Chromosomal Location and Genetic Relationship of Leaf Rust Resistance Genes *Rph9* and *Rph12* in Barley

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ABSTRACT

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Barley lines Hor 2596 and Triumph are the sources of leaf rust resistance genes *Rph9* and *Rph12*, respectively. An allelism test was performed with F_2 progeny of the cross Triumph/Hor 2596 inoculated with *Puccinia hordei*. No recombinants were found in a population of 3,858 progeny, indicating *Rph9* and *Rph12* are alleles. Molecular and morphological markers were used to identify the chromosomal location of these genes in the crosses Bowman/Hor 2596 and Triumph/I91-533-*va*. A linkage was detected between *Rph9* and the flanking sequence-tagged site (STS) markers *ABC155* and *ABG3* on chromosome 7(5H) at a distance of 20.6 and 20.1 centimorgans (cM), respectively, and to the microsatellite marker dehydrin-9 (*HVDHN9*) at a distance of 10.2 cM in the Bowman/Hor 2596 cross. Analysis of isozymes in bulks of the same population showed that *Rph9* may be closely linked to the *Est9* locus on chromosome 7(5H). The *Rph12* locus was linked to the morphological trait locus *va* (controlling variegated leaf color) on chromosome 7(5H) at a distance of 22.6 cM in the Triumph/I91-533-*va* cross. *Rph12* also was linked with STS marker *ABC155* (24.4 cM) and RAPD marker *OPA19 (1.5)* (17.8 cM). These data indicate that Hor 2596 and Triumph carry a leaf rust resistance gene at the same locus on the long arm of chromosome 7(5H) of barley.

Leaf rust of barley (*Hordeum vulgare* L.), caused by *Puccinia hordei* G. Otth, is considered one of the most important rust diseases of the crop. Although widespread and severe epidemics of leaf rust have historically been rare, local epidemics in the United States, Australia, and Europe have resulted in significant losses to barley crops (5,7,10). In the United States, sporadic outbreaks of leaf rust have occurred in the Mid-Atlantic and Upper Midwest regions (10,27). The use of resistant cultivars is the best control measure for the disease. To date, 14 genes for resistance to *P. hordei* (*Rph* genes, formerly *Pa*) have been identified in barley and its wild progenitor, *H. vulgare* ssp. *spontaneum* (C. Koch) Thell. (8,13,14,25). The line Hor 2596 (CI 1243) is the source of *Rph9* (29,31). This gene was thought to be present in some European barley cultivars, including the East German release Trumpf and its derived export selection Triumph (5). Further studies showed, however, that Hor 2596 and Triumph exhibited different infection types (ITs) in response to some *P. hordei* isolates (15). In a genetic study, Walther (33) detected three resistance genes in Trumpf: two with dominant gene action and one with recessive gene action. None of these genes were considered to be the same as *Rph9* in Hor 2596 (33). In a more recent genetic study, a single resistance gene was identified in Triumph and designated *Rph12* (14). This single-gene inheritance pattern of Triumph to leaf rust was corroborated in two subsequent studies (3,13).

Information regarding the chromosomal location of disease resistance genes could facilitate their efficient transfer in breeding programs. Unfortunately, definitive data on the chromosomal position of leaf rust resistance genes *Rph9* and *Rph12* are lacking. Jin et al. (14) identified chromosome 7(5H) as the putative location of *Rph12*, but this was based on distant linkage (recombination values of 26.1 and 39.3%, respectively) with the chromosome 7(5H) morphological trait loci *raw1* (controlling the degree of barbing on the awns) and *srh* (controlling the length of hairs on the rachilla). Thus, this study was undertaken to identify the chromosomal location of *Rph9* and *Rph12*, using both morphological and molecular markers. Additionally, we investigated the allelic relationship between the resistance gene in Triumph and *Rph9* in Hor 2596.

