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Identification of resistance genes against powdery mildew in four accessions of *Hordeum vulgare* ssp. *spontaneum*

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Summary

Four newly detected accessions of wild barley (*Hordeum vulgare* ssp. *spontaneum*) resistant to powdery mildew caused by *Blumeria graminis* f. sp. *hordei* were studied with the aim of finding the number of genes/loci conferring the resistance of individual accessions, the type of inheritance of the genes and their relationships to the *Mla* locus. F_2 populations after crosses between the winter variety 'Tiffany' and four wild barley accessions and use of microsatellite DNA markers were focused on the identification of individual resistance genes/loci by means of their chromosomal locations. In PI466495, one locus conferring powdery mildew resistance was identified in highly significant linkage with the marker *Bmac0213*. This location is consistent with the known locus *Mla* on chromosome 1HS. In the other three accessions the resistance was determined by two independent loci. In PI466197, PI466297 and PI466461, one locus was identified on chromosome 1HS and three new loci were revealed on chromosomes 2HS (highly significant linkage with *Bmac0134*), 7HS (highly significant linkage with *Bmag0021*) and 7HL (significant linkage with *EBmac0755*). Our prospective aim is identification of further linked DNA markers and the exact location of the resistance genes on the barley chromosomes.

Introduction

Powdery mildew caused by the fungus *Blumeria graminis* f. sp. *hordei* DC. f. sp. *hordei* Ém. Marchal (*Bgh*) is one of the most widespread diseases of barley (*Hordeum vulgare* L.). Its epidemics lead to the reduction of grain yield, feeding and malting quality, and profitability for growers.

Wild barley (*H. vulgare* ssp. *spontaneum*) proved to be a rich source of resistance genes against powdery mildew (Williams, 2003). The screening of wild barley accessions revealed that a high proportion of them expressed useful resistance to powdery mildew (Dreiseitl & Bockelman, 2003). This is a consequence of natural selection of the host under long-term occurrence of the pathogen, which generated increased polymorphism in wild barley populations at resistance (*R*) loci.

The knowledge about genetic determination of resistance to powdery mildew involves a multitude of genes (Jørgensen, 1994). The study of the presumed functions of the genes is in progress (Zhou et al., 2001; Panstruga & Schulze-Lefert, 2002; Williams, 2003; Ayliffe & Lagudah, 2004) and the different R proteins

required for defence activation have been characterized.

All seven barley chromosomes carry known disease resistance genes and new genes are continually being examined and positioned in the barley genome. Jørgensen (1994) summarized loci of race-specific genes conferring powdery mildew resistance on chromosomes 1H (*Mla*, *Mlk*, *Mlat*, *Mlnn*, *Mlra*, *MlGa*), 2H (*MlLa*), 4H (*Mlg*, *mlo*) and 6H (*Mlh*). Later, Schönfeld et al. (1996) mapped *Mlj* on chromosome 5H and *mlt* and *Mlf* on chromosome 7H. *Mla*, one of the genetically most thoroughly characterized race-specific loci conferring resistance to powdery mildew, spans 32 known alleles and represents a true allelic series on chromosome 1H (Kintzios et al., 1995). To date, two genes called *Rar1* and *Rar2* have been mapped on chromosome 2H (Lahaye et al., 1998) and were determined to be required for the function of many *Mla* resistance genes and for several unlinked *R* genes (Schulze-Lefert & Vogel, 2000). Two genes, *Ror1* and *Ror2*, are also required for full expression of *mlo* resistance (Freialdenhoven et al., 1996).

Plant breeders continuously implement new *R* genes derived from wild relatives (Noël et al., 1999). Efforts have been focused on monogenic as well as polygenic resistances. Various types of DNA markers proved to be a very efficient and promising way of identifying individual *R* genes and their diverse alleles, so that they could be combined into new barley varieties. In addition to hybridization-based RFLP markers, great interest has been devoted to PCR-based markers, in particular to those based on simple sequence repeats (SSR). A considerable number of microsatellites has been located in genetic maps of barley (Becker & Heun, 1995; Ramsay et al., 2000; Thiel et al., 2003), which has made them a significant tool for gene mapping. AFLP (amplified fragment length polymorphism) markers appear to be promising for the fine mapping of agronomically useful genes (Backes et al., 2003). Kleinhofs (2004) attempted to produce an integrated map of the morphological, physiological and disease resistance markers into the molecular Bin map.

