, which further confirmed that H₂O₂ accumulation and penetration resistance in barley are independent of salicylic acid. Expression analysis of stress and defense-related genes, including such being involved in reactive oxygen species production and cell death regulation, revealed stronger constitutive or pathogen-induced transcript accumulation in white leaves. We discuss the data on the basis of the finding that white *albostrians* leaves exhibit a supersusceptible interaction phenotype with the hemibiotrophic fungus *Bipolaris sorokiniana*.

Additional keywords: GDP-dissociation inhibitor, MLO, OXLP.

Plant mutants offer valuable biological variability in understanding plant processes and interactions. The *albostrians* mutant, derived from barley cv. Haisa, is such a mutant having different leaf phenotypes. The mutant was derived by X-ray-induced mutagenesis of a single recessive nuclear allele *albostrians*. The homozygous recessive allele *albostrians* blocks chloroplast development. This mutation of extranuclear hereditary factors is inherited purely maternally and is stable (Hagemann and Scholz 1962). The mutant produces entirely white and green leaves along with green and white striped leaves in various degrees. Both white and green leaves have the same nuclear genotype but differ from each other with respect to plastid differentiation. White leaves and white leaf parts contain undifferentiated plastids lacking internal membranes, which are photosynthetically inactive, have only trace amounts of chlorophylls, and lack 70S ribosomes (Hess et al. 1994a). The transcript levels of many photosynthesis-associated chloroplast and nuclear genes are extremely low in white

leaves (Hess et al. 1992; 1994b). In contrast, transcripts of many nuclear genes involved in the response against certain abiotic and biotic stresses were found to accumulate in much higher levels in white leaves (Hess et al. 1998).

In this study, we investigated the interaction of the *albostrians* mutant with the biotrophic fungal pathogen *Blumeria graminis* (D.C.) Speer f. sp. *hordei* Marchal, which causes powdery mildew on barley. Along with activation of defense-related genes (Gregersen et al. 1997), two main cytological responses are commonly recognized in the interaction of powdery mildew and barley. First, a papilla is formed directly beneath the site of attempted penetration. Second, a hypersensitive reaction (HR) is mounted, which is a cell death response of the attacked host cell, preventing the establishment of biotrophy (Koga et al. 1990). The successful penetration of a host cell is recognized by the formation of a haustorium, followed by formation of the elongated secondary hyphae (ESH) and fungal growth. One of the earliest events that occurs during host-pathogen interactions is generation of H₂O₂ and other reactive oxygen species (ROS), which play important roles in plant defense (Grant and Loake 2000; Hüchelhoven and Kogel 2003). Enhanced production of H₂O₂ during oxidative burst has been shown to be involved in a number of plant defense processes. It leads to rapid oxidative cross-linking of cell wall components (Bradley et al. 1992; Thordal-Christensen et al. 1997), activation of defense genes (Wu et al. 1997), triggers HR that causes blocking of biotrophic pathogen development (Levine et al. 1994), and can reach higher concentrations directly toxic to pathogens (Peng and Kuc 1992). In barley-powdery mildew interactions, accumulation of ROS has been studied during both incompatible and compatible interactions (Hüchelhoven and Kogel 1998; Hüchelhoven et al. 1999; Thordal-Christensen et al. 1997).

The present investigation was initiated by the previous observation that *albostrians* white leaves, unlike green *albostrians* leaves and leaves from parent cv. Haisa, were extremely susceptible (supersusceptible) (Schäfer et al. 2004) to the toxin producing fungal pathogen *Bipolaris sorokiniana*. Here, we analyze effects of the *albostrians* mutation on fungal development and plant defense responses in green and white leaves. We show that enhanced resistance to *B. graminis* f. sp. *hordei* is closely associated with enhanced papilla formation, H₂O₂ accumulation, enhanced expression of various defense genes previously implicated in powdery mildew resistance, as well as expression of the RAB-GDP-dissociation inhibitor (*GDI*) gene.

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RESULTS

Cytological analysis of the interaction of *albostrians* white and green leaves with *B. graminis* f. sp. *hordei*.