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MATERIALS AND METHODS

Plant materials. Barley line Hor 2596 (CI 1243) and cv. Triumph (provided by M. Johnston, Montana State University, Bozeman) were used as the donors of *Rph9* and *Rph12*, respectively, in crosses. Bowman (PI 483237) was used as the susceptible parent in the cross with Hor 2596. Line I91-533-*va*, a near-isogenic line of Bowman, which has the chromosome 7(5H) morphological trait locus *va* (controlling variegated leaf color), was crossed with Triumph. Line I91-533-*va* was used to test whether *va* might be linked to *Rph12*. Like Bowman, I91-533-*va* is susceptible to leaf rust. F_2 populations from the Bowman/Hor 2596 and Triumph/I91-533-*va* crosses were derived from single F_1 plants and consisted of 360 and 63 progeny, respectively. F_3 families of both crosses were evaluated for their leaf rust reaction to identify the genotypes of F_2 plants. Homozygous F_2 plants were used to create DNA bulks for bulked segregant analysis (24). For each population, two DNA bulks were made: one consisting of DNA from 10 homozygous resistant plants, and one consisting of DNA from 10 homozygous susceptible plants. Primers amplifying products that exhibited polymorphisms between the DNA bulks as well as between the parents subsequently were evaluated on all 63 progeny from the Triumph/I91-533-*va* population and on 134 randomly selected progeny from the Bowman/Hor 2596 population. In addition to the two mapping populations, a cross also was made between Hor 2596

and Triumph to test for allelism between *Rph9* and *Rph12*. This population consisted of 3,858 F_2 plants.

Wheat-barley addition lines and two doubled haploid populations of barley were utilized to position molecular markers on the barley chromosomes. The wheat-barley ditelosomic addition lines (except chromosome 5) of Islam (12) were used to determine the chromosome on which molecular markers were located. The doubled haploid populations of Steptoe/Morex (17) and Q21861/SM89010 (28) were used to further position some of the molecular markers on the identified chromosome. The Steptoe/Morex population is useful for this purpose because a highly saturated molecular map has been developed for it (17). Moreover, several key disease resistance genes and molecular markers have been positioned in the Q21861/SM89010 population (4,28).

Leaf rust evaluations. Seeds of parental, F_1, F_2, and F_3 plants were sown in plastic pots filled with a peat moss/perlite (3:1) potting mixture and grown at 22 ± 3°C in a greenhouse, with supplemental lighting provided by 1,000-W metal halide bulbs (530 to 710 µmol photon m^{-2} s^{-1}) for 13 h/day. One-week-old seedlings (primary leaves fully expanded) were inoculated with urediniospores of isolate ND8702 of *P. hordei* suspended in a light-weight mineral oil (Soltrol 170, Phillips Petroleum Company, Bartlesville, OK). In the allelism test, 996 progeny were inoculated with isolate ND8702, 1,420 were inoculated with isolate BLR4, and 1,442 were inoculated with isolate Aus220. These three *P. hordei* isolates are all avirulent on Hor 2596 and Triumph. A concentration of ≈3.5 mg of urediniospores per ml of oil was used. Inoculated plants were placed in chambers (20°C) maintained near saturation by periodic mistings from ultrasonic humidifiers. After a 16-h misting period in the dark, plants were allowed to dry slowly before being returned to the greenhouse. The ITs exhibited by plants in response to *P. hordei* were evaluated based on the rating scale of Levine and Cherewick (21) 12 to 14 days after inoculation.

Molecular markers. Several types of markers were used to position the leaf rust resistance genes on barley chromosomes, including random amplified polymorphic DNAs (RAPDs), sequence-tagged sites (STSs), simple sequence repeats (SSRs) or microsatellites, and isozymes. One hundred eighty arbitrary random primers sets, A, B, E, H, K, N, O, P, and T, from Operon Technologies (Alameda, CA) and one hundred fifty STS polymerase chain reaction (STS-PCR) primer sets from the Montana State University Barley Genetics Laboratory, Bozeman (provided by T. K. Blake), were used to identify markers linked to *Rph9* and *Rph12*. The STS primers (each ≈20 bp long) were developed from restriction fragment length polymorphism (RFLP) markers previously mapped to barley chromosomes. Sequences for the STS primers are available on-line at the Graingenes website (supported by the U.S. Department of Agriculture and the National Agricultural Library). Markers generated by STS primers that were not polymorphic in the parents after amplification were restricted with *Hae*III, *Hin*fI, *Hha*I, *Rsa*I, or *Taq*I endonuclease, according to the manufacturer's instructions (New England Biolabs Inc., Beverly, MA). Methods for DNA isolation from plants and PCR amplification were as previously described (4). Protocols for the STS primers were as described by Tragoonrung et al. (30). PCR amplification of microsatellites (SSRs) previously mapped to chromosome 7(5H) (22) was performed according to Yu et al. (34). The primers for *HVDHN9* (X152572, mRNA for dehydrin-9) and *HVDHN7* (X71362, gene for dehydrin-7) are described in Becker and Heun (1) and were synthesized by Gibco BRL Life Technologies (Gaithersburg, MD). PCR products were separated on a denaturing polyacrylamide gel with 6 M urea and on a nondenaturing 5% polyacrylamide gel in cases in which the PCR reaction did not contain a radioactive label. The nondenaturing gels were stained with ethidium bromide to visualize the DNA fragments on a UV transilluminator. Esterase isozymes in the primary leaves of 1-week-old plants from the Bowman/Hor 2596 population were analyzed in starch gels, according to the protocols of Hvid and Nielsen (11).

