Sequence Haplotypes Revealed by Sequence-Tagged Site Fine Mapping of the *Ror1* Gene in the Centromeric Region of Barley Chromosome 1H[1][w]

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We describe the development of polymerase chain reaction-based, sequence-tagged site (STS) markers for fine mapping of the barley (*Hordeum vulgare*) *Ror1* gene required for broad-spectrum resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*). After locating *Ror1* to the centromeric region of barley chromosome 1H using a combined amplified fragment length polymorphism/restriction fragment-length polymorphism (RFLP) approach, sequences of RFLP probes from this chromosome region of barley and corresponding genome regions from the related grass species oat (*Avena* spp.), wheat, and *Triticum monococcum* were used to develop STS markers. Primers based on the RFLP probe sequences were used to polymerase chain reaction-amplify and directly sequence homologous DNA stretches from each of four parents that were used for mapping. Over 28,000 bp from 22 markers were compared. In addition to one insertion/deletion of at least 2.0 kb, 79 small unique sequence polymorphisms were observed, including 65 single nucleotide substitutions, two dinucleotide substitutions, 11 insertion/deletions, and one 5-bp/10-bp exchange. The frequency of polymorphism between any two barley lines ranged from 0.9 to 3.0 kb, and was greatest for comparisons involving an Ethiopian landrace. Haplotype structure was observed in the marker sequences over distances of several hundred basepairs. Polymorphisms in 16 STSs were used to generate genetic markers, scored by restriction enzyme digestion or by direct sequencing. Over 2,300 segregants from three populations were used in *Ror1* linkage analysis, mapping *Ror1* to a 0.2- to 0.5-cM marker interval. We discuss the implications of sequence haplotypes and STS markers for the generation of high-density maps in cereals.

Markers based on PCR are favored over more conventional restriction fragment-length polymorphism (RFLP) markers because they do not require the use of radioactivity, they have a faster turn around, and they require smaller amounts of genomic DNA. Discreet DNA sequences that can be PCR-amplified from genomic DNA and that have defined genomic locations, termed sequence tagged sites (STSs), represent one type of PCR marker. Amplified STS products are assayed for sequence polymorphisms using direct sequencing or gel electrophoresis with or without prior restriction enzyme digestion. The development of detailed RFLP maps of cereal species such as maize, rice, and barley (*Hordeum vulgare*), together with the availability of the DNA sequences of the probes used to make these maps, form the basis for STS marker utilization in these species (Blake et al., 1996; Harushima et al., 1998; Davis et al., 1999; Michalek et al., 1999). In barley, STS markers have been developed to tag individual quantitative trait loci or genes (Horvath et al., 1995; Graner and Tekauz, 1996; Graner et al., 1996; Larson et al., 1996, Mohler and Jahoor, 1996; Borovkova et al., 1998; Ford et al., 1998) and for genome-wide mapping (Tragoonrung et al., 1992; Mano et al., 1999).

Barley lines homozygous for mutant alleles of the *Mlo* gene exhibit broad-spectrum resistance to all tested isolates of the barley powdery mildew fungus pathogen (*Blumeria graminis* f. sp. *hordei*; Jørgensen, 1992). This resistance is typically manifested in the failure of the fungus to penetrate the epidermal cell walls, and at these sites cell wall thickenings (papillae) are produced. The resistance response involves an enhanced accumulation of *PR* gene transcripts and a more intense cell wall-restricted oxidative burst (Peterhänsel et al., 1997; Hückelhoven et al., 1999). In the absence of the pathogen, *mlo* mutations result in pleiotropic effects including the spontaneous development of papillae and patches of mesophyll cell death similar to those seen in inoculated plants (Wolter et al., 1993; Peterhänsel et al., 1997). The product of the cloned *Mlo* gene has been shown to contain seven membrane spanning domains and to

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reside in the plasma membrane (Büschges et al., 1997; Devoto et al., 1999). However, the mechanisms by which the wild-type Mlo protein might carry out its proposed role as a negative regulator of a defense response have yet to be elucidated.

Two genes that are required for full expression of *mlo* resistance are *Ror* (required for *Mlo* resistance)*1* and *Ror2*. These two genes were identified as a result of a mutant screen performed in an *mlo*-resistant barley line (Freialdenhoven et al., 1996). Mutations in *Ror1* or *Ror2* partially disable *mlo* resistance and compromise all other known effects of *mlo* mutations (spontaneous and pathogen induced), identifying the *Ror* genes as important components of *Mlo*-modulated processes (Freialdenhoven et al., 1996; Peterhänsel et al., 1997). *Ror* mutations do not alter the function of race-specific powdery mildew resistance genes, and therefore define a resistance signaling pathway in barley that is distinct from another that involves the *Rar1* and *Rar2* genes (Peterhänsel et al., 1997).

The *Ror1* gene represents an attractive target for isolation since its isolation should reveal molecular factor(s) necessary for effective *mlo* resistance rather than defense modulation. With this in mind we first located *Ror1* to barley chromosome 1H using amplified fragment length polymorphism (AFLP) and RFLP markers, and then performed fine genetic mapping using STS markers as a first step toward isolating *Ror1* by a positional cloning approach. Sequence and mapping information from related grass species, as well as from barley, were used in STS development, allowing the *Ror1* region to be mapped to high resolution (average 0.5-cM marker interval). Sequence polymorphisms within individual STSs exhibited non-random distribution (haplotype structure). The significance of comparative mapping information and sequence haplotype structures for STS marker development is discussed.

RESULTS

AFLP Marker Screen

Three crosses were used for mapping *Ror1*: A89 (*mlo-5*; *ror1-2*) × BCPallas *mlo-5* (*mlo-5*; *Ror1*); C69 (*mlo-5*; *ror1-4*) × Grannenlose Zweizeilige (*mlo-11*; *Ror1*); and A89 (*mlo-5*; *ror1-2*) × Malteria Heda (*mlo-3*; *Ror1*). Because both parents of each cross were *mlo* resistant, resistance segregated as a monogenic trait at the *Ror1* locus in the F_2 and subsequent generations.

