The Ambivalence of the Barley *Mlo* Locus: Mutations Conferring Resistance Against Powdery Mildew (*Blumeria graminis* f. sp. *hordei*) Enhance Susceptibility to the Rice Blast Fungus *Magnaporthe grisea*

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Recessive alleles of the barley *Mlo* locus confer non-race-specific resistance against the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). Recently the *Mlo* gene has been isolated and it was suggested that the *Mlo* product is a negative regulator of cell death. Thus, loss of function can precondition cells to a higher responsiveness for the onset of multiple defense functions. Here, we document an enhanced susceptibility of barley *mlo* mutants to the rice blast fungus *Magnaporthe grisea*. The disease phenotype is independent of the barley cultivar in which the *mlo* allele has been introgressed and occurs in equal amounts in barley backcross lines of cv. Ingrid carrying the *mlo-1*, *mlo-3*, or *mlo-5* allele. *Ror* genes, which are required for the full expression of *mlo* resistance in barley against *Bgh*, do not affect the specific *mlo*-mediated phenotype observed after *M. grisea* infection. Formation of an effective papilla restricts blast development in epidermal cells of *Mlo* plants. In contrast, papillae are mostly penetrated in *mlo* mutants and, as a consequence, the fungus spreads into adjacent mesophyll cells. Both wild-type plants and *mlo* mutants did not differ in perception of a purified elicitor derived from *M. grisea*. Thus, we hypothesize that a functional *Mlo* protein is a prerequisite for penetration resistance of barley to fungal pathogens like *M. grisea*. The benefit of *mlo* alleles for durable resistance in barley and a proposed role of *mlo*-type-mutations in rice are discussed.

Among monocots, powdery mildew is one of the most destructive diseases. In barley, the disease is caused by the obligate biotrophic fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*; formerly known as *Erysiphe graminis* f. sp. *hordei*). Resistance of barley to the pathogen operates on several levels of specificity. A full range of dominant or partially dominant inherited resistance genes have been characterized (Wiberg 1974) and shown to depend on the gene-for-gene concept

(Flor 1971). Other cultivars have quantitative resistance that is at least partly under polygenic control (Jones et al. 1982). An additional type of disease resistance in barley is provided by the *mlo* alleles (Jørgensen 1994).

The resistance trait conferred by *mlo* is recessively propagated and non-race-specific since it is effective against all isolates of *Bgh* (Jørgensen and Mortensen 1977; Brown and Jørgensen 1991; Lyngkjaer et al. 1995). A characteristic feature of all *mlo* mutants is the spontaneous occurrence of lesions on the leaf surface that is a true pleiotropic effect of the *mlo* alleles (Jørgensen 1992). For that reason, *mlo* plants can be categorized with other lesion-mimic mutations such as *acd* (accelerated cell death) and *lsd* (lesions simulating disease) in Arabidopsis and the *lls* (lethal leaf spot) mutation in maize, all of which seem to be altered in the induction of cell death (Dangl et al. 1996). Recently, the *Mlo* gene of barley has been isolated and the deduced protein was predicted to be membrane associated. It was hypothesized that, in the absence of the *Mlo* protein, plants will be primed for the onset of multiple defense functions (Büschges et al. 1997). Breeding with *mlo* resistance has led to a considerable number of commercial spring barley varieties combining a moderate lesion type with extended yield capacity (Jørgensen 1992).

Our goal was to examine the *Mlo* function in barley after inoculation with *Magnaporthe grisea* (Hebert) Barr (anamorph, *Pyricularia grisea* (Cooke) Sacc., formerly *P. oryzae* Cavara) (Rossman et al. 1990). *M. grisea* is the causal agent of the rice blast disease, one of the most devastating crop diseases worldwide (Ou 1980). Breeding for resistance to this fungus is difficult and durability of new resistance genes under field conditions is very poor (Hamer et al. 1993). The fungus can be easily isolated from lesions of various hosts, the majority of which are monocotyledonous plants like wheat, barley, maize, goosegrass (*Eleusine indica* (L.) Gaertn.), and weeping-lovegrass (*Eragrostis curvula* (Schrud.) Nees) (Heath et al. 1991; Valent and Chumley 1991; Hamer et al. 1993), and some untypical hosts such as *Commelina* spp. (Purchino and Muchovej 1993). However, all isolates clearly represent a single species (Hamer et al. 1993).

