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Genetic characterization of resistance to wheat stem rust race TTKSK in landrace and wild barley accessions identifies the *rpg4/Rpg5* locus

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ABSTRACT

Mamo, B. E., Smith, K. P., Brueggeman, R. S, and Steffenson, B. J. 2015. **Genetic characterization of resistance to wheat stem rust race TTKSK in landrace and wild barley accessions identifies the *rpg4/Rpg5* locus**. Phytopathology 105(1):99-109.

Race TTKSK of the wheat stem rust pathogen (*Puccinia graminis* f. sp. *tritici*, *Pgt*) threatens the production of wheat and barley worldwide because of its broad-spectrum virulence on many widely grown cultivars. Sources of resistance against race TTKSK were recently identified in several barley landraces (*Hordeum vulgare* ssp. *vulgare*) and wild barley accessions (*H. vulgare* ssp. *spontaneum*). The objectives of this study were to characterize the inheritance of resistance to wheat stem rust race TTKSK in four barley landraces (Hv501, Hv545, Hv602, and Hv612) and two wild barley (WBDC213, and WBDC345) accessions, map the resistance gene(s), and determine the allelic relationships among the gene(s) in these accessions and the previously described *rpg4*/*Rpg5* locus. Resistant accessions were crossed with the susceptible cultivar Steptoe and resulting F_3 populations were evaluated for resistance to race TTKSK at the seedling stage. Segregation of F_3 families in populations involving the resistance sources of Hv501, Hv545, Hv612, WBDC213, and WBDC345 fit a 1:2:1 ratio for homozygous resistant (HR) : segregating (SEG) : homozygous susceptible (HS) progenies (with $\chi^2 = 2.27$ to 5.87 and $P = 0.053$ to 0.321), indicating that a single gene confers resistance to race TTKSK. Segregation of F_3 families in cross Steptoe/Hv602 did not fit a 1:2:1 ratio (HR 20 : SEG 47 : HS 43 with $\chi^2 = 11.95$ and $P = 0.003$), indicating that more than one gene is involved in imparting resistance to race TTKSK. Bulked

segregant analysis (BSA) using over 1,500 SNP markers positioned a resistance locus in all six populations on chromosome 5HL in very close proximity to the known location of the *rpg4/Rpg5* complex locus. Allelism tests were conducted by making crosses among resistant accessions Hv501, Hv545 and Hv612 and also Q21861 with the *rpg4/Rpg5* complex. No segregation was observed in F_2 families inoculated with race TTKSK, demonstrating that all Hv lines carry the same allele for resistance and that it resides at or very near the *rpg4/Rpg5* locus. Phenotype evaluations of the six barley accessions with wheat stem rust race QCCJ revealed resistant infection types (ITs) at low incubation temperature and susceptible ITs at high incubation temperature, similar to Q21861, which carries the temperature sensitive gene *rpg4*. The accessions also exhibited low ITs against the rye stem rust isolate 92-MN-90, suggesting they also carry *Rpg5*. This result was confirmed through molecular analysis, which revealed that all six barley accessions contain the STPK (serine/threonine protein kinase) domain that confers *Rpg5* resistance. These results indicate that cultivated barley is extremely vulnerable to African stem rust races like TTKSK because even these diverse selections of landrace and wild barley accessions carry only one locus for resistance.

INTRODUCTION

Stem rust, caused by *Puccinia graminis* Pers.:Pers., is one of the most important diseases of wheat, barley, oat, and rye, owing to its ability to completely destroy crops in a short period of time over a large scale. Wheat stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & Henn. (*Pgt*), attacks both wheat and barley in many regions of the world. Stem rust has caused multiple widespread epidemics in the northern Great Plains region of the United States and Canada, with the most recent occurring in the 1930s and 1950s (Roelfs, 1986). Losses due to stem rust have been greatly reduced in the Great Plains region of the United States since the late 1950s due to the wide scale deployment of resistant, early maturing cultivars, and also eradication of barberry (*Berberis vulgaris* L.), the alternate host of the stem rust pathogen (Kolmer, 2001; Roelfs, 1982). Since then, the incidence of new *Pgt* races and epidemics have been very infrequent (Roelfs, 1982). However, in 1998, a new race of *Pgt* now designated as TTKSK (also known as isolate Ug99) was detected in Uganda (Pretorius et al., 2000) and later found to be virulent against a

widely used resistance gene in wheat (*Sr31*) as well as many other commonly used resistance genes (Jin and Singh, 2006; Pretorius et al., 2000). Currently, race TTKSK threatens wheat and barley production worldwide. It is capable of attacking over 90% of the world's wheat cultivars (Singh et al., 2008). It is also widely virulent on barley, attacking over 97% of cultivars grown worldwide (Steffenson et al., 2013). Since its detection in Uganda, race TTKSK and its variants have spread to a number of other countries in Africa (Kenya, Ethiopia, Sudan, Tanzania, South Africa, Zimbabwe, Mozambique and Eritrea) and also the Middle East (Yemen and Iran) (Mukoyi et al., 2011; Nazari et al., 2009; Pretorius et al., 2010; 2012; Visser et al., 2011; Wanyera et al., 2006; Wolday et al., 2011). Variants in the "Ug99 lineage" are expected to spread to other cereal-producing regions of the world in the near future (Hodson et al., 2012; Singh et al., 2008).

While the damaging effects of stem rust, including race TTKSK, can be mitigated by fungicide applications, the extra input costs and potential negative consequences of chemical treatments on the environment warrant the use of host resistance genes to control stem rust. To date, eight stem rust resistance genes have been identified in different accessions of barley. Gene *Rpg1* was identified from barley accessions Chevron (CIho 1111) and Peatland (CIho 5267) (Powers and Hines, 1933; Shands, 1939), is effective against most wheat stem rust races, and has protected barley from significant stem rust losses for over 70 years (Steffenson, 1992). In another genetic study conducted with cv. Peatland, Fox et al. (1995) identified *RpgU* in addition to *Rpg1*. However, TTKSK and other related African races are highly virulent on *Rpg1*. Genes *Rpg2* and *Rpg3* were identified from the accessions Hietpas-5 (CIho 7124) (Patterson et al., 1957) and PI 382313 (Jedel, 1989; Jedel, 1990), respectively, and like *Rpg1* are not completely effective against race TTKSK (Steffenson et al., 2013). The recessive stem rust resistance gene *rpg4* was identified in breeding line Q21861 (PI 584766) and confers resistance to race QCCJ (Jin et al., 1994). More recently, a recessive stem rust resistance gene (*rpg6*) was identified in 212Y1, a barley line with an introgression of *Hordeum bulbosum* L. chromatin (Fetch et al., 2009).

Other resistance genes were described based on their reaction to the rye stem rust pathogen (*P. graminis* f. sp. *secalis* or *Pgs*). The dominant resistance gene *Rpg5* (initially designated *RpgQ*) was discovered in Q21861 (Brueggeman et al., 2008; Sun et al., 1996). Gene *Rpg5* is tightly linked to *rpg4* and is located on the long arm of barley chromosome 5H (Brueggeman et al., 2008; Druka

et al., 2000). A recessive gene designated as *rpgBH* also confers resistance to rye stem rust and was described from Black Hulless (CIho 666) (Steffenson et al., 1984).

In an expression QTL (eQTL) study of the barley population Q21861/SM89010 infected with *Pgt* race TTKSK at the adult plant stage in the field, Moscou et al. (2011) identified a chromosome 2H *trans*-eQTL that enhances resistance through transcriptional suppression of many genes. At seedling stage, the major effect locus identified was *rpg4*/*Rpg5* (*Rpg-TTKSK*) on chromosome 5H. Zhou et al. (2014) conducted an association mapping study of United States breeding germplasm to race TTKSK at the adult plant stage in the field and identified two QTL: one on chromosome 7H and the other on chromosome 5H, distantly proximal to *rpg4*/*Rpg5*. Given that *rpg4/Rpg5* is the only effective locus against race TTKSK (Steffenson et al., 2013), it is important to identify and genetically characterize new sources of resistance and transfer their genes into commercial cultivars.

The evaluation of a worldwide collection of barley germplasm identified a number of sources of seedling and adult plant resistance to race TTKSK in the *Hordeum* gene pool, comprising cultivars, landraces, and wild barley accessions (Steffenson et al., 2013; B. Steffenson, *unpublished*). Some of the most resistant accessions included landraces (*Hordeum vulgare* ssp. *vulgare*) from Switzerland and also accessions of wild barley (*H. vulgare* ssp. *spontaneum)* from the Wild Barley Diversity Collection (WBDC) (Steffenson et al., 2007). Six of these resistant barley accessions were chosen for detailed study to elucidate the genetic basis of race TTKSK resistance so as to enable more efficient use in breeding. Thus, the specific objectives of this study were to: (1) characterize the inheritance of resistance to race TTKSK in landrace and wild barley accessions at the seedling stage through bi-parental mapping; (2) determine the chromosomal locations of the resistance gene(s); and (3) elucidate the allelic relationships among the resistance gene(s) in these accessions and the previously described *rpg4*/*Rpg5* complex locus.

