

High-Resolution Mapping of the *Hor1/Mla/Hor2* Region on Chromosome 5S in Barley

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A high-resolution mapping population consisting of 270 individual lines, each representing an independent recombination event between the *Hor1* and *Hor2* loci, was used to construct a high-density restriction fragment length polymorphism (RFLP) map of the *Hor1/Mla/Hor2* region. To identify informative markers for screening the recombinant population, the Franger (containing *Mla6* and *Mla14*) and Rupee (containing *Mla13*) parental lines were screened for RFLPs using 12 restriction endonucleases and 35 cDNA and genomic clones, that had been shown to map to barley chromosome 5 or wheat group 1. As a result, 61 probe/enzyme combinations were chosen to screen the recombinant population. Analysis of the RFLP mapping data indicated nine of the 21 polymorphic probes mapped within the *Hor1/Hor2* interval. Map distance between *Hor1* and *Hor2* was calculated to be approximately 8.1 cM. Nine of the remaining probes mapped outside of the *Hor1/Hor2* interval and proximal to *Hor1*. The remaining three probes mapped to separate linkage groups. Sixteen of the recombinant lines had regions of DNA that were heterozygous at one or more RFLP loci within the *Hor1/Hor2* interval. Data from the high-resolution RFLP map of the *Hor1/Hor2* region was integrated with previous recombination data which positioned the *Mla6*, *Mla13*, and *Mla14*, resistance alleles, in relation to *Hor1* and *Hor2*. Five of the probes hybridized to multiple sites. BCD249, a cDNA-derived barley clone, hybridized to two sequences 0.3 cM apart, indicating a duplication event within the *Hor1/Hor2* interval. One of these sites, *Xbcd249.2*, was positioned within 1.6 cM from the *Mla* alleles.

Fundamental knowledge of the genetics of host-pathogen interaction is crucial to effectively utilize genetic resistance to control plant disease. Powdery mildew of barley is an ideal model to study these interactions. Because the genetics of the system have been extensively characterized, it is an excellent system for investigating specific recognition in gene-for-gene interactions among small grains and obligate fungal pathogens (Flor 1955; Keen 1990; Thompson and Burdon 1992). In

addition, there are many genes for resistance in the host, each giving a unique reaction to one or more isolates of the pathogen. This phenomenon results in a large supply of naturally occurring variation.

A number of *Ml* genes have been identified in barley, *Hordeum vulgare* L., which confer resistance to the powdery mildew fungus, *Erysiphe graminis* DC. Merat f. sp. *hordei* em. Marchal (Jørgensen and Moseman 1972; Jørgensen and Jensen 1976; Hinze *et al.* 1991; Görg *et al.* 1993). The *Mla* (powdery mildew resistance) locus, located near the telomeric end of chromosome 5S, homoeologous group 1 of the family *Gramineae*, is of particular interest because of its highly variable, multicomponent nature. There is a large cluster of alleles or tightly linked genes at the *Mla* locus, and at least twenty-three have been differentiated by their specific reaction to unique isolates of *E. graminis*, making this locus suitable to detailed genetic analysis (Jørgensen and Moseman 1972; Moseman and Jørgensen 1973; Giese 1981; Giese *et al.* 1981; Wise and Ellingboe 1983, 1985; Jahoor and Fischbeck 1987; Jahoor *et al.* 1993).

The specific interaction between different host alleles and their corresponding pathogen isolates has proven advantageous for the genetic analyses of many resistance gene clusters. These include the *Rp1* cluster in maize for resistance to the maize rust pathogen, *Puccinia sorghi* Schw. (Saxena and Hooker 1968; Pryor 1987; Hulbert and Bennetzen 1991; Sudupak *et al.*, 1993), the *L* and *M* gene clusters in flax (*Linum usitatissimum* L.) for resistance to the flax rust pathogen, *Melampsora lini* (Ehrenb.) Lev. (Shepherd and Mayo 1972; Mayo and Shepherd 1980; Islam and Shepherd 1991), a *Pc* gene cluster in diploid *Avena* for resistance to the oat crown rust pathogen, *Puccinia coronata* Corda f. sp. *avenae* Eriks (Rayapati *et al.* 1994), and various *Dm* loci in lettuce (*Lactuca sativa* L.) for resistance to the downy mildew pathogen, *Bremia lactucae* Regel (Hulbert and Michelmore 1985; Farrara *et al.* 1987; Paran *et al.* 1991). Research on the genetic mechanisms responsible for linkage between genes in resistance clusters is ongoing, but no specific process has been confirmed (Sudupak *et al.* 1993; Robbins *et al.* 1991). However, the clustering of many resistance gene families, often associated with seed storage protein gene families, suggests a conserved mechanism in which tandem duplication, followed by multiple recombination or mutation events, could be involved (Giese *et al.* 1981; Wise and Ellingboe 1985; Hulbert and Bennetzen 1991; Singh *et al.* 1990; Islam and Shepherd 1991; Paran *et al.* 1992; Sudupak *et al.* 1993). Similarly, a mechanism of unequal exchange may account for the observed instability of many race-specific resistance loci

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located within these clusters (Wise and Ellingboe 1985; Pryor 1987; Sudupak *et al.* 1993). These mechanisms, occurring singly or in combination may also account for the generation of new resistance specificities (Islam and Shepherd 1991).

