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ORIGINAL PAPER

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High-resolution mapping of the *Rym4/Rym5* locus conferring resistance to the barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2) in barley (*Hordeum vulgare* ssp. *vulgare* L.)

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Abstract Soil-borne barley yellow mosaic virus disease – caused by a complex of at least three viruses, i.e. *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV) and BaYMV-2 – is one of the most important diseases of winter barley in Europe. The two genes *rym4*, effective against BaMMV and BaYMV, and *rym5*, additionally effective against BaYMV-2, comprise a complex locus on chromosome 3HL, which is of special importance to European barley breeding. To provide the genetic basis for positional cloning of *the Rym4/Rym5* locus, two high-resolution maps were constructed based on co-dominant flanking markers (MWG838/Y57c10 - MWG010/Bmac29). Mapping at a resolution of about 0.05% rec., *rym4* has been located 1.07% recombination distal of marker MWG838 and 1.21% recombination proximal to marker MWG010. Based on a population size of 3,884 F_2 plants (0.013% recombination) the interval harbouring *rym5* was delimited to 1.49 ± 0.14% recombination. By testing segmental recombinant inbred lines (RILs) for reaction to the different viruses at a resolution of 0.05% rec. (*rym4*) and 0.019% rec. (*rym5*), no segregation concerning the reaction to the different viruses could be observed. AFLP-based marker saturation for *rym4*, using 932 *Pst*I + 2/*Mse*I + 3 primer combinations only resulted in three markers with the closest one linked at 0.9% recombination to the gene. Two of these markers detected epialleles arising from the differential cytosine methylation of *Pst*I sites. Regarding *rym5*, profiling of 1,200 RAPD primers (about 18,000 loci) and 2,048 *Eco*RI + 3/*Mse*I + 3 AFLP primer combinations (about 205,000 loci) resulted in one RAPD marker and seven AFLP markers tightly linked to the resistance gene. Flanking markers with the closest linkage to *rym5* (0.05% and 0.88% recombination) were converted into STS markers. These markers provide a starting point for chromosomal walking and may be exploited in marker-assisted selection for virus resistance based on *rym5*.

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Introduction

Barley yellow mosaic virus disease was first detected in Japan more than 60 years ago (Ikata and Kawai 1940), and due to a constant spread has become one of the most important diseases of winter barley (*Hordeum vulgare* ssp. *vulgare*) in Europe. There, the disease is caused by a complex of at least three viruses or virus strains, i.e. *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV), and BaYMV-2 (Huth 1989; Huth and Adams 1990), infecting barley individually or in combinations. Recent results suggested that differences in the pathogenicity between BaYMV and BaYMV-2 with respect to *rym4* are due to a polymorphism in the central coding region of the viral genome-linked protein (VPg) on RNA-1 (Kühne et al. 2003).

Besides this, studies conducted in France showed that additional variants of BaMMV and BaYMV exist (Hariri et al. 2000), and more recently, a new variant of BaMMV has been characterized (Hariri et al. 2003; Kanyuka et al. 2004). An even more complex situation exists in Japan, where seven strains of BaYMV and two strains of BaMMV have been described (Nomura et al. 1996). Together with *Wheat yellow mosaic virus*, *Wheat spindle streak mosaic virus*, *Oat mosaic virus* and *Rice necrosis mosaic virus*, these viruses belong to the genus *Bymovirus* within the family of the *Potyviridae* and are characterized by a bipartite, single-stranded (+)-sense RNA genome (Usugi et al. 1989). All these viruses are transmitted by the soil-borne fungus *Polymyxa graminis* Ledingham (Toyama and Kusaba 1970), which is a member of the *Plasmodiophorales*.

Until recently, breeding for resistance in Germany was mainly based on the recessive gene *rym4*. It confers resistance to BaMMV and BaYMV. Due to the increasing occurrence of BaYMV-2, the gene *rym5*, which, in addition to BaMMV and BaYMV, also confers resistance to BaYMV-2, has become the gene of choice in European barley breeding (Friedt et al. 2000). Both genes have been mapped in the same marker interval of the telomeric region on chromosome 3HL (Graner and Bauer 1993; Konishi et al. 1997; Graner et al. 1995, 1999) and are allelic with respect to resistance to BaMMV (Götz and Friedt 1993). The two resistance genes are of different origin: *rym4* is likely to have been introduced to European cultivars from the Dalmatian landrace 'Ragusa' (Huth 1984), while *rym5* derives from the Chinese landrace 'Mokusekko 3' (Konishi et al. 1997). Although *rym4* and *rym5* currently represent the genetic basis of resistance in European barley cultivars, nothing is known about the structure and function of these genes.

A high-resolution linkage map is a prerequisite for the positional or map-based cloning of any target gene. In recent years, this strategy has been successfully applied in several crop species including wheat and barley, which are characterized by comparatively large genomes (for review cf. Stein and Graner 2004). The process of map-based cloning can be structured into three steps, i.e. (1) high-resolution genetic mapping of the target locus, (2) construction of a physical map that carries the closest flanking markers on a single contig and (3) gene identification and functional analysis via genetic transformation or via the analysis of the corresponding mutants. Genetic mapping provides the crucial step in this process (for review cf. Ordon et al. 2000). The goal is to analyse a large mapping population to provide sufficient genetic resolution in the target region (based on recombination, centiMorgans) to facilitate the construction of a physical map.

In an attempt towards the construction of a physical map of the *Rym4*/*Rym5* locus, the present study aimed at high-resolution genetic mapping of this locus and the saturation of the flanking region with genetic markers. The results provide further evidence on the genetic fine structure of the locus and provide the basis for the map-based isolation of the corresponding resistance gene(s).

Materials and methods

Plant material and population development

Two independent high-resolution mapping populations were developed and analysed for the resistance genes *rym4* and *rym5*. For *rym4* a high-resolution mapping population was constructed on 1,040 F_2 individuals of the cross 'Igri' × 'Franka'. In the case of *rym5* a population of 3,884 F_2 individuals was developed by crossing the resistant doubled haploid (DH) breeding line 'W122/37.1' [derived from a cross between the Japanese breeding line 'Resistant Ym No. 1' (*rym5*, Muramatsu 1976) and cv. 'Igri' (Graner et al. 1999)] to the susceptible two-rowed cv. 'Alraune'. In order to identify plants carrying a recombination in the target interval, F_2 plants of both populations were analysed by co-dominant markers (MWG838/Y57c10-MWG010/Bmac29) flanking the resistance gene in an interval of 2.5 cM (*rym4*) and 2.1 cM (*rym5*), respectively (Graner and Bauer 1993; Graner et al. 1999). Homozygous recombinant inbred lines (RILs) were selected out of 12 progeny plants (F_3) of each heterozygous recombinant F_2 individual ($p > 0.95$, $x_{min} = 10.42$). These homozygous RILs were used for mapping newly developed markers. Furthermore, they were propagated by selfing and subjected to repeated phenotypic analysis for resistance to BaMMV, BaYMV and BaYMV-2 in F_4.

