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Molecular mapping of a new gene in wild barley conferring complete resistance to leaf rust (*Puccinia hordei* Otth)

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Abstract A dominant gene conferring resistance to all known races of *Puccinia hordei* Otth was identified in two accessions of *Hordeum vulgare* ssp. *spontaneum*. Using restriction fragment length polymorphism (RFLP) markers the gene was mapped on chromosome 2HS in doubled-haploid populations derived from crosses of both accessions to the susceptible cultivar L94. Until now, complete leaf rust resistance was not known to be conditioned by genetic factors on this barley chromosome. Therefore, the designation *Rph16* is proposed for the gene described in this study. A series of sequence tagged site (STS) and cleaved amplified polymorphic sequence (CAPS) markers were generated by conversion of RFLP probes which originate from the chromosomal region carrying the resistance gene. Two PCR-based markers were shown to co-segregate with the *Rph16* gene in both populations thus providing the basis for marker-assisted selection.

Key words CAPS · Disease resistance · *Hordeum vulgare* ssp. *spontaneum* · RFLP-mapping · STS

Introduction

Barley leaf rust, caused by the fungal pathogen *Puccinia hordei* Otth, represents an important foliar disease occurring in temperate regions throughout the world. The use of resistant barley varieties has proved an efficient way to control the disease and to prevent yield losses which may reach 32% in susceptible cultivars (Griffey et al. 1994). However, the deployment of resistance genes is counteracted by the occurrence of new, virulent, races of the pathogen thus causing a constant quest for the identification of new sources for resistance. To explore the genetic variability present in the barley gene pool extensive evaluations were performed. Resistance in *Hordeum vulgare* was shown to be very limited and mainly restricted to the genes *Rph3* and *Rph7* (Walther and Lehmann 1980, Jin et al. 1995). On the other hand, a large variability was found to exist in the wild progenitor *Hordeum vulgare* ssp. *spontaneum* C. Koch (in the following abbreviated as *H*. spontaneum) from Israel confirming that the Near East represents a major centre for the evolution of resistance to *P. hordei* Otth (Manisterski et al. 1986; Moseman et al. 1990).

Resistance to leaf rust has been intensively studied during the past decades and 15 genes, denoted *Rph1* to Rph*15*, have been described (Franckowiak et al. 1997). Except for three genes which are from *H. spontaneum* (*Rph10*, *Rph11, Rph15*) these have been identified in cultivated barley. By the use of morphological markers Jin et al. (1993) located *Rph3* and *Rph12* on barley chromosomes 7HL and 5HL, respectively. The *Rph3* locus apparently carries an allelic series, since a dominant as well as a recessive allele was detected (Jin and Steffenson 1994). By its linkage to the *Mla* mildew resistance locus, *Rph4* was mapped on chromosome 1HS (McDaniel and Hathcock 1969). Using trisomic analysis, the *Rph1* gene was assigned to chromosome 2H, and *Rph7*, which is still effective in Europe, was located on chromosome 3HS where it shows linkage to the *Rph5* gene (Tuleen and McDaniel 1971; Tan 1978). In *H. spontaneum* Feuerstein et al. (1990) were able to identify isozyme loci linked to two resistance genes (*Rph10*, *Rph11*) on chromosomes 3HL and 6H, respectively.

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Together with the availability of comprehensive molecular linkage maps the utilization of DNA markers provides the basis for both the accurate localization of resistance genes and the development of selectable markers. In this context, Poulsen et al. (1995) performed bulked segregant analysis and identified a random amplified polymorphic DNA (RAPD) marker linked to a resistance gene present in barley line Q21861. Borovkova et al. (1997) were able to locate this gene on chromosome 5HS at a distance of 1.6% recombination to the *Rrn2* locus. Moreover, these authors provided evidence that the gene from Q21861 is allelic to *Rph2*. Another RAPD marker was identified that maps at a 1.5% recombination distance to the allelic genes *Rph12* and *Rph9* on chromosome 5HL (Borovkova et al. 1998). Linkage was detected between the latter and *Rph13* with a recombination fraction of 30.4% (Jin et al. 1996).