Specific disease-resistant alleles have been mapped to general chromosomal regions in many crops. Construction of high-resolution restriction fragment length polymorphism (RFLP) maps has allowed the precise location of some of these disease resistance genes, including the *Pto* locus in

tomato (Martin *et al.* 1993), the *Rp1* locus in maize (Hulbert and Bennetzen 1991), the *Xa21* locus in rice (Ronald *et al.* 1993), and the *Dm3* locus in lettuce (Paran *et al.* 1991). Four RFLP maps have been reported for barley which assign locations to a number of molecular and morphological markers (Shin *et al.* 1990; Heun *et al.* 1991; Graner *et al.* 1991; Kleinhofs *et al.* 1993). Of the twenty-three *Mla* variants, the *Mla6* allele has been mapped near the *Xmwig036* RFLP locus (Graner *et al.* 1991; Schüller *et al.* 1992), and the *Mla12*

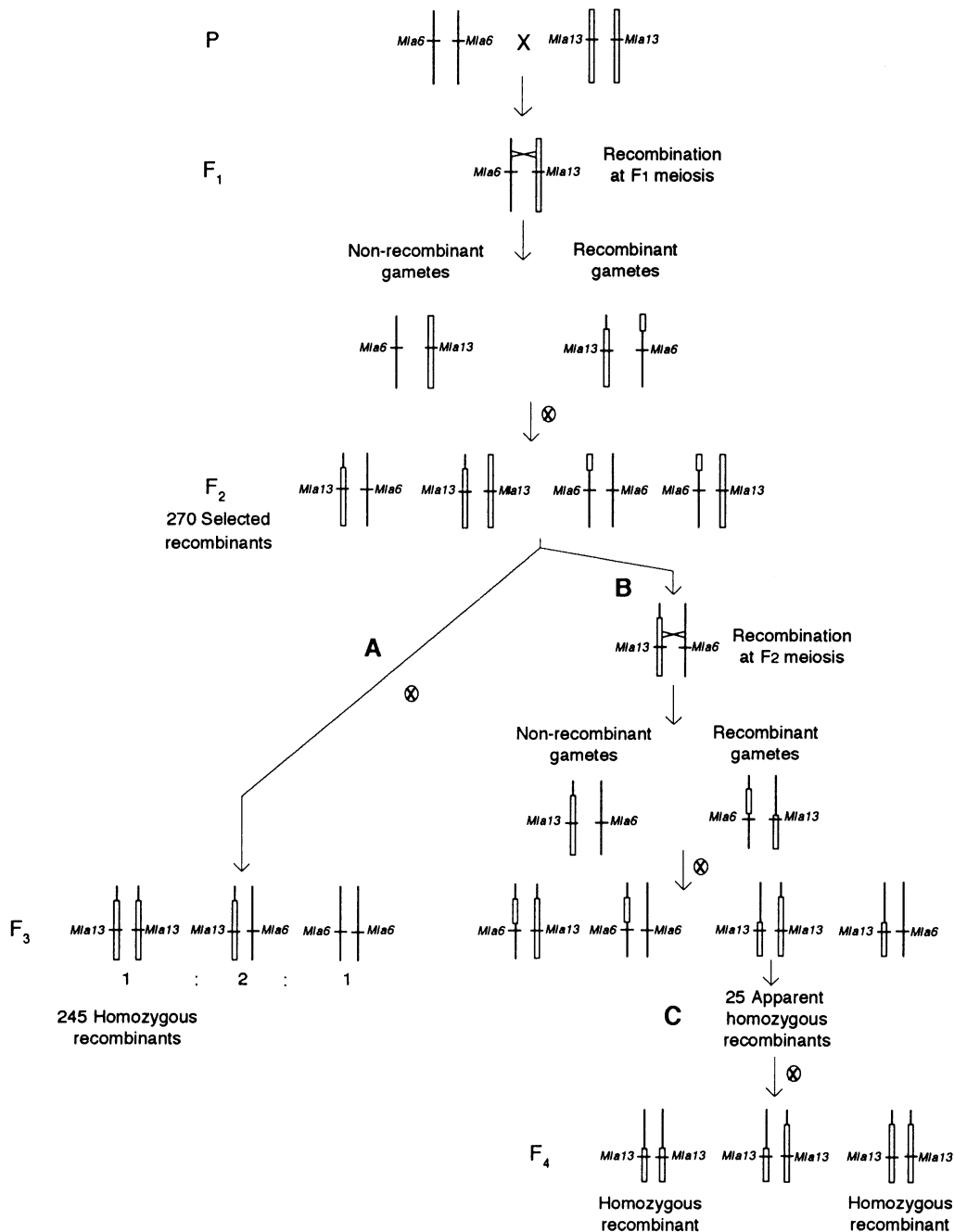


Fig. 1. Derivation of mapping population recombinant between *Hor1* and *Hor2*. The majority of the selected recombinants will be homozygous in the region spanning the *Hor1/Hor2* interval, indicated as derived via 1A. However, selection of homozygous recombinants based on the flanking *Hor1* and *Hor2* markers can result in the selection of apparent homozygous recombinants containing internal heterozygous regions. These are indicated as derived via 1B. The F₄ segregating progeny from selfed apparent homozygous recombinants were screened to select for true homozygous recombinants derived via 1C. The symbol ⊗ indicates self-pollination. The top and bottom of the vertical lines represent *Hor1* and *Hor2*, respectively.

allele was shown to be linked to the *Xbcd249* RFLP locus (Heun *et al.* 1991). However, a limited number of detailed, high-resolution RFLP maps have been developed for small grains.

