Genetics

Identification of Molecular Markers Linked to the Stem Rust Resistance Gene *rpg4* in Barley

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ABSTRACT

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The gene *rpg4* in barley confers resistance to pathotype QCC of *Puccinia graminis* f. sp. *tritici*. To facilitate the efficient transfer of *rpg4* into breeding lines, an investigation was initiated to identify molecular markers linked to the gene. A doubled haploid population (125 individuals) derived from Q21861, the source of *rpg4*, was subjected to bulked segregant analysis by using random amplified polymorphic DNAs (RAPDs) and restriction fragment length polymorphisms (RFLPs). From 500 10-mer primers evaluated, only three reliably amplified fragments in one of the DNA bulks and not in the other. Linkage of the three RAPD markers, OPP-05 (900), OPH-13 (1600), and OPH-15 (1000), with *rpg4* was confirmed by segregation analysis of the doubled haploid progeny. Primer OPH-15 generated the most closely linked (0.8 centimorgans [cM]) marker to *rpg4*. RFLP probes were used to map *rpg4* and associated RAPD markers to chromosome 7M. The RFLP markers, ABG390 and MWG740, mapped 5.7 and 4.9 cM distal and proximal to the resistance gene, respectively. The RAPD and RFLP markers identified for *rpg4* in this study should facilitate the efficient transfer of stem rust resistance in barley germ plasm.

Additional keywords: *Hordeum vulgare*, molecular marker-assisted selection.

For more than 50 yr, stem rust (caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn.) of barley (*Hordeum vulgare* L.) has been controlled through the wide deployment of the resistance gene *Rpg1* in cultivars of the northern Great Plains (23). However, during 1991 and 1992, minor epidemics of stem rust developed on barley, resulting from the appearance of a virulent *P. g. tritici* pathotype, Pgt-QCC. Pathotype QCC has increased in prevalence over the past 3 yr and is now one of the most common virulence types of *P. g. tritici* in the United States (20). Given the widespread distribution of pathotype QCC and the genetic uniformity of the host for susceptibility to this pathotype, epidemics may continue to threaten barley until adequate levels of stem rust resistance are transferred into new cultivars.

Several sources of resistance to pathotype QCC were recently identified from a diverse collection of barley germ plasm (7). One of the most resistant accessions identified from this study was Q21861, a breeding line originating from the program of the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Q21861 possesses a recessive gene (*rpg4*) that confers resistance to pathotype QCC at the seedling stage

(8,24) and presumably at the adult plant stage as well. An obvious strategy for combating the threat of stem rust on barley is to transfer both *Rpg1* and *rpg4* into new cultivars. The retention of *Rpg1* in cultivars is essential because this gene has proven durable against many pathotypes of the stem rust pathogen (23).

The transfer of both stem rust resistance genes into advanced barley lines will significantly complicate the breeding process. This is especially true for the development of cultivars used in malting, in which over 25 different quality traits must be considered in addition to yield and numerous agronomic traits (23). *Rpg1* and *rpg4* can be detected at the seedling stage, but only under fairly rigorous temperature-control conditions during incubation (8,24). The simultaneous detection of *Rpg1* and *rpg4* in barley plants is difficult because the resistance genes are best expressed under different incubation temperatures (8). The efficient transfer of resistance genes can be facilitated by the use of molecular markers, e.g., random amplified polymorphic DNAs (RAPDs) or restriction fragment length polymorphisms (RFLPs). If a close linkage is found between a molecular marker and a disease resistance gene, the marker can be exploited for the indirect selection of the gene without the need for disease testing (14). Several disease resistance genes so tagged may then be pyramided by means of molecular marker-assisted selection to increase the durability of resistance in the host.

Recently, a marker based on the polymerase chain reaction (PCR) was developed for the resistance gene *Rpg1* (9). This marker (ABG704) is 0.3 centimorgans (cM) distal to *Rpg1* on chromosome 1P and has proven useful for detecting the resistance gene in several different crosses (9). In this study, we report on the use of bulked segregant analysis for the identification of several RAPD and RFLP markers linked to the stem rust resistance gene *rpg4* in barley.