As a result of an ongoing program aimed at the evaluation of a comprehensive collection of *H. spontaneum* accessions for resistance to various pathogens, the present study reports the identification, genetic analysis and molecular mapping of a new gene conferring resistance to highly virulent isolates of *P. hordei*.

Materials and methods

Plant material

Two accessions of *H. spontaneum*. 078-2-0-002 9490 1831 and 084-2-0-016 6180 2002, which show complete resistance to leaf rust were crossed as male parents to the susceptible breeding line L94. The primary origin of both wild barley accessions, in the following designated as HS078 and HS084, which are conserved in the Gene Bank of the Institute for Plant Genetics and Crop Plant Research is unknown. Genetic mapping was performed in two populations of F_1-derived doubled-haploid (DH) lines comprising 46 (L94 × HS078) and 61 (L94 × HS084) lines, respectively. The DH-lines, which were produced by the *H. bulbosum* method, were kindly provided by Dr. R. Pickering (Crop and Food Research, Christchurch, N.Z).

Resistance tests

Wild barley accessions HS078 and HS084 were tested for resistance against leaf rust with a series of isolates, including two from Israel and one each from Morocco and the United States, being virulent on carriers of the resistance gene *Rph7*. Segregation analysis was performed in both DH-populations on five individuals per line using the standard rust isolate I-80, which is virulent on all resistance genes present in European barley, except for *Rph7*. Ten-day-old seedlings were incubated with a mixture of powder (white clay) and uredospores (3:1) for 48 h at 15–18°C in dark moist growth chambers. Subsequently, plants were grown at 18–20°C and 70–80% humidity and 16 h light in a growth chamber. Between 10 and 12 days after inoculation infection types were scored and classified according to a 0–4 scale (Levine and Cherewick 1952) with infection types 0, 0; nc, 1, 2 indicating a high level of resistance and types 2+, 3 and 4 with a high degree of susceptibility.

DNA and linkage analysis

Isolation of genomic DNA, Southern analysis and probe labelling were performed according to standard procedures, essentially as described by Graner et al. (1991). A set of chromosome-specific restriction fragment length polymorphism (RFLP) markers was used to perform bulked segregant analysis in pools formed of ten resistant and ten susceptible DH-lines, respectively. RFLP probes (prefix 'MWG') originated from genomic libraries as described in Graner et al. (1991). Linkage analysis was performed using the program MAPMAKER (Lander et al. 1987). A consensus map was constructed using the program Map Manager (Manly 1993) based on the combined data sets of both progenies. In case markers were polymorphic only in L94 × HS084 progeny, the remaining DH-lines of the second population were treated as missing values. Information about the DNA sequence from both ends of RFLP probes was used to design primer pairs. Oligonucleotide sequences and annealing temperatures (T_a) for PCR amplification of STSs are given in Table 1. PCR was performed in a volume of 20 µl containing 25 ng of DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 9.0, 200 µM of each dNTP, 0.5 µM of each primer and 1 unit of *Taq* polymerase (Boehringer Mannheim). The amplification profile was 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at T_a, and 1 min at 72°C. Monomorphic PCR products were digested with a set of eight restriction endonucleases to generate cleaved amplified polymorphic sequences (CAPS). The four-base cutters *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mse*I, *Msp*I, *Taq*I, and *Tsp*509 were used according to the manufacturers' instructions (New England Biolabs). DNA fragments were separated on agarose gels, stained by ethidium bromide and visualized under UV light. PCR analysis of the simple sequence repeat (SSR) marker HVKASI was performed as described by Liu et al. (1996).

