The Plant Cell, Vol. 6, 983–994, July 1994 © 1994 American Society of Plant Physiologists

Nar-1 and *Nar-2*, Two Loci Required for *Mla*$_{12}$-Specified Race-Specific Resistance to Powdery Mildew in Barley

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Previously isolated susceptible host mutants were used in a genetic and functional study of the resistance response of barley specified by resistance gene *Mla*$_{12}$ to the fungal pathogen *Erysiphe graminis* f sp *hordei*. Mutant M66 represents a defective allele of *Mla*$_{12}$, whereas M22, M82, and M100 represent mutations in loci unlinked to *Mla*$_{12}$. Intermutant crosses of the latter three show that susceptibility in M82 and M100 is caused by allelic, recessive mutations that define the *Nar-1* gene (necessary for *Mla*$_{12}$ resistance gene 1), whereas the semidominant mutation in M22 defines a second unlinked locus, *Nar-2*. We show that both genes are required for resistance specified by *Mla*$_{12}$ in different genetic backgrounds of barley. *Nar-1* maps on barley chromosome 2 within an ~6-centimorgan restriction fragment length polymorphism interval: this is 0.5 centimorgans from the anthocyanin pigmentation gene *Ant2*. Quantitative cytological analysis showed that functional alleles of *Mla*$_{12}$, *Nar-1*, and *Nar-2* are required for triggering a cell death reaction of attacked host cells at early stages during infection. Functional alleles of all three genes were also required for high-level transcript accumulation of barley defense-related genes that encode chitinase, peroxidase, and pathogenesis-related protein-1. The data support the hypothesis that host cell death and high-level accumulation of defense-related gene transcripts, which are under common control of *Mla*$_{12}$, *Nar-1*, and *Nar-2*, are crucial events of race-specific resistance to powdery mildew.

INTRODUCTION

Erysiphe graminis f sp *hordei* is an obligate biotrophic fungus that exclusively attacks epidermal leaf tissue of its host, barley. The outcome of this plant–fungus interaction, race-specific resistance or susceptibility, normally follows the rules of the gene-for-gene concept (Flor, 1971): resistance is specified by and dependent on the presence of two complementary genes, one from the host and one from the fungal pathogen. The complementary loci have been termed operationally a resistance and an avirulence gene, respectively. In barley, a multitude of race-specific powdery mildew resistance genes have been identified and characterized; they are predominantly inherited as dominant or semidominant acting traits (Wiberg, 1974; Søgaard and Jørgensen, 1988). Because all available biochemical data indicate that a resistance response is an active, complex, and coordinate process executed by the plant (Collinge and Slusarenko, 1987; Bowles, 1990; Dixon and Harrison, 1990; Collinge et al., 1994), it is of central interest to determine how resistance gene action is coupled to a complex series of biochemical defense events and whether different resistance genes trigger specific biochemical defense pathways. A genetic approach to these problems concerns the identification of genes required for expression of the resistant phenotype in addition to a race-specific resistance gene. The availability of appropriate mutants, the well-characterized genetics of the barley–powdery mildew system, the cytological peculiarities of this interaction, and the availability of molecular probes from defense-related genes allow us to address questions related to resistance gene function.

After contact of a fungal spore with the wax layer of a barley leaf, the following fungal structures differentiate successively within the first 24 hr (Ellingboe, 1972); the primary germ tube, the appressorial germ tube, and the haustorium, which invaginates an epidermal plasma membrane. Formation of aerial mycelium and sporulation represent late differentiation events between 4 to 7 days postinoculation. The establishment of the haustorium represents a key step for successful fungal reproduction because only this organ has direct contact to a host cell and is believed to supply the plant nutrients for further fungal growth. At early time points, when the interaction can be envisaged as an interaction between two single cells, quantitative cytological recordings of incompatible interactions have revealed putative host cell resistance responses conferring arrest of fungal development at distinct stages (Kita et al., 1981; Koga et al., 1988, 1990). Two of these putative host cell resistance responses are easily detected early during infection:

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a subcellularly restricted, highly localized cell wall reinforcement at attempted penetration sites (indicated by a wall apposition, which is often termed papilla) and/or the activation of cell death of the attacked epidermal cell (indicated by whole-cell autofluorescence, which is often termed single-cell hypersensitive response; Koga et al., 1988; Wolter et al., 1993). However, to date, only correlative data support a crucial role of these single-cell events in powdery mildew defense. It seems likely that, in addition to microscopically observed responses in the attacked epidermal cell, race-specific powdery mildew defense also includes host cells surrounding an interaction site at later time points because localized necrotic/chlorotic spots are usually observed with the naked eye in an incompatible interaction.

The *Mla*$_{12}$ resistance allele on chromosome 5 of barley represents one of several identified resistance "alleles" at the *Mla* locus, which specifies resistance only to powdery mildew isolates containing a complementary avirulence gene (Giese et al., 1981; Wise and Ellingboe, 1985). Previous work using near-isogenic barley lines has shown that *Mla*$_{12}$ triggers cell death of attacked epidermal cells concomitant with the establishment of the fungal haustorium early during infection (Görg et al., 1993). In a mutagenesis experiment involving mutagenized *Mla*$_{12}$-resistant barley seeds from the doubled-haploid line Sultan-5, Torp and Jørgensen (1986) isolated 25 susceptible M_2 individuals. Genetic backcrosses of the mutants with parental-resistant and parental-susceptible genotypes revealed that three of these susceptible mutants (M22, M82, and M100) are due to monogenically inherited mutations in loci unlinked to *Mla*$_{12}$, whereas all other mutants represent either mutations within or closely linked to *Mla*$_{12}$ (Jørgensen, 1988). The three mutants representing mutations in loci unlinked to *Mla*$_{12}$ are particularly suited to study *Mla*$_{12}$-mediated resistance gene function.

In this study, we provide a genetic and functional analysis of race-specific resistance controlled by *Mla*$_{12}$. This analysis is based on defective alleles from three genes that are all required for powdery mildew resistance. We show that mutations in each of these genes abolished activation of a cell death response in the attacked epidermal cell; this response was observed at an early time point in the *Mla*$_{12}$-resistant wild-type plant when the interaction was physically confined to two single cells. Our observation that the same three genes are also required for high-level transcript accumulation of barley defense-related genes is discussed in view of a spatially extended and temporarily late defense response. Thus, the mutational approach allows uncoupling of different components of the defense response.