The screen for *Ror1*-linked AFLP markers was performed using pools of DNA from individuals from the *Ror1* mapping families. Each pool was constructed using 10 F_2 segregants that had been determined to be homozygous resistant (*Ror1*/*Ror1*) or homozygous susceptible (*ror1*/*ror1*) by resistance assays performed on progeny of these plants (F_3 generation). A resistant and a susceptible pool for the A89 × BCPallas *mlo-5* cross and a susceptible pool for each of the other two crosses were analyzed alongside the parental lines. Potential *Ror1*-linked AFLP fragments were defined as those that were polymorphic between the resistant and susceptible pool (A89 × BCPallas *mlo-5* cross) or polymorphic between the susceptible pool and the resistant parent (other crosses).

All pools and parents were analyzed using 26 *Pst*I/*Mse*I primer combinations and 24 *Eco*RI/*Mse*I primer combinations, and the A89 × BCPallas *mlo-5* material was analyzed using a further 53 *Pst*I/*Mse*I primer combinations. Table I shows the levels of AFLP polymorphism observed between the parental barley lines. More than 30 potential *Ror1*-linked markers were identified from over 5,000 bands examined.

Locating *Ror1* to Barley Chromosome 1H

The AFLP primer pair *Pst*I+AA/*Mse*I+AGG amplified a fragment from the susceptible pool, but not the resistant pool, derived from the A89 × BCPallas *mlo-5* population. The AFLP marker, designated BPM101, cosegregated with the *Ror1* locus in the 20 individuals used to construct the A89 × BCPallas *mlo-5* pools (not shown). The fragment was cloned and was found to detect a *Bgl*II RFLP between the barley lines cv Igri and cv Franka (not shown), which are parents of a doubled haploid population used to construct a genetic map of the barley genome (Graner et al., 1994). A subset of 35 individuals from the cv Igri × cv Franka mapping population were assayed for the *Bgl*II polymorphism, and among these individuals the RFLP cosegregated with the MWG913 RFLP locus, which is positioned near the centromere of chromosome 1H (Graner et al., 1994). This pro-

Table I. *AFLP polymorphism between parent barley lines*[a]

Six parent barley lines were subjected to AFLP analysis using 50 AFLP primer combinations. The percentage of AFLP bands that were polymorphic for each pairwise barley line comparison is indicated for each restriction enzyme combination.

	BCIngrid/ Malt. Heda	BCIngrid/ BCPallas	BCIngrid/ Gran. Zweiz.	Malt. Heda/ BCPallas	Malt. Heda/ Gran. Zweiz.	Gran. Zweiz./ BCPallas
*Eco*RI/*Mse*I	5.1%	6.0%	11.8%	6.5%	10.8%	10.3%
*Pst*I/*Mse*I	11.4%	9.2%	20.6%	12.4%	19.9%	17.5%

[a] BCIngrid, BCIngrid *mlo*-5; BCPallas, BCPallas *mlo*-5; Gran. Zweiz., Grannenlose Zweizeilige; Malt. Heda, Malteria Heda. Data shown here are: 26 for *Pst*I/*Mse*I and 24 for *Eco*RI/*Mse*I primer combinations.

vided the first indication that the *Ror1* locus was located near the centromere on chromosome 1H.

STS Amplification

STS sequences in the *Ror1* region were PCR-amplified from the genomic DNA of the four *Ror1* mapping parent lines and the fragments were sequenced directly to identify polymorphisms to facilitate genetic mapping. To provide a broader view of sequence polymorphism in barley, sequences from the four parental lines were not only compared for combinations used in crossing, but for all six possible pairwise combinations. Table II lists the sequences used in the analysis. Further details regarding the analysis, including primers used for amplifying STSs, size of STS amplification products, and the locations of the sequence polymorphisms are provided

Table II. *Sequenced STSs*

Sequence of 22 STSs were determined from each of six barley parent lines. Most of the STSs were predicted to encode products with similarity to known or hypothetical proteins. The no. and types of polymorphisms observed for each marker/pairwise barley line comparison are shown.

STS[a]	Putative Product	Size[b]	Polymorphisms[c]					
			BCIngrid/ Malt. Heda	BCIngrid/ BCPallas	BCIngrid/ Gran. Zweiz.	Malt. Heda/ BCPallas	Malt. Heda/ Gran. Zweiz.	Gran. Zweiz./ BCPallas
ABC152	Histone H3	334 bp	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
ABG53	None	292 bp	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
ABG74	None	832 bp	3 subst.	n.p.	2 subst.	3 subst.	5 subst.	2 subst.
ABG373	S-like RNAse	449 bp	1 exchange	n.p.	n.p.	1 exchange	1 exchange	n.p.
ABG452	YABBY	1,671 bp	1 subst.	n.p.	1 subst.	1 subst.	1 subst.	n.p.
ABG494	Sodium proton exchanger	1,053 bp	2 subst. 1 indel	n.p.	n.p.	2 subst. 1 indel	2 subst. 1 indel	n.p.
ABG500	None	499 bp	10 subst. 4 indels	n.p.	n.p.	10 subst. 4 indels	10 subst. 4 indels	n.p.
BCD386	Hypothetical	1,231 bp	1 subst. 1 indel.	n.p.	1 subst. 2 indels	1 indel.	n.p.	2 indels
BCD454	Pyruvate orthophosphate dikinase	1,215 bp	n.p.	1 subst.	n.p.	1 subst.	n.p.	1 subst.
BCD1449	GTPase activator	260 bp	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
BPM101	None	2,538 bp	n.p.	1 indel 4 subst.	1 indel 5 subst.	1 indel 4 subst.	1 indel 2 subst.	2 subst.
CD098	mRNA transport regulator	1,270 bp	1 subst.	n.p.	1 subst.	1 subst.	n.p.	1 subst.
CD01173	None	2,156 bp	1 subst.	n.p.	1 subst.	1 subst.	n.p.	1 subst.
CDO1188	Alanine amino-transferase	3,294 bp	n.p.	n.p.	3 subst.	n.p.	3 subst.	3 subst.
COMT	Caffeic acid *O*-methyl-transferase	2,137 bp	n.p.	+/−	7 subst.	+/−	3 subst.	+/−
DGE18	Hypothetical	1,117 bp	n.p.	2 indels	n.p.	1 indel	n.p.	1 indel
MWG506	None	668 bp	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
cMWG758	Transcription factor	986 bp	n.p.	1 subst.	1 subst.	1 subst.	1 subst.	1 subst.
MWG800	Hypothetical	1,812 bp	n.p.	n.p.	3 subst.	n.p.	3 subst.	3 subst.
MWG896	Retroviral pol	698 bp	2 subst.	n.p.	4 subst.	2 subst.	2 subst.	4 subst.
MWG913	Lipo amide dehy-drogenase	1,522 bp	n.p.	4 subst.	n.p.	4 subst.	n.p.	4 subst.
MWG2056	None	2,164 bp	1 subst.	1 indel. 6 subst.	1 indel. 9 subst.	1 indel. 5 subst.	1 indel. 9 subst.	8 subst.
Total polymorphisms			29	21	42	46	49	34
Total sequence compared[d]			19,985 bp	23,702 bp	21,795 bp	18,499 bp	16,578 bp	18,633 bp
Sequence polymorphism frequency/basepair			1.5×10^{-3}	0.9×10^{-3}	1.9×10^{-3}	2.5×10^{-3}	3.0×10^{-3}	1.8×10^{-3}

