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Molecular mapping and genetic fine-structure of the *rym5* locus encoding resistance to different strains of the Barley Yellow Mosaic Virus Complex

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Abstract The genetic structure of the *rym5* locus was studied in a population comprising 391 doubled-haploid lines that were evaluated for resistance to two strains of Barley Yellow Mosaic Virus (BaYMV-1, 2) and to Barley Mild Mosaic Virus (BaMMV). The absence of recombinants that are able to differentiate between the reaction to these different bymoviruses provides evidence that *rym5* is a complex locus, which is either composed of several closely linked genes or of an allelic series of a single gene. For marker-assisted introgression of this locus into adapted barley germplasm, a CAPS (cleaved amplified polymorphic sequence) and a microsatellite marker were developed that flank the gene at distances of 0.8 and 1.3% recombination, respectively.

Key words Disease resistance · *Hordeum vulgare* · Marker-assisted selection · Molecular markers · PCR

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Introduction

Barley Mild Mosaic Virus (BaMMV) and Barley Yellow Mosaic Virus (BaYMV) represent a serious threat to the cultivation of winter barley in large areas throughout Central Europe and East Asia. They belong to the group of bymoviruses, which are characterized by a bipartite, single-stranded RNA genome and transmission by the soil-borne fungus *Polymyxa graminis* (Usugi et al. 1989). Upon infection through the root system, virus particles are translocated into the upper parts of the plant, causing typical mosaic symptoms on the leaves. As a result of infection, plants are particularly sensitive to cold stress, resulting in increased levels of winter killing and reduced vigour during the subsequent growing season.

Despite their similarities in transmission, host range, and symptom expression, BaMMV and BaYMV can be differentiated both serologically and by the primary structure of their genomes (Huth and Adams 1990; Kashiwazaki et al. 1992). Serological tests allow the detection and differentiation of both viruses in plants grown in infested fields that are frequently characterized by mixed infections. While BaMMV seems to be unique, two strains of BaYMV (BaYMV-1 and BaYMV-2) have been observed in Europe. The latter can be differentiated from BaYMV-1 based on its increased virulence (Huth 1989). An even more complex situation exists in Japan, where six different pathotypes of BaYMV and two different types of BaMMV have been identified based on their virulence on different barley cultivars (Kashiwazaki et al. 1989; Nomura et al. 1996).

Because of the soil-borne nature of the virus complex, the availability of disease-resistant varieties is of prime interest for the continuous cultivation of winter barley in infested areas. Knowledge of the chromosomal location of resistance genes, as well as the development of selectable DNA markers, are prerequisites

for the efficient manipulation of disease resistance in breeding programs. The *rym4* gene, derived from the South European landrace Ragusa, and, to a lesser extent, the *rym5* gene, derived from the Chinese landrace Mokusekko 3, form the genetic basis of resistance in European barley cultivars and breeding lines. Using the RFLP technique, *rym4* has been mapped to the distal region of the long arm of chromosome 3H (Graner and Bauer 1993). *Rym5* was mapped to the same chromosomal region based on its close linkage to the *Est1-Est2-Est4* isozyme locus, while another gene, *rym1*, which is also present in Mokusekko 3, has been assigned to chromosome 4H using morphological markers (Konishi et al. 1997).

Despite the fact that the number of resistance genes employed in breeding programs is limited, the genetic basis of resistance to the BaMMV/BaYMV complex is broad. In particular, barley accessions from East and Central Asia, as well as from Eastern Europe, provide a rich source of genetic variability (Ordon and Friedt 1993). Three novel genes originating from this source (*rym8*, *rym9*, *rym11*) were recently integrated into molecular-marker maps of chromosome 4HL (Bauer et al. 1997).