We evaluated interaction phenotypes of white and green first leaves of *albostrians*, of white and green parts of striped leaves, and of wild-type cv. Haisa leaves at 24, 48, and 72 hours after inoculation (hai) with *B. graminis* f. sp. *hordei* race A6. Evaluation at 24 hai showed that, in 59% of the interaction sites, *B. graminis* f. sp. *hordei* failed to penetrate cells of white leaves whereas only 35% of the interaction sites in green leaves showed unsuccessful penetration (Figs. 1 and 2A). Autofluorescent phenolics were seen in both white and green leaves around appressoria associated with penetration failure (Fig. 2B). In white leaves, successful penetration as evident by haustorium and ESH formation took place in 36% of attacked cells, whereas corresponding value for the green leaves was 58%. In green leaves, the frequency of haustorium formation remained at almost the same level through the time course up to 72 h, whereas in white leaves, the fungus showed a tendency for a delayed development, thereby penetration frequency increased to about 49 (after 48 h) and 54% (after 72 h) (Fig. 1). Frequency of nonpenetrated living cells with papilla formation was around 40% after 72 h in white leaves as compared with 25% in green leaves. The frequency of interaction sites showing epidermal HR of nonpenetrated cells (Fig. 2C) ranged from 7 to 12% in green leaves and from 3 to 6% in white leaves during the time course of the inspection. Interaction phenotypes of green leaves from *albostrians* and cv. Haisa were virtually identical. Both cv. Haisa and green *albostrians* leaves also showed a low frequency of mesophyll HR, which was not detected in white leaves (Fig. 1).

H₂O₂ localization at interaction sites.

H₂O₂ and other ROS accumulate in different amounts and patterns in susceptible and resistant barley lines after fungal attack (Hückelhoven and Kogel 2003). We analyzed the frequency of H₂O₂ at interaction sites in *albostrians* and cv. Haisa at 18, 24, and 48 hai (Fig. 3). After injection of diaminobenzidine (DAB), which polymerizes in the presence of H₂O₂ in a peroxidase-dependent manner, reddish-brown staining at interaction sites was observed. H₂O₂ accumulated beneath the primary germ tubes and appressoria (Fig. 2D through F), in anticlinal cell walls near penetration attempts, and in whole cells (Fig. 2H). Successful penetration, as evident by formation of haustorial initials, did not provoke H₂O₂ accumulation (Fig. 2D). In white leaves, the frequency of H₂O₂ accumulation beneath appressoria (AGT) was between 23 (48 hai) and 34% (18 and 24 hai) of all interaction sites (Fig. 3). In green leaves, these figures varied between 8 and 12%. Approximately 10% of epidermal cells showed whole cell DAB-staining in epidermal cells in green leaves, compared with only 2% in white leaves (Fig. 3). Whole cell DAB staining in mesophyll was observed exclusively in green but not in white leaves at about 3% of interaction sites. There were similar frequencies of DAB-staining in the anticlinal cell walls (ACW) without staining at the AGT in both white and green leaves. However at 24 hai, there was a tendency for more ACW staining in green leaves, coinciding with more frequent fungal penetration (Hückelhoven et al. 1999).

Protein cross-linking.

Oxidative cross-linking of proteins has been demonstrated in papillae and HR cells of barley upon fungal attack (Thordal-Christensen et al. 1997). Accordingly, we found Coomassie blue positive staining in papilla and HR cells of *albostrians* white and green leaves (Fig. 2I and J). The staining often was

not associated with visible fungal structures, probably because they were washed away after sodium dodecyl sulfate (SDS) treatment, which is necessary to remove unbound proteins. Therefore, we were unable to thoroughly quantify oxidative protein cross-linking. However, given that the inoculation density was equal, visible judgment of Coomassie blue spots per microscopic field (by 20-fold magnification) suggested that there were more interaction sites with Coomassie staining in and around papillae in white leaves. This accords with the

Fig. 1. Frequency of cytological responses of white and green *albostrians* leaves as well as leaves from wild-type barley cv. Haisa at 24, 48, and 72 h after inoculation (hai) with *Blumeria graminis* f. sp. *hordei* race A6. Nonpenetrated living cells refer to interaction sites where fungus failed to penetrate the foremost epidermal cell. HAU (haustoria) and ESH (elongated secondary hyphae) refer to successful penetration of the cells. HR (hypersensitive reaction) refers to interaction sites where the epidermal cells show whole-cell autofluorescence, and MSHR (mesophyll-HR) refers to autofluorescence of mesophyll cells. Each column represents the average of 500 interaction sites on five leaves. Repetition of the experiments led to very similar results.

finding that white leaves more frequently produce an oxidative environment at sites of attempted fungal penetration.