MATERIALS AND METHODS

Plant materials. Six *Hordeum* accessions exhibiting seedling and/or adult resistance to race TTKSK were crossed with the susceptible barley cultivar Steptoe (CIho 15229) to develop mapping populations for genetic analysis (Table 1). Four of the accessions (Hv501, Hv545,

Hv602, and Hv612) were landraces originally collected from the alpine regions of eastern Switzerland (Canton Graubünden), a country previously known to be a source of stem rust resistant barley germplasm (Steffenson, 1992). Seed was donated by the Station federale de recherches en production vegetale de Changins in Nyon, Switzerland, courtesy of Geert Kleijer. Selection of these four accessions from a total collection of 74 landraces was based on their genetic diversity as revealed by 12 simple sequence repeat (SSR) markers (P. Olivera, *unpublished*), geographic location within Graubünden, and their resistant stem rust phenotype. The two other accessions (WBDC213 and WBDC345) investigated were wild barleys collected from Samarkand and Kashkadarya provinces of Uzbekistan, respectively (Table 1). These accessions are part of the Wild Barley Diversity Collection (Steffenson et al., 2007), provided by the International Center for Agricultural Research in the Dry Areas in Aleppo, Syria, courtesy of Jan Valkoun.

Planting, inoculation and incubation of plants. F_2 plants from the crosses were not evaluated to race TTKSK because of space limitations for increasing to the next generation within the Biosafety Level-3 (BSL-3) Containment Facility at the University of Minnesota in St. Paul. Thus, 25-35 plants of each F_3 family were evaluated at the seedling stage for response to race TTKSK. The tests were conducted inside the BSL-3 facility during the winter months. F_3 families and parents were planted in plastic pots (7.6 × 7.6 × 10.8 cm, l × w × h) filled with a 50:50 mix of steam-sterilized native soil and Metro-Mix® 200 (Sun Gro Horticulture, Quincy, MI) (vermiculite, sphagnum peat moss, perlite, dolomitic limestone, and a wetting agent). After planting, all pots were watered and fertilized with Osmocote® controlled release fertilizer 14-14-14 (Scott's Company, Marysville, OH) (1.4 g/pot) and Peters Dark Weather fertilizer 15-0-15 (Scott's Company) (ca. 40 g/liter at 1/16 dilution). Populations derived from wild barley accessions were kept at 4°C for two weeks to break dormancy and facilitate uniform emergence and growth prior to inoculation. All populations were grown in a greenhouse at 19-22°C with a 14-16 hr photoperiod supplemented by 400 W high-pressure sodium lamps emitting a minimum of 300 µmol photons m^{-2} s^{-1}. When the plants began to emerge from the soil, they were brought into the BSL-3 facility for the remainder of the experiment. Stocks of rust isolate 04KEN156/04 of race TTKSK were prepared and utilized for inoculation following the protocols described in Steffenson et al. (2009)

149 with minor modifications. A rust spore suspension (14 mg urediniospores/0.7 ml oil) was applied
150 to 8 to 9-day-old plants with fully expanded primary leaves at a rate of ~0.09 mg/plant with an
151 atomizer pressured at 25-30 kPa (Sun and Steffenson, 2005). After inoculation, plants were placed
152 in chambers misted with ultrasonic humidifiers initially for 30-40 minutes of continuous misting
153 and thereafter for 4-8 minutes every hour for 16-18 hours in the dark. After the wetness period,
154 light was provided by 400 W sodium vapor lamps (150-250 μmol photon m^{-2} s^{-1}), and the mist
155 chamber doors were slightly opened to dissipate the heat. At this time, the humidifiers were set to
156 run for 4-8 minutes of misting every 15 minutes for the next 2 hours. After 2 additional hours, the
157 misters were turned off and the chamber doors opened halfway to facilitate slow drying of the
158 plant surfaces under continuous light for the next 3 to 4 hours. Finally, when the leaf surfaces were
159 completely dry, plants were returned to the greenhouse under the conditions previously described.
160 The parental accessions also were evaluated in limited field trials at the Kenya Agricultural
161 Research Institute in Njoro, Kenya according to the methods described by Zhou et al. (2014).
162 **Rust infection phenotyping.** At 12-14 days after inoculation, stem rust infection types
163 (ITs) were assessed on the first leaves of plants based on the 0 to 4 scale originally developed for
164 wheat by Stakman et al. (1962) and modified for barley (Steffenson et al., 1993). The IT scale used
165 for barley is based primarily on uredinial size as described by Miller and Lambert (1955). Plants
166 with ITs ranging from 0 to 2^{3-} were classified as resistant and those from $3^{-}2$ to 3^{+} as susceptible.
167 Individual F_3 families were grouped into three classes of homozygous resistant (HR), segregating
168 (SEG), or homozygous susceptible (HS) based on the reactions of individual plants.
169 **Statistical test.** Pearson's chi-square (χ^2) test was used to evaluate independence of
170 segregation for genetic ratios in the F_3 generation. The chi-square statistic and associated *P*-values
171 were calculated using the *chisq.test* function in Microsoft Excel.
172 **Sample preparation for bulked segregant analysis.** HR and HS F_3 families were used
173 for bulked segregant analysis (BSA), an efficient method for tagging and mapping disease
174 resistance genes (Hyten et al., 2009; Michelmore et al., 1991; Quarrie et al., 1999). One arbitrarily
175 selected plant from each HR and HS family was grown in the greenhouse and the leaf tissue
176 harvested for DNA extraction. Leaf tissue from plants representing eight F_3 families was bulked
177 to create sets of HR and HS bulks for BSA. Three independent sets of both the HR and HS bulks

were used in each population. For the Steptoe/Hv602 population, only 20 HR families were used to create the HR bulks because of the limited number of such families identified. Additionally, five seeds each of the resistant parents and Steptoe also were grown and leaf tissue harvested two weeks after sowing for DNA extraction. Samples were freeze-dried using a general purpose freeze dryer (Model 24DX48; Virtis Company, Gardiner, NY) according to the specifications of the manufacturer.

DNA extraction and genotyping. The freeze-dried tissue was used for total genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA sample quality was checked by separating and visualizing on a 1% agarose gel. The amount of DNA in each sample was quantified by measuring absorbance at 260 nm (A_{260}) with a spectrophotometer (Labomed, Inc., Culver City, CA). The DNA concentration of each sample was then normalized to 200 ng/µL, and 5 µL of each normalized sample was submitted for genotyping with 1,536 single nucleotide polymorphism (SNP) markers of the Barley Oligonucleotide Pooled Assay 1 (BOPA1) (Close et al., 2009; Rostocks et al., 2006). The 1,536 SNP markers were tested on the resistant parents, Steptoe, and the three independent HR and HS bulks per population using Illumina Bead Array Technology with the GoldenGate assay (Fan et al., 2003, 2006). The BOPA1 SNP markers were previously mapped onto the integrated molecular genetic linkage map of barley (Close et al., 2009; Muñoz-Amatriaín et al., 2011).

Genotype data analysis. Data generated by the GoldenGate assay were visualized and analyzed with the Genotyping Module of the GenomeStudio data analysis software (Illumina, San Diego, CA) GSGT version 1.8.4. Single nucleotide polymorphisms between the respective resistant parents and Steptoe were identified. All of the SNP call data were manually checked, and positive hits for BSA were noted when alleles of at least two of the resistant and/or susceptible bulks clustered close with alleles of the resistant parent or Steptoe, respectively, in the GenCall output.

Allelism tests. To determine the allelic relationships among stem rust resistance genes in the different resistant accessions and line Q21861, a half diallel was attempted. Successful crosses

206 were increased to the F_2 generation. F_2 seeds were sown, and plants were inoculated with race
207 TTKSK according to the protocol described above.

208 **Resistance spectrum of parents to other stem rust races.** To further characterize the
209 resistance spectrum of TTKSK-resistant accessions and also help resolve whether they may carry
210 the *rpg4*/*Rpg5* resistance gene complex, three additional races/isolates of the stem rust pathogen
211 were used. Race QCCJ was used because *rpg4* (and other genes in concert [Wang et al., 2013]) is
212 specifically effective against it at low incubation temperatures (18-20°C) (Jin et al., 1994). Since
213 *rpg4* is temperature sensitive, assays also were made with race QCCJ at 27-28°C, where the gene
214 is rendered completely ineffective (Jin et al., 1994). To assay for the presence of the closely linked
215 gene *Rpg5*, isolate 92-MN-90 of *Pgs* was used. In previous studies, *Rpg5* was found to confer a
216 clear low reaction type to this pathogen isolate (Steffenson et al., 2009; Sun et al., 1996). Finally,
217 race HKHJ of *Pgt* was used because it is capable of identifying *Rpg1* in the presence of other
218 resistance genes (Sun and Steffenson, 2005). Two replications of the parental accessions and
219 control lines were evaluated in experiments that were repeated at least twice in time against each
220 race or isolate. The conditions for plant growth and procedures for inoculation and IT assessment
221 were made according to the methods described previously. The only exception was for the
222 inoculation with race HKHJ where a concentration of 35 mg urediniospores/0.7 ml oil was used
223 due to a lower than normal germination rate.