The present study was undertaken to provide an understanding of four recently detected accessions of wild barley resistant to powdery mildew (Dreiseitl & Bockelman, 2003; Dreiseitl & Dinoor, 2004). The objectives of this investigation were: (1) to find the number of genes/loci conferring the resistance of individual wild barley accessions; (2) to find the type of inheritance of these genes; and (3) to find their relationships to the *Mla* locus. Microsatellite DNA markers were focused on the identification of individual resistance genes/loci by means of their chromosomal locations.

Materials and methods

Plant material and population development

Four wild barley accessions (PI466197, PI466297, PI466461 and PI466495), from the USDA National Small Grains Collection, with newly detected resistances to powdery mildew (Dreiseitl & Bockelman, 2003; Dreiseitl & Dinoor, 2004) and the two-row winter barley variety 'Tiffany', carrying the powdery mildew resistance gene *Mla7*, were used as the parental material. 'Tiffany', as a female parent, was crossed with the four resistant accessions. The dormancy of harvested seeds of the F_1 generation was routinely interrupted by keeping the seeds at 38 °C for 48 h, and the seeds were consecutively sown in vegetation pots. For vernalization young plants were grown in a cool room at 5 ± 2 °C for 42 days and then moved into a greenhouse until harvest. The seeds of F_2 generations were obtained after selfing of F_1 plants.

Pathogen isolates

Two pathotypes of *Bgh* held in the gene bank of the pathogen at the Agricultural Research Institute Kroměříž were used for the inoculation of the tested plants. A virulent (*Va7*) pathotype 0323 was used for the finding of the number of genes conferring the resistance of individual accessions and their mode of inheritance, and an avirulent (*Aa7*) pathotype 1002 for the tests of allelism for the *Mla* locus. Each pathotype had previously been purified, verified for the correct virulence phenotype on the differential hosts and increased on cvs. 'Tiffany' (0323) or 'Algerian' (1002).

Resistance tests

Four seeds per genotype (parental, F_1 and F_2 generations) were sown in pots (80 mm upper diameter) in the greenhouse at a continuous temperature of 17 ± 2 °C and under natural daylight. Four segments of about 25 mm in length were cut from the central part of each fully-expanded primary leaf of eighteen-day-old plants, and placed in four dishes on 0.6% water agar with 35 ppm of benzimidazole, for inoculation with each pathotype separately and in two replications. Testing procedure was described by Dreiseitl and Dinoor

(2004). Inoculum density was ca. 8 conidia mm^{-2}. Eight days after inoculation, reaction types (RTs) of leaf segments were scored by the 0–4 scale (Torp et al., 1978). Reaction types 3 and lower were considered resistant. Twenty to forty plants of each parent, 22 to 74 F_1 plants and 320 to 469 F_2 plants of individual crosses were evaluated.

Inheritance of resistance genes

The plant numbers of the two phenotypic categories (resistant and susceptible) found in F_2 populations were compared with theoretical Mendelian segregation ratios by a chi-square test, and the number of resistance genes in each resistance accession was estimated. The comparison of RTs between parental and F_1 generations enabled the determination of the type of inheritance of resistance genes (dominant, semi-dominant or recessive). The results of resistance tests obtained with *Va7* and *Aa7* pathotypes in F_2 populations were compared and the conclusions on allelism for the unknown and *Mla* locus were drawn. If the evaluation of phenotypic manifestation of the trait in the F_2 population revealed both resistant and susceptible plants after inoculation with the *Va7* pathotype and all plants showed only the resistant phenotype after inoculation with the *Aa7* pathotype, the resistance was considered to be determined by alleles of the *Mla* locus.

DNA analysis and linkage detection

At least 97 plants from each F_2 population ('Tiffany' × PI466197, 'Tiffany' × PI466297, 'Tiffany' × PI466461 and 'Tiffany' × PI466495) were used for molecular analyses. 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.), and DNA was quantified by spectrophotometer. A total of 117 microsatellite DNA markers, 111 by Ramsay et al. (2000), *MGB402* and *MGB371* by Korff et al. (2004) and *AWBMS80*, *AWBMS37*, *HvCSG* and *HvBAMY* from the Barley Consensus Map (http://www.graingenes.org/), were used for polymorphism evaluation between the two parental plants of each cross. The PCR amplified fragments were separated by electrophoresis either in 3% agarose gel or 10% polyacrylamide gel. The gels were visualized using ethidium bromide or silver-staining.