A hallmark of resistance to the BaYMV/BaMMV complex is the specificity of different resistance genes to individual virus isolates. Regarding European virus isolates, a germplasm survey led to the identification of accessions that exhibit resistance to BaMMV only, while others exhibit resistance to both BaMMV and BaYMV. In the latter group, resistance to either BaYMV-1 or to both BaYMV-1 and BaYMV-2 has been observed (Götz and Friedt 1993). Resistance to BaYMV-2 is of particular interest, because: (1) the acreage infested by this virus strain is gradually increasing, and (2) *rym4* (the most commonly deployed resistance gene) does not confer resistance to this strain. Since Mokusekko 3 and some of its derivatives show resistance to all European virus strains, they are being used increasingly to enhance resistance in European barley germplasm. However, to-date there has been no evidence that resistance to BaYMV-2 is encoded by the *rym5* locus.

The present study was initiated to perform a genetic analysis of the resistance specificities present at the *rym5* locus and to develop selectable PCR-markers, which may find application in practical marker-assisted selection.

Materials and methods

Plant material

Genetic analyses were conducted using anther-derived double-haploid progenies of the crosses listed in Table 1. Doubled-haploid breeding lines (W122/xy, Table 1) were employed as resistant parents, which were developed from a cross between the Japanese breeding line "Resistant Ym No. 1" (Muramatsu 1976), carrying the *rym5* gene, and cv Igri, a susceptible two-rowed winter barley.

Resistance tests

Mechanical inoculation with BaMMV was performed at the 4–5 leaf stage as described previously (Ordon and Friedt 1993). Briefly, inoculum was prepared from infected plants (cvs Gerbel, Corona) by adding carborundum (mesh 300) to an extract obtained from squashed leaves, which was subsequently diluted by adding 9 vol of K_2HPO_4 (0.1 M, pH 9.1). After spraying the two youngest leaves with a spraygun, plants were grown in a growth chamber for 4 weeks at 12°C under short-day conditions (10 h illumination). As an alternative to the use of a spraygun device, virus inoculum was rubbed manually onto the leaves. For correct phenotyping, mechanical inoculation was performed in two replications using five seedlings per DH-line. Since neither BaYMV-1 nor BaYMV-2 can be transmitted mechanically at sufficient rates, field experiments were performed at two locations, one infested with BaYMV-1/BaYMV-2 (Schladen, Lower Saxony) and another infested with BaMMV/BaYMV-1 (Höhefeld, Bavaria). The presence of virus particles was assessed by ELISA (enzyme linked immuno sorbent assay) according to Clark and Adams (1977). Using specific antisera raised against BaMMV and BaYMV (provided by Dr. W. Huth, BBA Braunschweig) the two viruses could be detected and differentiated unequivocally. For both mechanical inoculation and field experiments, infection frequencies as determined on susceptible standards (cvs Corona, Gerbel) were $> 90\%$. The combination of the results of mechanical inoculation and the two field trials allowed the inheritance of resistance to each virus strain (BaMMV, BaYMV-1, BaYMV-2) to be monitored individually.

Table 1 Barley germplasm used for genetic analysis of the *rym5* locus

Cross No.	Resistant parent	Resistant to	Suscept. parent	No. DH-progeny
1	W122/35.2	BaMMV, BaYMV-1, -2	Marinka	11
2	W122/35.2	BaMMV, BaYMV-1, -2	Harmonika	55
3	W122/35.2	BaMMV, BaYMV-1, -2	Cosima	29
4	W122/37.1	BaMMV, BaYMV-1, -2	Alraune	42
5	W122/37.1	BaMMV, BaYMV-1, -2	Posaune	65
6	W122/37.2	BaMMV, BaYMV-1, -2	Marinka	22
7	W122/37.2	BaMMV, BaYMV-1, -2	Cosima	25
8	W122/54.2	BaMMV, BaYMV-1, -2	Interbell	142
Total				391

