***RAR1*, *ROR1*, and the Actin Cytoskeleton Contribute to Basal Resistance to *Magnaporthe grisea* in Barley**

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The fungus *Magnaporthe grisea*, the causal agent of rice blast disease, is a major pathogen of rice and is capable of producing epidemics on other cultivated cereals, including barley (*Hordeum vulgare*). We explored the requirements for basal resistance of barley against a compatible *M. grisea* isolate using both genetic and chemical approaches. Mutants of the *RAR1* gene required for the function of major resistance gene-mediated resistance and mutants of the *ROR1* and *ROR2* genes required for full expression of cell-wall-penetration resistance against powdery mildew pathogens were examined for macroscopic and microscopic alterations in *M. grisea* growth and symptoms. *RAR1* contributed to resistance in epidermis and mesophyll at different stages of fungal infection dependent on the *MLO/mlo-5* status. Whereas no *ROR2* effect was detected, *ROR1* was found to contribute to cell-wall-penetration resistance, at least in the epidermis. Application of the actin agonist cytochalasin E promoted cell wall penetration by *M. grisea* in a dose-dependent manner, demonstrating an involvement of the actin cytoskeleton in penetration resistance.

Barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) is an agronomically important pathogen that has been used for the study of resistance in both barley (a host) and *Arabidopsis* (a nonhost). Cytological and genetic studies have revealed at least two fundamentally different types of powdery mildew resistance (Collins et al. 2003; Freialdenhoven et al. 1996; Peterhänsel et al. 1997). The first prevents penetration of the epidermal cell wall by the fungus, is not associated with death of the attacked host cell, and is generally broad-spectrum in effect. The second type is known to be triggered upon specific recognition between a cognate resistance (*R*) gene and an avirulence (*Avr*) gene in the host and pathogen, respectively, and is associated with suicide of the attacked host cell. The barley *MLO* gene acts as a negative regulator of penetration resistance, such that *mlo* mutants express highly effective broad-spectrum powdery mildew penetration resistance (Jørgensen 1992). Mutant searches identified the *ROR1* and *ROR2* genes required for full expression of *mlo* resistance (Freialdenhoven et al. 1996). *ROR1* and *ROR2* also contribute to the low level of ‘basal’ barley powdery mildew penetration resistance expressed in ‘susceptible’ (*MLO*) genotypes (Collins et al. 2003). These three genes influence penetration resistance being a

component of nonhost resistance of barley against the incompatible pathogen wheat powdery mildew (*B. graminis* f. sp. *tritici*) (Peterhänsel et al. 1997) and the *Arabidopsis* homologue of *ROR2* (*PEN1*) contributes to nonhost penetration resistance of *Arabidopsis* against barley powdery mildew (Collins et al. 2003). Similarly, a mutant search identified the *RAR1* gene, which is required for the action of a subset of barley powdery mildew *R* genes (Jørgensen 1988). *RAR1* homologues in *Arabidopsis* and tobacco were subsequently found to be required by particular *R* genes effective against bacterial, oomycete, and viral pathogens (Liu et al. 2002; Muskett et al. 2002; Tornero et al. 2002). Nongenetic approaches have highlighted the importance of the cytoskeleton in powdery mildew penetration resistance. Microtubules and actin microfilaments become spatially reorganized around sites of attempted penetration, and application of the actin and tubulin inhibitors allows cell wall penetration by powdery mildews in host-pathogen combinations that otherwise show essentially no penetration (Kobayashi et al. 1997a and b; Yun et al. 2003).

Another filamentous ascomycete cereal pathogen is *Magnaporthe grisea* (Herbert) Barr (anamorph *Pyricularia grisea* [Cooke] Sacc.), the causal agent of blast disease. *M. grisea* is a major pathogen of rice and can cause disease on other cultivated grasses (Ou 1980). Blast has been reported to produce epidemics on barley in some parts of Asia where barley grows close to rice (Mackill and Bonman 1986; Sato et al. 2001). While many *M. grisea* *R* genes of major effect have been characterized in rice, only one such gene has been reported in barley (*PHR-1*) (Yaegashi 1988), in addition, a number of barley quantified trait loci (QTL) for *M. grisea* resistance have also been identified (Chen et al. 2003; Sato et al. 2001). *M. grisea* uses considerable physical force to penetrate the cell walls of host epidermal cells (Howard et al. 1991), and compatible infections induce host cell death around the time the fungus emerges from the first invaded epidermal cell to begin colonization of neighboring epidermal and mesophyll tissue (Peng and Shishiyama 1988; Talbot 1995). Compatible infections of the obligate biotroph powdery mildew induce no host cell death and enzymatic digestion most likely assists in cell wall penetration (Pryce-Jones et al. 1999). Powdery mildew forms feeding structures (haustoria) within epidermal cells, but growth of the fungus otherwise occurs completely on the leaf surface (Kita et al. 1980). The contrasting lifestyle of powdery mildews and *M. grisea* make them potentially complementary pathogens in studies to characterize the extent of overlap between mechanisms of resistance against different fungi. Indeed, *mlo* mutations promote *M. grisea* infection of barley and therefore, intriguingly, have an effect on this pathogen that is essentially opposite to that observed for powdery mildews (Jarosch et al. 1999).

