Molecular Mapping of Leaf Rust Resistance Gene *Rph*5 in Barley

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ABSTRACT

Leaf rust caused by *Puccinia hordei* G. Otth is an important disease of barley (*Hordeum vulgare* L.) in many regions of the world. Yield losses up to 32% have been reported in susceptible cultivars. The *Rph*5 gene confers resistance to the most prevalent races (8 and 30) of barley leaf rust in the USA. Therefore, the molecular mapping of *Rph*5 is of great interest. The objectives of this study were to map *Rph*5 and identify closely linked molecular markers. Genetic studies were performed by analysis of 93 and 91 F_2 plants derived from the crosses 'Bowman' (*rph*5) × 'Magnif 102' (*Rph*5) and 'Moore' (*rph*5) × Virginia 92-42-46 (*Rph*5), respectively. Bulk segregant analysis (BSA) using amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and simple sequence repeat (SSR) markers was conducted. Linkage analysis positioned the *Rph*5 locus to the extreme telomeric region of the short arm of barley chromosome 3H at 0.2 centimorgans (cM) proximal to RFLP marker *VT1* and 0.5 cM distal from RFLP marker *C970* in the Bowman × Magnif 102 population. Map positions and the relative order of the markers were confirmed in the Moore × Virginia 92-42-46 population. RFLP analysis of the near isogenic line (NIL) Magnif 102/*8Bowman, the susceptible recurrent parent Bowman, and *Rph*5 donor Magnif 102, confirmed the close linkage of the markers *VT1*, *BCD907*, and *CDO549* to *Rph*5. Results from this study will be useful for marker-assisted selection and gene pyramiding in programs breeding for leaf rust resistance and provide the basis for physical mapping and further cloning activities.

Leaf rust caused by *P. hordei* is generally considered the most important rust disease of barley on a worldwide basis. Severe yield losses have been observed in Australia (31%) (Coterill et al., 1992) and Europe (17–31%) (King and Polley, 1976). In the USA, a 32% yield reduction was reported for susceptible cultivars under epidemic conditions in Virginia (Griffey et al., 1994).

Clifford (1985) listed two types of resistance against *P. hordei* in barley: partial resistance and race-specific resistance. Partial resistance is controlled by several to many genes and is generally considered more durable than the race-specific resistance (Qi et al., 2000; Kicherer et al., 2000). However, the quantitative expression of this trait and complex genetics make this type of resistance more difficult to use in breeding programs. Race-specific resistance is usually governed by single dominant genes. Although race-specific leaf rust resistance genes have not provided durable protection, they can be easily identified and transferred into appropriate germplasm (Parlevliet, 1976). To date, 16 major race-specific genes (designated as *Rph*1 to *Rph*16) for leaf rust resistance have been identified (Franckowiak et al., 1997; Ivandic et al., 1998).

Development of disease resistant barley cultivars has been the most efficient way to control leaf rust (Mathre, 1997; Zillinsky, 1983). The pyramiding of multiple *Rph* genes is expected to increase the durability of leaf rust resistance in cultivars. Although virulence for *Rph*5 is widely prevalent in Europe (Parlevliet, 1976) and South America (Brodny and Rivadeneira, 1996; Fetch et al., 1998), it has not been identified in North America. Thus, *Rph*5 could be used to protect barley cultivars from leaf rust damage in North America. However, a more sound gene deployment strategy would be to use this gene in combination with other effective genes such as *Rph*3 and *Rph*9 (Brooks et al., 2000).

