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# Allele Sequencing of the Barley Stem Rust Resistance Gene *Rpg1* Identifies Regions Relevant to Disease Resistance

A. Mirlohi, R. Brueggeman, T. Drader, J. Nirmala, B. J. Steffenson, and A. Kleinhofs

First author: College of Agriculture, Isfahan University of Technology, Esfahan, Iran; second, third, and fourth authors: Department of Crop and Soil Sciences, Washington State University, Pullman; fifth author: Department of Plant Pathology, University of Minnesota, St. Paul; and sixth author: School of Molecular Biosciences and Department of Crop and Soil Sciences, Washington State University, Pullman. The first two authors contributed equally to this work.

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## ABSTRACT

Mirlohi, A., Brueggeman, R., Drader, T., Nirmala, J., Steffenson, B. J., and Kleinhofs, A. 2008. Allele sequencing of the barley stem rust resistance gene *Rpg1* identifies regions relevant to disease resistance. *Phytopathology* 98:910-918.

The stem rust resistance gene *Rpg1* has protected North American barley cultivars from significant yield losses for over 65 years. The remarkable durability of this gene warrants further study as to its possible origin and allelic variation. Eight Swiss barley (*Hordeum vulgare*) landraces and eight wild barley (*H. vulgare* subsp. *spontaneum*) accessions from diverse geographic regions were analyzed to uncover new alleles of *Rpg1* and learn about its possible origin. The two germplasm groups included accessions that were resistant and susceptible to *Puccinia graminis* f. sp. *tritici* pathotype MCCF. Allele-specific primers were utilized to amplify 1 kbp overlapping fragments spanning the *Rpg1* gene and sequenced if a polymerase chain reaction (PCR) fragment was generated. Variation among the PCR products revealed significant polymorphisms among these *Hordeum* accessions. Landraces and wild barley accessions sus-

ceptible to pathotype MCCF exhibited the highest degree of *Rpg1* polymorphism. One resistant landrace (Hv672) and one resistant wild barley accession (WBDC040) yielded all seven *Rpg1*-specific PCR fragments, but only landrace Hv672 coded for an apparently functional *Rpg1* as determined by comparison to previously characterized resistant and susceptible alleles and also resistance to HKHJ, a stem rust pathotype that can specifically detect *Rpg1* in the presence of other resistance genes. Accessions resistant to stem rust pathotype MCCF, but completely lacking *Rpg1*-specific PCR amplification and hybridization with an *Rpg1*-specific probe, suggested the presence of stem rust resistant gene(s) different from *Rpg1* in the *Hordeum* germplasm pool. Some *Rpg1* alleles that retained the ability to autophosphorylate did not confer resistance to *Puccinia graminis* f. sp. *tritici* pathotype MCCF, confirming our previous observations that autophosphorylation is essential, but not sufficient for disease resistance. Thus, the Rpg1 protein plays a complex role in the stem rust disease resistance-signaling pathway.

*Additional keywords:* functional analysis, R genes.

Stem rust (caused by the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici*) has been one of the most important pathogens of barley (*Hordeum vulgare* L.) in the Upper Midwest region of the United States and Prairie Provinces of Canada over the past century. A single dominant gene (*Rpg1*) introgressed into barley cultivars has effectively controlled the stem rust epidemics which devastated the crop in the Northern Great Plains prior to 1942 (18). Many qualitative rust resistance genes in other cereal crops have been overcome by virulent pathotypes in a relatively short period of time (12); however, *Rpg1* has been durable, protecting barley from serious stem rust losses for more than 65 years. This remarkable durability warrants further characterization of *Rpg1*.

*Rpg1* was mapped to the short arm of barley chromosome 1(7H) and cloned from cultivar Morex by construction of high-resolution genetic and physical maps (2). Highly resistant transgenic plants were obtained by transforming the susceptible barley cultivar Golden Promise, devoid of *Rpg1*, with the Morex *Rpg1* gene, demonstrating that the gene isolated was functional in conferring stem rust resistance (9). *Rpg1* contains 14 exons in 4,466 bp of genomic sequence encoding an 837 amino acid protein with two tandem kinase domains, which is a unique resistance gene structure (2).

Sequence analysis of *Rpg1* from resistant North American barley cultivars revealed no amino acid sequence polymorphism (2). This suggests a singularity of the *Rpg1* source that can be

traced to an unimproved bulked seed lot (from which the sister selections of Chevron [PI 38061] and Peatland [PI 539109] were derived) obtained by the United States Department of Agriculture from Switzerland in 1914. Another potential source of stem rust resistance in barley was the cultivar Kindred (CIho 6969), a farmer selection of a single stem rust resistant plant from a field of susceptible Wisconsin 37 barley (18). The plant from which Kindred was derived was probably an admixture because it differed from Wisconsin 37 for many more traits than just stem rust resistance. Moreover, sequence analysis revealed that the *Rpg1* in silico translated amino acid sequence in Kindred was identical to that of Chevron and Peatland (2). The source of *Rpg1* in Kindred was likely Chevron because this cultivar had been used in the Wisconsin barley breeding program for a number of years prior to the release of Wisconsin 37.

During the map-based cloning effort of *Rpg1*, allele sequencing revealed three groups of stem rust susceptible barley lines (2). The first group contained susceptible *rpg1* genes with 3 base pairs (GTT) inserted in the sixth exon at nucleotide position 1,546 relative to the *Rpg1* sequence of Morex. The second group contained an *Rpg1*-like gene with a highly diverged 5' region (2), and the third group had no detectable *Rpg1* gene sequences. The second group, including barley line Sm89010, was recently characterized as containing a recombinant *Rpg1* gene with ABC1037, an *Rpg1* gene family member located  $\approx$ 50 kbp proximal to *Rpg1* (1). Lines with this recombinant *Rpg1* gene have a 5' end highly homologous to ABC1037 and a 3' end with high homology to *Rpg1* (1). Reverse transcription (RT)-PCR and sequence analysis indicated that the Sm89010 recombinant *Rpg1* gene is tran-

Corresponding author: B. Steffenson; E-mail address: bsteffen@umn.edu

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scribed, but does not function in conferring stem rust resistance (to pathotype MCCF) because it and two other barley cultivars (Dicktoo and Gobernadora) containing the Sm89010-like *Rpg1* gene were susceptible (2). It is possible that the Sm89010 *Rpg1* gene expresses novel specificities to other stem rust pathotypes, as unequal exchange events have been suggested as a means for the evolution of plant disease resistance genes (7,15). In flax, 13 alleles at the *L* rust resistance locus have been reported to express 10 different rust resistance specificities. These resistance specificities are presumed to have evolved through various mechanisms, including single base substitutions, insertion/deletions (indels), unequal recombination, and reassortment of variable regions within the alleles by multiple intra-genic sequence exchange events (7).

Naturally occurring allelic diversity in plants has been suggested to be an important genetic component for phenotypic variation and crop improvement. It is therefore important to characterize this diversity in order to identify economically important alleles and polymorphisms (3). In addition to landraces of domesticated barley (*H. vulgare*), the wild progenitor *H. vulgare* subsp. *spontaneum* is known to be a valuable source for alternative forms of disease resistance genes for barley improvement (8,22). Stem rust resistance has been reported in wild barley, but the frequency of resistance is often low (<10%) (8,20). Given the recent threat of new pathotypes such as *P. graminis* f. sp. *tritici* TTKS (i.e., isolate Ug99) in the world, wild barley may become an important new source of stem rust resistance genes (20).