Low salicylic acid contents detected in white leaves.

We addressed the question whether total salicylic acid (SA) content differs in white and green *albostrians* leaves. Seven-

day-old first leaves were inoculated with 50 conidia per mm² of *B. graminis* f. sp. *hordei*, and SA content was measured at 24 and 48 hai. In green leaves, SA concentration was 400 ng per gram of fresh weight and did not increase after inoculation. In white leaves, very low amounts of SA (<50 ng per gram of fresh weight) were detected in mock-inoculated

Fig. 2. Microphotographs of cytological responses of *albostrians* to *Blumeria graminis* f. sp. *hordei*. **A** through **C**, Defense responses expressed in epidermal cells in white leaves of *albostrians* after *B. graminis* f. sp. *hordei* infection. **A**, A fungal germling was stopped, and papillae formed beneath the primary germ tube and appressoria (arrows). Bar = 16 μ m, **B**, Same interaction site as shown in **A**. Phenolic compounds accumulated in and around papillae observed as autofluorescence under UV light excitation (arrows). Bar = 16 μ m, **C**, The nonpenetrated epidermal cell attacked by the appressorium (arrow) underwent a hypersensitive reaction (HR), as indicated by the whole-cell autofluorescence. Bar = 32 μ m. **D** through **H**, H₂O₂ accumulation patterns as observed by diaminobenzidine (DAB) staining in response to *B. graminis* f. sp. *hordei*. **D**, DAB-staining was not associated with successful penetration and formation of haustorial initial (arrow) (Bar = 16 μ m) but was restricted to sites (arrowhead) also in **E**, where fungus was prevented from penetration (arrow). Bar = 12 μ m, **F**, Interaction site with four unsuccessful penetration attempts (arrowheads); each of them are associated with H₂O₂ accumulation (48 hai). Bar = 24 μ m, **G**, Rare interaction site in a white leaf, where delayed penetration (arrow, haustorial initial) of DAB associated papilla (arrowhead) occurred at 48 hai. Bar = 16 μ m, **H**, Whole-cell H₂O₂ accumulation associated with epidermal hypersensitive cell death. Bar = 32 μ m. **I** and **J**, Coomassie blue staining for cross-linked proteins in papilla (arrow in **I**; Bar = 12 μ m) and in HR cell (**J**; Bar = 16 μ m). **K**, Micrograph of striped *albostrians* leaf after *B. graminis* f. sp. *hordei* inoculation (96 hai). In the green leaf area (upper part), fungal mycelia developed normally, whereas in the white leaf area (lower part), fungal growth was considerably retarded. Bar = 80 μ m.

leaves, and the SA concentration dropped upon inoculation below the detection limit (data not shown).

Gene expression patterns

in *albostrians*-*B. graminis* f. *sp. hordei* interactions.

We analyzed temporal expression patterns of genes, known to be differentially regulated in barley in response to *B. graminis* f. *sp. hordei*, in *albostrians* white and green first leaves at 0, 24, and 48 hai. Equal loading of RNA was achieved by adjusting 25S and 18S rRNA band intensities in gels (Zubko and Day 2002), since 23S and 16S rRNA bands

are lacking in white leaves. Higher gene transcript abundance was observed in white leaves for most of the genes (Fig. 4). The gene encoding basic pathogenesis-related 1b protein (*PR-1b*), a marker for pathogenesis-related gene expression, was induced by 24 and 48 hai in both white and green leaves (Fig. 4). Similarly, expression of a phenylalanine ammonia-lyase gene (*PAL*), a *14-3-3* gene, and a gene for oxalate oxidase-like protein (*OxLP*) was induced early upon fungal attack. The induction was always stronger in white leaves. Moreover, *OxLP* and *14-3-3* showed a greatly enhanced constitutive expression in white leaves.

We measured levels of putative anti- or proapoptotic genes. Expression of Bax inhibitor-1 (*BI-1*) and an aspartate protease (*Aspro*) were constitutively enhanced in white leaves. Upon

Fig. 3. H₂O₂ accumulation at interaction sites of white and green *albostrians* leaves as well as leaves from wild-type cv. Haisa at 18, 24, and 48 h after inoculation (hai) with *Blumeria graminis* f. *sp. hordei* race A6. H₂O₂ accumulation, visualized by diaminobenzidine staining, was detected beneath appressorial germ tubes (AGT), in anticlinal cell walls close to penetration attempts (ACW), in whole epidermal cells (WCE), or in whole mesophyll cells (WCM). Each column represents the average of 500 interactions on five leaves (100 sites on each leaf). Repetition of the experiments led to very similar results.