[a] Original RFLP probe type upon which STSs were based: ABC and ABG, barley cDNA and barley genomic DNA, respectively (Kleinhofs et al., 1993); BCD and CDO, barley cDNA and oat cDNA, respectively (Heun et al., 1991); COMT, barley genomic DNA (Lee et al., 1997); DGE18, *A. squarrosa* genomic DNA (also known as KSUDGE18 and KSUE18; Heun et al., 1991); and MWG and cMWG, barley genomic DNA and barley cDNA, respectively (Graner et al., 1994). [b] Sequences deposited under GenBank accession nos. AF309354 through AF309375. [c] For each barley line pairwise comparison. BCIngrid, BCIngrid *mlo*-5; BCPallas, BCPallas *mlo*-5; Gran. Zweiz., Grannenlose Zweizeilige; Malt. Heda, Malteria Heda; indel, insertion/deletions; subst., single or dinucleotide substitutions; exchange, exchange of 5 bp with another 10 bp; +/−, indel polymorphism of several hundred bp; n.p., no polymorphism; indels, single nucleotide substitutions, dinucleotide substitutions, and exchanges were each counted as one polymorphism. [d] Out of a total of 28,198 bp of STS sequence. Incomplete sequence of some STSs was obtained for some STS/barley line combinations.

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The 181-bp cloned BPM101 AFLP marker fragment was sequenced, and the sequences covering the ends of the BPM101 fragment and extending into neighboring 3′ and 5′ sequences were obtained by the method of Siebert et al. (1995), providing a total of approximately 1.9 kb of genomic sequence.

Other sequences were identified as a result of RFLP markers previously mapped near the centromere on genetic maps of barley chromosome 1H (Heun et al., 1991; Kleinhofs et al., 1993; Graner et al., 1994; Lee et al., 1997) or the corresponding regions in the related grass genomes of wheat and *Triticum monococcum* (Van Deynze et al., 1995a; Dubcovsky et al., 1996). Because this region of chromosome 1H has been shown to be related to oat chromosome A, RFLP markers from maps of oat chromosome A (Van Deynze et al., 1995b) were also used. Probe sequences that detected multiple loci on published maps or that showed complex hybridization patterns on Southern blots of cereal DNA (images obtained from GrainGenes web site) were avoided. Sequences of the RFLP probes were determined in our laboratory or were obtained from the GenBank or GrainGenes databases, and were used to design primers for the PCR amplification of the corresponding barley genomic DNA sequences.

Primers were based mostly on the sequences of barley cDNA or barley genomic DNA probes. However, for the CDO1188 marker that was originally mapped using an oat cDNA probe, primers were based on an orthologous 1.7-kb barley cDNA sequence present in the database (87% sequence identity; accession no. Z26322). The oat sequences CDO98 and CDO1174 and the *Aegilops squarrosa* sequence DGE18 did not identify orthologues in the database, but primers matching these sequences were successfully used to amplify barley orthologues (>85% sequence identity).

New sequence was generated as a result of completely sequencing markers for which only probe-end sequences were previously available. Extra sequence was also obtained in the form of introns, from markers that were originally based on cDNA probes. Additional primers were made to completely sequence PCR products that were too large to sequence using the original primer pair alone. The initial failure to identify polymorphism for some markers prompted us to obtain additional genomic sequence located 3′ or 5′ to probe sequences, using the method of Siebert et al. (1995). In this way, a further 5,349 bp of sequence was made available for analysis for markers ABG452, ABG500, cMWG758, MWG800, MWG913, and MWG2056.

Five different primer combinations that amplified fragments of the caffeic acid *O*-methyltransferase gene (COMT marker) from BCIngrid *mlo-5*, Grannenlose Zweizeilige, and Malteria Heda DNA templates failed to amplify fragments from BCPallas *mlo-5* DNA (not shown), indicating that at least 2.0 kb of the COMT gene is absent in the BCPallas *mlo-5* line. This is consistent with the reported absence of the COMT gene sequences from some barley lines (Lee et al., 1997). The other marker sequences shown in Table II were amplified from all four barley lines and were confirmed to be homologous to the original RFLP probe sequences by sequence alignment.

DNA Polymorphisms

Sequences spanning 28.2 kb from 22 markers were PCR amplified from the four barley parental lines for sequencing and polymorphism identification. The results are summarized in Table II. In addition to the large insertion/deletion (indel.) in COMT, 79 smaller unique sequence polymorphisms were identified from 18 of the marker sequences. These comprised 65 single-nucleotide substitutions, two dinucleotide substitutions, five single-basepair indels, six indels of three to 12 bp, and a replacement of AACAC with GTACTGAATG (in ABG373). Two of the indels represented variations in numbers of simple sequence repeats (microsatellites), namely $(AGG)_4/(AGG)_6$ (in DGE18) and $(TAAA)_3/(TAAA)_4$ (in BPM101).