RESULTS

The race-specific resistance response specified by resistance gene *Mla*$_{12}$ in the barley wild-type cultivar Sultan-5 with avirulent powdery mildew isolates is accompanied by very small necrotic leaf lesions. These lesions appeared around the sites of attempted infection late (7 days) after inoculation with avirulent powdery mildew isolates, for example, A6, and showed no detectable mycelium, as shown in Figure 1A. Growth of fungal mycelium and sporulation of isolate A6 occurred on all mutants (M66, M22, M100, and M82) with genotypes derived from the doubled-haploid *Mla*$_{12}$-resistant line Sultan-5 but conferred distinguishable infection types at late time points after inoculation (Figures 1B to 1E). Note that large necrotic and/or chlorotic lesions typically appeared around infection sites only in mutants M22 and M82 at this late time point. Preliminary genetic analysis of the mutants indicated that the M66 phenotype is caused by a mutation in the *Mla*$_{12}$ resistance gene, whereas M22, M82, and M100 phenotypes are due to monogenically inherited mutations unlinked to *Mla*$_{12}$ (Torp and Jørgensen, 1986; Jørgensen, 1988). The genetic data also indicated that the mutant genes act recessively with the exception of the mutant gene in M22, which is inherited as a semidominant trait.

Intermutant Crosses

In this study, intermutant crosses were performed only with mutants M22, M82, and M100. The infection phenotypes of the resulting F_1 and F_2 individuals after selfing have been analyzed after inoculation with isolate A6, as shown in Table 1. Because five F_1 individuals and all 229 F_2 individuals derived from the cross M100 × M82 yielded only susceptible infection types, we concluded that M82 and M100 represent noncomplementing recessive mutations and define two mutant alleles of the same gene. This result was surprising because the phenotypes of each mutant could be distinguished clearly from each other (Figures 1C and 1D). In contrast, the intermutant crosses M100 × M22 and M82 × M22 segregated resistant individuals in the F_2 population, indicating that M22 carries a mutation in a locus distinct from that defined for M82/M100. The susceptible F_1 phenotypes observed in these two crosses and the segregation ratio of susceptible and resistant F_2 individuals are compatible with previous data (Jørgensen, 1988), indicating that the mutation in M22 acts as a semidominant trait ($P > 0.5$ based on a χ^2 calculation of an expected 13:3 segregation ratio). Therefore, the three mutants M82, M100, and M22 enabled us to identify two genes *Nar-1* and *Nar-2* (necessary for *Mla*$_{12}$ resistance), whose respective wild-type alleles are required for phenotypic expression of *Mla*$_{12}$-specified race-specific powdery mildew resistance.

Action of M22, M82, and M100 in Different Genetic Backgrounds

Crosses were performed to address whether the mutant alleles in M22, M82, and M100 retain their function when transferred into different genetic backgrounds, as shown in Tables 2 and 3. In addition, the crosses were designed to

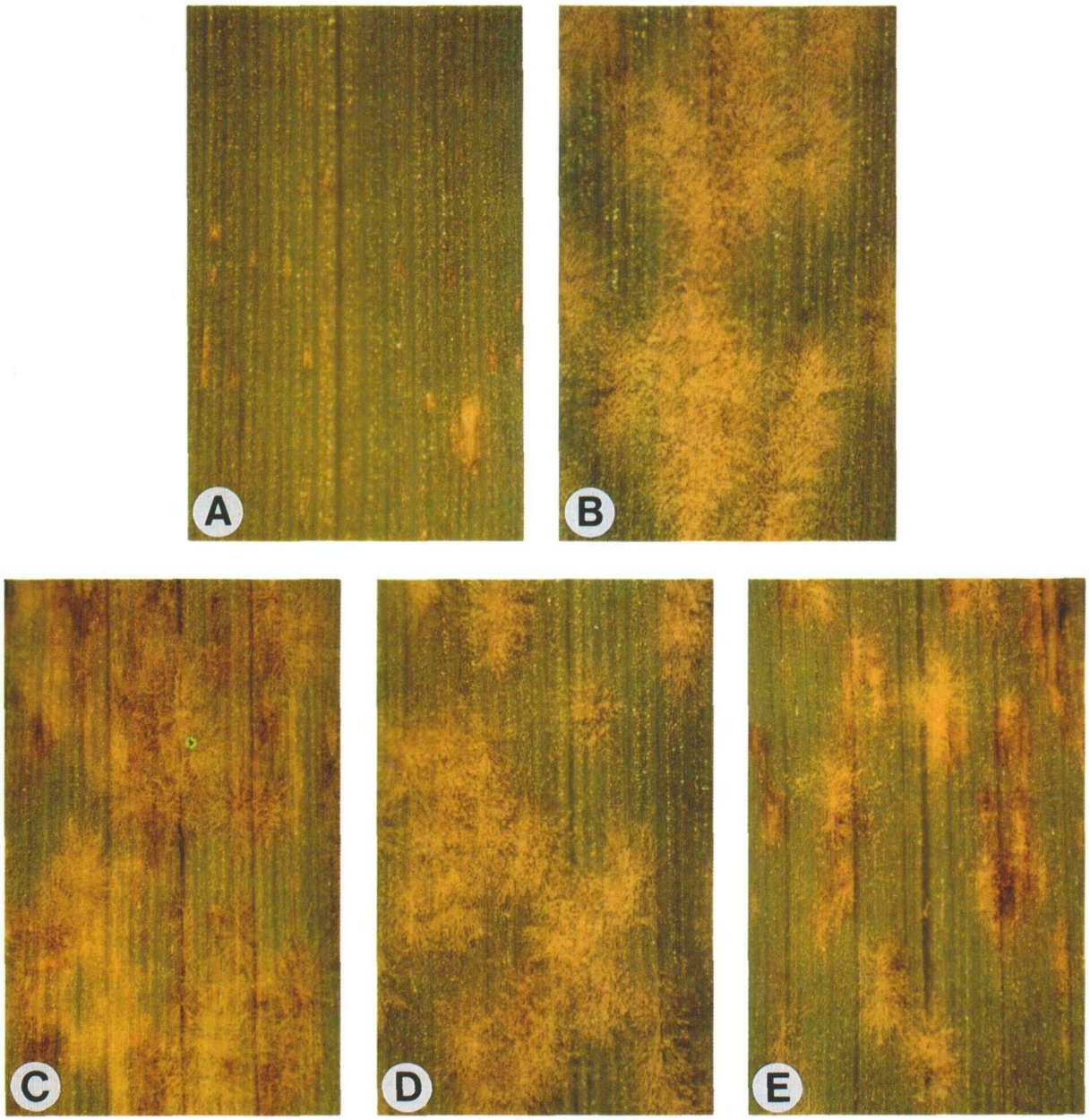

Figure 1. Phenotypes of the *Mla*$_{12}$-Resistant Wild-Type and Susceptible Mutant Genotypes 7 Days after Inoculation with Powdery Mildew Isolate A6.

(A) and **(B)** Shown are phenotypes of 14-day-old primary barley leaves of the *Mla*$_{12}$-resistant wild-type cultivar Sultan-5 in **(A)** and the susceptible mutant M66 in **(B)**, which carries a mutation in resistance gene *Mla*$_{12}$.