The mechanism of pathogenesis of *M. grisea* on rice includes elaboration of an appressorium that supports direct penetration through the cuticle of the host plant (Valent and

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Chumley 1991). So far, natural infections of barley with *M. grisea* have not been recorded. Nevertheless, *M. grisea* can infect barley under favorable laboratory conditions (Sweigard et al. 1998). To our knowledge, no form of resistance of barley to *M. grisea* has been documented. Here, we report on the infection process of different *M. grisea* races on barley plants carrying the *Mlo* wild-type gene or one of several *mlo* alleles.

RESULTS

Inoculation of barley cv. Ingrid (*Mlo*) with *Magnaporthe grisea* race 007 resulted in disease symptoms that were visible on the leaf surface after 5 to 7 days (Fig. 1A). Lesions were composed of a gray center surrounded by a brownish ring of necrotic cells and were therefore typical for blast. We addressed the question as to whether genes conferring resistance to powdery mildew have an effect on the development of *M. grisea*. A random screening of the infection phenotypes of barley cv. Ingrid (*Mlo*) and Ingrid backcross lines (BCIngrid) carrying the different powdery mildew resistance genes *Mlg*, *Mla-12*, and *mlo-5*, respectively, was carried out after inoculation with *M. grisea* race 007. Unexpectedly, only plants of the *mlo-5* genotype were highly susceptible to rice blast whereas the other backcross lines showed very slight symptoms and did not differ between each other (Fig. 1C). To exclude the possibility that susceptibility to *M. grisea* is specifically based on the function of the *mlo-5* allele of the *Mlo* locus, we inoculated barley backcross lines of cv. Ingrid carrying the *mlo-1* or *mlo-3* allele with *M. grisea* race 007 (Fig. 1A). Without exception, leaves of all *mlo* mutants were highly susceptible and became covered with blast symptoms including necrotic tips. In contrast, leaf tips of *Mlo* wild-type plants looked healthy and only a few symptoms were observed on the leaf surface. These findings strongly suggest that enhanced susceptibility of barley to *M. grisea* is dependent on a loss of function of the *Mlo* gene product. Similar results were obtained after inoculation of these barley lines with race 031 of *M. grisea* (Fig. 1B). In a further experiment, we addressed the question as to whether the genetic background might affect *mlo*-mediated susceptibility of barley against *M. grisea*. Therefore, we inoculated cv. Pallas (*Mlo*) and an *mlo-5* backcross line of Pallas with races 007 and 031 of *M. grisea*. Pallas showed very few blast symptoms, whereas the *mlo*-resistant backcross line was strongly infected by both races (data not shown).

Recently, genes have been identified that control *mlo*-mediated resistance in barley. Loss of function of these *Ror* (required for *mlo*-mediated resistance) genes results in successful infection of *mlo* genotypes by *Bgh* in a non-race-specific manner (Freialdenhoven et al. 1996). To test the effect of a mutation in the *Ror* locus on the development of rice blast, barley plants with the genotype *mlo-5/ror1* were inoculated with *M. grisea* race 007. Figure 1D shows that the *ror1* mutation did not affect the disease phenotype of barley mutant A98 (*mlo-5/ror1*) plants, compared with BCIngrid (*mlo-5/Ror1*) plants, thus indicating that the *Ror* gene does not control barley/*M. grisea* interactions.

We were interested in the cytological events accompanying the enhanced blast susceptibility of *mlo* mutants. Figure 2 shows the results of the microscopic survey of interactions. For the evaluation we followed the method of Koga et al.

