The Plant Cell, Vol. 8, 5–14, January 1996

RESEARCH ARTICLE

Identification of Genes Required for the Function of Non-Race-Specific *mlo* Resistance to Powdery Mildew in Barley

Andreas Freialdenhoven, Christoph Peterhänsel, Joachim Kurth, Fritz Kreuzaler, and Paul Schulze-Lefert[1]

Rheinisch-Westfälische Technische Hochschule Aachen, Department of Biology I, Worringer Weg 1, D-52074 Aachen, Germany

Recessive alleles (*mlo*) of the *Mlo* locus in barley mediate a broad, non-race-specific resistance reaction to the powdery mildew fungus *Erysiphe graminis* f sp *hordei*. A mutational approach was used to identify genes that are required for the function of *mlo*. Six susceptible M_2 individuals were isolated after inoculation with the fungal isolate K1 from chemically mutagenized seed carrying the *mlo-5* allele. Susceptibility in each of these individuals is due to monogenic, recessively inherited mutations in loci unlinked to *mlo*. The mutants identify two unlinked complementation groups, designated *Ror1* and *Ror2* (required for *mlo*-specified resistance). Both *Ror* genes are required for the function of different tested *mlo* alleles and for *mlo* function after challenge with different isolates of *E. g.* f sp *hordei*. A quantitative cytological time course analysis revealed that the host cell penetration efficiency in the mutants is intermediate compared with *mlo*-resistant and *Mlo*-susceptible genotypes. *Ror1* and *Ror2* mutants could be differentiated from each other by the same criterion. The spontaneous formation of cell wall appositions in *mlo* plants, a subcellular structure believed to represent part of the *mlo* defense, is suppressed in *mlo/ror* genotypes. In contrast, accumulation of major structural components in the appositions is seemingly unaltered. We conclude that there is a regulatory function for the *Ror* genes in *mlo*-specified resistance and propose a model in which the *Mlo* wild-type allele functions as a negative regulator and the *Ror* genes act as positive regulators of a non-race-specific resistance response.

INTRODUCTION

Most analyzed resistance reactions of barley against an attack by the obligate biotrophic fungal pathogen *Erysiphe graminis* f sp *hordei* are specified by dominantly or semidominantly inherited resistance genes (*Mlx*) that act race specifically (Jørgensen, 1994). Their triggering is dependent on the presence of complementary avirulence genes in the fungus, as described by Flor's gene-for-gene hypothesis (Flor, 1971).

An exceptional case of inherited resistance is exemplified by recessive alleles (*mlo*) of the *Mlo* locus. Each resistance allele of the locus acts in a non-race-specific manner and confers resistance to almost all isolates of *E. g.* f sp *hordei* (Jørgensen, 1977; Lyngkjaer et al., 1995), suggesting that the trigger of the defense response is independent of the presence of avirulence genes required for race-specific resistance responses. Resistance alleles of the *Mlo* locus can be induced by mutation of virtually any susceptible (*Mlo*) cultivar, and in the past, many *mlo* alleles have been isolated using diverse mutagens. In addition, at least one resistance allele (*mlo-11*) has been isolated from a natural habitat (Jørgensen, 1983). Thus, the genetic data are compatible with the assumption that *mlo* resistance is due to a loss of function of the *Mlo* wild-type allele.

The development of the fungus on *mlo*-resistant plants is arrested at the prehaustorial stage in a subcellularly restricted cell wall apposition (papilla) directly beneath the fungal appressorium (Jørgensen and Mortensen, 1977). A cell death response (single-cell hypersensitive response), frequently observed in race-specific resistance reactions to powdery mildew (Koga et al., 1990; Görg et al., 1993; Boyd et al., 1995), is almost absent in *mlo*-controlled defense. A striking feature of *mlo*-resistant plants is the spontaneous formation of cell wall appositions in the target tissue of the pathogen even under

[1] To whom correspondence should be addressed. Current address: The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom.

aseptic conditions (Wolter et al., 1993). This pleiotropic effect of *mlo* resistance alleles temporally precedes the formation of macroscopically visible necrotic and chlorotic leaf lesions, which are reminiscent of the phenotypes described for lesion mimic mutants in many plant species (Walbot et al., 1983; Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Jones, 1994). These findings suggest a negative regulatory function of the *Mlo* wild-type allele in the defense response (Wolter et al., 1993).

Recently, several race-specific resistance genes from different plant species have been isolated (reviewed in Dangl, 1995; Staskawicz et al., 1995). The striking similarity of related structural domains in the deduced gene products provides new insights into their function. However, it is currently not known how many components are involved in resistance gene–specified signaling to establish the resistant phenotype. This issue can be approached genetically through the identification of genes that are required for the function of a resistance gene. However, these studies have been limited to race-specific resistance genes (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994; Hammond-Kosack et al., 1994; Salmeron et al., 1994; Century et al., 1995).

Our objective was to provide new tools to investigate the molecular basis of *mlo*-specified resistance. The experiments were initiated by the assumption that the *Mlo* wild-type allele functions as a negative regulator. Following a mutational approach in *mlo*-resistant plants, we discovered two genes required for the function of *mlo* resistance. Extensive genetic and cytological analyses of the mutants provided evidence that these genes represent positive regulatory components and that a single resistance response is subject to both negative and positive genetic control.

RESULTS

The mutational approach for the identification of genes required for the function of *mlo* resistance alleles is outlined in Figure 1. The *mlo-5* allele used in this study was originally isolated after ethyl methanesulfonate (EMS) mutagenesis of a susceptible cultivar carrying the *Mlo* wild-type allele (Jørgensen, 1983). The mutagen-induced resistance allele was transferred into the genetic background of cultivar Ingrid by backcrossing (BCIngrid *mlo-5*). Seed of the resistant backcross (BC) line were mutagenized using EMS or NaN_3, and M_2 seedlings were screened for susceptibility 7 days after inoculation with spores of the powdery mildew isolate K1 (see Methods). Susceptibility in the seedlings was expected to be caused either by functional reversion events in the *Mlo* locus or by mutations in genes required for *mlo* function. No susceptible progenies were detected among 20,000 individuals from nonmutagenized selfed seed containing the *mlo-5* resistance allele.