(C), **(D)**, and **(E)** Shown are phenotypes of the three susceptible mutants: M82 in **(C)**, M100 in **(D)**, and M22 in **(E)**. Susceptibility in M82, M100, and M22 is caused by monogenically inherited mutations unlinked to *Mla*$_{12}$ (Jørgensen, 1988). All mutants were isolated from an M_2 population derived from mutagenized seeds of *Mla*$_{12}$-resistant, doubled-haploid cultivar Sultan-5. Powdery mildew isolate A6 carries the *Mla*$_{12}$ avirulence gene and is avirulent on *Mla*$_{12}$-resistant wild-type plants. Although all of the mutants allow sporulation of the fungus, each mutant confers a characteristic infection type at this late time point after inoculation. Note that only mutants M82 and M22 show extensive necrosis and/or chlorosis surrounding infection sites.

Table 1. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses between Mutants M22, M82, and M100

	F_1			F_2			
	Susc.						
	Moderate	Severe	Res.	Susc.	Res.	χ^2 (13:3)	χ^2 (3:1)
M100 × M82	0	5	0	229	0		
M100 × M22	5	0	0	239	59	$P > 0.5$	$P < 0.05$
M82 × M22	5	0	0	212	53	$P > 0.5$	$P < 0.10$

Susc., susceptible; Res., resistant.

provide the genetic material for genetic mapping of the mutant loci (see below). Because the mutant phenotypes are defined in the context of a functionally active Mla_{12} resistance allele in cultivar Sultan-5, we crossed each of the mutants with Mla_{12} backcross (BC) lines representing different barley genetic backgrounds (Mla_{12} BC Pallas, Mla_{12} BC Siri, and Mla_{12} BC Ingrid). In this particular type of cross, a simple monogenic segregation of the mutant alleles is expected in the resulting F_2 populations. Because the original wild-type cultivar from which the mutants are derived (Sultan-5) contains a pigmentation deficiency (*ant2*; anthocyanin deficiency in leaf sheath) as a phenotypic marker, it is expected that anthocyanin deficiency will also segregate as a monogenic recessive trait in the resulting F_2 populations from crosses of the mutants with the Mla_{12} BC-line cultivars.

The infection types of F_1 individuals and the segregation ratios of susceptible and resistant individuals in the F_2 populations from all crosses involving M82 and M100 confirmed that susceptibility is caused by monogenically inherited and recessively acting genes and showed that the respective wild-type gene is required for Mla_{12}-mediated resistance in the genetic backgrounds of cultivars Pallas, Siri, and Ingrid (Table 2). Inspection of the F_2 individuals for expression of the anthocyanin deficiency revealed the serendipitous observation that, with two exceptions (in cross M100 × Mla_{12} BC Pallas and M100 × Mla_{12} BC Ingrid), all of the susceptible F_2 individuals are anthocyanin deficient (*ant2*), whereas with few exceptions, all resistant individuals contain at least one *Ant2* allele. We concluded a tight genetic linkage of the *Ant2* locus with the mutant gene conferring susceptibility in M82 and M100 (*Nar-1*).

Crosses of M22 with the Mla_{12} BC lines revealed a mode of inheritance different from those involving M82 and M100 (Table 3). All F_1 individuals tested were susceptible, although of a lower infection type (type moderate), compared to homozygous M22 plants. Two classes of susceptible F_2 individuals could be differentiated, one class showing an infection type identical to homozygous M22 plants (type severe), the other class comparable to the infection type in F_1 plants (type moderate). The F_1 phenotypes and the F_2 segregation ratios of resistant and susceptible individuals strongly indicated that susceptibility in M22 is caused by a monogenically inherited semidominantly acting allele whose function is required for Mla_{12}-mediated resistance in the context of different genetic backgrounds. However, in contrast to the crosses including M82 and M100, no significant linkage could be observed between the locus segregating for resistance and/or susceptibility and the *Ant2* locus segregating for anthocyanin pigmentation. These data supported the results shown in Table 1 that M82 and M100 represent allelic mutations in one gene (*Nar-1*), which is different from that mutated in M22 (*Nar-2*). In particular, whereas *Nar-1* seems to be tightly linked to the *Ant2* locus, *Nar-2* seems to be unlinked to *Ant2*. Because the mutations conferring susceptibility in M22, M82, and M100 have been

Table 2. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses of Mutants M100 and M82 with Three Mla_{12} BC Line Cultivars

			F_2				
	F_1		Susc.		Res.		
	Susc.	Res.	*Ant2*	*ant2*	*Ant2*	*ant2*	χ^2 (3:1)
M100 (*ant2*) × Mla_{12} BC Pallas (*Ant2*)	0	5	1	18	62	3	$P > 0.5$
M100 (*ant2*) × Mla_{12} BC Siri (*Ant2*)	0	5	0	27	73	1	$P > 0.5$
M100 (*ant2*) × Mla_{12} BC Ingrid (*Ant2*)	0	5	1	21	64	2	$P > 0.9$
M82 (*ant2*) × Mla_{12} BC Pallas (*Ant2*)	0	5	0	23	75	1	$P > 0.5$
M82 (*ant2*) × Mla_{12} BC Siri (*Ant2*)	0	5	0	25	72	5	$P > 0.9$
M82 (*ant2*) × Mla_{12} BC Ingrid (*Ant2*)	0	5	ND	ND	ND	ND	

Susc., susceptible; Res., resistant; ND, not determined.

Table 3. Phenotypes and Segregation Ratios for the F_1 and F_2 Generations from Crosses of Mutant M22 with Three *Mla*$_{12}$ BC Line Cultivars

	F_1		F_2						
	Susc. Moderate	Res.	Susc. Moderate	Susc. Severe	Susc. (Moderate + Severe) *Ant2*	Susc. (Moderate + Severe) *ant2*	Res. *Ant2*	Res. *ant2*	χ^2 (1:2:1)
M22 (*ant2*) × *Mla*$_{12}$ BC Pallas (*Ant2*)	5	0	ND	ND	ND	ND	ND	ND	
M22 (*ant2*) × *Mla*$_{12}$ BC Siri (*Ant2*)	5	0	46	24	51	19	20	5	$P > 0.5$
M22 (*ant2*) × *Mla*$_{12}$ BC Ingrid (*Ant2*)	5	0	55	17	54	18	17	5	$P > 0.1$

Ant2 and *ant2*, wild-type and recessively inherited defect allele of a barley gene controlling anthocyanin pigmentation; Susc., susceptible; Res., resistant; ND, not determined.

shown previously to be unlinked to resistance gene *Mla*$_{12}$, all genetic data indicated that three genes (*Mla*$_{12}$, *Nar-1*, and *Nar-2*) are required for phenotype expression of *Mla*$_{12}$-specified, race-specific powdery mildew resistance in different genetic backgrounds of barley.

