Newly Discovered Genes for Resistance to Powdery Mildew in the Subtelomeric Region of the Short Arm of Barley Chromosome 7H

Martina SOLDÁNOVÁ[1], Jan IŠTVÁNEK[1], Jana ŘEPKOVÁ[1] and Antonín DREISEITL[2]

[1]Department of Experimental Biology, Masaryk University, Faculty of Science, Brno, Czech Republic; [2]Department of Integrated Plant Protection, Agricultural Research Institute in Kromeriz, Kroměříž, Czech Republic

Abstract

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Two dominant genes for resistance to powdery mildew (caused by *Blumeria graminis* f.sp. *hordei*) from the PI296825 and PI466461 accessions of wild barley (*Hordeum vulgare* subsp. *spontaneum*) were identified close to the subtelomeric region of the short arm of chromosome 7H. Genetic analyses predicted two resistance loci in F_2 populations established from crosses between each of the two accessions and the winter barley (*H. vulgare*) variety Tiffany. Genetic mapping revealed a highly effective (52% of phenotypic variation) resistance gene from PI296825 located between the markers *GBMS192* and *GBM1060*. In F_2 plants exhibiting resistance reaction types (RT) 0 to RT1–2, specific DNA fragments for co-segregating markers were amplified. In plants with RT2 and RT2–3, the resistance was conferred by another unidentified resistance gene. In PI466461, the resistance gene found on the short arm of chromosome 7H was flanked by the markers *GBM1126* and *GBM1060*. Another resistance gene coincided with the *Mla* locus. Resistance in RT0 plants was conferred by both resistance genes, which accounted for 58% of the total phenotypic variation. The two resistance genes with the same location on chromosome 7H have different phenotypic effects on the resistance in RT0 plants; therefore, the resistance alleles could be at different loci.

Keywords: DNA marker; genetic mapping; *Hordeum vulgare*; simple sequence repeats

Blumeria graminis DC. f.sp. *hordei* Ém. Marchal (*Bgh*) is an obligate biotrophic fungus that causes powdery mildew, a common disease in temperate climates and the most frequent disease affecting barley (*Hordeum vulgare* L.) in the Czech Republic (Dreiseitl 2011). Powdery mildew can be controlled with resistant varieties. However, the high mutation rate of the pathogen can give rise to new pathotypes able to overcome race-specific resistance genes in new cultivars within a few years (McDonald & Linde 2002). Non-specific and durable resistance to almost all known isolates of *Bgh* can be achieved by the recessive *mlo* allele of the *Mlo* locus. Just two isolates are known to produce moderate levels of the disease on *mlo*-plants: "Race 1" from Japan (Lyngkjaer *et al*. 1995) and HL3, produced in the laboratory from the avirulent GE-3 isolate by many generations of mass screening (Schwarzbach 1979). All other isolates of *Bgh* produce on *mlo*-plants a small

number of almost fully developed colonies, almost exclusively on cells in contact with the stomata (Anderson 1989). Dozens of cultivated spring barley varieties already contain alleles of the *Mlo* locus. Their growth and cultivation with winter barley varieties also possessing *mlo* alleles would lead to continuous, year-round and long-lasting propagation of *Bgh* on host plants with *mlo*. It could cause continuous adaptation of the pathogen to this unique resistance gene and a gradual increase of pathogenicity (Schwarzbach 1987). Therefore, a "gentleman's agreement" among breeders within EUCARPIA (Slootmaker *et al*. 1984) is still generally respected, keeping the winter and spring barley gene pools apart. The *mlo* allele is thus generally absent in commercial winter barley varieties.

Considering the limited number or complete lack of available new resistance genes for breeding of winter barley, related sources have been screened as potential donors of powdery mildew resistance genes. It was proved that cultivated barley varieties contain only 40% of alleles found in wild sources (Ellis *et al*. 2000) due to the loss of rare alleles during domestication. *H. vulgare* subsp. *spontaneum* (Jahoor & Fischbeck 1987; Řepková *et al*. 2006, 2009a, b; Řepková & Dreiseitl 2010; Tetu rová *et al.* 2010) appears to be a promising new source of resistance to important barley diseases, including powdery mildew. Barley landraces are a precious source of genes controlling important agronomic traits such as resistance to diseases. Novel *Bgh* resistance loci were recently detected in a Spanish barley landrace by Silvar *et al.* (2010).