Results

Genetic mapping

Complete resistance to a collection of known races of barley leaf rust, including those races virulent on the *Rph7* gene, was observed in HS078 and HS084. A dominant mode of inheritance for the leaf rust resistance was found to operate in both wild barley accessions since F_1 plants of crosses between both accessions and the susceptible cultivar L94 showed a resistant phenotype and the corresponding F_2 progeny segregated into resistant and susceptible cultivars in a 3 : 1 ratio. The classification of the DH-lines produced from the crosses L94 × HS078 and L94 × HS084 according to their reaction to the standard rust isolate I-80 is depicted in Fig. 1. As expected for the inheritance of a single gene a 1 : 1 segregation ratio was observed χ^2 2.2 and 1.3, 1d.f.). Similar to the reaction pattern of the parents, resistant and susceptible progeny lines could be clearly differentiated. All resistant lines displayed 0;nc infection type, while the susceptible portion of the progeny showed type-3 and type-4 reactions. With a set of previously mapped RFLP probes a new resistance gene, designated *Rph16*, was located to the proximal region of the short arm of chromosome 2H. As expected, the order of common markers was identical in both populations. Markers MWG874 and MWG2133 co-segregated in both populations with the resistance

Table 1 STS and CAPS markers of barley chromosome 2H: primer sequences, annealing temperatures, approximate sizes of amplification products in L94 and restriction endonucleases used for the generation of CAPS

Marker name	Prime sequence (5′–3′)	Annealing temperature (°C)	Size of amplicon in L94 (bp)	Polymorphism[a]
MWG 520	TCC TTG CTT CAC CTT TTC AC CAA AGT CAC GAA AGG GAG AA	60	840	30 bp
MWG 557	TCT CAA TTT GTA TCC AGG GC GAA CAA GCC AAA TGC TAA CC	60	800	*Taq*I
MWG 858	CCG CCT CGT CTG TCA TAC TC CTA CTA ACC CTG CCC GGA AG	60	1300	*Hinf*I
MWG 874	ATG GCA CAC AGG ATG CC AGC GCA CAC AGG ATG CC	62	2800	100 bp
MWG 889	CCA TGA ATT CAC GCG TTA TT TTT CTC GGG TGC TCT GAG AT	50	980	np
MWG 2054	CAG GCT TCC GTT AAA GTG TA GCT CCG TTC CTT TGA TTG TT	50	960	*Taq*I[b]
MWG 2067	GCG GAA AAT AAA TGG TCA TG GAC CGC CAA AGG TAA ATA CC	60	650	np
MWG 2133	TGT GAC GAC TAG TGG CTC G CAC AAG CTT TAC CAC GGT CT	60	2000	60 bp
MWG 2146	CGC GAG CTG GAT TCA TG TAC TGC TGC TAA TGG GTT GC	60	2000	np
MWG 2240	TGA TGT GAG GAT GTT GTG GA TTT CAG AAA CGT GTG CTT GT	55	440	*Taq*I[c]

[a] For STS markers the approximate size difference (in basepairs, bp) between amplicons of both wild barleys and L94 is given. For CAPS markers the enzyme used for mapping is indicated; np: non-polymorphic
[b] Restriction-site polymorphism between HS084 and L94 only;
[c] Restriction-site polymorphism between HS078 and L94 only