Preparation of genomic DNA

F_2 plants were grown in a greenhouse in 96 Quick-Pot plates, and DNA samples were taken at the two-leaf stage. For screening the population for recombinants, a semiautomatic SDS procedure using a 96 deep-well leaf crusher (CrushExpress, Saaten-Union Resistenzlabor, Hovedissen, Germany) was used for DNA extraction. The SDS protocol was provided by J. Foerster, Saaten-Union Resistenzlabor. DNA of segmental RILs was extracted using a modified CTAB procedure according to Doyle and Doyle (1987) for *rym5*, and large-scale DNA extractions for *rym4* were performed according to Saghai Maroof et al. (1984).

Marker analysis

RAPD primer screening and analysis employing 1,200 primers was performed as described previously (Ordon et al. 1995; Pellio et al. 2004). Fluorescence-labelled (Licor 4200) AFLP analysis (Zabeau and Vos 1993) for *Eco*RI + 3/*Mse*I + 3 primer combinations used to detect additional markers for *rym5* was carried out as described by Pellio et al. (2004). In total 32 *Eco*RI + 3 (A- and C-set)- and 64 *Mse*I + 3 (A-, C-, G- and T-set) primers were used, resulting in the analysis of 2,048 combinations. In the case of *rym4*, 932 *Pst*1 + 2/

*Mse*I + 3 primer combinations were analysed using radioactively labelled primers or fluorescence detection on an ABI-Prism 377XL according to Schwarz et al. (1999).

Amplification of the SSR marker Bmac29 and the cleaved amplified polymorphic sequence (CAPS) marker MWG838 was performed as described by Graner et al. (1999). PCR for CAPS analysis of marker Y57c10 (forward primer: CGC TCG CAA TAC CTT CTT TCT, reverse primer: CAT AGC CTT TGC GTC TAC TC) was performed in a volume of 20 µl [0.25 µM of each primer, 200 µM dNTPs, 1 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Qiagen) and its corresponding reaction buffer], using 50 ng DNA. The following cycling conditions were used: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and a final extension step (5 min at 72°C). PCR products were incubated for 1 h at 37°C after adding 1 U of *Eco*RV and subsequently separated on 2% (w/v) agarose gels (GIBCO-BRL). Analysis of the RFLP marker MWG010 for the construction of the *rym4* high-resolution mapping population was carried out according to Graner et al. (1999).

Bulked segregant analysis

Bulked segregant analysis (Michelmore et al. 1991) was employed as a strategy for marker saturation. DNA pools were built from 14 (*rym5*) and 10 (*rym4*) resistant and susceptible DH lines of the two mapping populations including lines carrying recombinations adjacent to the marker interval. In addition to the above-mentioned progeny pools, a set of near isogenic lines (NILs) was used for marker enrichment at the *Rym4* locus. These were derived from a resistant DH progeny line (no. 27) of the above-mentioned 'Igri' × 'Franka' mapping population by three backcrosses to the susceptible parent 'Igri'. Line no. 27 had been selected as the donor parent, since genome-wide fingerprinting revealed a high portion (85.2% vs an expected value of 50%) of 'Igri' genome present in this F_1 anther-derived DH line. After three backcrosses to cv. 'Igri', NILs were selected that contained small chromosome fragments with the resistance gene from cv. 'Franka', which varied in size between 6.5% and 15.5% recombination, with the rest of the genome being derived from cv. 'Igri'.

Besides being mapped on RILs, newly developed markers were mapped on an informative subset of 114 F_1 anther-derived DH lines of the standard mapping population (Graner et al. 1999) comprising lines carrying recombinations in the region of the *Rym5* locus. In the case of the *Rym4* locus, the reference population consisted of 71 F_1 anther-derived DH lines of the cross between 'Igri' and 'Franka'.

In order to get information on their specificity, STSs derived from closely linked RAPDs and AFLPs of chromosome 3HL were analysed on a set of disomic and ditelosomic wheat barley addition lines (derived from wheat cv. 'Chinese Spring' and barley cv. 'Betzes', Islam et al. 1981).

Marker conversion

In order to convert closely linked AFLP markers into STSs, AFLP fragments were separated on denaturing polyacrylamide gels, followed by silver staining or autoradiography of [γ-^{33}P]ATP-labelled fragments, respectively. Polymorphic fragments were excised directly from the dried gels and were eluted by incubation in 100 µl ddH_2O at 100°C for 10 min. Cloning of PCR fragments was carried out using the TOPO TA cloning vector system (pCRII-TOPO, Invitrogen). After transformation 21 independent bacterial colonies were picked and used for re-amplification. Three clones that yielded amplicons of the expected size were sequenced using a SequiTherm EXCEL II DNA-Sequencing Kit-LC (Epicentre Technologies) according to the manufacturer's instructions. Genomic DNA sequences that flank an AFLP marker of interest were recovered and cloned by using the Universal GenomeWalker Kit (BD Biosciences, Heidelberg, Germany). Repetitive DNA sequences were identified by BLASTN screening the NCBI data base (Altschul et al. 1990) prior to primer design employing the software package Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). To determine optimum PCR conditions, an Eppendorf Mastercycler Gradient programmed as follows was used: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C ± 10°C, 30 s at 72°C and extension of 7 min at 72°C.

Sequences of monomorphic STSs were analysed with the software WEBCUTTER (http://www.ccsi.com/first-market/cutter/cut2.html) in order to assess their possible conversion to CAPS markers.

Resistance tests

Resistance tests were carried out on F_4 progenies as described by Ordon and Friedt (1993). Reaction to BaMMV was estimated in the four- to five-leaf stage by mechanical inoculation in the greenhouse in two replications, each comprising ten plants of each segmental RIL (for details cf. Ordon and Friedt 1993). Since neither BaYMV nor BaYMV-2 can be transmitted mechanically at sufficient rates, field experiments were performed at four locations, two infested with BaYMV-2 (Schladen/Lower Saxony and Eikeloh/Northrhine-Westphalia) and the other ones infested with BaMMV/BaYMV (Höhefeld/Baden-Wuertemberg and Giessen/Hesse) by growing at least 30 plants per line and location. The presence of virus particles was assessed by DAS-ELISA according to Clark and Adams (1977), using specific antisera against BaMMV and BaYMV, kindly provided by Dr. F. Rabenstein, Federal Centre for Breeding Research on Cultivated Plants (BAZ), Aschersleben, Germany. Combining the results

of mechanical inoculation and field trials facilitates monitoring the inheritance of the individual resistances to BaMMV, BaYMV and BaYMV-2.