Most of the known barley leaf rust resistance genes have been described and mapped by means of morphological characters, biochemical markers, and cytogenetic stocks (Table 1). However, so far only five *Rph* genes have been mapped by means of molecular markers. Two alleles at the *Rph*9 locus, *Rph*9.*i* and *Rph*9.*z* (formerly designated as *Rph*12), were located on chromosome 5H using RFLP and sequence tagged site (STS) markers (Borovkova et al., 1997, 1998). STS and cleaved amplified polymorphic sequence (CAPS) markers were employed to map *Rph*16 onto barley chromosome 2H (Ivandic et al., 1998). Recently, *Rph*7 was mapped onto the short arm of chromosome 3H by means of RFLP markers (Brunner et al., 2000; Graner et al., 2000). Finally, *Rph*6 has been mapped onto chromosome 3HS (Steffenson, unpublished). The precise chromosomal position of *Rph*5 is not known, although the gene was assigned to chromosome 3H by trisomic analysis (Tan, 1978; Tuleen and McDaniel, 1971). Thus, the objectives of this study were to map *Rph*5 by means of molecular markers and develop closely linked markers for marker-assisted selection.

MATERIALS AND METHODS

Genetic Materials

Two F_2 populations derived from crosses Bowman (PI 483237) × Magnif 102 (PI 337140) and Moore (CI 7251) × Virginia 92-42-46 (hereafter, referred to as BM and MV populations, respectively) and consisting of 93 and 91 individuals, respectively, were used for molecular mapping. Magnif 102

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Abbreviations: AFLP, amplified fragment length polymorphism; BSA, bulk segregant analysis; CAPS, cleaved amplified polymorphic sequence; cM, centimorgan; NIL, near isogenic line; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat; STS, sequenced tagged site.

Table 1. Summary of described and mapped *Rph* genes.

Gene†	Number of alleles	Chromosome	Means of Positioning Locus	Reference
*Rph*1	1	2H	Trisomic analysis	Tuleen and McDaniel, 1971; Tan, 1978
*Rph*2	12	5H	Molecular markers	Franckowiak et al., 1997; Borovkova et al., 1997
*Rph*3	3	7H	Morphological markers	Jin et al., 1993
*Rph*4	1	1H	Trisomic analysis	Tuleen and McDaniel, 1971; Tan, 1978
*Rph*5	1	3H	Trisomic analysis	Tuleen and McDaniel, 1971; Tan, 1978
*Rph*6	1	3H	Molecular markers	Steffenson B.J. (unpublished)
*Rph*7	2	3H	Molecular markers	Brunner et al., 2000; Graner et al., 2000
*Rph*9.i	1	5H	Molecular markers	Borovkova et al., 1998
*Rph*9.*z*‡	1	5H	Molecular markers	Jin et al., 1993; Borovkova et al., 1998
*Rph*10	1	3H	Isozymes	Feuerstein et al., 1990
*Rph*11	1	6H	Isozymes	Feuerstein et al., 1990
*Rph*16	1	2H	Molecular markers	Ivandic et al., 1998

† Gene designations of *Rph*1 to *Rph*16 are from Franckowiak et al. (1997). The leaf rust resistance gene mapped by Borovkova et al. (1997) in Q21861 is thought to be *Rph*2 on the basis of allelism tests with the known *Rph*2 sources of Peruvian, PI 531840, and PI 531841.
‡ *Rph*9.*z* (formerly designated as *Rph*12) is based on Borovkova et al. (1998).

(Franckowiak et al., 1997) and Virginia 92-42-46 (Zwonitzer, 1999) carry *Rph*5 and provide the genetic sources of resistance to leaf rust in this experiment. To confirm the close linkage between *Rph*5 and flanking markers, the near isogenic line (NIL) Magnif 102/*8Bowman, together with recurrent parent Bowman and *Rph*5 donor Magnif 102, were used in this study. Seeds of the NIL were kindly provided by Dr. J.D. Franckowiak at North Dakota State University, Fargo.

