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Molecular mapping of the leaf rust resistance gene *Rph7* in barley

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With 2 figures

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Abstract

Leaf rust of barley, caused by *Puccinia hordei* Otth, is an important foliar disease in most temperate regions of the world. Sixteen major leaf rust resistance (*Rph*) genes have been described from barley, but only a few have been mapped. The leaf rust resistance gene *Rph7* was first described from the cultivar 'Cebada Capa' and has proven effective in Europe. Previously mapped restriction fragment length polymorphism (RFLP) markers have been used to determine the precise location of this gene in the barley genome. From the genetic analysis of a 'Bowman'/'Cebada Capa' cross, *Rph7* was mapped to the end of chromosome 3HS, 1.3 recombination units distal to the RFLP marker cMWG691. A codominant cleaved amplified polymorphic site (CAPS) marker was developed by exploiting allele-specific sequence information of the cMWG691 site and adjacent fragments of genomic DNA. Based on the large amount of polymorphism present in this region, the CAPS marker may be useful for the marker-assisted selection of *Rph7* in most diverse genetic backgrounds.

Key words: *Hordeum vulgare* — *Puccinia hordei* — bulked segregant analysis — molecular markers

Barley leaf rust, caused by the fungal pathogen *Puccinia hordei* Otth, is an important foliar disease in most temperate regions throughout the world. The use of disease-resistant barley cultivars has been an efficient means for controlling the disease and preventing yield losses, which may reach 32% in susceptible cultivars (Griffey et al. 1994). At present, 16 major genes for resistance to leaf rust (denoted *Rph1* to *rph16*) have been described in barley (for a recent review see Franckowiak et al. 1997). However, nearly all these genes have been overcome by the virulent pathotypes of *P. hordei* (Fetch et al. 1998), and only a limited number have been mapped to date. By the use of morphological markers Jin et al. (1993) positioned *Rph3* and *Rph12* on barley chromosomes 7HL and 5HL, respectively. Feuerstein et al. (1990) were able to identify isozyme loci which showed linkage to the resistance genes *Rph10* on chromosome 3H and *Rph11* on chromosome 6H. Poulsen et al. (1995) identified a random amplified polymorphic DNA (RAPD) marker linked to a gene for leaf rust resistance in the barley line Q21861. Later, Borovkova et al. (1997) mapped this gene on chromosome 5H using restriction fragment length polymorphisms (RFLPs) and sequence tagged site (STS) markers. The *Rph9* locus was also mapped to this chromosome using similar techniques (Borovkova et al. 1998). In the same study, the resistance gene *Rph12* from 'Triumph' was found to be an allele at the *Rph9* locus. Ivandic et al. (1998) localized a gene (*rph16*) from the wild barley *Hordeum vulgare* ssp. *spontaneum* on the short arm of barley chromosome 2H.

The semi-dominantly inherited gene *Rph7* was first described from the cultivar 'Cebada Capa' (PI 539113) (Roane and Starling 1970) and exhibited seedling resistance to all *P. hordei* isolates applied (Parlevliet 1976). Although *Rph7* has now been overcome by virulent pathotypes of *P. hordei* in the Near East and North America (Golan et al. 1978, Steffenson et al. 1993), it is still effective against the European population of the pathogen. Using trisomic analysis, *Rph7* was assigned to barley chromosome 3H (Tuleen and McDaniel 1971, Tan 1978). Attempts to position the gene more precisely using various morphological marker stocks did not provide further useful evidence (Jin et al. 1993). The putative location of *Rph7* on the barley consensus map was merely based on the observation that the gene is linked with 24% recombination to the *Ant17* locus (proto-anthocyanidine-free), which maps to the short arm of chromosome 3H (Falk 1985, Franckowiak 1996). The objectives of the study presented here were: (1) to determine the precise chromosomal location of *Rph7* using a set of previously mapped anchoring RFLP markers; and (2) to develop a codominant, polymerase chain reaction (PCR)-based assay for marker-assisted selection of the resistance gene.