The efficiency of the mutagenesis was monitored by the frequency of chlorophyll-defective M_2 seedlings (13×10^{-3} after EMS treatment and 31×10^{-3} after NaN_3 treatment). This frequency is within the range reported from extensive mutagenesis experiments in barley (Jende-Strid, 1978). Six susceptible seedlings were identified from among 54,410 M_2 plants after EMS treatment (frequency 0.11×10^{-3}). The same number were isolated from 39,759 M_2 plants after NaN_3 treatment (frequency 0.15×10^{-3}). Because the 12 M_2 individuals are derived from six different M_1 plants, they represent six independent mutational events. Fingerprint analysis with DNA markers verified that the susceptible seedlings originated from the genotype used in the mutagenesis (see Methods). The phenotypes of the independent mutants A39, A44, A89, C36, C69, and C88 at late stages after inoculation are shown in Figure 2. In each case, fungal colonies consisting of sporulating aerial mycelium were observed, indicating the completion of the powdery mildew life cycle. Significantly fewer colonies, however, were found on each of the mutants when compared with the *Mlo*-susceptible cultivar Ingrid. In addition, fewer colonies were reproducibly detected on mutant A44 compared with the other mutants.

Testcrosses were performed between each of the six independent mutants and the *mlo*-resistant line BCIngrid *mlo-5*. Table 1 shows that only resistant individuals were obtained in the F_1 generation. In the F_2 population, selfed F_1 plants revealed a ratio of susceptible and resistant individuals that is compatible with a 1:3 segregation. This finding excludes the possibility that susceptibility in the mutants was due to a reversion event that restored the dominant *Mlo* wild-type allele. (If this were the case, susceptible F_1 individuals and a 3:1 segregation of susceptible and resistant F_2 plants would be expected.) Testcrosses of the mutants with the susceptible near-isogenic cultivar Ingrid (*Mlo*) confirmed this interpretation, as summarized in Table 2. Only susceptible individuals were observed in the F_1 progeny. Selfed F_1 plants segregated sus-

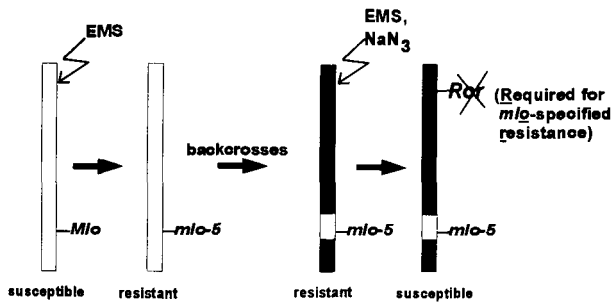

Figure 1. Schematic Representation of the Mutational Approach to Identify Genes Required for *mlo*-Specified Resistance.

The EMS-induced resistance allele *mlo-5* (Jørgensen, 1983) was transferred into the genetic background of cultivar Ingrid by repeated backcrossing. The resulting near-isogenic line served as the starting material for the second mutagenesis described in this study. Genetic backgrounds are represented by open bars for cultivar Carlsberg II and by closed bars for cultivar Ingrid.

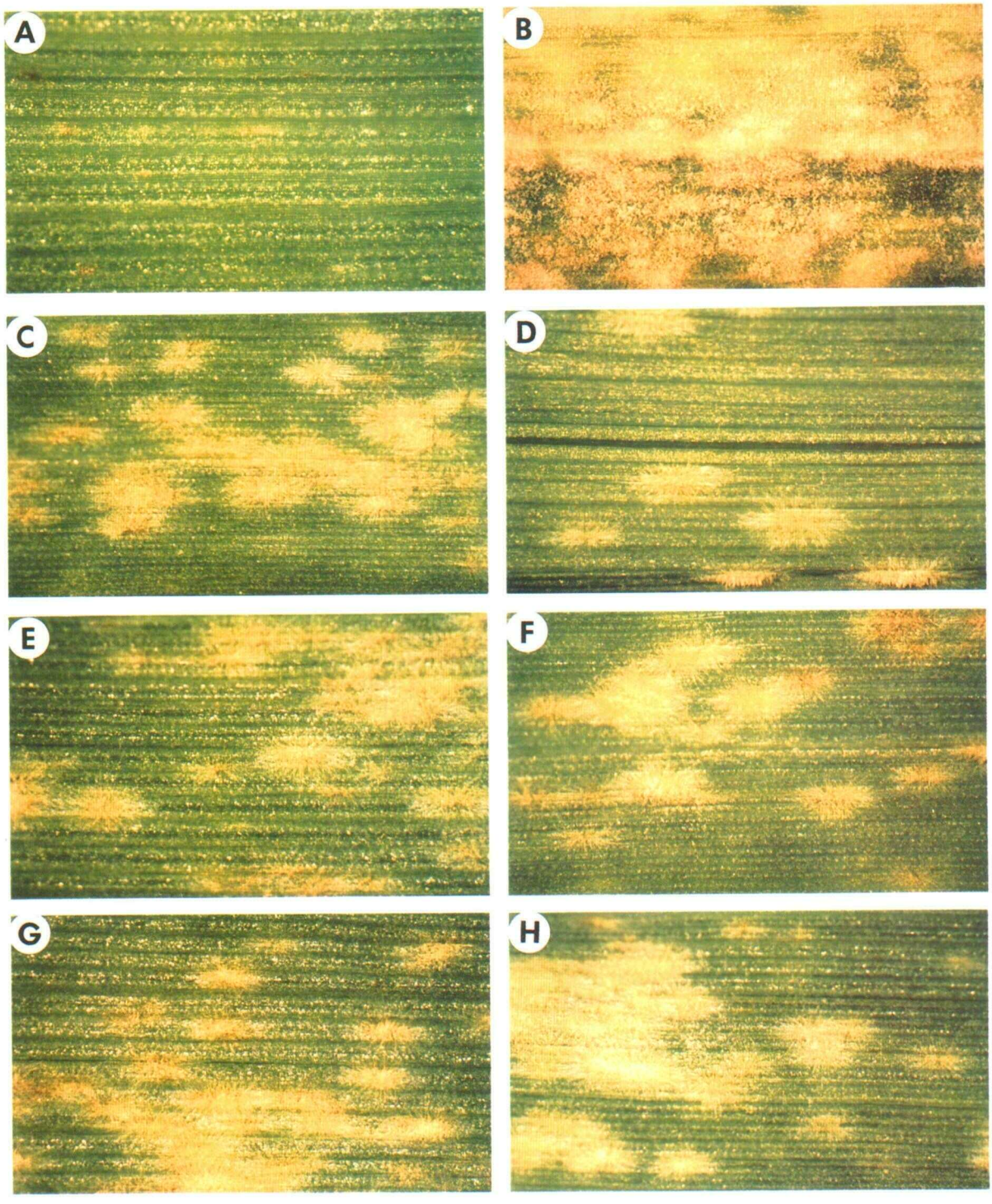

Figure 2. Phenotypes of *mlo*-Resistant, *Mlo*-Susceptible, and Mutant Seedlings 7 Days after Inoculation with *E. g.* f sp *hordei* Isolate K1.