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A *ror1* mutation has been shown to confer increased sensitivity to necrosis-inducing toxin from *Bipolaris sorokiniana* (Kumar et al. 2001), whereas the combined mutations of *mlo* and *ror1* had no influence on macroscopic symptoms of a *M. grisea* infection (Jarosch et al. 1999). But, to our knowledge, there have been no reported attempts to test whether *ROR1* or *ROR2* affect resistance to any pathogens other than powdery mildews with a broad genetic approach. In addition, a role of *RAR1* or the cytoskeleton in basal resistance in any compatible host-pathogen combination has not been established. The aims

of this study were, therefore, to investigate whether the genes *ROR1*, *ROR2*, and *RAR1* or the actin cytoskeleton influence basal resistance to a *M. grisea* isolate compatible on barley, using mutants and the actin depolymerizing factor cytochalasin E (Cooper 1987; Mathur et al. 1999). We also examined for possible effects of *ROR2* in interactions with an incompatible *M. grisea* isolate. Importantly, we demonstrate a role for *RAR1* in basal resistance and for *ROR1* in conferring resistance against a pathogen other than powdery mildew.

RESULTS

Macroscopic lesion development.

Effects of *ROR1*, *ROR2*, *RAR1*, and *MLO* genes on *M. grisea* infection were tested, using barley lines differing for mutations in these genes. Each line was homozygous for the mutant or wild-type allele at each locus and had been derived by self-pollination of an individual that was marker-selected from a segregating family. Tests were performed using *M. grisea* race 031, which originally derived from rice plants but which can grow and sporulate on barley (Jarosch et al. 1999, 2003). Primary leaves of 7-day-old seedlings were inoculated and, five days later, the inoculated leaves were assessed for the degree of macroscopically visible disease lesion development. The frequency with which spores can successfully establish a growing colony and the size of lesions that arise from established colonies can reflect independent components of the compatibility or resistance of *M. grisea* on different host genotypes (Heath et al. 1990). Accordingly, lesion development on the barley genotypes was assessed in two ways. First, the number of colonies that became visible on the inoculated leaves was recorded. Second, the inoculated leaves from the same experiment were also subjectively assessed for the amount of leaf area covered by lesions, which is a function of both lesion number and lesion size.

The lesion number measurements are presented in Figure 1. Consistent with previous findings (Jarosch et al. 1999), mutant *mlo-5* lines displayed six to ten times more lesions per leaf than did wild-type *MLO* lines (Fig. 1A and B). Therefore, the influence of mutations in *RAR1* and *ROR1* on lesion density was assessed independently for the *MLO* and the *mlo-5* background. Whereas, in the *MLO* background, a mutation in *RAR1* caused only a slight increase in lesion number, the effect in the *mlo-5* line was much more pronounced. Contrariwise, a mutation in *ROR1* displayed a small effect on lesion number in the *mlo-5* background and a significant increase in *MLO* plants. Combining *rar1-2* and *ror1-2* mutations had an approximately additive effect on lesion number only in the *MLO* background, whereas in the *mlo-5* plants the effect of a *rar1-2/ror1-2* double mutation did not differ from that caused by a single mutation in *RAR1*. Possible influence of the *ror2-1* mutation on lesion number was tested in the *MLO* background only, but no such effects were observed (Fig. 1C). These data are fully consistent with the previous study (Jarosch et al. 1999), in which a mutation in *ROR1* was found to have no significant effect on *M. grisea* macroscopic disease symptom development in *mlo-5*-containing genotypes.