224 **Molecular characterization of the *rpg4*/*Rpg5* region.** Genotyping for the presence or
225 absence of *rpg4*-mediated resistance was determined using PCR sequence tag site (STS) markers
226 specific to the *Rpg5* gene. Two pairs of PCR primers were designed to specifically amplify
227 functional or non-functional *Rpg5* alleles based on the presence of the serine threonine protein
228 kinase domain (STPK; *Rpg5*+) or the Protein Phosphatase 2C domain (PP2C; *rpg5*-). The
229 sequences of STS markers were designed based on the allele sequence data generated for the three
230 genes at the *rpg4/Rpg5* locus from multiple resistant and susceptible accessions (Brueggeman et.
231 al., 2008; Wang et al., 2013, Arora et. al., 2013). For each barley line, two separate PCR reactions
232 were performed using LRK-F1/LRK-R1 and RpgQ-F6/PP2C-R2 primer combinations. The 20 µl
233 PCR reactions consisted of approximately 100 ng of gDNA, 20 pmol of each forward and reverse
234 primers, 1X red Taq Buffer (Sigma, St. Louis, MO), 1 Unit of Red Taq DNA polymerase, and 0.2

mM dNTPs. Amplification was performed in a Mastercycler pro (Eppendorf, Hauppauge, NY) thermocycler using the following parameters; 95°C for 4 min, 35 cycles of 95°C for 30 sec, 62°C for 1 min, and 72°C for 1 min; followed by 72°C for 5 min. Primer sequences for the STS markers are given in Supplementary Table 1 along with the expected size of the PCR products. The *Rpg5* gene has three main domains (see Supplementary Fig. 1; Brueggeman et al., 2008). The *Rpg5* STS1 marker targets the LRR to the S/TPK region and is specific to the majority of resistant lines (e.g., line Q21861 as a reference genotype). The PP2C STS1 marker amplifies the LRR to the PP2C region and is specific to susceptible lines (e.g., Steptoe). Lines that gave a *Rpg5*+ result with the *Rpg5* STS1 marker were further analyzed for an infrequent allele that gives false positive results due to the presence of an intact protein kinase domain but a non-functional *rpg5* allele that contains a single C insertion causing a frame shift mutation and a predicted truncated non-functional RPG5 protein (Brueggeman et al., 2008; Arora et al., 2013). To test for this rare non-functional allele, a sequence was generated across the region by directly sequencing the amplicon produced using the *Rpg5*-F1 and *Rpg5*-R1 primer combination (Arora et al., 2013). The PCR conditions were the same as described above. The PCR reactions were purified with cycle pure spin columns (Omega Bio-Tek, Norcross, GA) and sequenced with the *Rpg5*-R1 primer. Sequencing was performed by GenScript on an ABI 3730xl (Applied Biosystems, Carlsbad, CA).

RESULTS

Genetics of resistance to race TTKSK of *Puccinia graminis* f. sp. *tritici*. The landrace (Hv501, Hv545, Hv602, and Hv612) and wild barley (WBDC213, and WBDC345) accessions exhibited highly resistant ITs (modes of 0; to 0;1) in response to race TTKSK at the seedling stage (Table 1). In contrast, Steptoe was susceptible, exhibiting an IT mode of 3^{+}. The resistant accessions also showed much lower rust severities and infection responses than Steptoe in the limited Kenyan field trials where they were included (Table 1). A total of 110 to 187 F_3 families from crosses between the resistant barley accessions and Steptoe were evaluated for stem rust reaction in this study (Table 2). Resistant F_3 plants exhibited ITs ranging from 0; to 12 (rarely 210; or 23^{-}) and could be easily differentiated from susceptible plants giving ITs of $3^{-}2$ to 3^{+} (Fig. 1). Thus, individual families of all populations could be confidently grouped into HR, SEG, or HS

263 categories. Segregation data for F_3 families of five populations fit a 1:2:1 ratio for HR : SEG : HS
264 (χ^2 ranging from 2.27 to 5.87 with respective *P*-values ranging from 0.321 to 0.053) (Table 2).
265 These data indicate that a single gene confers TTKSK resistance in Hv501, Hv545, Hv612,
266 WBDC213 and WBDC345. Segregation data for F_3 families in the Steptoe/Hv602 population did
267 not fit a 1:2:1 ratio ($\chi^2 = 11.95$ and $P = 0.003$), indicating that more than one TTKSK resistance
268 gene in Hv602 confer resistance to TTKSK.

269 To determine if resistance was dominant or recessive, several different aspects of the
270 segregating populations were investigated. First, a limited number of F_1 plants were phenotyped.
271 The IT mode of F_1 plants from the Steptoe/Hv545 population was similar to that of the susceptible
272 parent Steptoe (3^-2 vs. 33^+) (Fig. 1), suggesting a recessive or at least a partially recessive gene.
273 Unfortunately, no other F_1 seeds were available from the other crosses. Second, the composition
274 of individual plant reactions within each segregating F_3 family was tallied to assess possible gene
275 action (Mamo, 2013). At least half of the segregating families evaluated in each population (except
276 WBDC213/Steptoe and WBDC345/Steptoe) fit a single gene ratio (Mamo, 2013). Some of the
277 segregating F_3 families in the six populations did not follow a clear Mendelian ratio for an expected
278 single recessive gene as found for the Steptoe/Hv545 F_1 plant. For the Steptoe/Hv501,
279 Steptoe/Hv602 and WBDC345/Steptoe populations, 56% (27/48), 57% (27/47), and 48% (29/60)
280 of the segregating F_3 families, respectively, fit a 1:3 ratio for resistant to susceptible plants,
281 suggesting recessive gene action in the respective resistant parents (Mamo, 2013). Unexpectedly,
282 however, 23% (11/48), 32% (15/47) and 22% (13/60) of the segregating F_3 families of these three
283 respective populations showed dominant gene action, i.e. 3:1 ratio of resistant to susceptible plants
284 (Mamo, 2013). Only one segregating F_3 family among the Steptoe/Hv545, Steptoe/Hv612, and
285 WBDC213/Steptoe populations followed a recessive gene action ratio of 1:3 (Mamo, 2013). The
286 corresponding number of segregating F_3 families following a 3:1 ratio for resistant:susceptible
287 plants for these populations was 54% (13/24), 50% (15/30), and 45% (22/49), respectively (Mamo,
288 2013). For the Steptoe/Hv545 population, the dominant gene action discerned from the plants
289 within the segregating families contradicts the putative partially recessive resistance gene action
290 in Hv545 deduced from the IT of the single Steptoe/Hv545 F_1 plant (see below).

Genotyping of parents and bulks, and bulked segregant analysis. In order to determine the chromosomal location of the resistance genes, genetic analysis was conducted using BSA. A total of 525, 552, 564, 588, 568 and 561 SNPs were identified between the respective resistant parents (Hv501, Hv545, Hv602, Hv612, WBDC213 and WBDC345) and susceptible parent Steptoe after screening with 1,536 BOPA1 SNPs. Markers with possible linkage to the resistance locus were determined after establishing two broad criteria. First, SNPs differentiating at least two of the HR bulks (plus the resistant parent) and two of the HS bulks (plus Steptoe) were considered as putatively linked SNP markers to the resistance loci in the TTKSK-resistant barley accessions (Table 3). Accordingly, alleles of 18 SNPs in total (one in Steptoe/Hv501, three in Steptoe/Hv545, four in Steptoe/Hv602, and five each in Steptoe/Hv612 and WBDC345/Steptoe F_3 families) differed between two or more of the HR and HS bulks. Of these, one SNP each in all of the three resistant and susceptible bulks of Steptoe/Hv545 and Steptoe/Hv612, and two SNPs in all of the three resistant and susceptible bulks of WBDC345/Steptoe clustered together with the respective resistant parent (Table 3, SNPs in bold). Second, in cases where alleles of all the HR bulks were similar to the allele clusters of the resistant parent and alleles of the susceptible bulks had a different cluster from the susceptible parent, the SNP was considered as a putative "candidate" marker linked to the identified resistance gene in the populations (Table 3, SNPs in italic). This second criterion was considered because a mutation or some other genetic change might contribute to the shift of the allele cluster of the susceptible bulks away from the allele cluster of the susceptible parent. Based on this criterion, alleles of 12 SNP markers in total (two each in Steptoe/Hv501 and Steptoe/Hv545, three in Steptoe/Hv602, and five in WBDC213/Steptoe F_3 families) clustered with the resistant parent in all three resistant bulks (Table 3 SNPs in italic).