The lack of *Rpg1* polymorphism among previously-tested stem rust resistant barley cultivars suggests either a highly conserved or single source for this gene. High conservation of a protein kinase disease resistance gene has been shown with the tomato *Pto* gene, which confers resistance to *Pseudomonas syringae* pv. *tomato*. The wild tomato species *Lycopersicon pimpinellifolium* is the source of the *Pto* gene in cultivated tomato (*L. esculentum*). A *Pto* ortholog *LhirPto*, isolated from another wild tomato relative (*L. hirsutum*), shares 97% amino acid identity with the *Pto* from *L. pimpinellifolium* (16). Even more surprising, the domesticated tomato has a highly conserved, transcriptionally active *pto* allele that fails to confer disease resistance (10). Since the *Lycopersicon* species separated presumably millions of years ago, these findings suggest that the PTO kinase has been highly conserved, even when it does not retain disease resistance function.

The objectives of this study were to use molecular techniques to uncover new alleles of *Rpg1* and learn about its possible origin

in eight Swiss barley (*H. vulgare*) landraces and eight wild barley (*H. vulgare* subsp. *spontaneum*) accessions from diverse geographic regions. The presence/absence of *Rpg1*, its primary structure, and expression were analyzed by PCR of genomic DNA and mRNA (RT-PCR), Southern blotting, sequencing, western blotting, and autophosphorylation analyses.

## MATERIALS AND METHODS

**Plant materials and disease phenotyping.** From a germplasm collection of 74 barley landraces from Switzerland and 318 ecogeographically diverse wild barley accessions (20), 16 (eight from each group) were selected and used in this study (Table 1). Landraces from Switzerland were chosen for analysis because they are the donors of *Rpg1* (i.e., Chevron and Peatland) in North American barley cultivars and may possess additional allelic diversity for the gene. Wild barley accessions also were included because of their great allelic diversity and potential for identifying the origin of *Rpg1* in cultivated barley. Within each germplasm group, four resistant and four susceptible accessions to stem rust pathotype MCCF were chosen from ecogeographically diverse locations to identify functional *Rpg1* variants as well as spontaneous mutations that result in loss of function. Pathotype MCCF has been used for a number of years to select breeding lines with *Rpg1*, develop high resolution maps for map-based cloning of the resistance gene, and conduct functional analyses with the cloned gene (2,13,19). Recently, a comprehensive evaluation was made on the reaction of known stem rust resistance genes in barley to diverse pathotypes of *P. graminis* f. sp. *tritici* and isolates of *P. graminis* f. sp. *secalis* (the rye stem rust fungus) (21). This study revealed that *P. graminis* f. sp. *tritici* pathotype HKHJ was more effective than MCCF for detecting *Rpg1* in the presence of several other known stem rust resistance genes (21). Thus, the germplasm initially selected for this study based on reaction to pathotype MCCF was subsequently evaluated to pathotype HKHJ to further resolve the functionality of *Rpg1*. Cultivars Morex (with *Rpg1*) and Steptoe (without any known resistance genes) also were included as the resistant and susceptible controls, respectively. Plants were grown and assayed for their reaction to stem rust as previously described (19).

DNA and RNA were isolated from a single plant of each of the 16 selected accessions based initially on their stem rust phenotype. The plants were grown in plastic pots containing potting mix in a growth chamber under cool fluorescent lights with a 16 h

TABLE 1. Reaction of Swiss landrace and wild barley accessions to *Puccinia graminis* f. sp. *tritici* pathotypes MCCF and HKHJ at the seedling stage

Line	Origin	Pathotype MCCF		Pathotype HKHJ	
		IT range <sup>a</sup>	General reaction	IT range <sup>a</sup>	General reaction
Swiss Hv584	Fuormis, Switzerland	0;1 to 10;	Resistant	3 <sup>-</sup> to 3	Susceptible
Swiss Hv587	Platta, Switzerland	0;1 to 21	Resistant to moderately resistant	3 <sup>-</sup> to 3	Susceptible
Swiss Hv619	Fetan, Switzerland	10;	Resistant	3 <sup>-</sup> to 33 <sup>+</sup>	Susceptible
Swiss Hv672	Surava, Switzerland	10; to 12	Resistant	10; to 21	Resistant to moderately resistant
Swiss Hv489	Trans, Switzerland	3	Susceptible	3 to 33 <sup>+</sup>	Susceptible
Swiss Hv492	Scheid, Switzerland	3 to 33 <sup>+</sup>	Susceptible	3 <sup>-</sup> to 3	Susceptible
Swiss Hv611	Plan, Switzerland	3 <sup>-</sup> to 3	Susceptible	3 <sup>-</sup> to 3	Susceptible
Swiss Hv645	Scuol, Switzerland	3 <sup>-</sup> to 3	Susceptible	3 <sup>-</sup> to 3	Susceptible
WBDC040	Haifa, Israel	0;1 to 21	Resistant to moderately resistant	3 <sup>-</sup> to 3	Susceptible
WBDC214	Samarkand, Uzbekistan	0;1 to 213 <sup>-</sup>	Resistant to moderately resistant	3	Susceptible
WBDC220	Chimkent, Kazakhstan	10;2 to 213 <sup>-</sup>	Resistant to moderately resistant	3 to 33 <sup>+</sup>	Susceptible
WBDC225	Dushanbe, Tajikistan	0;1 to 21	Resistant to moderately resistant	3 to 3 <sup>+</sup>	Susceptible
WBDC019	West Azerbaijan, Iran	3 to 33 <sup>+</sup>	Susceptible	33 <sup>+</sup>	Susceptible
WBDC160	Sweida, Syria	3 to 3 <sup>+</sup> 3	Susceptible	3 to 3 <sup>+</sup>	Susceptible
WBDC269	Lebanon	3-2 to 33 <sup>+</sup>	Moderately susceptible to susceptible	3 <sup>-</sup> to 3	Susceptible
WBDC323	Ahalskii, Turkmenistan	3 to 33 <sup>+</sup>	Susceptible	3 to 3 <sup>+</sup>	Susceptible
Morex <sup>b</sup>	Minnesota, USA	0;1 to 213 <sup>-</sup>	Resistant to moderately resistant	12 to 21	Resistant to moderately resistant
Steptoe <sup>b</sup>	Washington, USA	3 to 33 <sup>+</sup>	Susceptible	3 to 33 <sup>+</sup>	Susceptible

<sup>a</sup> IT = infection types are based on a 0 to 4 scale, + and - denote more or less sporulation of uredinia, respectively. ITs of 0; 1, 2, and combinations thereof are indicative of resistance, whereas ITs of 3, 4 and combinations thereof are indicative of susceptibility (21).

<sup>b</sup> Morex (with *Rpg1*) and Steptoe (without any known stem rust resistance gene) are the resistant and susceptible controls, respectively.

photoperiod and day/night temperature of  $21 \pm 1^\circ\text{C}/18 \pm 1^\circ\text{C}$ . For protein analysis by western blot and autophosphorylation, a single plant from the same seed source was grown under the above conditions.