(A) and **(B)** Phenotypes of 14-day-old primary leaves of the *mlo*-resistant line BCIngrid *mlo-5*, which has been used for mutagenesis, and of the near-isogenic *Mlo*-susceptible cultivar Ingrid, respectively.

(C) to **(H)** Representative infection types of the M_2 individuals with A39 shown in **(C)**, A44 in **(D)**, A89 in **(E)**, C36 in **(F)**, C69 in **(G)**, and C88 in **(H)**. Barley powdery mildew isolate K1 is avirulent on *mlo*-resistant plants. Each mutant allows sporulation of the fungus, indicating completion of the asexual life cycle. The infection types of the mutants are between those of the *Mlo*-susceptible and *mlo*-resistant line, and fewer sporulating colonies are detectable on mutant A44 compared with each of the other mutants.

Table 1. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses of Susceptible Mutants with the Resistant Backcross Line BCIngrid *mlo-5*

	F_1		F_2		
Mutants	Susceptible Plants	Resistant Plants	Susceptible Plants	Resistant Plants	χ^2(3:1)
A39	0	15	107	388	$P > 0.05$
A44	0	5	18	78	$P > 0.1$
A89	0	10	79	246	$P > 0.5$
C36	0	5	35	110	$P > 0.5$
C69	0	6	34	121	$P > 0.1$
C88	0	5	36	128	$P > 0.1$

ceptible and resistant individuals in a 13:3 ratio with $P > 0.05$. This suggests a digenic control of the phenotype with one dominant and one recessive susceptibility allele. We conclude that susceptibility in each of the mutants is caused by mutations unlinked to *Mlo*.

Two Complementation Groups Are Required for *mlo* Function

Intermutant crosses were performed to determine the number of complementation groups represented by the mutants. Table 3 summarizes the number of resistant and susceptible progeny that were detected in the F_1 and F_2 generation from various crosses between the mutants. Except for two cases, no complementation of the susceptible phenotype in F_1 plants was detected. Selfings of these F_1 plants generated only susceptible progeny in the F_2 generation. In contrast, the resistant F_1 plants from the crosses A39 × A44 and C69 × A44 segregated susceptible and resistant F_2 progeny. In both cases, the observed segregation is compatible with a 7:9 ratio, indicating that susceptibility is caused by two unlinked, recessively inherited loci. In summary, we conclude that A39, A89, C36, C69, and C88 each represent recessively inherited mutations in a single complementation group conferring susceptibility to the powdery mildew fungus in the presence of the *mlo-5* resistance allele. Mutant A44 represents a recessively inherited mutation in a second complementation group. We have designated the respective loci *Ror1* and *Ror2* (required for *mlo*-specified resistance).

Ror Genes Are Required for the Function of Different *mlo* Resistance Alleles

Many *mlo* resistance alleles have been described previously. With few exceptions, the resistance alleles have been induced by mutagenesis of susceptible *Mlo* cultivars (Jørgensen, 1983). To determine whether the *Ror* genes are required for *mlo* function in the context of different *mlo* resistance alleles and/or different genetic backgrounds, crosses were performed with the mutagen-induced alleles *mlo-3* and *mlo-4* and with the *mlo-11* allele originally collected from a natural barley habitat. Crosses were also performed with the *mlo-5* allele originally used for *Ror* mutant identification but backcrossed into cultivar Pallas (BCPallas *mlo-5*). Qualitatively identical data were obtained with F_1 and F_2 progeny after inoculation with K1 spores for each of the respective crosses, as summarized in Table 4. The F_1 individuals exhibited only resistant phenotypes, and selfed F_2 individuals segregated susceptible and resistant phenotypes in a manner compatible with the expected 1:3 ratio. Thus, even in different genetic backgrounds, *Ror1* and *Ror2* are required for the function of all tested *mlo* resistance alleles.

Quantitative Cytological Analysis of Single Interaction Sites

A quantitative cytological analysis of single plant–fungus interaction sites on primary leaves of the *Ror* mutants was performed in the time course experiment shown in Figure 3. Previous studies have shown repeatedly that a cell wall apposition (CWA) is formed directly beneath the site of attempted penetration in both compatible and incompatible interactions (Zeyen and Bushnell, 1979; Aist and Israel, 1986). In the presence of *mlo* resistance alleles, however, fungal development is invariably arrested in this subcellular structure (Jørgensen and Mortensen, 1977). As expected, we found that *mlo*-associated CWAs in attacked epidermal cells of the resistant cultivar BCIngrid *mlo-5* were, with few exceptions, not penetrated by the fungus during the first 72 hr after inoculation (observed maximal penetration frequency, 0.5%). In contrast, an initial continuous increase in the number of penetrated CWAs between 15 and 36 hr after inoculation, followed by a high constant penetration frequency of ~70%, was observed in the susceptible (*Mlo*) near-isogenic cultivar Ingrid.

Table 2. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses of Susceptible Mutants with the Susceptible Near-Isogenic Line Ingrid (*Mlo*)

	F_1		F_2		
Mutants	Susceptible Plants	Resistant Plants	Susceptible Plants	Resistant Plants	χ^2(13:3)
A39	16	0	544	116	$P > 0.1$
A44	3	0	155	29	$P > 0.1$
A89	10	0	348	79	$P > 0.5$
C36	4	0	355	83	$P > 0.9$
C69	4	0	257	69	$P > 0.1$
C88	5	0	348	93	$P > 0.1$

Table 3. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses between Susceptible Mutants A39, A44, A89, C36, C69, and C88

	F_1		F_2		
Crosses	Susceptible Plants	Resistant Plants	Susceptible Plants	Resistant Plants	χ^2(7:9)
A39 × A44	0	4	105	113	P > 0.1
× C36	8	0	233	0	
× C69	4	0	53	0	
A89 × C36	4	0	41	0	
C36 × C69	14	0	95	0	
× C88	16	0	120	0	
C69 × A39	10	0	65	0	
× A44	0	10	126	164	P > 0.9
× C88	2	0	180	0	
C88 × A39	14	0	94	0	
× C36	10	0	60	0	

Each of the allelic *Ror1* mutants showed a comparable time course, characterized by an initial increase in the number of penetrated CWAs between 15 and 48 hr after inoculation, which approximated 20 to 30% and did not change significantly in the subsequent 24 hr. The time course of host cell penetration on the *Ror2* mutant A44 could be discriminated clearly from each of the *Ror1* mutants. Maximal recorded penetration frequencies in A44 approximated only 10%. Thus, the identification of two *Ror* complementation groups by genetic analysis was reflected at the cytological level by a separable efficiency of *ror1*- and *ror2*-defective plants to resist fungal penetration into the host cell attacked first.

