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Molecular mapping of the *Rph7.g* leaf rust resistance gene in barley (*Hordeum vulgare* L.)

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Abstract In many temperate areas of the world, leaf rust is becoming an important disease of barley. In the last decade, new races of *Puccinia hordei* G. Otth have emerged which are virulent against the so-far most-effective race-specific resistance genes, such as *Rph7*. Marker-assisted selection greatly facilitates the pyramidization of two or more resistance genes in a single variety in order to achieve a more comprehensive resistance. Such a strategy requires the development of efficient and reliable markers. Here, we have developed a linkage map and found RFLP markers closely linked to the *Rph7.g* resistance gene on chromosome 3HS of barley. The receptor-like kinase gene *Hv3Lrk* that maps at 3.2 cM from *Rph7.g* was used to develop a PCR-based marker by exploiting a single nucleotide polymorphism. This marker was detected in 11 out of 12 (92%) barley lines having *Rph7* and represents a valuable tool for marker-assisted selection. In addition, the identification of markers flanking *Rph7.g* provides the basis for positional cloning of this gene.

Key words Barley · Leaf rust · Marker-assisted selection · Resistance gene · Single-nucleotide polymorphism

Introduction

Leaf rust, caused by *Puccinia hordei* G. Otth is becoming an increasingly important pathogen of barley in temperate areas of the world. In Europe, North Africa, Australia, New Zealand, Eastern and Midwestern United States it can cause severe yield losses in susceptible varieties (Cotterill et al. 1992a). Until recently, breeding for resistance efficiently controlled barley leaf rust but in the last 20 years several resistance genes became ineffective. *Rph3* has been overcome in Europe (Clifford 1985) and *Rph12* in Europe and Australia (Clifford 1985; Cotterill et al. 1992b). *Rph7* was for a long time considered as the most effective resistance gene against leaf rust and has been widely used in breeding programs worldwide. At the beginning of the eighties, virulence against *Rph7* was reported in Israel and Morocco (Golan et al. 1978, Parlevliet et al. 1981) and more recently in the United States (Steffenson et al. 1993; Griffey et al. 1994). However, *Rph7* is still effective in Europe and therefore represents an interesting target for resistance breeding in combination with other genes. Until now, 16 barley leaf rust resistance (*Rph*) genes have been identified at the genetic level. Based on linkage or trisomic analysis, seven *Rph* genes have been assigned to barley chromosomes (Ivandic et al. 1998) but none has been isolated. *Rph7* has been assigned to chromosome 3HS (Tuleen and McDaniel 1971; Tan 1978) but no linkage map has been established so far at the *Rph7* locus. Molecular markers have been developed only for three leaf rust resistance genes: *RphQ* on chromosome 5HS which is most likely allelic to *Rph2* (Borovkova et al. 1997), the allelic genes *Rph9* and *Rph12* on chromosome 5HL (Borovkova et al. 1998), and *Rph16* (Ivandic et al. 1998) on chromosome 2HS. In breeding programs, one possibility to achieve more durable resistance consists of combining two or more different resistance genes in one elite line. The development of molecular markers for leaf rust resistance genes should support this strategy through marker-assisted selection.

In the absence of an efficient gene-tagging system, gene isolation in large genomes such as barley and wheat is limited to map-based cloning strategies. In recent years a considerable effort in developing molecular markers, together with the establishment of syntenic relationships between the grass genomes, has led to the development of comprehensive linkage maps in these species. Furthermore, several large insert genomic libraries including BAC and YAC libraries (Kleine et al. 1993) have been developed in barley. The recent isolation of

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the resistance gene *mlo* has shown that map-based cloning is feasible in barley (Büschges et al. 1997). Here, we report the genetic localization of molecular markers for the *Rph7.g* leaf rust resistance gene and the development of a PCR-based marker suitable for the detection of this gene in practical breeding programs. In addition, the finding of flanking markers around the *Rph7.g* resistance gene represents the first step in the isolation of this gene through map-based cloning.