DNA markers are the primary tools used for genetic mapping of powdery mildew resistance genes, and they are potentially useful for marker-assisted selection (MAS) and marker-assisted backcrossing (MAB). In the late 1980s and early 1990s, restriction fragment length polymorphism (RFLP) markers were developed for mapping. Many of them were linked and co-segregating with the known powdery mildew resistance genes (Görg *et al.* 1993; Kürth *et al.* 2001). Simple sequence repeat (SSR) markers randomly distributed in the genome have proved to be very useful for rough mapping.

Detection of individual resistance genes has been the target of many studies but there is a lack of knowledge of the effects of individual resistance genes on resistance phenotypes. In this paper, we describe the genetic characterisation and chromosomal locations of the powdery mildew race-specific resistance genes from the donors *H. vulgare* subsp. *spontaneum* PI296825 and PI466461. In the wild barley PI466461, two powdery mildew resistance genes on chromosomes 1H and 7H had previously been found (Řepková *et al*. 2006), but no detailed information about their positions and their effects on resistance was available at the time.

MATERIAL AND METHODS

Plant materials and segregating populations. The tested populations were obtained from two crosses between the Tiffany variety as a female parent and wild barley (*H. vulgare* subsp. *spontaneum*) accessions PI296825 and PI466461, which are resistant to powdery mildew. Tiffany is a two-rowed winter barley carrying the powdery mildew resistance genes *Mla7* and *MlaMu2* (Dreiseitl 2007) that have already been overcome. The F_2 populations were obtained after self-pollination of the F_1 plants.

Pathogen isolates and resistance tests. Two virulent pathotypes of *Bgh* held in the pathogen gene bank at the Agricultural Research Institute in Kromeriz (Czech Republic) were used to inoculate the young plants to be tested. A virulent (*Va7*, *VaMu2*) pathotype, 5715, was used for resistance tests with plants of the F_1 (30 plants) and F_2 (238 plants) populations derived from the cross with PI296825 as well as 12 plants from each parent. The resistance tests were performed on leaf segments detached from plants grown in the greenhouse as described by Řepková *et al*. (2006). The reaction types (RTs) of individual F_2 plants were scored on a scale of 0–4 (Torp *et al*. 1978) where RTs of 0, 0–1, 1, 1–2, 2 and 2–3 were considered resistant and RTs of 3, 3–4 and 4 were considered susceptible. The whole F_2 population derived from the cross with PI466461 consisted of 498 plants; a virulent (*Va7*) pathotype, 0323, was used for the resistance tests as described by Řepková *et al.* (2006). The tested populations were developed at the Agricultural Research Institute in Kromeriz.

Inheritance of resistance genes. The data for the observed and expected values for resistant and susceptible plants from the PI296825-derived F_2 population were compared with theoretical Mendelian segregation ratios by a chi-square test, and the number of resistance genes was estimated. For PI466461, two dominant resistance genes were determined as described by Řepková *et al*. (2006).

SSR and CAPS analyses and molecular procedures. A random selection of 128 plants from the PI296825-derived F_2 population was used for molecular analysis. For the PI466461-derived plants, a total of 143 F_2 plants were analysed. DNA extractions from the leaves of parental and F_2 plants were performed using the Gene Elute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co., Steinheim, Germany).

Altogether, 71 SSR markers (Table S1) polymorphic for both parents and encompassing all barley chromosomes were used to find markers linked to the gene of interest in the PI296825 accession. Linked markers were found using DNA from 18 individual susceptible F_2 plants (RTs 3–4 to 4) and 18 individual resistant F_2 plants (RT0) for dominant or recessive resistance alleles, respectively (recessive segregant analysis). The polymorphic SSR markers on chromosomes 1H and 7H used for the analysis of the Tiffany × PI466461 cross are summarised in Table S2. One CAPS marker designed from the *RGH1a* gene sequence from chromosome 1H (Řepková *et al*. 2009a) was used. SSR and CAPS analyses were performed in a volume of 10 µl with 10 pmol of each primer, 0.5 units of Go*Taq*® polymerase in 1× Green Go*Taq*® reaction buffer (Promega, Madison, USA), 0.2 µl of deoxynucleotide mix (10mM; Sigma-Aldrich Co., Steinheim, Germany) and 100 ng of barley DNA. Four PCR programmes for SSRs (Ramsay *et al*. 2000) and one for CAPS (Řepková *et al*. 2009a) were used for DNA fragment amplification. Primers were manufactured by EastPort (Prague, Czech Republic), PCR amplifications were performed in a gradient thermocycler (Biometra, Göttingen, Germany).