MATERIALS AND METHODS

Plant materials and stem rust evaluations. A doubled haploid (DH) population (125 lines in total) was produced by anther culture from F_1 plants derived from a cross between Q21861 and SM89010 (24). Q21861 is the source of *rpg4* (8) and was originally derived as a random head selection from a CIMMYT barley breeding nursery. SM89010 is a two-rowed malting line that is susceptible to pathotype QCC. This line was developed at the University of Saskatchewan from a cross between Nairn and Manley (B. L. Harvey, *personal communication*). Rust evaluations were made according to the methods of Steffenson et al (24). Briefly, parents and DH progeny were inoculated with a single uredinial culture (QCC-2) of pathotype QCC when the primary leaves of plants were fully expanded. Then, plants were placed in a mist chamber (20–21 C and 100% relative humidity) for 16 h in the dark, exposed to light (120–160 μmol photon $m^{-2}s^{-1}$) as the temperature increased from 21 to 24 C, and allowed to dry slowly for approximately 4 h before being placed in a greenhouse at 17–22 C. Two weeks after inoculation, the infection types of plants were assessed by using a 0–4 scale for stem rust (24). Progeny exhibiting infection types 0, 0;, 1, and 2 were considered to carry the *rpg4* allele for resistance, whereas those exhibiting infection types 3 and 4 were considered to carry the *Rpg4* allele for susceptibility to pathotype QCC (24). Infection phenotypes on the parents and DH progeny were distinct and allowed for the unequivocal identification of progeny carrying the *rpg4* allele for resistance. A 1:1 ratio of resistant to susceptible progeny was found in the DH population (24), thereby confirming the monogenic nature of resistance to pathotype QCC in Q21861 as originally reported by Jin et al (8). DH progeny suspected of having double crossovers between *rpg4* and flanking molecular markers were retested at least twice for their disease reactions.

RAPD analysis. Total genomic DNA was isolated by a combination of the proteinase K and CTAB (cetyltrimethylammonium bromide) methods (10). The concentration of DNA extracted from each genotype was determined by DNA fluorometry (model TKO100, Hoefer Scientific Instruments, San Francisco, CA), and samples were diluted to a concentration of 20 ng/μl for the PCR reactions. Aliquots of DNA (5 μl each) from 10–15 resistant or susceptible DH lines were combined to produce two sets of bulked DNA for bulked segregant analysis (15). During the course of this investigation, 500 10-mer primers (Operon Technologies Inc., Alameda, CA) were evaluated for their ability to produce polymorphic fragments in the DNA bulks.

PCR reactions were conducted according to the protocols of Williams et al (28) and were performed in a 10-μl total volume of 1× *Taq* polymerase buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin) with 0.1 mM of each dNTP, 0.2 μM primer, 20 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Promega Corporation, Madison, WI). Reaction mixtures were overlaid with one drop of mineral oil, and DNA was amplified in a programmable thermal controller (MJ Research, Inc. Watertown, MA). The temperature profile consisted of 45 cycles of 1 min at 94 C, 30 s at 36 C, and 2 min at 72 C. A final 7-min extension (72 C) was given at the end of the cycling period. Amplified DNA fragments were separated by electrophoresis in a 1.5% agarose gel in 1× TBE buffer (0.1 M Tris-HCl, 0.1 M boric acid, and 2 mM EDTA, pH 8.3) for 4 h at 100 V. After electrophoresis, the gels were stained with ethidium bromide and visualized on a UV transilluminator. Primers amplifying products that exhibited polymorphisms between the DNA bulks as well as between the parents were subsequently evaluated on all DH progeny.