The basis of the BPM101 AFLP polymorphism was found to be a sequence difference in BCPallas *mlo-5* that abolished the *Pst*I restriction site at the end of the *Pst*I-*Mse*I AFLP fragment, and other polymorphisms were identified elsewhere in the BPM101 marker sequence (Table II).

Barley cDNA sequence or annotated barley genomic DNA gene sequence spanning polymorphic sites were available on the database only for sites in ABG373 (AF182197), BCD386 (BE438153 and AA231678), CDO1188 (Z26322), and COMT (U54767). Of the nine polymorphic sites that were covered by these sequences, four were in exons and five were in introns. BLASTX searches of the database were also performed to identify coding potential of marker sequences. The results are included in Table II. Significant matches with known or hypothetical proteins in the database were obtained using 15 of the STS sequences, which included seven based on anonymous genomic DNA clones. The remaining polymorphic marker sequences for which no significant match was obtained (ABG74, ABG500, BPM101, and MWG2056) were all based on anonymous genomic DNA clones, indicating that these could be non-coding sequences. These four clones comprised 21% of the analyzed sequence, but yielded 56% of polymorphic sites, indicating a trend toward greater polymorphism in non-coding sequences.

The number of polymorphic markers identified for each barley line pair ranged from seven to 16, and the overall level of sequence polymorphism ranged from 0.9 to 3.0/kb for the different pairwise comparisons (Table II). Both of these measurements and the results

of the genome-wide AFLP analysis (Table I) indicate that the cross between C69 (BCIngrid *mlo-5* background) and Grannenlose Zweizeilige was the most polymorphic of the three *Ror1* mapping crosses.

It is interesting that many of the marker sequences assayed for multiple polymorphic sites showed clear haplotype structure, characterized by the presence of the same combination of bases at polymorphic sites in different barley lines over distances of several hundred basepairs, as illustrated in Figure 1. For example, marker sequence ABG500 was identical in lines BCIngrid *mlo-5*, BCPallas *mlo-5*, and Grannenlose Zweizeilige at all 14 polymorphic base positions, and marker sequence MWG913 was identical in BCIngrid *mlo-5*, Malteria Heda, and Grannenlose Zweizeilige at all four polymorphic base positions (Fig. 1). Markers such as ABG74, MWG896, and MWG2056 had sequences in some barley lines that appeared to consist of a patchwork of haplotypes (Fig. 1). Barley lines Malteria Heda, BCPallas *mlo-5*, and Grannenlose Zweizeilige, respectively, contained seven, five, and seven marker haplotypes that were unique among the four barley lines, whereas no polymorphism unique to BCIngrid *mlo-5* was identified.

Mapping

To map STSs, marker sequences were PCR amplified individually from segregants from the *Ror1* mapping populations, and the genotypes were determined by restriction digestion of the amplified products (cleaved amplified polymorphic sequence [CAPS] analysis) or by directly sequencing the fragments in cases when a polymorphism in a restriction site was not available. Primers, restriction enzymes, and sequence polymorphisms used for mapping are shown in Table III. The synthesis of new primers was required to obtain PCR/restriction products that were of a size that could be resolved on an agarose gel. In the case of the CAPS marker CDO98, a mismatch was introduced into one of the primers to eliminate a restriction site that would have otherwise impeded scoring (see "Materials and Methods").

Recessive *ror1* mutant alleles confer a partial loss of *mlo* resistance (Freialdenhoven et al., 1996). Use of the single-plant resistance assay to distinguish homozygous *ror1* plants from heterozygotes or homozygous *Ror1* plants was found to be about 95% reliable, based on the observed correlation between the resistance scores and subsequently determined genotypes for *Ror1*-linked STS markers. Individuals shown by marker analysis to be recombinant in the *Ror1* region were genotyped for the *Ror1* locus by performing resistance assays on 10 to 20 progeny. To provide additional confidence that the observed resistance reactions were due to the genotype at the *Ror1* locus, members of each inoculated family were individually analyzed with a *Ror1* marker that was segregating in the respective family.

Linkage analysis was performed using the three segregating populations, allowing the construction of three maps for the centromeric region of chromosome 1H, shown in Figure 2. With the exception of BCD454 and MWG896, all polymorphic markers were mapped in at least one of the populations. Except for ABG373, which mapped to a location not linked to *Ror1*, all mapped markers were found to be located close to *Ror1*. Markers BCD386, CDO98, and CDO1173 were originally examined because they were mapped to related chromosome segments in other grass species (i.e. centromeric regions of oat chromosome A and group 1 chromosomes of wheat and *T. monococcum*). Mapping of these markers to the *Ror1* region in barley confirmed the relationships between these chromosome segments. Positions of STS markers and *Ror1* obtained using the three populations were consistent (Fig. 2). Details of the mapping performed using the three different populations were as follows.

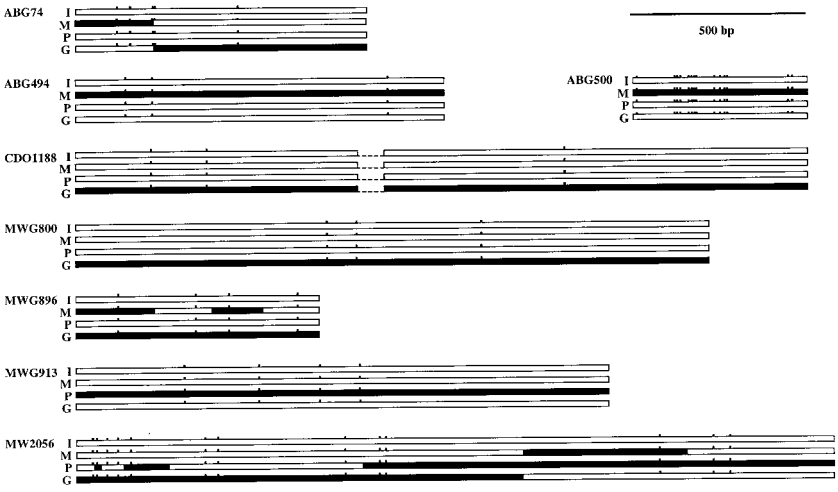

Figure 1. Haplotype structure of *Ror1*-linked marker sequences. *Ror1*-linked marker sequences were determined from each of the four parental barley lines BCIngrid *mlo-5* (I), Malteria Heda (M), BCPallas *mlo-5* (P), and Grannenlose Zweizeilige (G). Segments of the sequence defined by the polymorphic bases (tick marks) were classified as being the same as BCIngrid *mlo-5* (clear) or of a second type (colored) according to the nucleotides present at the polymorphic sites. The eight sequences shown are those for which two or more polymorphic sites were found and for which all four barley lines were assayed for all polymorphic sites. The interruption in the CDO1188 figure represents 1,200 bp of sequence containing no polymorphic sites.