Previous research in our laboratory was directed towards developing a high-resolution mapping population for the region between the *Hor1* and *Hor2* endosperm storage-protein genes on the short arm of barley chromosome 5. The *Hor1* and *Hor2* genes are approximately 8–10 centimorgans (cM) apart (Jensen *et al.* 1980), and flank the *Mla* powdery mildew-resistance locus. This high-resolution mapping population consists of 270 individual lines, each representing an independent recombination event between the *Hor1* and *Hor2* loci (Fig. 1). This population was used to map precisely the *Mla6*, *Mla13*, and *Mla14* alleles, and to investigate the genetic organization of the locus (Mahadevappa, DeScenzo, and Wise 1994). These experiments indicated that the *Mla6* and *Mla13* alleles are functionally distinct. In this report we describe the use of these recombinant lines to construct a high-resolution RFLP map of the *Hor1/Hor2* region. In addition, we define the recombination sites in each of the 270 lines at a more detailed level. Characterized in this manner, this high-resolution mapping population is an essential component towards our long term goal of map-based cloning at the *Mla* locus.

RESULTS

Identification of polymorphic markers in the (*Hor1/Hor2*) region.

To identify informative markers for screening the recombinant population, the Franger (containing *Mla6* and *Mla14*) and Rupee (containing *Mla13*) parental lines were screened for RFLPs by digesting their respective DNA with 12 restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I,

*Dra*I, *Xba*I, *Xho*I, *Apa*I, *Sty*I, *Bcl*II, and *Bgl*III). Southern filters carrying the digested DNA samples were hybridized with 35 cDNA and genomic clones, that had been shown to detect polymorphisms on barley chromosome 5 or wheat group 1 (Graner *et al.* 1991; Heun *et al.* 1991; Kleinhofs 1993). Numerous polymorphisms were detected between the two parental lines. As a result of the parental screening, 61 probe/enzyme combinations were chosen to screen the recombinant population; selected based on homozygosity at *Hor1* and *Hor2* (Table 1). The restriction endonucleases *Hind*III, *Dra*I, *Eco*RI, and *Eco*RV were selected for screening the mapping population because they, in conjunction with the available probes, yielded the greatest number of easily scored polymorphisms between the parental lines. Fifteen of the probes screened detected polymorphisms between the parents with at least one of these enzymes (Table 1, group A). Six additional probes detected polymorphisms with the restriction endonucleases *Bcl*II, *Bam*HI, *Xba*I, or *Sty*I (Table 1, group B). The remaining probes did not detect any easily scored polymorphisms.

Linkage analysis.

RFLP analysis within the *Hor1/Hor2* interval was completed on the mapping population of 270 F₃ recombinant lines (Fig. 1). The 270 individual recombinant lines were representative of an F₂ population size of 1,800 (3,600 gametes). Therefore, calculation of map distance within the *Hor1/Hor2* interval was based on an effective population size of 1,800. This population size was appropriate for calculating linkage distances within the *Hor1/Hor2* interval, because the 270 recombinant lines were obtained by screening 1,800 F₂ segregants and selecting the lines that contained a recombination event between these two markers (Mahadevappa *et al.* 1994). We could presume the remaining F₂ segregants had no recombination between *Hor1* and *Hor2* with the exception of

Table 1. Probes used to generate a high resolution map of the *Hor1/Mla/Hor2* region on barley chromosome 5S

Probe	Type	Enzymes that detect RFLPs*	Source
Group A			
CDO580	Oat cDNA	RV, H3	Sorrells and Tanksley, Cornell
CDO658	Oat cDNA	D1	Sorrells and Tanksley, Cornell
BCD98	Barley cDNA	R1, H3, RV, D1	Sorrells and Tanksley, Cornell
BCD249	Barley cDNA	H3, D1	Sorrells and Tanksley, Cornell
KSUDGE018	Wheat genomic	R1, H3, D1	B. Gill, Kansas St. Univ.
KSUDGG009	Wheat genomic	R1, RV, D1	B. Gill, Kansas St. Univ.
Hor 1	Barley cDNA	R1, RV, H3, D1	B. Ford, Rothamsted
Hor 2	Barley cDNA	R1, RV, H3, D1	B. Ford, Rothamsted
ABC160	Barley cDNA	R1, H3, D1	Kudrna and Kleinhofs, Wash. St.
pC.CHS 11	Barley cDNA	R1, RV, H3, D1	W. Rohde, Max-Planck-Institut
MWG036	Barley genomic	R1, RV, H3, D1	A. Graner, Grünbach
MWG060	Barley genomic	R1, RV, H3, D1	A. Graner, Grünbach
MWG068	Barley genomic	R1, RV, H3, D1	A. Graner, Grünbach
MWG075	Barley genomic	R1, H3, D1	A. Graner, Grünbach
MWG645	Barley genomic	R1, RV, H3	A. Graner, Grünbach
Group B			
WG180A	Wheat genomic	S1	Sorrells and Tanksley, Cornell
CDO534	Oat cDNA	BH1	Sorrells and Tanksley, Cornell
ABG373	Barley genomic	Bcl1	Kudrna and Kleinhofs, Wash. St.
ABG452	Barley genomic	Bcl1, X1	Kudrna and Kleinhofs, Wash. St.
ABC152	Barley cDNA	BH1, Bcl1, X1	Kudrna and Kleinhofs, Wash. St.
BCD371	Barley cDNA	BH1	Sorrells and Tanksley, Cornell

* Restriction enzymes: R1, *Eco*RI; RV, *Eco*RV; H3, *Hind*III; D1, *Dra*I; S1, *Sty*I; BH1, *Bam*HI; Bcl1, *Bcl*II; X1, *Xba*I.

rare double-crossover events. This permitted us to estimate the map distance between *Hor1* and *Hor2* to be 8.1 cM. Additional RFLP analysis for probes which mapped outside of the *Hor1/Hor2* interval was conducted on two discrete subsets of the mapping population and a linear order was determined (Fig. 2). DNA from the primary subset ($n = 155$) was digested with the restriction endonucleases *HindIII*, *DraI*, *EcoRI*, and *EcoRV*, whereas DNA from the secondary subset ($n = 115$) was digested with the restriction endonucleases *BclI*, *BamHI*, *XbaI*, or *StyI*.