RFLP analysis. Protocols for DNA extraction, prehybridization, hybridization, and washing of membranes were as described in Kleinhofs et al (10). Initially, Southern blots containing several restriction digests of DNA from Q21861 and bulks of resistant and susceptible DH lines were hybridized with several RFLP probes. Probes exhibiting possible linkage with *rpg4* were subsequently evaluated on all 125 DH progeny.

Genetic analysis. Segregating molecular markers were scored for each of the DH progeny, and linkage analyses were performed with MAPMAKER (12), version 3.0. A logarithm of the odds ratio (LOD) score of 3.0 or greater was established for linkage. The Haldane mapping function (12) was used to convert recombination frequencies to map distances in centimorgans.

RESULTS

Amplification products generated from 500 10-mer primers were evaluated with template DNA from resistant and susceptible bulks. The average number of products amplified per primer was approximately 10; the range was three to 16. Thirty primers generated at least one fragment that was present in one bulk but not

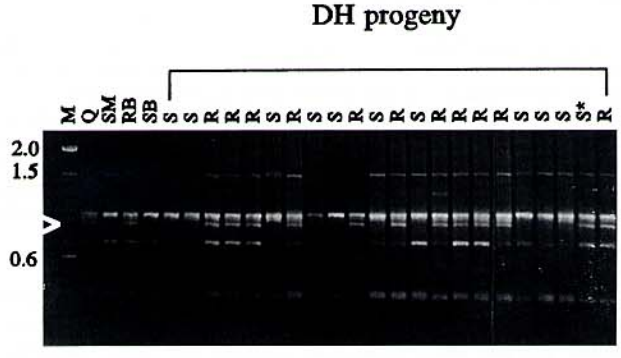

Fig. 1. Bulked segregant analysis with random amplified polymorphic DNA (RAPD) markers for *rpg4* in a doubled haploid population derived from Q21861 × SM89010. The RAPD products exhibit polymorphisms linked to *rpg4*. Lane 1 (M) is the 100-bp molecular weight ladder (GIBCO BRL). The sizes of the three brightest bands in the ladder are indicated in kilobase pairs. Products amplified by primer OPP-05 are from, lane 2, Q21861 (Q); lane 3, SM89010 (SM); lane 4, resistant bulk (RB); and lane 5, susceptible bulk (SB). Lanes 6–27 are individual doubled haploid progeny. R = resistant; S = susceptible; * = recombinant doubled haploid line; and the arrow indicates the 0.9-kb fragment that is linked to *rpg4*.

in the other. However, the vast majority of these primers were not investigated further because they either were not sufficiently reproducible or proved not to be linked with *rpg4*. Three primers, OPP-05 (5′ CCCCGGTAAC 3′), OPH-13 (5′ GACGCCACAC 3′), and OPH-15 (5′ AATGGCGCAG 3′), reliably produced fragments that exhibited polymorphism between bulked DNA samples and between the parents. Primer OPP-05 was the only one of the three to generate a product derived from the resistant parent, Q21861. Putative linkages between the RAPD markers and *rpg4* were corroborated by analyzing the segregation of the PCR products in relation to the infection phenotype class (resistant or susceptible) of individual DH lines. Polymorphisms generated by amplification with the three primers in the bulks, parents, and a portion of DH progeny are shown in Figures 1 and 2.