Lines containing wild-type *MLO* and the mutant *mlo-5* allele were assessed in separate experiments for leaf area covered by *M. grisea* lesions (Fig. 2A and B, respectively). Within each experiment, several lines of a given genotype were grouped into classes according to the infected area. Due to the greater susceptibility of *mlo-5* plants, the least affected class in the *mlo-5* background was more susceptible than the most affected one in the *MLO* background. In general, influences of *ROR1* and *RAR1* on leaf coverage resembled those revealed by lesion number (Fig. 1), as a mutation in *ROR1* had the most obvious

Fig. 1. Numbers of macroscopic *Magnaporthe grisea* lesions on barley leaves. Primary leaves of 7-day old barley seedlings of various genotypes were inoculated with spores of the compatible *M. grisea* isolate 031. After five days, the number of lesions per leaf was counted by eye. Lines were wild-type for defense genes unless indicated otherwise. **A**, Lines contained either wild-type or mutant alleles in *ROR1* and *RAR1* in a wild-type *MLO* background. **B**, Lines contained either wild-type or mutant alleles in *ROR1* and *RAR1* in a mutant *mlo-5* background. **C**, Lines contained either wild-type or mutant alleles for *ROR2* in a wild-type *MLO* background. Data for A, B, and C are representative of results obtained in four, five, and three independent replicate experiments, respectively, and one replicate was shown for each data set. The mean and standard errors represent data from 15 to 20 individuals per genotype scored from different lines.

effect in the *MLO* background whereas a mutation in *RAR1* was most effective in the *mlo-5* background. An exception was the *rar1-2* mutation exerting more influence on the area covered with lesions (Fig. 2A and B) than on lesion number (Fig. 1). This effect was most clearly manifested in the *mlo-5* background in which *rar1-2* genotypes are more frequently represented in the most susceptible classes than in the other ones. These observations indicate that wild-type *RAR1* acts on limiting colony size as well as number.

Microscopic evaluation of *M. grisea*–barley interaction sites.

To obtain further insights into the basis of the *ROR1*, *RAR1*, and *MLO* gene effects, we microscopically examined interactions of *M. grisea* isolate 031 on barley genotypes that differed for mutations in these three genes. Sites were examined both by bright-field and fluorescence microscopy, the latter being used to detect autofluorescence of host cells undergoing defense or compatibility responses. The appearance of fully compatible *M. grisea* infections resembled that which had been previously described for barley and other host plant species (Heath et al. 1990; Jarosch et al. 1999). Upon contacting the leaf surface, *M. grisea* conidiospores produce an appressorium to facilitate penetration into the epidermal cell. Fungal hyphae ramify in the penetrated epidermal cell, and hyphae exit the penetrated cell to invade neighboring tissue by growing intercellularly or by penetrating further epidermal and mesophyll cells. Spread of the fungus into the mesophyll is accompanied successively by cellular disorganization, cell collapse, and finally, occurrence of a strong autofluorescence, all of which are reminiscent of a hypersensitive response (HR). However, in case of the barley-*M. grisea* interaction, either the timing, the qualitative composition of this HR-like cell death in the mesophyll, or both is not capable of arresting fungal invasion. Contrariwise, arrest of the fungus within penetrated epidermal cells was correlated with autofluorescence in the epidermal cell, as previously shown for rice (Heath et al. 1990). Individual interaction sites were assigned to one of four classes, pictured in Figure 3. In order of increasing compatibility, these were i) no hyphae or autofluorescence visible even in the first challenged epidermal host cell, associated with a papilla visible opposite the attempted penetration site, ii) hyphae and autofluorescence visible in the initially penetrated epidermal cell only, iii) hyphae and autofluorescence in a few mesophyll cells, and iv) hyphae and autofluorescence in large patches of mesophyll tissue.

Observations with isolate 031 at 72 h after inoculation are summarized in Figure 4. Consistent with a previous report (Jarosch et al. 1999), mutation in *MLO* promoted *M. grisea* infection on barley at multiple stages of fungal development. In lines carrying the *mlo-5* mutation, fungal growth proceeded more frequently beyond the first attacked epidermal cell. Loss of wild-type *MLO* increased both the apparent frequency of successful initial epidermal cell wall penetration and the ability of hyphae to spread from the epidermal cell into adjacent mesophyll tissue. Because spread of the fungus from the initially challenged epidermal cell would be a prerequisite for formation of lesions visible to the naked eye, the ability of the *mlo-5* mutation to promote successful mesophyll invasion is consistent with the increased number of macroscopic lesions that develop on *mlo-5* lines (Fig. 1A and B). Patches of autofluorescing mesophyll cells were classified into two groups based on the extent of autofluorescence, enabling a more detailed characterization than in the previous study by Jarosch and associates (1999). Compared with the *MLO* lines, the mutant *mlo-5* lines showed a greater proportion of large versus small patches of mesophyll autofluorescence, indicating that

the *mlo-5* mutation also enabled greater expansion of those individual colonies that had successfully entered the mesophyll (Fig. 4).