The PCR-amplified fragments were separated by electrophoresis in either a 3% agarose gel (Agarose I, Amresco, Solon, USA) or a 10% polyacrylamide gel (Sigma-Aldrich Co., Steinheim, Germany). The DNA fragments were visualised by staining with ethidium bromide. In addition, an 8% denaturing polyacrylamide gel was used for DNA fragment separation (microsatellites *GBM1060* and *GBMS192*) by temperature gradient gel electrophoresis (TGGE Maxi System; Biometra, Göttingen, Germany). A GC clamp was designed on one primer (software available at http://www.biophys.uni-duesseldorf.de/POLAND/poland.html). The following PCR programme was designed for the amplification of the *GBM1060* DNA fragment: 3 min at 94°C; 10 cycles for 30 s at 94°C, 30 s at 60°C, using a touchdown programme with the annealing temperature decreasing by 0.5°C per cycle until 55°C was attained, and 15 s at 72°C; 35 cycles for 30 s at 94°C, 30 s at 55°C and 15 s at 72°C; and a final extension of 5 min at 72°C. The following PCR programme was designed for the amplification of the *GBMS192* DNA fragment: 3 min at 94°C; 45 cycles for 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; and a final extension of 10 min at 72°C. Perpendicular gels revealed the optimal temperature (T1 to T2), which ranged between 37°C and 52°C for *GBM1060* and between 39°C and 49°C for *GBMS192*. A buffered system consisting of 21 g of urea (Penta, Chrudim, Czech Republic), 13.3 ml of a 30% acrylamide and 0.8% bisacrylamide mixture (Sigma-Aldrich Co., Steinheim, Germany), 5 ml of 1× TBE, 2.5 ml of 40% glycerol and 29.2 ml of distilled deionised water was used. After the urea had been dissolved, 500 µl of 10% ammonium persulphate (APS; Amresco, Solon, USA) and 50 µl of TEMED (N,N,N',N'-tetramethylethylenediamine; Sigma-Aldrich Co., Steinheim, Germany) were added. One millilitre of Acryl-Glide (Amresco, Solon, USA) was applied on a glass with holes to prevent the gel from adhering to the glass. Iced BindSilane (3-Methacryloxypropyltrimethoxysilane; SERVA Electrophoresis GmbH, Heidelberg, Germany) was applied on both sides of the TGGE foil to increase gel adhesion. For DNA fragment visualisation silver staining of the gels with 0.2% $AgNO_3$ for 10 min was used after fixation (10 min; 10% ethanol in 0.5% acetic acid). The developer was prepared from 3% NaOH in 0.5% formaldehyde.

Linkage analysis and mapping. Recombination ratios for each individual marker and a particular resistance gene were inferred from marker genotypes of the 18 recessive segregants *rr*. For markers that were found to be linked with the resistance genes by recessive segregant analysis, the significance of the linkage was statistically evaluated by the MapQTL 6 software (Van Ooijen 2009) using 128 (PI296825) and 143 (PI466461) F_2 plants. The order and map distances for each linkage group of markers were established using the JoinMap 3.0 software (Van Ooijen & Voorrips 2001). Markers were grouped together using LOD > 3. The estimate of the map distance (*D*, cM) was determined using Kosambi's mapping function. Resistance genes were located in a genetic group by a composite interval mapping procedure. Significant gene positions were established where

LOD scores reached local maxima. The estimate of the overall amount of phenotypic variation attributed to the individual resistance genes was determined by the MapQTL 6 software.