Polymorphic DNA markers were used to find markers linked with the gene of interest. The genes at the *Mla* locus or on chromosome 1HS were identified by means of the markers *MGB402*, *GMS021*, *Bmac0213*, *Bmac0063, Bmac0032*, *EBmac0501* and *Bmac0090*. *R* genes with other chromosomal localization were searched by a modified bulk segregant analysis (BSA) where each resistant (RTs 1 and lower) and susceptible bulk (RTs 4 and 3–4) consisted of at least 27 individual plants without DNA pooling.

Identification of resistance genes on barley chromosomes

Once a linkage was identified by BSA, its verification was carried out using at least 97 F_2 plants of all genotypes of the particular F_2 population. The linkage between resistance genes and microsatellite markers found by BSA was detected with marker regression by means of Map Manager QTXb17 package (Meer et al., 2002). The significance of linkage was measured by a likelihood ratio statistic (LRS) and the probability of false linkage $P = 0.001$ was used as the criterion for evidence of linkage. Three threshold values were calculated by the permutation test (available in the Map Manager QTX software) for suggestive ($P < 0.05$), significant ($P < 0.01$) and highly significant ($P < 0.001$) evidence of linkage.

Results

Inheritance of resistance genes

The results summarizing data on gene number estimates and their allelic relationship to the *Mla* locus are presented in Table 1. The evaluation of plants of F_2 populations, after inoculation with the virulent pathotype, revealed the whole scale of RTs including the susceptible ones. Only resistant plants were found after inoculation with the avirulent pathotype except PI466297. The evaluation of the RTs of parental, F_1 and F_2 plants after the screening with the virulent pathotype is given in Figure 1. The comparison of the obtained data which enabled conclusions about the type of inheritance of particular *R* genes is given below.

PI466495 A ratio 3:1 in the F_2 population after inoculation with the virulent pathotype indicated one resistance gene allelic with the *Mla* (Table 1). The semi-dominance of the gene could be assumed with respect to RTs shifting from RT1 in resistant parent to RTs 1–2 and 2 in F_1 plants (Figure 1D).

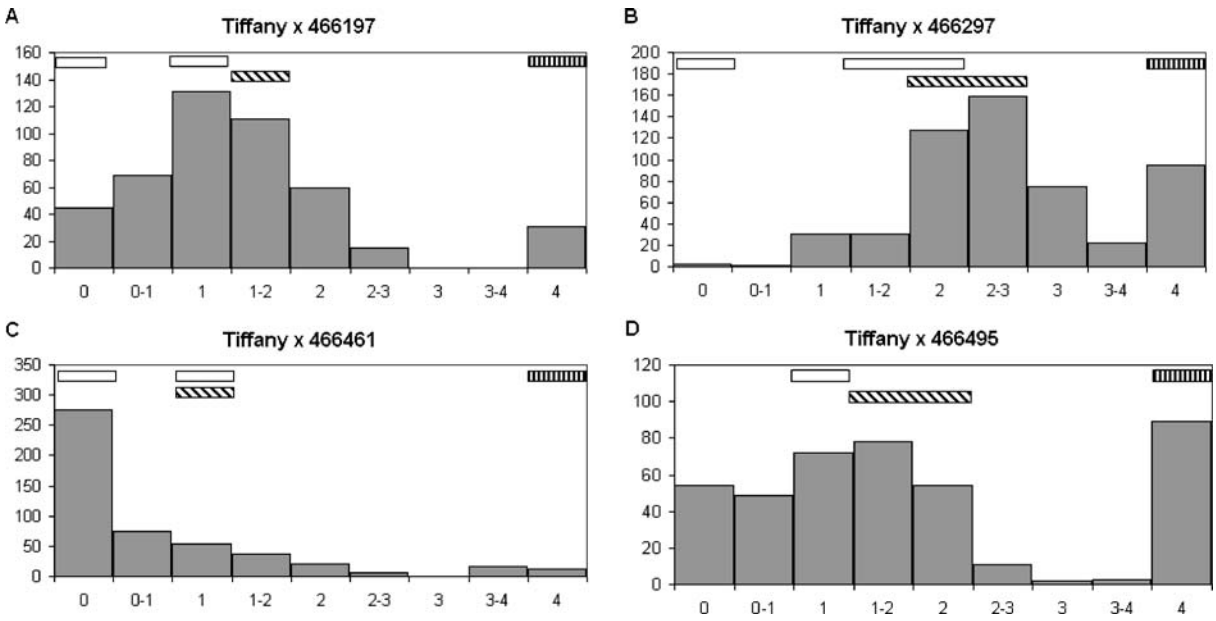

Figure 1. Distribution of reaction types of plants in the F_2 populations after screening with the *Va7* pathotype of *Blumeria graminis* f. sp. *hordei* and comparison with the parental (resistance accessions, variety 'Tiffany') and the F_1 generations. F_2 populations were obtained after the crosses of variety 'Tiffany' and individual resistance accessions of *Hordeum vulgare* ssp. *spontaneum* (A – PI466197, B – PI466297, C – PI466461, D – PI466495). X – scored reaction types (RTs) of leaf segments, Y – the number of plants for individual RTs Resistance accessions; Resistance accessons, Variety 'Tiffany', F_1 generations, F_2 populations