Material and methods

Plant material

Studies were performed with the near-isogenic lines (NILs) Bowman8*/3/ND771/Cebada Capa/MT81195 (referred as Bowman8*/Cebada Capa) and Bowman, as well as the *Rph7* donor spring barley line Cebada Capa. The NILs were developed (Chicaiza et al. 1996) and kindly provided by Dr. J.D. Franckowiak (Dakota State University, USA). Two segregating F_2 populations consisting of 112 individuals derived from a cross between Cebada Capa and Bowman, and of 109 individuals derived from a cross between the near isogenic lines (NILs) Bowman8*/Cebada Capa and Bowman, were used for linkage analysis. F_3 seeds were produced from each F_2 plant and 20 seeds per family were used for further phenotypic analysis of the F_3 progeny of the Cebada Capa×Bowman cross. Twelve barley breeding lines, Cebada Forrajera, Ab1122, Selection out of Cebada Capa, Dabat, La Estanzuela 76a, H2210, H2211, H2212 and Debra Sina kindly provided by Dr. A. Börner (IPK Gatersleben, Germany), Heris provided by Dr. A. Dreiseitl (Agricultural Research Institute Kromeriz, Czechoslovakia), Hanka from Saatzucht Hadmersleben (Germany) and Ellinor kindly provided by Dr. A. Falk (Upsala University, Sweden), known to posses *Rph7* were used to validate the PCR-based marker. Twenty five barley lines, Elisa, Michka, Meltan, Pallas, Golden Promise provided by the FAL (Zürich-Reckenholz, Switzerland), Gull, Lechtaler and Triumph provided by the NDSU (USA), Sundance, Trumpf, Peruvian, Egypt IV, Sudan, Bolivian, Magnif 102, Magnif 104, CI 1243, Quinn, Gold, Ribari, L94 and Tunisian 17 provided by Dr. R. Niks (Wageningen Agricultural University, The Netherlands), Morex, Franka and Igri provided by Dr. A. Graner (IPK Gatersleben, Germany), which have not been shown to contain the *Rph7* resistance gene, were also analyzed to validate the specificity of the marker for *Rph7.g*.

Artificial infection of barley plants with leaf rust

The phenotypes of the parental, F_2 and F_3 plants were analyzed by artificial infection with barley leaf rust isolate 1.2.1 avirulent on *Rph7* (kindly provided by Dr. R. Niks, Wageningen, The Netherlands). Plants were grown in a growth chamber at a 18°C/14°C day/night temperature, a 70/85% day/night relative humidity and a photoperiod of 16 h (360 µM $m^{-2}s^{-1}$ photosynthetic photon flux density). Ten-day old seedlings were inoculated with uredospores of the isolate 1.2.1 sprayed as a suspension with the mineral oil "Soltrol 170" (Philips Petroleum, Paris). Inoculated plants were kept at 16°C and 100% humidity for 24 h in the dark. Plants were then kept for another 9 days at growth conditions with a 16-h photoperiod, a 20°C/16°C day/night temperature and 90% relative humidity. The infection type on the F_2 and F_3 plants showing a hypersensitive reaction (black necrotic flecks) was attributed to resistant plants, while susceptible individuals showed large uredia with full sporulation and without any chlorosis or necrosis. Heterozygous plants showed tiny uredias in the center of necrotic flecks.

RFLP and linkage analysis

DNA isolation of mature leaves and Southern blotting was performed as described by Graner et al. (1990). Thirteen micrograms of genomic DNA of parental lines Cebada Capa, Bowman8*/Cebada Capa and Bowman were digested with seven restriction endonucleases (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I). Southern hybridization was performed with 75 RFLP probes mapping on chromosome 3HS of barley, chromosome group 3 of wheat, C of oat or 1S of rice. The cDNA and genomic probes derived from wheat (PSR, TAG, Ttksu and WG), oat (CDO), barley (BCD, MWG, ABC and ABG) and rice (RG and RZ) are described in the Graingene database (http://genome.cornell.edu); the rice cDNA probes (C, G, L, R, and S) are described at the Rice Genome Program database (http://www.staff.or.jp/index.html). Probes were kindly provided by the Cornell Research Foundation (Cornell University, USA), Dr. M. Schönfeld (Federal Centre for Breeding Research, Grünbach, Germany), Dr. M.D. Gale (John Innes Centre, Norwich, UK), Dr. A. Kleinhofs (Washington State University, USA) and Dr. T. Sasaki (National Institute of Agrobiological Resources, Japan).

Linkage estimation was based on the maximum-likelihood method using MAPMAKER (Lander et al. 1987) and the Kosambi function.