Materials and Methods

Plant materials: Genetic analysis was performed by analysis of 88 F_2/F_3 progenies derived from a cross between the original *Rph7* source 'Cebada Capa' and 'Bowman' (PI 483237), a leaf rust susceptible cultivar of *Hordeum vulgare* L. In addition to 'Cebada Capa' and 'Bowman', the following set of barley cultivars and accessions which differ in their allelic status at the *Rph7* locus were used for marker analysis: 'Hanka', 'La Estanzuela', 'Gondar', 'Cebada forrajera', 'Hor4517', 'Hor2564', 'Hor11387' (all *Rph7*/*Rph7*), 'Trumpf', 'Krona', 'Diamant', 'Steffi', 'Taiga', 'Hor4092', 'Hor5643', 'S3192' and 'S3170' (all *rph7*/*rph7*).

Resistance tests: Resistance tests were performed on individual F_2 progeny and subsequently F_3 families using the standard leaf rust isolate ND8702 (Borovkova et al. 1997), which is avirulent for *Rph2*, *Rph3*, *Rph5*, *Rph6*, *Rph7*, *Rph9*, *Rph12*, *Rph13*, *Rph14*, *Rph15 and rph16*. Seed of parents and F_2 and F_3 progeny were sown in pots filled with a peat moss–perlite (3 : 1) potting mixture and grown at $22 \pm 4°C$ in a greenhouse. Six-day-old seedlings (primary leaf fully expanded) were inoculated with urediniospores of *P. hordei* suspended in a lightweight mineral oil (3.5 mg/0.8 ml oil). Inoculated plants were then placed in chambers (at 20°C) maintained near saturation by periodic mistings

from ultrasonic humidifiers. After a 16-h misting period in the dark, plants were allowed to dry slowly before being placed in a greenhouse at 22 ± 4°C. Readings were taken 9–11 days after inoculation. Infection types were classified according to a 0–4 scale (Levine and Cherewick 1952) with infection types 0;n ('zero fleck with necrosis') and 3 indicating the presence and absence of the resistance gene *Rph7*. In order to determine the allelic state of resistant F_2 plants (i.e. *Rph7*/*Rph7* or *Rph7*/*rph7*), 16–29 plants from each F_3 family were tested for resistance as described above.

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). Marker-analysis was done in the F_3 progeny using DNA pooled from 20 plants per family. A set of chromosome-specific restriction fragment length polymorphism (RFLP) markers was used for bulked segregant analysis (Michelmore et al. 1991) with pools comprising 10 resistant and 10 susceptible F_3 families, respectively. RFLP probes prefixed 'MWG' and 'cMWG' originated from genomic and from cDNA libraries, as described in Graner et al. (1991). The barley cDNA probe ABC171 was kindly provided by A. Kleinhofs (Washington State University, Pullman, WA, USA). Autoradiography was carried out by exposing hybridized filters on imaging plates (Fuji, Straubenhardt, Germany) subsequently read by a phosphoimager (FujiX BAS 2000). Multipoint linkage analysis was performed using the Macintosh version of the program MAPMAKER (Lander et al. 1987). The sequences of all '*MWG' clones used in this study can be retrieved from the EMBL, Genbank, and DDBJ databases (Michalek et al. 1999).

Marker development: In order to convert the RFLP marker cMWG691, which is closely linked to the *Rph7* gene, into a codominant PCR-based marker, sequence information from its 5′- and 3′-flanking regions was gathered by application of the GenomeWalker kit (Clontech Laboratories, Palo Alto, Ca, USA) as outlined in the technical manual. 'GenomeWalker' libraries were constructed from the barley cultivar 'Franka'. For upstream walking, a *Hin*cII-library was used. Primers for preamplification were Adaptor Primer (AP) I and cMWG691as4 (5′-CGGGTGAGTGCCTCTTCG-3′). Nested PCR was performed with 2 µl of the preamplification reaction mixture using AP II and cMWG691as5 (5′-ATACGGCCCCAAGTGATCCACCTGC-3′). The PCR product was reamplified, cleaned via gel purification and directly sequenced. Downstream walking was executed with a *Ssp*I-library, as described above. Preamplification primers were AP I and cMWG691s3 (5′-CGGTTGTCCCAATAGGTGC-3′); nested primers were AP II and cMWG691s4 (5′-GTAGGCGATCCGCTTCCA-3′). After designing primers in the newly sequenced stretches, allele-specific sequences were obtained from the upstream and downstream regions of two susceptible ('Taiga' and 'Steffi') and two resistant ('Hanka' and 'Gondar') cultivars. Sequence comparison was performed with the DNASIS 2.5 sequence analysis program (Hitachi Software Engineering Co. Ltd, Maidenhead, UK).

