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# The Barley *Mlo* Gene: A Novel Control Element of Plant Pathogen Resistance

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## Summary

**Mutation-induced recessive alleles *(mlo)* of the barley *Mlo* locus confer a leaf lesion phenotype and broad spectrum resistance to the fungal pathogen, Erysiphe graminis f. sp. hordei. The gene has been isolated using a positional cloning approach. Analysis of 11 mutagen-induced *mlo* alleles revealed mutations leading in each case to alterations of the deduced Mlo wild-type amino acid sequence. Susceptible intragenic recombinants, isolated from *mlo* heteroallelic crosses, show restored *Mlo* wild-type sequences. The deduced 60 kDa protein is predicted to be membrane-anchored by at least six membrane-spanning helices. The findings are compatible with a dual negative control function of the Mlo protein in leaf cell death and in the onset of pathogen defense; absence of Mlo primes the responsiveness for the onset of multiple defense functions.**

## Introduction

In plants, resistance to specialized pathogens is frequently triggered by a recognition event followed by a coordinated complex defense response resulting in localized containment of the intruder (Hammond-Kosack and Jones, 1996). In this type of plant–pathogen interaction, resistance is specified by and dependent on the presence of two complementary genes, one from the host and one from the pathogen (Flor, 1971). The complementary genes have been termed race-specific resistance gene and avirulence gene, respectively. Several resistance genes have been isolated and appear to encode proteins that either contain a leucine-rich region (LRR), with or without an attached nucleotide binding site (NBS), indicative of ligand-binding and protein–protein interaction. Another class encodes a simple serine/threonine kinase (Dangl, 1995; Staskawicz et al., 1995; Zhou et al., 1995). The genetic and molecular observations are compatible with a specific receptor-mediated signal response triggering pathogen defense. The structural similarities in resistance gene products from different plant species to diverse pathogens such as bacteria, fungi, and viruses imply the existence of common "downstream" biochemical defense mechanisms. Although these mechanisms remain to be uncovered, localized death of host cells at the site of attempted infection, designated the hypersensitive response (HR), accompanies many incompatible race-specific interactions (Stakman, 1915; Staskawicz et al., 1995). Similarly, resistance in barley to the common biotrophic fungal pathogen Erysiphe graminis f. sp. hordei is in most analyzed cases specified by dominant or semidominant race-specific resistance genes and associated with a HR (*Mlx*; Jørgensen, 1994).

Monogenic resistance mediated by recessive (*mlo*) alleles of the *Mlo* locus is different. Apart from being recessive, it differs from race-specific incompatibility to single pathogen strains in that (1) it confers a broad spectrum resistance to almost all known isolates of the fungal pathogen, (2) *mlo* resistance alleles have been obtained by mutagen treatment of any tested susceptible wild-type (*Mlo*) variety, and (3) the resistance is apparently durable in the field despite extensive cultivation in Europe (Jørgensen, 1992). Finally, under pathogen-free or even axenic conditions, *mlo* plants exhibit a spontaneous leaf cell death phenotype, preceded by the appearance of characteristic cell wall appositions (Wolter et al., 1993).

Mutations have also been described in many other plant species in which cell death symptoms appear, resembling those in defense responses to plant pathogens (Walbot et al., 1983; Jones, 1994; Dangl et al., 1996). It has been suggested that at least some of these mutants, often collectively termed disease lesion mimics, affect control mechanisms of plant defense. Both recessively and dominantly inherited lesion mimic mutants have been analyzed for indicators of defense responses in Arabidopsis thaliana (Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al. 1995). Apart from the onset of cell death in the absence of pathogens, multiple defense functions such as plant cell wall modifications and the accumulation of defense-related gene transcripts and phytoalexins have been observed. The mutants (*lsd1* to *lsd7* and *acd2*) were found to exhibit elevated resistance to a bacterial (Pseudomonas syringae) and a fungal (Peronospora parasitica) pathogen. Lesion mimic mutants are not restricted to foliar tissue. Recessive alleles of the soybean *Rn* locus exhibit, under axenic conditions, HR symptoms in the root, accompanied by the accumulation of defense-related proteins

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and the pythoalexin glyceollin (Kosslak et al., 1996). Homozygous *rn* plants exhibit increased tolerance to root-borne infection by the fungal pathogen Phytophthora sojae. The findings suggest the activation of an at least partially overlapping set of biochemical events in pathogen-triggered race-specific resistance and during pathogen-independent cell death in several disease lesion mimic mutants.

We describe here the molecular isolation of the *Mlo* gene as a first step toward a molecular interpretation of broad spectrum resistance mediated by recessive host gene mutations. The gene encodes a member of a novel protein family apparently restricted to plants. We discuss the possible dual function of the Mlo protein in down-regulating leaf cell death and pathogen defense functions.

## Results

We had previously identified RFLP markers closely linked to *Mlo* on barley chromosome 4 in *mlo* backcross (BC) lines containing *mlo* alleles from six genetic backgrounds (Hinze et al., 1991). The identification of a 2.7 cM (centiMorgan) RFLP interval (bAL88–bAO11) containing *Mlo* based on the cross Carlsberg II *Mlo* × Grannenlose Zweizeilige *mlo-11* opened a route to isolate the gene via positional cloning (Figure 1; RFLP map). However, because the barley genome has a very unfavorable ratio of genetic and physical distances (approximately 3 Mb/cM; Bennett and Smith, 1991; Becker et al., 1995), we applied AFLP (Amplified Fragment Length Polymorphism) marker technology (Vos et al., 1995) to increase the DNA marker density around *Mlo* and to generate a genetic map with a resolution better than 0.05 cM. We aimed to physically delimit the gene with flanking DNA markers on single large insert size genomic clones, an approach that has been termed "chromosome landing" (Tanksley et al., 1995).

### Targeted Search for AFLP Markers

We selected AFLP markers around *Mlo* by searching for polymorphic DNA fragments between an *mlo* BC line ($BC_7$ Ingrid *mlo-3*) and DNA from the recurrent parent (Ingrid *Mlo*). The $BC_7$ Ingrid *mlo-3* line was previously shown to carry a small introgressed DNA segment on barley chromosome 4 (Hinze et al., 1991). The donor parent of the BC line represents a different genetic background (cultivar Malteria Heda *mlo-3*) in comparison to the recurrent parent line. In parallel, we established a second segregating F2 population from the cross Ingrid *Mlo* × $BC_7$ Ingrid *mlo-3*, formally representing an eighth backcross. To further narrow down the chromosomal interval for DNA marker identification to approximately 3 cM, pooled DNA from resistant (*mlo*) and susceptible (*Mlo*) F2 individuals were included in the search for AFLP markers besides DNA of the parental lines (see Experimental Procedures and Giovannoni et al., 1991). All possible PstI/MseI primer combinations (1,024) extending into genomic sequences up to nucleotide positions +2 and +3 and 880 EcoRI/MseI primer combinations (+3/+3) were tested. A total of 38 AFLP marker candidates were identified.

