

# Molecular Mapping of the Leaf Rust Resistance Gene *Rph6* in Barley and Its Linkage Relationships with *Rph5* and *Rph7*

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## ABSTRACT

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The barley cv. Bolivia carries two leaf rust (*Puccinia hordei*) resistance genes, *Rph2* and *Rph6*, and is the only known source of the latter gene. A resistant line (Bolivia-*Rph6*) carrying *Rph6* only was obtained in the F<sub>4</sub> generation of a cross between cv. Bolivia and the susceptible cv. Bowman via progeny testing with differential isolates of the leaf rust pathogen. Genetic analyses and bulk segregant analysis using amplified

fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers localized *Rph6* on the short arm of barley chromosome 3H at a distance of 4.4 centimorgans (cM) distal from RFLP marker MWG2021 and 1.2 cM proximal from RFLP marker BCD907. The allelic relationship of *Rph6* to other leaf rust resistance genes mapping to this region of chromosome 3H (namely *Rph5* and *Rph7*) were tested using crosses among cvs. Magnif 102 (carrying *Rph5*), Bolivia-*Rph6* (*Rph6*), and Cebada Capa (*Rph7*). Segregation analyses indicated that *Rph6* is allelic to *Rph5* and closely linked to *Rph7*. The data generated from this study will facilitate breeding for leaf rust resistance via marker-assisted selection and provide a starting point for positional gene cloning.

Leaf rust caused by *Puccinia hordei* G. Oth. is considered the most important rust disease of barley (*Hordeum vulgare* L.) worldwide (9). In the United States, leaf rust is a continual problem of barley grown in the Mid-Atlantic Region and California. Yield losses up to 32% have been reported in susceptible cultivars under epidemic conditions in Virginia (14). The deployment of resistant cultivars is the most effective means of control for this disease. Sixteen major race-specific genes (designated as *Rph1* to *Rph16*) for resistance to leaf rust have been identified from barley and its wild progenitor *H. vulgare* subsp. *spontaneum* (12,16); however, most of them have been rendered ineffective due to virulence changes in *P. hordei* populations (9,10,28). Transfer of multiple genes with different resistance spectra (i.e., gene pyramiding) into barley cultivars is one possible means by which more stable rust control might be achieved.

The mapping of leaf rust resistance genes and identification of molecular markers closely linked to them can facilitate the transfer and pyramiding of resistance genes in barley breeding programs through marker-assisted selection. It also may provide a foundation for gene cloning based on the positional cloning strategy. Most of the described *Rph* genes have been positioned to one of the seven barley chromosomes by various means, but specific chromosomal positions are lacking for some. *Rph1*, *Rph4*, and *Rph5* were localized on barley chromosomes 2H, 1H, and 3H, respectively, by trisomic analysis (30,31); *Rph10* and *Rph11* were positioned on chromosomes 3H and 6H, respectively, based on their linkage to previously mapped isozyme markers (11); and *Rph3* and *Rph12* were assigned to barley chromosomes 7H and 5H, respectively, based on their linkage with previously mapped morphological markers (17). The abundance of DNA markers has hastened the mapping of leaf rust resistance genes in barley. Using

this technique, *Rph2*, *Rph9*, and *Rph12* were mapped to chromosome 5H (1,2), *Rph16* to chromosome 2H (16), and *Rph7* to chromosome 3H (5,13). Recently, *Rph5* was mapped to the same general region of chromosome 3H where *Rph7* is located with amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers (21).

*Rph6* is one of only a few described leaf rust resistance genes that has not been fully characterized with respect to its chromosomal location and resistance spectrum to *P. hordei*. The reason is that cv. Bolivia, the only known source of the gene, also carries another resistance gene, *Rph2*. Thus, the objectives of this study were to (i) separate *Rph6* from *Rph2* in progeny derived from cv. Bolivia; (ii) determine the chromosome location of *Rph6* by means of molecular markers; and (iii) analyze linkage relationships between *Rph6* and two other leaf rust resistance genes, *Rph5* and *Rph7*.

