Chromosomal Location and Inheritance of Stem Rust Resistance Transferred from *Hordeum bulbosum* into Cultivated Barley (*H. vulgare*)

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Accepted for publication 31 October 2008.

ABSTRACT

Fetch, T., Jr., Johnston, P. A., and Pickering, R. 2009. Chromosomal location and inheritance of stem rust resistance transferred from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). *Phytopathology* 99:339-343.

Stem rust, caused by *Puccinia graminis* f. sp. *tritici*, is an important disease on barley (*Hordeum vulgare*). Host resistance has effectively controlled stem rust, primarily through use of gene *Rpg1*. However, virulence to *Rpg1* is present in North America, and a new race (TTKSK, or Ug99) from eastern Africa threatens barley production. A search for novel resistance was previously conducted, and an interspecific barley breeding line (212Y1) with introgressed chromatin from *H. bulbosum* was identified as carrying resistance to races MCCF and QCCJ. This study evaluated the inheritance of resistance in 212Y1 using populations from crosses to Morex (*Rpg1* donor) and Q21861 (*rpg4* donor) and the pathogen races MCCF (avirulent on *Rpg1* and *rpg4*) and QCCJ (virulent on *Rpg1* and avirulent on *rpg4*), and determined the chromosomal position

of the introgression using genomic in situ hybridization (GISH) and chromosome-specific polymerase chain reaction (PCR)-based markers. Progeny from the 212Y1/Q21861 F₂ population segregated for resistant and susceptible plants, indicating different gene loci. Genetic analyses of Morex/212Y1 F₃ families fit a 7 homozygous resistant (HR):8 segregating:1 homozygous susceptible (HS) family segregation ratio to race MCCF, indicating that two genes controlled resistance. Plants in segregating families were in 3R:1S (*Rpg1*), 13R:3S (*Rpg1*+212Y1), and 1R:3S (212Y1) ratios. Genetic analyses of the same F₃ families fit a 1HR:2 segregating:1HS family segregation ratio to race QCCJ, indicating monogenic inheritance. Plants in segregating families were in a 1R: 3S ratio, indicating recessive inheritance in 212Y1. The introgression from *H. bulbosum* into *H. vulgare* was positioned on chromosome 6HS based on GISH and the PCR-based markers. No known stem rust resistance gene has previously been mapped to that region. Thus, it is proposed to name this novel gene from *H. bulbosum* as *rpg6*.

Stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. is a disease that is found on barley (*Hordeum vulgare* L.) worldwide. Yield losses up to 15% have occurred in North America (15), but epidemics on barley have historically been rare unless nearby wheat fields are also severely rusted (16). However, recent epidemics of stem rust on barley in North America occurred from 1989 to 1991, with yield losses of 1 to 3%, attributable to race QCCJ (21). In this instance, spring wheat varieties in the northern Great Plains states were resistant, while barley varieties were susceptible and sustained loss. Race QCCJ carries virulence for *Rpg1*, which has conferred durable resistance in barley for over 50 years in North America until the appearance of this race in 1988 (21). Since then, it has been a frequent component in the *P. graminis* f. sp. *tritici* population in North America (3). Race QCCJ is virulent on all barley cultivars grown in Canada and the Upper Midwest region of the United States. Fungicide application is an effective method to reduce infection of stem rust, but is becoming more expensive due to increased petrochemical costs and also is not environmentally friendly. Thus, the most efficient and economic means of control is through the use of host resistance.

Five stem rust genes have been named in barley, of which four provide resistance to *P. graminis* f. sp. *tritici*. Gene *Rpg1* is an incubation-temperature insensitive gene derived from 'Chevron'