### High Resolution Mapping

A three step procedure was chosen to construct the high resolution AFLP map. First, we were able to position 21 of the identified candidate AFLP markers to opposite sides of *Mlo* by using recombinants for flanking RFLP markers that had been detected among a small number of 70 F2 individuals (data not shown). The remaining 17 AFLP markers could not be separated from *Mlo* using this population size. In a second step, two codominant AFLP markers on opposite sides of *Mlo* were chosen to screen 2,022 F2 segregants for recombination events in the interval. 76 recombinants were identified, and their genotype at *Mlo* was determined by testing selfed F3 families with powdery mildew isolate K1 that is not virulent on homozygous *mlo* genotypes. In a third step, an AFLP analysis was carried out with each of the remaining 17 candidate AFLP markers to determine their position relative to *Mlo* based on the 76 recombinants. The crucial result was the identification of a DNA marker cosegregating with *Mlo* (Bpm16) and two flanking markers (Bpm2 and Bpm9) at a distance of 0.24 and 0.4 cM, respectively (Figure 1; AFLP map).

### Physical Delimitation of *Mlo*

A large insert yeast artificial chromosome (YAC) library was constructed with genomic DNA of cultivar Ingrid *Mlo* using vector pYAC4 (Burke et al., 1987). The library comprises 40,000 clones with an average insert size of 500 kb and represents approximately four barley genome equivalents (construction and characterization of this library will be published elsewhere). Four YAC clones (YHV417-D1, YHV400-H11, YHV322-G2, and YHV303-A6) were isolated by an AFLP screen specific for marker Bpm16, which cosegregated with *Mlo*. AFLP analysis indicated that three of these clones (YHV400-H11, YHV322-G2, and YHV303-A6) also contained both flanking marker loci (Bpm2 and Bpm9). These findings implied physical delimitation of *Mlo* on three YAC clones.

We chose YHV303-A6 (insert size 650 kb; Figure 1) for subcloning experiments into bacterial artificial chromosome (BAC) vector pECSBAC4 containing a unique EcoRI site (Shizuya et al., 1992; see Experimental Procedures). Recombinant BAC clones containing the AFLP locus Bpm16 were subsequently identified using the cloned 108 bp PstI/MseI genomic Bpm16 fragment from cultivar Ingrid *Mlo* as a probe in colony hybridization experiments. One BAC clone, BAC F15, containing an insert of approximately 60 kb was chosen for further detailed studies (Figure 1; BAC F15). We found that the recombinant BAC clone contained locus Bpm2 in addition to the AFLP marker Bpm16, but not locus Bpm9, indicating physical delimitation in centromeric orientation to *Mlo*. Instead of constructing a BAC contig between Bpm16 and Bpm9, we developed new polymorphic markers from BAC F15 and mapped them using template DNA of 25 recombinants (derived from the high resolution mapping population described above) in the interval Bpm2–Bpm9. A codominant XbaI/MseI polymorphism (designated Bxm2) was identified between the parental lines Ingrid *Mlo* and $BC_7$ Ingrid *mlo-3*. The analysis of the 25 recombinant individuals revealed a position of Bxm2 in telomeric orientation from *Mlo* at a