Results

Development of high-resolution mapping populations for *rym4* and *rym5*

A high-resolution mapping population was constructed by screening for recombination events within the map interval delimited by the two co-dominant markers Y57c10 (proximal) and Bmac29 (distal) flanking *rym5* at a distance of 0.8 cM and 1.3 cM, respectively. In a first step, analysis of 1,026 F_2 plants resulted in the identification of 30 recombinant plants. Of these, 29 plants were heterozygous recombinants, since they carried one recombinant and one wild-type interval, while one plant showed two recombinant intervals. Thus, the genetic length of the interval amounted to 1.51% recombination. To generate RILs that carry the corresponding recombinations in a homozygous state, 12 F_3 plants from each of the originally identified F_2 plants were fingerprinted using the two flanking markers. Based on the analysis of these 30 genotypes (representing 31 recombination events), the *Rym5* locus was mapped $0.29 \pm 0.12\%$ recombination distal to Y57c10 and $1.22 \pm 0.24\%$ recombination proximal to Bmac29 (Fig. 1). To further increase the genetic resolution, an additional 2,858 F_2 plants were tested with the flanking markers, resulting in a resolution of 0.013% recombination based on a total population size corresponding to 3,884 F_2 plants. Out of these, 114 heterozygous recombinant plants and one homozygous recombinant (Table 1) were identified, resulting in a marker interval of $1.49 \pm 0.14\%$ recombination. The estimated segregation ratio between recombinant and non-recombinant plants was tested by a chi-square test ($\chi^2 = 17.97$, $df = 8$, $0.01 < p < 0.05$), indicating a slight deviation, which is caused by an excess of non-recombinant plants.

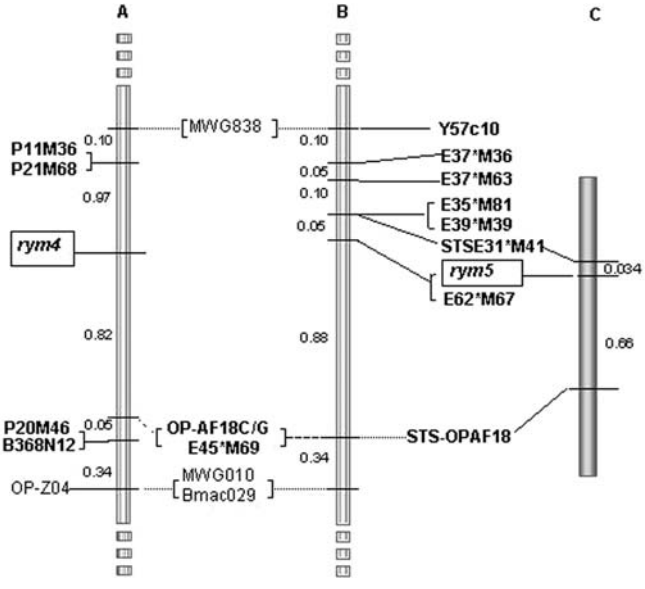

Fig. 1 Partial genetic maps of the distal portion of the long arm of barley chromosome 3H harbouring resistance genes *rym4* and *rym5*. **A** Cv. 'Igri' × cv. 'Franka' (*rym4*) high-resolution map based on the analysis of 1,040 F_2 individuals. **B** Cv. 'Alraune' × cv. 'W122/37.1 (*rym5*)' high-resolution map based on the analysis of 1,026 F_2 individuals. **C** High-resolution map of *rym5* based on the analysis of 86 recombinant inbred lines (RILs). Map positions (% recombination) of common markers are connected via *dashed lines*. Resistance genes are indicated in *boxes*. Newly mapped markers are printed in *boldface*

Similar results were obtained concerning *rym4*. Here, analysis of 1,040 F_2 plants led to the integration of the resistance locus at 1.07% recombination distal of MWG838 and 1.21% recombination proximal to Bmac29 (Fig. 1).

Marker saturation of the *Rym5* region

For marker saturation, DNA bulks derived from individuals of a DH mapping population (Graner et al. 1999) comprising both non-recombinant DH lines and lines carrying either a recombination distal or proximal to the target interval were employed for the identification of closely linked markers. For *rym5*, the analysis of 1,200 RAPD primers corresponding to about 18,000 RAPD fragments (on average 15 fragments/primer) led to the identification of 144 fragments differentiating between the resistant and the susceptible pool. Out of these, two new markers OP-AF18H971 (5′-GTG TCC CTC T-3′) and OP-AH12H550 (5′-TCC AAC GGC T-3′), closely linked to the *Rym5* locus, were identified. Only marker OP-AF18H971, amplifying an additional 971-bp fragment in susceptible lines, could be located in the interval Y57c10 – Bmac29 (Fig. 1), whereas OP-AH12H550 was mapped distal to Bmac29. Segregation analysis within the 30 RILs of the preliminary *rym5* high-resolution mapping population revealed that mar-

Table 1 Number of recombinants and non-recombinants between the markers Bmac029 and Y57c10

Genotype[a]	Observed frequency	Expected frequency
Non-Recombinant		
$Y_R\ Y_R\ B_R\ B_R$	936	930.61
$Y_S\ Y_S\ B_S\ B_S$	952	930.61
$Y_R\ Y_S\ B_R\ B_S$	1,882	1,861.99
Recombinant		
$Y_R\ Y_S\ B_S\ B_S$	31	40.01
$Y_R\ Y_S\ B_R\ B_R$	27	40.01
$Y_R\ Y_R\ B_R\ B_S$	31	40.01
$Y_S\ Y_S\ B_R\ B_S$	25	40.01
$Y_R\ Y_R\ B_S\ B_S$	0	0.39
$Y_S\ Y_S\ B_R\ B_R$	1	0.39
	$n = 3{,}884$	

[a] Y = Y57c10, B = Bmac029, R = allele of resistant parent, S = allele of susceptible parent. The expected frequency in the F_2 population is based on a distance of 2.1 cM between the markers Bmac029 and Y57c10 (Graner et al. 1999)

ker OP-AF18H971 is located 0.88% recombination distal of *rym5*.

The same DNA bulks as above have been used for AFLP-based marker saturation with 2,048 combinations of 32 *Eco*RI + 3 and 64 *Mse*I + 3 primers. They produced on average 100 fragments, thus leading to the analysis of about 205,000 loci. Seven AFLPs were mapped within the interval harbouring *rym5* (Y57c10 – Bmac29). Out of these markers E37/M63 (ACG/GAA), E35/M81 (ACA/TAG) and E31/M41 (AAA/AGG) showed an additional fragment in lines carrying *rym5*, while markers E39/M39 (AGA/AGA), E37/M36 (ACG/ACC), E62/M67 (CTT/GCA), and E45/M69 (ATG/GCG) were present in susceptible lines (*Rym5*). These markers could be integrated in the high-resolution genetic map as shown in Fig. 1. This could be confirmed by reassessment in 114 DH lines of the standard mapping population. In this case all markers located proximal of *rym5* co-segregated with this locus, while those located distally co-segregated with Bmac29 (Pellio et al. 2004). Furthermore, the same genetic position of the distally located co-segregating markers OP-AF18H971 and E45/M69 was verified within the high-resolution mapping population for the *Rym4* locus (Fig. 1). The other polymorphic markers for *rym5* turned out to be monomorphic on the *rym4* mapping population.