Ror Mutants Are Susceptible to Various Powdery Mildew Isolates

Because *mlo* resistance alleles confer a non-race-specific resistance reaction on almost all tested isolates of *E. g.* f sp *hordei*, we asked whether mutations in the *Ror* genes confer susceptibility on powdery mildew isolates different from K1 used in the initial screening of the M_2 populations. Two isolates, A6 and R146, each carrying characterized avirulence functions, were tested for this purpose (see Methods). Both isolates are avirulent on the BCIngrid *mlo-5* line but virulent on the near-isogenic *Mlo* cultivar Ingrid. A quantitative cytological analysis of single interaction sites on primary leaves of the various genotypes was performed 48 hr after inoculation. As expected, high and comparable penetration frequencies (64 to 79%) of CWAs were observed on the *Mlo* cultivar Ingrid, and a very low frequency (maximum 0.5%) was detected in the resistant BCIngrid *mlo-5* line after attempted attacks of each of the tested isolates (A6, R146, and K1). The representatively chosen *Ror1* mutant A89 showed mean penetration frequencies of 33% in interactions with A6, 29% in interactions with R146, and 17% in interactions with K1. A similar pattern, albeit with lower penetration frequencies (~10%), was found for the *Ror2* mutant A44 attacked by the tested isolates K1 and A6. With each fungal isolate, we detected sporulating aerial mycelium 7 days after inoculation on the leaf surface of the *Ror1* and *Ror2* mutants. Therefore, the susceptibility caused by defective *Ror* genes is not restricted to the fungal K1 genotype, but the mutations suppress *mlo*-mediated resistance in interactions with different isolates of *E. g.* f sp *hordei*.

Ror Mutants Suppress Spontaneous CWA Formation in Epidermal Tissue

We have reported previously that even aseptically grown *mlo*-resistant seedlings show a very high frequency of spontaneous CWA formation in a manner specific for the different cell types of a leaf epidermis (Wolter et al., 1993). In this study, we recorded the frequency of spontaneous CWA formation in the *Ror1* mutant A89 and the *Ror2* mutant A44 grown under mildew-free conditions. Table 5 summarizes the number of observed spontaneous CWAs per square centimeter of epidermal tissue from primary leaves of the relevant genotypes. Spontaneous

Table 4. Phenotypes and Segregation Ratios from Crosses of Susceptible *Ror* Mutants with Different *mlo*-Resistant Cultivars

	F_1		F_2		
Crosses[a]	Susceptible Plants	Resistant Plants	Susceptible Plants	Resistant Plants	χ^2(1:3)
A39 (*ror1-1*) × BCIngrid *mlo-4*	0	14	98	358	P > 0.05
A89 (*ror1-2*) × BCIngrid *mlo-4*	0	7	73	244	P > 0.1
A89 (*ror1-2*) × BCPallas *mlo-5*	0	10	114	374	P > 0.1
C36 (*ror1-3*) × Gr. Zw. *mlo-11*	0	5	7	24	P > 0.5
C69 (*ror1-4*) × Gr. Zw. *mlo-11*	0	5	12	27	P > 0.1
C88 (*ror1-5*) × M. Heda *mlo-3*	0	4	33	91	P > 0.5
A44 (*ror2*) × BCIngrid *mlo-4*	0	9	25	91	P > 0.1
A44 (*ror2*) × Gr. Zw. *mlo-11*	0	5	18	67	P > 0.1

[a] Gr. Zw., Grannenlose Zweizeilige; M. Heda, Malteria Heda.

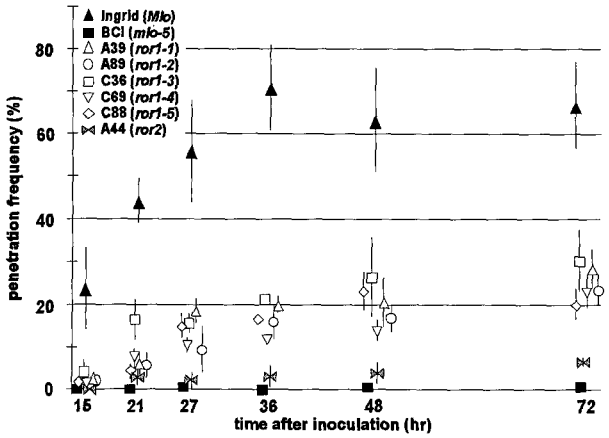

Figure 3. Frequency of Successful Penetration Attempts of Powdery Mildew Isolate K1 at Different Time Points after Inoculation.

Interaction sites were analyzed microscopically and scored only if the fungus had developed an appressorium and if the plant had reacted with the formation of a cell wall apposition. Very few interaction sites were found with an appressorium but without a cell wall apposition at the inspected time points. Penetration attempts were scored as successful if a haustorium was visible in the attacked cell. Each data point is the mean of at least 200 inspected interactions from four independent leaves. Vertical lines indicate standard deviations. Ingrid, near-isogenic susceptible cultivar (*Mlo Ror1 Ror2*); BCIngrid (BCI) *mlo-5*, resistant backcross line in cultivar Ingrid (*mlo Ror1 Ror2*); A39 to C88, *Ror1* mutants (*mlo ror1 Ror2*); A44, *Ror2* mutant (*mlo Ror1 ror2*).

CWA formation was recorded separately for different cell types of the epidermis (subsidiary cells of stomata, epidermal cells contacting subsidiary cells, and small epidermal cells not in contact with subsidiary cells). No spontaneous CWA formation was noticed in subsidiary cells of stomatae in any of the tested genotypes. A dramatic increase in CWA formation was detected in short epidermal cells contacting subsidiary cells in BCIngrid *mlo-5* (*mlo-5 Ror1 Ror2*) compared with the near-isogenic cultivar Ingrid (*Mlo Ror1 Ror2*). A clear suppression of constitutive CWA formation was found in this cell type in mutant A89 (*mlo-5 ror1 Ror2*). The suppression was also observed, albeit to a lesser extent, in mutant A44 (*mlo-5 Ror1 ror2*). A qualitatively similar distribution was found between the tested genotypes in short epidermal cells that do not contact subsidiary cells. The data show that defective *Ror* genes suppress both the resistance and the constitutive CWA formation mediated by *mlo* alleles.