Genetic Mapping of *Nar-1*

An interval mapping procedure (Hoisington and Coe, 1989) has been used to identify the genome map position of *Nar-1*, as summarized in Figure 2. Seventeen randomly chosen susceptible F_2 individuals have been analyzed from each of the crosses of mutants M22, M82, and M100 with the *Mla*$_{12}$ BC lines. Individuals have been included from more than a single cross because only ~25% of randomly tested restriction fragment length polymorphism (RFLP) markers detect DNA polymorphisms between two arbitrary barley cultivars (Graner et al., 1990). Because the *Ant2* locus is probably located on barley chromosome 2 (Danne, 1931; Jende-Strid, 1984), we focused our search for linked molecular markers on those that are known to reside on chromosome 2 (Graner et al., 1991). As shown in Table 4, marker MWG87 detected no recombination event in any analyzed susceptible F_2 individual (corresponding to 204 analyzed F_2 gametes) derived from crosses including mutants M82 and M100. Only three recombination events were detected, each with markers MWG503 and cMWG694.

In contrast, MWG87 and cMWG694 both detected 20 recombination events in the genetically analyzed cross involving M22. Because the RFLP loci MWG503-MWG87-cMWG694 are spaced at ~3 centimorgans from each other and therefore define an interval of ~6 centimorgans on chromosome 2, according to the general barley RFLP map (Graner et al., 1991), we concluded that only the mutations causing susceptibility in M82 and M100 map within this chromosomal interval. The mapping data support the conclusion that susceptibility in M82 and M100 is due to allelic mutations that defined a single gene, *Nar-1*, which is tightly linked to the *Ant2* locus on chromosome 2 but unlinked to resistance gene *Mla*$_{12}$, which is known to be located on chromosome 5 (Jahoor et al., 1993). Furthermore, the data confirmed that M22 defines a third gene, *Nar-2*, required for race-specific mildew resistance that is unlinked to *Nar-1*.

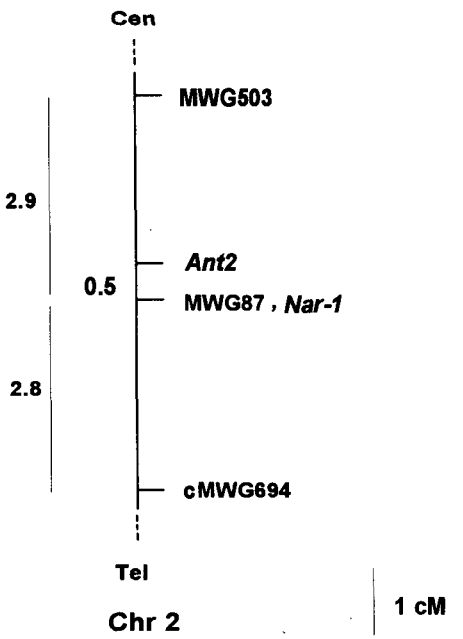

Figure 2. Genetic Map Position of *Nar-1* on Barley Chromosome 2.

The position of RFLP loci relative to *Nar-1* on barley chromosome 2 (Chr2) is based on data shown in Table 4. Genetic distances between MWG503, MWG87, and cMWG694 are based on data derived from the general barley RFLP map (Graner et al., 1991) and were calculated using multipoint estimates. If the distances are calculated using data shown in Table 4 on the basis of two-point estimates, similar numerical values are obtained. The distance between the *Ant2* locus and *Nar-1* was calculated on the basis of two-point estimates. cM, centimorgan; Cen, centromere; Tel, telomere.

Table 4. Number of Recombination Events between RFLP Markers MWG503, MWG87, and cMWG694 and the Locus *Nar-1* as Evaluated in 17 Susceptible F_2 Individuals from Crosses of Mutants M82, M100, and M22 with Three Resistant Mla_{12} BC Line Cultivars

	M82			M100			M22		
	× Mla_{12} BC Ingrid	× Mla_{12} BC Pallas	× Mla_{12} BC Siri	× Mla_{12} BC Ingrid	× Mla_{12} BC Pallas	× Mla_{12} BC Siri	× Mla_{12} BC Ingrid	× Mla_{12} BC Pallas	× Mla_{12} BC Siri
MWG503	0	0	NI	2	1	NI	ND	ND	NI
MWG87	0	0	0	0	0	0	ND	ND	20
cMWG694	0	NI	3	0	NI	0	ND	ND	20

NI, noninformative marker; ND, not determined.

The tight genetic linkage of *Ant2* and *Nar-1* prompted us to search for additional rare recombinants between the two genes. For this purpose, the F_2 population size of the mapping crosses involving mutants M82 and M100 was increased substantially. Of 2600 F_2 individuals, a total of 639 susceptible seedlings were identified in the various crosses after inoculation with powdery mildew isolate A6. A score for presence of anthocyanin pigmentation (*Ant2*) among the susceptible F_2 class identified only eight *Ant2* individuals, indicating recombination events between *ant2* and *nar-1*. Plants were grown to maturity, and DNA from each of the resulting F_3 families was analyzed with probes MWG503, MWG87, and cMWG694 (data not shown). The genetic analysis inferred a position of *Ant2* centromeric to *Nar-1* at a distance of ~0.5 centimorgans between loci MWG503 and MWG87 (Figure 2).

Quantitative Cytological Analysis

Quantitative cytological recordings were performed from time course experiments at early stages during the infection process, including the Mla_{12}-resistant wild-type plant and susceptible mutants M66, M22, M82, and M100. The parameters cell wall appositions (CWA; papillae and associated haloes) without cell death and cell death of attacked epidermal cells (single-cell hypersensitive response) represent recorded plant reactions only from the epidermal cell first attacked, as shown in Figures 3A and 3B. In parallel, undifferentiated and differentiated haustoria served to monitor fungal development in each of the analyzed plant genotypes (Figures 3C and 3D). In Mla_{12}-resistant wild-type plants, CWA formation could be detected as early as 15 hr postinoculation after formation of fungal appressoria (Figure 3A) but prior to initial haustorium formation (Figure 3C). The proportion of attacked cells containing only a CWA increased to 40% at 24 hr postinoculation and dropped thereafter to ~20%, presumably a result of fungal penetration of part of the CWAs. The onset of plant cell death of the attacked epidermal cell was detected at 36 and 48 hr postinoculation (Figure 3B) and therefore occurred after the onset of CWA formation. At these time points, the fungus had already penetrated and established haustoria in the majority of attacked epidermal cells. Note, however, that the actual number of differentiated fungal haustoria was masked at 48 hr postinoculation through the simultaneous onset of plant cell death, which in many cases is accompanied by a rupture of haustorial membranes (Figure 3D). This makes it impossible to quantitate precisely the frequency of differentiated haustoria at 48 hr postinoculation (or later) in the Mla_{12}-resistant wild-type plant.

No significant differences with respect to timing and frequency of CWA formation are detectable among the Mla_{12}-resistant wild-type plant and susceptible mutants M66, M82, and M100 (Figure 3A). Unexpectedly, however, mutant M22 showed a significant reduction in the efficiency of CWA formation in response to fungal attack. Mutant alleles in Mla_{12}, *Nar-1*, or *Nar-2* (mutants M66, M82 and M100, M22) completely abolished the ability of the plant to trigger a cell death reaction in response to fungal attack (Figure 3B). From these studies, we concluded that defective alleles of Mla_{12} and *Nar-1* selectively affect the ability of the plant to trigger an early cell death response of the initially attacked cell, whereas the defective allele in *Nar-2* affects both plant cell death and the ability to establish a CWA prior to initial haustorium formation.