Marker and linkage analysis

DNA extraction, digestion, and hybridization were performed as described previously (Graner et al. 1991) except for replacing the restriction endonuclease *Sac*I by *Dra*I. Linkage analysis was conducted using Mapmaker and MapManager computer software (Lander et al. 1987; Manley 1993). Phenotypic pools for bulked segregant analysis (Michelmore et al. 1991) comprised equal amounts of ten susceptible and ten resistant DH-lines, each. RAPD primer screening and RAPD analysis followed the protocol described by Weyen et al. (1996). Amplification of the microsatellite marker BMac29 (primer sequences available from R.W.) was carried out in a reaction volume to 20 µl containing 20 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µm primer, and 1 U *Taq* polymerase (Gibco BRL). The following cycling conditions were applied: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final extension step (5 min at 72°C). Electrophoretic separation of the resulting amplicons was performed either in 2% agarose (Metaphor™, Biozym) or by running 8% denaturing polyacrylamide gels on a LICOR 4200S-2 sequencing machine. A CAPS (cleaved amplified polymorphic sequence) marker was developed from RFLP probe MWG838 (primer 1: ctg cag atg acc cta aga cg; primer 2: cag ggg aat aca ggg acc tt). Amplification was performed as outlined above. Upon completion of the PCR reaction the amplification products were digested by adding 3 U of the restriction endonuclease *Hin*fI to the reaction mixture. Restriction fragments were separated on 2% agarose (Biozym).

Results

Fine-mapping of the *rmy5* locus

The breeding lines, W122/35.2, W122/37.1, W122/37.2 and W122/54.2, which were used for fine mapping in this study, represent full sibs whose pedigrees trace back to Mokusekko 3 via their common parent "Resistant Ym No. 1". Doubled-haploid progeny lines derived from crosses with susceptible winter barley cultivars were used for genetic analysis. As expected for a monogenic trait, resistance to BaMMV segregated in the DH-progeny of cross #8 (Table 1) in a 1:1 ratio ($\chi^2 = 0.34$, 1 *df*). Segregation in the remaining progenies was highly biased towards the occurrence of resistant lines. This distortion is due to pre-selection for resistance to BaMMV, which was performed in the course of a breeding program prior to the genetic analysis presented in this study. Marker analysis revealed that, except for crosses #1 and 6 which were monomorphic for marker MWG838, the *rym5* locus could be placed into an interval flanked by markers MWG10 and MWG838 (Fig. 1). In crosses #1 and 6, the locus was bordered on its proximal side by the RAPD marker OP-O20. Final map construction was performed by combining the data sets of individual progenies (391 DH-lines in total) yielding a resolution of 0.26% recombination (Fig. 1).

Genetic structure of the *rym5* locus

While fine-mapping was performed on the basis of resistance to BaMMV only, the parental lines used for these experiments also display immunity to BaYMV-1 and BaYMV-2. To investigate whether the corresponding resistance genes all reside at the *rym5* locus, all 391 progeny lines were planted in different fields infested by either BaYMV-2 only, or co-infested by BaYMV-1 and BaMMV. In the latter case the use of specific antibodies allowed the differentiation between BaMMV and BaYMV-1. Thus, by combining the results of all three resistance tests (BaMMV, BaYMV-1, and BaYMV-2), the inheritance of individual resistance genes could be followed separately. However, no recombination event was detected in any DH-line, i.e. plants were either completely resistant or fully susceptible to all three virus types. According to Hanson (1959) this means that, in the case of tight linkage between any two genes at a locus, these are separated by a distance of no more than 1.2 cM.

Development of PCR-based markers

To facilitate marker-assisted selection, PCR-based markers were developed for the chromosomal region carrying the *rym5* locus. Bulked segregant analysis using 500 different decamer primers led to the identification of five RAPD markers that gave a differential signal on both pools. Fine-mapping revealed that none of these mapped closer than 3.2 cM to *rym5*. In an alternative approach, RFLP marker MWG838, a genomic *Pst*I probe, was sequenced and primers were developed for both ends. Digestion of the corresponding PCR-amplicons using a series of restriction enzymes with a 4-bp recognition sequence led to the identification of co-dominant polymorphisms in the case of *Rsa*I and *Hin*fI (Fig. 1). However, these polymorphisms were detected only in crosses #2, 3, 4, and 7, with the remaining parental combinations being monomorphic due to the presence of "resistant alleles" in the susceptible cvs Interbell, Marinka and Posaune at this marker locus. In contrast, a co-dominant microsatellite marker, BMac29, isolated from a genomic library enriched for AC/GT repeats (Ramsay et al., in preparation), was informative in all parental combinations used to map the *rym5* locus. Carriers of this gene are characterized by a DNA fragment of 160 basepairs (bp) in length, while all susceptible barley accessions that were tested showed a 176-bp fragment. Those cultivars carrying *rym4*, which is located in the same chromosomal interval, displayed a diagnostic 158-bp fragment (Table 2).