Table III. *Primers and polymorphisms used for STS mapping*

For linkage analysis of STS loci, primer pairs were used to PCR amplify STS sequences from *Ror1* segregants and to segregate sequence polymorphisms within the fragments scored by restriction digestion (CAPS analysis) or by direct sequence of the PCR products.

STS	Primers (5′ to 3′)[a]	Assay[b]
ABG74	CTGCAGGAGCCTCCTGACCTGCTGC GTATGATGCTCAAGCCCCCGGAATG	*Xho*I
ABG452	GCCGGCCATTGTTGCAGCTTCC CCTTCTTCTCCTGTTGATTGCCAAGTT	*Dde*I
ABG452	GGGAGTATGTGATATTGTGGGCATC GCATGGAGTGTGCAAGTACGTGGTC	CAG(A/G)CAC[c]
ABG494	TGCCAGGTCTTGGTGGGACGAGGAG CAATGGTGAGACTACCATACCTTACTGTCTTC	CTT(A/G)TTT[d] and ATT(A)$_{7/8}$TCC[d]
ABG500	CCTTCCACTCTCGACCTCGCCCGTCC GAGACCACACACTGCAGCATAAGCCAG	*Bgl*I
BCD386	GTGAGCAGTGCAACATGTATAGAG CTGGAGAATGCGGAGGTATCATCAG	*Afl*III
BPM101	CAGCAACGCACGGGGTATCATCTAG GCTGCCTCAAGAAGTACACCCTGTACC	*Pst*I
BPM101	GAAATGCATTTCCCTGCGATCAGGATCAATG GCTGACATTTGCTCCGTACCTCATCC	*Xba*I
CDO98	AATGAGTTGTTTAAGCACACGAGAAGAG CTTGTGCTTATGTTGTCTACAACGTATG	*Rsa*I
CDO1173	TGCACTGAAGCTCTGAATCTGGATG GGATCACTTGGTGCATGAGAAGTGG	*Eco*RV
CDO1188	GGCCACAAAACCGCGGAAAGAGATAG AGATCTACCTGTTGCCTAGTTTCTC	*Bsp*143I
DGE18	CCTGCTGCTGCTGGAGAACCAGATC GTCTGCTCGAACGCGATCAGGTTCC	*Hae*III
COMT	ACAAGCTGGATTTACCGGTGGCATC GCGTGTGAGATCACGGTAAGCAAGG	*Nco*I
cMWG758	TCTTGTCGCCAGCGATGTCTGTCTG TCGCCGTTCTCCTCTACCTCTAGTG	*Sac*II
MWG800	GCTCTAGCCAACTTAGCTGCACATGGAG GATAGGTTCCACAAAGCACCAGCAGC	*Alu*I
MWG913	GAGCAGAGCACCCTTGAAGCAGAC GCTTCATGGATGATCTCTCCAGCA	*Bsp*143I
MWG2056	TCATCATGCATTATATGTTCAGGAGATG ATGTCGTTTTGGACTTTGCCAATG	*Rsa*I
MWG2056	CAAGCAAGCTGTTTTTTCTTTTCTTACGTAC GCACAGGCATTGGTTTTATGTGAG	*Sst*I

[a] Forward and reverse primers are shown first and second, respectively. [b] Polymorphisms were assayed by CAPS analysis with the restriction enzymes indicated or by assaying the indicated sequence polymorphisms by direct sequencing of PCR products. [c] Sequencing performed using forward primer. [d] Sequencing performed using reverse primer.

A89 × BCPallas mlo-5 Population

All 709 segregants were scored for resistance and for markers BPM101, MWG913, and MWG2056, whereas only a fraction of these were scored for the more distant marker DGE18. MWG2056 was the marker that mapped the nearest to *Ror1* in this population, at a distance of 1.0 cM (Fig. 2).

C69 × Grannenlose Zweizeilige Population

A subset of the 1,301 individuals was analyzed using all markers to establish a preliminary order for *Ror1* and the molecular markers. The remainder of the population was then assayed using the *Ror1* flanking marker pair CDO1188/cMWG758 (Fig. 2), and only those that were found to be recombinant were assayed for CDO98 and BCD386. All five identified CDO98-BCD386 recombinants and three randomly chosen recombinants for the cMWG758-BCD386 interval (Fig. 2) were scored for the ABG452 marker by direct sequencing, and in these individuals ABG452 cosegregated with BCD386. Inoculation tests, including progeny tests for all five identified CDO98-BCD386 recombinants, located *Ror1* to the 0.2-cM interval between ABG452/BCD386 and CDO98, in which two and three recombination events were identified above and below *Ror1*, respectively (Figure II).