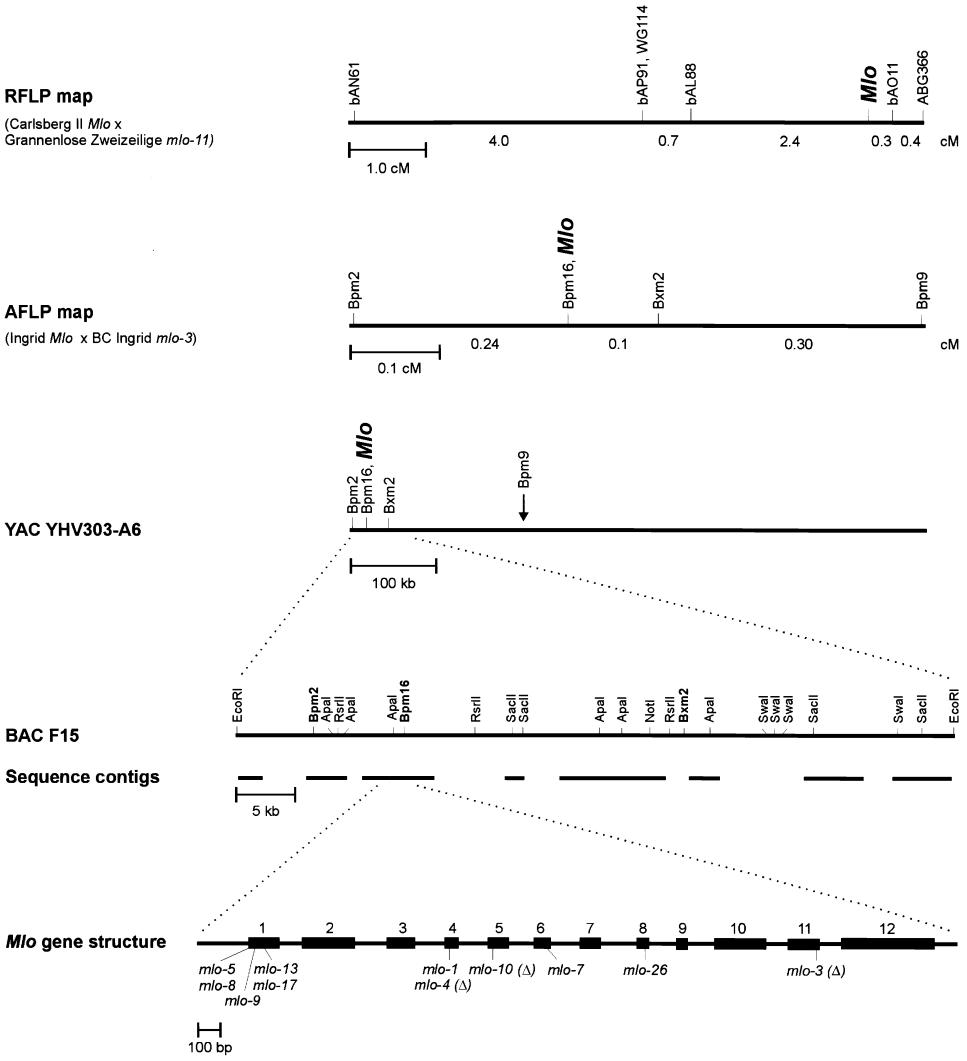


Figure 1. Positional Cloning of *Mlo*

The *Mlo* locus has been mapped with increasing precision on the long arm of barley chromosome 4 using RFLP and AFLP markers. The upper part of the figure presents the genetic linkage maps of these markers relative to *Mlo*. Genetic distances are indicated in centiMorgans (cM); the RFLP map is based on multipoint linkage analysis; and the AFLP map was calculated by two point estimates. The RFLP marker map is based on the analysis of 257 F2 individuals derived from the cross Carlsberg II *Mlo* × Grannenlose Zweizeilige *mlo-11*. The previously published RFLP map (see Results) of the same cross was based on only 44 F2 individuals. The gene was delimited to a 2.7 cM interval bordered by markers bAO11 (in telomeric orientation) and bAL88 (in centromeric orientation). AFLP markers (Bpm2, Bpm9, Bpm16, and Bxm2) were identified and mapped as described in Experimental Procedures. Their genetic distance to *Mlo* is based on the cross Ingrid *Mlo* × $BC_7$ Ingrid *mlo-3*. One YAC clone, YAC YHV303-A6 (insert size 650 kb), containing the cosegregating marker Bpm16 and two flanking loci (Bpm2 and Bpm9) is shown in the middle section of the figure. The position of marker Bpm9 was only roughly estimated within the YAC clone as indicated by the arrow. The insert of BAC F15 represents a 60 kb subfragment of this YAC as indicated in the lower part of the Figure. The approximate physical positions of AFLP markers Bpm2, Bpm16, and Bxm2 (spanning an interval of approximately 30 kb) as well as the location of some rarely occuring restriction sites are indicated. Dashed lines below the schematic representation of BAC F15 DNA show the position of the largest established DNA sequence contigs. The structure of the *Mlo* gene is given schematically in the bottom line of the figure. Exons are highlighted by closed boxes. Positions of mutational events are indicated for the eleven tested *mlo* alleles. Mutant alleles carrying deletions in their nucleotide sequences are marked with a (Δ).

distance of 0.1 cM (Figure 1; AFLP map). We concluded that *Mlo* had been physically delimited on BAC F15 between marker loci Bpm2 and Bxm2.

### A Candidate *Mlo* Gene

DNA sequences of the approximately 60 kb insert of BAC F15 were obtained from randomly chosen clones of a plasmid sublibrary (see Experimental Procedures). In parallel, a physical map was generated (Figure 1; BAC F15). The map indicated that the flanking markers Bpm2 and Bxm2 are separated by approximately 30 kb. Rare cutting restriction sites enabled us to assign larger sequence contigs within BAC F15. We searched the available sequence contigs for regions of high coding probability (see Experimental Procedures). Only one sequence contig of 5.8 kb, including the cosegregating marker

Bpm16, revealed an extensive region of high coding probability.

We performed reverse transcriptase–polymerase chain reactions (RT–PCR) with total leaf RNA derived from cultivar Ingrid *Mlo* using a series of primers deduced from regions that indicated high coding probabilities and obtained in each case a distinct amplification product (Experimental Procedures). Sequencing of the largest RT–PCR products revealed a single extensive open reading frame of 1,599 bp (Figure 2). 5′ and 3′ ends of the gene transcript were identified using rapid amplification of cDNA ends (RACE) technology. The deduced putative protein of 533 amino acids has a molecular weight of 60.4 kDa. No significant homologies were found to any other described protein in the various databases, but at least six putative membrane-spanning helices indicated membrane association (for details, see Discussion). We were unable to detect a signal in Northern blot experiments containing total RNA with the labeled RT–PCR probe, but a rare RNA transcript of approximately 2.0 kb length was clearly visible in the tested *Mlo*, *mlo-1*, and *mlo-3* genotypes when poly(A)$^+$ RNA was used (Figure 3). This transcript size is in agreement with the combined data from RT–PCR and RACE analysis. A comparison of the genomic DNA and RT–PCR-derived sequences revealed 12 exons, each flanked by the consensus splice site sequences (Figures 1 and 2). Since marker Bpm16 is part of exon 11 and intron 11 and, as shown above, cosegregated with the resistance phenotype, it represented a candidate *Mlo* gene. We started genomic PCR-based sequencing of eleven mutagen-induced *mlo* resistance alleles and their corresponding wild-type DNAs (Experimental Procedures). These mutants had been isolated within six different genetic backgrounds. We identified nucleotide alterations (point mutations or deletions) in all tested mutant alleles that at the amino acid level result either in single amino acid substitutions or truncated versions of the predicted wild-type protein (Table 1). Surprisingly, a comparison among the wild-type gene sequences of seven tested barley cultivars (Carlsberg II, Diamant, Foma, Haisa, Ingrid, Malteria Heda, and Plena) indicated not a single amino acid difference. Moreover, we observed that at the nucleotide level the wild-type gene is identical among 6 tested cultivars both in exon and intron sequences, whereas cultivar Foma revealed 7 nucleotide substitutions (2 in exon and 5 in intron sequences). In conclusion, the comparative sequencing of genomic DNA from various mutant *mlo* lines and their respective *Mlo* wild-type cultivars supported our assumption that we had identified *Mlo*.

### Characterization of Intragenic Recombinants

It had been our intention to provide a chain of evidence for the molecular isolation of *Mlo* that is not dependent upon complementation experiments by the time-consuming production of transgenic barley plants. We reasoned that recombination events between two physically separated mutation sites within the gene should give rise to a wild-type allele and an allele carrying both mutant sites. The former product of such rare intragenic recombination events is predicted to confer susceptibility upon powdery mildew attack only if the inactivation of the described candidate gene above is a requirement for resistance.

Based on this assumption, we performed intermutant crosses with lines containing alleles *mlo-1*, *mlo-5*, and *mlo-8*, generating in each case at least 10 F1 plants (Table 2; note that mutant sites in *mlo-1* and *mlo-5* as well as *mlo-1* and *mlo-8* are each separated by approximately 820 bp, as shown in Figure 1). The mutant alleles originate from the genetic backgrounds Haisa (*mlo-1*) and Carlsberg II (*mlo-5* and *mlo-8*). F2 populations were obtained by self-fertilization. F2 seedlings were screened for rare disease–susceptible individuals after inoculation with powdery mildew isolate K1, which is virulent on each of the parental *Mlo* wild-type cultivars (note that we were unable to select for products of intragenic recombination events carrying both mutagenic events because they are expected to exhibit a resistant phenotype). Susceptible F2 individuals were identified with an average frequency of $6 \times 10^{-4}$. This frequency is of the same order of magnitude as in previous reports of intragenic recombination events in plant genes (Salamini and Lorenzoni, 1970; Freeling, 1978; Koornneef et al., 1983; Dooner and Kermicle, 1986; Mourad et al., 1994). In contrast, when comparable numbers of progeny from selfings of each of the three *mlo* mutants were tested, no susceptible seedlings were identified (Table 2). This finding strongly indicated that the susceptible individuals derived from the intermutant crosses were not due to spontaneous reversion of the *mlo* alleles.