Most of the 1,536 SNP markers used in this study were previously mapped onto the integrated consensus map of barley (Close et al., 2009; Muñoz-Amatriaín et al., 2011; Kleinhofs and Graner, 2011). Almost all SNPs that had their alleles clustered with alleles of the respective parent in all three resistant and susceptible bulks were located within a 10 cM region of the long arm of chromosome 5H between SNP markers 11_10528 and 11_10869 (Table 3; Figs. 2 & Fig. 3;). Likewise, most SNPs that had their alleles clustered with the resistant parent in all three resistant bulks (positive SNPs)—irrespective of their pattern in the

HS bulks of all six populations—were located within the same region of chromosome 5H. Other SNPs where two of the resistant bulks clustered with the resistant parent or where two or more of the susceptible bulks clustered with the susceptible parent also were located in the same interval of chromosome 5H in all populations (Table 3; Fig. 2 & Fig. 3). This result suggests that all six barley accessions contain a resistance gene mapping to the same region of chromosome 5H. The *rpg4/Rpg5* complex locus in Q21861 also maps to this same region of chromosome 5H (Steffenson et al., 2009). One exception to note is that in the Steptoe/Hv602 population, a SNP (11_21061) that maps at 99.39 cM on the same arm of chromosome 5H had all the three resistant bulks clustered with the resistant parent (Table 3; Fig. 2). This region is interesting because Zhou et al. (2014) identified a novel adult plant resistance locus against race TTKSK in the 69.3-103.9 cM interval of 5HL. A SNP marker (11_21472) mapping to the long arm of chromosome 3H at 66.62 cM had three of the resistant bulks clustered with the resistant parent in the Steptoe/Hv612 population.

Allelism tests. Crosses for the half diallel among the resistance sources and to Q21861 were only obtained for six of the 21 possible combinations: Hv545/Hv602 (300 progeny), Hv545/Hv612 (140 progeny), Hv602/Hv612 (280 progeny), Q21861/Hv501 (680 progeny), Q21861/Hv545 (500 progeny), and Q21861/Hv612 (760 progeny) (Supplementary Table 2). Successful crosses were not obtained for the other combinations due mostly to flowering time differences and poor pollen production. All progeny from crosses Hv545/Hv602, Hv545/Hv612, and Hv602/Hv612 exhibited resistant ITs to race TTKSK indicating that the same allele imparts resistance in the three Swiss landraces. Similar results were obtained in populations involving landraces Hv501, Hv545 and Hv612 crossed to Q21861, indicating the resistance gene(s) were allelic to the *rpg4/Rpg5* complex. One F_2 plant derived from Q21861/Hv501 and three F_2 plants derived from Q21861/Hv612 showed an intermediate IT, but these were retested and later confirmed to be resistant to race TTKSK. Attempts at other crosses for the half diallel were not successful; however, some deductions may be made based on the current results. Although Hv602 was not successfully crossed to Q21861, it likely carries a resistance gene allelic to the *rpg4/Rpg5* complex based on the allelism of Hv602 to Hv612 and Hv612 to Q21861.

348 **Resistance spectrum of parents to other stem rust races.** Landrace and wild barley
349 accessions were inoculated with three additional races/isolates of *Puccinia graminis* to help
350 resolve whether they contain the same resistance gene complex of *rpg4*/*Rpg5* and also to profile
351 their resistance spectrum. Steptoe, the susceptible control, gave high ITs to all races tested (Table
352 4). The landrace and wild barley accessions reacted the same as the resistant controls of Q21861
353 (with *rpg4*/*Rpg5* and *Rpg1*) and QSM20 (with *rpg4*/*Rpg5*) to race QCCJ at low (ITs of 0; to 0;1)
354 and high temperature ($3^{-}2$) incubation. The TTKSK-resistant accessions also were evaluated
355 against *Pgs* isolate 92-MN-90 to determine if they carry *Rpg5*. All accessions exhibited low ITs
356 (0; to 0;1) similar to those of Q21861 and QSM20, suggesting they also carry *Rpg5*. Finally, the
357 six TTKSK-resistant accessions were evaluated to race HKHJ to assess whether they might carry
358 *Rpg1*. Accessions Hv501, Hv612, WBDC213, and WBDC345 exhibited high ITs ($3^{-}2$ to $3^{-}3$) to
359 race HKHJ, suggesting they lack *Rpg1*. Accessions Hv545 and Hv602 gave intermediate ITs of
360 210; and 213^{-}, respectively. These two accessions exhibited similar ITs in repeated evaluations
361 and therefore possess a resistance spectrum that is different from the other studied accessions.
362 Q21861 and QSM20 gave low (0;) and high (33^{+}) ITs to race HKHJ, confirming the presence and
363 absence of *Rpg1* in the respective accessions.
364 **Molecular characterization of the *Rpg5* region.** The parental lines were genotyped at the
365 *rpg4*/*Rpg5* region using sequence tagged site (STS) markers (Brueggeman et. al., 2008; Wang et
366 al., 2013; GenBank accession number EU812563) to assay for the presence of *Rpg5*. All accessions
367 contained the nucleotide binding site (NBS) of the *Rpg5* gene (Table 5). Additionally, all resistant
368 parents and Q21861 contained an intact STPK (serine/threonine protein kinase) domain at the 3'
369 end of the *Rpg5* gene, indicating that the gene is functional in all resistant accessions. Steptoe
370 contained the non-functional allele of *Rpg5* as it lacks the STPK domain.

371 **DISCUSSION**

372 Race TTKSK is a serious threat to wheat and barley production worldwide because of its
373 virulence on multiple resistance genes of agricultural importance. Steffenson et al. (2009)
374 previously reported that the stem rust resistance locus *rpg4/Rpg5* in Q21861 was the only one
375 described in barley that confers resistance against race TTKSK. In an effort to identify additional

376 genes for resistance to race TTKSK, a large and diverse collection of *Hordeum* germplasm was
377 evaluated at the seedling stage (Steffenson 2013). Several Swiss landrace (Hv501, Hv545, Hv602
378 and Hv612) and wild barley (WBDC213 and WBDC345) accessions were among the most
379 resistant identified to race TTKSK. To fully characterize the genetics of resistance in these
380 accessions, the following studies were conducted: 1) bi-parental populations were developed to
381 determine the inheritance of resistance and define the chromosomal locations of the resistance loci
382 using BSA; 2) allelism tests were made to resolve the relationships of genes among some of the
383 resistance sources; 3) accessions were tested to other stem rust races to characterize the resistance
384 spectrum of the gene(s) and postulate their possible identity; and 4) specific primers were used for
385 detecting *Rpg5*, a gene implicated with *rpg4* in conferring resistance to race TTKSK.
386 Genetic analysis of the segregating populations clearly indicated that a single gene confers
387 seedling resistance to race TTKSK in accessions Hv501, Hv545, Hv612, WBDC213 and
388 WBDC345. In accession Hv602, more than one gene was involved in conferring seedling
389 resistance (Table 2). Monogenic inheritance for resistance to different races/isolates of the wheat
390 and rye stem rust pathogens have been reported in a number of barley accessions in previous
391 studies (Fetch et al., 2009; Fox and Harder, 1995; Jin et al., 1994; Jedel, 1990; Patterson et al.,
392 1957; Shands, 1939; Steffenson et al., 1984; Sun et al., 1996). Steffenson et al. (2009) reported
393 that resistance to race TTKSK in Q21861 segregates as a single gene that lies at the *rpg4*/*Rpg5*
394 region. It appears that this same gene complex confers resistance in the accessions characterized
395 in this study. Perhaps this complex locus and some other gene is involved in conferring resistance
396 in Hv602. The population Steffenson et al. (2009) evaluated had only 129 progeny and therefore
397 segregation of the two closely linked genes was unlikely. The recessive gene *rpg4* controls
398 resistance to race QCCJ in Q21861 at low incubation temperatures (18−21°C) (Jin et al., 1994).
399 This gene also was thought to confer resistance to rye stem rust in a partially dominant fashion
400 (Sun et al., 1996) until Brueggeman et al. (2008) identified recombinants exhibiting resistance to
401 *Pgs* isolate 92-MN-90 and not race QCCJ in a single large segregating population
402 (Steptoe/Q21861). However, the second tightly linked locus containing the *Rme1* (*rpg4*-modifier
403 element 1) gene, identified in the Steptoe/Q21861 population that is required for *rpg4*-mediated
404 resistance, is only polymorphic in this population and not in the other two large populations

405 analyzed (MD2/Q21861 and Harrington/Q21861). These data suggested that the *Rpg5* gene
406 conferring resistance to rye stem rust is partially dominant and lies only a few recombination units
407 from *Rme1*, yet is the polymorphic *R*-gene at the locus and the major determinant of *rpg4*-mediated
408 resistance (Brueggeman et al., 2008; Wang et al., 2013).