Plant genotyping and effects of resistance genes on phenotype. The genotype of each F_2 plant was determined by evaluating the tightest DNA marker determined by mapping. Owing to the co-dominant mode of inheritance of all markers, these genotypes were determined as *RR* (homozygous for the amplified fragment from the resistant parent), *Rr* (heterozygous) or *rr* (homozygous for the amplified fragment from the susceptible parent). The cross with two resistance genes at least enabled us to estimate whether a particular plant possessed only one or both resistance genes. The *RR* or *Rr* genotypes indicated the presence of a particular dominant resistance gene, while *rr* indicated the absence of a particular dominant resistance gene. Molecular data for each plant were compared with the phenotype of leaf segments from the same F_2 plant from the cross Tiffany × PI296825 (128 plants) or Tiffany × PI466461 (143 plants).

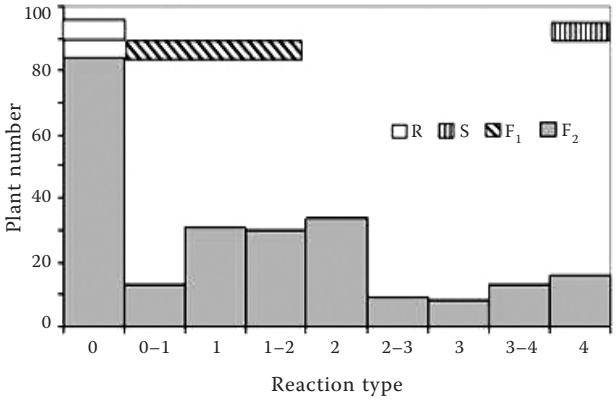

Figure 1. Reaction types of parents, the F_1 hybrid and F_2-plants of the cross Tiffany × PI296825 following inoculation with the pathotype 5715 of *BGH,* virulent to *Mla7* (R – resistant, S – susceptible)

RESULTS

Inheritance of resistance genes in PI296825

Only the RT0 phenotype was found in the resistant parent PI296825; in the F_1 generation, the RT ranged from 0–1 to 1–2. Evaluation of F_2 plants following inoculation with the virulent pathotype revealed the entire range of RTs (Figure 1). A ratio of 13:3 (χ^2 = 1.60, P = 0.21) resulted from the segregation of 201 resistant and 37 susceptible plants in the F_2 population, and this result is consistent with a model of two independent genes with one dominant/semi-dominant and one recessive allele of resistance. This model can also be deduced from Figure 1, where the phenotypic effect of a dominant resistance gene coincides with RT0 and phenotypes encoded by the other locus range around RT2.

Linkage detection and identification of resistance genes on barley chromosomes

Resistance donor PI296825. Five markers (*Bmag0767*, *Bmag0206*, *Bmag0007*, *Bmag0189* and *Bmag0507*) on chromosome 7H displayed recombination ratios under 0.5 (Table 1) using 18 susceptible plants (recessive segregants *rr*). The

Table 1. Recessive segregant analysis and linkage detection in the F_2 population derived from the cross of the winter barley Tiffany and the powdery mildew resistant accession PI296825 of *Hordeum vulgare* subsp. *spontaneum*

Markers on 7H	Genotypes[a]			r[b]
	R_2R_2	R_2r_2	r_2r_2	
Bmag0767	1	2	15	0.11
Bmag0206	1	6	11	0.22
Bmag0007	1	6	11	0.22
Bmag0189	5	0	13	0.28
Bmag0507	2	6	10	0.28
GBM1060	2	2	14	0.17
GBMS192	1	3	14	0.14

[a]based on DNA fragments amplified for a particular marker; inferred from the marker genotype; [b]recombination ratio

tightest linkage was determined with the *Bmag0767* marker; therefore, two other markers in its vicinity, *GBM1060* and *GBMS192*, were included in the recessive segregant analysis (Table 1). These microsatellites were polymorphic in TGGE analysis after a GC clamp was added to one of the primers (Table S3). Figure 2 shows the position of the QTL for powdery mildew resistance on the short arm of chromosome 7H. The linkage between markers *Bmag0189* and *Bmag0507* and the resistance gene indicated by the recessive segregant analysis could not be confirmed. The QTL peaked in the marker interval *GBMS192–GBM1060* with a LOD score of 20.2 and accounted for 52.4% of the total variance.