A89 × Malteria Heda Population

Only a fraction of the 312 segregants was scored for the more distant markers ABG74 and ABG500,

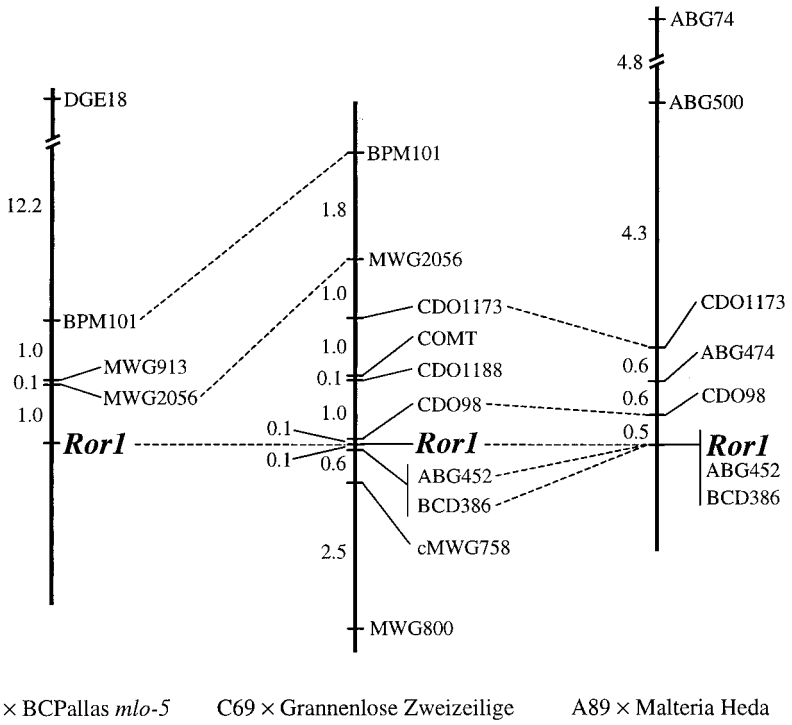

Figure 2. Genetic maps of the centromeric region of barley chromosome 1H constructed using three *Ror1* mapping populations. Distances are shown in centiMorgans. Dotted lines join markers common between the maps.

whereas the entire population was screened with the CAPS markers CDO1178 and ABG452, shown in the C69 × Grannenlose Zweizeilige population to flank *Ror1* (Fig. 2). All CDO1178-ABG452 recombinants were scored for CDO98 and BCD386. ABG494, which was scored by direct sequencing, was mapped to the CDO98-CDO1173 interval using a few recombinants for the region, and was then scored for all eight identified recombinants for this interval (Fig. 2). Resistance assays were performed on approximately one-half of the segregants in the first generation, and on progeny of selected recombinants, including two of the three identified for the interval ABG452/BCD386-CDO98. In these individuals characterized for *Ror1* genotype, *Ror1* cosegregated with the markers ABG452 and BCD386.

DISCUSSION

Polymorphism in Barley

With the exception of the COMT marker sequence, which was absent in the BCPallas *mlo-5* line, all marker sequences were present in all four barley parental lines tested. Comparisons of the sequences from the different barley lines identified 79 other unique sequence polymorphisms, comprising 65 single-basepair substitutions, 11 small indels, two dinucleotide substitutions, and one 5-bp/10-bp substitution. The *ror1* mutant lines A89 and C69, produced by mutagenesis with the chemical ethyl methanesulfonate (EMS; Freialdenhoven et al., 1996), were used as the source of sequences from the BCIngrid *mlo-5* background. No sequence variants were identified in A89 or C69 that were not also present in one or more of the other barley lines, indicating that EMS treatment was not responsible for any of the observed polymorphisms. These data indicate that the vast majority of naturally occurring polymorphisms present in single- or low-copy sequences in barley are single-nucleotide substitutions and small indels.

The polymorphism frequencies observed here in pair-wise comparisons of sequences from different barley lines varied from between 0.9 to 3.0/kb, depending on the lines being compared. Polymorphism observed in barley was lower than that revealed between maize lines in a number of genes (2.0–25/kb; White and Doebley, 1999 and refs. therein), and was lower than that observed between the two Arabidopsis ecotypes Columbia and Landsberg *erecta* in a survey of a large number of low-copy sequences (3.8/kb; Konieczny and Ausubel, 1993).

In *Drosophila melanogaster* and in plants, including *A. squarrosa*, which is a close relative of barley, natural polymorphism levels within individual chromosome regions have generally been found to be proportional to the frequency of recombination within these regions (Begun and Aquadro, 1992; Dvořák et al., 1998; Kraft et al., 1998; Stephan and Langley, 1998). This correlation has been proposed to be due to the purifying effect of genetic linkage to individual amino acid-altering polymorphisms that influence Darwinian fitness. Similar to other cereal chromosomes, barley chromosome 1H generally shows suppressed recombination nearer to the centromere (Künzel et al., 2000). Markers mapped in the present study were previously located to within 5.0 cM of

the centromere using linkage analysis and cytogenetic stocks (Ragowsky et al., 1993; Van Deynze et al., 1995a; Dubcovsky et al., 1996; Korzun and Künzel, 1996; Künzel et al., 2000), although inconsistencies in these published data prevented us from locating the centromere to an exact marker interval on the maps shown in Figure 2. Taking into account the location of these STS marker sequences in a region of low recombination and the general influence of recombination on polymorphism (see above), it would seem probable that the levels of sequence polymorphism observed in this study represent an underestimate of the levels of sequence polymorphism in the barley genome as a whole.

Comparisons between the lines BCIngrid *mlo-5* and BCPallas *mlo-5* revealed the lowest levels of sequence polymorphism and also showed the fewest differences of all the barley pairs in the genome-wide AFLP screen (Tables I and II). BCIngrid *mlo-5* and BCPallas *mlo-5* were made by backcrossing the *mlo-5* allele from CarlsbergII into the genetic backgrounds of the cultivars Ingrid and Pallas, respectively. Cultivars Pallas and Ingrid are Swedish cultivars, and each has the cultivars Gull, Maja, and Opal in its parentage, in addition to other barley lines that are unique to each pedigree (Aufhammer et al., 1968). The overlapping pedigrees of BCIngrid *mlo-5* and BCPallas *mlo-5* provides a likely explanation for the relatively high degree of relatedness observed between these lines by molecular analysis. No polymorphisms were observed for this barley pair for the region located below MWG913 on the genetic map (Fig. 2), raising the possibility that this chromosome region may be of identical origin in the two lines.