Conversion of the *Hv3Lrk* gene into a PCR-based marker for *Rph7.g*

Two primers Lrk3S1 (5´GAGCACCTGGCTTGGAGCT3´) and Lrk3S2 (5´TTGAACACAGGTACGCTTGG3´) corresponding to nucleotide positions 181–199 and 936–953, respectively, were designed from the sequence of the receptor-like kinase gene *Hv3Lrk* (Feuillet and Keller 1999). PCR was performed on 50 ng of genomic DNA isolated from the susceptible parent Bowman and the resistant parent Cebada Capa. PCR-amplification was performed in 25-µl reaction containing 0.5 units of *Taq* DNA-polymerase (Sigma, Switzerland), 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin), 100 µM of dNTPs and 400 nM of primers. Amplifications were performed in a PTC-200 thermocycler (MJ Research, Bioconcept, Switzerland) as follows: one cycle of 3 min at 94°C, 30 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C. The extension of the amplified products was achieved at 72°C for 5 min. The fragments of 772 bp, amplified from both parents, were cloned into the pGEMT vector (Promega, Switzerland) and subsequently sequenced on an automatic sequencer Licor DNA4200 (MWG, Germany). Comparison of the sequences (AF187301 and AF187302) revealed a single nucleotide polymorphism at position 225. The Lrk3SNP3 primer (5´ATCCGCAGGATGCCC3´) was designed from position 211 to position 225 on the Cebada Capa sequence (AF187301) with the SNP (C) at the 3´ end of the primer. The primer Lrk3S4 (5´TTGGCCCAATCTCTTGC3´) was designed in a conserved sequence (position 456 to 472) in order to have exactly the same melting temperature as Lrk3SNP3. PCR-amplifications were performed on 50 ng of genomic DNA in the same conditions as described above except for the annealing temperature which was set to 52°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV light after ethidium bromide staining.

Results

Segregation analysis of the *Rph7.g* resistance gene

The phenotype of 112 F_2 individuals derived from the cross between Cebada Capa and Bowman was determined after artificial leaf rust infection. Eighty four F_2 plants were scored as resistant and 28 as susceptible. The

resistance gene followed a 3:1 segregation indicating the dominant action of *Rph7.g* in this cross. Artificial infection performed on 20 F_3 seedlings of each 112 F_2 individuals allowed us to distinguish homozygous resistant from heterozygous resistant F_2 plants for a more-accurate linkage analysis. The F_2 population was then characterized as 38 homozygous resistant, 46 heterozygous resistant and 28 homozygous susceptible individuals ($\chi^2_{0.05,2}$=5.36). In the second F_2 population, 109 individuals out of a cross between the NILs Bowman8*/Cebada Capa and Bowman were analyzed. Thirty plants showed the same resistant phenotype as Cebada Capa, 22 showed a susceptible reaction, while 57 showing an intermediate phenotype with tiny uredinias in the center of a necrotic fleck were considered as heterozygotes ($\chi^2_{0.05,2}$=1.36). These data showed a 1:2:1 segregation in this population suggesting that *Rph7.g* has an intermediate action in this cross.

Identification of molecular markers linked to *Rph7.g*

The hybridization pattern of 75 RFLP probes from barley, wheat, oat and rice was analyzed on Southern blots containing the two resistant parental lines, Cebada Capa and Bowman8*/Cebada Capa, and the susceptible line Bowman. Forty nine probes that showed at least one polymorphic fragment between the resistant and susceptible parents were tested on the segregating populations. In the F_2 population originating from the cross Cebada Capa×Bowman, ten barley, one oat and two rice RFLP probes showed linkage to *Rph7.g*. The barley probes ABC171 and CDO395 mapped at 16 cM and 22.4 cM, respectively, on the proximal side of *Rph7.g*, while the rice probe S1543 mapped at 2.3 cM distal to the gene (Fig. 1). A cluster of three cosegregating barley markers (Hv3Lrk, cMWG691 and MWG848) was mapped at 0.9 cM distal to S1543 while MWG2158 mapped 1.3 cM from this cluster. The rice probe C970 mapped at 0.9 cM from MWG2158, and BCD907 at 5 cM from C970 in the distal telomeric region (Fig. 1).

In the F_2 population originating from the NILs Bowman8*/Cebada Capa×Bowman cross the four probes S1543, cMWG691, MWG2158 and Hv3Lrk cosegregated at 7 cM from *Rph7.g* (data not shown). In the first mapping population, recombination was found between these markers (Fig. 1) suggesting a reduced recombination in the cross between the NILs compared to the Cebada Capa×Bowman cross. Based on these results we focused our study on the Cebada Capa×Bowman segregating population.