## MATERIALS AND METHODS

**Plant materials.** Cv. Bolivia (PI 36360) was used as the donor of the leaf rust resistance gene *Rph6* and was crossed with the susceptible cv. Bowman (PI 483237) for genetic analyses. A homozygous resistant line carrying only *Rph6* (designated as Bolivia-*Rph6*) was obtained in an F<sub>4</sub> family from the original cross. The Bolivia-*Rph6* line was then used as a male parent in a cross with cv. Bowman to produce a mapping population of 122 F<sub>2</sub> plants and F<sub>3</sub> families (derived from the F<sub>2</sub> plants). Preliminary mapping data suggested that *Rph6* was located on the short arm of chromosome 3H, a region where *Rph5* from cv. Magnif 102 (CIho 13806) and *Rph7* from cv. Cebada Capa (PI 539113) were recently mapped (5,13,21). To test the linkage relationships among these leaf rust resistance genes, crosses were made among the three barley lines carrying the respective genes. The number of F<sub>2</sub> progeny evaluated in each cross is listed in Table 1.

Also included in the inoculation tests were cvs. Peruvian (CIho 935), which served as a reference source for an *Rph2* allele

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thought to act in a similar manner to the one in Bolivia (B. Steffenson, unpublished data), and Moore (CIho 7251), a susceptible check. The wheat cv. Chinese Spring (CItr 14108), barley cv. Betzes (PI 129430), and derived wheat-barley addition lines 1HS, 2H, 3H, 4H, 5H, 6H, and 7H (15) were used to assign cloned AFLP markers to specific barley chromosomes. The sowing and growing of plant materials were as previously described (29).

**Leaf rust evaluations.** Leaf rust inoculations were made according to the methods described by Steffenson et al. (29). Twelve days after inoculation, the infection types (ITs) of plants were assessed using the rating scale of Levine and Cherewick (20). ITs of 0, 0;, 1, 2, or combinations thereof and 2,3- were considered indicative of host resistance (i.e., low ITs), whereas ITs of 3, 4, or combinations thereof were considered indicative of host susceptibility (i.e., high ITs). Two isolates of *P. hordei* (Tel Aviv and ND8702) were used in this study. The ITs they elicit on the pertinent *Rph* sources and controls are given in Table 2. Isolate Tel Aviv elicited an IT of 2,3- on cv. Peruvian (which carries an allele at the *Rph2* locus) and an IT of 0;,1 on cv. Bolivia (*Rph2* + *Rph6*); thus, it was useful for differentiating F<sub>2</sub> plants carrying only *Rph2* from those carrying *Rph6* or *Rph2* + *Rph6* in the original Bowman/Bolivia cross. Isolate ND8702 was used in all other leaf rust evaluations because it is avirulent on *Rph5*, *Rph6*, and *Rph7*.

**Molecular mapping of *Rph6*.** A single newly expanded leaf was harvested from 16 plants of each F<sub>3</sub> family (122 in total) from the Bowman/Bolivia-*Rph6* cross and bulked for DNA extraction using the protocols of Riede and Anderson (24). For bulked segregant analysis (BSA) (22), equal amounts of DNA from 20 homozygous resistant F<sub>3</sub> families were combined for the resistant bulk, and equal amounts of DNA from 20 homozygous susceptible F<sub>3</sub> families were combined for the susceptible bulk. For BSA with AFLP markers, template DNA (0.25 µg) from the parents and bulks was digested using the restriction enzymes *EcoRI* and *MseI*, according to the manufacturer's protocol (New England Biolabs Inc., Beverly, MA). The adapter ligation, preamplification, and selective amplification were carried out using an AFLP kit (Gibco BRL, Grand Island, NY). Selective amplification products were separated in a denaturing polyacrylamide gel (7M Urea, 0.5× TBE, 6% Long Ranger Gel Solution [BMA, Rochland, ME],

0.05% TEMED, and 0.05% ammonium persulfate), and AFLP bands were detected using the DNA silver-staining system (Promega, Madison, WI). AFLP markers were cloned using the method described by Brigneti et al. (3).