The inheritance of the susceptible F2 individuals was tested after selfing in F3 families. Each of the F2 individuals segregated in the F3 in the predicted ratio of 3 susceptible to 1 resistant, indicating heterozygosity for alleles conferring resistance and susceptibility in the F2. Homozygous susceptible F3 progeny were isolated for the majority of susceptible F2 individuals (see Experimental Procedures). A molecular analysis of these was performed using RFLP markers tightly linked ($<$4 cM) on each side of the *Mlo* locus to determine if restoration of *Mlo* function was accompanied by flanking molecular marker exchange (Figure 4). A compilation of the detected RFLP alleles of all relevant genotypes is given in Table 3. The compilation reveals that seven susceptible individuals exhibited flanking molecular marker exchange, indicating reciprocal crossover events (CO), whereas five susceptible individuals revealed no flanking marker exchange and therefore a non-crossover type of recombination (NCO). The latter class could be explained by a gene conversion or double crossover event. The ratio of the two observed classes (7:5) is compatible with the double-strand break repair model for recombination (Szostak et al., 1983). The relative position of the mutant sites in *mlo* alleles used in both heteroallelic crosses (Figure 1) predicts that the CO type recombination events are resolved unidirectionally with respect to flanking marker alleles in order to restore the *Mlo* wild-type allele. This is the case for all seven analyzed CO type recombinants (Table 3).

DNA of the CO type recombinants was tested for the presence of wild-type or mutant sequences. Genomic

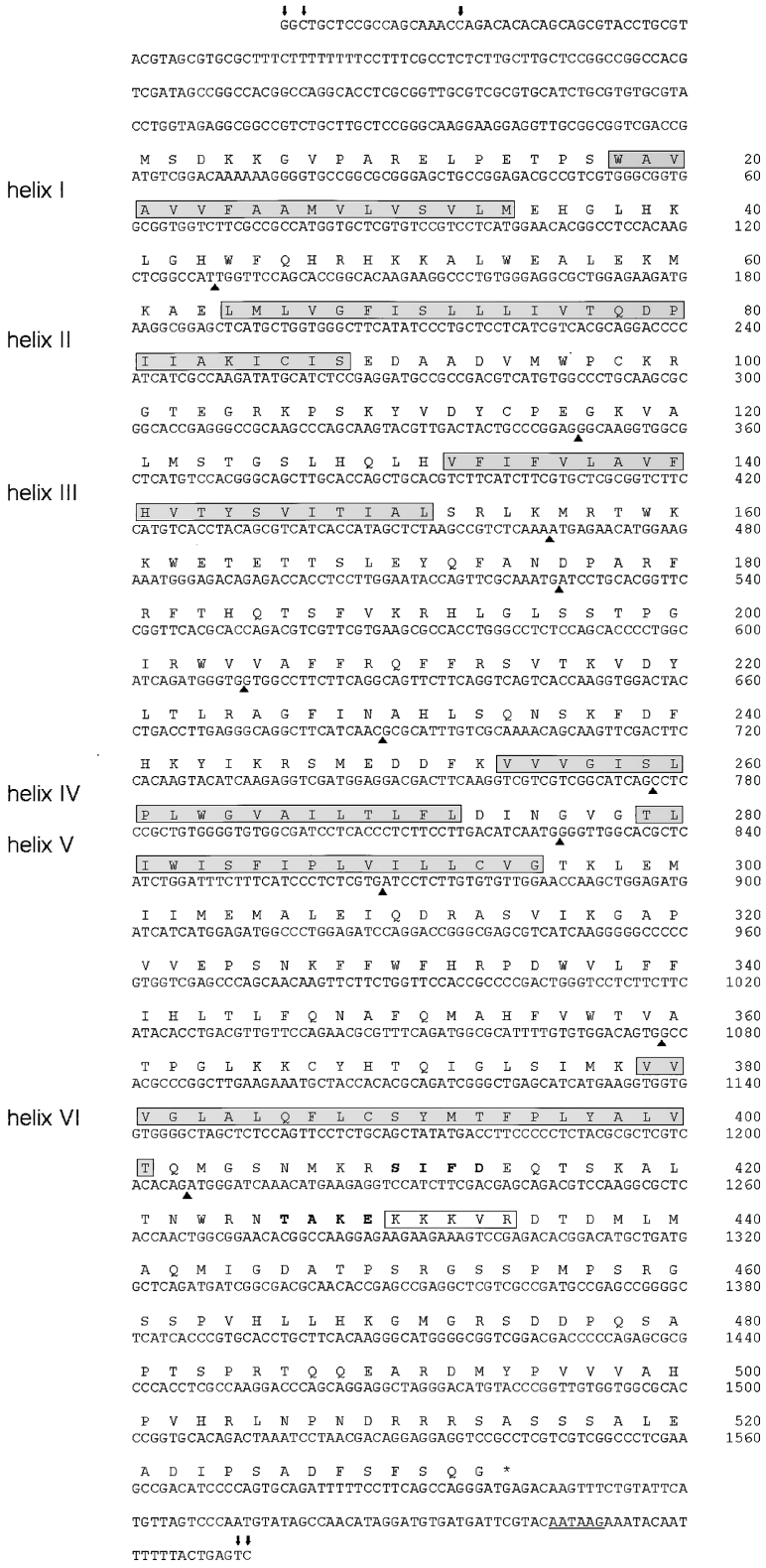


**Figure 2. Nucleotide and Deduced Amino Acid Sequence of the Barley *Mlo* cDNA**

The nucleotide and the deduced amino acid sequence are based on the combined data of RT–PCR and RACE obtained from experiments using RNA of cultivar Ingrid *Mlo*. The stop codon is marked by an asterisk, the putative polyadenylation signal is underlined, and the detected termini of RACE products are indicated by arrows above the sequence. Positions of introns as identified by comparison with corresponding genomic clones are labeled by triangles below the nucleic acid sequence. Six membrane-spanning helices predicted according to the MEMSAT algorithm (see Discussion) are boxed in gray. A putative nuclear localization signal (K-K-K-V-R) is boxed, and two casein kinase II sites (S/T-X-X-D/E) are shown in bold type.