Fig. 4. Northern blot analysis of *PR-1b*, phenylalanine ammonia-lyase (*PAL*), *14-3-3*, oxalate oxidase-like-protein (*OxLP*), Bax inhibitor-1 (*BI-1*), and aspartate protease (*Aspro*) transcript accumulation after inoculation with *Blumeria graminis* f. *sp. hordei* race A6 in white and green leaves of the barley *albostrians* mutant. Seven-day-old first leaves were inoculated with 50 conidia per square mm, and leaves were harvested at 0, 24, and 48 h from mock-inoculated and inoculated plants. Total RNA was extracted, and 10 µg and 5 µg of total RNA for green and white leaves, respectively, were separated on denatured agarose gel and blotted onto nylon membranes. Blots were hybridized with digoxigenin-labeled antisense probes. Ethidium bromide staining of the gel served as loading control. Independent experiments showed similar expression patterns.

inoculation with *B. graminis* f. sp. *hordei*, *BI-1* was slightly up-regulated only in the green leaves, while *Asprot* was induced only in the white leaves (Fig. 4). Expression of the powdery mildew resistance regulator *Mlo*, cytosolic ascorbate peroxidase (*cAPX*), barley putative NADPH oxidase (*pNAox*), and GDP dissociation inhibitor (*GDI*) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 5). Total RNA was extracted from white and green leaves at 0, 3, 6, 12, 24, and 48 hai. Template concentration was again adjusted using 25S and 18S rRNA. For final adjustment, the RT-PCR expression pattern of a barley ubiquitin gene was taken as an additional constitutive control to correct variation in RNA template loading. As with several but not all other genes, expression of ubiquitin was higher in white leaves (Fig. 5). Similar to *BI-1*, expression of *Mlo* was slightly induced only in green leaves, though expression levels appeared constitutively higher in white leaves. Expression of *cAPX* was strongly induced by *B. graminis* f. sp. *hordei* in green leaves, though its expression level was constitutively high in white leaves, also. Expression of *pNAox* was up-regulated at early timepoints in both green and white leaves after *B. graminis* f. sp. *hordei* infection but was somewhat delayed in the white leaves. In contrast, expression of *GDI* was constitutively higher in white leaves and up-regulated in response to *B. graminis* f. sp. *hordei* in both types of leaves.

DISCUSSION

In the present study, we analyzed defense responses of white and green leaves of the barley mutant *albostrians*. We found that resistance to powdery mildew fungus is enhanced in white leaves. These results must be interpreted in the light of the finding that the same leaves are extremely susceptible to the hemibiotrophic fungus *Bipolaris sorokiniana* (Schäfer et al. 2004). A comparable ambivalence of defense characters in biotrophy and hemibiotrophy (or necrotrophy) has already been described in barley mutants with allelic variation at the *Mlo* locus (Jarosch et al. 1999; Kumar et al. 2001).

Resistance to *B. graminis* f. sp. *hordei* was associated with higher frequencies of H₂O₂ accumulation beneath the appressorial germ tubes, protein cross-linking in papillae, and a con-

stitutively enhanced expression of various defense genes, such as those encoding a 14-3-3 protein and OXLP, the latter having been previously shown to be causally implicated in powdery mildew resistance (Schweizer et al. 1999).