The absence of plant cell death is not a result of delayed development of the fungus in the mutant plant genotypes because differentiated fungal haustoria were detected for each mutant at the majority of infection sites (Figure 3D). Although the overall developmental time frame of the fungus was similar in plants with resistant wild-type and mutant genotypes, fungal development may proceed slightly faster in the mutants. Thus, maximum numbers of undifferentiated haustoria were detected in all mutants at 18 hr postinoculation, rather than at 21 hr postinoculation in the resistant wild-type plant. In addition, the onset of the haustorial differentiation process in mutants M66, M82, and M100 was already observed at 21 hr postinoculation, rather than at 24 hr postinoculation in the resistant wild-type plant and mutant M22.

Transcript Accumulation of Defense-Related Genes

Transcript accumulation in response to pathogen attack of "defense-related genes" has been repeatedly described for various plant–pathogen interactions (Bowles, 1990; Collinge et al., 1994), including the barley–powdery mildew system (Bryngelsson and Collinge, 1992; Gregersen et al., 1993). To

assess the potential role of barley defense-related genes in Mla_{12}-mediated powdery mildew resistance, transcript accumulation was studied in time course experiments at early time points during infection of resistant wild-type and all susceptible mutant genotypes, as shown in Figure 4. It should be noted that at these early stages in the infection process, only traces of fungal mycelium can be detected under the microscope; these traces are not significantly different in wild-type resistant and susceptible mutant genotypes (see above). In addition, during this early phase, the interaction is physically confined to a single fungal cell attacking a single host epidermal cell.

The constitutive transcript levels detected with a barley glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Martin et al., 1989) served as an internal control for equal loading of total plant RNA from each genotype at each time point. In contrast to the constitutive glyceraldehyde-3-phosphate dehydrogenase transcript levels, two maxima of transcript accumulation are detected with a chitinase cDNA probe in all five genotypes tested: a first peak at 16 hr postinoculation and

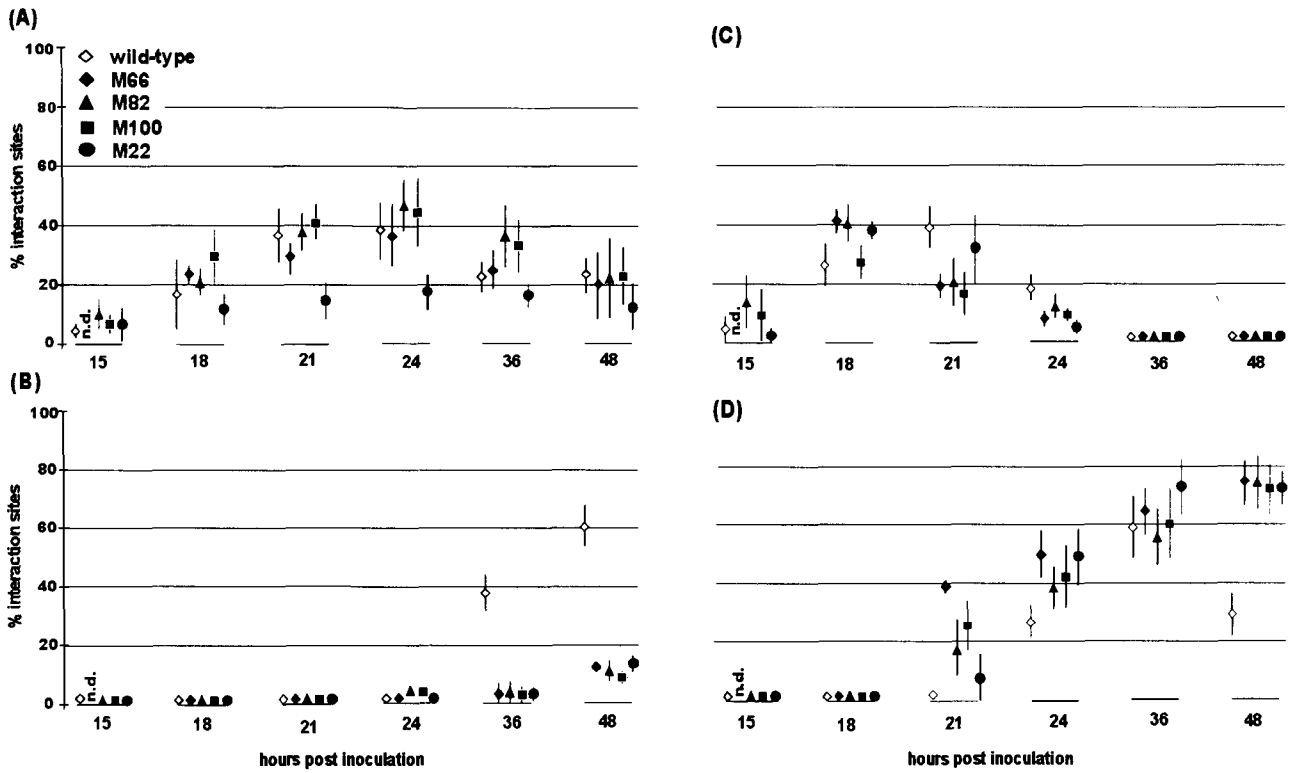

Figure 3. Putative Resistance Responses of the Host Epidermal Cell First Attacked at Early Time Points during Infection in the Mla_{12}-Resistant and -Susceptible Mutant Genotypes after Inoculation with A6 Spores.

Each data point represents the mean value and corresponding standard deviation based on an inspection of 50 single-cell interaction sites of primary leaf segments from at least five individuals per time point and per genotype.

(A) The frequency of interaction sites is restricted to a single host cell in which fungal attack is arrested within a cell wall apposition (papilla and associated halo). None of these interaction sites exhibited whole-cell autofluorescence (single-cell hypersensitive response). Therefore, data are based on an inspection of ~7500 interaction sites. Because the time course of fungal penetration into short and long epidermal cell types is different (Koga et al., 1990), only interaction sites of short epidermal cells have been evaluated. Note that only M22 shows a reduced frequency of cell wall apposition formation during the time course.

(B) Frequency of interaction sites restricted to a single host cell in which the attacked cell shows a characteristic yellow whole-cell autofluorescence after excitation with UV light (λ = 310 nm). Whole-cell autofluorescence is a reliable measure of cell death in the barley–powdery mildew interaction (Koga et al., 1990; Görg et al., 1993).

(C) Frequency of established nondifferentiated haustoria in the host cell first attacked during the first 48 hr after inoculation with A6 fungal spores.