For CAPS marker development, primers cMWG691forward1 (5′-GCTCGGTTGCTGCTTTTAGC-3′) and cMWG691reverse1 (5′-CCATCGCGAACAGTACATCC-3′) were set in domains conserved in both the susceptible and the resistant allele. The amplification product was restricted directly in the PCR mixture by adding 10 U of *Rsa*I (Life Technologies, Karlsruhe, Germany) and 1 µl of 10x *Rsa*I buffer per reaction.

PCR was performed in a volume of 20 µl containing 25 ng 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 0.5 units *Taq* polymerase (Qiagen, Hilden, Germany). The amplification profile was a modified touchdown programme: initial denaturation for 5 min at 94°C; 35 cycles of 30 s at 94°C; 30 s at annealing temperatures of 62, 60 and 58°C, respectively (5 cycles each) and 55 and 50°C, respectively (10 cycles each); 30 s at 72°C, terminated by a final extension step of 10 min at 72°C. DNA fragments were separated on 1% agarose gels, stained by ethidium bromide and visualized under UV light.

Results

The number of resistant (65) and susceptible (23) F_2 progeny observed was consistent with a 3 : 1 segregation pattern ($\chi^2 = 0.06$, df = 1), indicating that resistance is conferred by a single dominant gene (*Rph7*) in 'Cebada Capa'. This result was confirmed in the F_3 generation as the number of homozygous resistant (17), segregating (48) and homozygous susceptible (23) families approximated a 1 : 2 : 1 ratio, ($\chi^2 = 1.55$, df = 2). Bulked segregant analysis using a set of markers previously mapped on chromosome 3HS led to the identification of RFLP markers linked with the resistance gene. Subsequent genetic fine mapping based on the entire population allowed the construction of a partial RFLP map of the telomeric portion of the short arm of chromosome 3H (Fig. 1). Here, *Rph7* was positioned at the end of the chromosome arm with 1.3% recombination distal from cMWG691.

Genetic mapping and the identification of closely linked RFLPs form a prerequisite for the marker-assisted selection for Rph7. To circumvent the time- and labour-consuming DNA-hybridization procedures required for RFLP detection, we attempted to convert probe cMWG691 into a PCR marker. Since the cDNA probe cMWG691 is only 292 bp long, the 'GenomeWalker' technique was applied to extend sequence information to more than 600 additional bp into its 5′-flanking region and 800 bp into its 3′-flanking region. While distal parts of the newly sequenced stretch were monomorphic in the two resistant and the two susceptible cultivars analysed, the internal portion of the sequence is extremely variable owing to the presence of numerous point mutations that formed the basis for the development of a CAPS marker (Fig. 2a).

Congruent segregation was observed for both the cMWG691 RFLP and the corresponding CAPS marker, confirming their genetic identity. In contrast to other restriction

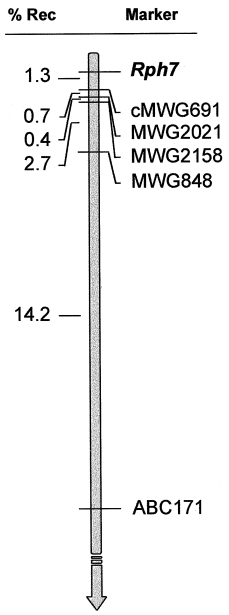

Fig. 1: Genetic map of *Rph7*. The linkage map represents the distal portion of barley chromosome 3HS and is based on 88 F_3 progenies. The arrow points towards the centromere. Restriction fragment length polymorphism probe MWG2021 represents part of a putative resistance gene (*Lrk10*)