The receptor-like kinase gene Hv3Lrk as a PCR-based marker for the *Rph7.g* resistance gene

With the aim of using PCR to support the marker-assisted selection of *Rph7* in breeding programs, we converted the barley RFLP marker Hv3Lrk that mapped at 3.2 cM from the *Rph7.g* gene into a PCR-based marker. The receptor-like kinase gene sequence *Hv3Lrk* (Feuillet and Keller 1999) was used to design two primers (Lrk3S1 and Lrk3S2) which led to the amplification of a single PCR fragment with an expected size of 772 bp in the parents Bowman and Cebada Capa. Sequencing of the respective fragments (AF187301 and AF187302) revealed single nucleotide polymorphisms (SNPs). A SNP at position 225 was then used to design a primer (Lrk3SNP3) which perfectly matched the sequence amplified from the resistant parent Cebada Capa (AF187301) but had a single mismatch at the 3′ end of the sequence corresponding to the Bowman allele. PCR performed on genomic DNA with the Lrk3SNP3 and Lrk3S4 primers resulted in the amplification of a polymorphic fragment (Lrk3ph7) with an expected size of 261 bp only in the resistant parents Cebada Capa and the NIL Bowman8*/Cebada Capa. Two additional fragments of about 550 bp and 800 bp were also amplified in both susceptible and resistant genotypes. The polymorphic 261-bp fragment segregated with the *Hv3Lrk* gene in the 112 individuals of the segregating population (Fig. 2a). Thus, Lrk3ph7 is a dominant PCR-based marker that segregates at 3.2 cM from the *Rph7.g* resistance gene.

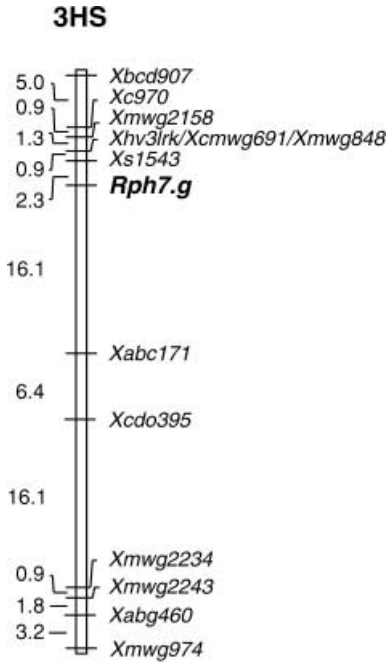

Fig. 1 Genetic map of the *Rph7.g* locus on barley chromosome 3HS. Linkage analysis was performed on 112 F_2 plants from a cross between Cebada Capa and Bowman with RFLP probes from barley (BCD, ABC, MWG, ABG), rice (S, C) and oat (CDO). Distances (in cM) between loci are listed on the left side of the diagram

Lrk3ph7 as a tool to detect *Rph7.g* in breeding programs

The breeding value of the Lrk3ph7 marker was tested on 12 barley lines containing the *Rph7* resistance gene in