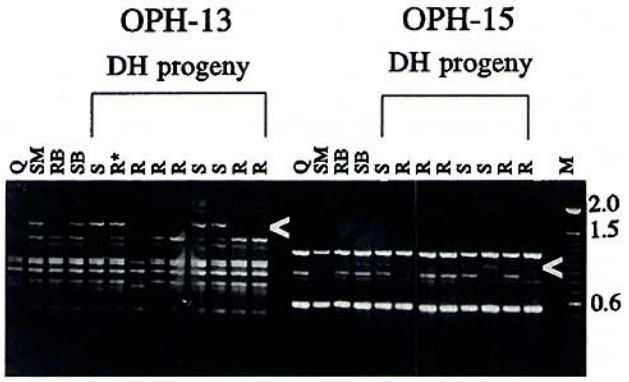

Fig. 2. Bulked segregant analysis with random amplified polymorphic DNA (RAPD) markers for *rpg4* in a doubled haploid population derived from Q21861 × SM89010. The RAPD products exhibit polymorphisms linked to *rpg4*. Products amplified by primer OPH-13 are from, lane 1, Q21861 (Q); lane 2, SM89010 (SM); lane 3, resistant bulk (RB); lane 4, susceptible bulk (SB); and lanes 5–13, individual doubled haploid progeny. R = resistant, and S = susceptible. Products amplified by primer OPH-15 are from, lane 14, Q21861 (Q); lane 15, SM89010 (SM); lane 16, resistant bulk (RB); lane 17, susceptible bulk (SB); and lanes 18–25, individual doubled haploid progeny. Lane 26 (M) is the 100-bp molecular weight ladder (GIBCO BRL). * = Recombinant doubled haploid line, and arrows indicate the 1.6-kb (left) and 1.0-kb (right) fragments that are linked to *rpg4*.

To determine the chromosome location of *rpg4* and its orientation with linked RAPD markers, RFLP analyses were made. Southern blots were hybridized with several probes specific to barley chromosome 7M. The selection of probes from this chromosome arm was based on prior data suggesting a putative association between *rpg4* and *R*, a gene on 7M that controls the degree of barbing on the awns (22). Initially, the probe ABG314 was used because it yields a marker that is just 5 cM proximal to the putative telomere marker in the cross between Steptoe and Morex of the North American Barley Genome Mapping Project (NABGMP) (A. Kilian and A. Kleinhofs, *unpublished*). The 5.5-kb band of the resistant parent gave a signal at least two times stronger in the resistant bulk than in the susceptible bulk (Fig. 3, group 5). From this result, it was reasoned that the location of *rpg4* was about 20–30 cM proximal from ABG314. To test this hypothesis, probes located proximal to ABG314 (in approximately 10- to 15-cM intervals) on the basis of marker locations from two NABGMP populations (A. Kilian and A. Kleinhofs, *unpublished*) were chosen for the next hybridizations. In total, seven RFLP probes were tested in the bulked segregant analysis. Probe CDO484, which is about 10 cM proximal to ABG314, showed very little of the resistant parent band (7.0 kb) in the susceptible bulk (Fig. 3, group 4), thereby confirming the assumption regarding the proximal location of *rpg4* with respect to ABG314. The presence of the resistant parent bands only in the resistant bulk (as well as the absence of these bands in the susceptible bulk) for ABG390 (4.5-kb band) and MWG740 (7.0-kb band) suggested their close linkage with *rpg4* (Fig. 3, groups 3 and 2, respectively). The pattern obtained for CDO504 (9.5-kb band), which is more than 50 cM away from ABG314 (A. Kilian and A. Kleinhofs, *unpublished*), indicated its proximal location with respect to *rpg4* (Fig. 3, group 1).

Markers ABG390 and MWG740 were mapped with respect to *rpg4* and the three linked RAPD markers in the Q21861 × SM89010 DH population. Careful checking of all segregation data revealed very few putative double crossovers. All data were rigorously rechecked both for molecular markers and disease reaction. A linkage map for these loci was constructed by using MAPMAKER (12) and is shown in Figure 4. The map spans a region of approximately 23.1 cM of chromosome 7M and is anchored by the flanking RFLP markers ABG390 and MWG740, which are 5.7 and 4.9 cM distal and proximal to *rpg4*, respectively.