PCR-based sequencing demonstrated in all cases restored wild-type sequences. This observation strongly suggested that the intragenic crossover event occured between nucleotides +1 and +821 in the cross *mlo-1* × *mlo-8* and between +3 and +821 in the cross *mlo-1* × *mlo-5* (numbers refer to genomic DNA sequences). Due

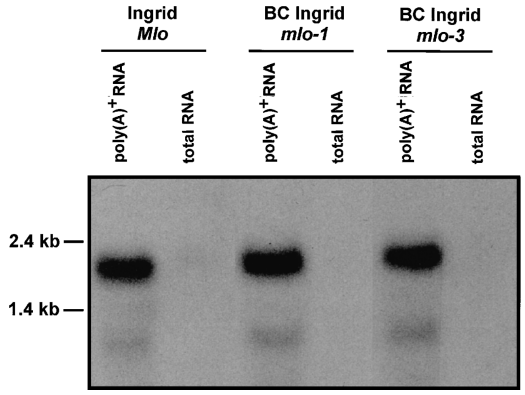


Figure 3. Northern Blot Analysis of *Mlo* Transcript Accumulation

Total RNA (20 μg) and poly(A)$^+$ RNA (5 μg) of 7 day old uninfected barley primary leaves of one wild-type (cultivar Ingrid *Mlo*) and two mutant (BC Ingrid *mlo-1*, BC Ingrid *mlo-3*) cultivars were isolated, separated on a 1.2 % formaldehyde gel, and transferred to a nitrocellulose membrane (Hybond). The filter was probed under stringent conditions with the radioactively labeled full size RT–PCR product derived from Ingrid *Mlo* (Figure 2). A clear signal is detected only in the lanes containing poly(A)$^+$ RNA. The signal corresponds to a size of approximately 2 kb.

to homomorphism of the DNA in these two intervals, we were unable to further delimit the intragenic recombination sites. In sum, the molecular analysis of seven intragenic recombinants from two heteroallelic crosses provides final proof that the above described candidate gene represents *Mlo*.

## Discussion

We have described here an isolation procedure for the *Mlo* gene that is not dependent upon complementation experiments via transgenic barley plants. The chain of evidence rests on physical delimitation of *Mlo* to an approximately 30 kb interval by using flanking molecular markers and high resolution genetic mapping, identifying mutation sites in all tested *mlo* mutants, and demonstrating that susceptibility to the pathogen is coincident with restoration of the *Mlo* wild-type gene. Thus, the gene identification relies on reciprocal molecular tests involving both gene inactivation and restoration events and is conceptually similar to a transposon-tagging-mediated gene isolation approach based on gene inactivation by transposon insertion and restoration of gene function via transposon excision (Osborne and Baker, 1995).

The study has shown the feasibility of a chromosome landing approach (Tanksley et al., 1995) in the largest genome for which a positional cloning approach has been completed so far ($5.3 \times 10^9$ bp/haploid genome equivalent, which is almost double the size of the human genome size; Bennett and Smith, 1991). Important elements were the construction of a local high resolution genetic map and the application of the AFLP marker technology (Vos et al., 1995), enabling us to delimit the target physically to 30 kb. The data reveal the variability of genome-wide and local ratios of genetic and physical distances. Within the *Mlo* gene, the recombination frequency was found to be 0.04 cM/kb (7 reciprocal crossover events among 20,670 F2 individuals in the 820 bp interval covering the first four exons, assuming that a comparable number of crossover events will have generated recombinant chromosomes carrying both mutant sites but were not detectable in the screen for susceptible intragenic recombinants). Thirteen recombinants were identified within the 30 kb interval bordered by markers Bxm2 and Bpm2 corresponding to 0.01 cM/kb, similar to the frequency found within the gene. These ratios deviate from the genome-wide estimate (0.0003 cM/kb) by one to two orders of magnitude (Bennett and Smith, 1991; Becker et al., 1995). Although the ratio of genetic/physical distances is generally believed to be much higher in telomeric regions of plant chromosomes (Heslop-Harrison, 1991), this argument does not apply to *Mlo* because of its location in the middle of the long arm of barley chromosome 4. Thus, the findings could be better explained with the reported exceptionally high frequencies of recombination within genes resulting in a scattered distribution of high and low recombination frequencies along a chromosome, as has been shown for the Arabidopsis thaliana chromosome 4 (Salamini and Lorenzoni, 1970; Freeling, 1978; Koornneef et al., 1983; Dooner and Kermicle, 1986; Schmidt et al., 1995).

The deduced amino acid sequence of *Mlo* reveals no homologies to any other described plant resistance gene so far, supporting the idea of a distinct mechanism triggering pathogen defense. Moreover, the gene shows no striking similarities to any characterized plant, animal, or prokaryotic gene in the various data bases (EMBL,

Table 1. *mlo* Mutant Alleles

| Allele | Mother Variety | Mutagen | Mutational Event at *Mlo* | Effect on Amino Acid Level |
|---|---|---|---|---|
| *mlo-1* | Haisa | X-rays | $T^{484} \rightarrow A$ | $Trp^{162} \rightarrow Arg$ |
| *mlo-3* | Malteria Heda | γ-rays | Deletion of 2 nucleotides (1188–1189) | Frame shift after $Phe^{395}$ |
| *mlo-4* | Foma | X-rays | Deletion of 11 nucleotides (478–488) | Frame shift after $Trp^{159}$ |
| *mlo-5* | Carlsberg II | EMS | $G^3 \rightarrow A$ | $Met^1 \rightarrow Ile^a$ |
| *mlo-7* | Carlsberg II | EMS | $G^{677} \rightarrow A$ | $Gly^{226} \rightarrow Asp$ |
| *mlo-8* | Carlsberg II | EMS | $A^1 \rightarrow G$ | $Met^1 \rightarrow Val^a$ |
| *mlo-9* | Diamant | EMS | $C^{28} \rightarrow T$ | $Arg^{10} \rightarrow Trp$ |
| *mlo-10* | Foma | γ-rays | Deletion of 6 nucleotides (543–548) | 2 amino acids ($Phe^{182}$, $Thr^{183}$) missing |
| *mlo-13* | Plena | EMS | $T^{89} \rightarrow A$ | $Val^{30} \rightarrow Glu$ |
| *mlo-17* | Plena | EMS | $C^{92} \rightarrow T$ | $Ser^{31} \rightarrow Phe$ |
| *mlo-26* | Plena | EMS | $T^{809} \rightarrow A$ | $Leu^{270} \rightarrow His$ |

Numbers of nucleotides and amino acids are given according to the translational start site (see Figure 2). EMS = ethylmethane sulfonate.
[a] Next start codon is at nucleotide positions 79–81 and is in frame with the coding sequence.