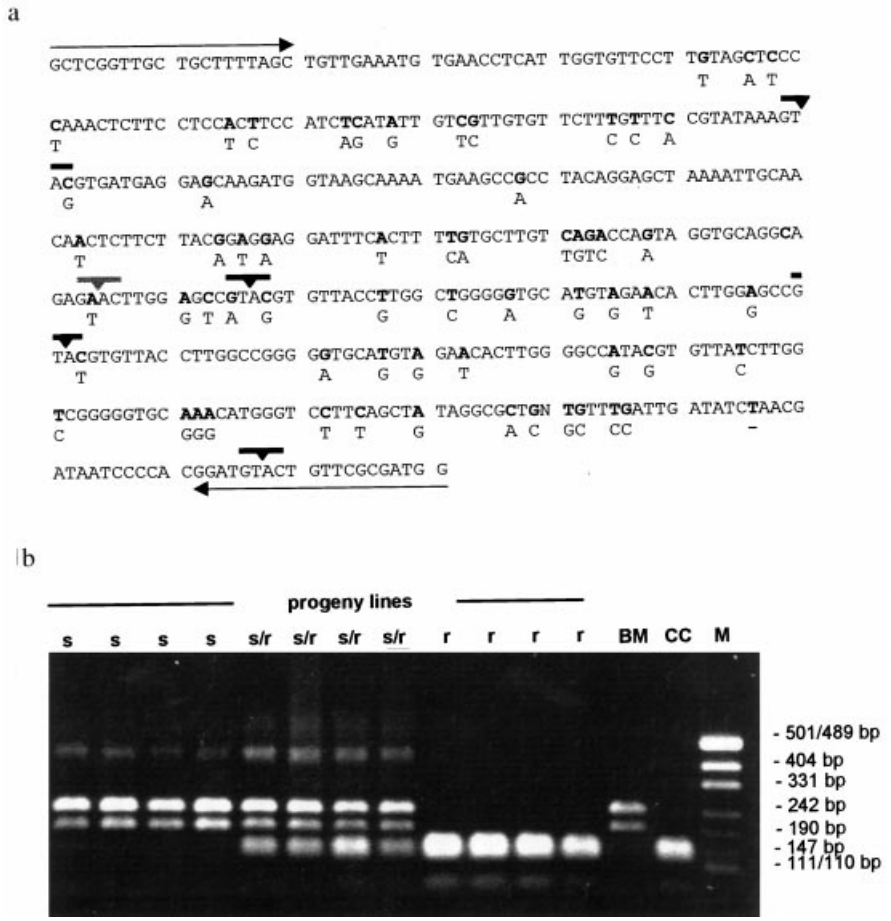

Fig. 2: Conversion of restriction fragment length polymorphism probe cMWG691 into a codominant cleaved amplified polymorphic site marker linked to *Rph7*. a. DNA sequence of the amplicon of a resistant plant. The sites of point mutations are indicated by giving the base substitution of the susceptible allele below the sequence. The *Rsa*I site tinted grey is unique for the susceptible allele, whereas *Rsa*I sites marked in black are cleaved in the resistant allele. The *Rsa*I site located in the reverse primer's sequence is present in both alleles. b. Size fractionation of *Rsa*I-digested amplicons generated by the primers as shown in panel a. Pooled DNA derived from 20 F_3 individuals was analysed in each track. The marker pattern shows homozygous susceptible (s), segregating (s/r) and homozygous resistant (r) F_3 families along with the parents 'Bowman' (BM, susceptible) and 'Cebada Capa' (CC, resistant). According to the sequence data given in A, the restriction pattern of resistant plants comprises fragments of 136 bp (2×) and 120 bp in length, which comigrate as a single band under the conditions applied. The expected 45 bp fragment occurs as a faint signal. Susceptible plants show two fragments of 244 bp and 193 bp. Faint bands of approximately 450 bp represent residual, undigested amplicon. M: DNA-length standard

endonucleases that are of potential use in the present case, *Rsa*I yields DNA-fragments that show a clear difference in mobility between susceptible and resistant individuals, which allows accurate identification of heterozygous progeny plants (Fig. 2b). Moreover, this enzyme can be added directly to the PCR reaction mixture, thus saving extensive manipulation during sample analysis.