(1988, 1990). Two differentiation classes were defined: one classifies the type of penetrated epidermal cells and the other takes into account the type of resistance responses performed by the cells. Cells were grouped as (i) so-called long cells located above vascular bundles and (ii) short cells including those surrounding the stomata. Host defense responses were classified according to two criteria: (i) highly localized cell wall appositions (papillae) at sites of fungal attack; and (ii) a whole cell autofluorescence as an indicator for the hypersensitive response (HR; Görg et al. 1993; Kita et al. 1981). An interaction site was defined as a successful attempt of the fungus to invade a cell or by the onset of a visible defense response. We analyzed interaction sites in cv. Ingrid (*Mlo*) and BCIngrid *mlo-5* 72 h post inoculation with *M. grisea* race 007 (Fig. 2A). On both genotypes, 1 to 5% of attacked short cells did not show any visible defense response and fungal hyphae grew unrestrictedly in these cells. On *Mlo* plants, about 44% of attacked cells formed an effective papilla whereas only 11% did so in the *mlo-5* backcross line. These papillae were judged to be effective in that no fungal development occurred in these cells. The frequency of HR of penetrated epidermal cells was comparable (about 45%) in both genotypes. However, in attacked cells of cv. Ingrid (*Mlo*) fungal hyphae were much shorter than those in *mlo-5* plants, where hyphae took up the whole cells (Fig. 3). Hence, it appears as if HR in cv. Ingrid (*Mlo*) is more effective at restricting fungal growth than in BCIngrid *mlo-5*. As an explanation, we assume that HR in cv. Ingrid (*Mlo*) occurs earlier or more rapidly in time to stop the fungus. A further class of cellular host responses is the concomitant occurrence of epidermal and mesophyll HR (Fig. 3B). This phenotype was found mostly on BCIngrid *mlo-5* plants (44 versus 9% of interaction sites). This shows that in BCIngrid *mlo-5* about half of all penetration attempts were successful, with the consequence that the infection proceeded to the mesophyll tissue. A similar distribution of cellular responses was found in the *ror1* mutant A89 (*mlo-5/ror1*) (data not shown). Analysis of interaction sites on long cells compared with short cells did not reveal qualitative differences between the genotypes in plant responses or fungal development (Fig. 2B). We addressed the question as to whether increased lesion size correlated with further development of fungal biomass. Microscopic analysis of interaction sites by 96 h after inoculation showed further growth of fungal hyphae penetrating several epidermal cells accompanied by an increased number of mesophyll cells undergoing HR and thus leading to larger lesions in BCIngrid *mlo-5* (Table 1).

Because it appears that the *mlo* mutant lines are defective in the onset of effective resistance responses, we addressed the question as to whether the genotypes differ in the perception of signal molecules derived from *M. grisea*. For that purpose, we used an elicitor that has been shown to induce multiple resistance responses in rice (Schaffrath et al. 1995). Aqueous solutions of elicitor were infiltrated into the intercellular spaces of barley leaves. As a marker for the host response, peroxidase (POX) activity was measured. Enzyme activity increased in both genotypes upon infiltration with varying concentrations of elicitor, but activities showed the same dose dependency (Fig. 4). The kinetics of POX activity followed a similar pattern in cv. Ingrid (*Mlo*) and in the BCIngrid *mlo-5* (Fig. 5). Thus, we conclude that perception of the elicitor is not altered by the *mlo* mutation.

Fig. 1. Leaves from infection assays 7 days after inoculation. Barley plants of cv. Ingrid (*Mlo*) and backcross lines of cv. Ingrid carrying the *mlo-1*, *mlo-3*, or *mlo-5* allele of the *Mlo* locus, were inoculated with race (A) 007 or (B) 031 from *Magnaporthe grisea*. C, Leaves from barley plants with different powdery mildew resistance genes after infection with race 007 from *M. grisea*. D, Phenotypes of barley plants carrying different alleles of the *Mlo* and *Ror1* locus after infection with race 007 from *M. grisea*. The *Ror1* locus defines a gene that is required for *mlo*-mediated resistance. All experiments were performed (A, B, and D) at least twice or (C) in triplicate with similar results.

DISCUSSION

The present study was basically aimed at the question as to whether *mlo* resistance, which is extremely effective in controlling powdery mildew in barley, might be agronomically useful for the control of the rice blast disease. We demonstrated here that such an approach probably would fail.