**DNA and RNA isolation.** Genomic DNA for Southern hybridization was isolated from young barley leaves with a modified CTAB method (11). For PCR amplification, genomic DNA was isolated from young barley leaves according to Edwards et al. (6). Total RNA was isolated from young seedling leaves using Trizol reagent (4). Integrity and quantity of the RNA samples were checked by  $1\times$  TBE (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) agarose gel electrophoresis.

**PCR and RT-PCR.** PCR primers were designed from cultivar Morex and line Sm89010 *Rpg1* allele sequences (GenBank accession numbers AF509748 and AF509762, respectively) to amplify approximately 1-kbp overlapping fragments covering the entire *Rpg1* gene (Fig. 1 and Table 2). Primer set 228F6/228R6 amplified a fragment from both cultivars Morex and Sm89010; however, the Sm89010 fragment is of higher molecular weight (Table 2). Primer combination 37F2/228R6 amplified only the Sm89010 *Rpg1* allele and resulted in a 1,678-bp band. DGK-F1 and DGSex2 primers are internal to 37F2/228R6 and were used only in the sequencing reaction in order to cover the gap between the forward 37F2 and reverse 228R6 primers. PCR reactions and analyses were as previously described (2).

Approximately 1 $\mu$ g of total RNA was used for oligo dT-primed first-strand cDNA synthesis using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's recommended conditions followed by PCR reactions (2). Primers EX3cw2/228R6 and 37-F2/228R7 were used for RT-PCR to amplify Morex or Sm89010 *Rpg1*-specific cDNA, respectively.

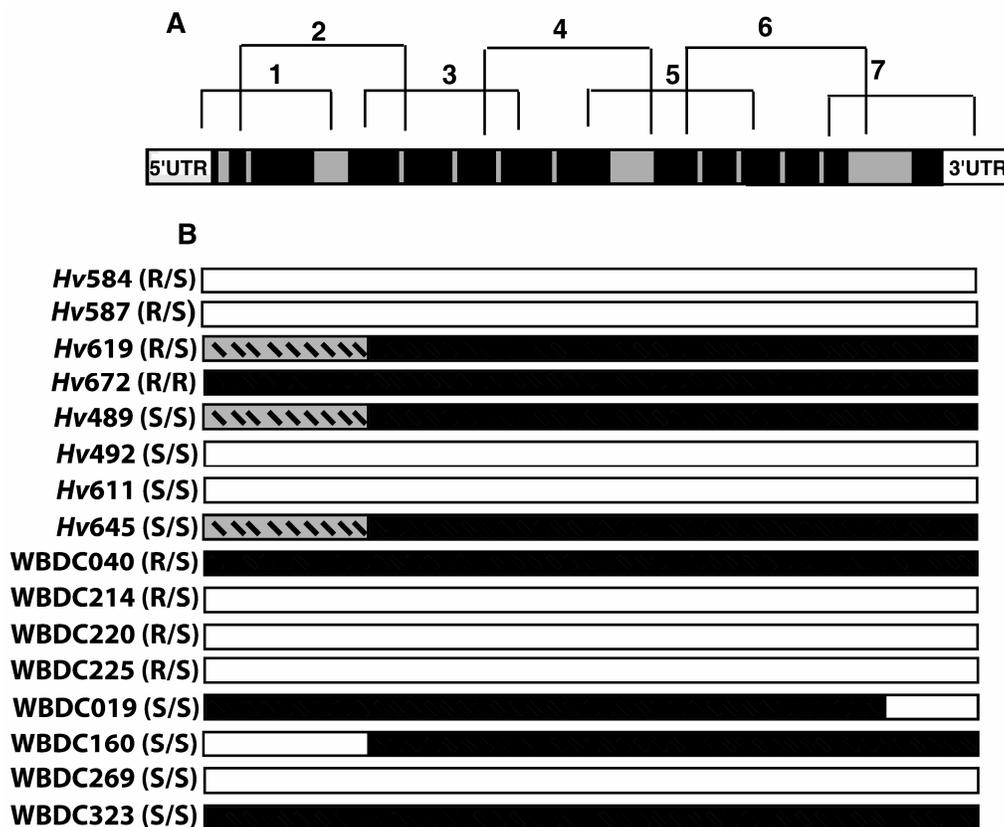
**Southern blot analysis.** Southern blotting and hybridization were as previously described (11). Five micrograms of *Hind*III-digested barley genomic DNA was separated by agarose gel electrophoresis, blotted onto nylon membranes, and hybridized with the *Rpg1*-specific DNA probe RSB228 (2).

**Sequencing.** PCR fragments were eluted from the agarose gel, purified, concentrated by ethanol precipitation, and used as templates for sequencing reactions. Sequencing reactions were performed using the Big-Dye Terminator kit (Applied Biosystems, Foster City, CA) and sequenced at the Bioanalytical Center, Washington State University, Pullman, WA. Sequences were analyzed and contigs assembled using ContigExpress from the Vector NTI software package.

**cDNA cloning and sequencing.** Oligo dT-derived cDNA from Morex and WBDC040 was used as the template to amplify the ninth intron/exon junction of *Rpg1*. RT-PCR products were cloned in the pGEM-T vector (Promega) following the manufacturer's protocol. Clones were confirmed by restriction digestion with *Eag*I (New England Biolabs, Beverly, MA) and *Nco*I (Fermentas, Hanover, MD) and sequenced using T7 and SP6 universal primers.

**Protein analysis.** Polyclonal antibody was raised in New Zealand white rabbits at the Washington State University animal facility against a recombinant His-RPG1 protein purified from *Pichia pastoris* (13). Ribi adjuvant was used according to the manufacturer's recommendation (Corixa, Hamilton, MO). Western blotting and autophosphorylation assays were performed as previously described (13).

**Rapid amplification of cDNA 3' ends (3' RACE).** 3' RACE was carried out using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX) and the 3' RACE System (Invitrogen, Frederick,



**Fig. 1.** Schematic representation of the *Rpg1* gene structure. **A**, The relative position of the seven cultivar Morex *Rpg1*-specific primer pairs and overlapping fragments generated in relation to the *Rpg1* gene structure. Introns are shown in gray and exons in black shading. The 5' and 3' untranslated transcribed regions (UTRs) are indicated. **B**, Black bars indicate fragments amplified using the Morex-specific set of primer pairs, hatched bars indicate fragments amplified using the barley line Sm89010 allele-specific primers, and white bars indicates no amplification. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. *tritici* pathotypes MCF/HKHJ, respectively.

MD) under the manufacturer's recommended standard conditions. The nested gene-specific primers used for both systems were 228-F3 and 228-F4 as previously described in Brueggeman et al. (2).

## RESULTS

**Stem rust reaction.** The controls reacted as expected with Morex exhibiting resistant to moderately resistant infection types and Steptoe susceptible infection types to pathotypes MCCF and HKHJ (Table 1). Hv584, Hv587, Hv619, and Hv672 were among the most resistant accessions found to *P. graminis* f. sp. *tritici* pathotype MCCF in the Swiss landrace germplasm, giving infection types ranging from 0;1 to 21 (Table 1). In contrast, Hv489, Hv492, Hv611, and Hv645 were among the most susceptible accessions found, giving infection types of 3– to 33+. Resistance to wheat stem rust in wild barley is fairly rare (8,20). The resistant wild barley accessions used in this study (WBDC040, WBDC214, WBDC220, and WBDC225) were selected from a previous preliminary study examining a large wild barley germplasm collection (20) and were among the most resistant found to pathotype MCCF, giving infection types ranging from 0;1 to 213–. Accessions WBDC019, WBDC160, WBDC269, and WBDC323 were representative of the most susceptible accessions found and ex-

hibited infection types ranging from 3–2 to 3+3. All Swiss landraces and wild barley accessions also were evaluated to HKHJ, a pathotype that can effectively detect *Rpg1* in the presence of other stem rust resistance genes (21). The Swiss landrace Hv672 was the only accession found with resistance (IT range of 10; to 21) to pathotype HKHJ. This suggests that it may be the only accession with a functional *Rpg1* gene (Table 1).