Disease Screening

To determine infection type (disease reaction phenotype), F_2 plants from both populations were inoculated with race 8 as described by Brooks et al. (2000). To confirm the genotype for resistance in F_2 plants (i.e., whether *Rph*5/*Rph*5, *Rph*5/*rph*5, *rph*5/*rph*5), 50 seeds from each $F_{2:3}$ family were planted, inoculated, and evaluated for their leaf rust reaction. A set of host differential lines including 'Sudan' (*Rph*1), 'Peruvian' (*Rph*2), 'Aim' (*Rph*3), 'Estate' (*Rph*3), 'Gold' (*Rph*4), 'Bolivia' (*Rph*2 +*Rph*6), 'Cebada Capa' (*Rph*7), 'Egypt 4' (*Rph*8), 'Hor 2596' (*Rph*9.i), 'Triumph' (*Rph*9.z), 'Clipper BC8' (*Rph*10), 'Clipper BC67' (*Rph*11), Berac*3/HS2986 (*Rph*13), 'PI 531901-1' (*Rph*14) and Bowman*4/PI 3555447 (*Rph*15) were included as checks in the experiments. The virulence/avirulence formula of race 8 is *Rph*1, 4, 8, 10, 11/*Rph*2, 3, 5, 2+6, 7, 9.i, 9.z, 13, 14, 15 (Griffey et al., 1994). Infection types were scored by the 0-to-4 scale of Levine and Cherewick (1952). Infection types of 0, 1, or 2 were considered indicative of host resistance, whereas infection types 3 or 4 were considered indicative of host susceptibility. Disease assessments were performed 10 to 14 d after inoculation. Infection types of F_2 progeny were compared with infection types of the parental lines and host-differentials to assure proper scoring and assignment into resistant and/or susceptible classes.

Molecular Mapping

Genomic DNA from 91 individual MV F_2 plants and 93 BM $F_{2:3}$ families was processed for molecular marker analysis. DNA was extracted from freeze-dried leaf tissue as described by Saghai Maroof et al. (1984). For BSA (Michelmore et al., 1991), DNA from six MV F_2 individuals identified as homozygous resistant or homozygous susceptible, on the basis of $F_{2:3}$ disease phenotype data, as well as six BM $F_{2:3}$ homozygous resistant and homozygous susceptible families were pooled to form resistant and susceptible bulks. For RFLP analysis, DNA samples from the susceptible and resistant bulks, parental samples, NIL Magnif 102/*8Bowman, and 91 individual MV F_2 plants and 93 BM $F_{2:3}$ families were digested with six restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Sst*I, and *Xba*I according to the manufacturer's protocol (Gibco BRL, Rockville, MD). RFLP analysis was performed as previously described (Biyashev et al., 1997).

In addition to RFLP analysis, a set of microsatellite markers was used for mapping purposes. PCR amplification and microsatellite analysis were carried out according to published procedures (Liu et al., 1996; Ramsey et al., 2000). AFLP analysis was conducted following the protocol described previously (Vos et al., 1995; Maughan et al., 1996). Conversion of AFLP markers to RFLP markers was performed as described by Upender et al. (1995) and Hayes and Saghai Maroof (2000).

Sequence Analysis

DNA was sequenced with an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA). Plasmid template was prepared by standard alkaline-lysis method followed by purification with QiaexII (Qiagen Inc., Valencia, CA). Dye-terminator cycle sequencing was done on the basis of the manufacturer's protocols (Perkin Elmer, Foster City, CA). Sequence analysis, including primer design was conducted with Lasergene software (DNASTAR, Madison, WI).

Linkage Analysis

The computer program MAPMAKER version 3.0b was used for genetic mapping and linkage analysis (Lander et al.,

Table 2. Segregation for leaf rust resistance in F_2 plants and $F_{2:3}$ families in MV and BM populations.

Cross	Number of F_2 plants			Ratio	χ^2	P-value
	Resistant	Susceptible				
MV	71	20		3:1	0.44	0.506
BM	70	23		3:1	0.01	0.952
	Number of $F_{2:3}$ families					
	Homozygous resistant	Segregating	Homozygous susceptible			
MV	22	49	20	1:2:1	0.63	0.731
BM	22	47	24	1:2:1	0.10	0.953

1987). Linkage maps were constructed on the basis of a LOD threshold of 3.0 and maximum Haldane distance of 50 cM.