For RFLP analysis, 20 µg of DNA from the parents, resistant bulk, and susceptible bulk were digested with five restriction enzymes (*BamHI*, *DraI*, *EcoRI*, *EcoRV*, and *HindIII*) according to the manufacturer's protocol (New England Biolabs Inc.). Filters with the digested DNA from parents and bulks were screened with RFLP clones that were previously mapped to barley chromosome 3H (18). When polymorphisms between the parents and also between the bulks were identified, the entire mapping population of 122 F<sub>3</sub> families was subjected to RFLP analysis to confirm the linkage relationship of these markers with the resistance gene. Southern blotting, hybridization, and membrane washing and stripping procedures were carried out as described by Riede and Anderson (24). RFLP clones were provided by A. Kleinhofs (Washington State University, Pullman), A. Graner (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany), and M. A. Saghai-Marouf (Virginia Polytechnical Institute and State University, Blacksburg).

The computer program MAPMAKER (version 2.0) was used for linkage analysis and genetic map construction (19). Linkage maps were constructed based on a LOD (logarithm of odds) threshold of 3.0 and maximum Kosambi distance of 40 centimorgans (cM).

## RESULTS

**Separation of *Rph6* from *Rph2*.** Cv. Bolivia possesses two leaf rust resistance genes, *Rph2* and *Rph6* (25,26). To separate *Rph6* from *Rph2*, cv. Bolivia was crossed with cv. Bowman, and 150 F<sub>2</sub> seedlings were evaluated with the *P. hordei* isolate Tel Aviv (Table 2). The ratio of resistant (ITs of 0, 0;, 1, 2, or combinations thereof and 2,3-) to susceptible (ITs of 3, 4, or combinations thereof) F<sub>2</sub> plants was 136:14, which fits an expected two-gene segregation ratio of 15:1 ( $\chi^2 = 2.43$ ,  $P = 0.12$ ). Twenty F<sub>2</sub> plants exhibiting very low ITs of 0, 0;, or 1 were selected to produce F<sub>3</sub> families for further genetic analysis. These F<sub>2</sub> plants were presumed to carry either *Rph2* + *Rph6* or *Rph6* alone based on their

TABLE 1. Results of allelism tests among three leaf rust resistance genes (*Rph5*, *Rph6*, and *Rph7*) on chromosome 3H of barley

Cross	Parental ITs <sup>x</sup>	Number of F <sub>2</sub> plants evaluated	Observed ratio <sup>y</sup>	Linkage distance <sup>z</sup>
Magnif 102 ( <i>Rph5</i> )/Bolivia- <i>Rph6</i> ( <i>Rph6</i> )	(0;n)/(0;n)	2,133	2,133:0	0
Magnif 102 ( <i>Rph5</i> )/Cebada Capa ( <i>Rph7</i> )	(0;n)/(0;n)	775	774:1	7.2 ± 3.5%
Cebada Capa ( <i>Rph7</i> )/Bolivia- <i>Rph6</i> ( <i>Rph6</i> )	(0;n)/(0;n)	850	849:1	6.9 ± 3.4%

<sup>x</sup> Parental infection types (ITs) were in response to leaf rust isolate ND8702.

<sup>y</sup> The observed ratio is the number of resistant plants versus susceptible plants.

<sup>z</sup> The linkage distance was estimated using the maximum likelihood method.

TABLE 2. Infection types (ITs) of selected barley cultivars to *Puccinia hordei* isolates used in differentiating leaf rust resistance genes and in evaluating progenies of crosses

Isolates	Cultivar (Recognized <i>Rph</i> gene)					
	Bolivia ( <i>Rph2</i> + <i>Rph6</i> )	Bolivia- <i>Rph6</i> ( <i>Rph6</i> )	Cebada Capa ( <i>Rph7</i> )	Peruvian ( <i>Rph2</i> )	Bowman (None)	Moore (None)
Tel Aviv	0;,1 <sup>z</sup>	0;,1	0;,1	2,3-	3	3
ND8702	0;,1	0;,1	0;,1	2,1	3	3

<sup>z</sup> Infection types were based on the 0 to 4 rating scale of Levine and Cherewick (20).