**Lines with an apparently intact *Rpg1* gene.** Seven primer pairs, designed to amplify the entire *Rpg1* coding region, were used to amplify *Rpg1* genes from all accessions. All expected DNA fragments were successfully amplified from Swiss Hv672, WBDC040, and WBDC323 (Fig. 1B), suggesting the presence of an intact *Rpg1* gene in these genotypes. Southern blots of genomic DNA digested with *Hind*III and hybridized with the *Rpg1*-specific probe RSB228 confirmed these results (Fig. 2). The hybridizing band was the same size (approximately 4.5 kbp) as observed in cultivar Morex and the wild barley accession OSU6 (2) containing an intact *Rpg1* gene. These data were not in agreement with the stem rust phenotypes as only Hv672 was resistant to both pathotypes MCCF and HKHJ. OSU6 was previously characterized as resistant to stem rust pathotype MCCF, but multiple subsequent assays with both pathotypes MCCF and HKHJ on the selected plants used in the molecular analysis showed that

TABLE 2. Primers used to amplify Morex-like and Sm89010-like gene fragments for genotyping and sequencing

Fragment name <sup>a</sup>	Primer name	Primer sequence	Location and product size <sup>b</sup>	
			Sm89010	Morex
1 Sm	37F2/228R6	cttccacttctctctcagatc/cggaaggcgtcaaacagctctc	–52–1,626/1,678 bp	None
1	228F11/228R11	tgccagctactctgccacttgag/gggatgggtgctttgtgacaac	None	–135–736/870 bp
2	228F6/228R6	cggaaaggcgtcaaacagctctc/cggaaggcgtcaaacagctctc	209–1,624/1,414 bp	130–1,090/959 bp
3	228F1/228R1	gccgggctggagatgaggaattc/gaacctgaatgcaaacctctgtc	1,420–2,506/1,085 bp	884–1,967/1,082 bp
4	228F2/228R2	tgttagacattccaccgaagag/catctgtcccaatcaagttgcagga	2,151–3,247/1,095 bp	1,612–2,708/1,095 bp
5	228F3/228R3	catgcaacgaggaagccttgacgac/acccgatctactggtgatctc	2,893–3,980/1,086 bp	2,353–3,440/1,086 bp
6	228F4/228R4	ggactactccaactatgcacac/gcagctgatgatgatggcagctgac	3,629–4,561/931 bp	3,090–4,020/929 bp
7	228F12/228R12	agatttccaggctacttctccag/gaccctctatttctctctcctc	4,349–5,100/750 bp	3,810–4,561/750 bp
RT-PCR1	EX3cw2/228R6	gccggtgtactactccttc/cggaaggcgtcaaacagctctc	None	465–1,088/396 bp
RT-PCR2	37-F2/228R7	cttccacttctctctcagatc/tactgatgtaaagaccaccgtata	52–2,065/879 bp	None
Seq 1 Sm	DGK-F1	cctcggatgctactctcagcatgac	875	NA <sup>c</sup>
Seq 1 Sm	DGSex2	tgggtaatgagaaaggtgtagatg	953	NA <sup>c</sup>
Ubiquitin	Hvu_Ubi1_cw1/Hvu_Ubi1_ccw1	gccgcacctcgtccgactac/cggcgttggggcactccttc	219 bp	219 bp

<sup>a</sup> Fragments 1 to 7 primers were used to amplify the Morex *Rpg1* coding regions as shown in Figure 1. Fragment 1 Sm primers amplified fragment 1 only from the Sm89010-like *Rpg1* allele. Fragment 2 primers amplified fragment 2 from both Morex and Sm type *Rpg1* alleles, but a higher molecular weight fragment from the Sm89010-like allele. Reverse transcription-polymerase chain reaction (RT-PCR) 1 and 2 primers were used to amplify a specific *Rpg1* mRNA fragment from either Morex or Sm89010, respectively. Seq1Sm primers DGK-F1 and DGSex2 are internal to the location of primers 37F2 and 228R6, and were used to cover the sequencing gap in fragment 1Sm, which was approximately 1.7 kbp.

<sup>b</sup> Bases upstream of the start codon are designated with minus (–) values. The product size is given in base pairs and the location indicated by base pair position in Morex or Sm89010 with the first base in the ATG start codon designated as 1. Ubiquitin primers were used to amplify a 219 bp fragment of the ubiquitin 1 gene from all cDNAs as shown in Figure 4.

<sup>c</sup> NA, not applicable.

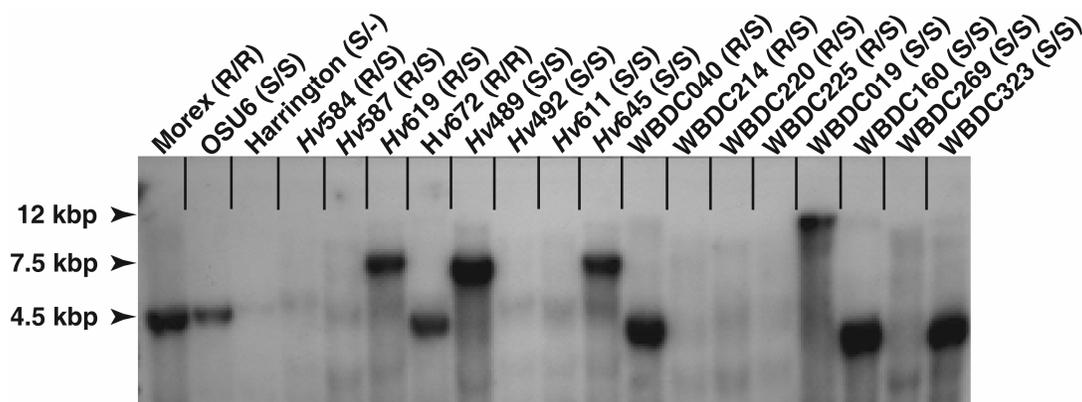


Fig. 2. Southern blot analysis of *Hordeum* accessions. Genomic DNA of each line was digested with *Hind*III and hybridized with the *Rpg1*-specific probe RSB228. The Morex-specific *Rpg1* band is approximately 4.5 kbp. The Sm89010-specific *Rpg1* band is approximately 7.5 kbp (not shown) and of similar size to fragments hybridizing with DNA of Swiss landraces Hv619, Hv489, and Hv645. Harrington does not contain *Rpg1*. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. *tritici* pathotypes MCCF/HKHJ, respectively.