409 Efforts to characterize the gene action (recessive vs. dominant) of TTKSK resistance in the
410 resistance sources did not yield clear-cut results. The IT mode of a single F_1 plant from the
411 Steptoe/Hv545 population and the ratio of plant reaction types within 48 to 57% of segregating F_3
412 families of the Steptoe/Hv501, Steptoe/Hv602, and WBDC345/Steptoe populations were
413 suggestive of a recessive or at least partially recessive acting resistance gene. On the contrary, the
414 ratio of plant reaction types in other segregating F_3 families was suggestive of a dominant gene.
415 One explanation for the lack of conclusive results concerning gene action based on segregating F_3
416 families is that multiple genes are likely involved in the phenotype as recently found by Wang et
417 al. (2013), and therefore some F_3 plants might represent various recombinations of genes at other
418 loci that interact with the *rpg4/Rpg5* complex of genes. The contention of additional genes being
419 involved was supported by the IT data observed in the Steptoe/Hv501 population, where some
420 resistant families exhibited intermediate ITs instead of the typical 0; to 0;1 types shown by the
421 resistant parent. In addition, some plants in the susceptible families of the Steptoe/Hv602
422 population had quite variable ITs, ranging from $3^{-}2$ to 3^{+}. This suggests again that there might be
423 complementary resistance genes involved in the population(s). In fact, segregation data of the
424 Steptoe/Hv602 population indicated that TTKSK resistance involves more than one gene. Indeed,
425 the recessive nature of *rpg4* is determined at the *Rpg5* locus by the HvPP2C dominant
426 susceptibility factor (R. Brueggeman, *unpublished*). Preliminary expression analysis data suggest
427 that the expression levels of *Rpg5* and the interaction of *Rpg5* with HvPP2C may determine the
428 recessive vs. dominant nature of *rpg4/Rpg5*-mediated resistance, suggesting that other genes
429 segregating in these populations, possibly transcriptional regulators similar to those proposed by
430 Moscou et al., (2011), could be influencing the nature of gene action in the resistance sources (R.
431 Brueggeman, *unpublished*). Further evaluation of the *rpg4/Rpg5* locus and other loci influencing
432 the resistance in the populations reported here may help determine the dominant/recessive nature
433 of *rpg4*/*Rpg5*-mediated resistance.

The *rpg4* locus (now the *rpg4/Rpg5* complex locus) was mapped to the long arm of barley chromosome 5H using molecular markers (Borovkova et al., 1995; Druka et al., 2000). *Rpg5* was later isolated through positional cloning (Brueggeman et al., 2008). Recent high-resolution recombinant analysis by Wang et al. (2013) indicated that the *rpg4/Rpg5* region spans a ~290 kbp physical region and contains several candidate genes. Wang et al. (2013) have implicated *Rpg5*, along with other tightly linked genes in the region, in *rpg4*-mediated resistance against races TTKSK and QCCJ. The multiple genes in the *rpg4/Rpg5* region required for resistance to TTKSK and QCCJ often segregate as a single locus because they are very closely linked, i.e. within a ~70 kbp genetic interval. Research is underway to characterize additional informative recombinants in the region to resolve which gene(s) are essential for conferring TTKSK resistance (Wang et al., 2013). This information will be useful for identifying the genes needed to confer TTKSK resistance in the landrace and wild barley accessions.

After demonstrating a monogenic inheritance pattern for all but one of the resistant accessions, BSA was used to approximate the chromosomal location(s) of the TTKSK resistance loci. Several SNP markers identified using BSA in the six populations were previously mapped to the subtelomeric region of the long arm of chromosome 5H in the 158 to 165 cM interval between SNP markers and11_10869 (Muñoz-Amatriaín et al., 2011). The SNPs identified by BSA lie in a region coincident with *rpg4/Rpg5*. A previous but lower resolution mapping study of the resistance gene *rpg4* in the Q21861/SM89010 (Q/SM) population using restriction fragment length polymorphism (RFLP) markers identified MWG740 and ABG390 as linked markers (Borovkova et al., 1995). That study mapped *rpg4* 5.7 cM distal from the RFLP marker ABG390. On recent consensus maps, ABG390 lies in the same genomic region with the SNP markers detecting the TTKSK resistance locus in the current study (Muñoz-Amatriaín et al., 2011; http://wheat.pw.usda.gov/GG2/index.shtml). Steffenson et al. (2009) mapped the gene(s) conferring TTKSK resistance in Q21861 to the *rpg4/Rpg5* complex locus based on the cosegregation of this resistance with the previously mapped *rpg4* locus conferring resistance to race QCCJ and also resistance to *Pgs* isolate 92-MN-90 (Sun et al., 1996). Moscou et al. (2011) mapped qualitative TTKSK seedling stage resistance in the Q/SM population to 146.78 cM on chromosome 5H using mRNA transcript abundance with the Barley1 Affymetrix array. Based on

463 the chromosomal position in consensus maps of RFLP markers linked to the *rpg4* locus,
464 specifically ABG390, the TTKSK resistance locus detected by BSA analysis in this study lies very
465 near the *rpg4/Rpg5* complex locus. In BSA, SNP markers that were positive in each of the three
466 resistant and susceptible bulks and also detected in more than one population were positioned at a
467 more proximal location on chromosome 5H, closer to the putative location of *rpg4/Rpg5* (see SNPs
468 in bold in Fig. 2). Other markers, not positive in all three resistant and susceptible bulks and
469 detected in only one of the populations, were positioned at more distal locations on the
470 chromosome. The other interesting region to note is the 69.3-103.9 cM interval of chromosome
471 5HL where a novel adult plant TTKSK resistance locus was identified through association
472 mapping in United States barley breeding germplasm (Zhou et al., 2014). BSA of the
473 Steptoe/Hv602 population identified a marker (SNP 11_21061) that maps at 99.39 cM on the same
474 arm of chromosome 5H. This region of chromosome 5HL may contain a gene that interacts with
475 the *rpg4*/*Rpg5* complex locus to impart TTKSK resistance in Hv602.
476 Steffenson et al. (2007) identified DArT markers significantly associated with wheat stem
477 rust (race MCCF) resistance in the *rpg4/Rpg5* gene complex region of chromosome 5H through
478 association mapping in the WBDC. A bi-parental mapping study with one of the resistant wild
479 barley accessions (WBDC348 also known as 'Damon') identified a single major gene conferring
480 resistance to stem rust races MCCF and QCCJ in the same bin as *rpg4*/*Rpg5* (Alsop, 2009).
481 Research is underway to continue high-resolution recombinant analysis in the *rpg4/Rpg5* region
482 to precisely map these resistant loci (Wang et al., 2013).
483 To provide additional data regarding the relationships among the resistance genes
484 identified in the six accessions and also the *rpg4/Rpg5* complex in Q21861, tests of allelism were
485 made. No segregation was observed in crosses between the Swiss landraces (Hv501, Hv545 and
486 Hv612) and Q21861 with the *rpg4/Rpg5* complex (Supplementary Table 2). This indicates that the
487 gene(s) conferring TTKSK resistance in the landraces are either allelic with those at the *rpg4/Rpg5*
488 locus or are closely linked to it. Further confirmation of this finding was obtained from the allelism
489 tests among selected Swiss landraces (Supplementary Table 2). No segregation was observed in
490 any of these populations, demonstrating that Hv501, Hv545, Hv612 and Hv602 all carry the same
491 allele for resistance to race TTKSK and that it resides at *rpg4/Rpg5* locus.