The F_3 intercross function of Mapmaker/Exp version 3.0b (Lander *et al.* 1987; Lincoln *et al.* 1992) was used to establish genetic linkage. This function was used because the recombinant population had been through an additional meiosis in the F_2 prior to selecting the individuals homozygous at both flanking markers. Nine of 21 markers were placed within the *Hor1/Hor2* interval (Fig. 2). Nine of the remaining markers were mapped outside of the *Hor1/Hor2* interval and proximal to *Hor1*. The remaining three probes did not map to barley chromosome 5 in our mapping population. Within the *Hor1/Hor2* interval the framework assigned to chromosome 5 was *Hor1*, *Xmwg068*, *Xbcd249.1*, *Xbcd249.2*, *Xmwg036*, *Xmwg060*, *Xmwg645.2*, and *Hor2*. Markers outside the interval were positioned using a combination of the "place" command along with ordering and overlapping additional subsets. Linkage analysis indicated four loci, *Xmwg645.2*, *Xmwg075.2*, *XChs1.1*, and *XChs1.2* were unlinked to the *Hor1/Hor2* interval.

The *Mla6*, *Mla13*, and *Mla14* disease resistance alleles were previously ordered in the recombinant population (Mahadaveppa *et al.* 1994). Data from these experiments were integrated with the results from the present work in order to position the *Mla* locus relative to the RFLP markers.

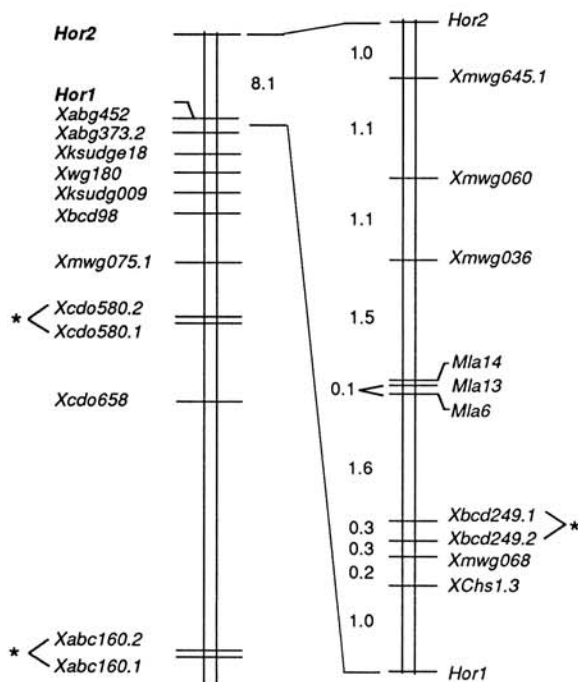


Fig. 2. High-resolution map of the introgressed region spanning the *Hor1/Hor2* region located at the telomeric end of barley chromosome 5S. Distances are in centimorgans rounded off to the nearest tenth. * Indicates closely linked duplicate sequences.

Sequence duplication.

Five of the probes used in this study hybridized to multiple sites (Fig. 2). BCD249, a cDNA derived barley clone, hybridized to two sequences 0.3 cM apart (*Xbcd249.1* and *Xbcd249.2*), indicating a sequence duplication event within the *Hor1/Hor2* interval (Fig. 3). Duplicated sequences of ABC160 (*Xabc160.1* and *Xabc160.2*) and CDO580 (*Xcd0580.1* and *Xcd0580.2*) were detected at two sites, each duplicate pair closely linked, outside the *Hor1/Hor2* interval. Similarly, two other probes, pc.CHS11 and MWG645 detected one site, *XChs1.3* and *Xmwg645.2*, respectively, within the *Hor1/Hor2* interval and one or more sites that were unlinked. The probe MWG075 hybridized to one site on chromosome 5 (*Xmwg075.1*) and one unlinked site.

Heterozygous internal regions with homozygous flanking markers.

Sixteen of the recombinant lines had regions of DNA that were heterozygous at one or more RFLP loci within the *Hor1/Hor2* interval. These were most likely the result of an additional recombination event in the F_2 meiosis (Fig. 1B). Analysis of RFLP data with the output from Map Manager v2.5 indicate these regions were variable in length, but were clustered around the *Xbcd249.1/Xbcd249.2* loci within the *Hor1/Hor2* interval. In addition, heterozygous regions were found associated with *Xmwg060*, and *Xmwg068*. Two classes of heterozygous regions were observed (Fig. 4). The type 1 class contained heterozygous regions that were flanked by one parental sequence on one side and the other parental sequence on the opposite side (Fig. 4; columns 2, 7, and 12). The type 2 class contained heterozygous regions that were inserted into and flanked by a single parental sequence (Fig. 4; column 28). Eleven of the type 1 and five of the type 2 heterozygous regions were observed in the mapping population.