(D) Frequency of identifiable differentiated haustoria (with fingerbuds) in the host cell first attacked during the first 48 hr after inoculation with A6 fungal spores. Note that the low value observed at 48 hr postinoculation in Mla_{12}-resistant wild-type plants is most likely a result of rupture of haustorial membranes as a consequence of host cell death at this time point. In many cases, fragments of haustorial membranes are still identifiable in this genotype, but they have not been recorded as differentiated haustoria.

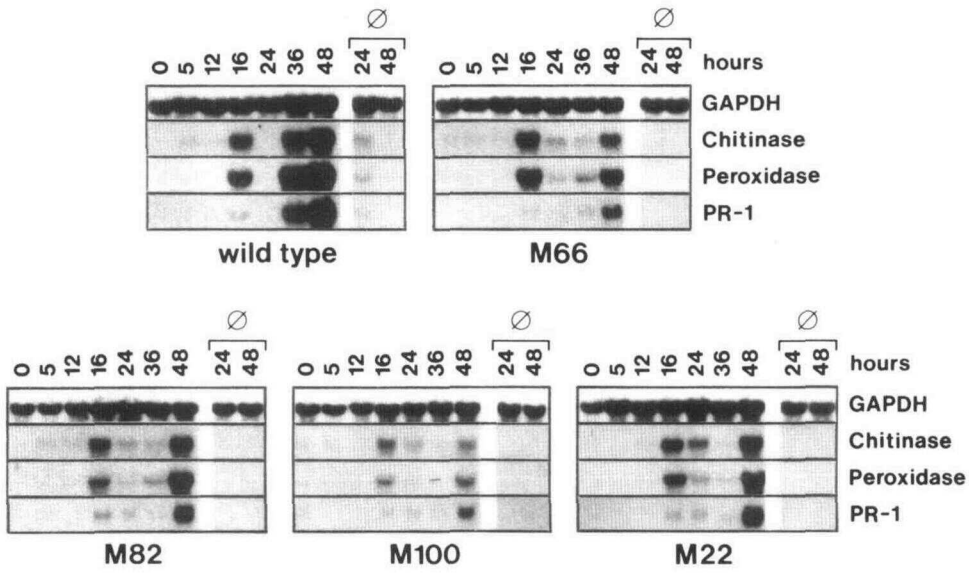

Figure 4. Time Course of Transcript Accumulation Early after Inoculation with Powdery Mildew Isolate A6 in Primary Leaf Segments of Mla_{12}-Resistant Wild-Type and Mutant Genotypes.

Data shown are representative of four replicate experiments. Each lane contains 15 μg of total RNA extracted from primary leaf segments from 15 individuals per time point and per genotype. Filters were subsequently hybridized with cDNA probes encoding barley glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Martin et al., 1989), barley chitinase (B. Gummesson, T. Bryngelsson, and P.L. Gregersen, unpublished results), barley peroxidase (Thordal-Christensen et al., 1992), and barley PR-1 (Bryngelsson et al., 1994). Ø, noninoculated controls.

a second peak at 48 hr postinoculation. Whereas the amount of transcript at approximately the 16-hr peak was obviously similar in all analyzed genotypes, significantly less transcript accumulated at 48 hr postinoculation in all susceptible mutant genotypes in comparison to the Mla_{12}-resistant wild-type plant. Noninoculated control plants corresponding to the 24 and 48 hr time points showed no significant transcript accumulation in any of the genotypes. Essentially indistinguishable accumulation profiles were observed for barley peroxidase transcripts. Again, transcript accumulation at about 36 and 48 hr postinoculation was drastically lowered in all mutants. Finally, for the barley defense-related pathogenesis-related protein-1 (*PR-1*) gene, we also observed an accumulation profile of transcripts that was very similar to those for chitinase and peroxidase. We concluded that high-level transcript accumulation of these barley defense-related genes is under control of Mla_{12}, *Nar-1*, and *Nar-2*.

DISCUSSION

In this study, we provided genetic, quantitative cytological, and molecular data that demonstrate that at least three genes (Mla_{12}, *Nar-1*, and *Nar-2*) are required to execute a race-specific resistance response against powdery mildew attack. Whereas the positions of Mla_{12} and *Nar-1* are now precisely mapped to barley chromosomes 5 (Jahoor et al., 1993) and 2, respectively, the genome position of *Nar-2* remains to be determined. We established that mutant M22 defines *Nar-2* as being unlinked to *Nar-1* (defined by mutants M82 and M100) because the M22 mutation was complemented in crosses with M82 or M100 and because tightly linked molecular markers to *Nar-1* detected no linkage to the mutation causing susceptibility in M22 (Tables 1 and 4). The conclusion that M22 defines a mutation in a gene unlinked to Mla_{12} was derived from the fact that a backcross of M22 with a susceptible wild-type parent (mla_{12}) segregates resistant F_2 individuals (Jørgensen, 1988). An independent molecular confirmation is difficult to obtain from our populations segregation for M22: because the crosses were performed with Mla_{12} BC lines (Table 3), any tested molecular marker that is tightly linked to resistance gene Mla_{12} on chromosome 5 displays homomorphisms in all F_2 segregants (data not shown). Preliminary data indicated, however, that an RFLP marker (AT13), which maps at 17.5 centimorgans from the *Mla* locus and detects parental polymorphisms between M22 and Mla_{12} BC Siri, segregated randomly in the susceptible F_2 individuals. Therefore, all available data indicated that the mutation conferring susceptibility in M22 is not only unlinked to *Nar-1* but also unlinked to Mla_{12}.

Recently, two loci, *Rcr-1* and *Rcr-2* (required for *Cladosporium* resistance), have been identified in tomato via a mutagenesis approach; both are required for race-specific resistance against the fungal pathogen *Cladosporium fulvum* specified by resistance gene *Cf-9*. These genes are unlinked to each other and to *Cf-9* (Hammond-Kosack et al., 1994). There is, however, an

important difference between mutant alleles of *Rcr-1* and *Rcr-2* in tomato and mutant alleles of *Nar-1* and *Nar-2* in barley: whereas the available defect alleles of *Rcr-1* and *Rcr-2* weaken the wild-type resistance response without permitting sporulation, mutant alleles of *Nar-1* and *Nar-2* confer sporulation and therefore a completion of the *E. g. hordei* life cycle. Whether this reflects qualitative differences of *Rcr* and *Nar* genes or merely represents the result of partially inactivated gene product activity of the mutant alleles in tomato awaits the isolation of additional mutant alleles (see below).

One of the key findings reported here is the fact that mutant alleles either in Mla_{12} or in *Nar-1* or *Nar-2* resulted in a deficiency of the host plant to trigger a cell death response in the attacked epidermal cell early during the interaction (Figure 3B). Although cell death has been inferred in this study only on the basis of whole-cell autofluorescence measurements, we (Görg et al., 1993) and others (Koga et al., 1990) have shown repeatedly that in the barley–powdery mildew interaction whole-cell autofluorescence precisely parallels independent measures, such as the capability of live cells to plasmolyse in the presence of α-methyl-D-glucose or to accumulate the vital dye neutral red. The fact that wild-type alleles of all three genes Mla_{12}, *Nar-1*, and *Nar-2* were required for the cell death response of the initially attacked host cell implies that the process is under control of all three genes and that it represents a crucial mechanism of Mla_{12}-specified powdery mildew resistance.