492 To obtain more data as to whether the resistant accessions contain the same resistance gene
493 complex of *rpg4*/*Rpg5*, additional phenotype evaluations were made with *Pgt* races QCCJ and
494 HKHJ and *Pgs* isolate 92-MN-90. These tests were critical because the genes have unique
495 hallmarks: *rpg4* is temperature sensitive (Jin et al., 1994) and *Rpg5* specifically confers resistance
496 to rye stem rust without the need for other genes (Sun et al., 1996). The Swiss landraces and wild
497 barleys exhibited resistant ITs against race QCCJ at low temperature and susceptible ITs at high
498 temperature, similar to those exhibited by Q21861 with the *rpg4/Rpg5* complex (Steffenson et al.,
499 2009; Brueggeman et al., 2009). The resistant accessions also gave low ITs against *Pgs* isolate 92-
500 MN-90, similar to those exhibited by Q21861, suggesting they also carry *Rpg5* (Table 4). These
501 results strongly support our hypothesis that the six resistance sources contain the *rpg4/Rpg5* locus.
502 Tests with race HKHJ indicated that Hv501, Hv612, WBDC213 and WBDC345 likely lack *Rpg1*.
503 This result is in agreement with *Rpg1*-specific marker analysis that revealed the absence of this
504 gene in the Swiss landraces (B. Steffenson and R. Brueggeman, *personal communication*). Two
505 accessions (Hv545 and Hv602) exhibited unexpected ITs in response to race HKHJ over multiple
506 evaluations. Known controls with *Rpg1* gave classical ITs of 0; to 10; when tested with race
507 HKHJ. In contrast, Hv545 and Hv602 exhibited low to intermediate ITs of 210; and 213^{-},
508 respectively, to this race. *Rpg1*-specific marker analysis indicated that these two landraces lack
509 *Rpg1* (B. Steffenson and R. Brueggeman, *personal communication*). However, they may carry a
510 partially effective gene against race HKHJ. This result should be verified further, including testing
511 the two accessions with stem rust races under different temperature conditions.
512 Molecular haplotyping provided another strong piece of evidence concerning the presence
513 of a functional *Rpg5* gene in the six resistant accessions. *Rpg5* encodes a protein with nucleotide
514 binding-site, leucine-rich, and protein kinase domains (Brueggeman et al., 2008). Molecular
515 characterization of the *Rpg5* region with STS markers indicated that all six resistant accessions
516 contain a functional *Rpg5* gene (Table 5). A sequenced portion of the allele also revealed that the
517 serine/threonine protein kinase (STPK) domain at the C-terminus end of the *Rpg5* gene is intact.
518 The STPK domain is a functionally crucial unit of *Rpg5* for conferring resistance. These data
519 demonstrate that TTKSK resistance in landrace and wild barley accessions likely involves *Rpg5*.
520 By lieu of its close linkage to other genes, the presence of *Rpg5* in these sources also strongly

suggests the presence of *rpg4* and other nearby genes needed for conferring TTKSK resistance. The latest research on stem rust resistance mediated by the *rpg4/Rpg5* region suggests that *Rpg5* is the R-gene that is responsible for the gene-for-gene interaction determining *rpg4*-mediated resistance and is the only reliable gene with polymorphism that can be used to determine the presence of *rpg4*-mediated resistance (Arora and Brueggeman, 2013). In the future, any newly discovered barley accessions with TTKSK resistance should be initially screened for the presence of the functional *Rpg5* gene to determine whether or not the resistance might be novel. This test will likely serve to detect other genes at the locus since *rpg4* and *Rpg5* are likely conserved as revealed by their discovery both in landrace and also wild barley accessions.

In summary, segregation data from F_3 families developed from crosses of landrace and wild barley accessions with the susceptible cultivar Steptoe indicated that a single locus confers resistance to race TTKSK in five of the six populations and that more than one locus govern resistance in Hv602. Molecular genetic mapping by BSA, together with molecular haplotyping for a functional *Rpg5* gene and screening with rye stem rust demonstrate that the TTKSK resistance gene in the landrace and wild barley accessions map to the *rpg4/Rpg5* region. Taken together, these data indicate that the TTKSK resistance genes in the barley accessions are simply alleles of the *rpg4/Rpg5* gene complex. Q21861 is the original source of the *rpg4/Rpg5* gene complex and is one of the best known accessions possessing a high level of adult plant resistance against race TTKSK. Several barley breeding programs in North America are introgressing *rpg4/Rpg5* into elite breeding lines for resistance to race TTKSK. However, future work should be done to identify additional sources of resistance so that barley cultivars can be developed with a broad spectrum of resistance to *Pgt* races, including race TTKSK and its variants.

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TABLE 1. Reaction of Swiss barley landraces, wild barley accessions, and susceptible control Steptoe to stem rust race *Pgt*-TTKSK at the seedling and adult plant stages

			Seedling reaction		Kenya 2008	Kenya 2009	Kenya 2010
Accession[a]	**Origin**	**Location**	**IT mode[b]**	**IT range[c]**	**Sev % / IR[d]**	**Sev % / IR**	**Sev % / IR**
Hv501	Switzerland	Near Bonaduz	0;1	0; to 0;1	2 MR	--	0 R
Hv545	Switzerland	Near Disentis	0;1	0; to 0;1	5 MS	--	0.5 R/5MS
Hv602	Switzerland	Unknown	0;	0; to 10;	1 R	--	0 R
Hv612	Switzerland	Near Laret	0;	0; to 0;1	20 MS-MR	--	0 R
WBDC213	Uzbekistan	Samarkand	0;	0; to 0;1	--[e]	10 MR/20 MS-S	--
WBDC345	Uzbekistan	Kashkadarya	0;1	0; to 210;	--	Trace MS/Trace R	--
Steptoe	USA	Washington	3^+	3^-2 to 3^+	40 S	--	20 S-MS

[a] Hv (*Hordeum vulgare*) number assigned by the Station federale de recherches en production vegetale de Changins in Nyon, Switzerland; WBDC: Wild Barley (*H. vulgare* ssp. *spontaneum*) Diversity Collection accession described by Steffenson et al. (2007).
[b] Infection type (IT) mode represents the one or two most commonly observed ITs on plants, listed in order of their relative prevalence.
[c] Infection type (IT) range represents the lowest and highest ITs observed on plants. Plants were evaluated for their ITs based on the 0 to 4 scale originally developed for wheat (Stakman et al., 1962) and modified for barley (Steffenson et al., 1993). ITs 0, 0;, 1, 2, and 2^3 were considered indicative of host resistance, whereas types 3^-, 3, and 3^+ were considered indicative of susceptibility.
[d] Terminal rust severity (0-100%) at the adult plant stage was based on the modified Cobb scale (Peterson et al., 1948; Stubbs et al., 1986). The infection responses (IR) were based on the size and type of uredinia observed where R: resistant; MR: moderately resistant; MS: moderately susceptible, and S: susceptible (McIntosh et al., 1995; Roelfs et al., 1992). Severity readings of "Trace" denote very low rust infection (<0.5%) in the field. The slash symbol (/) indicates the variation in stem rust severity at different times of disease rating during the growing season.
[e] Not Tested.

728 **TABLE 2.** Segregation for resistance in F_3 families from crosses of landrace and wild barley accessions with Steptoe to race TTKSK
729 of *Puccinia graminis* f. sp. *tritici* at the seedling stage

	Number of F_3 families					
Cross	**Homozygous resistant**	**Segregating**	**Homozygous susceptible**	**Expected ratio**	**χ^2 value (2 df)**	**Probability (>χ^2)[a]**
Steptoe/Hv501	32	48	40	1:2:1	5.87	0.053 ns
Steptoe/Hv545	48	98	35	1:2:1	3.11	0.211 ns
Steptoe/Hv602	20	47	43	1:2:1	11.95	0.003 **
Steptoe/Hv612	39	88	60	1:2:1	5.36	0.068 ns
WBDC213/Steptoe	30	52	36	1:2:1	2.27	0.321 ns
WBDC345/Steptoe	32	76	52	1:2:1	5.40	0.067 ns

730 [a] ns = not significant at 0.05; ** = significant at 0.01.

731 **TABLE 3.** Chromosomal position of markers associated with resistance against stem rust race
732 TTKSK in landrace and wild barle accessions as determined by bulked segregant analysis

Chrom.	Arm	Position along chrom. (cM)[a]	SNP designation[b]	Mapping pop. in which associations were identified	No. HR bulks[c]	No. HS bulks[d]
3H	L	66.62	11_21472	Steptoe/Hv612	2	3
5H	L	51.51	11_20501	Steptoe/Hv602	1	3
		99.39	*11_21061*	*Steptoe/Hv602*	*3*	*1*
		152.93	*11_10901*	*WBDC213/Steptoe*	*3*	*1*
		155.45	*11_10528*	*WBDC213/Steptoe*	*3*	*1*
		155.66	*11_21024*	*WBDC213/Steptoe*	*3*	*0*
		157.61	11_10336	Steptoe/Hv545	3	2
		157.61	11_10336	Steptoe/Hv602	2	2
		157.61	11_10336	Steptoe/Hv612	3	2
		157.61	11_10336	WBDC345/Steptoe	2	3
		157.61	*11_20646*	*Steptoe/Hv545*	*3*	*1*
		157.61	11_20646	Steptoe/Hv602	2	2
		157.61	11_20646	Steptoe/Hv612	2	2
		157.61	**11_20646**	**WBDC345/Steptoe**	**3**	**3**
		157.61	*11_21018*	*Steptoe/Hv545*	*3*	*0*
		158.28	11_11464	WBDC345/Steptoe	2	3
		162.98	11_11216	**Steptoe/Hv545**	**3**	**3**
		162.98	11_11216	Steptoe/Hv612	3	2
		162.98	11_11216	*WBDC213/Steptoe*	*3*	*1*
		162.98	11_11216	**WBDC345/Steptoe**	**3**	**3**
		163.72	11_20546	Steptoe/Hv501	2	2
		163.72	11_20546	Steptoe/Hv602	2	2
		163.72	11_20546	Steptoe/Hv612	2	3
		163.72	11_20686	WBDC345/Steptoe	2	3
		164.15	*11_20644*	*Steptoe/Hv602*	*3*	*1*
		164.15	*11_20644*	*WBDC213/Steptoe*	*3*	*1*
		165.28	*11_10869*	*Steptoe/Hv501*	*3*	*1*
		165.28	11_10869	Steptoe/Hv545	2	2
		165.28	11_10869	Steptoe/Hv602	2	2
		165.28	11_10869	**Steptoe/Hv612**	**3**	**3**
		168.44	*11_20536*	*Steptoe/Hv501*	*3*	*0*
		168.44	*11_20536*	*Steptoe/Hv602*	*3*	*0*