(CIho 1111) and 'Peatland' (CIho 5267) and has provided durable resistance to most races of *P. graminis* f. sp. *tritici* in North America (including MCCF) since the release of 'Kindred' barley in 1942 (14,21), but is ineffective to race QCCJ (23). Gene *Rpg2* is an adult-plant resistance gene derived from 'Heitpas-5' (9). Heitpas-5 has also displayed seedling resistance to stem rust races HTMJ and JCMN, but is susceptible to most races of stem rust at the seedling stage (23). Gene *Rpg3* is derived from PI 382313 and has been reported to possess intermediate levels of seedling resistance (5). It is susceptible to race QCCJ, but is resistant to race MCCF and several other races (23). The only described gene in barley providing resistance to QCCJ is *rpg4*, from the breeding line Q21861 (6,7). Q21861 has displayed a high level (IT = 0;1) of resistance to all tested races of wheat stem rust including the highly virulent African race TTKSK (Ug99) (T. Fetch et al., unpublished data), but is sensitive to incubation at high temperatures (7). Gene *Rpg5*, a dominant gene very closely linked to *rpg4* in Q21861, provides resistance to races of rye stem rust (*P. graminis* f. sp. *secalis*) (2).

Since the only gene in barley that conditions resistance to race QCCJ is *rpg4*, a study was conducted in an attempt to identify stem rust resistance in barley lines with introgressions of *H. bulbosum* L. chromatin (4). *H. bulbosum* is the only species in the secondary gene pool of cultivated barley (1), and resistance to diseases such as powdery mildew (10), leaf rust (13), Septoria speckled leaf blotch (24), leaf scald (20), and barley yellow mosaic virus (17,18) has previously been transferred from *H. bulbosum* into *H. vulgare*. Fetch et al. (4) evaluated 43 recombinant *H. vulgare* lines with introgressions of *H. bulbosum* chromatin and found two (119Y4 and 212Y1) displaying resis-

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doi:10.1094/PHYTO-99-4-0339

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tance to race QCCJ. Resistance to race QCCJ is very rare (6); thus the stem rust resistance that was introgressed into 119Y4 and 212Y1 from *H. bulbosum* may be conditioned by novel gene(s). The seedling infection type (IT) observed in 212Y1 (10;2⁻) was lower than 119Y4 (120;), thus 119Y4 was dropped from further testing. The objectives of this study were to (i) determine if the resistance in 212Y1 was different from Q21861 (has *rpg4*); (ii) determine if the resistance in 212Y1 was different from 'Morex' (has *Rpg1*); (iii) characterize the inheritance of resistance to stem rust in 212Y1; and (iv) determine the chromosomal location of the *H. bulbosum* introgression in 212Y1.

MATERIALS AND METHODS

Germplasm. 212Y1 was a doubled haploid line derived from a triploid hybrid-*H. vulgare* 'Golden Promise' ($2n = 2 \times = 14$)/*H. bulbosum* Cb2920/4/Colch ($2n = 4 \times = 28$), which was backcrossed to Golden Promise. 212Y1 was found to possess resistance to stem rust races MCCF (avirulent on *Rpg1* and *rpg4*) and QCCJ (virulent on *Rpg1* and avirulent on *rpg4*) at the seedling stage in previous glasshouse tests (T. Fetch, unpublished data). Crosses were subsequently made between 212Y1 and both Morex (*Rpg1* [21]) and Q21861 (*rpg4* [21]) in Lincoln, New Zealand in 2001. In previous tests, the seedling IT displayed by 212Y1 (10;2) was similar to that of Q21861 (0;1) when inoculated with race QCCJ (4) at 20°C. Thus, F₂ seed from 212Y1/Q21861 was generated at Lincoln and sent to Winnipeg for allelism testing using race QCCJ. For evaluation of inheritance of resistance, five F₁ seeds from Morex/212Y1 were sent to Winnipeg. Four F₁ seeds (designated A, B, C, and D) were planted and increased to the F₂ generation. Of these, F₂ progeny from plants A (187 progeny) and B (188 progeny) were initially selected for testing at the seedling stage for stem rust reaction, with individual F₂ plants transplanted into 10 × 10 cm² pots and selfed to produce F₃ seed for subsequent rust evaluation to confirm the F₂ seedling reaction. The 212Y1, Morex, and Q21861 parents along with 'Wolfe' (susceptible check) were planted in each experiment as controls. The barley accessions Heitpas-5 (*Rpg2* [24]) and PI 382313 (*Rpg3* [21]) were also evaluated for their reaction to races MCCF and QCCJ in separate experiments to provide information on their phenotypic response compared to line 212Y1.