Papillae in white and green leaves accumulated phenolic compounds, as evidenced by microscopic observation of auto-fluorescence (Fig. 2B), but significantly more nonpenetrated papillae were found in white leaves at any given timepoint (Fig. 1). At later timepoints (48 and 72 hai), a considerable proportion of papillae in white leaves were penetrated, as evidenced by an enhanced proportion of ESH (Fig. 1). This indicates that papilla formation per se may not be sufficient to mediate penetration resistance to *B. graminis* f. sp. *hordei*. The tendency of delayed penetration may be due to lack of photosynthetic activity and limited supply of nutrients in white leaves. This may ultimately result in slow development of mycelium (Fig. 2K) and extremely rare formation of *B. graminis* f. sp. *hordei* pustules, development of which was delayed by 1 or 2 days (data not shown). Consistently, delayed development of *B. graminis* on white (albino) leaves from *Triticum timopheevii* and *Hordeum vulgare* (cv. Trebi I) has been previously demonstrated (Nashaat and Moore 1992; Shioji et al. 1982). A lower frequency of interaction sites with HR of nonpenetrated epidermal cells were observed in white leaves (Fig. 1), probably reflecting a higher penetration resistance of white leaves. Notably, white leaves showed more epidermal HR against the hemibiotrophic *Bipolaris sorokiniana* (Schäfer et al. 2004). In contrast to our results, Nashaat and Moore (1992) observed a lower percentage of papillae in response to the wheat powdery mildew fungus on albino leaves of *Triticum timopheevii*. These authors suggested that low photosynthetic activity in white leaves may have reduced the efficiency of papilla formation. Thus, enhanced penetration resistance in white *albostrians* leaves may be specific for the *albostrians* mutation. This notion is supported by the finding that white leaves show a higher number of sites with H₂O₂ accumulation at papillae, clearly suggesting an active defense mechanism (Fig. 3).

Protein cross-linking in and around papillae and in HR cells, as evident by a positive Coomassie blue stain (Fig. 2I and J), is suggested to be H₂O₂-mediated (Thordal-Christensen et al. 1997). The emergence of immobile proteins in papillae was more intense and frequent in white leaves, thus correlating with a higher frequency of H₂O₂ accumulation and higher penetration resistance (Figs. 1 and 3).

Previous studies have shown that SA does not accumulate in barley after powdery mildew inoculation (Hückelhoven et al. 1999; Vallelleian-Bindschedler et al. 1998). Moreover, H₂O₂ generation, formation of effective papillae, and HR are independent of SA accumulation (Hückelhoven et al. 1999). In agreement with these findings, SA did not accumulate in response to *B. graminis* f. sp. *hordei*. Interestingly, white leaves showed very low amounts of basal SA, though they more frequently accumulated H₂O₂ in papillae. The pathway of SA synthesis in plants is not completely elucidated. SA is suggested to be synthesized from either phenylalanine, chorismate, or both (Shah 2003). White leaves of *albostrians* contain higher amounts of phenylalanine but drastically reduced amounts of the chorismate-precursor shikimate (data not shown), i.e., SA might indeed be formed from chorismate via isochorismate in barley. Many studies have shown positive correlation between *PR-1* transcript induction and SA content (Nawrath and Métraux 1999). However, expression of genes encoding basic PR1 proteins have been shown to be induced by jasmonic acid and ethylene and, in some cases, repressed by SA (Santamaria et al. 2001). In our experiments, pathogen-induced expression of basic *PR-1b* that apparently contributes in barley to penetration resistance against *B. graminis* f. sp.

Fig. 5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of barley ubiquitin, *MLO*, cytosolic ascorbate peroxidase (*APX*), putative NADPH oxidase (*pNAox*), and GDP-dissociation inhibitor (*GDI*) in white and green leaves of the barley *albostrians* mutant after inoculation with *Blumeria graminis* f. sp. *hordei* race A6. Total RNA (4 µg for green and 2 µg for white leaves) was used for first-strand cDNA synthesis using SuperScript reverse transcriptase. Expression levels of the genes were determined using 1 µl of cDNA for PCR amplification with the gene-specific primers in a 25-µl PCR reaction. Expression of ubiquitin (*Ubi*) was used as control for comparison over timepoints within green and white leaves.

hordei (Schultheiss et al. 2003) was higher in white leaves (Fig. 4). This suggests that background resistance of white leaves and the activation of *PR-1b* in response to *B. graminis* f. sp. *hordei* do not require SA.

Along with a constitutively enhanced gene expression level and in accordance with the higher resistance, we found in white leaves a stronger increase of defense-related genes *PAL*, *OXL*P, and *14-3-3*. The role of *PAL* (Boyd et al. 1994; Clark et al. 1994; Dixon and Paiva 1995) and *OXL*P (Schweizer et al. 1999; Wei et al. 1998) in plant defense has been demonstrated previously. Early and strong expression of *OXL*P was found in barley lines resistant to penetration by *B. graminis* f. sp. *hordei* (Hückelhoven et al. 2001). Thus, stronger expression of *OXL*P in white leaves (Fig. 4) agrees with these results and is one possible explanation for the higher resistance of these leaves.