Unexpectedly, barley plants carrying an *mlo* allele for powdery mildew resistance are much more susceptible than *Mlo* plants to *M. grisea*. Although sporulation occurred on both *Mlo* and *mlo* genotypes, the degree of sporulation was much higher in *mlo* plants, which showed enlarged lesions (data not shown). It has been documented that host specificity among *M. grisea* isolates from different plant species is rather narrow for any particular isolate (Valent and Chumley 1991; Hamer et al. 1993). Therefore, the moderate number of blast symptoms on *Mlo* plants may be explained by the fact that races 007 and 031 are isolates from rice with unknown virulence on barley. Recent studies on pathogenicity genes with different races of *M. grisea* and other barley cultivars have revealed that, in principle, the fungus is capable of causing devastating symptoms on barley (Sweigard et al. 1998).

By using near-isogenic barley lines bearing different recessive alleles of the *Mlo* locus, we demonstrate unequivocally and for the first time that the *Mlo* locus controls the interaction of *M. grisea* and barley. An oligogenic effect has been excluded by analyzing the *mlo* alleles introgressed into different genetic backgrounds. Furthermore, other agronomically

relevant powdery mildew genes such as *Mlg* and *Mla-12* do not affect blast disease development.

The infection process of *M. grisea* on barley covers a series of temporal sequences reflecting germination, successful invasion, and induction of host defense responses. Plants of the *Mlo* genotype counteract penetration attempts by a highly localized apposition of cell wall material. In half of all incidences the formation of a papilla was able to effectively arrest further fungal invasion of the epidermal cells. However, if the fungus penetrates into a cell (approximately 40% of interaction sites), then development of intracellular mycelium is still very poor. Limitation of fungal growth in the first invaded cell was accompanied by a strong autofluorescence of cell walls

Fig. 2. Frequency of interaction sites on barley cv. Ingrid (*Mlo*) and the barley backcross line carrying the *mlo-5* allele (BCImlo-5) of the *Mlo* locus infected with race 007 from *Magnaporthe grisea*. Leaves were harvested 72 h after inoculation and prepared for microscopy as described in the text. Single-cell responses were discriminated as indicated. Data from a single experiment shown are based on (A) 100 infection sites on short cells and (B) 50 infection sites on long cells from four leaves.

Fig. 3. Light micrographs depicting plant responses to infection with race 007 from *Magnaporthe grisea* in barley 72 h after inoculation on short cells. **A**, Epidermal cells of cv. Ingrid (*Mlo*). Illumination with UV light results in a strong autofluorescence of cell walls (indicative for hypersensitive reaction) of the penetrated cell (upper part). A short hyphae can be observed in the same cell with transmission bright light (lower part). **B**, Leaf of a barley backcross line of cv. Ingrid carrying the *mlo-5* allele of the *Mlo* locus. The fungus has penetrated successfully into the first epidermal cell and has started to grow into the surrounding tissue (lower part). Under UV light (upper part) a strong autofluorescence was observed in epidermal and mesophyll cells.

indicative for an HR (Koga et al. 1990, 1994; Peterhänzel et al. 1997). Only in less than 10% of all incidences could epidermal and mesophyll HR be observed simultaneously. This is likely due to fungal spread into neighboring cells. In these few incidences an HR does not effectively restrict fungal development and sporulation. In contrast, *M. grisea* develops undisturbed on *mlo* barley and in half of all interaction sites spreads into mesophyll cells. Therefore, the *mlo* phenotype is due to massive fungal growth rather than to expression of hypersensitivity.

These events can be more easily interpreted in the light of what is going on in the rice/rice blast interaction. For rice, it has been shown that development in the first penetrated cell is a very critical step for the outbreak of blast disease (Peng and Shishiyama 1988; Thieron 1993; Koga 1994). In incompatible interactions, fungal growth is stopped in the first attacked cell by deposition of lignin or lignin-like substances (Thieron 1993). If the fungus is not arrested, invasion proceeds into adjacent epidermal and mesophyll cells; this happens predominantly in compatible interactions (Thieron 1993). The HR usually occurs in such cells but this is probably too late to prevent disease. The HR, in this situation, was considered to be a consequence of progressive cellular destruction associated with susceptibility rather than a cause of resistance (Thieron 1993), i.e., it is probably not what is usually consid-

ered to be an HR. It is a striking similarity of incompatible interactions of *M. grisea* on rice and on barley that restriction of fungal invasion depends on the efficiency of a resistance response in the first penetrated cell.