Table 1. The number of resistant and susceptible plants in the F_2 populations of particular powdery mildew resistance accessions of *Hordeum vulgare* ssp. *spontaneum* after inoculation with virulent (*Va7*) and avirulent (*Aa7*) pathotypes of *Blumeria graminis* f. sp. *hordei* and significance of considered segregation ratio

Resistance accession	*Va7* pathotype No. plants		Segregation ratio	χ^2	*Aa7* pathotype No. plants	
	Resistant	Susceptible			Resistant	Susceptible
PI466197	431	31	15:1	0.17*	462	0
PI466297	425	117	13:3	2.86*	532	10
PI466461	469	29	15:1	1.15*	498	0
PI466495	320	92	3:1	1.57*	412	0

Reaction types 3 and lower were considered resistant, 3–4 and 4 susceptible.
*Note.**Significance of the tested segregation ratio was confirmed at $P > 0.05$.

PI466197 A ratio 15:1 in the F_2 population showed segregation consistent with two independent dominant resistance genes (Table 1). Their inheritance was semi-dominant because RTs were 0 and 1 in the resistant parent and 1–2 in the F_1 plants (Figure 1A). Allelism test revealed one of the resistant genes at the *Mla* locus.

PI466461 A ratio 15:1 in the F_2 population revealed two independent resistance genes; one was allelic with the *Mla* locus (Table 1). Two RTs, 0 and 1, were determined in the resistant parent. Only RT1 was determined in the F_1 generation, which indicated semi-dominant inheritance for one gene and dominant for the other (Figure 1C).

PI466297 The resistant parent resembled three different RTs (Figure 1B). A ratio 13:3 in the F_2 population indicated two independent genes, one probably semi-dominant and the other recessive. A

very low portion of susceptible plants after inoculation with the avirulent pathotype did not confirm unique allelism with the *Mla* (Table I).

Polymorphism testing and linkage detection

Using nine recommended protocols (Ramsay et al., 2000), 109 microsatellites were amplified. Additional eight SSR markers producing no PCR products were excluded from the subsequent analysis of polymorphism and linkage detection.

Of 109 microsatellite markers used for polymorphism testing, 59 (54%) turned out to be polymorphic between 'Tiffany' and PI466197. Between 'Tiffany' and PI466297, PI466461 and PI466495 polymorphism was displayed for 65 (60%), 67 (61%) and 59 (54%) microsatellites, respectively.

The linkage was revealed by means of BSA by recessive segregants *rr* (susceptible plants) when resistance was dominant over susceptibility. When resistance was recessive (PI466297), *rr* were resistant plants. For 'Tiffany' × PI466197, 27 microsatellite primer pairs were screened using modified bulk segregant analysis with 31 susceptible plants. *Bmac0213* on chromosome 1HS (5 recombinants out of 56 chromosomes tested) and *Bmag0134* on chromosome 2HS (2 recombinants out of 62 chromosomes) were indicated for linkage with the resistance. In 'Tiffany' × PI466297, linkage with the marker *Bmac0213* (28 recombinants out of 76 chromosomes) was revealed by the screening of 38 susceptible plants. The other gene was found as linked with *EBmac0755* on chromosome 7HL after a screening of 36 resistant plants (18 recombinants out of 72 chromosomes). Altogether 34 markers were screened for these linkage detections. Modified BSA with susceptible plants 'Tiffany' × PI466461 and with 29 markers revealed linkage with *EBmac0501* on chromosome 1HS (14 recombinants out of 54 chromosomes) and with *Bmag0021* on chromosome 7HS (4 recombinants out of 54 chromosomes). Once the linkage was identified for two independent loci, as the genetic analysis indicated, particular F_2 population did not have to be screened with all polymorphic markers. In 'Tiffany' × PI466495, linkage with *Bmac0213* (1 recombinant out of 38 chromosomes) was displayed after the screening of 19 susceptible plants. Subsequently, when the number of susceptible plants was enlarged to 72, 12 recombinants were revealed of 144 chromosomes screened. Other independent loci showed a frequency of recombinants of about 0.5.