RESULTS

In both crosses, the number of resistant and susceptible F_2 progeny approximated a 3:1 ratio, indicating that a single dominant gene (*Rph5*) conferred resistance in Magnif 102 and Virginia 92-42-46 (Table 2). This result was confirmed by the 1:2:1 ratio of homozygous resistant, segregating, homozygous susceptible $F_{2:3}$ families (Table 2). Infection types of resistant parents and resistant progeny are summarized in Table 3.

Trisomic analysis of Tuleen and McDaniel (1971) and Tan (1978) indicated that *Rph5* was located on barley chromosome 3H. Therefore, we selected previously reported RFLP and SSR markers from chromosome 3H for BSA. Six RFLP markers (*CDO549*, *BCD907*, *C970*, *MWG2021*, *MWG848*, and *TAG683*) in the BM population were mapped in the vicinity of the *Rph5* locus (Fig. 1A). RFLP clone *MWG691*, originally mapped to the telomeric region of barley chromosome 3HS (Graner et al., 1994), was monomorphic in the BM population. To map *MWG691*, we converted it into a PCR-based marker. An insert fragment of 290 bp from the *MWG691* clone was sequenced. The sequence information was used to design a pair of primers (5′gatcacttggggccgtatgtgtta3′ and 5′aattccgggtgagtgcctcttc 3′) to PCR amplify the DNA from parental forms and bulk segregants of both populations. As a PCR-based marker, *MWG691* revealed polymorphism between Bowman and Magnif 102. In the BM F_2 population, this marker segregated in a codominant fashion and was mapped 0.9 cM proxi-