Data analysis. The chi-square method was used to test the hypothesis of independent segregation in F_2 populations, and the method of maximum likelihood was used to calculate linkage distances from F_2 data. Segregating molecular markers were scored for each progeny, and linkage analyses were performed with the MAPMAKER software program, version 3.0 (20). The Kosambi (18) mapping function was used to convert recombination frequencies to map distances in centimorgans.

RESULTS

Genetic studies. In the Bowman/Hor 2596 cross, the total F_2 progeny population segregated 268:92 (resistant/susceptible) to isolate ND8702 of *P. hordei*, indicating resistance to leaf rust was conferred by a single gene ($\chi^2_{3:1}$ = 0.06; P = 0.81) in Hor 2596. Similar results were obtained in the Triumph/I91-533-*va* cross, in which F_2 progeny segregated 43:20 for resistance and susceptibility ($\chi^2_{3:1}$ = 1.53; P = 0.22). No segregation for leaf rust reaction was observed in the cross between Triumph and Hor 2596 for the allelism test. All 3,858 F_2 plants inoculated with isolate ND8702, BLR4, or Aus220 exhibited resistant ITs (0; to 2), indicating *Rph9* and *Rph12* are alleles.

Mapping of *Rph12*. In the Triumph/I91-533-*va* cross, *Rph12* was linked with the morphological trait locus *va* on chromosome 7(5H) at a distance of 22.6 centimorgans (cM). An attempt was made to saturate this region with additional markers by evaluating 180 RAPD primers in the bulks; however, only 1 RAPD marker, *OPA19 (1.5)*, was found to be linked (17.8 cM) to *Rph12*. The linkage distance between *va* and *OPA19 (1.5)* was 11.4 cM. One hundred fifty STS-PCR primer sets for different chromosomes

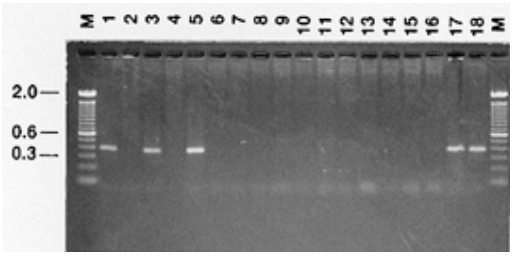

Fig. 1. Analysis to determine the chromosomal location of the *ABC155* sequence-tagged site marker generated by polymerase chain reaction with wheat-barley addition lines. Lanes 1 through 5, DNA of barley lines Bowman (lane 1), Hor 2596 (lane 2), Steptoe (lane 3), Morex (lane 4), and Betzes (lane 5). Lane 6, DNA of wheat parent Chinese Spring. Lanes 7 through 18, DNA of wheat lines carrying barley chromosome arms 1α (lane 7), 1β (lane 8), 2S (lane 9), 2L (lane 10), 3S (lane 11), 3L (lane 12), 4S (lane 13), 4L (lane 14), 6S (lane 15), 6L (lane 16), 7L (lane 17), and 7L (lane 18). Lane M, DNA size markers (100-bp ladder).

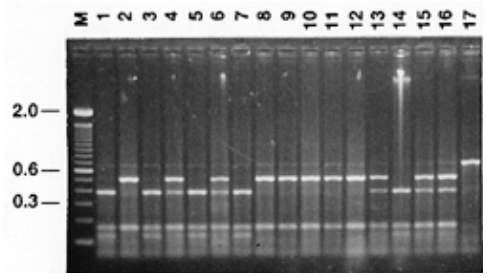

Fig. 2. Ethidium bromide-stained agarose gel of polymerase chain reaction amplification generated by ABG3 STS primers in the Bowman/Hor 2596 barley population after *Hin*fI restriction. Lane 1, Bowman parent; lane 2, Hor 2596 parent; lanes 3 through 7, homozygous susceptible F_2 plants; lanes 8 through 12, homozygous resistant F_2 plants; lanes 13 through 16, heterozygous F_2 plants (lanes 6 and 14 represent recombinants); lane 17, nonrestricted fragment; and lane M, DNA size markers (100-bp ladder).