Identification of resistance genes on barley chromosomes

Linkage analysis with the polymorphic markers for which a linkage with individual resistance genes was traced by BSA was carried out with 107, 209, 97 and 108 plants of the F_2 populations of 'Tiffany' × PI466197, 'Tiffany' × PI466297, 'Tiffany' × PI466461 and 'Tiffany' × PI466495, respectively. All loci associated with resistance genes in these four accessions are summarized in Table 2. Markers with the highest LRS were included here after marker regression analysis. Significant linkages were revealed for all markers given in Table 2, with the only exception of *Bmag0090* in PI466461, for which only suggestive linkage was found. In 'Tiffany' × PI466461, BSA revealed the linkage between *R* gene on chromosome 1HS and *EBmac0501*, for which LRS reached 7.5 and indicated only suggestive linkage. Therefore, F_2 population was analyzed also for other polymorphic markers on chromosome 1HS, *Bmac0032*, *Bmac0063*, *Bmac0090*. *Bmac0090* showed a little higher LRS value and therefore was included in Table 2.

An expected position of one resistance locus of the cross 'Tiffany' × PI466197 was established on chromosome 1HS between the microsatellites *Bmac0213*

Table 2. The survey of all microsatellite loci associated with resistance loci in particular powdery mildew resistance accessions of *Hordeum vulgare* subsp. *spontaneum*

Resistance accession	Chr	Locus	LRS	P	Significance[a]
PI466197	1HS	*Bmac0213*	33.1	0.00000	***
	1HS	*MGB402*	17.8	0.00014	***
	2HS	*Bmac0134*	23.6	0.00001	***
PI466297	1HS	*Bmac0213*	11.6	0.00303	**
	7HL	*EBmac0755*	17.8	0.00014	**
	7HL	*EBmac0764*	16.7	0.00024	**
PI466461	1HS	*Bmac0090*	8.8	0.01251	*
	7HS	*Bmag0021*	30.6	0.00000	***
PI466495	1HS	*Bmac0213*	43.1	0.00000	***

*Note.*Chr – chromosome (S short arm, L long arm).
Locus – microsatellite marker.
LRS – likelihood ratio statistic.
P – the probability of an association between the trait and SSR marker.
[a] the significance thresholds $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for suggestive, significant and highly significant evidence of linkage, respectively.

and *MGB402*, which was also included in regression analysis (Table 2). The location of the other *R* locus was determined on chromosome 2HS, in association with *Bmac0134*. The locations of two *R* loci in 'Tiffany' × PI466297 were assigned to chromosome 1HS in association with the microsatellite *Bmac0213* and on chromosome 7HL between the SSR markers *EBmac0755* and *EBmac0764* (also included in regression analysis; Table 2). In 'Tiffany' × PI466461, the first association of the trait was suggested with *Bmac0090* on chromosome 1HS. Due to the absence of further polymorphic markers, this location could not be more specified. The other resistance locus was associated with *Bmag0021* on chromosome 7HS. In 'Tiffany' × PI466495, the association of the resistance locus with *Bmac0213* on chromosome 1HS was highly significant.

Discussion

Blumeria graminis f. sp. *hordei* ranks high among all barley pathogens for its adaptability and likelihood to cause crop loss (McDonald & Linde, 2002). The step of crucial importance in barley breeding for resistance against powdery mildew is to obtain new fully effective resistance resources, mainly from wild barley, and to identify new resistance genes. DNA markers have provided a way to combine more fully effective resistance genes in one variety.

Inheritance of resistance genes

Four original accessions of wild barley resistant to powdery mildew were subjected to genetic and molecular analyses. Firstly, genetic analysis in the segregating F_2 populations was performed, and it indicated approximately how many genes and/or loci it would be necessary to look for in individual resistance accessions. A good fit of ratios 3:1, 15:1 and 13:3 for the two tested phenotypic categories showed that the trait was determined by one or two loci. Nevertheless, more or less continuous phenotypic distribution of the trait in F_2 populations indicated the possibility of its more complex inheritance; this means that more linked genes could be localized in one determined locus. Contemporary performed allelism tests simplified the later molecular analysis and gene identification and resulted in finding that one resistance allele in three accessions might be allelic with the *Mla* locus. A very low portion of susceptible plants PI466297 could suggest the presence of a resistant allele in another linked locus.