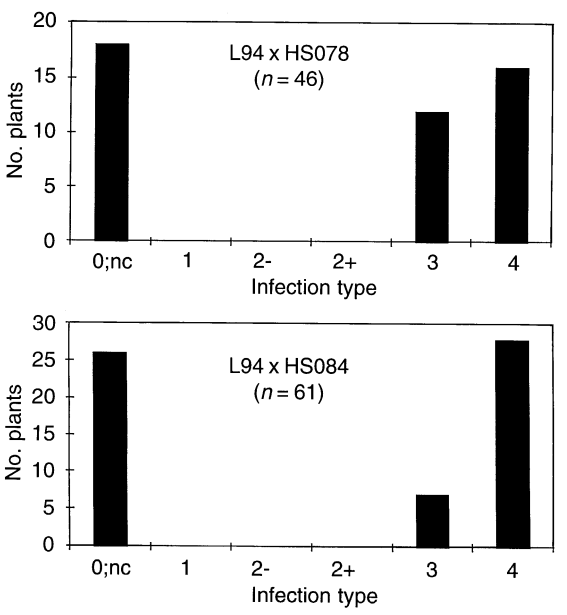

Fig. 1 Reaction to leaf rust isolate I-80 of 61 double-haploid lines derived from the cross L94 × HS084 and 46 DH-lines from L94 × HS078. The parental infection types were 0;nc (HS078, HS084) and 3, 4 (L94)

gene. Therefore, it was decided to combine both progenies to construct consensus map, based on a total of 107 individuals (Fig. 2).

Development of selectable PCR markers

The *Rph16* gene was tagged by a series of closely linked RFLPs which were partly converted into STS- and CAPS markers. In total, sequence information of ten RFLP probes was used to design primer pairs. In Table 1, information for PCR amplification of STSs and the restriction enzymes used for the generation of CAPS is given. The product sizes of three amplicons were found to be distinct between both wild barleys and L94. As an example, Fig. 3 shows the segregation of STS marker MWG2133 alleles in DH-lines produced from F_1 progeny of L94 × HS084. PCR amplification of parental genomic DNA yielded two DNA fragments of different size. While a fragment of about 2 kb in length was obtained using wild barley DNA, cultivar L94 displayed a clearly discernable band of slightly higher molecular weight. For the generation of CAPS markers seven monomorphic amplicons ranging in size from 440 bp to 2 kb were subjected to digestion with eight different endonucleases. Out of these, four amplicons yielded polymorphic fragments upon digestion with at least one out of the eight restriction endonucleases. Two restriction-site polymorphisms were detected between both wild barleys and L94. CAPS markers MWG2240 and MWG2054 were polymorphic only between one accession and cultivar L94. For all CAPS and STS markers represented in Fig. 1 the map positions of the PCR and the corresponding RFLP markers were identical.

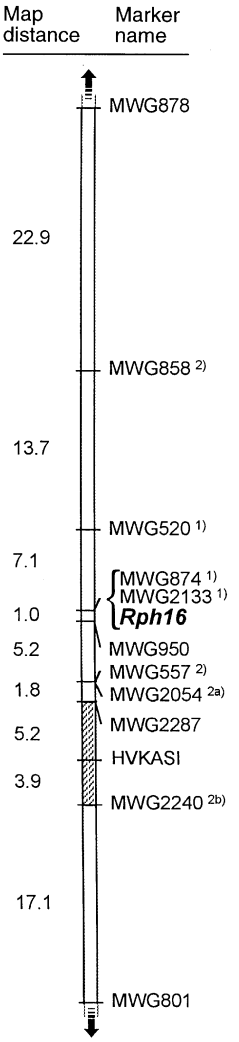

Fig. 2 Partial map of barley chromosome 2H depicting the genetic location of the leaf rust resistance gene *Rph16*. The map shown is based on combined data sets of the two progenies L94 × HS078 and L94 × HS084. Map distances are given in % recombination. The *shaded region* denotes the approximate position of the centromere.[1)] RFLP markers that were converted into STS markers; [2)]RFLP markers which were converted into CAPS markers; [2a)]CAPS marker which which was polymorphic only in DH-population L94 × HS084; [2b)]CAPS marker which was polymorphic only in DH-population L94 × HS078