In the *mlo-5* background, the *rar1-2* mutation was correlated with a modest increase in the frequency of mesophyll invasion, particularly accompanied by large patches of autofluorescence at 72 h after inoculation (Fig. 4). This observation is consistent with the ability of the *rar1-2* mutation to increase the leaf area covered by *M. grisea* lesions (Fig. 2B). In contrast, a mutation in *ror1-2* has no substantial effect on the frequency of cellular interaction phenotypes in the mesophyll compared with *mlo-5ROR1RAR1* plants (Fig. 4), and this is consistent with the absence of a clear impact of *ror1-2* on number and size of macroscopically visible *M. grisea* lesions (Fig. 1B and 2B). Plants of the *mlo-5* genotype combining a mutation in both *RAR1* and *ROR1* displayed the highest frequency of large patches of HR-like cell death in the mesophyll. This observation is consistent with the grouping of this genotype into the most susceptible classes (Fig. 2B). Similarly, the highest number of macroscopically visible lesions was found on this particular genotype (Fig. 1B).

In contrast to the situation observed in *mlo-5* plants, the *MLO* genotypes showed only slight variation in the frequency of mesophyll invasion, which was not correlated with the *rar1-2* or the *ror1-2* mutations (Fig. 4). However, in the *MLO* background, a mutation in *RAR1* clearly increased the proportion of

Fig. 2. Leaf coverage by *Magnaporthe grisea* lesions. Inoculated leaves were assessed for the degree of coverage by *M. grisea* lesions five days after inoculation, taking into account lesion size as well as number. In each experimental replicate, several lines of a given genotype were grouped into classes of susceptibility, depending on the analysis of 20 to 30 leaves. Due to hypersusceptibility of *mlo-5* genotypes, classification was relative only within groups of plants with the same A, *MLO* and B, *mlo-5* status, e.g. plants in the most susceptible class in B were covered with more lesions than those in A. Data in A and B were obtained by summarizing four and five experiments, respectively.

sites showing papillae and decreased the frequency of hyphae and autofluorescence limited to penetrated epidermal cells. Mutations in *ROR1* had a reverse influence, namely a decrease in papilla formation and an increase in autofluorescing epidermal cells (Fig. 4). In plants carrying a mutation in both *RAR1* and *ROR1*, these oppositional effects were additive, reminiscent of the situation in wild-type plants. In addition, a slight increase in the frequency of large patches of mesophyll HR-like cell death was found on these double mutants.

Obviously the status of the *MLO* allele influenced the impact of the *rar1-2* as well as the *ror1-2* mutation on the frequency

of cellular interaction phenotypes (Fig. 4). Whereas a *rar1-2* mutation enhanced penetration resistance in the *MLO* background, in *mlo-5* plants, it promoted spreading in the mesophyll, as evidenced by the increased proportions of large relative to small patches of mesophyll cell autofluorescence. In contrast, the *ror1-2* mutation lowered penetration resistance in the presence of a functional *MLO* protein. This effect could not be observed in *mlo-5* genotypes. Interestingly, in each of the *MLO* and *mlo-5* sets, the greatest proportion of sites with large autofluorescing mesophyll patches were encountered for those lines containing mutations in both *RAR1* and *ROR1* (Fig. 4).

Fig. 3. Different types of microscopically observable outcomes of interactions between *Magnaporthe grisea* (isolate 031) and barley (cvs. Ingrid and BCIngridmlo5) 72 h after inoculation. **A, C, E, and G,** Four interaction sites viewed by bright-field microscopy. **B, D, F, and H,** The same four sites viewed by fluorescence microscopy. **A and B,** The penetration attempt is effectively stopped by a papilla under the fungal appressorium. **C and D,** Successful penetration into the first epidermal cell, which showed hypersensitive response (HR)-like cell death in response to infection. **E and F,** Secondary hyphae started to branch and penetrate neighboring epidermal cells. Adjacent mesophyll cells showed strong autofluorescence. **G and H,** Secondary hyphae spread intensely into surrounding tissue. Several mesophyll cells responded with HR-like cell death. For quantitative microscopic evaluation, interaction sites shown in A and B were categorized as class “PAP”, in C and D, as “SHY/HREPI”, in E and F, as “SHY/HRMESO,” and those in G and H as “SHY/HRMESO+”. APP = appressorium; CO = conidia; N = nucleus; PAP = papilla; SHY = secondary hyphae; HREPI = HR-like cell death in epidermal cell; and HRMESO = HR-like cell death in mesophyll cell. Scale bar = 40 μ m.