In order to examine the diagnostic value of the CAPS marker, a set of 18 (mostly unrelated) barley accessions was analysed. All of the eight accessions known to be homozygous carriers of the *Rph7* gene (U. Walther, pers. comm.) were characterized by a restriction pattern identical to that of the cultivar 'Cebada Capa'. Conversely, marker patterns of susceptible plants were mainly split into two groups. Out of nine susceptible accessions that were tested in addition to the susceptible mapping parent, 'Bowman', four showed the banding pattern of the susceptible parent, while the pattern of another four accessions was identical to that of the resistant parent. The susceptible accession 'Hor 5643' showed a pattern distinct from all the other lines tested. Thus, marker-assisted selection using *Rsa*I-CAPS cMWG691 can only be performed in a limited number of combinations between resistant and susceptible parents (60% in the present sample). Given the large degree of polymorphism observed in the genomic region surrounding the cMWG691 locus, it seems likely that the use of additional restriction enzymes will increase the number of polymorphic parental combinations.

Discussion

Despite the large number of resistance genes that have been located in the barley genome (Graner 1996), *Rph7* is the only one that maps in the distal part of chromosome 3HS, a region characterized by a general paucity of mapped traits and markers. However, recently a receptor-like kinase (*Lrk10*) has been identified and isolated from wheat cosegregating with the leaf rust resistance gene *Lr10* on chromosome 1AS (Feuillet et al. 1997). The RFLP clone MWG2021 used in this study represents a 1-kb genomic *Pst*I fragment of the *Lrk10* gene. In addition to homoeologous group 1, this clone also maps to the distal end of the short arm of homoeologous group 3 in both wheat and barley, suggesting the presence of a resistance gene in this chromosomal region (Gallego et al. 1998). *Lrk10* was therefore considered as a possible candidate gene for *Rph7*. However, because of to the detection of two recombination events between MWG2021 and *Rph7*, *Lrk10* (MWG2021) must be ruled out as a candidate for the *Rph7* gene (Fig. 1). Interestingly, the DNA sequence obtained in the context of converting cMWG691 into a PCR-marker revealed homology ($2.5e^{-5}$–$1.5e^{-8}$) to receptor-like kinases (RLKs) from several

plant species including *Oryza sativa*, *Triticum aestivum* and *Nicotiana tabacum*. Hence, it is possible that this chromosomal region harbours a series of RLKs. Since RLKs (1) have been shown to encode race-specific disease resistance genes in several plant species and (2) are frequently organized in gene families, the identification of homologues in the *Rph7* region suggests that RLKs may represent possible candidates for the *Rph7* gene (Michelmore and Meyers 1998). The favourable ratio between genetic and physical distances in the distal region of barley chromosome 3HS (0.1–2.1 Mb/cM) will prompt efforts for the map-based isolation of *Rph7* in order to test this hypothesis (Künzel et al. 2000).

The pyramiding of resistance genes represents a straightforward strategy to increase the durability of protection from a given pathogen. In a conventional breeding programme, this would require extensive resistance tests using the appropriate leaf rust isolates. Time-consuming progeny tests would be inevitable if the presence of the resistance genes could not be differentiated by this means (i.e. if a combination of two genes conferring complete resistance were attempted). In the case of leaf rust, tightly linked markers are now available for a series of major genes including *Rph7* and *rph16* (Ivandic et al. 1998). As long as pathotypes of *P. hordei* with virulence for *Rph7* and *rph16* have not been detected in Europe, marker-assisted selection represents an effective and convenient way to combine reliably the two genes in barley cultivars within a short period of time.

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