Analysis of F_4 lines derived from selfed F_3 lines containing type 1 heterozygous regions.

The F_3 lines containing type 1 heterozygous regions were analyzed to determine their haplotypes. Homozygous F_4 lines

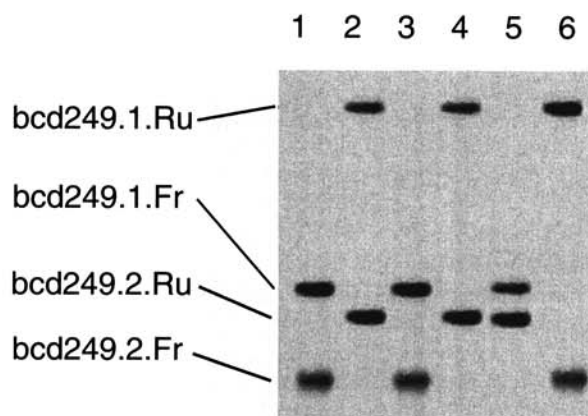


Fig. 3. Southern analysis of probe BCD249 hybridizing to two loci (*Xbcd249.1* and *Xbcd249.2*) within the *Hor1/Hor2* interval. The two parental types, observed in lane 1, Franger (*Mla6*), and lane 2, Rupee (*Mla13*), were the predominant pattern observed. Two allelic sites were detected at each locus, and designated *bcd249.1.Fr/bcd249.1.Ru*, and *bcd249.2.Fr/bcd249.2.Ru*. Eleven lines, out of the 270 analyzed, had recombinant RFLP patterns indicating duplication of the sequence hybridizing with BCD249 (lanes 5 and 6).

were selected by screening segregating progeny from selfed F₃ plants containing type 1 heterozygous regions (Fig. 1). Both RFLP analysis and resistance phenotypes were used to confirm the identity of homozygous lines. At least 16 F₄ progeny derived from each selfed F₃ line were screened for disease resistance. Eight of these progeny were selected for RFLP analysis based on their disease resistance phenotype. Two classes of homozygous recombinants were recovered from each of the selfed F₃ lines. Each class of homozygous recombinants contained one of the two recombination sites which flanked the heterozygous region in the F₃ line. Data obtained from analysis of F₄ segregating progeny from two type 1 heterozygous lines (column 7; line H92S6447 and column 12; line H92S6453) (Mahadevappa *et al.* 1994) are shown in Figure 4. At the present time, F₄ progeny from selfed F₃ lines containing type 2 heterozygous regions have not been analyzed.

DISCUSSION

The objectives of this study were to develop a high-resolution RFLP map of the *Hor1/Hor2* region on barley chromosome 5, and to integrate this RFLP map with previous recombination data which positioned the three *Mla* resistance alleles, *Mla6*, *Mla13*, and *Mla14*, in relation to *Hor1* and *Hor2*. In this report, markers from three previously reported maps (Heun *et al.* 1991; Graner *et al.* 1991; Kleinhofs *et al.* 1993) have been integrated with three alleles of the *Mla* locus into one map using our high-resolution recombinant population. In addition, we positioned three RFLP markers (*Xcdo658*, *Xcdo580.1*, and *Xcdo580.2*) proximal to the

Hor1/Hor2 interval, which have not been previously reported as mapped to chromosome 5S.

Due to the near-isogenic nature of the two parental lines, we anticipated the majority of the probes detecting polymorphisms would be located in the introgressed region centered around the *Hor1/Hor2* interval. Theoretically, each of the recombinant lines contain approximately 88% of the recurrent parent germplasm, and 12% from the respective donor parents, based on the selection method utilized to generate the lines used as parents for the mapping population (Hanson 1959). However, nine of the probes mapped outside the *Hor1/Hor2* interval proximal to *Hor1*. These RFLP data indicate the introgressed region extends to marker *Xcdo658* and probably extends to *Xabc160.1* and *Xabc160.2* (Fig. 2). Alternatively, the polymorphisms observed at *Xabc160.1* and *Xabc160.2* could be associated with a second introgressed region.

Fifteen of 155 recombinant lines in the primary mapping subset contained additional recombination events in the *Hor1/Xcdo658* segment adjacent to the *Hor1/Hor2* interval. We would expect that interference from a crossover within the *Hor1/Hor2* interval would affect subsequent crossovers adjacent to the *Hor1/Hor2* interval during a single meiosis. However, crossovers occurring in the F₁ meiosis would not interfere with crossovers in the F₂ meiosis. We are not able to determine if the additional recombination events close to *Hor1* occurred during the F₁ meiosis, and were subject to interference, or if they occurred in the F₂ meiosis. However, analysis of these 15 recombinant lines suggest the possibility of interference occurring in this region. For example, lines in which the within-interval recombination occurred close to