Stem rust phenotyping. For 212Y1/Q21861 allelism tests, F₂ seeds were planted in 10 × 10 cm² pots (20 seeds/pot) filled with Sunshine mix No. 5 (SunGro Horticulture Inc., Seba Beach, Alberta, Canada), and seedlings inoculated at the first leaf fully emerged stage (7 to 8 days old) with race QCCJ according to Sun and Steffenson (23). For Morex/212Y1 genetic experiments, F₂ progeny from plants A and B were planted into plastic conetainers (21 cm long and 3.8 cm diameter) filled with Sunshine mix No. 5 (1 seed per cone). Progeny from plant A (187) were inoculated with race MCCF and progeny from plant B (188) were inoculated with race QCCJ, using previously described methods (23). Subsequently, seeds of F₃ families were planted in 14 × 20 cm² fiber flats (20 seeds/flat) and F₃ families derived from both plants A (187 families) and B (188 families) were evaluated with races MCCF and QCCJ. All seedling plants were inoculated at the first leaf stage using a concentration of 4.0 mg urediniospores/0.7 ml Bayol oil and incubated using standard protocols (23). Seedlings were rated 14 days later using a modified 0 to 4 IT scale (25)

where combinations of ITs 0, 1, and 2 were classified as a resistant host response, and combinations of 23⁻ or higher as a susceptible host response. The stem rust reaction in barley is typically mesothetic (has several pustule types), so the IT ratings described in this study indicate the most common pustule type first, followed by the next most common types (22). All F₃ families were inoculated separately with races MCCF and QCCJ at least twice, with some families repeated a third time so that a final designation (homozygous resistant, segregating, or homozygous susceptible) could be determined.

Statistical tests. Chi-square tests were used to evaluate the independence of segregation for genetic ratios in the F₂ and F₃ generations. Homogeneity of segregation ratios among F₃ families from plants A and B for each race was tested using contingency tables (2 × 3) and chi-square statistics generated by Fisher's exact test in PROC FREQ using SAS (19). Data for F₃ families from plants A and B were pooled when the chi-square values were not significant.

Chromosomal location of *H. bulbosum* introgression. Genomic in situ hybridization was done on actively dividing root tip cells to locate the introgression on the short arm of barley chromosome 6H (6HS) according to Pickering et al. (11). To confirm the 6HS location, five polymerase chain reaction (PCR) markers were used to characterize the *H. bulbosum* introgression located in 212Y1 (Primer sequences in Table 1). All PCR markers were amplified using the following cycling conditions: an initial denature of 94°C for 2 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension of 72°C for 5 min. Three PCR markers (H31_2522, H31_2318, and H31_24136) were developed from assembly No. 31 of the HarVEST database and were mapped to chromosome 6HS using the Steptoe/Morex doubled haploid mapping population (8). Primer sequences of two additional markers (KS171, Professor Kazuhiro Sato, Research Institute for Bioresources, Okayama University, Kurashiki, Japan; and GBM1021 [25]) were kindly provided by collaborators. PCR products from all five markers were amplified from four barley lines and four *H. bulbosum* genotypes and were directly sequenced on an ABI3100 Genetic Analyser (Applied Biosystems) using BigDye v3.1 (Applied Biosystems), according to the manufacturer's instructions. Conserved single nucleotide polymorphisms (SNP) between the species were identified and targeted to develop species-diagnostic markers after restriction enzyme digestion for H31_2522 (*Tru1I*) and H31_2318 (*MboI*). Restriction digests were performed by adding 5 µl of 1× Buffer R (Fermentas, Inc., Glen Burnie, MD) containing 1 U of the restriction enzyme *Tru1I* or *MboI*, Fermentas, Inc., Glen Burnie, MD) to a 10-µl PCR reaction and incubating at the appropriate temperature (65°C *Tru1I* or 37°C *MboI*) for 4 h. An aliquot (5 µl) of this reaction was then added to an agarose loading buffer and run on 2% agarose gels to separate the restriction products. The remaining three markers were sequenced directly from PCR products amplified from 212Y1 to determine whether the barley (Golden Promise) or *H. bulbosum* (Cb2920/4/Colch) allele was present at these loci.