Discussion

Resistance to soil-borne viruses is a prerequisite for the continued cultivation of winter barley in infested areas. The successful generation of resistant cultivars by cross

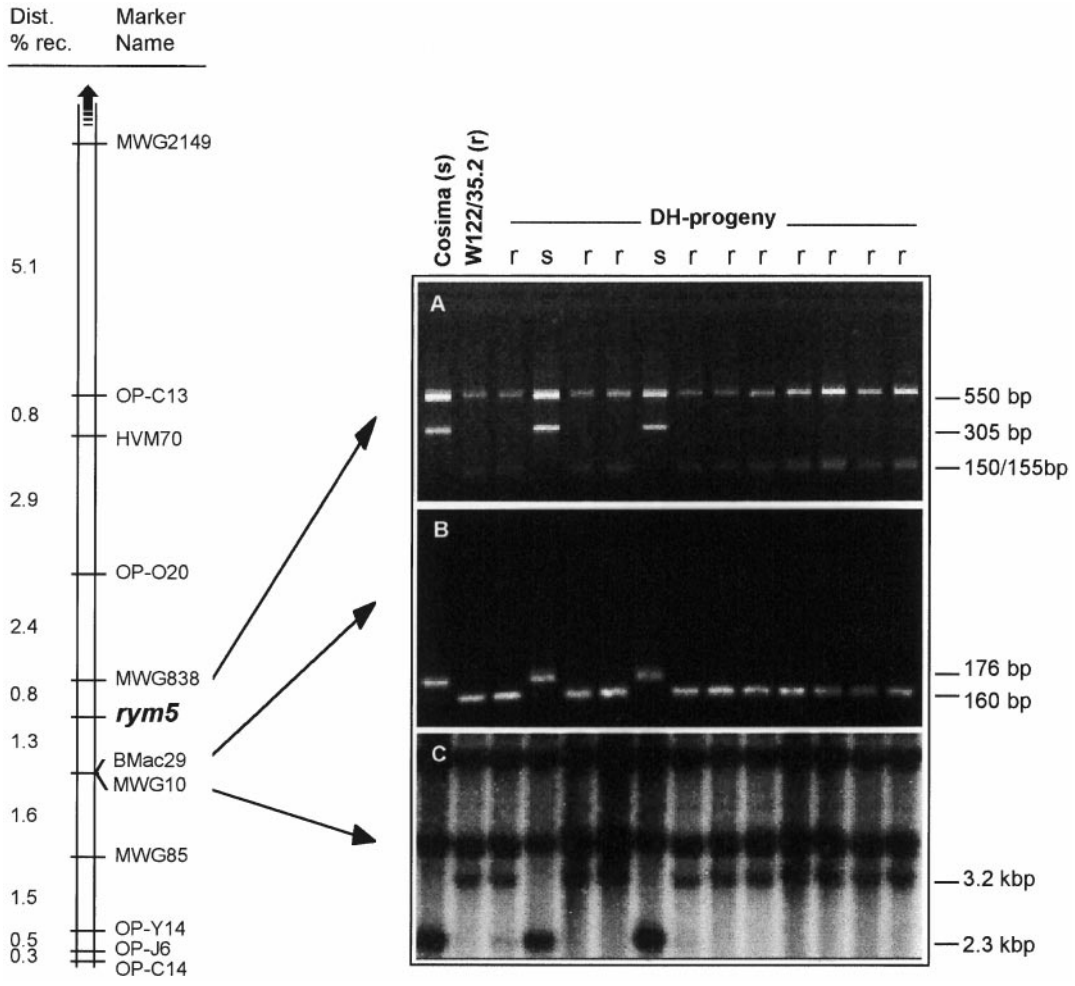

Fig. 1A–C Molecular map of the *rym5* resistance region. *Left*: consensus map of the distal portion of barley chromosome 3HL based on 391 DH-progeny comprising crosses no. 1–8 as given in Table 1. Since RFLP-marker MWG838 was not polymorphic in crosses including cv Marinka as a susceptible parent (#1, 6) its map position is based on the analysis of a subset consisting of 358 DH-lines. *OP-xx*: RAPD-marker; *MWGxx*: RFLP-marker; *BMac*29, HVM70: barley microsatellite markers. *Right*: DNA-fragment patterns of selected progeny plants along with their parents. **A** CAPS-MWG838 (*Hin*fI-digested amplicons). Resistant progeny lines (*r*) are characterized by the presence of two restriction fragments of 150 bp and 155 bp in size which have not been resolved under the conditions applied to run the gel. Susceptible lines (*s*) show a typical restriction fragment of 305 bp. **B** Barley microsatellite marker BMac29. Resistant progeny lines show a 160-bp and susceptible lines a 176-bp DNA fragment, respectively. **C** Gel-blot analysis with RFLP-marker MWG10. Resistant lines show a 3.2-kilobasepairs (kbp) and susceptible lines a 2.3-kbp *Bam*HI restriction fragment

breeding depends on: (1) the identification of resistant germplasm, (2) the localization of the corresponding genes, and (3) the development of selectable markers to accelerate the breeding process. In this context, Mokusekko 3 and its various descendants have been