	12345	67890	11111 12345	11112 67890	22222 12345	22223 67890	33333 12345	33334 67890	F4 progeny from column 7 44444444 12345678	F4 progeny from column 12 45555555 90123456
<i>Hor2</i>	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBBBBBB	BBBBBBBB
									X	
<i>Xmwg645.2</i>	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBBB	BBBAB	BBBBB	BBBBBBBB	BBBBBBBB
	Xx			X	XX		X			
<i>Xmwg060</i>	AHBBB	BBBBB	BBBBB	BBBAB	BBBAA	BBBBB	BABAB	BBBBB	BBBBBBBB	BBBBBBBB
	x X	X	x	X X		X X		X X		XX
<i>Xmwg036</i>	AABAB	BBABB	BHBBB	BABAA	BBBAA	ABBAB	BABAB	BABAB	BBBBBBBB	AABBBBBB
		x			X	X	X X	X	Xxx	
<i>Mla14</i>	AABAB	BHABB	BHBBB	BABAA	ABBAA	ABAAB	AAAAB	AABAB	AHHBBBBB	AABBBBBB
<i>Mla13</i>	AABAB	BHABB	BHBBB	BABAA	ABBAA	ABAAB	AAAAB	AABAB	AHHBBBBB	AABBBBBB
<i>Mla6</i>	AABAB	BHABB	BHBBB	BABAA	ABBAA	ABAAB	AAAAB	AABAB	AHHBBBBB	AABBBBBB
		X	X	XxX X	X	X	x	X		XXXXXX
<i>Xbcd249.1</i>	AABAA	BHAAB	AAABA	BAAAA	AABAA	ABHAB	AAAAA	AABAB	AHHBBBBB	AAAAAAA
						Xx				
<i>Xbcd249.2</i>	AABAA	BHAAB	AAABA	BAAAA	AABAA	AAAAA	AAAAA	AABAB	AHHBBBBB	AAAAAAA
		x				X		X	x xXXXXX	
<i>Xmwg068</i>	AABAA	BAAAB	AAABA	BAAAA	AABAA	AAAAA	AAAAA	AAAAB	AAAAAAA	AAAAAAA
			X	X						
<i>XChs1.3</i>	AABAA	BAAAB	AAAAA	AAAAA	AABAA	AAAAA	AAAAA	AAAAB	AAAAAAA	AAAAAAA
	X	X	X		X			X		
<i>Hor1</i>	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAA	AAAAAAA	AAAAAAA

Fig. 4. Map Manager v2.5 output illustrating 40 of the 270 recombinant lines used in this study. An "A" denotes the Franger (*Mla6*) allele, "B" denotes the Rupee (*Mla13*) allele, and an "H" denotes a heterozygote for the RFLP or resistance locus indicated on the left side of the table. Individual accessions are in numerical order across the top of the figure. Columns 2, 7, and 12 represent examples of lines containing type 1 heterozygous regions which occur at the junction between the two genotypes. Column 28 represents a line containing a type 2 heterozygous region in which the heterozygous region is flanked by a single parental genotype. Data from RFLP and disease resistance analysis for selfed segregating progeny from the lines represented in columns 7 (line H92S6447) and 12 (line H92S6453) (Mahadevappa *et al.* 1994) were used to generate haplotypes which are shown in columns 41-48 and 49-56, respectively.

Hor2, exhibited the additional recombination close to *Hor1*, within the *Hor1/Xcdo658* segment. Likewise, lines in which the within-interval recombination occurred near *Hor1*, exhibited the additional recombination in the *Hor1/Xcdo658* segment closer to *Xcdo658*.

The calculated map distance between *Hor1* and *Hor2* of 8.1 cM is within the range of previous reports: 8.5 cM (Siedler and Graner 1991); 13.91 map units (Shin *et al.* 1990); approximately 10.0 cM (Graner *et al.* 1991); 12.5 cM (Kleinhofs *et al.* 1993); and 16.4 cM (Schondelmaier *et al.* 1993). The majority of the markers we have mapped within the *Hor1/Hor2* interval are consistent with orders reported in the previously published maps. However, marker *Xabg373.2*, which we mapped to a site proximal to *Hor1*, had been previously placed 153.8 cM proximal to *Hor1* (Kleinhofs *et al.* 1993). Similarly, *Xabg452*, which mapped identically to *Hor1*, was previously placed approximately 53.7 cM proximal to *Hor1* (Kleinhofs *et al.* 1993). In addition, the *Xbcd249* locus had not previously been positioned on a map with RFLP markers located in the *Hor1/Hor2* interval. The remaining markers placed on the same linkage group were mapped outside the *Hor1/Hor2* interval, proximal to *Hor1* (Fig. 2), and correspond to the map order previously reported. Of particular interest, towards our long-term goals, are the markers *Xbcd249.2* and *Xmwig036* that flank *Mla6* and *Mla13*, by 1.6 and 1.5 cM, respectively, on either side. Identifying flanking RFLP markers in this proximity to the *Mla* resistance locus has enabled us to investigate the correlation between distinct RFLP alleles and resistance phenotype. The flanking markers are invaluable in the investigation of lines with altered resistance phenotypes.

A recent report (Ahn and Tanksley 1993) indicates gene duplication frequently occurs throughout the genome in maize as well as wheat, oats, and barley. High-resolution populations enable the detailed genetic investigation of duplicated regions within linkage groups. We have positioned five cDNA probes that hybridize to multiple sites, indicating duplicated regions. The probes hybridized to duplicate sites within and outside the *Hor1/Hor2* interval. Identification of duplicate sequences in this interval has been previously reported for sites within the *Hor1* and *Hor2* loci (Forde *et al.* 1985), and Schondelmaier *et al.* (1993) observed a duplicated sequence, separated by 20.2 cM, on barley chromosome 5S adjacent to the *Hor1/Hor2* interval. We observed several duplicate DNA sequences in the *Hor1/Xcdo658* segment of the introgressed region. Some pairs of duplicate sequences (*Xcdo580.1* and *Xcdo580.2*) were tightly linked, while other pairs were not linked. Sudupak *et al.* (1993) hypothesized that there were duplicate sequences carried in some of the *Rp1* alleles in maize, which determine resistance to races of the maize rust fungus, *Puccinia sorghi*. Further analysis of markers in the *Rp1* region revealed an additional duplication of the closely linked NP1285 RFLP locus (Hong *et al.* 1993). In addition, Paran *et al.* (1992) observed duplicate sequences associated with the glycolytic enzyme, triose phosphate isomerase (TPI). The duplicate TPI sequences mapped to separate clusters associated with downy mildew resistance. Martin *et al.* (1993) observed numerous cross-hybridizing sequences at the tomato *Pto* locus, indicating sequence duplication occurred at this resistance cluster. Although these observations suggest the areas associated with clusters of