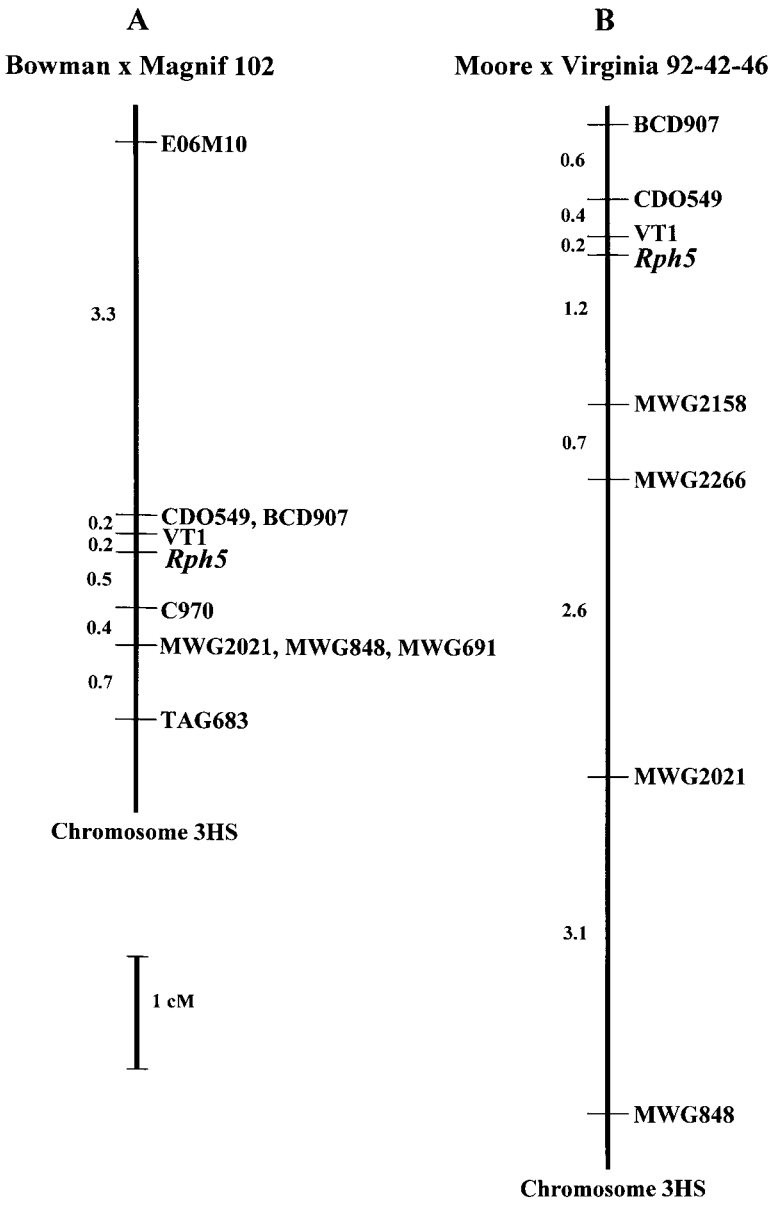

Fig. 1. Partial molecular maps of barley chromosome 3H showing the genetic location of leaf rust resistance gene *Rph5*. Markers were mapped in two segregating populations: (A) Bowman (*rph5*) × Magnif 102 (*Rph5*); (B) Moore (*rph5*) × Virginia 92-42-46 (*Rph5*), respectively. Map distances are given in centimorgans (cM).

Table 3. Infection types of barley parents to *Puccinia hordei* race 8.

Line	Accession #	Infection type†
Moore	CI 7251	4
VA92-42-46		0;
MV resistant progeny		0;N
Bowman	PI 483237	33$^-$
Magnif 102	PI337140	0;
BM resistant progeny		0;N

† Infection type (IT) ratings are based on the 0-to-4 scale of Levine and Cherewick (1952). IT designations of plants refer to: 4 = extremely susceptible with large uredia; 33$^-$ = moderately susceptible with combination of medium size (3) and small size (3$^-$) uredia; 0; = resistant with necrotic flecks; and 0;N = resistant with necrotic lesions.

mal to *Rph*5. Map positions and the relative order of the markers *CDO549*, *BCD907*, *MWG2021*, *MWG848* were confirmed on the MV population (Fig. 1B). In addition to the above-mentioned markers, two RFLP markers, *MWG2158* and *MWG2266*, were mapped 1.5 and 1.9 cM proximal to *Rph*5, respectively, in this population.

To identify more closely linked markers, AFLP analysis on parental lines and bulks from both populations was conducted. As a result, an AFLP fragment of 120 bp was detected with the primer combination *Eco*+ACA/*Mse*+AGG in both populations. This AFLP marker was converted to an RFLP probe (hereafter, referred to as *VT1*) and mapped 0.2 cM distal to *Rph*5 in both populations. Also, an AFLP (E06M10) fragment of 200 bp was detected with the primer combination *Eco*+AGA/*Mse*+ATA and mapped to the most telomeric region of barley chromosome 3HS 3.7 cM distal from *Rph*5 in the BM population. This DNA fragment was cloned and sequenced. A BLAST search detected high similarity with the wheat telomere-specific DNA fragment (GenBank accession #AF004950).

The close linkage of the RFLP markers flanking *Rph*5 was confirmed by RFLP analysis of NIL Magnif 102/*8Bowman, recurrent parent Bowman and the *Rph*5 donor Magnif 102 as well as the other known source of *Rph*5 'Quinn' (PI39401). The markers *VT1*, *BCD907* and *CDO549* detected DNA fragments of the same size in NIL Magnif 102/*8Bowman, Magnif 102 and Quinn, while a different size fragment was observed in Bowman. As an example, RFLP patterns with the *VT1* marker is shown in Fig. 2. In total, 16 RFLPs, four SSRs, and one AFLP marker were placed on chromosome 3H in the BM population, and 15 RFLP and five SSR markers were mapped on the same linkage group in the MV population. Established maps share 13 common markers, including 10 RFLPs and three microsatellites, and cover 172.7 and 105.8 cM of the barley chromosome 3H in BM and MV populations, respectively (whole chromosome 3H maps are not shown).