Marker saturation of the *Rym4* region

AFLP-marker saturation of the *Rym4* locus was performed by using the restriction enzyme/primer combination (PC) *Pst*I + 2/*Mse*I + 3. Analysis of 350 PCs on resistant and susceptible pools of DH progeny lines led to the identification of differential AFLPs in more than 10% of the assayed primer combinations. Further analysis of a sub-sample of 32 differential AFLPs on a single-plant level, using freshly isolated DNA samples, revealed that the expected AFLP fragments were no longer detectable in 20 cases. In the remaining cases, differential fragments were still present but detected map positions randomly distributed across the genome. However, in most cases markers could only be placed by accepting double-crossover events in some of the DH lines of the mapping population. Because of inconclusive results, marker saturation was repeated using a set of well-characterized NILs. Here, the analysis of 932 primer combinations revealed 32 differential AFLPs that could be assigned to three defined map intervals based on the information obtained from the analysis of three NILs that contained an introgressed fragment of different size. Genetic mapping revealed three markers, encoded P21M68, P11M36, and P20M46, that were flanking the *Rym4* locus at 0.97% and 0.82% recombination, respectively (Fig. 1).

Since none of these markers mapped closer to the gene than those markers developed for *rym5* (see previous paragraph; Fig. 1), no attempts were carried out to transfer them to the *rym5* population.

Epigenetic changes of AFLP patterns

While markers P11M36 and P20M46 could be integrated without further problems on the high-resolution map, marker P21M68 repeatedly was scored as heterozygous in 4 of the 45 RILs of the *rym4* high-resolution mapping population. To further investigate this observation, these AFLPs were converted into STS markers by cloning the corresponding AFLP fragments along with their flanking genomic DNA. Sequences were obtained for the two parents 'Franka' and 'Igri'. None of the fragments contained internal *Pst*I or *Mse*I restrictions sites, which, in theory, could cause differential AFLP patterns based on incomplete digestion. In all cases the fragment length deduced from the DNA sequence corresponded to that calculated for the AFLP band. The sequence of P20M46 revealed a 3-bp deletion in the *Pst*I recognition site present in cv. 'Franka', being the cause for the AFLP. In addition, the *Pst*I/*Mse*I fragment of 'Igri' contained four single-nucleotide polymorphisms (SNPs), a 6-bp insertion and a 6-bp deletion. In contrast to these findings, unaltered *Pst*I and *Mse*I sites were detected in 'Igri' and 'Franka' for markers P11M36 and P21M68. Moreover, no sequence polymorphism was detected within the AFLP fragments. Therefore, the sequence data obtained for P11M36 and P21M68 suggest that the observed AFLPs could have been caused by differential cytosine methylation of the *Pst*I recognition site. To test this hypothesis, *Pst*I-digested DNA of 'Igri' and 'Franka' was probed with the labelled *Pst*I/*Mse*I fragments of both markers (Fig. 2). The P11M36 fragment revealed a clear single-copy polymorphism between the two genotypes. The larger fragment of 'Franka' could be due to an insertion event, to the presence of a sequence polymorphism or to the methylation of one of the *Pst*I sites bordering the smaller 'Igri' fragment. While the first two hypotheses could not be tested because of the limited sequence information available, methylation of the *Pst*I site in 'Franka' would be in accordance with the presence of unaltered *Pst*I sites in both parents and the absence of the 175-bp *Pst*I/*Mse*I fragment in its AFLP pattern. When using the P21M68 DNA fragment as a hybridization probe, a DNA ladder pattern was observed for 'Igri'. This may reflect a partial methylation of several adjacent *Pst*I sites. In contrast to 'Igri', 'Franka' showed a clear single-copy fragment of higher intensity, which probably originated from complete digestion and the absence of *Pst*I methylation in the corresponding region. Methylation in 'Igri' was also supported by absence of the 171-bp *Pst*I/*Mse*I fragment in its AFLP pattern. Taken together, the results obtained from AFLP, Southern and PCR analyses provided evidence for the presence of cytosine methylation of the *Pst*I sites flanking the markers P11M36 and P21M68, respectively.

Marker conversion

As a prerequisite for efficient screening of a barley BAC library, attempts were carried out to convert the above-