Enhanced susceptibility of *mlo* barley to the rice blast fungus is an outstanding phenomenon that does not support the hypothesis that *mlo* mutants react to diseases other than powdery mildew in the same way as non-*mlo* varieties (Jørgensen 1992). Furthermore, we show that the *mlo* mutation does not confer resistance against all other pathogens that penetrate living epidermal cells, as hypothesized by Jørgensen. Although *M. grisea* is a facultative, biotrophic fungus that is able to produce toxins and that may kill penetrated cells before colonization (Umetsu et al. 1974; Lebrun et al. 1990), our results indicate that the restriction of fungal growth is due to an active defense.

A non-race-specific elicitor derived from *M. grisea* that induces various resistance responses in rice is also active in barley. But the induction pattern of enzyme activity is similar in both *Mlo* wild-type plants and *mlo* mutants. Therefore, it seems that in the barley/rice blast interaction the perception of this signal molecules is not altered.

It was hypothesized that recessive *mlo* alleles similar to lesion-mimic mutants in Arabidopsis represent cell death control mutants that define wild-type genes playing a role in the

Table 1. Frequencies of papillae and disease symptoms on barley cv. Ingrid (*Mlo*) and a barley backcross line carrying the *mlo-5* allele (BCIngrid *mlo-5*) infected with race 007 from *Magnaporthe grisea* 96 h after inoculation^a

Barley line	Papillae	Interaction sites (%)			
		Hypersensitive reaction in epidermis and mesophyll (diameter of lesions, in mm)			
		<0.2	<0.6	0.6 to 1.2	>1.2
Ingrid (<i>Mlo</i>)	42.6	28.9	21.1	3.7	3.7
BCIngrid <i>mlo-5</i>	7.1	5.1	18.6	31.1	38.1

^a Papillae were evaluated with a magnification of $\times 250$ whereas the diameter of necrosis was analyzed with a magnification of $\times 100$. Data from the single experiment shown are based on evaluation of four leaves with 100 infection sites per leaf.

Fig. 4. Dose-response curves for the induction of peroxidase (POX) activity in barley leaves of cv. Ingrid (*Mlo*) and a backcross line of cv. Ingrid carrying the *mlo-5* allele (BCImlo-5) treated with an elicitor from *Magnaporthe grisea*. Vertical bars represent SD about the mean of three samples from a representative of two independent experiments.

Fig. 5. Induction of peroxidase (POX) activity in barley plants of cv. Ingrid (*Mlo*) and a barley backcross line carrying the *mlo-5* allele (BCImlo-5) of the *Mlo* locus. Leaves were treated by injection of an elicitor of *M. grisea* (1 mg/ml) into the intercellular spaces. Leaves were harvested at time points indicated (hours after inoculation, h p.i.). Vertical bars represent SD about the mean of three samples from a representative of two independent experiments.

control of the HR (Dangl et al. 1996; Büschges et al. 1997). Loss of function of *Mlo* should prime the responsiveness of barley *mlo* mutants for the onset of multiple defense reactions (Büschges et al. 1997). Our data may indicate that the *Mlo* locus plays a complex role during infection of barley with various pathogens on the level of basic resistance, supporting the speculative role of *Mlo* as an evolutionary early defense trait (Wolter et al. 1993). In barley, resistance conferred by *mlo* mutations seems to be an exception limited to powdery mildew diseases and apparently uses a specific signal transduction pathway. This assumption is underlined by the fact that *Ror* genes taking part in the *mlo* resistance pathway do not influence the *M. grisea* phenotype on barley.

Our work provokes a critical reconsideration of the benefit of the *mlo* resistance in spring barley breeding programs. This is especially true for regions, like the north of Italy, the south of France, or Asia, where rice and barley might grow together. It will be of great interest to see whether rice plants carrying a mutation in *Mlo*-like genes are altered in blast resistance.

MATERIALS AND METHODS

Plants, pathogens, and inoculation.