RESULTS

Allelism tests with Q21861. Infection levels on 212Y1/Q21861 F₂ plants, 212Y1, Q21861, and Wolfe were uniform, but

TABLE 1. Primer sequences of barley chromosome 6HS polymerase chain reaction markers

Marker name	Forward primer	Reverse primer
H31_2522	GTTGGAGTKATTGGAGCTG	GTGGAYTGGTAMAGATCAAAGTAG
H31_2318	CCGGTGAAGATCTCCAAGAA	GTGTAGACGAGGCGCTTGAC
H31_24136	GGTCTCAGCTCCTTCTACCTC	AATGCAGATAAAATATGAAGAGCTTG
KS171	CGTCGATGACTCTTCTTCAGG	TGTGATGAGGCACCTTTATTTTC
GBM1021	GTTTCTGATCAAGGAACCTTTT	TCAAATATACGCCCTCCGTC

ITs were lower than normal due to cool mean (18°C) incubation temperatures in the greenhouse that occurred in January. Q21861 displayed a highly resistant seedling response (IT = 0;1, indicating mostly 0; reactions with some small IT = 1 pustules present), while 212Y1 had a moderately resistant response (IT = 10;2, indicating mostly IT = 1 pustules were present along with some 0; and some IT = 2 pustules). Wolfe had a moderately susceptible response (IT = 23-1, indicating mostly IT = 2 type pustules along with IT = 3 and some IT = 1 pustules) due to incubation at 18°C. Although most progeny had resistant to intermediate responses to infection with race QCCJ, susceptible F₂ progeny were clearly identified from the 212Y1/Q21861 population.

Inheritance of resistance in 212Y1. Infection on F₂ progeny from Morex/212Y1 F₁ plants A and B was very poor with many plants escaping infection, which was attributed to an inadvertent exposure to propiconazole that was applied to plants in an adjoining greenhouse room. Thus, F₂ progeny from two additional F₁ plants (C and D) were inoculated with races MCCF and QCCJ, respectively. F₂ Progeny from plant C (90 lines) that were inoculated with race MCCF fit a 13 resistant: 3 susceptible ratio, and F₂ progeny from plant D (90 lines) inoculated with race QCCJ fit a 1 resistant: 3 susceptible ratio (Table 2). Infection on Morex/212Y1 F₃ families was generally uniform and results are summarized in Table 2. In some cases, a third replicate of testing on specific F₃ families was conducted in order to determine a final classification. Tests of homogeneity of segregation ratios among replicated F₃ populations were nonsignificant using 2 × 3 contingency tables ($\chi^2 = 0.009$, $P = 0.995$ for race QCCJ, $\chi^2 = 0.026$, $P = 0.987$ for race MCCF), so F₃ data could be pooled by race.

Both Morex (IT = 0;1-) and 212Y1 (IT = 120;) had low responses to race MCCF at 22°C, and Wolfe showed a susceptible (IT = 3-2) response. Segregation data for F₃ families evaluated with race MCCF fit a 7:8:1 (homozygous resistant [HR]: Segregating [Seg]: homozygous susceptible [HS]) ratio (Table 2). Segregating families (20 plants per family evaluated) with 3R:1S (resistant plants with IT = 0;1), 13R:3S (resistant plants with mostly IR = 0;1 but some IT = 120;), and 1R:3S (resistant plants with only IR = 120;) ratios were found. About three in four families were 3R:1S or 13R:3S, and one in four F₃ families were 1R:3S.

Seedling responses at 21°C to race QCCJ were differential between Morex (IT = 3-32) and 212Y1 (IT = 120;), and Wolfe showed a susceptible (IT = 3-23) response. Segregation data for F₃ families evaluated with race QCCJ fit a 1:2:1 (HR: Seg: HS) ratio (Table 2). Segregating families (20 plants per family evaluated) from both populations generally followed a 1R:3S ratio.