This study may add an additional facet to the regulation of CWA formation (papillae and associated haloes) and host cell death response (single-cell hypersensitive cell death). Mla_{12}, *Nar-1*, and *Nar-2* are all required to control cell death of the attacked host cell. Interestingly, the mutation in M22, which defines gene *Nar-2*, also affected, at least quantitatively, the formation of CWAs in response to pathogen attack (Figure 3A). This suggests that the wild-type allele of *Nar-2* has a dual function: it controls CWA formation and cell death in response to powdery mildew attack. Further experiments with different mildew isolates are, however, necessary to clarify this aspect of *Nar-2* action.

One central question in race-specific resistance concerns whether putative common and/or separate defense pathways are triggered by different resistance genes. This question has been addressed recently through genetic experiments in which mutants M22, M82, and M100 have been combined with different powdery mildew resistance genes (H.J. Jørgensen, personal communication). Whereas the resistance response specified by resistance genes Mla_{13}, Mla_{23}, and *Mlat* is obviously suppressed in the presence of either M22, M82, or M100, *Mlg*-, *Mlp*-, and *mlo*-specified resistance remains unaffected. These findings may help to resolve seemingly conflicting data with respect to the function of the cell death response of the initially attacked cell in *Mlg*- and Mla_{12}-controlled resistance (Görg et al., 1993). Although cell death of the initially attacked cell is triggered in the presence of resistance allele *Mlg*, gene dosage experiments revealed that this response is not causally required for arrest of fungal growth. Assuming that *Nar-1* and *Nar-2* are both also required to trigger the cell death response of the attacked host cell in the context of *Mlg*, a retained *Mlg* function in conjunction with defective alleles of *Nar-1* or *Nar-2* (H.J. Jørgensen, personal communication) would argue in the same direction.

These controversial findings with respect to the cell death function in *Mlg*- and Mla_{12}-resistant plants may be resolved by the fact that *Mlg*, in contrast to Mla_{12}, triggers, in addition, the formation of efficient CWAs (papillae), which result in an abrogation of fungal development prior to haustorium differentiation (Görg et al., 1993). All these observations are compatible with the hypothesis that CWA formation and the cell death response of attacked host cells represent key single-cell events in powdery mildew defense, which are differentially controlled by Mla_{12} and *Mlg*. It appears in particular that host cell death is likely to be crucial in the presence of Mla_{12}, whereas it can be omitted in the presence of *Mlg*.

Data from the intermutant crosses (Table 1) and the RFLP mapping of *Nar-1* have demonstrated that M82 and M100 represent allelic mutations. However, both mutants clearly conferred distinguishable infection types (Figures 1C and 1D). Infection sites in M82 differed from those of M100 in that they were surrounded by extended necrotic and chlorotic tissue at late time points during the infection process. Less aerial mycelium and sporulation were observed in M82 than M100 plants (Figure 1). We interpret these findings by inferring that the *Nar-1* gene product of M82 (and probably also M100) has retained residual activity. This supposition must be opposed to the observation that both mutants are indistinguishable with respect to their complete inability to trigger a cell death response of the initially attacked host cell (Figure 3B). An explanation for the differential phenotypes of the allelic mutations in M82 and M100 probably lies in a residual resistance response, which is spatially extended and temporarily late compared to the events in the initially attacked epidermal cell. This implies that Mla_{12} and *Nar-1* are not only required for a cell death response of the initially attacked cell but also for a spatially extended defense control in neighboring cells (see below).

Accumulation of all studied defense-related gene transcripts in the Mla_{12}-resistant wild-type plant was characterized by two maxima at ~16 and 48 hr postinoculation (Figure 4). We cannot yet provide a definitive explanation for this phenomenon. However, the timing of the maxima corresponds approximately to penetration of the fungus into the host cell (formation of undifferentiated haustoria; Figure 3C) and to maturation of fungal haustoria (formation of differentiated haustoria; Figure 3D). Nevertheless, there is reason to argue against a simple and direct connection between fungal differentiation events in the initially attacked host cell and the biphasic accumulation profile of defense-related gene transcripts. It seems unlikely that the observed massive transcript accumulation reflects only the changes of mRNA steady state levels in the initially attacked epidermal cell because only a fraction of epidermal cells was in physical contact with the fungus and because the epidermal tissue represents itself only a minor fraction of whole leaf tissue, which was used for RNA extraction. Indeed, the peroxidase (pBH6-301) and the PR-1 homolog proteins are secreted

into the apoplast (Kerby and Somerville, 1989; Thordal-Christensen et al., 1992; Bryngelsson et al., 1994).

It is therefore more likely that the observed transcript accumulation in response to powdery mildew attack originates from cells surrounding the interaction sites. An analogous situation with respect to transcript accumulation surrounding infection sites has been demonstrated in the parsley/*Phytophthora megasperma* interaction (Schmelzer et al., 1989). Furthermore, any connection of events in the initially attacked cells with transcript accumulation profiles of defense-related genes should take into account that the penetration processes of the two cell types of the epidermis (small and long cell type) take place with a different kinetics (Koga et al., 1990; Görg et al., 1993). It seems therefore possible that the characteristic two maxima observed in the time course early during infection also reflect events in leaf tissue surrounding short and long epidermal cell-type infection sites.

It is tempting to correlate the differential infection phenotypes of M82 and M100 with the seemingly differing transcript accumulation at 48 hr postinoculation from those defense-related barley genes tested (Figure 4). In accordance with this interpretation is the observation that M22, which confers a similar necrotic infection type as M82 (Figure 1), also showed a comparable residual transcript accumulation of defense-related genes (Figure 4). Moreover, the weakest transcript accumulation of defense-related genes, observed in M66 and M100, paralleled the fact that neither showed significant necrosis around infection sites at later stages (Figure 1). We are aware that only in situ hybridizations with cDNA probes from defense-related genes will resolve directly the mechanisms by which the mutated genes affect the spatial extent of transcript accumulation during the later stages of infection.