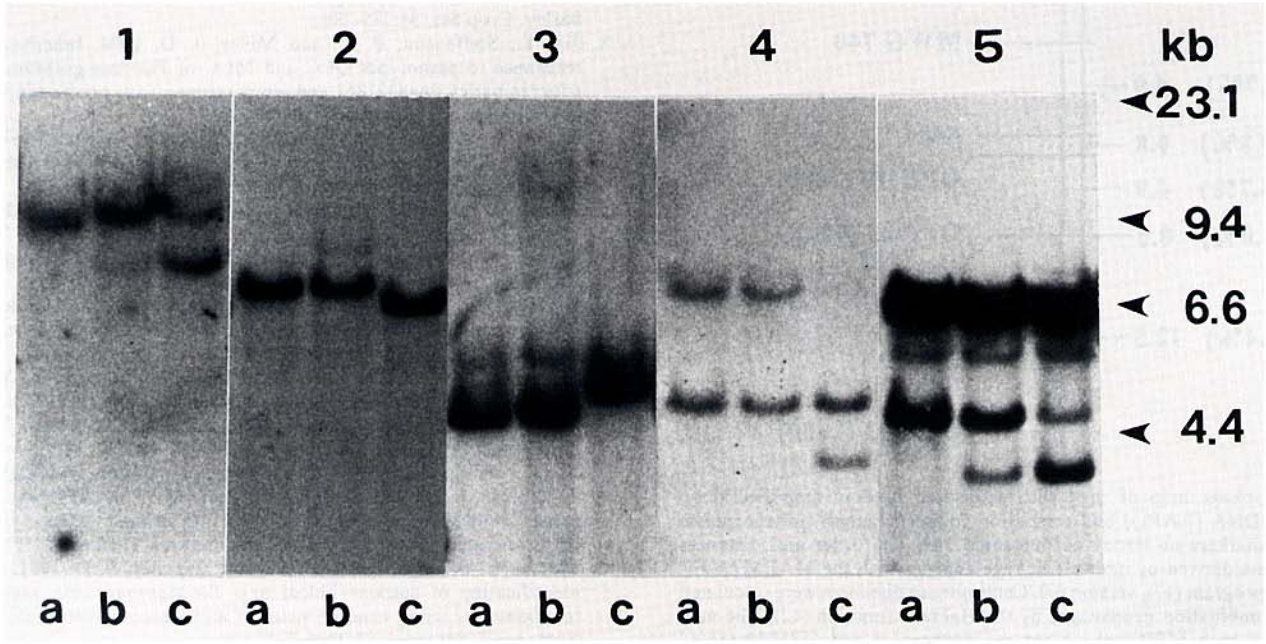

Fig. 3. Bulked segregant analysis with restriction fragment length polymorphism (RFLP) markers for *rpg4* in a doubled haploid population derived from Q21861 × SM89010. Lane a, DNA from resistant parent, Q21861; lane b, DNA from resistant bulk; and lane c, DNA from susceptible bulk. Probes and restriction enzyme combinations were as follows: group 1, CDO504 and *Eco*RV; group 2, MWG740 and *Dra*I; group 3, ABG390 and *Eco*RI; group 4, CDO484 and *Dra*I; and group 5, ABG314 and *Dra*I.

The closest marker to *rpg4* was generated by primer OPH-15; this RAPD marker, OPH-15 (1000), mapped 0.8 cM distal to the *rpg4* locus. RFLP marker ABG390 cosegregated with the RAPD marker OPP-05 (900) in this population.

DISCUSSION

Q21861 is one of only a few barley accessions that possess resistance to pathotype QCC of *P. g. tritici* (7). The gene (*rpg4*) conferring resistance to this pathotype was recently described (8) but had not been assigned to a specific chromosome of the barley genome. Using bulked segregant analysis, we identified several RAPD and RFLP markers linked to *rpg4* and were able to identify its location on barley chromosome 7M. These results further validate the utility of bulked segregant analysis (15) in tagging disease resistance genes as reported by other workers (1,5,16,18,19).