also were analyzed in the bulks, with special emphasis on possible chromosome 7(5H) STS markers. STS marker *ABC155* was linked with *Rph12* at distance of a 24.4 cM. STS marker *ABC155* was mapped to chromosome 7(5H) by analysis of the wheat-barley addition lines (Fig. 1). The position of this marker on chromosome 7(5H) was determined by its cosegregation with RFLP marker *ABC155* in the Steptoe/Morex population (data not shown). Seven other chromosome 7(5H) STS markers (*ABG3*, *ABC717*, *ABG473*, *ABC302*, *ABG712*, *ABG390*, and *ABC168*) were evaluated in this study but were not polymorphic in this cross after amplification and restriction with *Hae*III, *Hin*fI, *Hha*I, *Rsa*I, or *Taq*I endonuclease.

Mapping of *Rph9*. Because the allelism test revealed that *Rph12* and *Rph9* were either allelic or very closely linked, the same chromosome 7(5H) markers used to map *Rph12* in the Triumph/I91-533-*va* cross also were used to map *Rph9* in the Bowman/Hor 2596 cross. No linkage was found between any of the 180 RAPD markers and *Rph9* in the Bowman/Hor 2596 population. RAPD marker *OPA19 (1.5*) was not polymorphic in this population. However, a linkage was found between *Rph9* and STS markers *ABC155* and *ABG3*. STS markers *ABC155* and *ABG3* flank *Rph9* at a distance of 20.6 and 20.1 cM, respectively. Both markers are located on chromosome 7(5H). STS marker *ABG3* (Fig. 2) was polymorphic after restriction with *Hin*fI in both the Bowman/Hor 2596 and Q21861/SM89010 populations. Its chromosomal location was determined in the latter population by linkage with stem rust resistance gene *rpg4* (31.7 cM) and RFLP marker *MWG740* (30.6 cM), which were previously positioned on chromosome 7(5H) (4).

The putative location of *Rph9* on chromosome 7(5H), as determined by linkage with the STS markers, placed the gene in the vicinity of the *Est9* locus, based on the Steptoe/Morex map (17). Analysis of esterase isozymes in Bowman and Hor 2596 revealed polymorphisms for *Est2*, *Est4*, and *Est9*. A distinct band intensity polymorphism for *Est9* was found in the bulks of homozygous resistant and homozygous susceptible F_3 families from the Bowman/Hor 2596 cross (F_2 plants were not available), suggesting *Rph9* may be closely linked to the *Est9* locus on chromosome 7(5H). No polymorphism for *Est2* or *Est4* was detected in these bulks. Analysis of 40 F_3 families revealed that *Est9* and *Rph9* were linked at a distance of 9.3 cM, although the activity of esterase 9 was low and the fragments were not always easy to score. To further corroborate the chromosome 7(5H) location of *Rph9*, we evaluated the *HVDHN7* and *HVDHN9* microsatellites, which were previously mapped to this chromosome by Liu et al. (22). *HVDHN7* and *HVDVN9* were analyzed in the parental lines in both a nondenaturing and a denaturing polyacrylamide gel. *HVDHN7* was not polymorphic in the Bowman/Hor 2596 population. In contrast, the *HVDHN9* primers generated several fragments, one of which (a 0.128-kb fragment) was polymorphic and distinct in both gels. Thus, the entire population was analyzed for this SSR in a 5% nondenaturing polyacrylamide gel. *Rph9* was linked to *HVDHN9* at a distance of 10.2 cM. Linkage maps showing the relationship between the two leaf rust resistance genes and associated markers in the Bowman/Hor 2596 and Triumph/I91-533-*va* populations are given in Figure 3.