Table 2. F2 Progeny Obtained from *mlo* Heterallelic Crosses and Corresponding *mlo* Selfings

| Testcrosses[a] | Selfings | Resistant Individuals | Susceptible Individuals | Frequency of Susceptible F2 Progeny |
|---|---|---|---|---|
| *mlo-8* × *mlo-1* | | 5,281 | 3 | $5.7 \times 10^{-4}$ |
| *mlo-1* × *mlo-5* | | 915 | 0 | — |
| *mlo-5* × *mlo-1* | | 14,474 | 9 | $6.2 \times 10^{-4}$ |
| | *mlo-1* | 12,634 | 0 | — |
| | *mlo-5* | 5,498 | 0 | — |
| | *mlo-8* | 8,435 | 0 | — |

[a] Crosses are given female × male.

GenBank, SWISS-PROT). However, highly significant homologous sequences have been identified both in the EST databases from rice and Arabidopsis thaliana (EMBL/GenBank accession numbers D24131, D24287, N37544, H76041, T22145, T22146, and T88073). In addition, we have isolated cross-hybridizing genomic clones from barley and rice containing highly homologous DNA sequences (data not shown). This strongly suggests that the Mlo protein is likely to represent a member of a novel protein family and implies a conserved function among monocot and dicot plants. We failed to detect homologous sequences in either the human, mouse, or Caenorhabditis EST data bases. Homologous sequences were also not detected in the Saccharomyces cerevisiae genome for which complete DNA sequence information is available (Dujon, 1996). Thus, Mlo is likely to represent a member of a novel protein family restricted to the plant kingdom.

A close inspection of the predicted amino acid sequence reveals six hydrophobic stretches that are likely to form at least six transmembrane helices (Figure 2). The significance of this finding is supported by applying three different algorithms for assessment of membrane-anchored proteins, indicating in each case six membrane-spanning helices (ALOM, Klein et al., 1985; MEMSAT, Jones et al., 1994; TMpred [http://ulrec3.unil.ch/software/TMPRED_form.html]). In addition, a putative nuclear localization motif (NLS) was found in exon 12, indicating a possible transport of the protein into the nucleus (K-K-K-V-R; Nigg et al., 1991). Two casein kinase II motifs (S/T-X-X-D/E; Rihs et al., 1991) are located immediately upstream of the NLS. Casein kinase II sites are frequently found at distances between 10 and 30 amino acids from NLS motifs and have been shown to determine the rate of nuclear transport (Rihs et al., 1991). However, because NLS motifs appear to be insufficient to target membrane-bound proteins to the nucleus (Soullam and Worman, 1995), detailed functional studies are necessary for subcellular localization of the protein.

The apparent clustering of the mutations in *Mlo* (Figure 1) may be the first hint that functionally sensitive domains in the protein can be delimited. The *mlo-4* allele, characterized by an 11 bp deletion in exon 4, might have a special implication. The resulting frameshift is predicted to shorten the length of the expressed Mlo

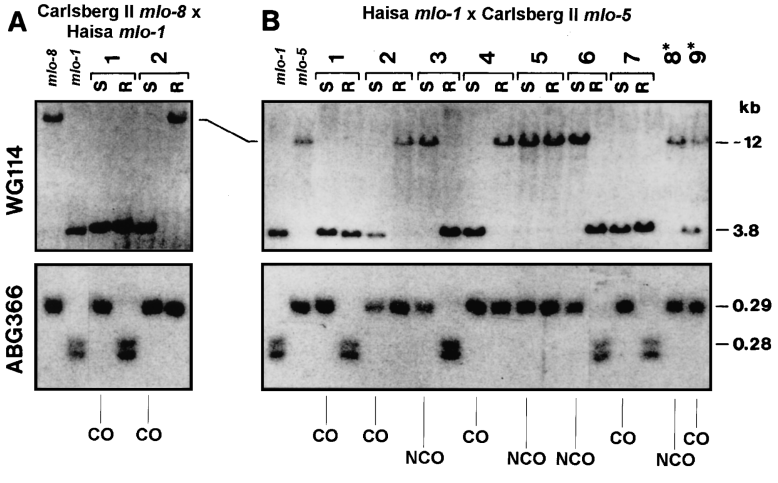


Figure 4. Southern Blot Analysis of Intragenic Recombinants Derived from *mlo* Heteroallelic Crosses

The alleles of two RFLP markers flanking *Mlo* on opposite sides of either susceptible F2 individuals or homozygous susceptible and homozygous resistant progeny were determined by Southern blot analysis. Plant DNA (10 μg) of the individuals were digested with PstI (A) or HaeIII (B) and hybridized with the radioactively labeled RFLP markers WG114 (upper panel; maps 3.1 cM in centromeric orientation to *Mlo*; see Figure 1) and ABG366 (lower panel; maps 0.7 cM in telomeric orientation to *Mlo*; see Figure 1) according to standard procedures. CO, crossover type of recombinants; NCO non-crossover type of recombinants.

(A) DNA of the parental lines *mlo-8* and *mlo-1* as well as 2 homozygous susceptible (S, *Mlo Mlo*) and 2 resistant (R, *mlo mlo*) progenies derived from 2 susceptible F2 plants (designated 1 and 2) were tested. The DNAs in lanes S and R represent selected F3 individuals from F3 families obtained by selfing the susceptible F2 individuals 1 and 2. Note that susceptible F2 individuals are expected to be heterozygous at *Mlo* in this selection scheme. Infection phenotypes were scored 7 days after inoculation with the *mlo* avirulent isolate K1. DNA from a third susceptible individual of this heteroallelic cross (see Table 3) is not included in this Figure.

(B) DNA of the parental lines *mlo-5* and *mlo-1* and 7 homozygous susceptible (S, *Mlo Mlo*) and 7 resistant (R, *mlo mlo*) progeny derived from 7 susceptible F2 plants (designated 1 to 7) were tested. The DNAs in lanes S and R represent selected F3 individuals from F3 families obtained by selfing the susceptible F2 individuals 1–7. DNA was analyzed from 2 further susceptible individuals of this heteroallelic cross only in the F2 generation (8* and 9*).