Location of introgression of *H. bulbosum* chromatin. After doing genomic in situ hybridization on root tip cells of 212Y1, two signals were seen on the short arm of two chromosomes with large satellites (Fig. 1). The size of the satellites indicated that the chromosome was *H. vulgare* 6H, and the introgression was distally located on the short arm (6HS). PCR markers distally located on chromosome 6HS confirmed this finding. DNA from

212Y1 produced homozygous *H. bulbosum* patterns for both H31_2522 (digested with *Tru*II [TTAA] and H31_2318 [digested with *Mbo*I GATC]) (Fig. 2). Direct sequencing of PCR amplification products from two more proximal markers (H31_24136 [KS338] and KS171, Fig. 3) and multiple sequence alignment using SeqScape (Applied Biosystems) indicated that sequences from 212Y1 completely matched the sequences generated from its *H. bulbosum* parental genotype (2920/4) for these markers (data not shown). Only GBM1021 was determined to be proximal to the introgressed chromatin in 212Y1, as its amplification product resembled that of the barley parent Golden Promise (data not shown). This indicates that the genetic size of the introgressed region from *H. bulbosum* on chromosome 6HS was less than 40 cM, or approximately 29% of the length of barley chromosome 6H (Fig. 3).

DISCUSSION

This is the first report of the transfer of stem rust resistance from *H. bulbosum* into *H. vulgare* and adds to the extensive suite of resistance genes that are available to breeders from a species in the secondary gene pool. Previous assessment of barley lines with *H. bulbosum* introgressions found that 212Y1 possessed levels of resistance to race QCCJ that was similar to line Q21861 (4). The allelism test in this study between Q21861 and 212Y1 found susceptible F₂ progeny, so the resistance gene in 212Y1 is different from *rpg4*. Furthermore, since the gene in 212Y1 was positioned to chromosome 6HS and gene *rpg4* is on 5HL (2), they are independently inherited. This means that a novel source of resistance to race QCCJ from 212Y1 is available to barley breeders.

The Morex/212Y1 cross was made both to perform allelism tests to *Rpg1* and for characterization of the inheritance of resistance in 212Y1. Segregation of F₂ progeny and F₃ families fit a two-gene ratio with dominant and recessive ratios in segregating F₃ families when inoculated with race MCCF. Morex has *Rpg1*, which is a dominant gene that is located on chromosome 7HS (26), thus the second gene conditioning recessive inheritance is attributed to 212Y1. Segregation of F₂ progeny and F₃ families fit a one-gene ratio with a recessive ratio in F₃ families when inoculated with QCCJ. Since *Rpg1* is ineffective to race QCCJ (21) but 212Y1 was resistant, this provides further evidence for a single recessive gene in 212Y1. Additionally, since the gene in 212Y1 is on chromosome 6HS and *Rpg1* is on 7HS, they are independent.

The locus *rpg6* is proposed for the novel recessive gene in 212Y1. In this study, 212Y1 was tested for allelism to *Rpg1* and *rpg4* and found to be independent to both genes. Allelism tests between the recessive gene in 212Y1 with *Rpg2* and *Rpg3* were not conducted because we did not have an avirulent pathotype of *P. graminis* f. sp. *tritici* to either gene at the seedling stage at the start of the study. In our study, Heitpas 5 (*Rpg2*) was susceptible (IT = 3-2) to both races QCCJ and MCCF, while PI 382313 (*Rpg3*) was susceptible (IT = 3-2) to race QCCJ and intermediate (IT = 213-) to race MCCF. Sun and Steffenson (23) recently

TABLE 2. Segregation of F₂ progeny and F₃ families of a Morex/212Y1 barley population to *Puccinia graminis* f. sp. *tritici* races MCCF and QCCJ at the seedling stage in greenhouse trials conducted at the Cereal Research Centre, Winnipeg from 2002 to 2007

Generation ^a	F ₁ Plant	Race	Resistant	Segregating	Susceptible	Model	χ^2 value	P value
F ₂	C	MCCF	70		20	13:3	0.712	0.3988 NS
F ₃	A	MCCF	87	88	12	7:8:1	0.661	0.7186 NS
F ₃	B	MCCF	86	90	12	7:8:1	0.347	0.8407 NS
F ₃	Pooled	MCCF	173	178	24	7:8:1	0.982	0.6120 NS
F ₂	D	QCCJ	19		71	1:3	0.726	0.3942 NS
F ₃	A	QCCJ	40	103	44	1:2:1	2.102	0.3496 NS
F ₃	B	QCCJ	40	103	45	1:2:1	1.989	0.3699 NS
F ₃	Pooled	QCCJ	80	206	89	1:2:1	4.083	0.1298 NS

^a Since infection on progeny from F₁ plants A and B was poor at the F₂ generation, progeny from F₁ plants C and D was also tested at the F₂ generation to obtain segregation data.