Ror Mutants Retain the Capability to Accumulate Major Structural Components of CWAs

In contrast with constitutive CWA development, initial microscopic inspections revealed that pathogen-triggered CWA formation was not suppressed by *Ror*-defective alleles. We therefore asked whether qualitative alterations of pathogen-induced CWAs would be detectable by histochemical methods. Major components of CWAs are phenolic-like substances (Mayama and Shishiyama, 1978; Aist and Israel, 1986) and β-1,3-glucans (Smart et al., 1985). Figure 4A shows the yellow autofluorescence characteristic of a CWA of the *Ror1* mutant A89, indicating a retained capability to accumulate phenolic-like substances in appositions. As shown in Figure 4B, the *Ror1* mutant also accumulated β-1,3-glucans, detected by sirofluor-mediated fluorescence in penetrated CWAs. Identical results were obtained with the *Ror2* mutant A44 (data not shown). The findings suggest that susceptibility in the *Ror1* mutant A89 and the *Ror2* mutant A44 is not associated with major structural alterations or a major disturbance in the accumulation of the tested compounds.

DISCUSSION

This study describes the successful identification of two genes required for the function of a resistance gene (*mlo*) acting in a non-race-specific manner. Similar analyses have identified genes required for the function of race-specific resistance genes in tomato, barley, and Arabidopsis (Freialdenhoven et al., 1994; Hammond-Kosack et al., 1994; Salmeron et al., 1994; Century et al., 1995). All previous studies have shown susceptible mutants affected in the resistance genes and in genes

Table 5. Cell Type–Specific Evaluation of the Number of Spontaneous CWAs per cm^2 Epidermal Tissue of Primary Leaves in Wild-Type and *Ror* Mutant Genotypes

	BCIngrid *mlo-5* (*mlo Ror1 Ror2*)			Ingrid (*Mlo Ror1 Ror2*)			A89 (*mlo ror1 Ror2*)			A44 (*mlo Ror1 ror2*)		
Cell Type[a]	1	2	3	1	2	3	1	2	3	1	2	3
a cells	0	0	0	0	0	0	0	0	0	0	0	0
b cells	1364	1446	2571	70	26	45	202	461	276	834	726	1057
c cells	350	403	720	26	11	13	104	115	78	363	311	429

[a] a cells, subsidiary cells of stomata; b cells, cells contacting subsidiary cells; c cells, other cells (only short type; <400 μm in length).

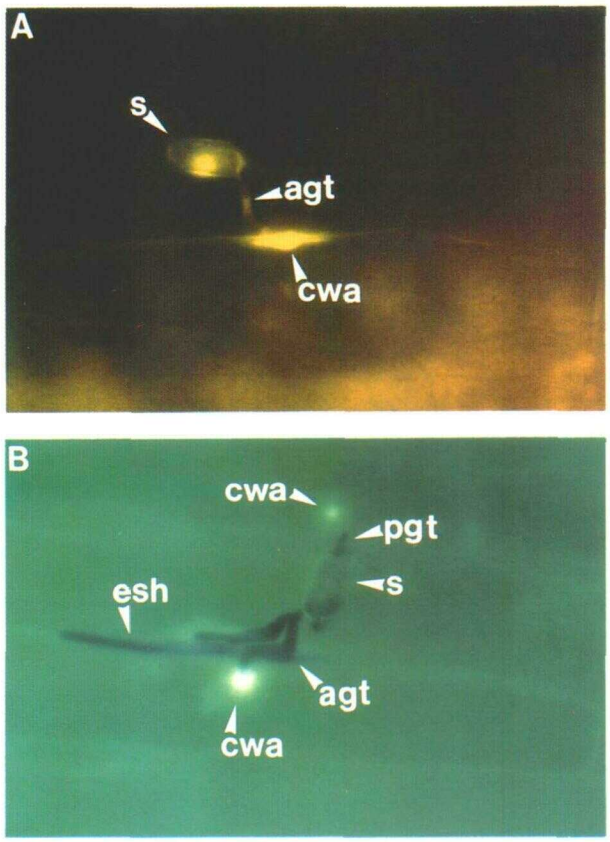

Figure 4. Detection of Phenolics and β-1,3-Glucans in Cell Wall Appositions of the *Ror1* Mutant A89 by Fluorescence Microscopy.

Single interaction sites on primary leaves 27 hr after inoculation with powdery mildew isolate K1 are shown.

(A) The characteristic yellow autofluorescence in the CWA indicates the presence of phenolics (excitation wavelength λ of 470 nm). The autofluorescence under the spore is a result of a CWA formed in response to the primary germ tube.

(B) The whitish yellow sirofluor-mediated fluorescence in the CWA indicates the presence of β-1,3-glucans (excitation wavelength λ of 360 nm).

Magnification is ×400. agt, appressorial germ tube; cwa, cell wall apposition; esh, elongated secondary hypha; pgt, primary germ tube; s, spore.

required for their function. In contrast, in this investigation we detected only mutants affected in genes required for *mlo* function (Tables 2 and 3). This was expected because previous genetic evidence suggested that the EMS-derived *mlo-5* resistance allele represents a mutation-induced loss of function in the *Mlo* wild-type allele (Jørgensen, 1983). Therefore, it is more likely to isolate loss-of-function alleles in genes required for *mlo* function than to isolate a gain of function in *Mlo*. The observation that each of the six isolated susceptible mutants represents a recessively inherited defective allele in a *Ror* gene supports our assumption. Thus, the susceptible M_2 plants isolated here represent double mutants: they carry a defect in the dominant *Mlo* wild-type allele and defects in either the *Ror1* or the *Ror2* gene.

It is important to determine how many host genes contribute to the function of resistance genes. This study revealed five allelic mutants of the *Ror1* locus and a single mutant of the *Ror2* locus. In general, it is believed that the entire number of nonredundant genes with nonlethal defective alleles controlling the expression of a phenotype would have been uncovered if multiple mutant alleles from each locus had been isolated. This is obviously not the case in our study, but we believe that it would be arduous to identify additional mutant genes conferring higher infection types because the five mutants with the most drastically altered infection types (Figures 2 and 3) are allelic. However, we are aware that the accessibility of different loci to identification by mutagenesis can vary substantially (Lundqvist, 1991). Mutants exhibiting very low infection types similar to the only *ror2* allele in A44 have probably escaped our stringent screening procedure, which relies on the macroscopic detection of sporulating colonies on the leaf surface. More sensitive screening procedures that involve β-glucuronidase–mediated detection of few fungal hyphae (Hammond-Kosack et al., 1994) or haustoria, for example, should be helpful for this purpose. Thus, an exhaustive detection of loci required for the function of a resistance gene will depend primarily on the sensitivity and stringency of the screening procedure to detect modified infection types.

None of the isolated *Ror* mutants showed the fully susceptible infection type as defined by the *Mlo* genotype (Figures 2 and 3). Similarly, the *Rar1* and *Rar2* mutants (former designation *Nar-1* and *Nar-2*; Freialdenhoven et al., 1994), which are required for the function of *Mla-12*–specified resistance in barley, and the *Rcr-1* and *Rcr-2* mutants, which are required for *Cf-9* function in tomato (Hammond-Kosack et al., 1994), have shown new infection types on a scale between the resistant and susceptible wild-type parents. Whether this indicates residual gene product activities of the isolated mutant alleles or bypass mechanisms that can partially compensate the functional defects remains an open question.