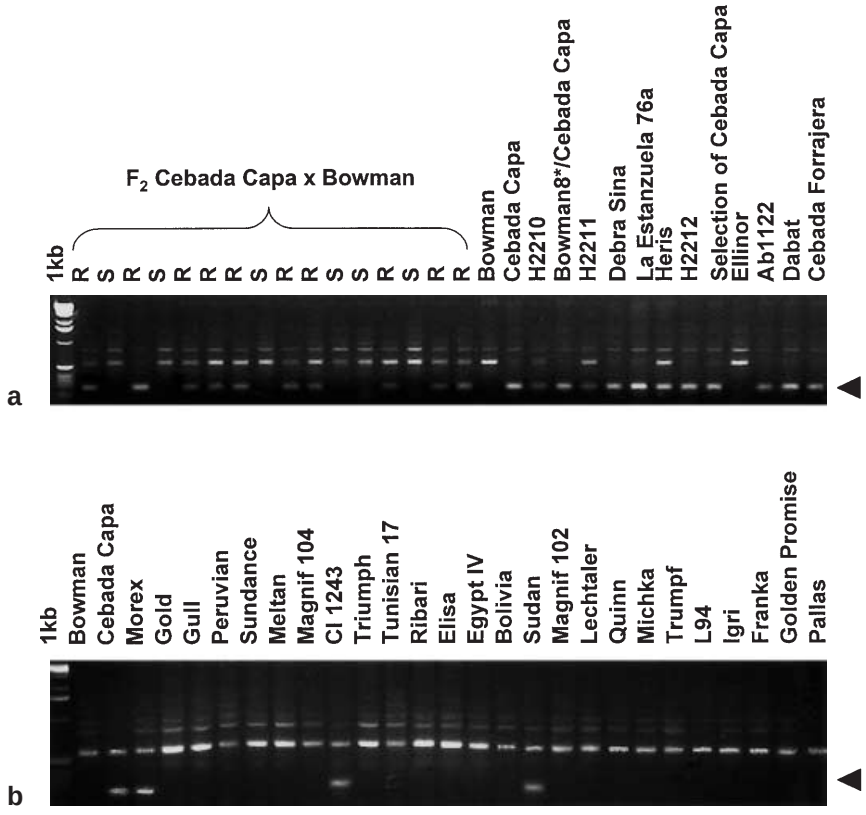

Fig. 2a, b Detection of the *Rph7.g* leaf rust resistance gene with the PCR-based marker Lrk3ph7. **a** Segregation pattern of the Lrk3ph7 marker in a set of F_2 plants derived from the cross Cebada Capa×Bowman and detection of Lrk3ph7 in 11 out of 12 barley breeding lines containing the *Rph7* resistance gene. **b** The Lrk3ph7 marker is absent in 22 out of 25 barley lines without the *Rph7* resistance gene. The *arrowheads* indicate the 261-bp fragment corresponding to the Lrk3ph7 marker. The size marker is the 1-kb ladder

different genetic backgrounds. PCR on genomic DNA resulted in the amplification of the 261-bp polymorphic fragment in all the lines except Ellinor (Fig. 2a). This result suggests that recombination has occurred in this line between the resistance gene and *Hv3Lrk*. We have also tested 25 barley lines from different origins not known to contain the *Rph7* resistance gene. The Lrk3ph7 marker was amplified only in the three lines Morex, CI1243 and Sudan (Fig. 2b). An artificial infection performed with the leaf rust isolate 1.2.1 avirulent on *Rph7* did not induce any resistance reaction in these three lines. This suggests that either recombination occurred between the resistance gene and *Hv3Lrk* or that these lines contain another *Rph7* allele that is not recognized by the isolate 1.2.1.

Linkage analysis showed that the rice probe S1543 was the closest marker to the resistance gene. However, the conversion of S1543 into a PCR-based marker was not feasible due the presence of several bands and a high background signal obtained after hybridisation of this probe with barley DNA. Hybridisation of S1543 on the barley lines with *Rph7* showed that the fragment that segregated at 2.3 cM from the resistance gene was present in all these lines (data not shown). Interestingly, the segregating fragment was present in Ellinor, the only line containing *Rph7* that did not show the Lrk3ph7 product. Thus, S1543 can be used in combination with Lrk3ph7 to assess the presence of the *Rph7* resistance gene in barley breeding lines.

Discussion

Development of a PCR-based marker and detection of *Rph7* in breeding lines

Molecular markers can support classical breeding in crop plants by shortening the time of selection in some phases of the breeding programs (Mohan et al. 1997). Only very recently, PCR-based markers have been developed for major leaf rust resistance genes in barley, i.e. *RphQ* (Borovkova et al. 1997), *Rph12* (Borovkova et al. 1998) and *Rph16* (Ivandic et al. 1998). The development of easy and reliable markers is of great importance for the efficient introgression of leaf rust resistance genes into barley breeding material. This is particularly true in barley growing regions where virulence has been found for major resistance genes such as *Rph7*. In general, the combination of genes such as *Rph16* and *Rph7* (Ivandic et al. 1998) through marker-assisted selection is highly suitable to achieve more-durable resistance. Here, we have developed a dominant PCR-based marker for the *Rph7.g* resistance gene on chromosome 3HS. We successfully converted the Hv3Lrk RFLP probe into a PCR-based marker by exploiting the presence of SNP in the sequence amplified from the parents of our mapping population. Lrk3ph7 has been amplified from more than 90% of the lines known to contain *Rph7*. Only 3 out of 25 lines without *Rph7* showed the same allele as lines containing the gene. In those lines, we cannot exclude that recombination occurred between the Lrk3ph7 mark-