Our macroscopic analysis revealed no effect of the *ror2-1* mutation on the barley-compatible *M. grisea* isolate 031 (Fig. 1C). While the *ROR2* gene contributes to basal penetration resistance against compatible barley powdery mildew isolates, *ROR2* and a close *Arabidopsis* homologue (*PEN1*) had also been shown to contribute to nonhost resistance of barley against wheat powdery mildew and of *Arabidopsis* against barley powdery mildew, respectively (Collins et al. 2003; Peterhansel et al. 1997). Consequently, to look at potential effects of *ROR2* by microscopic analysis, we chose an isolate of *M. grisea* that is incompatible on barley. The isolate CD86 had been collected from *Pennisetum thyphoides* specimens growing in the Ivory Coast and produced no visible symptoms following test inoculations of five wild-type *MLO* barley cultivars (including cv. Ingrid and Sultan5) (data not shown). The results of the microscopic investigation are summarized in Figure 5.

M. grisea isolate CD86 was able to frequently penetrate barley epidermal cells to form intracellular hyphae in all genotypes tested (65 to 75% of sites) (Fig. 5). However, growth very seldom proceeded into the mesophyll (1 to 3% of sites) (Fig. 5) and never resulted in macroscopic lesions, consistent with the finding that CD86 failed to reproduce on barley. As with the compatible isolate 031, the *mlo-5* mutation resulted in an apparent decrease in the frequency of effective papillae that arrest fungal penetration and a concomitant increase in the frequency of sites showing autofluorescence and hyphae limited to the initial epidermal cell (Fig. 5). However, the *ror2-1* mutation had no discernable effect on any stage of attempted infection of barley by CD86, neither in the *MLO* nor in the *mlo-5* background (Fig. 5).

Cytochalasin E treatment promotes cell wall penetration by *M. grisea*.

To test the role of papillae in the resistance of barley to *M. grisea*, two concentrations (5 and 10 $\mu\text{g ml}^{-1}$) of the microfilament depolymerization factor cytochalasin E were infiltrated into intercellular spaces of *MLO* and *mlo-5* barley leaves, respectively, 3 to 4 h prior to inoculation with *M. grisea* race 031. Since it was previously shown (Jarosch et al. 2003) that

acquired resistance of barley against *M. grisea* is due to enhanced levels of papilla formation, the influence of cytochalasin E was also tested on plants treated with the resistance-inducing chemical DCINA (2,6-dichloro-isonicotinic acid). Although floating leaves on a solution was used as method for application of cytochalasin E in previous studies (Kobayashi et

Fig. 5. Microscopic analysis of interactions of barley with an incompatible *Magnaporthe grisea* isolate. Barley plants containing different combinations of wild-type or mutant alleles for the *MLO* and *ROR2* genes were analyzed for microscopic interaction with the incompatible *M. grisea* isolate CD86 72 h after inoculation. Individual sites were classified into one of four classes: PAP, papilla; SHY/HREPI, secondary hyphae together with HR-like cell death in an epidermal cell; SHY/HRMESO, secondary hyphae and HR-like cell death in a few mesophyll cells; SHY/HRMESO+, secondary hyphae and with HR-like cell death in large mesophyll patches. One representative result is shown from two replicates with a minimum of five leaves analyzed per genotype with 150 interaction sites per leaf. BCImlo5 is the *mlo-5* backcross line of cv. Ingrid; A44, the *ror2-1* mutant of BCImlo5; and siblings of the cross A44 with cv. Sultan5.

Fig. 4. Microscopic analysis of interactions of barley with a compatible *Magnaporthe grisea* isolate. Barley plants containing different combinations of wild-type or mutant alleles for the *MLO*, *RAR1*, and *ROR1* genes were analyzed for microscopic interactions with the compatible *M. grisea* isolate 031 at 72 h after inoculation. Individual sites were classified into one of four classes: PAP, papilla; SHY/HREPI, secondary hyphae together with HR-like cell death in an epidermal cell; SHY/HRMESO, secondary hyphae and HR-like cell death in a few mesophyll cells; SHY/HRMESO+, secondary hyphae and with HR-like cell death in large mesophyll patches. One representative result from two replicates is shown, with bars representing means and standard errors from five leaves analyzed per genotype with 150 interaction sites per leaf.

al. 1997b), the injection method was found to be effective as well, as verified by the ability of cytochalasin E to break the strong barley powdery mildew resistance expressed in *mlo-5* lines (data not shown). The results of the microscopic analyses are shown in Figure 6.