Table 3. Genotypes at Flanking RFLP Markers in Susceptible Progeny Derived from Heteroallelic *mlo* Crosses

| Testcrosses[a] | Susceptible Plants | Parental Genotype in Centromeric Orientation to *Mlo*[b] | Parental Genotype in Telomeric Orientation to *Mlo*[c] | Type of Recombination |
|---|---|---|---|---|
| *mlo-8* × *mlo-1* | 1 | *mlo-1* | *mlo-8* | CO |
| | 2 | *mlo-1* | *mlo-8* | CO |
| | 3 | *mlo-8* | *mlo-8* | NCO |
| *mlo-1* × *mlo-5* | 1 | *mlo-1* | *mlo-5* | CO |
| | 2 | *mlo-1* | *mlo-5* | CO |
| | 3 | *mlo-5* | *mlo-5* | NCO |
| | 4 | *mlo-1* | *mlo-5* | CO |
| | 5 | *mlo-5* | *mlo-5* | NCO |
| | 6 | *mlo-5* | *mlo-5* | NCO |
| | 7 | *mlo-1* | *mlo-5* | CO |
| | 8[d] | *mlo-5* | *mlo-5* | NCO |
| | 9[d] | *mlo-1* + *mlo-5* | *mlo-5* | CO |

CO = crossover type, NCO = noncrossover type of recombination.
[a] Crosses are given female × male.
[b] Deduced from alleles of RFLP marker WG114 (see Figure 1).
[c] Deduced from alleles of RFLP marker ABG366 (see Figure 1).
[d] Genotypes of flanking RFLP markers have been determined in heterozygous susceptible F2 individuals; in all other cases, homozygous susceptible F3 progeny derived from the susceptible F2 individuals were tested.

protein by 75%. We assume that at least this resistance allele represents a complete functional inactivation of the protein.

This study has shown that broad spectrum resistance to the powdery mildew fungus is caused by a defective *Mlo* gene. One of the key questions concerns function(s) of the Mlo protein and its homologues. Based on experimental evidence, we propose two hypotheses. In the first model, Mlo would have a negative control function in leaf cell death since punctate dead cell leaf lesions appear even in axenically grown seedlings carrying different *mlo* alleles (Wolter et al., 1993). In analogy to pcd in animals (Raff, 1992; White, 1996), Mlo would suppress a default cell suicide program in foliar tissue. In this scenario, resistance would have to be envisaged as a consequence of deregulated pcd. The intimate link between resistance and extent of spontaneous leaf lesions has been studied on the basis of an allelic series of 95 chemically induced *mlo* alleles (Habekuss and Hentrich, 1988). The defective alleles could be classified according to gradually different infection phenotypes upon infection of a mixture of nine powdery mildew isolates. Only three mutant alleles were found to exhibit an intermediate infection phenotype (i.e., a considerable number of sporulating fungal colonies on the leaf surface) and revealed no macroscopically detectable leaf lesions. In contrast, the most efficient resistance alleles exhibited pronounced necrosis. Another line of evidence for a connection between resistance and deregulated cell death control in *mlo* mutants comes from recently identified genes (*Ror1* and *Ror2*) that are required for *mlo* function, i.e., resistance to the pathogenic fungus (Freialdenhoven et al., 1996). The dead cell leaf lesion phenotype in pathogen-free grown *Ror mlo* mutants is abolished in *ror mlo* double mutants as determined by a failure for trypan blue uptake in the latter genotype (P. S.-L. et al., unpublished data).

In our second model, the Mlo protein would have a specific negative regulatory function by down-regulating multiple defense-related functions. Spontaneous cell death in *mlo* genotypes would merely represent the fatal end of an accumulating activation of defense responses. It is supported by the chronological order of defense-related events in *mlo* genotypes in the absence of pathogens. Cell wall appositions (CWAs) appear spontaneously in epidermal tissue of approximately 14 day old seedlings (Wolter et al., 1993). CWAs are always found in response to an authentic penetration attempt of the pathogen directly beneath the fungal appressorium and are believed to form a physical barrier against pathogen ingress (Bayles et al., 1990). In 18 day old seedlings, trypan blue positive leaf cell patches appear, indicating commitment to cell death, and 2–3 days later, necrotic flecks become macroscopically detectable (P. S.-L. et al., unpublished data). However, powdery mildew resistance is fully functional in 5 day old *mlo* seedlings, the earliest time point to carry out a resistance test for technical reasons. Thus, establishment of defense-associated events in pathogen-free *mlo* genotypes is not a requirement for effective resistance upon attempted powdery mildew attack. We conclude that a complete or partial inactivation of the Mlo protein "primes" or up-regulates the responsiveness of the seedling for the onset of pathogen defense.

This is an important difference to all but one characterized lesion mimic in Arabidopsis as well as to the soybean *rn* mutants, in which elevated resistance to pathogens is either dependent on lesion formation (*acd2*, *lsd2*, *lsd3*, *lsd4*, *lsd5*, and *rn*; Greenberg et al., 1994; Dietrich et al., 1994; Kosslak et al., 1996) or is expressed concomitantly with the appearance of dead cell lesions (*lsd6* and *lsd7*; Weymann et al. 1995). So far, only the Arabidopsis *lsd1* lesion mimic mutant appears to exhibit elevated pathogen resistance at the prelesion state (Dietrich et al., 1994). In contrast to the determinate and punctate growth of lesions in *mlo* leaves, lesion formation is indeterminate in *lsd1*, consuming the entire leaf. However, host cell death cannot be required for the early developmental arrest of the powdery mildew fungus in CWAs in the genuine interaction on *mlo* genotypes since

the host cell survives the attack (Jørgensen and Mortensen, 1977; Wolter et al., 1993). A priming of defense functions in *mlo* plants would make it possible that inefficient defense responses in the *Mlo* genotype (e.g., CWA formation) become efficient (e.g., through increased speed and/or reduced response times) but that the early developmental arrest of the pathogen is insufficient to trigger a signal for the execution of the host cell death reaction.

Both of these two seemingly different functions of the Mlo protein could be explained by assuming that the protein has a dual function in down-regulating onset of leaf cell death and onset of multiple defense functions. We expect that the biochemical characterization of Mlo, the analysis of the unique collection of *mlo* mutants, and the identification of proteins with which it physically interacts should provide further insight into the molecular relationship between the control of plant cell death and plant defense functions.

## Experimental Procedures

### Plant Material

A compilation of the *mlo* mutants and their mother varieties analyzed in this study has been described by Jørgensen (1992) (*mlo-1*, *mlo-3*, *mlo-4*, *mlo-5*, *mlo-7*, *mlo-8*, *mlo-9*, *mlo-10*, and *mlo-11*) and by Habekuss and Hentrich (1988) (mutants in cultivar Plena 2018 [*mlo-13*], 2034 [*mlo-17*], 2118]. Since mutant 2118 has not been assigned to an allele number so far, we designate the allele here as *mlo-26*, according to current numbering in the GrainGene database (gopher://greengenes.cit.cornell.edu). All *mlo* BC lines in cultivar Ingrid were a gift from Prof. James McKey, Uppsala, Sweden.