Resistance donor PI466461. Figure 3 shows the partial genetic map for chromosomes 1H and 7H for the cross Tiffany × PI466461. The CAPS marker *Rgh1aI1a* (Řepková *et al.* 2009a) was mapped to chromosome 1H, distal to *K06257*. The LOD score maximum (6.25) was determined to occur between *K06257* and *Bmac0153*. The estimated position of the resistance locus was found to be 4 cM proximal to *K06257*. The resistance locus on chromosome 7H was positioned between *GBM1126* and *GBM1060* with a LOD score of 7.11. MapQTL 6 revealed that

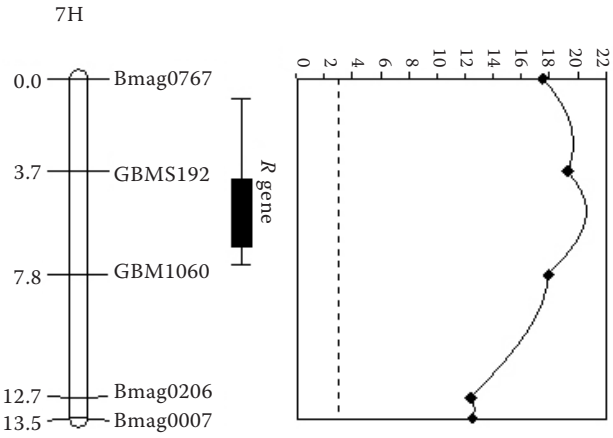

Figure 2. A partial genetic map of the barley chromosome 7HS based on the analysis of the F_2 plants from the cross Tiffany × *Hordeum vulgare* subsp. *spontaneum* PI296825 showing the position of the putative locus, *R*, conferring resistance to powdery mildew

Map intervals in centiMorgans are given to the left of the chromosome using Kosambi's mapping function; DNA marker loci are assigned to the right of the chromosome; the bar to the right of the chromosome map indicates the *R* locus, with confidence interval positioned by the likelihood mapping