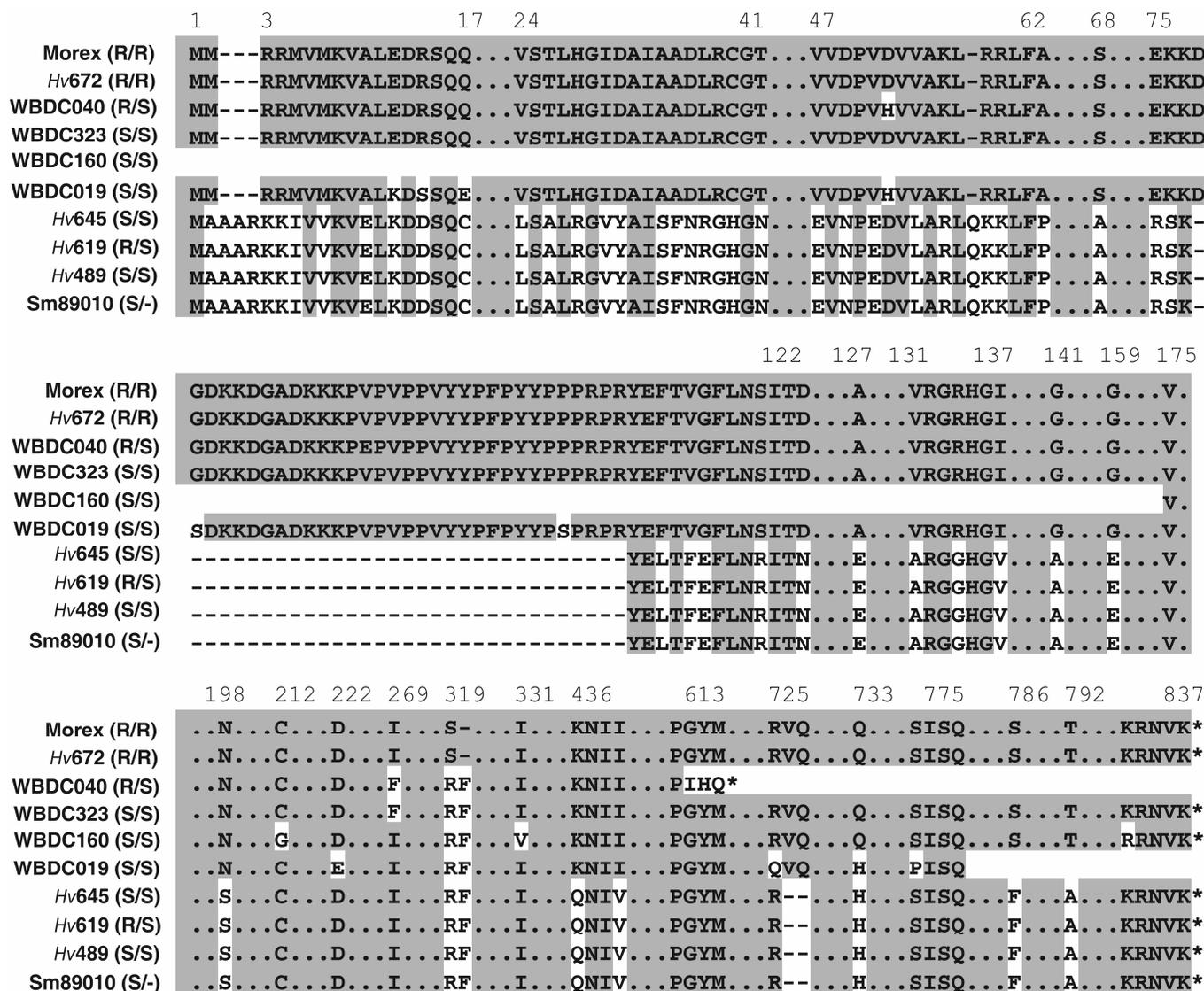
it is susceptible (B. Steffenson, unpublished data). Accession WBDC040 was resistant to moderately resistant to pathotype MCCF and susceptible to pathotype HKHJ, while WBDC323 was susceptible to both pathotypes.

All of the PCR-amplified genomic fragments were sequenced and aligned to resolve the phenotype versus genotype discrepancy. The predicted amino acid sequence of the resistant Swiss landrace Hv672 was identical to that of cultivar Morex RPG1, supporting the phenotype results to both stem rust pathotypes.

*Rpg1* sequence analysis of wild barley accession WBDC040 suggested the presence of an inactive *Rpg1* gene since it had a GTT nucleotide insertion at nucleotide position 1,546 relative to the Morex sequence, a motif indicative of a susceptible allele (5). The GTT insertion resulted in an S to R amino acid substitution at position 319 and an F insertion at 320 (Fig. 3). Initial RT-PCR analysis suggested that WBDC040 produced an *Rpg1* mRNA similar to Morex (Fig. 4). However, sequencing of several cDNA clones of the ninth exon/intron junction of *Rpg1* from Morex and the resistant wild barley accession WBDC040 showed that there

was a substitution altering the conserved 3' AG splice site of intron 9 to AA at position 3251 resulting in the next downstream AG being used as the 3' splice site. This resulted in a 17-nucleotide deletion in the mRNA of the WBDC040 allele, causing a frame shift and a stop codon at amino acid position 617 (Fig. 5). Therefore, the resistance of WBDC040 to pathotype MCCF could not be due to the *Rpg1* gene. This was confirmed by protein analyses that showed that the RPG1 protein failed to accumulate in WBDC040 and autophosphorylation was not detected (Fig. 6). These results suggest the presence of a gene different from *Rpg1* conferring resistance to pathotype MCCF in this accession. The susceptibility of WBDC040 to pathotype HKHJ supports this result.

*Rpg1* sequence analysis of wild barley accession WBDC323 identified a few nucleotide substitutions scattered throughout the nucleotide sequence in addition to the GTT insertion as previously described. At the amino acid level, however, there are only two differences in the *Rpg1* sequence between WBDC323 and Morex. The GTT insertion results in an S to R substitution at position 319, an F insertion at position 320, and an I to F

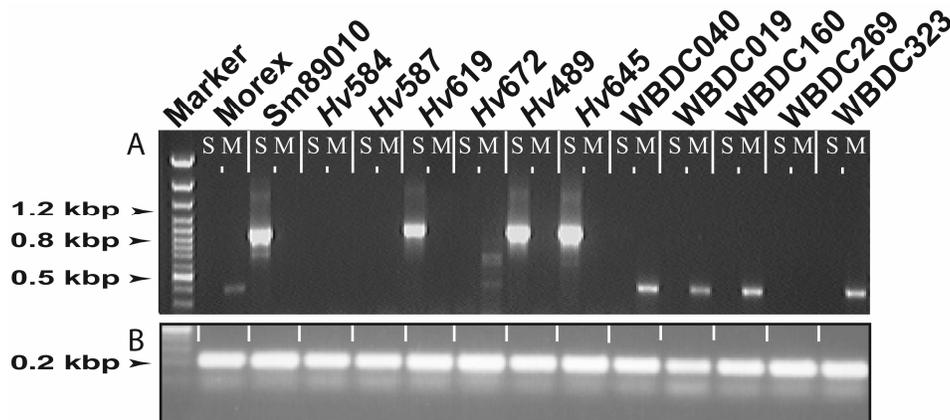


**Fig. 3.** Predicted amino acid alignment of *Rpg1* alleles from Swiss barley landraces and wild barley accessions compared with Morex and Sm89010. Gray boxes represent identical residues and white areas represent diverged residues compared to the resistant Morex *Rpg1* allele. Dashes represent gaps in the alignment. Dots represent blocks of conserved amino acid sequences between all alleles. Asterisk indicates stop codon. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. *tritici* pathotypes MCCF/HKHJ, respectively. Sequence information has been deposited in the GenBank database under the following accession numbers: Swiss Hv489, DQ854803; Swiss Hv619, DQ854802; Swiss Hv645, DQ854804; Swiss Hv672, DQ657356; WBDC019, DQ854805; WBDC040, DQ657357; WBDC160, DQ854806; and WBDC323, DQ657358. The Morex and Sm89010 sequences have been previously deposited and can be found under accession numbers AF509748 and AF509762, respectively (2).

substitution at position 269 (Fig. 3). RT-PCR analysis showed that the *Rpg1* gene was expressed in this accession at the mRNA level (Fig. 4), and western blot and autophosphorylation assays showed that the RPG1 protein is present and capable of autophosphorylation (Fig. 6). Though the GTT insertion and/or the I to F

substitution did not affect the autophosphorylation activity, the protein was not functional in conferring resistance to either pathotype MCCF or HKHJ.