CWA formation is a ubiquitous phenomenon accompanying host wall penetration attempts in interactions between cereal hosts and powdery mildews (Aist, 1976). It is believed that this structure represents a physical reinforcement of the host cell wall directly beneath the fungal appressorium. Because CWA formation is detectable in both susceptible and resistant plants and because the CWAs from both genotypes cannot be qualitatively discriminated at the ultrastructural or histochemical level (Smart et al., 1985; Zeyen and Ahlstrand, 1993), it is not known whether the temporal and spatial correlation between CWA formation and *mlo*-mediated defense indicates a causal relationship. Physiological experiments with *mlo*-resistant plants have shown that micromolar concentrations of both 2-deoxy-D-glucose, an effective inhibitor of callose

deposition in plants, and chlortetracycline, a Ca^{2+} chelator, drastically decrease CWA formation in response to pathogen attack. Correspondingly, the inhibitors increase penetration rates of the residual CWAs. These investigations show that the timing of CWA formation is likely to be crucial for the outcome of the interaction (Gold et al., 1986; Bayles et al., 1990). This hypothesis is compatible with our data from histochemical analysis of penetrated and nonpenetrated CWAs in the susceptible *Ror* mutants that revealed neither significant major structural alterations nor major disturbances in the accumulation of CWA compounds (Figure 4). However, a detailed biochemical analysis is necessary to substantiate the absence of qualitative differences and to prove the existence of a differential timing of CWA formation in *mlo*-resistant plants and susceptible *mlo/ror* mutants.

Although *mlo*-resistant plants confer a broad, non-race-specific resistance response to *E. g.* f sp *hordei*, they are susceptible to infection by *Rhynchosporium secalis*, a pathogen of barley that uses the identical target tissue (leaf epidermis) to colonize the host (W. Knogge and P. Schulze-Lefert, unpublished results). The same holds true for barley leaf rust and stem rust, which attack mesophyll cells (Jørgensen, 1977). Therefore, the effectiveness of the *mlo* resistance exhibits at least some level of pathogen specificity. It was important to demonstrate in this context that the susceptibility of the *mlo-5/ror* genotypes is not restricted to the fungal isolate K1. Thus, mutations in the *Ror* genes seemingly confer a similar broad susceptibility to powdery mildew isolates compared with the resistance mediated by *mlo* alleles.

Recently, it has been proposed that the *Mlo* wild-type allele might function as a susceptibility factor that interacts with a putative compatibility factor from the biotrophic fungus to establish basic compatibility (Johal et al., 1995). Compatibility is thought to result from the suppression of a defense reaction through the interaction between *Mlo* and the fungal compatibility factor. Accordingly, *Ror* genes could represent any positive regulatory component of the suppressed defense. Alternatively, it is conceivable that the *Mlo* wild-type allele functions as a negative regulator of a defense response without interacting with a basic compatibility factor. Consistent with this hypothesis is the observation that *mlo*-resistant plants exhibit a constitutive expression of the resistance response, as indicated by a spontaneous CWA formation in the absence of the pathogen (Wolter et al., 1993). The reduction of spontaneous CWA formation in *ror mlo* genotypes (Table 5) provides additional evidence to support the latter model. The finding that *ror* alleles inactivate the function of different *mlo* alleles is expected if resistance in *mlo* plants is due to a loss of the negative regulator *Mlo* (Table 3).

The strongest argument that the *Ror* genes represent positive regulatory genes and not effector components at the end of a putative signaling pathway is based on the fact that both *Ror1* and *Ror2* are required for high-level, constitutive CWA formation (Table 5). Until now, our study has been confined to the quantitative analysis of plant/fungus interaction sites on genotypes carrying either *ror mlo*, *Ror Mlo*, or *Ror mlo* allele combinations (Figure 3). In context with the proposed negative regulatory function of *Mlo* in a defense response, it would be interesting to test whether a *ror Mlo* genotype increases the penetration frequency of CWAs even above the level observed in susceptible *Mlo Ror* plants (Figure 3). Such a "supersusceptibility" has been reported in compatible barley/powdery mildew interactions after application of α-aminooxy-β-phenylproprionic acid–inhibiting phenylalanine ammonia-lyase (Carver et al., 1992).

We have recently identified two genes, *Rar1* and *Rar2*, that are required for the function of the race-specific resistance gene *Mla-12* (previous gene designation *Nar-1* and *Nar-2*; Freialdenhoven et al., 1994). The *mlo* resistance is retained in plants with defective *Rar* genes, suggesting that the *Rar* genes are not required for *mlo* function (A. Freialdenhoven, unpublished results). Identification of the *Ror* genes allows us to address the question of whether defects in *Ror* abolish the function of the various race-specific resistance genes to powdery mildew in barley. However, such experiments require DNA markers that are linked to the *Ror* genes for marker-assisted selection of the appropriate genotypes. Thus, the availability of mutants required for the function of either a race-specific (*Mla-12*) or non-race-specific (*mlo*) resistance gene allows us to direct our attention to questions concerning separate, shared, or common pathways for resistance gene function to the same pathogen.

METHODS

Plant Material

The *mlo-5* allele has been isolated through ethyl methanesulfonate (EMS) mutagenesis in *Hordeum vulgare* subsp. *vulgare* cultivar Carlsberg II, as described by Jørgensen (1983). The *mlo* backcross (BC) lines in the genetic background of cultivar Ingrid were kindly provided by James McKey (Uppsala, Sweden). They were generated through seven backcrosses with cultivar Ingrid, followed by at least six selfings. The generation of the *mlo-5* backcross line in cultivar Pallas has been described by Kølster et al. (1986). The allele *mlo-3* has been induced by γ-rays in the genetic background of cultivar Malteria Heda, whereas the allele *mlo-11* has been collected from a natural habitat (Jørgensen, 1983). Mutant plants were pollinated with pollen derived from male parent plants, as listed in Tables 1 to 4. F_1 progeny and F_2 plants generated by selfings were grown to maturity in the greenhouse. We have designated the *ror1* alleles in mutants A39, A89, C36, C69, and C88 *ror1-1* to *ror1-5* and the defective allele in A44 *ror2*.