TABLE 3. Segregation of F<sub>2</sub> progeny and F<sub>3</sub> families of the barley cross Bowman/Bolivia-*Rph6* to *Puccinia hordei* isolate ND8702 at the seedling stage

Number of F <sub>2</sub> plants	Number of F <sub>3</sub> families	Expected ratio	$\chi^2$	Probability
90:32 (R:S) <sup>y</sup>	...	3:1	0.10	0.78
...	41:49:32 (HR:SEG:HS) <sup>z</sup>	1:2:1	6.05	0.49

<sup>y</sup> R = resistant, S = susceptible.

<sup>z</sup> HR = homozygous resistant, SEG = segregating, and HS = homozygous susceptible.

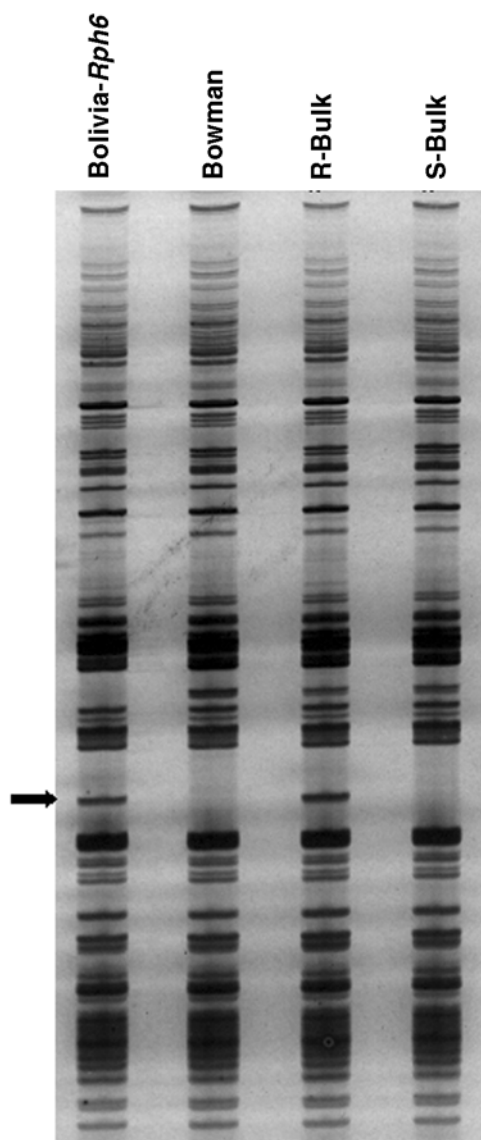
reaction to isolate Tel Aviv. Fifty seedlings from each of the 20 selected F<sub>3</sub> families were then evaluated with isolate ND8702. Seven families were homozygous resistant and 13 segregated for resistance and susceptibility (data not shown). Families segregating in a clear 3:1 ratio for resistance and susceptibility were presumed to carry only *Rph6*. Five resistant plants were selected from each of these families and grown to produce seed. Subsequent testing of F<sub>4</sub> seedlings from each of these resistant selections to isolate ND8702 allowed us to select a single plant (designated Bolivia-*Rph6*) that was homozygous for resistance, presumably due to *Rph6*.

One hundred twenty-two F<sub>2</sub> seedlings derived from a cross between cv. Bowman and Bolivia-*Rph6* were evaluated for their reaction to *P. hordei* isolate ND8702. The number of resistant (ITs of 0, 0, 1, 2 or combinations thereof) to susceptible (ITs of 3 or 4) F<sub>2</sub> progeny approximated a 3:1 ratio (Table 3), indicating that a single dominant gene (presumably *Rph6*) conferred leaf rust resistance in the line Bolivia-*Rph6*. This result was confirmed from the evaluation of F<sub>3</sub> families, which segregated in an approximate 1