DISCUSSION

With two segregating populations, the leaf rust resistance gene *Rph*5 was precisely mapped to the extreme telomeric region of chromosome 3HS by means of molecular markers. Mapping results were confirmed by NIL analysis. Several closely linked molecular markers

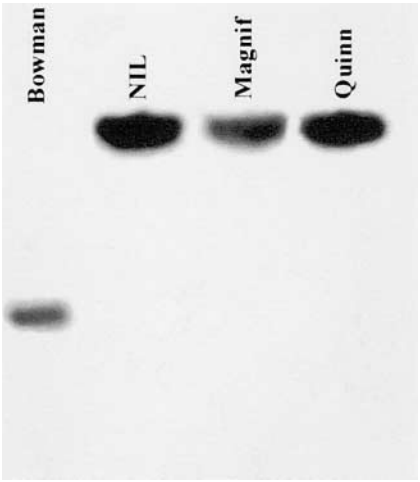

Fig. 2. RFLP analysis of Bowman (*rph*5), the NIL Magnif 102/8*Bowman (*Rph*5), Magnif 102 (*Rph*5) and Quinn (*Rph*5 and *Rph*2) with *VT1*.

were identified for *Rph*5. In the BM cross, the bracketing markers are *VT1* (at 0.2 cM distal) and *C970* (at 0.5 cM proximal) and in the MV cross, *VT1* (also at 0.2 cM distal) and *MWG2158* (at 1.2 cM proximal) (Fig. 1A and B). The closely linked markers, identified in this study, may be useful as probes for detecting the barley lines carrying resistance alleles of *Rph*5. The other benefit derived from comprehensive mapping is the possibility of positional cloning of *Rph*5 in the future. One of the most crucial steps in positional cloning is the discovery of molecular markers bracketing the gene of interest as demonstrated here for *Rph*5. Map-based cloning has been successfully applied for several disease resistance genes in barley (Buschges et al., 1997; Wei et al., 1999).

As was mentioned above, the *Rph*7 locus was also mapped to the extreme telomeric region of barley chromosome 3HS (Brunner et al., 2000; Graner et al., 2000). Interestingly, this part of chromosome 3HS does show an increased recombination rate that indicates a relatively high level of genetic activity in the region (Kunzel et al., 2000). The availability of molecular maps with common markers allowed comparison of map positions and estimation of relative locations of other loci. In this study, we compared three molecular maps of the *Rph*5 and *Rph*7 flanking regions: two of the maps were developed in this study and the third by Brunner et al. (2000). On the basis of the positions of common markers, we estimate that *Rph*5 is located about 6 cM distally from *Rph*7 on barley chromosome 3HS (Fig. 3).

Another interesting finding is the positioning of the AFLP marker E06M10 on the extreme telomeric region of chromosome 3HS. It was mapped 3.3 cM distal to the RFLP markers *CDO549* and *BCD907* in the BM cross. Sequence analysis of the DNA fragment detected by E06M10 revealed a high level of similarity with wheat telomere-associated DNA (GenBank accession #AF-004950). In this regard, it is interesting to note that Kilian et al. (1999) generated marker Tel3S from a telomere-associated sequence of barley and mapped it to the most terminal region of barley chromosome 3HS, which is located ~4.5 cM away from the *MWG691*/