The number of reports documenting the linkage of molecular markers with genes conferring resistance to phytopathogenic fungi (2,5,6,16,21,32,33), bacteria (13,30), viruses (3,4,25,31), and nematodes (11,19,26,27) has increased markedly over the past several years. With proper validation, many of these markers will prove valuable for the selection of resistant progeny in segregating populations from breeding programs. For *rpg4* in barley, any one of the several markers identified could serve in such a capacity. The closely linked RAPD markers generated by OPH-15 and OPP-05 could be used for the indirect selection of *rpg4* in barley; however, more reliable and specific assays for the gene can be made by converting the markers into sequence tagged sites (STSs) or sequence characterized amplified regions (SCARs) (17,21,29). The RFLP markers found linked to *rpg4* in this study both detect a single band in most of the restriction enzyme digestions tested, and both hybridize well with barley DNA. These attributes make ABG390 and MWG740 ideal markers for assisted selection at the *rpg4* region but only in laboratories where Southern hybridizations are feasible. In an effort to develop a simple and rapid technique for detecting *rpg4* in breeding programs, we are converting the RAPD and RFLP markers into STSs and SCARs.

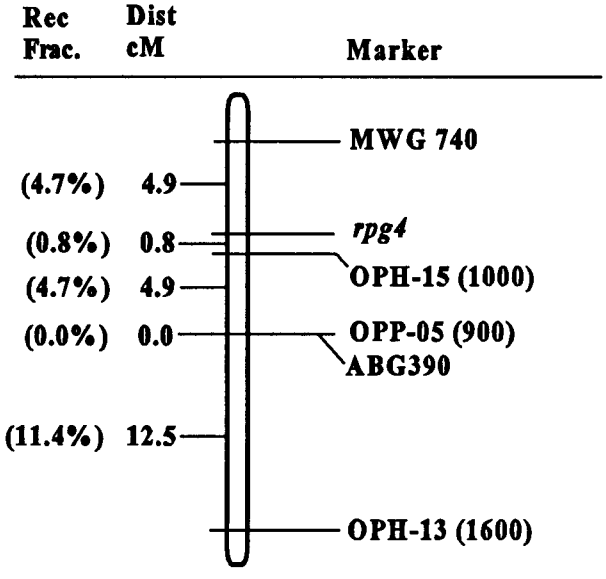

Fig. 4. Linkage map of *rpg4* with associated random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers on barley chromosome 7M. The order and distances of loci were derived by multiple linkage analysis with the MAPMAKER software program (12), version 3.0. Centimorgan distances were calculated from recombination percentages by the Haldane function (12). The map is oriented with MWG740 toward the centromere and OPH-13 (1600) toward the telomere of chromosome 7M. RAPD marker designations were made according to Michelmore et al (15). The size of the polymorphic DNA fragment amplified by the primer is rounded off to the nearest 100 bp and is given in parentheses.

To reduce the threat of the stem rust pathotype QCC on barley, *rpg4* should be incorporated with *Rpg1* in cultivars for the northern Great Plains. The simultaneous detection of these genes by infection type assessments is laborious because it requires the inoculation of plants with two different pathotypes under two different incubation temperatures (8,24). The pyramiding of these resistance genes can be greatly facilitated through molecular marker-assisted selection. Q21861 carries both *rpg4* and *Rpg1* for stem rust resistance (8,24). In a preliminary blind test with an F_2 population (150 progeny) derived from Q21861 × SM89010, the PCR-based markers OPH-05 (900) and ABG704 (9) reliably detected individuals carrying *rpg4* and *Rpg1*, respectively (I. G. Borovkova and B. J. Steffenson, *unpublished*). In addition to the wheat stem rust pathogen, Q21861 also possesses resistance to the leaf rust (*P. hordei* G. Otth) and powdery mildew (*Blumeria graminis* (DC.) E. O. Speer f. sp. *hordei* Ém. Marchal) pathogens. Research is underway to identify molecular markers for these other resistance genes by bulked segregant analysis. Since Q21861 is being used as a parent in a number of breeding programs, the tagging of additional resistance genes from it will provide an efficient means of transferring multiple disease resistance into barley.

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