Consistent with a previous study of *M. grisea* on barley (Jarosch et al. 2003), DCINA treatment both increased the frequency of papillae that cannot be penetrated and decreased the extent to which the fungus spread within the mesophyll (Fig. 6). Apparently, the application of cytochalasin E lowers the frequency of effective papillae in a dose-dependent manner (up to about 10% of sites) in both the *MLO* and the *mlo-5* background, except in *MLO* leaves without DCINA treatment (Fig. 6).

DISCUSSION

Previous reports described the requirement of *RAR1* for strong resistance types, which depends on the recognition of incompatible pathogen isolates by single *R* genes (Shirasu et al. 1999). However, *RAR1* did not affect the growth of virulent bacterial isolates in *Arabidopsis* (Muskett et al. 2002). The finding that *RAR1* contributes to resistance against *M. grisea* extends the known range of influences of *RAR1* in plant defense. Similarly, we show that the *ROR1* gene influences resistance to a pathogen other than a powdery mildew. The abilities of *rar1-2* and *ror1-2* mutations to enhance *M. grisea* symptoms were tested in experiments involving marker-selected lines deriving from a cross between A89 (*mlo5rar1-2RAR1* background Ingrid) and M100 (*MLOROR1rar1-2* background Sultan5). To assure that the effects observed were not due to natural *ROR1*- and *RAR1*-linked QTL-dependent resistance that might segregate in the parental lines, we compared infection phenotypes on cultivars Ingrid and Sultan5 and the *MLOROR1RAR1* progeny from the A89 × M100 cross (data not shown).

Microscopic analysis gave more insight into the basis of *RAR1* and *ROR1* effects. The *MLO* gene strongly promoted resistance in both the epidermis and the mesophyll, which is not the case for *mlo-5* plants. Thus, the alternating effect of the

two *MLO* alleles highlighted influences of *RAR1* and *ROR1* on different stages of colonization. In lines containing wild-type *MLO* fungal growth was slower or was halted more frequently within the epidermis, enabling analysis of *RAR1* and *ROR1* effects on resistance in this tissue. In the *mlo-5* background, fungal growth was faster or more frequently overcame resistance barriers in the epidermis, allowing *RAR1* and *ROR1* effects on growth into and within the mesophyll to be more readily observed.

In compatible infections by powdery mildew, cell wall penetration takes place only at the surface of the epidermis, and basal resistance to this penetration has a partial requirement for *ROR1*. Here, we reveal that the as-yet-uncloned *ROR1* gene also limits entry of *M. grisea* into barley epidermal cells, most evident in *MLO* genotypes, as the *ror1-2* mutation was correlated with a significant increase in lesion number and a reduced formation of effective papillae. The combination with the *rar1-2* mutation appeared to result in a slightly increased spread within the mesophyll, pointing to the possibility that *ROR1* may also have a direct effect in limiting subsequent penetrations or intracellular growth stages.

In the *mlo-5* background, loss of *RAR1* promoted disease by significantly increasing lesion size and number, correlating with enhanced progression of *M. grisea* out of the epidermis and spread within the mesophyll. In contrast, an influence of a mutation in *ROR1* on penetration resistance could not be observed in the *mlo-5* background, possibly due to the lack of a functional *MLO* protein being already sufficient to lower penetration resistance. This, in turn, might be responsible for the most extensive hyphal growth in the mesophyll and the severe disease phenotype found on *mlo-5* genotypes with a concomitant mutation of both *ROR1* and *RAR1*.

The dependence of *RAR1* in both *R* gene-mediated resistance and basal defense hints at a possible overlap between the two types of resistance. This overlap has also been suggested by the requirement of the *Arabidopsis* gene *EDS1* in *R* gene-mediated resistance and basal defense (Parker et al. 1996). Since different *R* genes from the barley *MLA* locus allow dif-

Fig. 6. Effects of the actin agonist cytochalasin E on interactions between barley and a compatible *Magnaporthe grisea* isolate. Lines containing wild-type *MLO* or the mutant *mlo-5* allele in the cv. Ingrid background were treated with the chemical inducer of resistance 2,6-dichloro-isonicotinic acid (DCINA) and the appropriate control solution (WP = wetttable powder) by soil drench, three days prior to inoculation with the compatible *M. grisea* isolate 031. Cytochalasin E (Cyt. E) was applied at concentrations of 0, 5, and 10 µg ml⁻¹ by infiltration into intercellular spaces, 3 to 4 h prior to inoculation. Individual sites were classified into one of four classes 72 h after inoculation: PAP, papilla; SHY/HREPI, secondary hyphae together with HR-like cell death in an epidermal cell; SHY/HRMESO, secondary hyphae and HR-like cell death in a few mesophyll cells; SHY/HRMESO+, secondary hyphae and with HR-like cell death in large mesophyll patches. Means and standard errors derived from three independent experiments with a minimum of 11 leaf segments analyzed per treatment and 150 interaction sites per segment.