The *14-3-3* proteins form a conserved gene family in animals and plants and have been associated with several stress responses. Their role in plant defense is proposed to be in signal transduction and gene regulation (Finnie et al. 1999; Roberts 2003). Induction of *14-3-3* expression in response to *B. graminis* f. sp. *hordei* results in an activation of the plasma membrane H^+ -ATPase in barley epidermal cells (Finnie et al. 2002). Modulation of plasma membrane H^+ -ATPase activity has been reported to be involved in defense and general stress signaling in plants. A role for the activation of the H^+ -ATPase in the induction of the HR of barley to *B. graminis* f. sp. *hordei* has been suggested (Zhou et al. 2000).

We found increased expression of the *Aspro*t gene in response to *B. graminis* f. sp. *hordei* in white leaves (Fig. 4). The gene was implicated in developmentally regulated barley cell death (Runeberg-Roos and Saarna 1998), and expression of *Aspro*t was up-regulated after powdery mildew infection in barley lines showing either penetration resistance or mesophyll cell death but not in susceptible barley (Hückelhoven et al. 2001).

A striking result of this study is the finding that expression of *BI-1* and *Mlo* is constitutively enhanced in white leaves (Fig. 5). Both genes exert cell death and defense-suppressing capacity, and their expression can be associated with biotic and abiotic stresses (Kawai et al. 1999; Piffanelli et al. 2002; Sanchez et al. 2000). *BI-1* has been described as a regulator of cell death, and its expression is up-regulated after pathogen inoculation and wounding (Hückelhoven et al. 2001; Kawai et al. 1999; Sanchez et al. 2000). Overexpression of barley *BI-1* induces supersusceptibility to *B. graminis* f. sp. *hordei* and breaks *mlo*-mediated penetration resistance (Hückelhoven et al. 2003). *Mlo* is a negative regulator of defense reactions (penetration resistance, necrosis) to *B. graminis* f. sp. *hordei* and mutations in *Mlo* confer race-nonspecific resistance to the fungus. A transiently increased expression of *Mlo* has been shown in response to *B. graminis* f. sp. *hordei* and *Magnaporthe grisea* infection and in response to abiotic stresses such as wounding and paraquat treatment (Piffanelli et al. 2002). We detected slightly induced *Mlo* expression by 6 h after *B. graminis* f. sp. *hordei* inoculation in green leaves. Strikingly and unexpectedly, high *Mlo* and *BI-1* expression in white leaves was insufficient to induce enhanced susceptibility to *B. graminis* f. sp. *hordei*, though overexpression of *Mlo* (Kim et al. 2002) and *BI-1* (Hückelhoven et al. 2003) in barley epidermal cells greatly enhanced cell accessibility for this fungus. In a control experiment, we confirmed that transient overexpression of both *BI-1* and *MLO* in white leaves resulted in increased susceptibility to *B. graminis* f. sp. *hordei* (data not shown). Thus, *MLO* and *BI-1* are obviously functional in white leaves, and enhanced resistance of *albostrians* seems to be independent of *BI-1* and *MLO*.

Interestingly, we detected a high frequency of H_2O_2 accumulation at epidermal sites of attempted infection but a

strongly reduced potential to accumulate H_2O_2 in mesophyll tissue of white leaves (Schäfer et al. 2004). One of the major H_2O_2 scavenging enzymes in the plant is ascorbate peroxidase, which is found throughout the cell (Jespersen et al. 1997). Increased expression and activity of a *cAPX* isoform has been detected upon *B. graminis* f. sp. *hordei* infection, especially at late timepoints of the compatible interaction (Burhenne and Gregersen 2000; Hückelhoven et al. 2001). Notably, strong *cAPX* expression in white leaves did not compromise apoplastic H_2O_2 accumulation. One reason for this could be tissue-specific or posttranscriptional regulation of *cAPX* (Mittler et al. 1998).

NADP(H) oxidase generates superoxide anions ($O_2^{\cdot-}$) that become readily dismutated into H_2O_2 either spontaneously or by superoxide dismutase (Grant and Loake 2000). There is increasing evidence that, in the barley–powdery mildew interaction, $O_2^{\cdot-}$ accumulation at infection sites, in contrast to H_2O_2 , is associated with successful cell wall penetration rather than defense (Hückelhoven and Kogel 2003). In accordance with these findings, a pathogen-induced early increase of NADP(H) oxidase expression was found in green leaves, while white leaves showed a delayed response of NADP(H) oxidase expression. Notably, this late response correlates with delayed penetration events in white leaves.