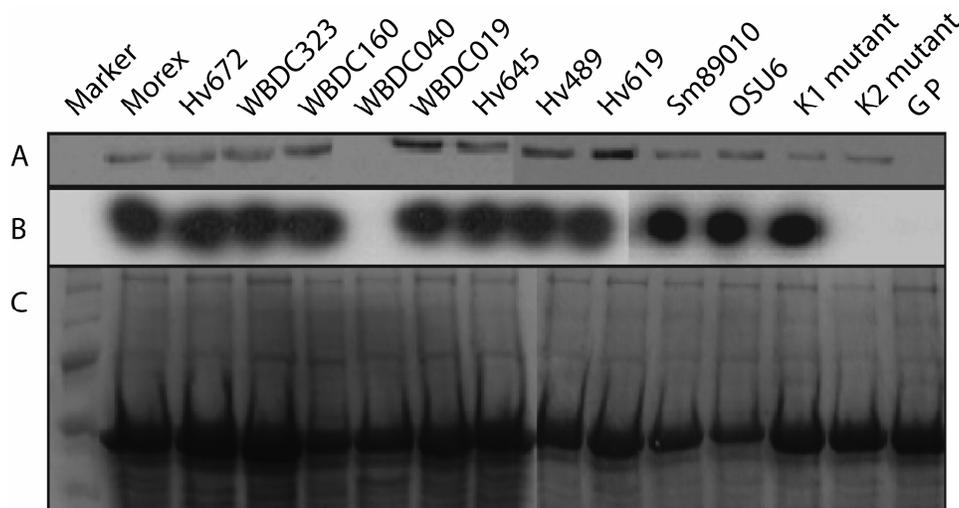
**Lines with an apparent unequal recombination.** We previously described an apparent unequal recombination event in



**Fig. 4.** Reverse transcription-polymerase chain reaction showing Morex-like or Sm89010-like bands. **A**, Oligo dT-primed cDNA derived from total RNA was amplified with either Morex-like *Rpg1*-specific primers (right lane designated M) or Sm89010-like *Rpg1*-specific primers (left lane designated S). The accessions used for cDNA isolation are listed above. The Morex band is approximately 0.4 kbp and the Sm89010 band is approximately 0.9 kbp. Lines devoid of *Rpg1* did not yield a band with either set of primers. **B**, Oligo dT-primed cDNA of each was amplified using ubiquitin-specific primers to demonstrate loading and RNA quality.

	Exon 9	Intron 9	Exon 10	
	611			623
Morex aa	...G T P /	. . . . .	G Y M S P E Y I N E	
Morex cDNA	...GGAACACC/	-----	TGGATACATGTCACCAGAATACATCAATGAA..	
Morex gDNA	...GGAACACC/	...GATGTATTGCA <b>AG</b>	TGGATACATGTCACCAGAATACATCAATGAA..	
WBDC040 gDNA	...GGAACACC/	...GATGTATTGCA <b>A</b>	TGGATACATGTCACC <b>AG</b> AATACATCAATGAA..	
WBDC040 cDNA	...GGAACACC/	-----	AATACATCAATGAA..	
WBDC040 aa	...G T P /	. . . . .	. . . . . I H Q *	
	611			617

**Fig. 5.** Sequence comparison of Morex and WBDC040 *Rpg1* intron 9/exon 10 junction and predicted amino acid sequence. The WBDC040 cDNA sequence is based on three cloned reverse transcription-polymerase chain reaction generated fragments. Note the G to A substitution at the 3' end of intron 9 (white A) in the WBDC040 genomic sequence and the subsequent use of the next AG pair (**AG** bold) for splicing of the mRNA. The resulting cDNA is missing 17 nucleotides from exon 10 causing a frame shift and a stop codon at position 617. The truncated translation product failed to accumulate a protein detectable by the RPG1-specific antibody.



**Fig. 6.** Western blot and autophosphorylation assays. **A**, RPG1 protein visualized with an RPG1-specific antibody. WBDC040 and GP (cultivar Golden Promise, which does not have the *Rpg1* gene) failed to produce a detectable RPG1 protein. **B**, Autophosphorylation assay corresponding to RPG1 western blot. WBDC040 and GP did not autophosphorylate. The K2 (kinase domain 2) mutant also failed to autophosphorylate, while the K1 (kinase domain 1) mutant autophosphorylated as previously reported (13). **C**, Coomassie blue staining of total protein to demonstrate protein loading in each lane.

barley line Sm89010, resulting in a chimeric gene between ABC1037, a closely linked *Rpg1* homologue, and *Rpg1* (1). In the Swiss landraces Hv619, Hv489, and Hv645, amplification of fragments 3 to 7 with the Morex-specific primers was successful and of the expected size, but fragment 1 failed to amplify (Fig. 1). Fragment 2 amplified, but was larger than from Morex (1.6 kbp versus 1.0 kbp). This was similar to the situation previously observed with the Sm89010 allele. Successful amplification of fragment 1 using Sm89010 recombinant allele-specific primers confirmed the presence of an Sm89010-like recombinant allele in these lines (2). Southern blot hybridization of *Hind*III digested Hv619, Hv489, and Hv645 DNA yielded a band similar to that previously observed with Sm89010 (1) and with a higher molecular weight than from the lines containing the Morex-like allele (Fig. 2). RT-PCR amplification using primers specific to Morex or Sm89010 alleles resulted only in a band similar to Sm89010 in these lines and no amplification with Morex-specific primers (Fig. 4). Nucleic acid sequence in silico translation predicted amino acid identity among the three Swiss landraces Hv619, Hv489, and Hv645 and with the Sm89010 *Rpg1*-like recombinant gene (Fig. 3). At the nucleotide level, there were only three differences between the entire Sm89010 *Rpg1* gene sequence and the sequences of the three Swiss landraces (2). There was a G to A substitution at position 2,183 as well as single nucleotide deletions of A at 2,199 and C at 4,713, with numbering based on the Sm89010 *Rpg1* sequence (data not shown). These single nucleotide polymorphisms (SNPs) did not change the amino acid sequence since the G 2,183 A substitution was in the third nucleotide in the codon and A 2,199 and C 4,713 deletions were in introns. The recombinant *Rpg1*-like gene is not expected to provide stem rust resistance as previously demonstrated with the Sm89010 line (1). Landraces Hv489 and Hv645 were indeed susceptible to stem rust pathotypes MCCF and HKHJ as expected; however, Hv619 was resistant to pathotype MCCF, suggesting the presence of a gene different from *Rpg1* conferring the resistance. The lack of a functional *Rpg1* gene in Hv619 also was confirmed by susceptibility to pathotype HKHJ. All three lines with the recombinant type *Rpg1* allele produced an RPG1 antibody positive protein and autophosphorylated (Fig. 6).