ferent amounts of fungal establishment before growth is halted (Boyd et al. 1995), it is possible that basal resistance of barley to *M. grisea* may be conferred by one or more *RAR1*-dependent *R* genes of relatively weak or late effect.

Successful *M. grisea* colonization of barley was accompanied by autofluorescence and HR-like cell death in the mesophyll. This host response may favor the development of fungi like *M. grisea* that have a necrotrophic habit at later stages of infection. In contrast, in interactions of *M. grisea* with host species other than barley, the frequency of autofluorescence in penetrated epidermal cells is negatively correlated with compatibility (Heath et al. 1990). Indeed, lignification of the first penetrated epidermal cell, as monitored by the occurrence of autofluorescence, was shown to be the crucial defense response that accompanies *R* gene-triggered resistance of rice to the blast pathogen (Koga 1994). However, in the barley-*M. grisea* interaction, timecourse studies revealed that HR-like cell death in epidermal cells does not arrest fungal growth (data not shown). Thus, if cell death response has any influence on fungal development, it might be a slowdown of hyphal spread into the mesophyll.

Microscopy in the *MLO* background showed that the *rar1-2* mutation increased the proportion of sites with effective papillae in the epidermis and decreased the ones with hyphae and autofluorescence in the epidermis. One interpretation for this finding is that the *RAR1* gene may negatively regulate resistance to the initial penetration by this fungus. However, *RAR1* does not quantitatively influence basal or penetration resistance to powdery mildew, at least in the *mlo-5* genotype (Freialdenhoven et al. 1996; Peterhansel et al. 1997). In contrast to the results obtained in the *MLO* background, the *rar1-2* mutation in *mlo-5* plants allowed more frequent progression of the fungus into the mesophyll. Regarding these differences, an alternative hypothesis is that the presence of a functional *MLO* protein modulates the effect of *RAR1* on defense responses.

In contrast to the effects of *ROR1*, mutants of *ROR2* showed unaltered interactions with *M. grisea*. The solitary *ror2-1* mutant allele in barley encodes a stable protein containing an internal deletion of 31 amino acids that enables 10% or less penetration of powdery mildew on *mlo* barley, whereas a knockout allele of the *Arabidopsis* *ROR2* homologue (*PEN1*) increased barley powdery mildew penetration of *Arabidopsis* by around 60% (Collins et al. 2003; Freialdenhoven et al. 1996). Although the relative contributions of *PEN1/ROR2* in the two host-pathogen systems may be different, this comparison raises the possibility that the *ror2-1* allele retains partial function and that, for this reason, minor effects on *M. grisea* could not be detected.

A requirement for the actin cytoskeleton in fungal penetration resistance on nonhost species has been well established with the use of chemicals such as the actin depolymerization factor cytochalasin E (Kobayashi et al. 1997a and b; Yun et al. 2003). Remodeling of the cytoskeleton is involved in targeted vesicle exocytosis in plant cells (Foissner et al. 1996) and may, therefore, be required to direct defense compounds to interaction sites. This study showed a small but dose-dependent effect of cytochalasin E as penetration of barley epidermal cells by a compatible isolate of *M. grisea* was increased. This effect is reminiscent of those observed in *ror1-2* mutants and may hint at the *ROR1* mode of action. The finding of a role of the actin cytoskeleton in basal penetration resistance further reinforces the notion that basal and nonhost penetration resistance against fungi has a common mechanistic basis (Collins et al. 2003). The absence of a clear reduction of papilla formation by cytochalasin E in the non-DCINA-treated *MLO* genotype may hint at qualitative differences in the composition or formation of papillae.

MATERIALS AND METHODS

Barley genotypes.

Barley (*Hordeum vulgare* L.) genotypes were selected from segregating families, using polymerase chain reaction-based markers, assayed using methods described (Collins et al. 2001). Markers were based on the *mlo-5* mutation (Peterhansel et al. 1997), the *ror2-1* mutation (GenBank accession AY246906), MWG913 located 1.1 cM from *ROR1* (Collins et al. 2001), and OK1114 located less than 0.1 cM from *RAR1* (Shirasu et al. 1999).