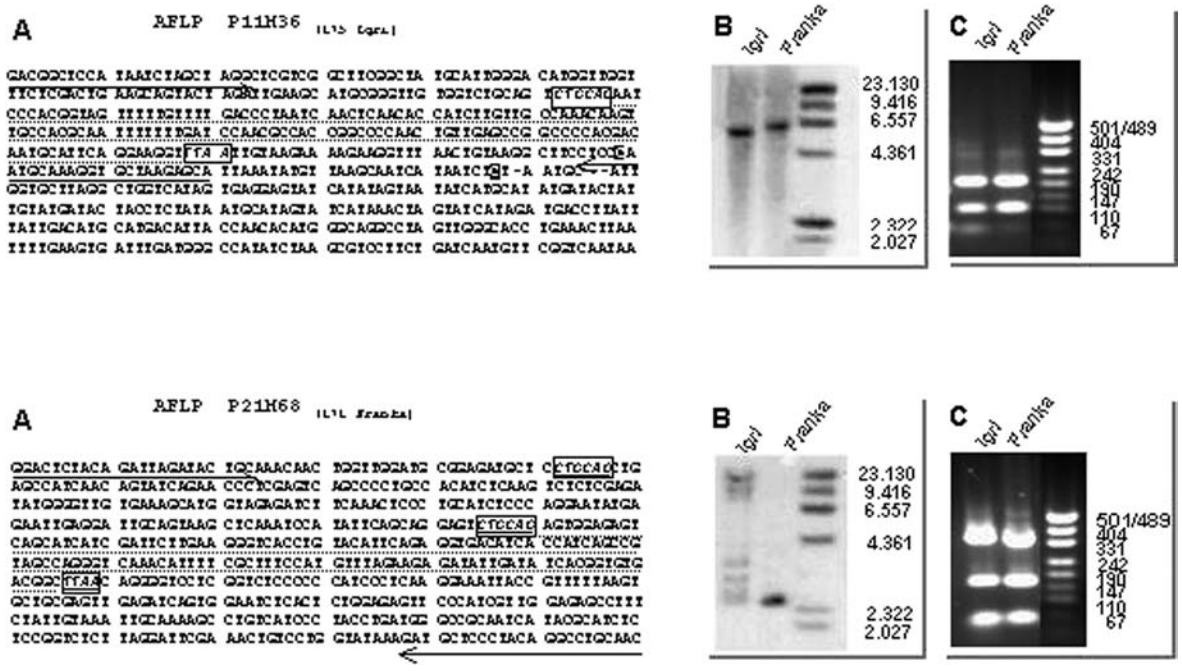

Fig. 2 Differential methylation of *Pst*I sites flanking AFLP markers P11M36$_{(175\ \text{Igri})}$ and P21M68$_{(171\ \text{Franka})}$. **A** Genomic DNA sequences (cv. 'Igri') of the AFLP fragments and the bordering regions. *Pst*I (CTGCAG) and *Mse*I (TTAA) sites are indicated by *boxes*, and AFLP fragments are marked by a *dotted line below the sequence*. Deletions and insertions relative to the sequence of cv. 'Franka' are indicated by *underlining* or *inverted colours* of the inserted nucleotide, respectively. *Boldfaced letters* mark single-nucleotide polymorphisms. *Arrows* highlight the primers used for PCR amplification. **B** Southern hybridization of *Pst*I-digested genomic DNA of 'Igri' and 'Franka' probed with the AFLP fragment P11M36 (*top*) and P21M68 (*bottom*). The *right lane* shows a lambda *Hin*dIII fragment size marker. **C** PCR amplicons obtained from the primers indicated in **A** after digestion with *Pst*I. Both mapping parents showed identical restriction patterns, which were also obtained for the 45 RILs of the high-resolution mapping population

mentioned markers to specific STSs. In order to improve the performance of polymorphism detection of RAPD primer OP-AF18, each of the four bases (A, C, G, T) was added to the 3′ end of the original decamer primer. The combination of OP-AF18 C and OP-AF18 G showed a clear-cut, easily detectable dominant polymorphism between resistant (*rym5*) and susceptible lines, while the other elongated primers did not exhibit any discriminating fragment (Pellio et al. 2004). Based on the sequence of this fragment, the primers (fwd: 5′-GTG TCC CTC TGA ACC ACA AG-3′, rev: 5′-GTG TCC CTC TCC TGT TTC TG-3′) were deduced, giving a monomorphic but specific fragment as revealed by testing wheat barley addition lines. By allele-specific sequencing, five SNPs were identified, among which the SNP at position 926 (T → C) created an additional *Eco*R105 restriction site in the susceptible parent cv. 'Alraune'. Re-mapping of this CAPS marker on the 115 recombinant lines revealed perfect co-segregation with OP-AF18.

With respect to converting AFLPs ranging from 74 bp to 609 bp, E39/M39 was excluded due to 91% sequence homology to repeats of the *Afa* family (Nagaki et al. 1998). The primers deduced from E45/M69 amplified a fragment present in wheat and barley and STSs derived from E62/M67, E35/M81 and E37/M36 generated fragments on all barley wheat addition lines However, the primers derived from E31/M41 (fwd: 5′-GAG TCG TCA CAA CGT ACC TTG C-3′, rev: 5′-GTG GCT GTA AAT AGG CTA AGG CC-3′) led to the amplification of a single 435-bp fragment on the resistant parental line being specific for chromosome 3HL (Fig. 3). Re-mapping of this STS resulted in co-segregation with the original polymorphic AFLP fragment E31/M41. Analysis of these newly developed STS markers on the 115 recombinant plants of the high-resolution mapping population led to the localisation of STS E31/M41 at 0.37% recombination distal of Y57c10, and STS-OPAF18 was mapped at 0.34% recombination proximal to Bmac29, resulting in an interval of 0.78% recombination harbouring *rym5*.

Unfortunately, out of the identified 115 recombinants, 29 were lost due to severe BYDV infection in the greenhouse. However, 86 recombinants corresponding to a resolution of 0.017% recombination have been analysed for BaMMV resistance, leading to mapping of *rym5* 0.034% recombination distal of STS-E31/M41 and 0.66% recombination proximal to STS-OPAF18 (Fig. 1).

Genetic fine structure of the Rym4/Rym5 locus

As outlined above, parental plants carrying the *Rym5* locus show immunity to BaMMV, BaYMV and BaYMV-2. In order to investigate whether this multiple

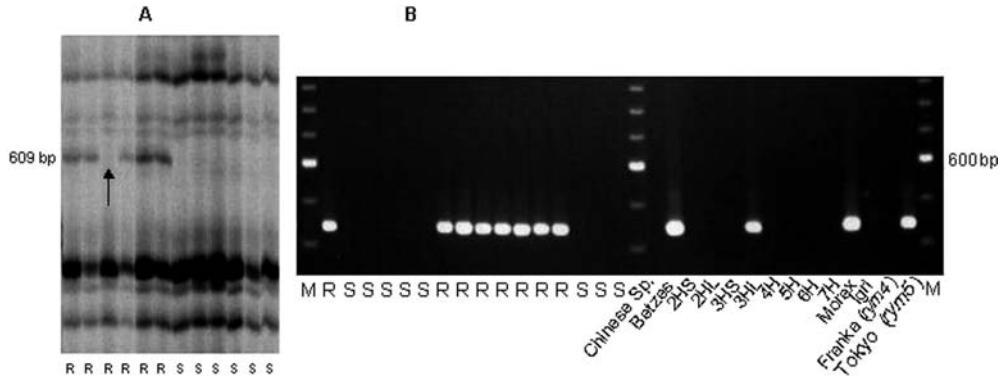

Fig. 3 **A** Banding pattern of AFLP E31/M41 on resistant and susceptible RILs. The absence of the 609-bp fragment in one of the resistant individuals is marked by an *arrow*, indicating the solely recombination event in the *rym5*-Y57c10 interval (1,026 F_2 plants). **B** STS-E31/M41 differentiating between resistant (*rym5*) and susceptible lines of the high-resolution mapping population (*left*). Analysis of STS-E31/M41 on a set of wheat barley addition lines indicating localisation on chromosome 3HL (*right*)

resistance is due to the presence of only one or several closely linked genes, 75 RILs corresponding to a genetic resolution of 0.019% recombination were tested individually for resistance to BaMMV (artificial inoculation) as well as to BaYMV and BaYMV-2 (field tests). No altered patterns of virus resistance could be detected, i.e. plants were either susceptible to all viruses or completely resistant (32r:43s, $\chi^2_{(1r:1s)} = 1.72$, 1*df*), leading to the conclusion that the *Rym5* locus comprised one gene effective against all viruses or two or three genes each effective against a single virus but closer linked than 0.019% recombination. Similarly, no intra-locus recombinants were observed for the *Rym4* locus, since a population of 45 RILs corresponding to 1,040 F_2 plants and a genetic resolution of 0.05% recombination either showed complete resistance to both BaMMV and BaYMV or complete susceptibility to the two viruses (19r:25s, $\chi^2_{(1r:1s)} = 0.818$, 1*df*).Thus, a total of 2,080 (*rym4*) and 5,022 (*rym5*) gametes have been analysed without revealing any intra-locus recombination.

Discussion

High-resolution genetic mapping of the recessive virus resistance locus *Rym4*/*Rym5* of barley in conjunction with marker saturation of the target interval has been achieved by molecular mapping in two large F_2 populations. A genetic resolution of 0.013% and 0.05% recombination could be established for *rym5* and *rym4*, respectively. Thus, the presented data provide an essential step towards the map-based cloning of the virus resistance genes *rym4*/*rym5*.