The high resolution map is based on a cross between Ingrid *Mlo* × $BC_7$ Ingrid *mlo-3* . F1 plants were selfed generating a segregating F2 population of approximately 600 plants. Phenotypically susceptible F2 plants that showed heterozygosity for RFLP markers on opposite sites of *Mlo* were selfed and generated further segregants in the F3 generation for high resolution mapping.

### Powdery Mildew Infection Tests

The fungal isolate K1 (Hinze et al., 1991) is virulent on all cultivars used in this study carrying the *Mlo* allele and avirulent on all tested *mlo* genotypes. Plant growth and inoculation with Erysiphe graminis f. sp. hordei were carried out as described previously (Freialdenhoven et al., 1996). The genotype at *Mlo* of recombinants used for the high resolution map were determined after selfing and subsequent inoculation experiments in F3 or F4 families comprising at least 24 individuals.

### AFLP Analysis

Genomic DNA for AFLP analysis was isolated according to Stewart and Via (1993). AFLP analysis was carried out as described by Vos et al. (1995). A set of four DNA templates has been used: from the susceptible parent cultivar Ingrid *Mlo*, the resistant parent $BC_7$ Ingrid *mlo-3*, a pool of 2 resistant F2 individuals (*mlo-3 mlo-3*), and a pool of 9 susceptible F2 individuals (*Mlo Mlo*) derived from the cross Ingrid *Mlo* x $BC_7$ Ingrid *mlo-3* . Amplified genomic fragments representing AFLP markers Bpm2, Bpm9, and Bpm16 (Figure 1) were cloned and sequenced as follows: gel pieces (fixed by vacuum drying to Whatman 3MM paper) containing the amplified genomic fragments were identified via autoradiography and subsequently excised. 100 μl water were added and boiled for 10 min, and after centrifugation, 5 μl of the supernatant was used as a template for nonradioactive reamplification (30 cycles) with the selective AFLP primers. Amplification products were isolated after agarose gel electrophoresis and subsequently cloned in the EcoRV site of pBluescript SK (Stratagene). Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer) and resolved either on an ABI 373 or 377 (Applied Biosystems) automated sequencer.

### Barley YAC Library and BAC Sublibrary Construction of YAC YHV303-A6

The YAC library of barley cultivar Ingrid was established using the pYAC4 vector (Burke et al., 1987) and yeast strain AB 1380. Details of the library construction and its characterization will be described elsewhere. Screening for YAC clones containing marker Bpm16 was done by AFLP analysis. For construction of a BAC sublibrary of YAC YHV303-A6, total DNA of this yeast clone was used. After partial EcoRI digestion and preparative pulsed-field gel electrophoresis, DNA fragments in the size range of 50 kb were recovered and subcloned in the pECSBAC4 vector. Clones carrying YHV303-A6-derived inserts were identified by a two step colony hybridization procedure using first, labeled total DNA of the nonrecombinant yeast strain AB1380 and subsequently, labeled recombinant chromosome YHV303-A6 after enrichment by preparative pulsed-field gel electrophoresis.

### DNA Sequencing of BAC F15

DNA of BAC F15 was isolated by an alkaline lysis large scale plasmid preparation according to Sambrook et al. (1989). 50 μg of purified DNA were nebulized by high pressure treatment with argon gas in a reaction chamber for 150 s. The ends of the sheared and reprecipitated DNA were blunt-ended by a T4 DNA polymerase–mediated fill-in reaction. DNA fragments in the size range of 800 bp to 3 kb were isolated from agarose gels using a DNA isolation kit (Jetsorb, Genomed Inc., USA), subcloned into the pBluescript SK vector (Stratagene), and propagated in E. coli DH5α. Clones carrying BAC F15–derived inserts were selected by hybridization using the labeled sheared DNA of BAC F15 as a probe. Sequencing reactions were performed as described above. Evaluation of the sequencing data, construction of sequence contigs, and estimation of coding propabilities were done by means of the STADEN software package for Unix users (fourth edition, 1994). Homology searches were done using the BLAST software.

### PCR-Based Sequencing of Alleles at *Mlo*

Plant chromosomal DNA for this purpose was isolated according to Chunwongse et al. (1993). DNA sequences of *Mlo* alleles of the different barley varieties, *mlo* mutants, BC lines, and intragenic recombinants used in this study were obtained by PCR-based sequencing. Using sets of specific primers, seven overlapping subfragments of the gene (each 400–600 bp in length) were amplified by PCR (35 cycles, 60°C annealing temperature). After preparative agarose gel electrophoresis and isolation of the amplification products using the Jetsorb kit (Genomed Inc., USA), fragments were reamplified. The resulting products were subsequently purified from nucleotides and oligonucleotides (Jetpure, Genomed Inc., USA) and used as a template in DNA sequencing reactions (see above). All DNA sequences of mutant alleles and corresponding regions of the parental lines and the intragenic recombinants were derived from both strands and confirmed in independent sets of experiments.

### RT–PCR and Rapid Amplification of cDNA Ends (RACE)

RT–PCR was performed using the SUPERSCRIPT preamplification system for first-strand cDNA synthesis (Gibco BRL). Total RNA (1 μg) of 7 day old primary barley leaves (cultivar Ingrid) served as template. First-strand cDNA synthesis was primed by an oligo(dT) primer. The putative coding region of the *Mlo* gene was subsequently amplified using oligonucleotides 25L (GTGCATCTGCGTGTGCGTA) and 38 (CAGAAACTTGTCTCATCCCTG) in a single amplification step (35 cycles, 60°C annealing temperature). The resulting product was analyzed by direct sequencing. 5′ and 3′ ends of the *Mlo* cDNA were determined by RACE using the MARATHON cDNA amplification kit (Clontech). To obtain specific RACE products, two consecutive rounds of amplification (35 cycles, 55°C annealing temperature) were necessary. Two sets of nested primers were used in combination with the adapter primers of the kit: oligonucleotides 46 (AGGGTCAG GATCGCCAC) and 55 (TTGTGGAGGCCGTGTTCC) for the 5′ end and primers 33 (TGCAGCTATATGACCTTCCCCCTC) and 37 (GGA CATGCTGATGGCTCAGA) for the 3′ end. RACE products were subcloned into pBluescript SK (Stratagene). Ten 5′ end and eight 3′ end clones were chosen for DNA sequence analysis.

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The accession number for the sequence of *Mlo* is Z83834.