Fig. 1. Genomic in situ hybridization image of a mitotic cell preparation taken from an actively dividing root tip cell of 212Y1 showing two signals on the short arms of two barley chromosomes with large satellites (chromosome 6HS). Bar = 10 µm.

Fig. 2. Cleaved amplified polymorphic sequence (CAPS) marker H31_2522 which amplifies a 218 bp product in all barley cultivars and all *Hordeum bulbosum* genotypes tested. Restriction digestion with the enzyme *TruI* (TTAA) produces two products (146 + 72 bp) in the *H. bulbosum* genotypes 2920/4 and A17 (lanes 9 and 10) with the barley cultivars Golden Promise and Emir remaining undigested (lanes 1 and 2). Lanes 3 and 4 are replicates of 212Y1, lanes 5 and 6 are unrelated 6HS introgression lines 119Y4 and 174Q1, lane 7 is a 6Hb chromosome substitution line (34L71), lane 8 is the inter-specific hybrid 204K3 (heterozygotes displaying an additional heteroduplex band larger than the barley parental band), lane 11 is a no template control, and flanking lanes are DNA ladder.

described pathotype JCMN as avirulent to both *Rpg2* and *Rpg3*, thus this race could be used for future allelism and genetic studies. Since the genes *Rpg2* and *Rpg3* are dominant and both are ineffective to races MCCF and QCCJ (*Rpg3* is intermediate), and gene *rpg6* is recessive and effective to races MCCF and QCCJ, it is unlikely that *rpg6* is allelic to either *Rpg2* or *Rpg3*.

Additional seedling tests have indicated that *rpg6* is not effective against the highly virulent African race TTKSK (T. Fetch, unpublished data), but we have no data on the adult plant response of 212Y1 to this race. In previous work with *Puccinia hordei* G. Otth., some *H. vulgare*-*H. bulbosum* introgression lines exhibited a susceptible seedling infection type, but a low incidence of disease at a later developmental stage (12). Hence, it is conceivable that 212Y1 might possess similar resistance and needs to be evaluated at the adult plant stage to particularly virulent stem rust races. The durability of these resistances will only become clear after extensive growing over a period of years of new barley cultivars containing these genes. Because linkage drag of deleterious traits is often associated with introgressed DNA from wild related species, it would be advantageous to shorten the length of the introgressed segment from *H. bulbosum* if possible. However, the precise location of the resistance locus will only be determined by screening a large segregating population derived from 212Y1 and a susceptible barley cultivar. Studies are in progress to resolve this, and when finished will be

Fig. 3. Genetic linkage maps indicating the relative positions of markers used during analysis (indicated in bold). Connectors indicate markers common between maps, and dashed line indicates the likely proximal end of the *Hordeum bulbosum* introgression in 212Y1 (A, consensus map [28], B, Steptoe × Morex map [9], and C, doubled haploid population [n = 93]) between the barley cv. Haruna Nijo and the *H. spontaneum* genotype H602 (K. Sato, personal communication).

beneficial to barley breeding programs in providing information that will aid in reducing the size of the introgression while retaining the novel gene *rpg6* that is described in this study.

ACKNOWLEDGMENTS

We thank K. Sato, Research Institute for Bioresources, Okayama University, Kurashiki, 710-0046, Japan for permission to use the chromosome 6HS map in Figure 3, and his primer sequences to carry out the molecular marker analyses. We also thank K. Dunsmore and C. LeClerc for their excellent technical assistance in the stem rust phenotyping of the Morex/212Y1 populations. This is contribution number 1966 of the Cereal Research Centre

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