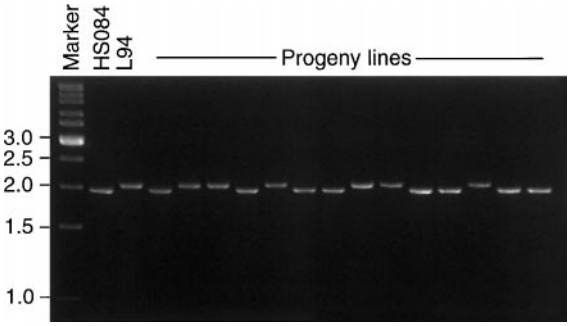

Fig. 3 Segregation pattern of STS marker MWG2133 in a set of DH-progeny lines derived from the cross L94 × HS084. PCR amplification products were separated on a 1% agarose gel. DNA fragment sizes are given in kilobase pairs (size marker: 1-kb DNA ladder; MB1 Fermentas)

Discussion

Similar to powdery mildew the leaf rust disease provides an instructive example for the gradual depletion of useful resistance genes within the gene pool of cultivated barley. Consequently, increasing efforts need to be invested into the exploration of genetic resources of the wild barley *H. spontaneum* for additional genetic variability with relation to disease resistance. Systematic approaches to increase the level of resistance require: (1) the evaluation of the genetic variability present in the gene pool of barley, (2) the localization of resistance genes, and (3) the development of selectable markers. In this context, a gene conferring resistance to all known races of leaf rust, including those overcoming the gene *Rph7*, was detected in *H. spontaneum* accessions HS078 and HS084. Analysis of genetic data from crosses with the susceptible cultivar L94 revealed the presence of a single dominant gene on the short arm of chromosome 2H. Although the results presented in this study refer to seedling resistance only, identical reaction patterns were obtained in field experiments indicating that the *Rph16* gene is also effective during later growth stages. Of all the barley leaf rust resistance genes characterized to-date, only *Rph1* was associated with chromosome 2H (Tuleen and McDaniel 1971). Since this gene does not confer resistance to the rust isolates used in the present study, the gene described herein is distinct and has therefore been designated *Rph16*. No recombination was observed between markers MWG2133, MWG874 and the *Rph16* gene within the two populations analyzed, comprising 107 DH-lines in total. For these and other tightly linked markers identical banding patterns were observed in HS078 and HS084 (data not shown). Given the congruency of the marker positions and the identity of the RFLP patterns we presume that allelic forms of *Rph16* are present in both wild barley accessions.

One objective of barley breeding programs is to increase resistance to leaf rust via introgression of new genes into adapted material. As a first step to this end, the two STS markers developed from RFLP probes MWG2133 and MWG874 will facilitate a rapid and reliable marker-assisted introgression of this gene within few backcross steps. This is of particular relevance for geographical regions, like the United States, where the *Rph7* gene has been recently overcome (Steffenson et al. 1993). In Europe, where *Rph7* is still effective, the use of a molecular markers provides the possibility to efficiently combine *Rph7* and *Rph16* without extensive progeny testing. The application of this strategy requires, however, the precise localization of *Rph7* and the development of selectable markers for that gene.

Like other *Triticeae* chromosomes those of barley are characterized by reduced recombination of proximal regions resulting in apparently high marker densities around the centromere and low marker densities in distal chromosome portions (Korzun and Künzel 1996). Although this effect may be expected to be even more pronounced in maps derived from crosses between genetically divergent parents, the data obtained from our study do not support this hypothesis. With 27% recombination in the present consensus map the interval delimited by markers MWG858 and MWG557 compares well to the 26.2 cM in the Steptoe × Morex map (Kleinhofs et al. 1993). Together with the availability of co-segregating markers, this ensures the transfer of only limited amounts of unadapted *H. spontaneum* chromatin surrounding *Rph16* in the course of marker-assisted selection. In conclusion, the results obtained from the present study further complete the picture of the genetic basis of leaf rust resistance in barley and provide the basis for the rapid deployment of *Rph16* in the context of a breeding program.

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Note added in proof According to the revised recommendations for nomenclature of barley leaf rust resistance genes (Franckowiak et al. 1997) the designation *Rph16* was recently extended into *Rph16.a.e.*

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