DISCUSSION

No recombinants were detected in the allelism test between the sources of *Rph9* (Hor 2596) and *Rph12* (Triumph) when 3,858 progeny were evaluated. This result indicates *Rph9* and *Rph12* are allelic. Clifford and Jones (6) were the first to suggest that Hor 2596 and Triumph may have different leaf rust resistance genes, after showing that the two lines exhibited distinctly different reactions to isolate 673 of *P. hordei*. This result was subsequently confirmed by others (33; B. J. Steffenson and Y. Jin, *unpublished data*). Based on the analysis of reciprocal crosses of Hor 2596 and Trumpf, Walther (33) concluded that different genes were present in the two lines, despite the fact that they gave similar seedling

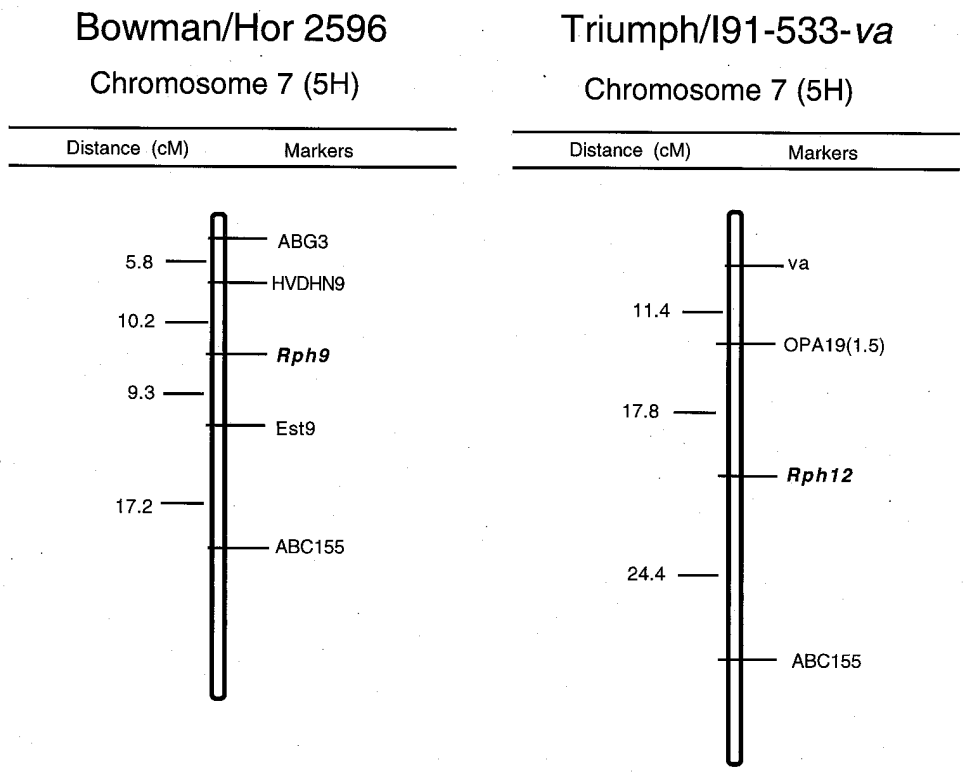

Fig. 3. Linkage maps showing the relationship between two leaf rust resistance genes (*Rph9* and *Rph12*) and associated markers in the Bowman/Hor 2596 and Triumph/I91-533-*va* barley populations. Distances (centimorgans) were calculated from recombination percentages by the Kosambi function (18). The map is oriented with the upper portion of the chromosome toward the centromere of chromosome 7(5H).

reactions to *P. hordei*. The genes in Hor 2596 were detected against *P. hordei* pathotypes that were either avirulent or virulent for Trumpf, indicating none of the dominant genes in Trumpf were identical to *Rph9* in Hor 2596 (33). Walther (33) suggested the presence of two dominant or partially dominant resistance genes in Trumpf detected against avirulent pathotypes and one recessive gene detected against virulent pathotypes. Two additional recessive genes acting complementary to each other were hypothesized in Hor 2596 (33). Jin et al. (14) detected one incompletely dominant gene in Triumph against isolate ND8702 of *P. hordei.* This result was confirmed in this and two other studies involving Triumph (3,13). The different results obtained by Walther (33) and our group may be explained by the use of two different barley lines, each with a different number of leaf rust resistance genes. Walther (33) used the East German cv. Trumpf in her studies, whereas we used Triumph, an export selection from Trumpf. Our data clearly show the presence of a single leaf rust resistance gene in both Hor 2596 and Triumph to isolate ND8702.