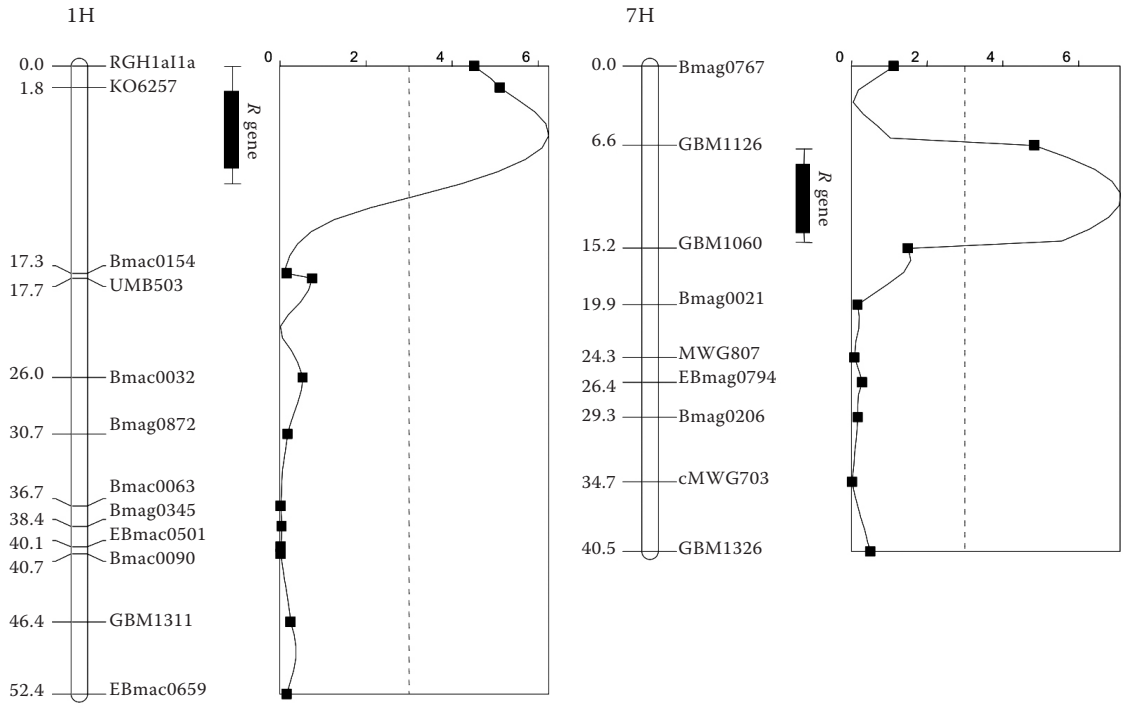

Figure 3. A partial genetic map of the barley chromosomes 1HS and 7HS based on the analysis of the F_2 plants from the cross Tiffany × *Hordeum vulgare* subsp. *spontaneum* PI466461 showing the positions of two putative loci, *R*, conferring resistance to powdery mildew; map intervals in centiMorgans are given to the left of chromosomes using Kosambi's mapping function; DNA marker loci are assigned to the right of the chromosomes; the bars to the right of chromosome maps indicate the *R* loci, with confidence intervals positioned by the likelihood mapping

the QTL on chromosome 1H explained 33.4% of the phenotypic variance, whereas the contribution of the QTL on chromosome 7H was 24.5%.

Plant genotyping and effects of resistance genes on phenotype

The resistance gene from PI296825 co-segregated with the markers *GBMS192* and *GBM1060* on chromosome 7H. Its position was 1.5 cM proximal to *GBMS192* and 2.6 cM distal to *GBM1060*. Out of 83 F_2 plants exhibiting RT0 to RT1–2, DNA fragments of *GBMS192* and *GBM1060* indicative of the presence of the resistance gene were amplified in 79 and 78 plants, respectively. Plants that had undergone crossing-over in the chromosomal region between the resistance locus and a particular marker were deemed susceptible at the molecular level. Four and five plants satisfied this criterion for the *GBMS192* and the *GBM1060* markers, respectively. Molecular analysis showed that the resistance was conferred by an unidentified resistance gene in 17 of the plants with RT2 and RT2–3. In these plants, *GBMS192* and *GBM1060* did not co-segregate with the resistance gene, and these markers mostly identified the plants as susceptible. As expected, no resistance gene was identified in 28 of the susceptible plants with RT3, RT3–4 or RT4.

The phenotypic effects of the two identified *R* genes were studied in PI466461. The two identified DNA markers that linked tightest with the two resistance genes were 4 cM proximal to *K06257* on chromosome 1H and 4 cM proximal to *GBM1126* on chromosome 7H. These two markers were analysed in 143 F_2 plants. The DNA fragments indicative of the presence of the two resistance genes were determined in 83 RT0, RT0–1 and RT1 plants; DNA fragments indicating presence of a single resistance gene were observed in 47 plants with RT0 to RT2, and no resistance allele was found in 13 RT4 plants (Table 2). The ratio of these plant numbers (83:47:13) fits well with a segregation ratio of 9:6:1 ($\chi^2 = 3.18$, $P > 0.05$) and indicates an additive effect of the two genes. The observed deviation could be due to the incomplete co-segregation of the resistance genes with the markers.

Table 2. The evaluation of resistance gene numbers from wild barley (*Hordeum vulgare* subsp. *spontaneum*) accession PI466461 in individual F_2 plants using markers *RGH1aI1a*, *K06752*, *GBM1126* and *GBM1060*

RT[a]	Number of plants with		
	2 genes	1 gene	0 genes
0	60	13	
0–1	16	7	
1	7	12	1[b]
1–2		14	
2		1	
4			12

[a]reaction type determined as phenotypic manifestation of the resistance genes; [b]recombination

DISCUSSION

Plant diseases significantly limit crop production. QTLs offer resistance with durable effects; however, breeding for QTLs is difficult and time-consuming. Some possible solutions involve molecular methods such as MAS, which is effective for major genes and for major QTLs, which explain >10% of the phenotypic variation (Kou & Wang 2010). Therefore, an estimate of the effect of a gene or a QTL identified in resistance donors is of primary importance. Durable resistance can be supported by gene pyramiding. Identification of new resistance genes and alleles in genetic sources is a continuous process. This report presents the detection of highly effective resistance genes against powdery mildew, one on chromosome 1H and two on chromosome 7H.