733 [a] SNP marker position according to Muñoz-Amatriaín et al. (2011).
734 [b] SNP marker BOPA_C nomenclature according to Close et al. (2009); SNP markers that were positive in
735 each of the three resistant and susceptible bulks are indicated in bold; SNP markers that were positive in
736 each of the three resistant bulks and in none or one of the susceptible bulks were considered as putative
737 "candidate" SNPs. Such SNPs are indicated in italics.
738 [c] Number of homozygous resistant (HR) bulks (out of three) which have alleles identical to the respective
739 resistant parent.
740 [d] Number of homozygous susceptible (HS) bulks (out of three) which have alleles identical to the respective
741 susceptible parent.

TABLE 4. Seedling infection types (IT) of parental lines and controls in response to wheat stem rust races *Pgt*-QCCJ and *Pgt*-HKHJ and rye stem rust isolate *Pgs*-92-MN-90

	Infection type mode[b]			
	Pgt-QCCJ		*Pgt*-HKHJ	*Pgs*-92-MN-90
Accession[a]	**21°C[c]**	**28°C**	**21°C**	**21°C**
Hv501	0;1	3⁻2	3⁻2	0;
Hv545	0;1	3⁻2	210;	0;
Hv602	0;1	3⁻2	213⁻	0;1
Hv612	0;1	3⁻2	3⁻2	0;
WBDC213	0;	3⁻2	3⁻3	0;
WBDC345	0;1	3⁻2	3⁻2	0;1
Q21861	0;1	3⁻2	0;	0;
QSM20	0;1	3⁻2	33⁺	0;
Steptoe	3⁻2	33⁺	3⁻3	3⁻2

[a] Hv (*Hordeum vulgare*) number assigned by the Station federale de recherches en production vegetale de Changins in Nyon, Switzerland; WBDC: Wild Barley (*H. vulgare* ssp. *spontaneum*) Diversity Collection accession described by Steffenson et al. (2007).

[b] Infection type (IT) mode represents the one or two most commonly observed ITs on plants, listed in order of their relative prevalence. Plants were evaluated for their ITs based on the 0 to 4 scale originally developed for wheat (Stakman et al., 1962) and modified for barley (Steffenson et al., 1993). ITs 0, 0;, 1, 2, and 23⁻ are considered indicative of host resistance, and types 3⁻, 3, and 3⁺ are indicative of susceptibility.

[c] Incubation temperature under which plants were grown after stem rust inoculation.

TABLE 5. Genotyping and sequencing of landrace and wild barley accessions for the wheat stem rust resistance gene *Rpg5*

Accession	*Rpg5*[a]	Kinase[b]	5' end insertion[c]	Sequence[d]
Hv501	+	+	-	Unknown
Hv545	+	+	-	Unknown
Hv602	+	+	-	+
Hv612	+	+	-	Unknown
WBDC213	+	+	-	Unknown
WBDC345	+	+	-	Unknown
Q21861	+	+	-	+, D
Steptoe	+	-	Unknown	-, D
Steptoe	+	-	Unknown	-, D

[a] *Rpg5* NBS (nucleotide binding site-leucine rich repeat) region was present.
[b] The C-terminal protein kinase is intact (+ for resistant genotype) or replaced by the PP2C gene (- for susceptible genotypes).
[c] The 5' end of the allele does not contain an insertion that results in a frame shift and truncated *Rpg5* protein (-).
[d] Resistant genotype (+) or susceptible genotype (-) based on the sequenced portions of the allele; the entire allele has been sequenced (D) (Wang et al., 2013).

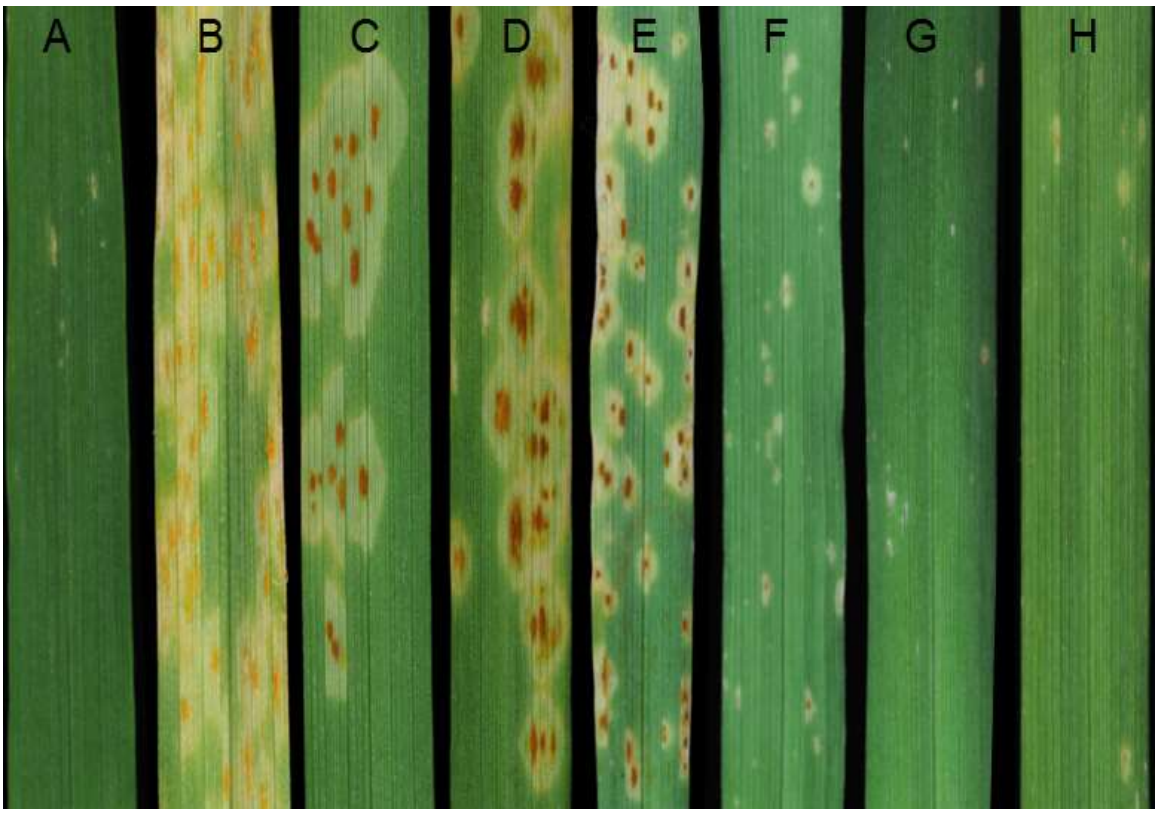


Fig. 1. Disease phenotypes of barley landraces in response to wheat stem rust race TTKSK (**A**) highly resistant reaction of Hv545, (**B**) susceptible reaction of F_1 plant from the Steptoe/Hv545 cross, (**C**) susceptible reaction of Steptoe parent, (**D**) susceptible plant from a homozygous susceptible F_3 family of the Steptoe/Hv612 cross, (**E**) moderately susceptible plant from a segregating F_3 family of the Steptoe/Hv612 cross, (**F**) resistant plant from a segregating F_3 family of the Steptoe/Hv612 cross, (**G**) resistant plant from a homozygous resistant F_3 family of the Steptoe/Hv612 cross, and (**H**) highly resistant reaction of Hv612.