#### Lines with apparent 3' and 5' deletions in the *Rpg1* genes.

The wild barley accessions WBDC019 and WBDC160 were susceptible to stem rust pathotypes MCCF and HKHJ and had apparent 3' and 5' deletions in the *Rpg1*-like genes. For WBDC019, fragment 7 (the most 3' *Rpg1* PCR fragment) failed to amplify and for WBDC160, fragments 1 and 2 (the most 5' *Rpg1* PCR fragments) failed to amplify with either the Morex- or Sm89010-specific primers (Fig. 1B). Southern blot analysis showed that both lines had *Rpg1* probe-positive hybridizing fragments; however, accession WBDC019 showed a very high molecular weight fragment, while the WBDC160 fragment was about the same size as Morex (Fig. 2). Since WBDC019, the only accession lacking amplification for fragment 7, had a unique high molecular weight fragment hybridized, we assumed that there might be a large insertion in this region. Additional efforts using amplification conditions for long range PCR ( $\approx 10$  kbp fragments) and fragment 7 primers (228F12/228R12) did not result in amplification. Long range PCR also was applied for amplification of fragment 1 and 2 from WBDC160 using primers 228F11/228R11 and 228F6/228R6, respectively, but without success. RT-PCR produced a band similar to Morex (Fig. 4), indicating that the gene is expressed and suggesting the possibility of an mRNA similar to the Morex-like allele. The RT-PCR was carried out with primers that amplified the internal Morex-like region of the gene. Therefore, the bands are approximately the same size as the Morex *Rpg1* mRNA.

The amplified genomic DNA fragments 1 to 6 from WBDC019 and fragments 3 to 7 from WBDC160 were sequenced and aligned with the other RPG1 derived amino acid sequences (Fig.

3). The susceptible *Rpg1* allele from WBDC019 had diverged, including the GTT insertion, but still was very similar to the Morex RPG1 protein sequence until the C terminus where it could no longer be amplified and recognized as a Morex *Rpg1* homologue. All attempts at extending the sequence with 3' RACE failed.

The in silico translated sequence of the susceptible wild barley WBDC160 RPG1 starts at amino acid position 162 and is fairly similar to the Morex sequence from then on (Fig. 3). This gene also has the GTT insertion resulting in substitution of S 319 R and insertion of F 320. Both lines produced RPG1 antibody-positive protein and autophosphorylated (Fig. 6).

***Rpg1* gene PCR amplification deficient lines.** Of the 16 lines analyzed, 8 failed to produce any amplification products with either Morex-specific or Sm89010-specific *Rpg1* primers (Fig. 1B). The eight lines were evenly divided between the germplasm groups with four Swiss landraces and four wild barley accessions failing to yield any amplification products. To confirm the absence of *Rpg1* alleles in the eight genotypes, an additional 27 combinations of primers designed from the Morex and Sm89010 nucleotide sequences were tested on this group. No amplification products were obtained with these sets of primers, confirming the absence of a Morex-like or Sm89010-like *Rpg1* in these lines (data not shown). In accordance with the PCR amplification results, no hybridization to the RSB228 probe was observed with DNA from lines lacking PCR amplification (Fig. 2). To further confirm the lack of an *Rpg1*-like gene, two Swiss landraces resistant (Hv584 and Hv587) and one wild barley accession susceptible (WBDC269) to pathotype MCCF from this group were included in RT-PCR analysis. No amplification products were obtained using Morex or Sm89010 *Rpg1* primers (Fig. 4). Five of these accessions (Hv584, Hv587, WBDC214, WBDC220, and WBDC225) were resistant to pathotype MCCF and three (Hv492, Hv611, and WBDC269) were susceptible. All of these accessions were susceptible to pathotype HKHJ. These results clearly indicate the presence of a stem rust resistance gene(s) different from *Rpg1* in these *Hordeum* accessions.

## DISCUSSION

To investigate the diversity and possible origin of the barley stem rust resistance gene *Rpg1*, we analyzed two subsets of *H. vulgare* and *H. vulgare* subsp. *spontaneum* germplasm: one comprised of eight Swiss landraces and the second of eight wild barley accessions from diverse geographic regions. Accessions resistant and susceptible to stem rust pathotype MCCF were chosen in order to identify functional *Rpg1* variants as well as spontaneous mutations that result in loss of function (Table 1). Additionally, pathotype HKHJ also was used in this study because it is highly effective for detecting *Rpg1* in the presence of other stem rust resistance genes.

The Swiss landraces used in this study were a potential source of stem rust resistant germplasm and possibly new *Rpg1* alleles. However, only one accession (Hv672) with an intact *Rpg1* gene was found to confer resistance to both *P. graminis* f. sp. *tritici* pathotypes MCCF and HKHJ (Fig. 1; Table 1). The gene in Hv672 was identical in predicted amino acid sequence to the cultivar Morex RPG1 protein (Fig. 3). Thus, Swiss landraces appear to be the sole source for *Rpg1* as both cultivars Chevron and Peatland from Vorrenwald Eich in Canton Lucerne (18) and accession Hv672 from Surava in Canton Graubünden (B. Steffenson, unpublished data) have intact *Rpg1* genes that encode a protein with amino acid sequences identical to Morex, and are all functional in conferring resistance to pathotype HKHJ. It is interesting to note that Hv672 (together with its duplicate selection Hv671) was the only accession among 74 Swiss landraces tested that exhibited resistance to pathotype HKHJ (B. Steffenson, unpublished data). Thus, the presence of *Rpg1* also is

apparently rare in Swiss landraces. The *Rpg1*-derived amino acid sequence in Kindred was previously shown to be identical to that of Chevron and Peatland (2). As mentioned previously, the donor of *Rpg1* in Kindred was likely Chevron.

Wild barley is known to be a rich source of different disease resistance alleles for cultivated barley improvement (8). However, to stem rust, the frequency of resistance is relatively low, even in a large (318 accessions) and diverse collection (20). To identify the possible source of *Rpg1* in cultivated barley, eight ecogeographically diverse wild barley accessions that were resistant or susceptible to stem rust pathotype MCCF were selected for this study. Analysis of the *Rpg1* alleles from the eight wild barley accessions failed to identify any with an intact and functional *Rpg1* gene as was found for the Swiss landrace accession Hv672 (Fig. 1 and Table 1). Surprisingly, most of the resistant wild barley accessions contained either a defective *Rpg1* gene or did not contain a recognizable *Rpg1* gene. This result indicates that other stem rust resistance gene(s) effective against *P. graminis* f. sp. *tritici* pathotype MCCF exist in wild barley. The fact that defective *Rpg1* alleles were found among the wild barley accessions indicates that the gene exists or has existed in these populations.