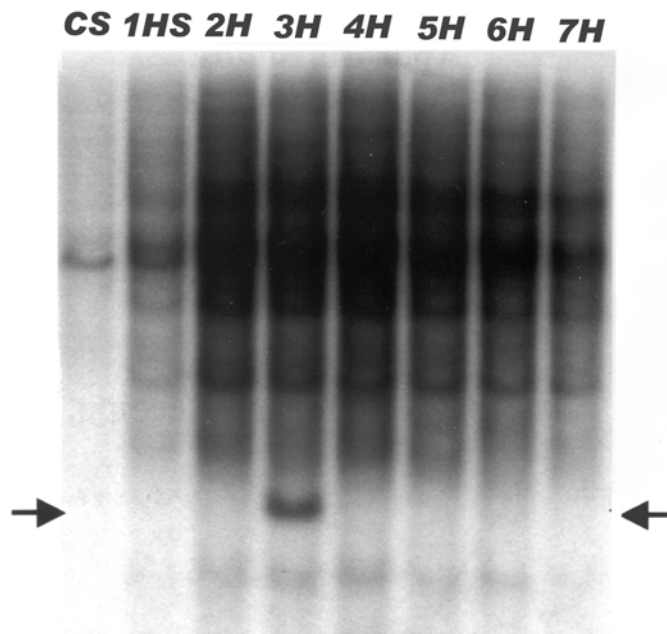
homozygous resistant:2 segregating:1 homozygous susceptible ratio ( $\chi^2 = 6.05, P = 0.49$ ) (Table 3).

**Molecular mapping of *Rph6*.** BSA with AFLPs identified a polymorphic band between cv. Bowman and Bolivia-*Rph6* as well as between the two bulks using primers E-ACC and M-CAT (Fig. 1). This band was cloned and hybridized with filters made from a *Dra*I digest of the wheat-barley addition lines. A unique hybridization band was observed only in the lane from the addition line of barley chromosome 3H (Fig. 2). This result suggested that *Rph6* is located on chromosome 3H. Unfortunately, the cloned AFLP band was not polymorphic when used as an RFLP probe in the F<sub>3</sub> population with the five restriction enzymes tested (data not shown). Thirty-two RFLP markers previously mapped to chromosome 3H of barley were then selected for BSA. Three markers (ABG70, BCD907, and MWG848) located on the short arm of chromosome 3H generated a polymorphism between the two parents and also the resistant and susceptible bulks. RFLP analysis of the entire F<sub>3</sub> population with these RFLP markers further confirmed their close linkage to the *Rph6* locus (Fig. 3). Since markers BCD907 and MWG848 were previously found closely linked to *Rph5* (21) and *Rph7* (5,13) on the short arm of chromosome 3H, other RFLP markers (MWG691, MWG2021, MWG2158, MWG2266, and CDO549) mapping to the same region were evaluated for polymorphism between cv. Bowman and Bolivia-*Rph6*. Of this group of RFLP markers, only MWG2021 and CDO549 generated a polymorphism between the two parents and segregated in the F<sub>3</sub> population of the cross. The clone VT1, which was converted from an AFLP marker linked with *Rph5* (21), also produced a polymorphism between cv. Bowman and Bolivia-*Rph6* and segregated in the progeny population. The map position of *Rph6* in relation to these RFLP markers on the short arm of chromosome 3H is shown in Figure 3.

**Linkage tests.** *Rph6* was found to map near two other leaf rust resistance genes (*Rph5* and *Rph7*) on chromosome 3H. To test the linkage relationships among the three genes, crosses were made among cvs. Magnif 102 (carrying *Rph5*), Bolivia-*Rph6* (carrying only *Rph6*), and Cebada Capa (carrying only *Rph7*) and their F<sub>2</sub> progeny tested for reaction to isolate ND8702 (Table 1). No segregation was observed among 2,133 F<sub>2</sub> progeny derived from the



**Fig. 1.** Amplified fragment length polymorphism (AFLP) analysis with susceptible parent Bowman, resistant parent Bolivia-*Rph6*, and DNA bulks prepared from resistant (R-bulk) and susceptible (S-bulk) F<sub>3</sub> families. The AFLP bands were amplified with primer pairs E-ACC and M-CAT and detected using the DNA silver-staining system. The polymorphic band only present in the resistant parent Bolivia-*Rph6* and the resistant bulk is indicated with an arrow.