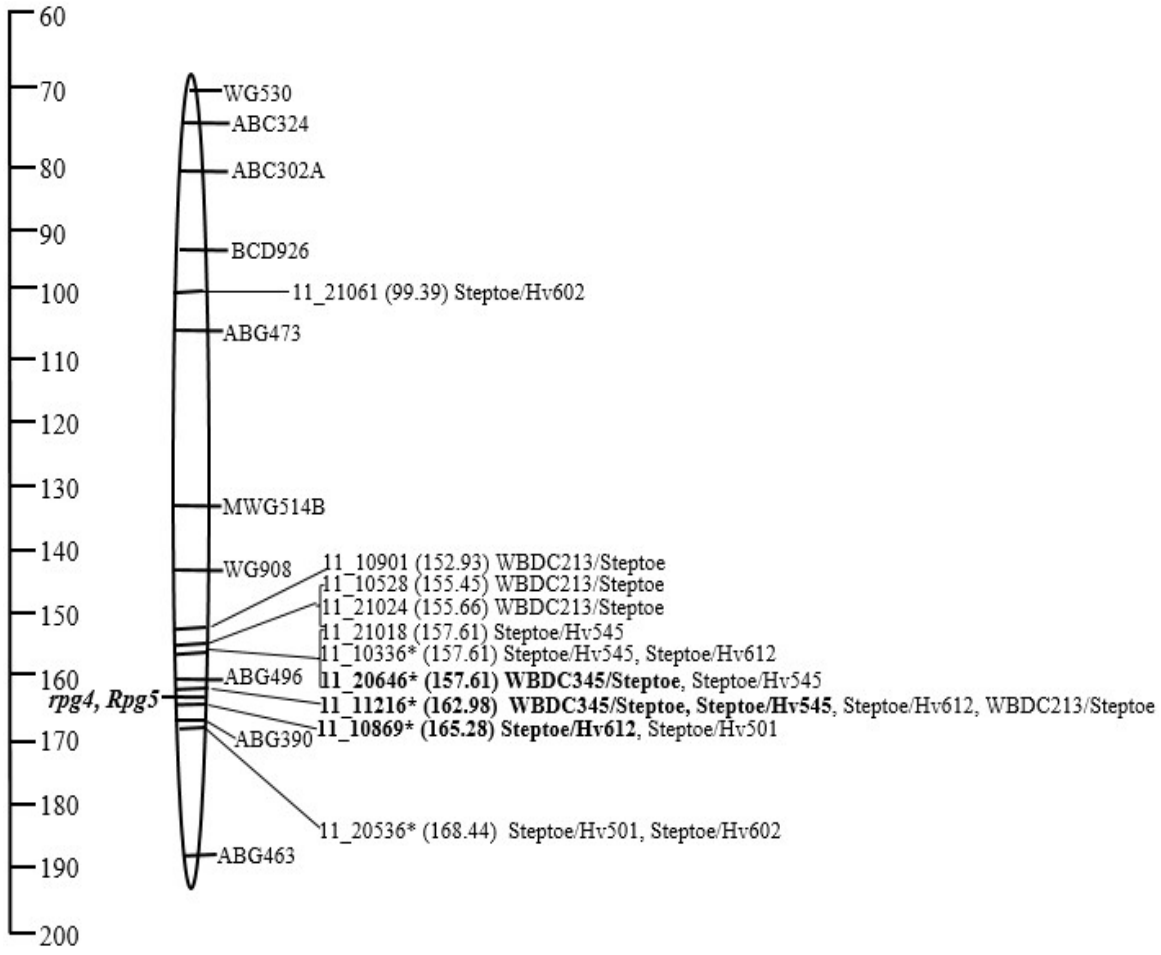
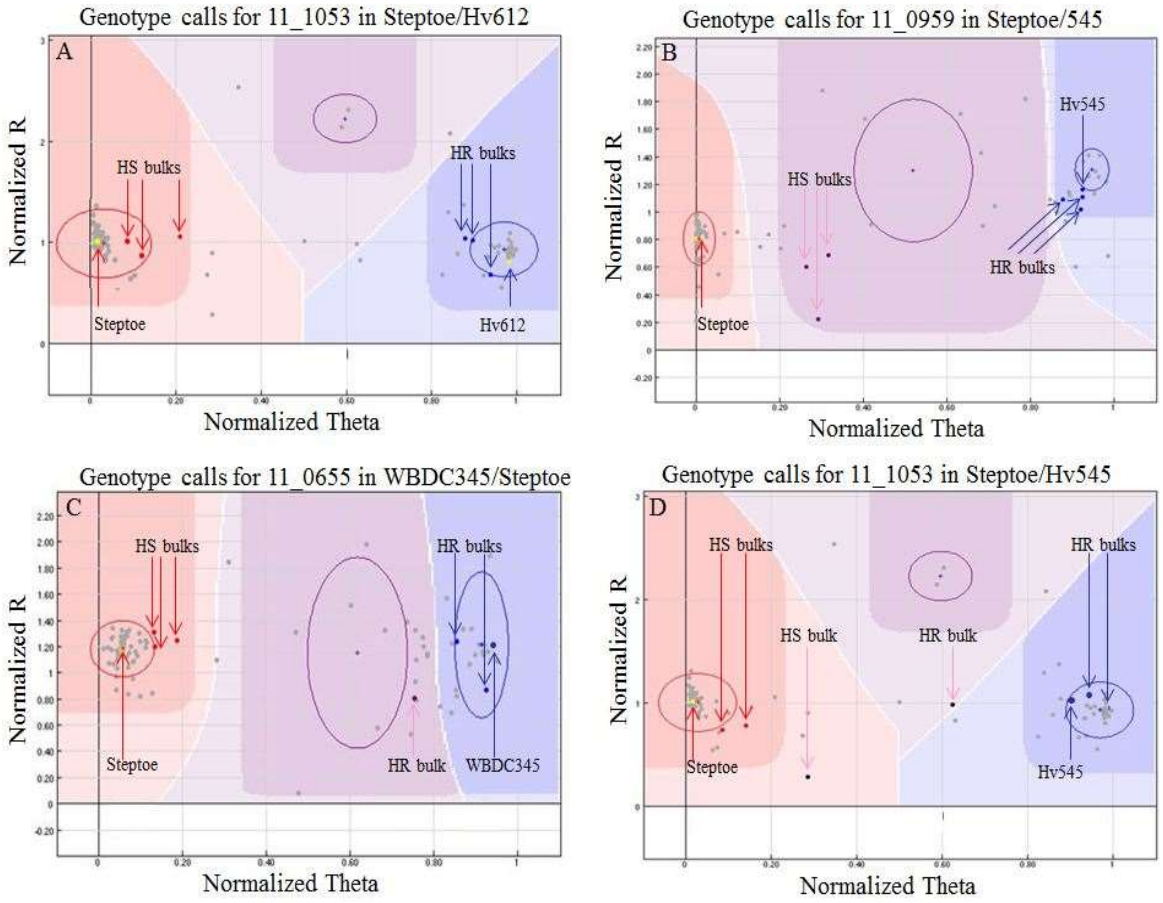


Fig. 2. Consensus genetic map of the long arm of chromosome 5H of barley. The consensus map was constructed from the Steptoe/Morex (SM) doubled haploid line population (Kleinhofs and Graner, 2001) and modified from a presentation by R. Brueggeman (*unpublished*). SNP markers detected in the subtelomeric region of the long arm of chromosome 5H through bulked segregant analysis are indicated with the thinner and longer line. SNP marker names begin with numbers according to the BOPA_C nomenclature used in Close et al. (2009) followed by the name of the cross in which they were detected. Cumulative cM distances are in parenthesis next to the marker name as given by Muñoz-Amatriaín et al. (2011). The stem rust resistance gene complex *rpg4/Rpg5*, given in bold italics on the left-hand side of the subtelomeric region of the hypothetical chromosome arm, was previously mapped to this region (Borovkova et al., 1995; Steffenson et al., 2009). The cM position of the *rpg4/Rpg5* locus and the linked RFLP marker ABG390 is approximate based on the map position of the latter in recent barley consensus maps (GrainGenes, 2013). SNP markers that were positive in each of the three resistant and susceptible bulks are indicated in bold. SNPs detected in more than one population are followed by an asterisk. The line scale on the left-hand side of the figure gives approximate map distances in Kosambi cM at 10 cM interval.



828

829 **Fig. 3.** The clustering of sample GoldenGate assay results from GenomeStudio that were
830 considered a positive hit for SNP markers linked with TTKSK resistance in landrace and wild
831 barley accessions using bulked segregant analysis. (**A**) the three susceptible bulks clustered with
832 the susceptible genotype Steptoe and the three resistant bulks clustered with the resistant genotype
833 Hv612, (**B**) the three resistant bulks clustered with the resistant genotype Hv545, but the three
834 susceptible bulks did not cluster with Steptoe, (**C**) the three susceptible bulks clustered with
835 Steptoe and two of the three resistant bulks clustered with the resistant genotype WBDC345, and
836 (**D**) two of the susceptible bulks clustered with Steptoe and the two of the resistant bulks clustered
837 with Hv545. The cluster position of the bulks (resistant vs. susceptible) and the parents (resistant
838 vs. Steptoe) are indicated with arrows (HR: homozygous resistant; HS: homozygous susceptible).
839 The *X* axis is normalized theta. A normalized theta value nearest 0 is homozygous for allele A, and
840 a theta value nearest 1 is homozygous for allele B. The *Y* axis is normalized R (Fan et al., 2006).