GDI is one of the regulatory proteins of the RAB GTPase cycle involved in vesicular membrane traffic regulation (Pfeffer et al. 1995; Ueda et al. 1998). Involvement of RAB proteins and vesicle transport in plant defense has been suggested (Bogdanove and Martin 2000; Collins et al. 2003; Cooper et al. 2003; Sano and Ohashi 1995). Hence, regulation of RAB-GDI might be critical for effective defense. In rice, expression of two RAB-specific *GDI* genes (*OsGDIs*) was substantially induced after treatments with an elicitor of *M. grisea* (Kim et al. 1999), suggesting GDI may have an important role in the plant response to fungal infection. We found a strong induction of the barley *GDI* to *B. graminis* f. sp. *hordei* inoculation, and in accordance with an earlier report (Hess et al. 1998), a stronger overall expression in white leaves (Fig. 5). Single-cell gene silencing of barley *GDI* slightly enhanced susceptibility of white leaves to *B. graminis* f. sp. *hordei* (data not shown), suggesting that *GDI* might be one element of enhanced resistance. The mechanism of RAB and GDI function in resistance is the subject for further research, since plants have many RAB proteins but many fewer RAB-GDIs (Kim et al. 1999; Vernoud et al. 2003).

MATERIALS AND METHODS

Plant material, pathogen, and inoculation.

The barley (*Hordeum vulgare* L.) mutant *albostrians* was derived from spring barley cv. Haisa by irradiation (Hagemann and Scholz 1962). This mutant produces plants with white, green, and white and green striped leaves, respectively. Seedlings were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h.

Race A6 of *Blumeria graminis* f. sp. *hordei* was used for inoculation of first leaves (Wiberg 1974). For cytological analyses, a conidial density of about 15 to 20 spores per square mm, and for gene expression and salicylic acid measurement, 50 conidia per square mm were used.

Microscopy for host-pathogen interactions.

Seven-day-old first leaves were inoculated on the adaxial surface with 15 to 20 conidia per square mm. Leaves were harvested at 24, 48, and 72 hai. Leaf fixation and clearing, bright field microscopy, and fluorescence microscopy were performed as described by Hückelhoven and Kogel (1998). Quan-

tification of penetration resistance, HR, and penetration was performed in 100 interactions per leaf. Because the rate of fungal penetration of barley leaf epidermal cells vary depending on the cell types, only short epidermal cells (A and B cells covering parenchymatic mesophyll tissue) were evaluated for cellular phenotypes (Koga et al. 1990). Interactions were scored only when the conidium had developed a mature appressorium and host cells were attacked by a single conidium.

H₂O₂ detection by DAB infiltration method.

Detection of H₂O₂ was performed using a DAB infiltration method adapted from Thordal-Christensen and associates (1997). The first leaves from 7-day-old seedlings were inoculated on the adaxial surface with a spore density of 15 conidia per square mm and were incubated in the growth chamber. Inoculated leaves were infiltrated with 0.7 mg of DAB per ml (pH 3.8, adjusted with HCl) and were incubated in the growth chamber for 2 h before fixation at indicated timepoints. Inoculated leaves were harvested and fixed at 18, 24, and 48 h. Different patterns of DAB-staining were evaluated quantitatively. If more than one cellular structure stained with DAB at the same interaction site, the most intensively stained interaction was counted.

Protein cross-linking by Coomassie blue staining.

In order to visualize protein cross-linking in cell walls, we performed Coomassie blue staining as described by Thordal-Christensen and associates (1997) with slight modification. Seven-day-old first leaves were inoculated on the adaxial surface. At specific timepoints, inoculated leaves were fixed and cleared in 0.15% trichloroacetic acid (wt/vol) in ethyl alcohol/chloroform (4:1; vol/vol) (Hückelhoven and Kogel 1998), then shook in 1% SDS at 80°C for 24 h to remove unbound proteins. Subsequently, leaves were stained at 90°C for 30 min in 0.1% Coomassie brilliant blue (in 40% ethanol and 10% acetic acid) and were destained in a solution of 40% ethanol and 10% acetic acid at 90°C for 10 min. The samples were examined by light microscopy.

Determination of SA.