In two different populations (Bowman/Hor 2596 and Triumph/I91-533-*va*), *Rph9* and *Rph12* were linked to a common molecular marker, *ABC155*, at a distance of 20.6 and 24.4 cM, respectively. STS marker *ABC155* was positioned on chromosome 7(5H), using the wheat-barley addition lines (T. K. Blake, *personal communication*; and this study, Fig. 1). The linkage identified with *ABC155* places both *Rph9* and *Rph12* on the long arm of chromosome 7(5H). *Rph9* also showed linkage (20.1 cM) with STS marker *ABG3*. The primers for this STS marker were developed from the *ABG3* RFLP clone, which produces a marker on chromosome 4(4H) in the Steptoe/Morex population. However, this clone also produces RFLP marker *ABG3B* that maps to chromosome 7(5H) in other barley crosses (e.g., Harrington/Morex, [16]). Thus, *ABG3* identifies two distinct genetic loci on chromosomes 4(4H) and 7(5H) in barley. Linkage with markers on chromosome 7(5H) (i.e., *rpg4* and *MWG740*) in the Q21861/SM89010 population led us to conclude that the STS marker generated by the ABG3 primers is located on chromosome 7(5H) and not on chromosome 4(4H).

The putative location of the *Rph9* locus on chromosome 7(5H) was determined by analysis of linkage with *Est9*. A distinct band intensity polymorphism for *Est9* in resistant and susceptible F_3 family bulks strongly suggested that *Rph9* may be closely linked to the *Est9* locus. The location of *Rph9* in that region was confirmed by linkage with the dehydrin-9 (*HVDHN9*) microsatellite marker. *HVDHN9* recently was mapped to chromosome 7(5H) in the Harrington/TR306 population (22) and is positioned ≈12 cM from RFLP marker *CDO504* (M. Saghai Maroof, *personal communication*).

In a previous study, *Rph12* was shown to be linked with the chromosome 7(5H) morphological trait loci *raw1* and *srh*, with recombination values of 26.1 ± 2% and 39.3 ± 3%, respectively (14). In this study, *Rph12* was linked with the *va* locus at a distance of 22.6 cM. Loci *va* and *raw1* were positioned on the chromosome 7(5H) map of morphological markers at a distance of 25.3 and 29.9 cM from the *srh* locus, respectively (32). Recently, the morphological marker *va* was integrated into the molecular marker map of chromosome 7(5H) in the Steptoe/Morex population (19). This marker showed linkage to *ABC717* in bulked segregant analysis. These data, together with those generated in the current study, place *va* in a region of chromosome 7(5H) with a 33-cM gap with respect to mapped RFLP markers in the Steptoe/Morex population (17). Amplified fragment length polymorphisms (AFLPs) can generate more polymorphic markers than PCR or RFLP markers (26). However, in a recent study, AFLPs failed to generate additional markers in this gap in the Proctor/Nudinka barley population (2). *HVDHN7* and *HVDHN9* are two additional markers recently mapped to this chromosome 7(5H) region (22); unfortunately, they were not polymorphic in the Triumph/I91-533-*va* population.

Although 14 leaf rust resistance genes have been identified in barley (13), only a few of them have been mapped to specific regions on the chromosomes. *Rph4* from barley line Gold has been placed in the chromosome 5(1H) linkage group, using the *Reg1 (Ml-a)* powdery mildew resistance gene as a genetic marker (23). Leaf rust resistance genes *Rph10* and *Rph11* from *H. vulgare* ssp. *spontaneum* were mapped to chromosomes 3(3H) and 6(6H), respectively, using their linkage with isozymes markers (8). Recently, *Rph2* was mapped to the centromeric region of chromosome 7(5H), using molecular markers (3). The evidence reported here indicates Hor 2596 and Triumph carry a leaf rust resistance gene at the same locus on the long arm of chromosome 7(5H) of barley. The locus designation of *Rph12* was assigned to the gene in Triumph (14) before proper allelism tests were conducted. From a classical genetic point of view and considering the large number of F_2 progeny evaluated in the allelism test, it appears that the gene in Triumph is indeed an allele at the *Rph9* locus. Thus, the *Rph12* designation should be changed to the allele designation of *Rph9.z* according to the proposed nomenclature system of Frankowiak et al. (9) for leaf rust resistance genes in barley. The recommended allele designation for the *Rph* gene in Hor 2596 is *Rph9.i*.

Unlike the unimproved line Hor 2596, Trumpf and its export selection Triumph have been widely used in commercial barley production in several regions of the world. However, both of them have succumbed to damage by virulent pathotypes of *P. hordei* (7,15). The resistance found in Triumph may be useful in North America, however, because pathotypes of *P. hordei* with virulence for *Rph12* have not been detected (B. J. Steffenson and T. G. Fetch, *unpublished data*).

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