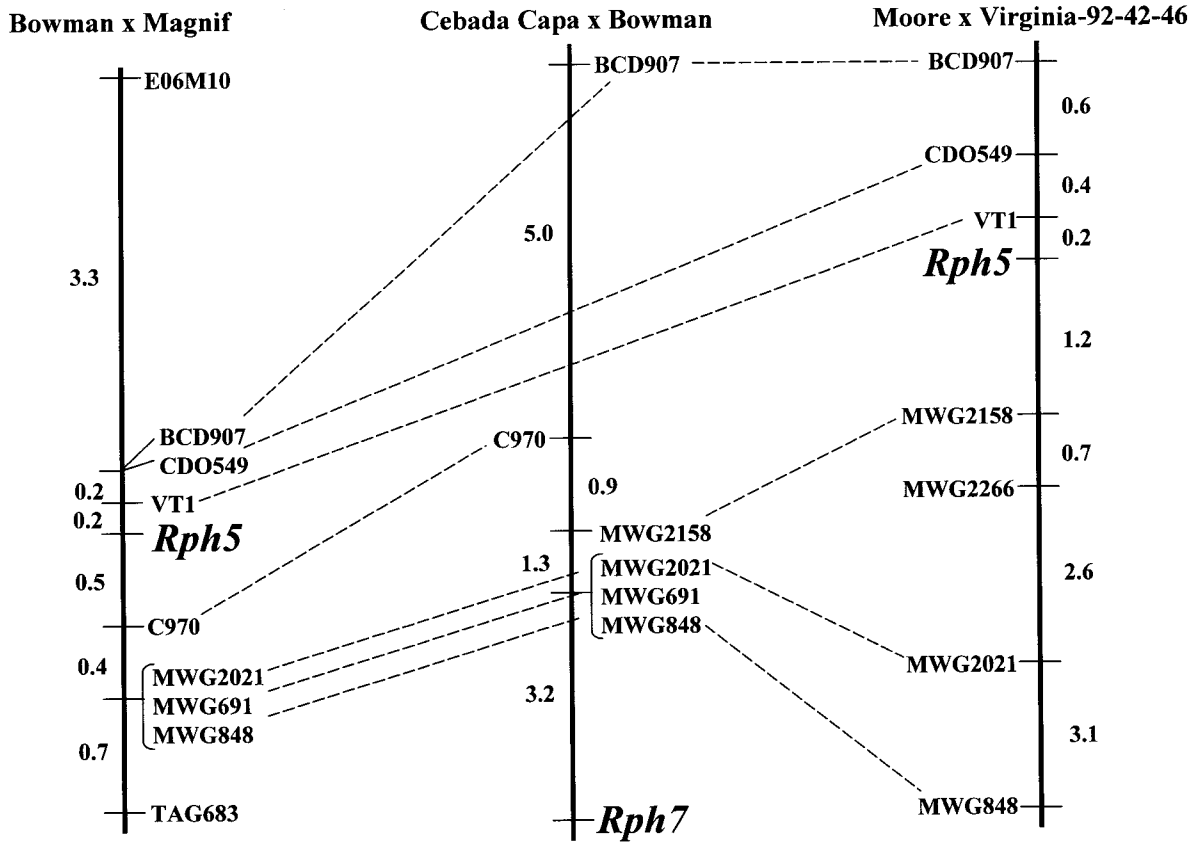

Fig. 3. Estimation of the relative locations of *Rph*5 and *Rph*7 leaf rust resistance genes on the basis of comparison of three maps with common markers. Maps of Moore × Virginia 92-42-46 and Bowman × Magnif 102 are from this study, and Cebada Capa × Bowman map was published recently (Brunner et al., 2000).

ABG316A cluster. In our map, the distance between *MWG691* and E06M10 is approximately the same—4.6 cM (Fig. 1A). This observation confirmed the position of marker Tel3S in the terminal region of barley chromosome 3HS.

Precise mapping of *Rph*5 has resulted in the identification of closely linked molecular markers that are potentially suitable for marker-assisted selection and pyramiding of genes confirming more durable resistance to leaf rust. Also, the results provide the basis for physical mapping and map-based cloning of the *Rph*5 gene.

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