The present data indicate that host cell death in response to fungal attack and a high-level accumulation of transcripts from defense-related genes represent "downstream events," which are under common control of genes Mla_{12}, *Nar-1*, and *Nar-2*. In view of a hypothetical linear signal pathway defined by these three genes, it is of considerable interest to establish a linear order of gene action. Because the first molecularly isolated race-specific resistance gene *Pto* (resistance to *Pseudomonas syringae* pv *tomato*) in tomato against the bacterial pathogen *P. s. tomato* represents a biochemically and intracellularly localized putative serine-threonine kinase (Martin et al., 1993) and because Flor's gene-for-gene hypothesis permits that any step in a series of events leading to the recognition of the pathogen may represent a resistance gene, there is no argument against placing Mla_{12} "upstream" from *Nar-1* and *Nar-2*. Epistatic interactions of mutant genes may assist in establishing gene orders of putative pathways. Preliminary data indicate, however, that mutant alleles of *Nar-1* and *Nar-2* act additively because the proportion of F_2 individuals showing a high infection type in the susceptible class derived from intermutant crosses M22 × M82 and M22 × M100 was larger than expected for independent action of M22 and M82/M100. The fact that all three relevant genes act independently of their genetic background allowed us to perform marker-assisted introgression of mutant alleles and the construction of double and triple mutants, which is likely to reveal further insights into gene interactions between Mla_{12}, *Nar-1*, and *Nar-2*.

METHODS

Plant Material

Seeds of doubled-haploid barley (*Hordeum vulgare*) line Sultan-5 and mutants M22, M66, M82, and M100 were generously provided by J. Helms Jørgensen, Risø National Laboratory, Risø, Denmark (Torp and Jørgensen, 1986). Sultan-5 and the mutants that were originally isolated from this line contain the phenotypic marker gene *ant2*. The Mla_{12} backcross (BC) lines in cultivars Siri and Pallas were a gift from Lisa Munk, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Their generation has been described previously (Kølster et al., 1986; Kølster and Stolen, 1987). The Mla_{12} BC line in cultivar Ingrid is a gift from James McKey, University of Upsala, Upsala, Sweden. The line was generated through seven backcrosses with *H. vulgare* cv Ingrid followed by at least seven selfings.

Each of the mutants M22, M82, and M100 was pollinated with pollen derived from the Mla_{12} BC line cultivars. Five F_1 plants from each cross were grown to maturity; they generated an F_2 population of at least 1200 individuals from each cross.

Tests for Resistance

Powdery mildew infections were performed in a phytochamber with *Erysiphe graminis* f sp *hordei* isolate A6 expressing the Mla_{12} avirulence function (Wiberg, 1974). Single F_2 kernels were sown in a peat–clay mixture, grown at 15°C and a relative humidity of 80%. Seven days later, primary leaves were inoculated on adaxial and abaxial surfaces at conidial densities of 100 to 200/cm^2. This low density reduced the likelihood that host epidermal cells would be attacked by more than one spore. Seven days later, the infection type of each seedling was classified on a rating scale as described by Torp et al. (1978).

Score of Anthocyanin Pigmentation and Identification of Recombinants between *Nar-1* and *Ant2*

Susceptible F_2 individuals derived from the crosses with the Mla_{12} BC line cultivars were treated with fungicide and grown to maturity. The score for presence of only *Ant2* alleles or presence of at least one *Ant2* allele was carried out with 6-week-old plants. The absence of detectable red color in the leaf sheath epidermis indicated the presence of *ant2* homozygous alleles. Recombination events between *nar-1* and *ant2* (susceptible and white leaf sheath) were verified using F_3 family tests: all F_3 family individuals were susceptible when inoculated with isolate A6 but segregated *Ant2* and *ant2* plants in an expected ratio of 3:1.

Quantitative Cytological Analysis

For microscopic analysis of early infection stages, primary leaves of 7-day-old plants were inoculated in an identical manner as described above. At the indicated times, leaves were harvested, placed

in alcoholic lactophenol (96% ethyl alcohol-lactophenol, 2:1 [v/v]), and boiled for 2 min to remove all chlorophyll. The boiling step was repeated, and the leaves were transferred to fresh alcoholic lactophenol and stored in stoppered glass tubes. The apical 1 cm of each leaf was discarded, and the next 4 cm was inspected under the microscope. Whole-cell autofluorescence was observed by incident-light fluorescence microscopy (λ_{max} = 310 nm), and haustoria were visualized by bright-field microscopy. Haustoria without and with fingerbuds were classified as undifferentiated and differentiated haustoria, respectively. At the indicated times, functional cell wall appositions (CWAs) (papillae and surrounding haloes) were recorded if both a high refractive index using bright-field microscopy and a yellow autofluorescence using incident-light fluorescence microscopy were observed directly beneath sites of attempted fungal penetration in the absence of detectable haustorial initials. To facilitate identification and differentiation of extracellular and intracellular fungal structures, fixed leaf segments were stained with Coomassie blue (0.6% Coomassie Brilliant Blue R 250, 15% trichloroacetic acid, 1:1 [v/v]) for 5 sec, washed in distilled water, and mounted in 50% glycerol (v/v).

RNA Gel Blot Analysis

Inoculation and growth conditions of the plants were identical to those used in the quantitative cytological study. Total RNA was extracted from 4-cm-long primary leaf segments at the indicated time points after inoculation with A6 conidia as described previously (Collinge et al., 1987; Gregersen et al., 1990). The apical 1 cm of each primary leaf was discarded. For each time point and genotype, primary leaf segments from 15 to 20 individuals were included. Fifteen micrograms of total RNA was loaded on 1.2% denaturing formaldehyde-agarose gels and transferred after electrophoretic size separation to nylon membranes (Hybond N; Amersham Corp.). Hybridizations with ^{32}P-labeled cDNA probes (Feinberg and Vogelstein, 1984) were performed using standard conditions (Maniatis et al., 1982).

Linkage Analysis

Linkage between the *Nar-1* and *Ant2* locus is based on a two-point linkage calculation according to Ritter et al. (1990) and Allard (1956).

DNA Hybridizations

Mapping filters were prepared essentially as described by Graner et al. (1990) or Gebhardt et al. (1989). DNA restriction enzymes used for restriction fragment length polymorphism (RFLP) analysis were HindIII and XbaI detecting RFLPs for MWG87, EcoRI detecting an RFLP for MWG503, and HaeIII, HpaII, and RsaI detecting RFLPs for cMWG694. Probe labeling and hybridization were performed as previously described (Hinze et al., 1991).

ACKNOWLEDGMENTS

We are indebted to our colleagues, in particular Drs. Jeff Dangl, Per Gregersen, Francesco Salamini, Marietta Wolter, Rainer Büschges, and V. Smedegaard for their critical contributions and encouragement. We thank Barbara Förster, Jeanett Christiansen, and the late Heilke Klatt for their invaluable technical support. We are grateful to Drs. Tomas Bryngelsson, PeO Gummesson, and Per Gregersen for permission to use the chitinase clone prior to publication. We thank Dr. Andreas Graner for his willingness to exchange probes from the general RFLP map. This work was supported by a grant from the German Research Society (DGF) Schwerpunktprogramm "Molekulare Phytopathologie" to P. S.-L. and by the Framework Program for Disease Resistance, Nordic Council of Ministers (via the Nordic Contact Organ for Agriculture) and SJVF, the Danish Research Council for Agricultural Research.

Received February 14, 1994; accepted May 3, 1994.

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