Lines containing combinations of mutations in *MLO*, *ROR1*, and *RAR1* were derived from a cross between the *rar1-2* mutant line M100 (Shirasu et al. 1999) (*MLO*, *ROR1*, *rar1-2*, background Sultan5) and the *ror1-2* mutant line A89 (Freialdenhoven et al. 1996) (*mlo-5*, *ror1-2*, *RAR1*, background Ingrid). Two F₃ families segregating for *ROR1* and *RAR1*—one homozygous *mlo-5* and the other homozygous wild-type *MLO*—were identified, and F₃ individuals were analyzed to identify individuals homozygous for different allele combinations at the *ROR1* and *RAR1* loci. The F₃ plants were allowed to self-pollinate to generate seed for use in pathogen testing.

The *MLO**ROR2* and *MLO**ror2-1* lines derived from a cross between Sultan5 (*MLO**ROR2*) and the *ror2-1* mutant line A44 (Freialdenhoven et al. 1996) (*mlo-5*, *ror2-1*, background Ingrid). Plants homozygous for the different allele combinations were selected in the F₂ generation, and seed was obtained by self-pollination.

Seeds of other barley lines were provided by P. Schulze-Lefert (Max Planck Institute for Plant Breeding Research, Cologne, Germany).

Fungi and inoculations.

The *M. grisea* race 031 (TH6772) was provided by the Institute of Biochemistry, Faculty of Agriculture, Tamagawa University, Machida-shi, Tokyo, Japan. The isolate CD86, collected in the Ivory Coast from *Pennisetum thyphoides*, was received from D. Tharreau (CIRAD, Montpellier, France). Both fungi were maintained alternately on potato dextrose agar and rice leaf agar (water extract of 50 g of rice leaves, 2 g of Faex medicinales per liter [yeast extract; Biolabor, Bremen, Germany], 10 g of water-soluble starch per liter, 15 g of agar-agar per liter) at 27°C under a 16-h day and 8-h night regime using black light (310 to 360 nm) for two weeks. Conidia were harvested by rinsing with distilled water and filtering through three layers of gauze. This suspension was diluted 1:1 (vol/vol) with the spraying solution (0.1 % [wt/vol] gelatin, 0.05 % [wt/vol] sodium oleate), and the concentration was adjusted to 2 × 10⁵ conidia per milliliter. Seedlings were grown in a growth chamber at 16 to 18°C, 50 to 60% relative humidity, and a 16-h photo period and were inoculated by spraying primary leaves seven days after sowing. After incubation for 24 h in a moist chamber in the dark (26°C, up to 100% relative humidity), plants were kept at high humidity under the same temperature and light regime described above.

Macro- and microscopic evaluation of interaction sites.

Disease symptoms on leaves were macroscopically evaluated six to seven days after inoculation with *M. grisea* and barley powdery mildew, respectively. For microscopy, leaves were harvested at the timepoints indicated by placing them in a clearing solution (0.15% [wt/vol] trichloroacetic acid in 4:1 [vol/vol] ethanol/chloroform) for 48 h. Leaf segments were subsequently stored in 50% (vol/vol) glycerol. Fungal structures were observed by bright-field microscopy. Host cell autofluorescence was observed by fluorescence microscopy (excitation filter 485 nm, dichroic mirror 510 nm, barrier filter 520 nm; TCS

SP; Leica, Bensheim, Germany). Images were taken using the digital camera JVC KYF 750. A total of 100 to 200 infection sites were inspected per 5-cm leaf segment. Three to five seedlings were analyzed for each data point. Because defense reactions of short and long epidermal cells against *Magnaporthe grisea* are nearly identical (Jarosch et al. 1999), no distinction was made in their evaluation.

Treatment with DCINA and cytochalasin E.

DCINA (CGA41396; Novartis, Basel, Switzerland), formulated as 25% (wt/wt) active ingredient with a wettable powder (WP) carrier, was dissolved in water to a final concentration of 20 ppm of active ingredient. The appropriate amount of WP was dissolved in water for the control treatment. The solutions (10 ml) were applied to 4-day-old barley seedlings by soil drench and three days prior to infiltration of cytochalasin E and to inoculation.

Cytochalasin E was diluted to concentrations of 5 and 10 µg/ml in 10 mM calcium chloride, respectively. Solutions were infiltrated into leaf intercellular spaces of intact 7-day-old barley seedlings, using a syringe 3 to 4 h prior to inoculation. Leaf segments (2 cm) surrounding the infiltration site were harvested for microscopic evaluation as described above.

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