resistance genes are prone to gene duplication and rearrangement, there is no direct evidence that these processes function at a higher rate in these areas than in other areas of the genome. However, sequence duplication has been postulated as a component of the mechanism by which new disease resistance alleles are generated (Hulbert and Bennetzen 1991; Sudupak *et al.* 1993).

The homozygous recombinant population was developed by selecting individual lines in which a recombination event had occurred between the *Hor1* and *Hor2* loci. Although the mapping population is homozygous at the flanking selected markers, *Hor1* and *Hor2*, heterozygous regions internal to the flanking markers were detected in 16 of the lines during RFLP analysis on the mapping population. The presence of internal heterozygous regions was not detected when the flanking markers were screened to select the recombinant population. In six of the sixteen recombinant lines containing internal heterozygous regions, one or more of the adjacent *Mla* alleles are heterozygous. In addition, nine other recombinant lines are heterozygous at one or more of the *Mla* alleles (Mahadevappa *et al.* 1994). Internal heterozygous regions were observed either at the junction of the two parental sequences (type 1), or they occurred inserted into and flanked on both sides by a single parental sequence (type 2) (Fig. 4). Type 1 heterozygous regions are likely the result of an additional recombination occurring in the F₂ meiosis (Fig. 1B). Although the presence of the additional recombination events was not unexpected, it was advantageous to resolve the heterozygous regions in order to obtain a single defined recombination site in these lines. To verify this, F₄ progeny were selected from selfed F₃ plants containing type 1 heterozygous regions to yield true homozygous recombinant lines (Fig. 1C and Fig. 4).

The mechanisms underlying formation of the type 2 heterozygous region are not presently understood. However, these could be the result of a non-crossover or gene conversion event, as several of the type 2 heterozygous regions only affect one locus. Similar unusual events have been observed to accompany unequal exchange and gene conversion in *Saccharomyces cerevisiae* (Maloney and Fogel 1987), in *Drosophila melanogaster* (Peterson and Laughnan 1963; Goldberg *et al.* 1983; Davis *et al.* 1987; Hipeau-Jacquotte *et al.* 1989), and in maize (Laughnan 1961; Dooner and Kermicle 1970; Sudupak *et al.* 1993). The lines containing type 2 heterozygous regions are a valuable source of germplasm to further investigate the formation of these regions.

Genetic rearrangements as the result of base mispairing and unequal crossing-over have been suggested to explain a number of unusual recombination events observed using high-resolution genetic analysis on genomic regions containing disease resistant loci (Hulbert and Bennetzen 1991; Ronald *et al.* 1992; Sudupak *et al.* 1993). The results of this research have enabled us to obtain more detailed information on the location of recombination events occurring in the *Hor1/Hor2* interval flanking the *Mla* locus. We have defined the recombination sites in each of the 270 lines in our mapping population using both RFLP and disease resistance analysis. We hope to overcome some of the inherent difficulties associated with chromosome walking in cereal crops by utilizing select lines containing defined recombination sites between specific loci.

In the process of developing a high-resolution mapping population and completing a fine structure map of the *Hor1/Hor2* interval, we have generated recombinants within the *Mla* complex and flanked it with probes no more than 1.6 cM on either side. The data from these experiments indicate we need to place more markers in this region prior to initiating physical mapping. It is advantageous to have markers mapped within 0.1 cM of each other to determine the relationship between genetic and physical distance. However, with the current markers we should be able to resolve recombination sites between closely linked loci using pulsed-field-gel-electrophoresis analysis. We have 11 lines containing a recombination event between *Xbcd249.1* and *Xbcd249.2*, which are separated by 0.3 cM. We also have 10 lines containing recombination events between *Xmwig068* and *Xbcd249.2* which are separated by 0.3 cM. The data obtained from these experiments will be used to determine the resolution of the mapping population for physical mapping of this region.

MATERIALS AND METHODS

RFLP probes.

The RFLP probes and their sources used in these experiments are listed in Table 1.

Plant material.

The F₃ recombinant mapping population utilized in this study was developed within our research group (Mahadevappa *et al.* 1994). The recombinant lines selected were derived from a cross between nearly isogenic barley lines developed for reaction to *E. graminis* f. sp. *hordei* (Moseman 1972). Initial crosses were constructed between the Franger (C.I. 16151) and Rupee (C.I. 16155) accessions containing the *Mla6* and *Mla13* alleles, respectively (Moseman 1972). The mapping population was selected by analyzing endosperm extracts from 1,800 F₂ seeds for recombinant C and B hordein polypeptide patterns (Fig. 1). These recombinant patterns indicate genetic recombination between the *Hor1* and *Hor2* loci, which flank the *Mla* region. Embryo halves from the selected F₂ heterozygous recombinants were planted in the greenhouse to generate recombinant F₃ families. The recombinant F₃ families were screened to identify homozygous lines, via analysis of the C and B hordein proteins. These F₃ homozygous recombinant lines were planted and leaf tissue was harvested for DNA extraction. F₄ seeds were collected and analyzed to confirm that these recombinant lines were homozygous.