The other two barley lines used in the comparisons were the Argentinian cultivar Malteria Heda containing the γ-ray-induced *mlo-3* resistance allele (Favret, 1965), and the Ethiopian wheat landrace (i.e. primitive cultivated barley line) Grannenlose Zweizeilige, which contains the natural *mlo-11* resistance allele (Jørgensen, 1976; Meyer and Lehmann, 1979). Comparisons between Grannenlose Zweizeilige and the other lines generally revealed the highest levels of polymorphism, in the AFLP screen (at least 50% more; Table I) and in the comparisons of chromosome 1H marker sequences (Table II). Grannenlose Zweizeilige also possessed a large number (seven) of marker sequence haplotypes that were unique among the four lines (BCIngrid *mlo-5*, Malteria Heda, and BCPallas *mlo-5* had zero, seven, and five, respectively). The development of modern cultivars, essentially from selections of landrace barleys, has led to a narrowing of the genetic base of barley presently under cultivation (Nevo, 1992). The relatively high levels of polymorphism observed between Grannenlose Zweizeilige and the other lines reflects this history and confirms the usefulness of landrace × cultivar crosses in providing high levels of molecular variation for mapping studies.

Haplotype Structures and Implications for Mapping

The conservation of particular combinations of polymorphic bases at adjacent polymorphic sites (haplotype structure) was observed for STS sequences over stretches of several hundred basepairs (Fig. 1). Haplotype structure of allelic DNA sequences has been well documented in plants (e.g. Ellis et al., 1999). Population subdivision and the predominantly inbreeding nature of barley could facilitate the development of haplotypes by maintaining reproductively isolated lineages in which different mutations could accumulate. However, the significance of inbreeding in generating haplotype structure may not be critical, as haplotype sequence structure has also been observed in maize, an outbreeding species (Henry and Damerval, 1997). Genetic drift, population bottlenecks, or selection for or against individual polymorphisms could be forces influencing haplotype structure by reducing the number of different combinations in which polymorphic bases occur. Sequences of the markers ABG74 MWG896 and MWG2056 appeared to consist of chimeras of different haplotypes in some barley lines (Fig. 1). These patterns could reflect occasional outcrossing followed by recombination or gene conversion between different haplotypes. In an alternate manner, these could represent intermediate forms in the development of one haplotype into another by mutation, which have not been eliminated by drift, selection, or a population bottleneck.

Haplotype sequence structure in barley reduces the probability that extending analysis of a sequence outward (e.g. by the method of Siebert et al., 1995) will reveal polymorphism if the sequence has not already been shown to be polymorphic between the two barley lines being compared. Instead of investing time and effort obtaining longer sequences, examining sequences from different markers, if these are accessible, would be a more efficient strategy of obtaining a high density of polymorphic STS markers in a region of interest. In addition, the use of multiple mapping populations derived from different crosses should increase the chance of identifying a mappable polymorphism for any given marker.

The sequence information obtained in this and similar studies should facilitate STS marker deployment for mapping genes of interest by providing a record of known variable base positions that can be tested for polymorphism in specific mapping populations. The listing of barley STS polymorphism data on web sites (e.g. http://www.plantphysiol.org/for this study and also http://hordeum.oscs.montana.edu/) makes this information readily accessible. Individual polymorphisms present in restriction enzyme recognition sequences could be particularly useful because they can be tested by CAPS analysis. The narrow genetic base of modern barley cultivars (Nevo, 1992) predicts that sequence variants observed in cultivars or cultivar-derived material such as BCIngrid *mlo-5*,

Malteria Heda, or BCPallas *mlo-5* will also be present in other barley cultivars. Consistent with this prediction, Waugh et al. (1997) reported that AFLP fragments of the same size and map position could be polymorphic in multiple barley populations derived from different crosses (Waugh et al., 1997). Although the Ethiopian landrace Grannenlose Zweizeilige possessed some haplotypes that were not found in any of the three modern cultivated genetic backgrounds, for other markers such as ABG494, ABG500, and MWG913, Grannenlose Zweizeilige contained sequence variants that were also present in the modern cultivated germplasm (Fig. 1). These observations are consistent with the expectation that haplotype variants present in modern barley cultivars will be a subset of those present in landrace barleys.

STS Markers Versus RFLP Markers

Marker colinearity between barley and wheat, *T. monococcum*, and oat made it possible to use knowledge of RFLP markers on related chromosome regions from these grasses as a further source of STS sequences in the barley *Ror1* region. In many cases, sequences of RFLP probes derived from barley were available for STS primer design. However, primers based on sequences of RFLP probes from oat or *A. squarrosa* (markers CDO98, CDO1174, and DGE18) were used successfully in the PCR amplification of orthologous STS sequences from barley, presumably due to DNA sequence conservation with barley in the primer regions. A considerable marker density was obtained and the markers defining the 0.2- to 0.5-cM interval containing *Ror1* were effectively used to identify recombinants from large mapping populations, which will be critical in the project to isolate the *Ror1* gene. Use of across-species sequence information for STS marker generation should be further facilitated by the increasing numbers of random expressed sequence tags available on the database (e.g. from the International Triticeae EST Cooperative initiative; http://wheat.pw.usda.gov/genome/index.html), which will allow identification of homologous sequences on which to base species-specific STS primers. The growth of this common pool of information, together with the development of more efficient procedures for the detection of STS polymorphisms (Hauser et al., 1998; Cho et al., 1999), should increase the ease with which STS markers can be developed for cereal chromosome regions of interest.

RFLP and STS markers are similar in that their potential to be used for targeted generation of high density linkage maps in regions of interest will increase as the sequencing and mapping information produced by the scientific community accumulates. The relative ability of these two marker types to detect polymorphism is, therefore, of interest. STS sequencing would be expected to be more effective in detecting the small substitutions and indel polymorphisms that represent the bulk of polymorphism in single- or low-copy sequences in barley. However, RFLP analysis, but not STS sequencing, has the potential to detect polymorphism several kilobases from the single-copy sequences, potentially within high-copy retrotransposon-like sequences that account for over 80% of the barley genome and that are responsible for considerable intraspecific genome plasticity in the genus *Hordeum* (Kalendar et al., 2000). A screen for RFLPs between the four barley-mapping parents using 20 restriction enzymes and the probes cMWG758, MWG800, MWG896, and MWG2056 revealed polymorphisms for only two of the 24 probe/barley line combinations (not shown), whereas sequencing of these markers revealed polymorphisms for 19 of the marker/barley line comparisons (Table II). Although the sequences examined here from the *Ror1* region may be less variable than barley sequences taken from random genomic locations (see above), the average frequency with which a marker could distinguish any two of the spring barley lines by sequencing was 0.54 (sequencing on average 900 bp per marker). Using different barley lines and probes to the present study, Graner et al. (1990) found that the genome-wide average frequency with which an RFLP probe was capable of distinguishing any two spring barley lines using three restriction enzymes was 0.34. These data suggest that STS sequencing is more effective than RFLP marker analysis at detecting polymorphisms in barley.