**Fig. 2.** Southern hybridization of a cloned amplified fragment length polymorphism marker linked with *Rph6* to DNA of the wheat-barley addition lines of Islam (15). The hybridization band indicated with an arrow is only present in the addition line with barley chromosome 3H. CS = Chinese Spring wheat control.

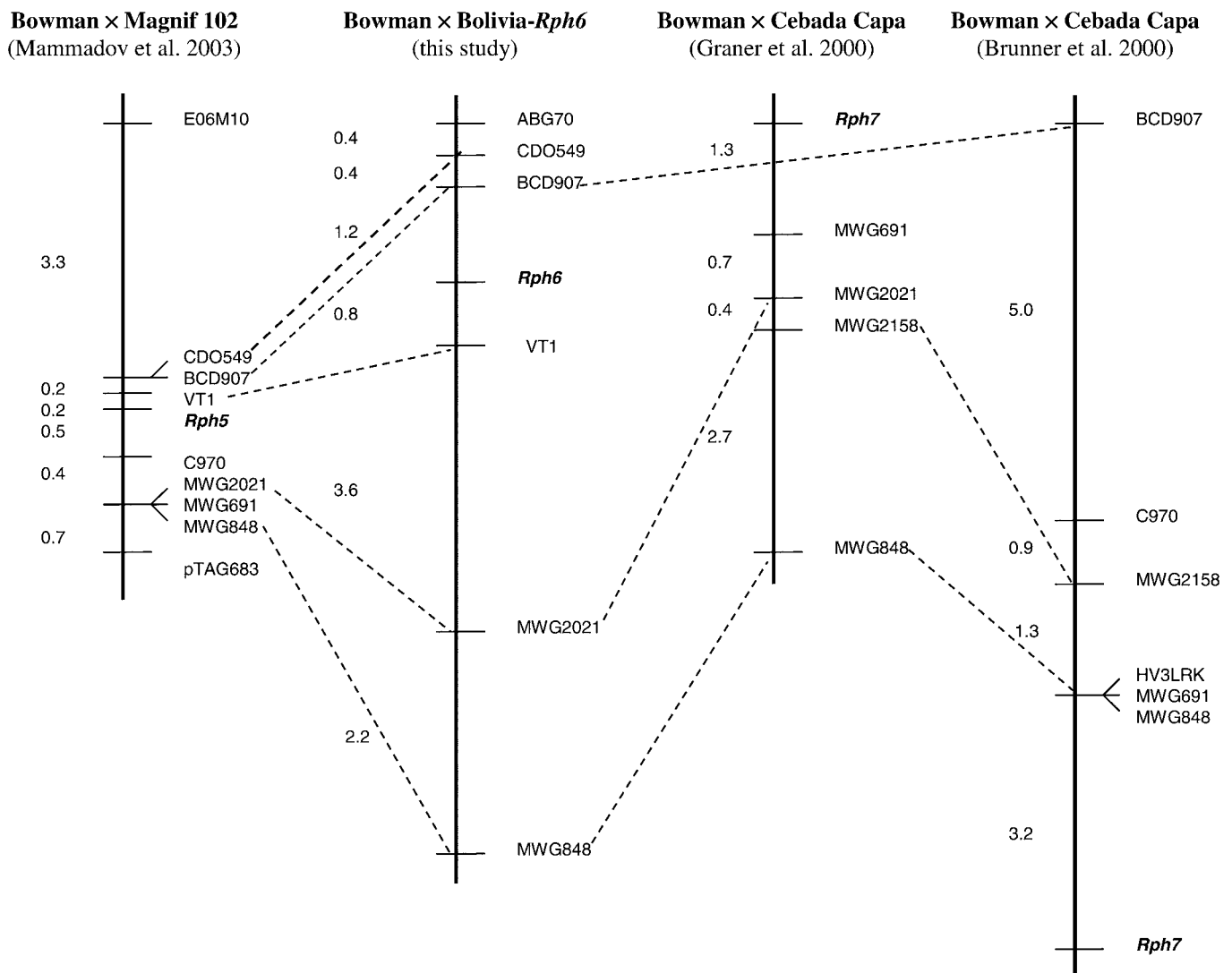
Magnif 102/Bolivia-*Rph6* cross, suggesting that *Rph5* and *Rph6* are allelic. One susceptible recombinant plant was found among 775 F<sub>2</sub> progeny from the Bolivia-*Rph6*/Cebada Capa cross, indicating that *Rph6* is likely not allelic, but closely linked to *Rph7*. In the Magnif 102/Cebada Capa cross, one susceptible plant was found in 850 F<sub>2</sub> progeny, indicating that *Rph5* and *Rph7* are closely linked and likely not allelic. The maximum likelihood estimate of the recombination frequency between *Rph6* and *Rph7* and *Rph5* and *Rph7* was  $6.9 \pm 3.4\%$  and  $7.2 \pm 3.5\%$ , respectively.