Table 2 Diagnostic DNA-fragment pattern of microsatellite marker BMac29. Fragment sizes are given for a set of randomly selected barley accessions being susceptible to BaMMV, BaYMV and BaYMV-2 or having different resistance genes on the long arm of barley chromosome 3H (*rym4*, *rym5*)

Accession	Phenotype	SSR-fragment size
Alraune	Susceptible	176
Corona	Susceptible	176
Gerbel	Susceptible	176
Igri	Susceptible	176
Trixi	Susceptible	176
Ragusa	Resistant (*rym4*)	158
Brunhild	Resistant (*rym4*)	158
Colambo	Resistant (*rym4*)	158
Franka	Resistant (*rym4*)	158
Labea	Resistant (*rym4*)	158
Mokusekko 3	Resistant (*rym5*)	160
Resistant Ym No. 1	Resistant (*rym5*)	160
Japan S-1001	Resistant (*rym5*)	160
Misato Golden	Resistant (*rym5*)	160
Kanto Nijo 19	Resistant (*rym5*)	160

recognized as a valuable source for resistance in breeding programs since they confer resistance against all European strains of the BaMMV/BaYMV complex (Götz and Friedt 1993). The results of the present study provide evidence that the individual specificities of this broad-spectrum resistance resides exclusively at the *rym5* locus since no recombinant line showing an altered resistance spectrum could be identified in a population consisting of 391 DH-lines. However, it should be noted that the *rym5* locus does not confer resistance to the Japanese strain III of BaYMV. To obtain complete resistance to all Japanese BaYMV strains, the presence of an additional gene (*rym1*) is required (Konishi et al. 1997). The fact that the *rym5* gene does not confer resistance to all Japanese virus isolates leaves open the possibility that new, virulent strains may occur in Europe thus underscoring the need to identify additional resistance genes.