er and the *Rph7* gene. Alternatively, they may contain other alleles than *Rph7.g* that are not detected by the 1.2.1 isolate. Indeed, there is evidence for the existence of two or more resistant alleles at the *Rph7* locus (Dr. Franckowiack, personal communication). We conclude that the Lrk3ph7 marker has a good specificity for the *Rph7* resistance gene and can be used as a diagnostic tool in breeding programs. However, when using this marker, possible recombination events occurring between the marker and the gene have to be considered.

The *Rph7.g* resistance gene segregates differently in both F_2 populations

Phenotypic analysis revealed a difference in the *Rph7* gene behavior between the two segregating populations we used in this study. Indeed, in the cross between the NILs, *Rph7* shows intermediate inheritance while in the cross between Cebada Capa and Bowman we could not accurately detect heterozygous individuals, suggesting a dominant action of the gene. Such effects of the genetic background on gene expression have been already observed in wheat with leaf rust resistance genes such as *Lr35* (R. Seyfarth, personal communication) where the gene was dominant in the Thatcher*Lr35*×Frisal cross and codominant in the Thatcher*Lr35*×Thatcher cross. It has also been reported that *Lr2c* in the background of Prelude was recessive when crossed with Thatcher, and dominant in crosses with Prelude and Red Bobs (Dyck and Samborski 1974).

Linkage analysis showed a discrepancy in the recombination rate between the two segregating populations. There was a lower frequency of recombination between the markers in the F_2 population originating from the cross between the NILs. Near-isogenic lines represent some of the best material for finding polymorphisms which are closely linked to the gene of interest. However, it has been suggested in tomato (Grandillo et al. 1996), and observed in wheat (unpublished data), that after some backcrosses the rate of recombination can decrease up to 6-fold. Thus, in a map-based cloning approach it is advantageous to develop several mapping populations in order to avoid marker clustering and problems in estimating the ratio of genetic to physical distance.

A strategy for the map-based cloning of *Rph7.g*

In this study, we have established a linkage map at the *Rph7* locus which covers about 50 cM on chromosome 3HS. We have found two RFLP markers flanking the *Rph7.g* resistance gene. A barley cDNA (ABC171) encoding a heat-shock protein was located at 16 cM on the proximal side of the gene and a rice cDNA (S1543) with no homology to any known protein in the database was mapped 2.3 cM distal to *Rph7.g*. S1543 maps at 0.9 cM from a cluster of genes containing the receptor-like kinase *Hv3Lrk* (Feuillet and Keller 1999) and at 2.1 cM from another rice cDNA C970 (Fig. 1). In rice a receptor-like kinase gene family homologous to *Hv3Lrk* has been mapped at the distal end of chromosome 1 where it cosegregated with C970 and S1543 (Gallego et al. 1998). This region of rice has already been shown to be in synteny with the Triticeae chromosomes 3 (Ahn et al. 1993). Interestingly, the allele that was detected in Cebada Capa with the rice gene S1543 was also found in all the barley lines having the *Rph7* resistance gene. These results suggest that the synteny between barley and rice can be exploited in this region to support the map-based cloning of *Rph7.g*. The benefit of using a small-genome grass like rice as a source of new probes to saturate larger genomes such as the one of barley has already been shown (Kilian et al. 1995; Han et al. 1998). We are currently further investigating the synteny between rice and barley around S1543 by using rice YACs spanning this region. This should allow us to generate new probes, as well as to study the conservation of physical to genetic relationship in this region. Based on recent cytogenetic studies that have established precise relationships between the physical and genetic distances along the barley chromosomes (Künzel et al. 2000) a ratio of 100 kb/cM can be expected in the *Rph7* region.

So far, powdery mildew has been the most important disease in barley and much effort has been invested in studying the genetic basis of powdery mildew resistance and isolating *Ml* resistance genes (Büschges et al. 1997). As barley leaf rust becomes an important disease, increased efforts in resistance breeding, e.g. through the development of molecular markers for *Rph* resistance genes, are needed. In addition, the isolation of resistance genes should contribute to an understanding of the basis of leaf rust resistance in barley and to achieve more durable resistance.

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