### DISCUSSION

To develop a line from cv. Bolivia (*Rph2+Rph6*) carrying only the resistance gene *Rph6*, it was essential to have a *P. hordei* isolate (i.e., Tel Aviv) that exhibits differential virulence on *Rph2* and *Rph6* (Table 2). Within the resistant class of the F<sub>2</sub> population from the original Bowman/Bolivia cross, the ITs of plants ranged from 0 to 2,3-. Plants exhibiting an IT of 2,3-, similar to cv. Peruvian, were presumed to carry *Rph2* alone. In contrast, plants exhibiting very low ITs of 0, 0;, 1, or combinations thereof were presumed to carry either *Rph2 + Rph6* or *Rph6* alone. By selecting resistant plants exhibiting very low ITs in the F<sub>2</sub> population, we eliminated plants carrying *Rph2* only. Subsequent phenotyping of F<sub>3</sub> and F<sub>4</sub> progeny to isolate ND8702 allowed us to select a line

(Bolivia-*Rph6*) carrying a single resistance gene, which was presumed to be *Rph6*. Two lines of evidence support the contention that Bolivia-*Rph6* carries *Rph6* rather than *Rph2*. First, Bolivia-*Rph6* confers a spectrum of resistance that is different from *Rph2* sources (e.g., cv. Peruvian) against pathotypes of *P. hordei*. We tested 373 *P. hordei* isolates on Bolivia-*Rph6* and cv. Peruvian and found that they differed in their reaction to 50% of the isolates (B. Steffenson and T. Fetch, unpublished data). Second and more compelling is the fact that the two resistance genes map to different chromosomes. An allele at the *Rph2* locus (temporary designation: *RphQ*) from barley line Q21861 was mapped to chromosome 5H (2), whereas *Rph6* was positioned on chromosome 3H in this study.

Several recent studies have shown that the short arm of barley chromosome 3H is also the location of *Rph5* (21) and *Rph7* (5,13). Since several common RFLP markers were used in mapping *Rph5* (21), *Rph6* (this study), and *Rph7* (5,13), the relative positions of these three leaf rust resistance genes can be estimated and compared. In this study, *Rph6* was positioned at a locus flanked by the RFLP markers BCD907 and MWG2021 within a distance of 5.6 cM. Interestingly, *Rph5* was also mapped at a position flanked by these two markers, but within a distance of 1.3 cM (21). Based on this mapping data, we decided to conduct an allelism test between *Rph5* and *Rph6*. No susceptible recombinant plants were



**Fig. 3.** Molecular linkage maps of the three leaf rust resistance genes, *Rph5* (21), *Rph6* (this study), and *Rph7* (5,13) mapping on the short arm of chromosome 3H. Common restriction fragment length polymorphism markers are indicated with dash lines.

identified among 2,133 F<sub>2</sub> progeny from a Bolivia-*Rph6*/Magnif 102 cross, suggesting that *Rph6* is allelic with *Rph5*. According to the locus/allele nomenclature given by Franckowiak et al. (12), *Rph6.f* (i.e., the original locus and allele designation of the gene) should be renamed as *Rph5.f*.