In the case of the BaMMV and BaYMV, complex resistance loci have been identified at two sites in the barley genome; *rym8* and *rym9* map in the distal portion of chromosome 4HL (Bauer et al. 1997), while the *rym4* and the *rym5* loci map in an interval measuring about 2.1 cM in size on chromosome 3HL (Graner and Bauer 1993; this study). The latter two loci carry an allelic gene conferring resistance to BaMMV since F_1 progeny plants of a cross between W122/37.1 (*rym5*) and cv Franka (*rym4*) were resistant after mechanical inoculation with BaMMV. In addition, another gene from the cultivar Hiberna maps to the same chromosomal region. Unlike *rym4* and *rym5*, this gene conditions resistance to BaYMV-1 and BaYMV-2 but not to BaMMV (data not shown). Finally, Iida and Konishi (1994) mapped a gene termed *ym6* in this chromosomal region. In this case, however, no data are available on its allelism to any of the genes mentioned above.

Increasing knowledge on the molecular structure and the genomic organization of resistance genes, as well as the analysis of resistance gene analogues, provides evidence that the presence of complex loci might be the rule rather than the exception. In a series of plant species, complex loci carrying resistance genes against various fungal pathogens have been discovered and analyzed at the molecular level. In most cases they consist of a series of closely related and tightly linked genes. Examples are the *M* locus in flax (Anderson et al. 1997), the *Rp1* rust-resistance complex in maize (Sudupak et al. 1993), the *Dm3* locus in lettuce (Anderson et al. 1996), the *Cf2* locus (Dixon et al. 1996) and the *Cf4*/*Cf9* locus in tomato (Parniske et al. 1997). On the other hand, the *L* locus in flax represents an example of an allelic series of a single gene, giving rise to resistance against various races of the fungal pathogen according to a gene-for-gene interaction (Ellis et al. 1995).

Due to the finite resolution of genetic experiments, the lack of detectable recombination within the *rym5* locus can not provide conclusive evidence about its physical structure. Hence, the present data allow the formulation of three hypotheses in terms of the structure of the *rym5* locus: (1) the locus consists of three individual genes linked at a distance of less than 1.2 cM (Hanson 1959), with each gene showing specificity to a different virus strain; (2) the locus carries a single gene displaying an allelic series, with individual alleles displaying different specificities; and (3) the locus is composed of two genes, one conferring resistance to BaMMV and the other to BaYMV-1 and BaYMV-2. The latter hypothesis is supported by the fact that BaMMV and BaYMV are clearly distinct viruses and the corresponding resistance responses might be caused by two different genes. To further test the above mentioned hypotheses, attempts towards map-based cloning of the *rym5* locus have been initiated.

Regardless of the molecular structure of the *rym5* locus, the availability of closely linked markers forms the basis to efficiently manipulate resistance in the course of a breeding program. Since resistance conferred by the *rym5* locus is a recessive trait, the availability of a co-dominant marker is a prerequisite for the identification of heterozygous carriers. Two such PCR markers linked at a distance of < 1.5 cM to the resistance gene have been developed. The use of either of these markers will make the introgression of corresponding resistance genes independent of environmental factors, which range from low infection frequencies, as they are observed after a cold and dry autumn, to increased winter killing under optimal conditions for virus infection. Due to the tight association of the *rym4* and the *rym5* loci, both markers can also be used to monitor the inheritance of the *rym4* resistance gene. Since MWG838 and BMac29 show closer linkage to the *rym4*/*rym5* complex than the *Est1-Est2-Est4* locus, their deployment is expected to result in an improved selection accuracy. Moreover, PCR technology provides a general platform for marker-assisted selection, accessible to automation, whereas the analysis of isoenzymes requires the application of elaborate protocols. On a genome-wide level only a restricted number of isoenzyme loci are known for barley, which, apart from the *Est1-2-4* locus, showed low, or even no, allelic variation in a series of unrelated accessions of cultivated barley that were investigated to map additional genes for resistance to the BaMMV/BaYMV complex (Le Gouis et al. 1995).

In conclusion, the results of the present study show that the *rym5* locus carries multiple specificities, similar to resistance genes against various fungal pathogens. These multiple specificities could either be the result of close linkage or of multiple allelism. To study this issue further, molecular mapping has provided a basis for the positional cloning of these genes and for an investigation of their function at the molecular level. In the context of a breeding program, closely linked PCR-markers afford a basis for efficient marker-assisted selection.

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