Mutagenesis

Seed of the backcross line BCIngrid *mlo-5* were presoaked in water at 4°C overnight. Subsequently, they were treated with a solution of 1% (v/v) EMS (Sigma, Munich, Germany) or 10^{-3} M NaN_3 for 2 hr. EMS treatment was performed in 0.1 M sodium phosphate buffer, pH 7.4, using a volume of ~1 mL per seed. NaN_3 treatment was performed in 0.1 M potassium phosphate buffer, pH 3.0, under extensive aeration. The treated M_1 kernels were rinsed thereafter in water for

12 hr, sown in soil, and grown to maturity in the greenhouse. One spike was harvested from each M_1 plant. Therefore, mutants derived from different M_1 spikes must represent independent mutational events. Intact M_1 spikes were sown in a peat–clay mixture, and the 7-day-old M_2 seedlings were tested for susceptibility in inoculation experiments, as described below.

Tests for Resistance

Powdery mildew inoculations were performed with *E. g.* f sp *hordei* isolates K1, A6, and R146. K1 is a field isolate collected near Cologne (Germany) that has been used in previous studies (Hinze et al., 1991). Isolate A6 and isolate R146 were provided by H.P. Jensen (Risø National Laboratory, Risø, Denmark). Tests for resistance were performed in a phytochamber at 15°C, 70% relative humidity, and a photoperiod of 16 hr. F_1 and F_2 individuals were sown in a peat–clay mixture, and primary leaves were inoculated at day 7 on the adaxial and abaxial surfaces with spore densities of 100 to 200 per cm^2. Plants were scored for resistance 7 days after inoculation.

DNA Fingerprint Analysis

Restriction fragment length polymorphism markers bAL88/2, bAP91, Bmy1, and bBE54 (Hinze et al., 1991) were used to test the genotype origin of the isolated susceptible M_2 individuals. The first three markers detect each a single-copy locus, whereas the latter marker detects at least 10 loci on different barley chromosomes. Markers bAL88/2 and bAP91 are tightly linked to the *Mlo* locus and are located within the introgressed chromosomal segment of the BCIngrid *mlo-5* line (Hinze et al., 1991). DNA from the resistant BCIngrid *mlo-5* line, the isolated susceptible M_2 mutants, and the near-isogenic susceptible *Mlo* cultivar Ingrid were included in the restriction fragment length polymorphism analysis. Hybridization filters were prepared using methods described by Gebhardt et al. (1989), using a high-resolution polyacrylamide-based electrophoretic separation of DNA fragments. Each of the 13 inspected loci in the susceptible mutants showed identical hybridization patterns when compared with the BCIngrid *mlo-5* line. As expected, the mutants and the BCIngrid *mlo-5* line were polymorphic in comparison with DNA from cultivar Ingrid (*Mlo*) for loci bAL88/2 and bAP91.

Microscopic Analysis and Histochemical Tests

Barley primary leaves were harvested at the indicated time points, fixed, and cleared in alcoholic lactophenol (ethanol-lactophenol 2:1 [v/v]). The solution was changed twice after 1 day and then after an additional 2 days. Specimens were stored in stoppered tubes at room temperature in the dark. Fungal structures were inspected by bright-field microscopy (Leitz Dialux 20; Leica Instrs. GmbH, Cologne, Germany) using segments from the middle part of the leaf. The segments were stained for 5 sec in Coomassie blue (0.6% [w/v] Coomassie Brilliant Blue R 250 [Sigma] in methanol), rinsed in distilled water, and mounted in 50% (v/v) glycerol.

The presence of phenolics in cell wall appositions was tested by incident-light fluorescence microscopy, using a mercury vapor lamp (model No. HBO 50 W; Osram, Niederau, Germany), 450-nm exciter filter, 510-nm dichroic mirror, and >515-nm barrier filter.

Detection of β-1,3-glucans in the cell wall appositions was performed mainly as described by Stone et al. (1984) and Bayles et al. (1990). Briefly, cleared primary leaves were treated with periodic acid and Schiff's reagent to degrade β-1,4-glucans and to mask autofluorescence of phenolic compounds. Leaf segments were then mounted in 0.1 M potassium phosphate buffer, pH 11, containing the highly specific fluorochrome sirofluor (Biosupplies, Parkville Victoria, Australia) at a final concentration of 0.25 mg/mL. Incident-light fluorescence microscopy was performed as described above but with a 360-nm exciter filter, 400-nm dichroic mirror, and >430-nm barrier filter.

The frequency of spontaneous cell wall appositions in epidermal cells was determined by fluorescence microscopy, as described above for the detection of phenolics. Plants were grown under mildew-free conditions, and primary leaves were fixed 23 days after sowing. The different epidermal cell types (Koga et al., 1990) were scored independently.

ACKNOWLEDGMENTS

We thank James McKey for kindly providing seed from several *mlo* BC lines. Sonja Töpsch, Barbara Förster, Manfred Vater, and the late Heilke Klatt are acknowledged for their expert technical assistance. We thank Jörg Nähring for his kind assistance in computing. Marietta Wolter, Edda von Röpenack, Rainer Büschges, and Thomas Lahaye are acknowledged for critical comments during manuscript preparation. We are grateful to Francesco Salamini for his kind willingness to provide greenhouse facilities for plant growth and a phytochamber for inoculation experiments at the Max Planck Institut für Züchtungsforschung, Cologne, Germany. This work was supported by a grant from the Deutsche Forschungsgemeinschaft Schwerpunktprogramm Molekulare Phytopathologie to P.S.-L.

Received August 7, 1995; accepted October 31, 1995.

REFERENCES

Aist, J.R. (1976). Papilla and related wound plugs of plant cells. Annu. Rev. Phytopathol. **14,** 145–163.

Aist, J.R., and Israel, H.W. (1986). Autofluorescent and ultra-violet absorbing components in cell walls and papillae of barley coleoptiles and their relationship to disease resistance. Can. J. Bot. **64,** 266–272.

Bayles, C.J., Ghemawat, M.S., and Aist, J.R. (1990). Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew in an *mlo* barley mutant. Physiol. Mol. Plant Pathol. **36,** 63–72.

Boyd, L.A., Smith, P.H., Foster, E.M., and Brown, J.K.M. (1995). The effect of allelic variation at the *Mla* resistance locus in barley on the early development of *Erysiphe graminis* f sp *hordei.* Plant J. **7,** 959–968.

Carver, T.L.W., Zeyen, R.J., Robbins, M.P., and Dearne, G.A. (1992). Effects of the PAL inhibitor, AOPP, on oat, barley and wheat cell responses to appropriate and inappropriate formae speciales of *Erysiphe graminis* DC. Physiol. Mol. Plant Pathol. **41,** 397–409.

Century, K.S., Holub, E.B., and Staskawicz, B.J. (1995). *NDR1,* a locus of *Arabidopsis thaliana* that is required for disease resistance

to both a bacterial and a fungal pathogen. Proc. Natl. Acad. Sci. USA **92,** 6597–6601.