Genetic versus physical resolution

The genome size of barley comprises roughly 5,000 Mb DNA (Bennett and Leitch 1995). Given a genetic map length of about 1,300 cM and an even distribution of recombination across the genome, it would require analysis of some 4,000 meiotic events to resolve a recombination at every 100 kb, which corresponds to the average insert length of a BAC clone (Yu et al. 2000). However, recombination is not evenly distributed over the barley genome. Hot and cold spots/regions of recombination have been detected for the barley genome based on the physical mapping of STS markers. For the telomeric region of chromosome 3HL around the *Rym4*/*Rym5* locus, a ratio of 400 kb/cM was determined. Here, the resistance genes are located in a telomeric region that makes up only 5% of the physical length but 35% of the length of the genetic map of chromosome 3H (Künzel et al. 2000). This suggests that the analysis of 500 to 1,000 meiotic events could be sufficient to reach the necessary genetic resolution for chromosome landing at the *Rym4*/*Rym5* locus. However, these considerations remain speculative, since ratios of physical to genetic distances are subject to abrupt local changes. For example, at the *Lr10* leaf rust resistance locus in wheat, recombination frequencies change from 0.6 Mb/cM to 12 Mb/cM within a 230 kb physical distance (Stein et al. 2000). Similar observations were made at the *Mla* locus and the *Rpg1* locus in barley (Wei et al. 1999; Brueggeman et al. 2002). Thus, even if a gene is located in a favourable region of the genome, e.g. close to the telomeres, it still depends on the genomic context around that gene, whether map-based cloning by chromosome walking will be successful. This is also illustrated by the fact that about 8,000 gametes were analysed to reach sufficient genetic resolution for the map-based isolation of the two barley genes *rar1* and *Rpg1* (Shirasu et al. 1999; Brueggeman et al. 2002). On the other hand, only 520 F_2/F_3 plants were sufficient to allow for chromosome landing at the wheat *Lr21* locus, due to the availability of a co-segregating marker, which was part of the finally cloned disease resistance gene (Huang et al. 2003). As to the present study, it can only be speculated whether the combined genetic resolution from both crosses (1/9,848 = 0.01% recombination) will be sufficient to support for chromosome walking towards the *Rym4*/*Rym5* locus. However, first results from physical map construction showed that the resolution was sufficient to orient a 100-kb BAC selected by an STS derived

from AFLP-marker E31/M41 on the high-resolution genetic map (results not shown).

Genetic fine structure of the *Rym4*/*Rym5* locus

Many of the resistance genes that have been cloned to date represent members of gene families that are organized as clusters of closely linked genes. It has been shown that different members of these clusters can give rise to different specificities, i.e. confer resistance to different races of a given pathogen such as *RPP*1 (Botella et al. 1998) or *Cf*-4 (Takken et al. 1999). Alternatively, two homologous genes, which form members of a small gene family present on a 115-kb genomic fragment in potato, have been shown to confer resistance to different pathogens of potato (van der Vossen et al. 2000). In the case of tomato, a single gene (*Mi*) has been shown to confer resistance to two different pathogens (Rossi et al. 1998; Vos et al. 1998). BaYMV and BaYMV-2 are two strains of a virus that differ in their virulence, while BaMMV represents a different virus. Regarding the *Rym4*/*Rym5* locus, the question arises whether the resistance is encoded by different genes, with each carrying a different specificity or by a single gene, characterized by the presence of at least three alleles (susceptible, *rym4*, *rym5*, Graner et al. 1999). Fine mapping of the *Rym4*/*Rym5* locus based on more than 7,000 meiotic events did not reveal any intra-locus recombinants that would provide experimental evidence for the presence of a gene family. Thus, our results lend strength to the hypothesis that a single gene encodes resistance at the *Rym4*/*Rym5* locus. However, due to the finite resolution of genetic experiments and because of the possibility that recombination may be suppressed around the resistance locus, only the physical isolation of the locus will provide final evidence on the molecular basis of the individual resistance specificities.

Marker saturation

PCR-based marker technologies in combination with bulked segregant analysis have proven useful for marker saturation of target intervals (e.g. Simons et al. 1997; Lahaye et al. 1998a). The efficiency of this procedure can be enhanced by the use of marker pre-selected pools incorporating recombinants adjacent to the interval of interest (e.g. Cnops et al. 1996; Lahaye et al. 1998b). Using this approach, out of about 18,000 RAPD fragments and 205,000 *Eco*RI/*Mse*I AFLP fragments analysed, seven additional AFLP and one additional RAPD marker could be incorporated into the 1.5 cM interval between Y57c10 and Bmac29 in the case of the *rym5* population. This corresponds reasonably to the predicted frequencies, based on a genome-wide polymorphism of 9% for AFLPs (18,500 polymorphic fragments) and 12% for RAPDs (2,200 polymorphic fragments) for the *rym5* population and an assumed genetic map length of about 1,400 cM. At a given equal distribution of recombination frequencies, about 20 AFLP and two RAPD markers are expected within the target interval. Wei et al. (1999) reported three polymorphic RAPD fragments in a 3 cM interval at the *Mla* locus of barley out of 739 analysed random primers. In the same analysis, they generated 132 polymorphic AFLP fragments out of a total of 256 primer combinations, leading to the identification of seven polymorphic markers. This is less than what has been observed in the present study and what could be predicted. However, the different success of AFLP-based marker saturation at the *Rym5* and the *Rym4* locus, as has been observed in this study, implies that the parental combination chosen to build up a mapping population is of critical importance for the degree of polymorphism and for the recombination frequency within a certain target region of the barley genome. Therefore, it seems crucial to have available more than a single mapping population derived from genetically distant parents. Our results also show that the choice of the restriction enzymes used for marker saturation may be of crucial importance for the successful marker saturation of a target region. The low marker coverage that was obtained by *Pst*I/*Mse*I AFLPs may be due to the bias of *Pst*I to cut hypomethylated CG or CNG sites, which are supposed to be associated with genes (e.g. Lindsay and Bird 1987). Indeed, sequence analysis of a random set of RFLP probes derived from *Pst*I-restricted genomic DNA of barley revealed a significant portion of known genes (Michalek et al. 1999). Southern analysis of the three *Pst*I/*Mse*I fragments that were placed on the high-resolution map of the *rym4* population revealed single-copy fragments that could be easily converted into STS markers, suitable for screening of a barley BAC library. Two of the three markers showed BLASTN homologies to ESTs from wheat ($E < 10–30$), providing further evidence that *Pst*I/*Mse*I AFLPs are biased to originate from transcribed regions in the barley genome. It should be noted in this context that *Eco*RI/*Mse*I fragments usually comprise repetitive DNA fragments and pose more difficulties regarding STS-marker development.