A curious GTT indel occurs in the *Rpg1* gene of several Swiss landraces and wild barley accessions. This indel results in an S 319 R substitution and insertion of F at position 320 (Fig. 3). In the wild barley accession WBDC323, the only other amino acid change relative to the Morex RPG1 sequence is an I 269 F substitution resulting in a stem rust susceptible phenotype. We have previously observed the GTT indel in the wild barley accession OSU6 (PBI004-7-0-015 accession no. 8321) (2). OSU6 was previously characterized as resistant to stem rust pathotype MCCF, but multiple subsequent assays with both pathotypes MCCF and HKHJ on the selected plants used in the molecular analysis showed that it is susceptible (B. Steffenson, unpublished data). Except for the amino acid changes resulting from the GTT indel, OSU6 does not have any other differences in the RPG1 amino acid sequence compared to the Morex RPG1 sequence. The S to RF change due to the GTT indel appears in seven out of the eight accessions containing an *Rpg1*-like gene analyzed here and thus is very common (Fig. 3). These seven accessions (Hv619, Hv489, Hv645, WBDC040, WBDC019, WBDC160, and WBDC323) do not show a consistent pattern of resistance or susceptibility to the stem rust pathotype MCCF, but all were susceptible to HKHJ, the best pathotype for indicating the functionality of *Rpg1*. Thus, it seems that the GTT insertion affects *Rpg1*-mediated stem rust resistance as indicated by reaction to pathotype HKHJ, but may not affect resistance conferred by other genes, i.e., those conferring resistance to pathotype MCCF. The potential role of the GTT insertion is unknown. It occurs at position 319 in the RPG1 amino acid sequence, placing it near the end of the kinase 1 domain. This domain is not essential for RPG1 autophosphorylation activity, but plays an important, yet undefined role in the overall stem rust resistance reaction (13). The fact that the RPG1 protein with the GTT insertion is active in autophosphorylation suggests that the kinase 2 domain, which is essential for autophosphorylation and disease resistance, is intact and the gene is expressed and may retain some unknown function (Fig. 6).

A set of primers that can differentiate barleys with and without *Rpg1* was developed based on the gene's nucleotide sequence and the GTT insertion (5). The authors tested 42 different barley cultivars and showed that the primers correctly identified the phenotype in all cases, but one. The one exception was cultivar Maud, originally classified as resistant (with a question mark), but grouped with susceptible lines that do not contain an *Rpg1* gene (5). We repeatedly tested Maud at both the seedling and adult plant stages and found it to be susceptible to stem rust pathotype MCCF (B. Steffenson, unpublished data). These results support our conclusion that the GTT indel is associated with a nonfunc-

tional *Rpg1* gene, at least in terms of *P. graminis* f. sp. *tritici* resistance to pathotypes MCCF and HKHJ.

Another interesting group of *Rpg1* alleles are those represented by an apparent unequal recombination between the *Rpg1* gene and a closely linked paralog designated ABC1037 (1). This recombination was observed only in the Swiss landraces and not in any of the wild barley lines tested. The gene is expressed at the mRNA level (Fig. 4). Moreover, an RPG1 antibody positive protein is produced and is active in autophosphorylation (Fig. 6). The function, if any, of this recombinant allele is unknown, but we speculate that it may involve recognition of a putative *AvrRpg1* effector of the pathogen. Recombination among homologous disease resistance genes has been observed and can result in the creation of novel resistance phenotypes (14,17).

The two wild barley accessions WBDC019 and WBDC160 with apparent 3' and 5', respectively, incomplete *Rpg1* genes were susceptible to both stem rust pathotypes as expected (Fig. 1 and Table 1). Both genes, however, are expressed at the mRNA level (Fig. 4) and produce an RPG1 antibody positive protein that undergoes autophosphorylation (Fig. 6). The genomic nucleotide sequence of the *Rpg1* gene in these lines is apparently similar enough to the Morex *Rpg1* gene in the interior regions to produce mRNA and protein with *Rpg1* properties and an intact kinase domain 2 that is active in autophosphorylation. The failure to amplify *Rpg1* fragment 7 from WBDC019 and fragments 1 and 2 from WBDC160 suggest that those regions of the *Rpg1* gene may be deleted, harbor a large insertion, or are highly diverged. A 19 nucleotide insertion and 11 nucleotide deletion in intron 12, plus a 17 nucleotide deletion at the 3' end and 25 nucleotide deletion at the 5' end of exon 13 of the WBDC019 allele, indicated high divergence in this region of the gene. However, these changes apparently are far enough downstream of the kinase 2 domain and do not affect the protein's autophosphorylation activity (Fig. 6). The reason for the extensive divergence of the sequence at the 3' end while the rest of the *Rpg1* gene primary structure is mostly conserved is unknown.

The origin of the widely used and durable *Rpg1* gene bred into North American barley cultivars remains a mystery since we have yet to discover a wild barley accession containing an intact and functional *Rpg1* gene. Although the number of wild barley accessions examined by complete sequencing to date is small, they nevertheless represent some of the most stem rust resistant germplasm found to date in extensive evaluations of *H. vulgare* sp. *spontaneum* germplasm (8,20). In wild barley, we repeatedly found an *Rpg1* gene with a very similar primary gene structure, but containing a GTT insertion that apparently renders the gene nonfunctional in providing stem rust resistance. Is this the progenitor resistance gene that later developed specificity for the stem rust pathotypes MCCF/HKHJ by deletion of the GTT triplet in the cultivated species? A similar, but inversed situation has been described with the *Pto* disease resistance gene (10). In that case, the wild tomato relative *L. pimpinellifolium* contributed the functional resistance gene, but the domesticated *L. esculentum* has maintained highly related and expressed *pto* and *fen* alleles. These alleles encode predicted proteins of 87 and 98% identity with the PTO and FEN kinases and are active in in vitro autophosphorylation, but not functional in disease resistance. The answer to the question of the *Rpg1* origin will require a more extensive search of wild barley germplasm for a potential *Rpg1* donor.

Eight of the 16 *Hordeum* accessions analyzed in this study did not have an *Rpg1*-like gene recognizable by multiple PCR primers or Southern blot analysis, yet five exhibited some resistance to pathotype MCCF (Fig. 1 and Table 1). These results clearly indicate the presence of a gene(s) different from *Rpg1* conferring resistance to stem rust pathotype MCCF. The observed resistance could be due to the *rpg4/Rpg5* complex as there is some evidence that one or both of these genes confers resistance to pathotype

MCCF in addition to other pathotypes (21)(B. Steffenson, *unpublished data*). Additional studies are underway to elucidate the genetics of stem rust resistance in these accessions.

In summary, *Rpg1* alleles with a primary structure different from the one found in cultivar Morex, but apparently functional in conferring stem rust resistance to pathotypes MCCF and HKHJ were not discovered in this study. Moreover, we failed to find a progenitor *Rpg1* gene among the wild barley accessions examined. A common potential progenitor gene has a GTT indel that apparently results in failure to provide resistance to pathotype MCCF. Further, examples of an apparent unequal recombination were discovered, but only in the domesticated *Hordeum* group (i.e., Swiss landraces) suggesting that this may be a relatively recent event. We have previously shown that RPG1 autophosphorylation is essential, but alone is not sufficient for conferring stem rust resistance (13). The observation that all the lines examined in this study, except WBDC040, were active in autophosphorylation reinforces the previous data that it is not sufficient for resistance. Thus, RPG1 plays a complex role in the stem rust disease resistance-signaling pathway.

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