The *mlo-1* allele was induced by X-rays in the genetic background of cv. Haisa, whereas the allele *mlo-3* was generated in the background of cv. Malteria Heda through X-ray mutagenesis. For generation of the *mlo-5* allele, cv. Carlsberg II was chemically mutagenized with ethylmethane sulfonate (EMS) as described by Jørgensen (1983). The backcross lines in the genetic background of cv. Ingrid (BCIngrid *mlo-1*, BCIngrid *mlo-3*, BCIngrid *mlo-5*, BCIngrid *Mlg*, and BCIngrid *Mla-12*) were kindly provided by J. McKey (University of Uppsala, Uppsala, Sweden), whereas the barley cultivar Pallas and the *mlo-5* backcross line in cv. Pallas were obtained from L. Munk (Copenhagen). The generation of the *mlo-5* backcross line in cv. Pallas (BCPallas *mlo-5*) has been described by Kølster et al. (1986). The *Ror* mutant was obtained by P. Schulze-Lefert (Sainsbury Laboratory, Norwich). The mutant *ror1* (i.e., *ror1-2*, A89) was generated through EMS mutagenesis in BCIngrid *mlo-5* as described in Freialdenhoven et al. (1996). Plants were grown in a growth chamber at 16°C, 60% relative humidity (RH), and a photoperiod of 16 h.

The *M. grisea* race 007 (H-373) was a kind gift of Y. Sekizawa (Tamagawa University, Tokyo). The fungus was cultivated on an oat-meal starch agar (30 g liter⁻¹ oat flakes, 20 g liter⁻¹ agar-agar, 10 g liter⁻¹ starch, and 2 g liter⁻¹ yeast extract). After incubation at 27°C for 2 weeks, aerial mycelia were removed with a sterile spatula and synchronous sporulation was induced by further incubation under blacklight (310 to 360 nm). For inoculation the concentration of conidia was adjusted to 1 × 10⁶ ml⁻¹ in a spraying solution (1 g liter⁻¹ gelatin, 0.1% Tween 20). Plants were inoculated 12 to 14 days after sowing by spraying the conidial suspension onto the leaves. After an incubation for 24 h in a dark moist chamber (26°C, up to 100% RH), plants were kept in a humid atmosphere under the same temperature and light regime as described above.

Macroscopic and microscopic analysis.

Infection on the secondary leaves was macroscopically evaluated 7 days after inoculation. For microscopic analysis,

leaves were harvested at different time points after inoculation and cleared in 0.15% (wt/vol) trichloroacetic acid in ethylalcohol/chloroform 4:1 (vol/vol). The solution was changed once during the next 48 h of incubation, then leaves were rinsed in water and mounted in 50% (vol/vol) glycerol. Whole cell autofluorescence was observed by fluorescence microscopy (excitation filter 485 nm, dichroic mirror 510 nm, barrier filter 520 nm; Axioplan, Zeiss, Germany). At least five seedlings were analyzed for each data point. A total of 150 infection sites were inspected per leaf and scored independently taking into account the different cell types of the adaxial epidermis (Koga et al. 1990).

Elicitor treatment and measurement of POX activity.

For elicitor treatments, a crude elicitor fraction (CEF) prepared from vegetative mycelium of *M. grisea* as described by Schaffrath et al. (1995) was used. The elicitor solution was injected into extracellular spaces of barley leaves with a Hagborg device with a hypodermic syringe (Hagborg 1970). Leaves were harvested at the time points indicated and immediately frozen in liquid nitrogen. Each sample consisted of six leaves of approximately 4 cm in length. After harvesting, three replicates consisting of two leaves each were ground in a mortar containing 2 ml of phosphate buffer (50 mM, pH 5.2). The homogenate was transferred to a micro test tube and centrifuged at 13,800 × g for 10 min. The supernatant was immediately placed into a new tube and served as enzyme extract.

For determination of total POX activity a modified method of Pütter (1970) was used. The enzyme extract (25 µl) was incubated with 2.875 ml of phosphate buffer (50 mM, pH 5.2) containing 18 mM guaiacol and the reaction was started by adding 100 µl of H₂O₂ solution (0.1% vol/vol). Production of tetraguaiacol was measured in a spectrophotometer at 470 nm and enzyme activity was expressed in nkat mg⁻¹ protein. Protein concentrations were determined with the Bio-Rad protein kit (Bio-Rad, Richmond, CA).

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