Polymorphism testing and linkage detection

The screening of SSR markers based on a modified BSA analysis without the pooling of F_2 individuals, proved to be convenient and useful for assessment of the presence and/or absence of linkage. To reveal a linkage between the *R* gene of interest and a DNA marker, we restricted the molecular typing efforts to only recessive homozygote plants *rr*. This method of mapping is commonly used in *Arabidopsis thaliana* as well as in other plant species (Fabri & Schäffner, 1994) because it has the advantage that dominant and recessive alleles of genes can be mapped in the same way. Manipulation with recessive homozygotes can also solve the problem of possible gene interaction. The distances between markers should not be more than 30 cM, optimally 20 cM. Considering the genome size of barley (approximately 1,106 cM; Kleinhofs & Graner, 2001), about 40 markers are theoretically sufficient for such preliminary linkage detection. However, we have to assume the possibility of, for instance, segregation distortion. Mendelian segregation (i.e. a 1:2:1 ratio) was verified for all markers. Markers that proved to have segregation distortion were excluded from molecular analysis. Modified BSA without DNA pooling enabled the finding of a marker in a less tight linkage with the resistance gene than BSA with DNA pooling. BSA, usually performed with DNA pooling, requires many more DNA markers. Polymorphism between individual pairs of our parental lines ranged from 54% to 61%, which corresponded to 59 to 67 SSR markers. To reveal unknown loci in our accessions, about 30 markers were sufficient.

Identification of resistance genes on barley chromosomes

Regression analysis found those markers whose presence was significantly associated with the phenotypic difference, i.e. powdery mildew resistance. The preliminary expectation concerning the gene numbers was confirmed and molecular analyses succeeded in the equivalent phenotype-marker associations and the rough identification of the individual resistance loci according to the known localization of linked SSR markers on barley chromosomes. Knowing the chromosomal localization of the resistance genes is a prerequisite for gene identity assessment. Two genes were

identified in the distinct positions of previously known loci of powdery mildew resistances. In PI466197, aside from one resistance gene on chromosome 1HS, with high significance the *Mla*, another one was localized on chromosome 2HS. In this position no powdery mildew resistance gene has yet been detected. Only *MlLa* and also *Rar1* and *Rar2* were localized on chromosome 2H, but on its long arm (Jørgensen, 1994; Lahaye et al., 1998). The other new locus was identified on chromosome 7HS in PI466461.

Molecular analysis of 'Tiffany' × PI466297 led to the location of a resistance gene on chromosome 7HL linked with the marker *EBmac0755*. The *Mlf* locus for powdery mildew resistance in barley was previously located on the long arm of chromosome 7H. Schönfeld et al. (1996) identified a relatively tight linkage between the *Mlf* gene and the *MWG539* RFLP marker on chromosome 7HL, which is consistent with position 135 cM (Backes et al., 2003). For the present, it cannot be concluded if the resistance gene position is different or not. However, molecular analysis was consistent with genetic analysis and indicated a recessive mode of its inheritance. One resistance gene with a recessive mode of inheritance, *mlt*, was already mapped on the short arm of chromosome 7H (Schönfeld et al., 1996). However, no recessive powdery mildew resistance gene has been assigned to chromosome 7HL.

Besides the major genes, quantitative genes encoding resistance to powdery mildew were also detected on six barley chromosomes (Backes et al., 2003). Three of the five powdery mildew related quantitative trait loci (QTL) share their chromosomal position with already identified qualitative resistance genes. It is known that more than a half of mapped resistance genes are found as clusters containing several closely related genes or as mixed clusters containing members of different gene families (Leister et al., 1998; Wei et al., 1999; Williams, 2003).

Screening *H. vulgare* ssp. *spontaneum* accessions from the centre of barley origin proved to be a good way to obtain new resistance accessions. This approach has been successful in new gene identification. Direct mapping using resistant accessions is only one of the possible approaches. Other eventualities are comparative mapping and mapping of resistance gene analogs, which is done using information on conserved domains in the resistance genes already cloned (Irigoyen et al., 2004).

In summary, three of four wild barley accessions contained two powdery mildew resistance genes with new positions in the genetic map of barley on chromosomes 2HS, 7HS and 7HL. Therefore, further study will be devoted to the finding of additional linked DNA markers in these chromosomal regions. It is necessary to develop new, reliable, PCR based markers for fine-map construction. The availability of molecular markers tightly linked to the resistance genes will greatly facilitate gene transfer into desirable varieties.

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