MATERIALS AND METHODS

Plant Material

Mutant barley (*Hordeum vulgare*) lines A89 (*ror1-2*) and C69 (*ror1-4*) were respectively derived by EMS and NaN_3 mutagenesis of the line BCIngrid *mlo-5* containing the *mlo-5* resistance allele from cv CarlsbergII backcrossed into the cv Ingrid (Freialdenhoven et al., 1996). Line BCPallas *mlo-5* contains the *mlo-5* resistance allele backcrossed into cv Pallas (line P22 in Kølster et al., 1986) and Malteria Heda contains the *mlo-3* resistance allele induced by γ irradiation (Favret, 1965). Grannenlose Zweizeilige is a landrace wheat line collected from Ethiopia by German expeditions in 1937 and 1938, and contains the natural *mlo-11* resistance allele (Jørgensen, 1976, 1992; Meyer and Lehmann, 1979).

Each of the three *Ror1*-mapping populations was derived from a cross between a partially susceptible *ror1* mutant line (*ror1*, *mlo*) and a fully resistant line carrying a wild-type *Ror1* allele and an *mlo* resistance allele (*Ror1*, *mlo*): A89 (*mlo-5*; *ror1-2*) × BCPallas *mlo-5* (*mlo-5*; *Ror1*); C69 (*mlo-5*; *ror1-4*) × Grannenlose Zweizeilige (*mlo-11*; *Ror1*); and A89 (*mlo-5*; *ror1-2*) × Malteria Heda (*mlo-3*; *Ror1*). Segregation of resistance in the progeny of these crosses (F_2 and beyond) was therefore expected to be controlled by the *Ror1* locus only. In our hands, lines containing the *mlo-11* resistance allele allow growth of occasional fungal colonies at frequencies above those seen in lines carrying other *mlo* resistance alleles (data not shown). To avoid potential com-

plications caused by the incomplete resistance of the *mlo-11* allele, mapping with the C69 (*mlo-5; ror1-4*) × Grannenlose Zweizeilige (*mlo-11*; *Ror1*) population was confined to progeny of F_2 individuals that were determined to be homozygous for the *mlo-5* allele, but heterozygous for *Ror1* using CAPS markers for the *Mlo* (Peterhänsel et al., 1997) and *Ror1* (this study) loci. Individuals used for mapping in the other two populations were from the F_2 generation, or from F_3 and F_4 families determined to be segregating for *Ror1*.

Resistance Assays

Conditions for maintenance of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) isolate K1 and barley plants for resistance assays were essentially as described (Peterhänsel et al., 1997). Ten-day-old seedlings were inoculated, and after approximately 10 d, resistance was assessed by eye on the basis of the number of colonies and the amount aerial mycelium produced.

AFLP

AFLP analysis was performed as described (Vos et al., 1995). In the majority of cases a pre-amplification step was included using primers with a single base extension, prior to the selective PCR using the +3/+2 primers (Vos et al., 1995). Enrichment of fragments resulting from *Eco*RI or *Pst*I restriction at least at one end was also performed in some cases before selective amplifications, using biotinylated *Eco*RI or *Pst*I adapter primers and selection with streptavidin-coated beads (Dynal Biotech, Norway). Primers used for selective PCRs (using primer nomenclature at http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.htm) were six *Pst*I primers (P11–P16), two *Eco*RI primers (E40 and E43), and 16 *Mse*I primers (M31–M46).

DNA Sequencing and CAPS Analysis

Barley genomic DNA for use as PCR template was prepared by the method of Stewart and Via (1993) or by using the DNeasy 96 Plant Kit DNA extraction system (Qiagen, UK). PCR was performed in 50-μL volumes for direct sequencing or 20-μL volumes for CAPS analysis, using *Taq* DNA polymerase and standard amplification conditions with a 60°C annealing temperature for all primer combinations. PCR products were separated by agarose gel electrophoresis, purified from excised gel pieces using the QIAquick Gel Extraction Kit (Qiagen), and used as template for BigDye Terminator sequencing (Applied Biosystems, Foster City, CA). The direct sequencing of the PCR products would not be subject to errors introduced by *Taq* because the 100 ng of genomic DNA template used in each reaction represents multiple genome equivalents (more than 20,000 copies, based on a 1C DNA content of 5.0 pg for barley; Arumuganathan and Earle, 1991).

For each STS the sequence representative of the BCIngrid *mlo-5* background was obtained from the A89 or the C69 mutant (both BCIngrid *mlo-5* background). Marker sequences were extended outward to obtain new genomic DNA sequence using the method of Siebert et al. (1995). Sequence comparisons and analyses were performed using the GCG package of software (Genetics Computer Group, Madison, WI; Devereux et al., 1984), and the programs GAP4 (Bonfield et al., 1995), BLASTN (Altschul et al., 1990) and BLASTX (Gish and States, 1993).

Primers for CAPS analysis were placed to allow clear resolution of alternative digestion products. In marker CDO98, an invariant *Rsa*I site located 14 bp from the polymorphic *Rsa*I site was eliminated by designing a mismatch in the reverse primer (containing the sequence CTAC instead of the GTAC *Rsa*I recognition sequence; Table III), thus increasing the size difference between alternative digestion products from 14 to 32 bp to assist scoring. CAPS analysis was performed by digesting 10 μL of PCR product in a 20-μL volume containing 2.5 units of enzyme and the recommended buffer for 3.0 h, and then analyzing the products on 1.5% to 3.0% (w/v) agarose gels.

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