It is well known that *Pst*I/*Mse*I AFLPs detect epigenetic variation caused by the presence of 5-methylcytosine in the recognition sequence of *Pst*I (5′-CTGCAG-3′, Knox and Ellis 2001). The observed polymorphisms of two out of three closely linked markers in the present study were based on such epigenetic changes. Although knowledge on the Mendelian inheritance of methylation patterns is still sparse, the presence of C-methylation polymorphisms and their stable inheritance has been exploited for linkage mapping in maize, using a modified AFLP procedure (Vuylsteke et al. 1999, 2000). However, since environmental factors are also known to highly influence DNA methylation, it is not surprising that we found evidence for partial methylation present at AFLP P21M68. Similar observations have been made in pea, where 2 out of 24 progeny lines showed AFLP patterns indicative of partial methylation (Knox and Ellis 2001).

In any progeny line affected by partial methylation, the corresponding locus is isolated by flanking crossover events. The result is an increase in genetic map length, or if the "double crossovers" are too numerous, the incorrect arrangement of a marker. The presence of partial methylation in those progeny lines that were pooled in the initial bulked segregant analysis of the *rym4* population could explain the many wrong positive AFLP fragments that differentiated the bulks but subsequently did not map to the target region. Since population development for high-resolution mapping frequently extends over periods of several years, it seems important to isolate DNA from plants that have been grown under standardized conditions, at defined growth stages and from defined tissues. Otherwise, partial cytosine methylation might influence the genetic analysis when methylation-sensitive marker systems are to be used.

Marker-assisted selection

Due to their very close linkage to the resistance locus several markers are of potential use for marker-assisted selection (MAS) in practical barley breeding. With respect to resistance genes for the BaYMV complex, many easy-to-handle PCR-based markers have been developed (for overview cf. Werner et al. 2003; Le Gouis et al. 2004). Due to its diagnostic character, the barley microsatellite marker Bmac29 is widely applied in European barley breeding programmes (Schiemann and Backes 2000). However, the two Austrian cultivars 'Prima' and 'Petra' exhibited the *rym4*-specific allele of marker Bmac29 while being tested susceptible in phenotypic screens (J. Lafferty, personal communication). Thus, recombination is likely to have occurred between Bmac29 and the resistance gene, which is not unexpected, based on the genetic distance of 1% recombination between this marker and the *Rym4*/*Rym5* locus. Analysis of the two cultivars with marker OPAF18H971C/G revealed the correct marker allele indicating the potential value of this marker to further improve MAS for this trait.

Conclusions

In the present study, a high-resolution map of the *Rym4*/*Rym5* locus was constructed, and closely linked markers have been developed. These will be instrumental for the isolation of seeding BACs and the subsequent development of a physical BAC contig map of this chromosomal region. The paucity of *Pst*I/*Mse*I AFLPs in the *Rym4* region prompted us to speculate that the locus is in a chromosomal region characterized by a low density of transcribed genes. Moreover, isolation of the *Rym4*/*Rym5* locus will reveal whether it consists of a single gene with multiple specificities or whether it comprises two or three genes genetically linked closer than 0.019% recombination.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Mol Biol 215:403–410

Bennett MD, Leitch IJ (1995) Nuclear DNA amounts in angiosperms. Ann Bot 76:113–176

Botella MA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JDG (1998) Three genes of the *Arabidopsis* RPP1 complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. Plant Cell 10:1847–1860

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A (2002) The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc Natl Acad Sci USA 99:9328–9333

Clark MJ, Adams AN (1977) Characteristics of the microplate method of the enzyme linked immunosorbent assay for the detection of plant viruses. J Gen Virol 34:475–483

Cnops G, den Boer B, Gerats A, Van Montagu M, Van Lijsebettens M (1996) Chromosome landing at the Arabidopsis *TORNADO1* locus using an AFLP-based strategy. Mol Gen Genet 253:32–41

Doyle JF, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bull 19:11–15

Friedt W, Werner K, Ordon F (2000) Genetic progress as reflected in highly successful and productive modern barley cultivars. In: Proceedings of the 8th international barley genetics symposium, Adelaide, 22–27 Oct 2000, Inv. Papers I, pp 271–279

Götz R, Friedt W (1993) Resistance to the barley yellow mosaic virus complex—differential genotypic reactions and genetics of BaMMV-resistance of barley (*Hordeum vulgare* L.). Plant Breed 111:125–131

Graner A, Bauer E (1993) RFLP mapping of the *ym4* virus resistance gene in barley. Theor Appl Genet 86:689–693

Graner A, Bauer E, Kellermann A, Proeseler G, Wenzel G, Ordon F (1995) RFLP analysis of resistance to the barley yellow mosaic virus complex. Agronomie 15:475–479

Graner A, Streng S, Kellermann A, Schiemann A, Bauer E, Waugh R, Pellio B, Ordon F (1999) Molecular mapping and fine structure of the *rym5* locus encoding resistance to different strains of the Barley Yellow Mosaic Virus Complex. Theor Appl Genet 98:285–290

Hariri D, Meyer M, Le Gouis J, Bahrman N, Fouchard M, Forget C, Andre A (2000) Characterisation of BaYMV and BaMMV pathotypes in France. Eur J Pathol 106:365–372

Hariri D, Meyer M, Prud'homme H (2003) Characterization of a new barley mild mosaic virus pathotype in France. Eur J Pathol 109:921–938

Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. Genetics 164:655–664

Huth W (1984) Die Gelbmosaikvirose der Gerste in der Bundesrepublik Deutschland—Beobachtungen seit 1978. Nachrichtenbl Deut Pflanzenschutzd 36:49–55

Huth W (1989) Ein weiterer Stamm des Barley yellow mosaic virus (BaYMV) gefunden. Nachrichtenbl Deut Pflanzenschutzd 41:6–7

Huth W, Adams MJ (1990) Barley yellow mosaic virus (BaYMV) and BaYMV-M: two different viruses. Intervirology 31:38–42

Ikata S, Kawai I (1940) Studies on wheat yellow mosaic disease. Noji Kairyo Shiryo 154:1–123

Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. Heredity 46:161–174

Kanyuka K, McGrann G, Alhidaib K, Hariri D, Adams MJ (2004) Biological and sequence analysis of a novel European isolate of Barley mild mosaic virus that overcomes the barley *rym5* resistance gene. Arch Virol 149:1469–1480

Knox MR, Ellis THN (2001) Stability and inheritance of methylation states at *Pst*I sites in Pisum. Mol Genet Genomics 265:497–507

Konishi T, Ban T, Iida Y, Yoshimi R (1997) Genetic analysis of disease resistance to all strains of BaYMV in a Chinese barley landrace, Mokusekko 3. Theor Appl Genet 94:871–877

Kühne T, Shi N, Proeseler G, Adams MJ, Kanyuka K (2003) The ability of a bymovirus to overcome the *rym4*-mediated resistance in barley correlates with a codon change in the *Vpg* coding region on RNA1. J Gen Virol 84:2853–2859

Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Lahaye T, Shirasu K, Schulze-Lefert P (1998a) Chromosome landing at the barley *Rar1* locus. Mol Gen Genet 260:92–101

Lahaye T, Hartmann S, Topsch S, Freialdenhoven A, Yano M, Schulze Lefert P (1998b) High-resolution genetic and physical mapping of the *Rar1* locus in barley. Theor Appl Genet 97:526–534

Le Gouis, J, Devaux P, Werner K, Hariri D, Bahrman N, Beghin D, Ordon F (2004) *rym15* from the Japanese cultivar 'Chikurin Ibaraki 1' is a new Barley Mild Mosaic Virus (BaMMV) resistance gene mapped on chromosome 6H. Theor Appl Genet 108:1521–1525

Lindsay S, Bird AP (1987) Use of restriction enzymes to detect potential gene sequences in mammalian DNA. Nature 327:336–338

Michalek W, Künzel G, Graner A (1999) Sequence analysis and gene identification in a set of mapped RFLP markers in barley (*Hordeum vulgare*). Genome 42:849–853

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using bulked segregant analysis. Proc Natl Acad Sci USA 88:9828–9832

Muramatsu M (1976) Breeding of malting barley which is resistant to barley yellow mosaic. In: Proceedings of 3rd international barley genetics symposium, Garching, Germany, Barley Genet III:476–485

Nagaki K, Tsujiomoto H, Sasakuma T (1998) Dynamics of tandem repetitive *Afa*-family sequences in Triticaea, wheat related species. J Mol Evol 47:183–189

Nomura K, Kashiwazaki S, Hibino H, Inoue T, Nakata E, Tsuzaki Y, Okuyama S (1996) Biological and serological properties of strains of Barley Yellow Mosaic Virus. J Phytopath 144:103–107

Ordon F, Friedt W (1993) Mode of inheritance and genetic diversity of BaMMV-resistance of exotic barley germplasms carrying genes different from '*ym4*'. Theor Appl Genet 86:229–233

Ordon F, Bauer E, Friedt W, Graner A (1995) Marker-based selection for the *ym4* BaMMV-resistance gene in barley using RAPDs. Agronomie 15:481–485

Ordon F, Köhler H, Pellio B, Friedt F (2000) Recombination: From genetic towards physical distances: High resolution mapping of plant resistance genes. Prog Bot 61:37–53

Pellio B, Friedt W, Graner A, Ordon F (2004) Development of PCR-based markers closely linked to *rym5*. J Plant Disease Prot 111:30–38

Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc Natl Acad Sci USA 95:9750–9754

Saghai MMA,. Biyashev RM, Yang GP, Zhang Q, Allard RW (1984) Extraordinarily polymorphic microsatellite DNA in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 91:5466–5470

Schiemann A, Backes G (2000) The use of molecular markers in practical barley breeding. In: Proceedings or the 8th international barley genetics symposium, vol 3, Adelaide, pp 42–44

Schwarz G, Michalek W, Mohler V, Wenzel G, Jahoor A (1999) Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high resolution mapping with AFLP markers. Theor Appl Genet 98:521–530

Shirasu K, Lahaye T, Tan MW, Zhou FS, Azevedo C, Schulze-Lefert P (1999) A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell 99:355–366

Simons G, van der Lee T, Diergaarde P, van Daelen R, Groenendijk J, Fritjers A, Büschges R, Hollrichter K, Töpsch S, Schulze-Lefert P, Salamini S, Zabeau M, Vos P (1997) AFLP-based mapping of the *Mlo* gene to a 30-kb DNA segment of the barley genome. Genomics 44:61–70

Stein N, Graner A (2004) Map-based gene isolation in cereal genomes. In: Gupta PK, Varshney RK (eds) Cereal genomics. Springer, Berlin Heidelberg New York

Stein N, Feuillet C, Wicker T, Schlagenhauf E, Keller B (2000) Subgenome chromosome walking in wheat: a 450 kb physical contig in *Triticum monococcum* L. spans the *Lr10* resistance locus in hexaploid wheat (*T. aestivum* L.). Proc Natl Acad Sci USA 97:13436–13441

Takken FLW, Thomas CM, Joosten MHAJ, Golstein C, Westerink N, Hille J, Nijkamp HJJ, De Wit PJGM, Jones JDG (1999) A second gene at the tomato *Cf*-4 locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulence determinant. Plant J 20:279–288

Toyama A, Kusaba T (1970) Transmission of soil-borne barley yellow mosaic virus. 2. *Polymyxa graminis* Led. as vector. Ann Phytopath Soc Jpn 36:223–229

Usugi T, Kashiwazaki S, Omura T, Tsuchizaki T (1989) Some properties of nucleic acids and coat proteins of soil-borne filamentous viruses. Ann Phytopath Soc Jpn 55:26–31

Vos P, Simons G, Jesse T, Wijbrandi J, Heinen L, Hogers R, Frijters A, Groenendijk J, Diergaarde P, Reijans M, Fierens-Onstenk J, de Both M, Peleman J, Liharska T, Hontelez J, Zabeau M (1998) The tomato *Mi*-1 gene confers resistance to both root-knot nematodes and potato aphids. Nat Biotechnol 16:1365–1369

van der Vossen EAG, van der Voort JNAMR, Kanyuka K, Bendahmane A, Sandbrink H, Baulcombe DC, Bakker J, Stiekema WJ, Klein-Lankhorst RM (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. Plant J 23:567–576

Vuylsteke M, Mank R, Antonise R, Bastiaans E, Senior ML, Stuber CW, Melchinger AE, Lubberstedt T, Xia XC, Stam P, Zabeau M, Kuiper M (1999) Two high-density AFLP (R) linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theor Appl Genet 99:921–935

Vuylsteke M, Mank R, Brugmans B, Stam P, Kuiper M (2000) Further characterization of AFLP (R) data as a tool in genetic diversity assessments among maize (*Zea mays* L.) inbred lines. Mol Breed 6:265–276

Wei F, Gobelman-Werner K, Moroll SM, Kurth J, Mao L, Wing R, Leister D, Schulze-Lefert P, Wise RP (1999) The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR Gene Families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of Barley. Genetics 153:1929–1948

Werner K, Friedt W, Laubach E, Waugh R, Ordon F (2003) Dissection of resistance to soil-borne yellow-mosaic-inducing viruses of barley (BaMMV, BaYMV, BaYMV-2) in a complex breeder cross by means of SSRs and simultaneous mapping of BaYMV/BaYMV-2 resistance of var. 'Chikurin Ibaraki 1'. Theor Appl Genet 106:1425–1432

Yu Y, Tomkins JP, Waugh R, Frisch DA, Kudrna D, Kleinhofs A, Brueggeman RS, Muehlbauer GJ, Wise RP, Wing RA (2000) A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor Appl Genet 101:1093–1099

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European patent application number 92402629.7, publication number 0 534 858 A1