Dangl, J.L. (1995). Pièce de résistance: Novel classes of plant disease resistance genes. Cell **80,** 363–366.

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994). Arabidopsis mutants simulating disease resistance response. Cell **77,** 565–577.

Flor, H.H. (1971). Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9,** 275–296.

Freialdenhoven, A., Scherag, B., Hollricher, K., Collinge, D.B., Thordal-Christensen, H., and Schulze-Lefert, P. (1994). *Nar-1* and *Nar-2*, two loci required for Mla_{12}-specified race-specific resistance to powdery mildew in barley. Plant Cell **6,** 983–994.

Gebhardt, C., Ritter, E., Debener, T., Schachtschabel, U., Balkemeier, B., Uhrig, H., and Salamini, F. (1989). RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. **78,** 65–75.

Gold, R.E., Aist, J.R., Hazen, B.E., Stolzenburg, M.C., Marshall, M.R., and Israel, H.W. (1986). Effects of calcium nitrate and chlortetracycline on papilla formation, *mlo* resistance, and susceptibility of barley to powdery mildew. Physiol. Mol. Plant Pathol. **29,** 115–129.

Görg, R., Hollricher, K., and Schulze-Lefert, P. (1993). Functional analysis and RFLP-mediated mapping of the *Mlg* resistance locus in barley. Plant J. **3,** 857–866.

Greenberg, J.T., and Ausubel, F.M. (1993). *Arabidopsis* mutants compromised for the control of cellular damage during pathogenesis and aging. Plant J. **4,** 327–341.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. Cell **77,** 551–563.

Hammond-Kosack, K.E., Jones, D.A., and Jones J.D.G. (1994). Identification of two genes required in tomato for full *Cf*-9–dependent resistance to *Cladosporium fulvum*. Plant Cell **6,** 361–374.

Hinze, K., Thompson, R.D., Ritter, E., Salamini, F., and Schulze-Lefert, P. (1991). Restriction fragment length polymorphism–mediated targeting of the *mlo* resistance locus in barley (*Hordeum vulgare*). Proc. Natl. Acad. Sci. USA **88,** 3691–3695.

Jende-Strid, B. (1978). Mutation frequencies obtained after sodium azide treatment in different barley varieties. Barley Genet. Newslett. **8,** 55–57.

Johal, G.S., Gray, J., Cruis, D., and Briggs, S.P. (1995). Convergent insights into mechanisms determining disease and resistance response in plant–fungal interactions. Can. J. Bot. **73,** 468–474.

Jones, J.D.G. (1994). Paranoid plants have their genes examined. Curr. Biol. **4,** 749–751.

Jørgensen, J.H. (1977). Spectrum of resistance conferred by *Mlo* powdery mildew resistance genes in barley. Euphytica **26,** 55–62.

Jørgensen, J.H. (1983). Experience and conclusions from the work at RISØ on induced mutations for powdery mildew resistance in barley. In Induced Mutations for Disease Resistance in Crop Plants II (Vienna: International Atomic Energy Agency), pp. 73–87.

Jørgensen, J.H. (1994). Genetics of powdery mildew resistance in barley. Crit. Rev. Plant Sci. **13,** 97–119.

Jørgensen, J.H., and Mortensen, K. (1977). Primary infection by *Erysiphe graminis* f. sp. *hordei* of barley mutants with resistance genes in the *mlo* locus. Phytopathology **67,** 678–685.

Koga, H., Bushnell, W.R., and Zeyen, R.J. (1990). Specificity of cell type and timing of events associated with papilla formation and the hypersensitive reaction in leaves of *Hordeum vulgare* attacked by *Erysiphe graminis* f sp *hordei*. Can. J. Bot. **68,** 2344–2352.

Kølster, P., Munk, L., Soglen, O., and Løhde, J. (1986). Near-isogenic barley lines with genes for resistance to powdery mildew. Crop Sci. **25,** 903–907.

Lundqvist, U. (1991). Swedish mutation research in barley with plant breeding aspects. In Plant Mutation Breeding for Crop Improvement (Vienna: International Atomic Energy Agency), pp. 135–148.

Lyngkjaer, M.F., Jensen, H.P., and Østergård, H. (1995). A Japanese powdery mildew isolate with exceptionally large infection efficiency on *mlo*-resistant plants. Plant Pathol. **44,** 786–790.

Mayama, S., and Shishiyama, J. (1978). Localized accumulation of fluorescent and UV-absorbing compounds at penetration sites in barley leaves infected with *Erysiphe graminis hordei*. Physiol. Mol. Plant Pathol. **13,** 347–354.

Salmeron, J.M., Barker, S.J., Carland, F.M., Mehta, A.Y., and Staskawicz, B.J. (1994). Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. Plant Cell **6,** 511–520.

Smart, M.G., Aist, J.R., and Israel, H.W. (1985). Structure and function of wall appositions. 1. General histochemistry of papillae in barley coleoptiles attacked by *Erysiphe graminis* f sp *hordei*. Can. J. Bot. **64,** 793–801.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G., and Jones, J.D.G. (1995). Molecular genetics of plant disease resistance. Science **268,** 661–667.

Stone, B.A., Evans, N.A., Bonig, I., and Clarke, A.E. (1984). The application of sirofluor, a chemically defined fluorochrome from aniline blue for the histochemical detection of callose. Protoplasma **122,** 191–195.

Torp, J., and Jørgensen, J.H. (1986). Modification of barley powdery mildew resistance gene Mla_{12} by induced mutation. Can. J. Genet. Cytol. **28,** 725–731.

Walbot, V., Hoisington, D.A., and Neuffer, M.G. (1983). Disease lesion mimic mutants. In Genetic Engineering of Plants, T. Kosuge, C.P. Merdith, and A. Hollaender, eds (New York: Plenum Press), pp. 431–442.

Wolter, M., Hollricher, K., Salamini, F., and Schulze-Lefert, P. (1993). The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defense mimic phenotype. Mol. Gen. Genet. **239,** 122–128.

Zeyen, R.J., and Ahlstrand, G.G. (1993). X-ray microanalysis of frozen-hydrated, freeze-dried, and critical point dried leaf specimens: Determination of soluble and insoluble chemical elements at *Erysiphe graminis* epidermal cell papilla sites in barley isolines containing *Mlo* and *mlo* alleles. Can. J. Bot. **71,** 284–296.

Zeyen, R.J., and Bushnell, B.R. (1979). Papilla response of barley epidermal cells caused by *Erysiphe graminis*: Rate and method of deposition determined by microcinematography and transmission electron microscopy. Can. J. Bot. **57,** 898–913.