One susceptible recombinant plant was found among 850 F<sub>2</sub> progeny from the Bolivia-*Rph6*/Cebada Capa cross, indicating that *Rph6* and *Rph7* are closely linked and not allelic. This result is in agreement with the study of Roane and Starling (26) who found two susceptible recombinant plants in a population of 295 F<sub>2</sub> progeny derived from a Cebada Capa/Bolivia cross. In a cross between cv. Magnif 102 (*Rph5*) and cv. Cebada Capa (*Rph7*), we found one susceptible recombinant plant in 775 F<sub>2</sub> progeny. This result provides further evidence that the *Rph5/Rph6* locus is closely linked and not allelic with *Rph7*. By comparing two molecular maps of *Rph5* (one from a Moore/Virginia 92-42-46 cross and another from a Bowman/Magnif 102 cross) with the molecular map of *Rph7* established by Brunner et al. (5), Mammadov et al. (21) suggested that *Rph5* is approximately 5 cM distal from *Rph7* on the short arm of barley chromosome 3H. The estimated genetic distance between *Rph6* and *Rph7* is approximately 9.8 cM based on molecular mapping data. This estimate is in close agreement with the linkage test data, which gave an estimated distance of 6.9 ± 3.4 mapping units between *Rph6* and *Rph7* and 7.2 ± 3.5 mapping units between *Rph5* and *Rph7*. Graner et al. (13) mapped *Rph7* 5.1 cM distal from marker MWG848. Their result is in contrast to Brunner et al. (5) who localized *Rph7* 3.2 cM proximal from MWG848. Comparison of our molecular map with that of Graner et al. (13) gives an estimated distance of approximately 2.4 cM between *Rph6* and *Rph7*. The discrepancy in the estimated genetic distances may be due to different mapping populations and their sizes.

Several investigators have proposed various sets of barley lines with different *Rph* genes for differentiating virulence phenotypes of *P. hordei* (8,27). Cv. Bolivia has been included in these sets (9,27; B. Steffenson, unpublished data) because it is the only known source of *Rph6*. Unfortunately, cultivars like Bolivia that possess two or more resistance genes are inefficient for detecting virulence polymorphisms in pathogens (23). The successful isolation of *Rph6* from *Rph2* in cv. Bolivia will facilitate the development of a more efficient differential host line that carries only *Rph6*. Indeed, development of a set of near-isogenic lines (NILs) for *Rph1-15* in a cv. Bowman background is approaching completion (7; J. D. Franckowiak, unpublished data). This differential set will be valuable for researchers conducting virulence surveys of *P. hordei*.

The map-based cloning technique has been successfully applied for isolating several disease resistance genes in barley including *mlo* and *Mla* for powdery mildew resistance (6,32) and *Rpg1* for stem rust resistance (4). Mapping of leaf rust resistance genes and identification of closely linked molecular markers are essential steps for the map-based cloning approach. A project to clone the leaf rust resistance gene *Rph5* from cv. Magnif 102 by this method has been initiated (M. Saghai Maroof and B. Steffenson, unpublished data). Success in this endeavor will help to resolve the molecular basis of specificity in the *Rph5* and *Rph6* alleles at this complex locus. In addition to providing a starting point for map-based cloning attempts, the mapping information generated from this study may also facilitate breeding for leaf rust resistance via marker-assisted selection. Gene pyramiding is one possible strategy that may lead to more stable leaf rust resistance in barley. A collection of 373 *P. hordei* isolates from around the world was tested on the sources of *Rph5* (cv. Magnif 102), *Rph6* (Bolivia-*Rph6*), and *Rph7* (cv. Cebada Capa) and 15.0, 20.3, and 16.4% carried virulence for the three respective genes (B. Steffenson and T. Fetch, unpublished data). However, only eight of the 373 isolates (2.1%) were virulent for both *Rph5/Rph6* and *Rph7*. None of these isolates were from the United States; thus, the transfer of

both *Rph5/Rph6* and *Rph7* in barley cultivars may offer some promise for more stable resistance in this country.

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