DNA preparation.

Barley genomic DNA was isolated from leaves of 3- to 5-wk-old greenhouse grown plants using a modified hexadecyl trimethylammonium bromide (CTAB) method (Saghai-Maroof *et al.* 1984, Wise and Schnable 1994). DNA suspensions were quantified on a Hoefer mini-fluorometer according to manufacturers instructions.

Southern analysis.

For gel blot analysis, 10 µg of DNA was restricted according to suppliers instructions, using the buffer supplied with the enzyme. Gels (20 × 24 cm) were poured using 0.9%

SeaKem LE agarose in 250 ml 1× TPE buffer (36 mM Tris-HCl, 30 mM NaH₂PO₄, 1 mM EDTA). DNA was transferred for 16–20 hr onto Hybond N⁺ nylon membrane using 20× SSC (1× SSC is 15mM trisodium citrate, 150 mM NaCl) as the transfer solution in a BIOS blotting unit. DNA was fixed to the membranes by treating for 20 min with 0.4 N NaOH followed by a brief wash in 5× SSPE (1× SSPE, 10 mM NaH₂PO₄, 180 mM NaCl, 1 mM EDTA, pH 7.4) to neutralize the membranes.

Preparation of probe and hybridization.

Probes were radiolabeled by the random hexamer primer method (Feinberg and Vogelstein 1983). Hybridizations and initial washes were done in a Robbins Scientific Hybridization Oven (Robbins Scientific Corporation, Sunnyvale, CA). The blots were prehybridized in 1.0% BSA, 1 mM EDTA, 0.5 M NaHPO₄, pH 7.2, 7.0% sodium dodecyl sulfate (Church and Gilbert 1984) for a minimum of 2 hr prior to adding the probe, and then hybridized for 16 hr. Initial washes were done in the hybridization tubes and consisted of two 30-min washes in 1× SSPE, 0.1% SDS, at 65° C followed by transfer to a plastic tray and washed in 1× SSPE, 0.1% SDS for 1 hr at 65° C with shaking. The final wash consisted of 15 min at 65° C in 0.2× SSPE, 0.1% SDS. Membranes were exposed to Kodak X-Omat AR film for 3–7 days at –70° C using two Dupont Cronex Lightening Plus intensifying screens.

Characterization of disease resistance phenotype.

Planting, inoculation with *Erysiphe graminis* f. sp. *hordei*, and scoring of disease resistance phenotype for the F₄ progeny from selfed F₃ lines containing type 1 heterozygous regions were performed as described previously (Mahadevappa *et al.* 1994).

Data analysis.

RFLP and disease resistance phenotype data were recorded, edited, and transformed into Mapmaker format using the Macintosh compatible program Map Manager (version 2.5), developed by Kenneth Manly at the Roswell Park Cancer Institute (Manly 1993). Map Manager is a program for genetic mapping with selfed recombinant inbred strains or backcrosses. The file output is invaluable for analysis of cross-overs and heterozygous regions. The F₃ intercross (self) function of Mapmaker/Exp version 3.0b (Lander *et al.* 1987; Lincoln *et al.* 1992) was used to analyze the RFLP data to determine linkage. Genetic linkage was calculated by ordering subsets of the linkage group and then overlapping those subsets, mapping any remaining markers relative to those already mapped. The “suggest subset” command was used to produce the initial subset for ordering. Additional subsets were ordered by overlapping existing markers and a framework was produced. The framework was assigned to chromosome 5 and the “place” command was used to position remaining markers within the interval.

The F₃ mapping population was selected by screening only for recombinants between *Hor1* and *Hor2* and, therefore, was not representative of the entire F₃ population. Because the population was not a true F₃, but had undergone an additional round of meiosis in the F₂, neither the F₂ nor F₃ analysis functions from Mapmaker 3.0b were considered appropriate for determining the distance between markers in the *Hor1/Hor2*

interval. To determine distance between markers within the *Hor1/Hor2* interval, the distance was calculated between *Hor1* and *Hor2* in the F₂ population. Subsequently, the distribution of recombinants in the F₃ was used to define the subintervals within the *Hor1/Hor2* interval. Distance between the markers was determined using the following formula:

$$n \times d / t = s$$

where *n* is equal to the number of recombinants in the subinterval between two markers; *d* is equal to the distance between *Hor1* and *Hor2*, calculated from the analysis of the F₂ population; *t* is equal to the total number of homozygous recombinants in the *Hor1/Hor2* interval, and *s* is equal to the distance between the markers in a particular subinterval.

Markers outside the interval were positioned using a combination of the "place" command along with ordering and overlapping additional subsets. To accurately map markers outside of the *Hor1/Hor2* interval, the mapping population was adjusted from 1,800 to 155 or 115, i.e., the size of the primary and secondary subset, respectively. This was done to compensate for the change from a sample of 270 recombinant lines representing a population of 1,800, to a selected sample of 155 or 115 lines used as the mapping population. Analysis on markers outside the interval was done on the primary and secondary data sets consisting of the same 270 individuals used for mapping within the *Hor1/Hor2* interval.

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