Many resistance genes have been identified by means of various types of DNA markers. The DNA sequences of the three resistance gene homologues (*RGH*) at the *Mla* locus are useful for the development of new CAPS markers, which exploit point mutations. Based on the known *RGH1a* gene sequence, the polymorphic marker *RGH1aI1a* was obtained for the distal part of chromosome 1HS and revealed linkage with the putative resistance gene on chromosome 1HS that had been introduced from wild barley accession PI466461. In addition, the *K06257* marker was found to be the most tightly linked with this resistance gene (4 cM). Based on our allelism test (Řepková *et al*. 2006) and genetic mapping, this detected resistance gene might correspond to the previously identified *Mla* locus. This gene corresponding to the *Mla* locus possesses high efficiency of resistance (33.4% of the phenotypic variance) and is valuable for

introgression into elite breeding lines for disease improvement. A large phenotypic effect in combination with a cost-effective system of diagnosis is a prerequisite for breeding resistant varieties. Locus complexity due to allelism and/or tight linkage of individual genes further complicates gene identification. Only DNA resequencing of the gene and comparison with known references could answer the question of gene identity.

Our results indicated that the two other powdery mildew resistance genes from *H. vulgare* subsp. *spontaneum* PI466461 and PI296825 mapped closely to the subtelomeric region of barley chromosome 7HS and were flanked by the markers *GBM1126* (4 cM) and *GBM1060* (4.6 cM) and *GBMS192* (1.5 cM) and *GBM1060* (2.6 cM), respectively. The positions of these resistance genes correspond to that of the known gene *mlt* (Schönfeld *et al*. 1996). Its recessive mode of inheritance excludes an identity with the dominant resistance genes from PI466461 and PI296825. On the upper part of the short arm of chromosome 7H, Silvar *et al*. (2010) detected a major QTL with a large effect that accounted for 3.8–18.5% of the total phenotypic variance for *Bgh* resistance. In our study, the major resistance gene from PI296825 accounted for 52% of the phenotypic variation, and both resistance genes from PI466461 accounted for 58% of the phenotypic variation.

The phenotypic effects of powdery mildew resistance genes from PI466461were estimated using the four identified DNA markers linked with the two resistance genes. A good correspondence with a modified Mendelian ratio of 9 (functional proteins conferred by both *R* genes):6 (functional proteins conferred by either *R* gene):1 (non-functional proteins at both *R* genes) was confirmed for the phenotypic manifestation of resistance by molecular data using plants of the segregating F_2 population. We showed that the resistance of RT0, and possibly of RT0–1, was conferred with an additive effect by alleles of the two resistance genes (Table 2). The phenotypic manifestation of resistance in plants with RT1 to RT3 is determined by one resistance gene, and plants evaluated as susceptible (RT3–4 and RT4) possessed no resistance gene. As for PI296825, resistance in plants with RT0 to RT1–2 was conferred exclusively by one dominant resistance gene. The other resistance gene with a lesser phenotypic effect was sufficient for determining the resistance in plants with RT2 and RT2–3. It can be concluded that the two resistance alleles of genes with the same chromosomal location on chromosome 7H have different phenotypic effects on the resistance in RT0 plants and different interallelic relationships; therefore, the resistance alleles could be at different loci. This conclusion could not be drawn only by gene mapping.

In addition to powdery mildew resistance at the *mlt* locus, *Rpg1* stem rust resistance and *Rh2* scald resistance loci have been located in the subtelomeric region of chromosome 7H and the detected genes from PI466461 and PI296825 are components of this known resistance gene cluster. Knowledge of the phenotypic effects of the determined resistance genes together with environmentally insensitive DNA markers could enhance the efficiency of powdery mildew resistance breeding.

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Corresponding author:

Doc. RNDr. Jana Řepková, CSc., Masarykova univerzita, Přírodovědecká fakulta, Ústav experimentální biologie, Kampus Bohunice, Kamenice 5, 625 00 Brno, Česká republika
e-mail: repkova@sci.muni.cz