Total SA was extracted and quantified following the protocol described by Hückelhoven and associates (1999). First-leaf tissue samples were ground to a fine powder in liquid nitrogen, and 0.4 g of leaf powder (fresh weight) each in three replicates from the pool was used for sample preparation. SA was determined by fluorescence (excitation 298 nm, emission 400 nm) after separation on a C18 reverse phase HPLC (high-pressure liquid chromatography) column (LiChroCart 250-4, LiChrosphere C18, 5 µm; Merck, Darmstadt, Germany) using a trichloroacetic acid and methanol gradient. The SA content in the sample extracts was corrected for the recovery of internal standard (O-methoxy benzoic acid) (Meuwly and Métraux 1993).

RNA extraction and gene expression analyses.

Total RNA was extracted from first leaves using an RNA extraction buffer, peqGold RNAPure, according to the manufacturer's instructions (Peqlab Biotechnology GmbH, Erlangen, Germany). RNA concentrations were determined spectrophotometrically. For Northern analysis, 10 µg of total RNA from each sample of green leaves and 5 µg from white leaves were fractionated by electrophoresis on denaturing agarose gels. Ethidium bromide staining revealed similar amounts of total RNA in samples from green and white leaves, as judged by the intensities of the bands representing 25S and 18S rRNAs (Zubko and Day 2002). 23S and 16S rRNA bands were not detectable in the white leaves, as they are deficient in plastid

ribosomal RNAs. These gels were blotted by capillary transfer to positively charged nylon membranes. The RNA was cross-linked under UV light. Membranes were hybridized under stringent conditions in Dig Easy Hyb buffer (Roche, Mannheim, Germany) with digoxigenin or fluorescein labeled probes. Immunodetection of transcript with chemiluminescence was performed after two stringent washes for 20 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS according to manufacturer's protocols for anti-digoxigenin- or anti-fluorescein-AP, Fab fragments, and CDP-Star (Roche, Mannheim, Germany). Plasmids containing cDNAs from *PR1b* (Bryngelsson et al. 1994), *PAL* (GenBank accession number AJ427587; K.-H. Kogel, unpublished data), *14-3-3* (GenBank accession number P29305; Brandt et al. 1992), and *OXLP*, *BI-1*, and *Aspro1* (Hückelhoven et al. 2001) were used for generating antisense digoxigenin-labeled RNA probes for Northern analysis.

RT-PCR was performed to analyze the expression of *MLO*, *APX*, *pNAox*, and *GDI*. Based on the EST sequence AJ000240 (Hess et al. 1998), a full-length cDNA clone for a barley *GDI* was isolated and sequenced during the course of this study (GenBank accession number AY346010). First leaves from white and green leaves at 0, 3, 6, 12, 24, and 48 h from mock-inoculated and inoculated plants were harvested, and total RNA was extracted. The RNA was reverse transcribed in a 20-µl reaction using the SuperScript cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). Equal amounts of RNA from green and white leaves (4 and 2 µg, respectively, resulted in equal amounts of mRNA, as judged by ethidium bromide staining) were used to synthesize first strand cDNA by using SuperScript II reverse transcriptase and oligo(dT) in the presence of ribonuclease inhibitor. After annealing at 42°C, 200 units of Superscript II reverse transcriptase was added, and the reaction was left for 50 min at 42°C, followed by inactivation of the enzyme at 70°C for 15 min. Expression levels of these genes was determined using 1 µl of cDNA from each sample for PCR amplification with the gene-specific primers in 25 µl of PCR reaction. For putative barley *pNAox* and for barley *APX*, primers described in Hückelhoven and associates (2001) were used. Primer pairs 5'-CAGCTGCACGCTCTTCATCTT-3' and 5'-AAATCTGCACTGGGGATGTC-3' for the 1,200-bp-long *Mlo* fragment (Büschges et al. 1997) and 5'-CACCCCCTGC AAATATAGGA-3' and 5'-AATTCGACCTTGCACTCTGG-3' to obtain the 601-bp fragment of *GDI* (Hess et al. 1998) were used. Barley ubiquitin (GenBank accession number M60175) was used as a control for constitutive gene expression, primers for which were 5'-CCAAGATGCAGATCTTCGTGA-3' (5' primer) and 5'-TTCGCGATAGGTAAAAGAGCA-3' (3' primer) for a 513-bp fragment.

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