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ORIGINAL PAPER

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Fine-mapping of the BaMMV, BaYMV-1 and BaYMV-2 resistance of barley (*Hordeum vulgare*) accession PI1963

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Abstract Barley yellow mosaic disease caused by the bymoviruses barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV) is one of the economically most important diseases of winter barley in Europe. In European barley breeding programmes, resistance is currently due to only two genes—*rym4*, which is effective against viruses BaMMV and BaYMV-1, and *rym5*, which is effective against BaYMV-2. Diversification of resistance is therefore an important task. Because the accession PI1963 confers immunity against all European strains of barley yellow mosaic disease and is not allelic to *rym5*, we have attempted to develop closely linked markers in order to facilitate the efficient introgression of this resistance into adapted germplasm. By means of restriction fragment length polymorphism analysis, we located a gene locus for resistance to BaMMV, BaYMV-1 and BaYMV-2 of PI1963 on chromosome 4HL using a mapping population (W757) comprising 57 doubled haploid (DH) lines. Subsequent tests for allelism indicated that the BaMMV resistance gene in PI1963 is allelic to *rym11*. Two DH populations, IPK1 and IPK2, comprising 191 and 161 DH lines, respectively, were derived from the initial mapping population W757 and used for further analysis. As random amplified polymorphic DNA development did not facilitate the identification of more closely linked markers, simple sequence repeat (SSR) analyses were conducted. For population IPK1, the closest SSRs detected were Bmac181 and Bmag353, which flank the gene at 2.1 cM and 2.7 cM, respectively. For the IPK2 population, the SSR markers HVM3 and Bmag353 are located proximally at 2.5 cM and distally at 8.2 cM, respectively. In order to develop markers more tightly linked to *rym11*, a targeted amplified fragment length polymorphism (AFLP) marker identification approach was adopted using bulks comprising lines carrying recombination events proximal and distal to the target interval. Using this approach we identified six AFLP markers closely linked to *rym11*, with the two markers, E56M32 and E49M33, co-segregating with *rym11* in both populations. The SSRs and AFLPs identified in this study represent useful tools for marker-assisted selection.

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Introduction

Barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV), both belonging to the bymoviruses (Usugi et al. 1989) and transmitted by the soilborne fungus *Polymyxa graminis* (Toyama and Kusaba 1970), are the causal agents of barley yellow mosaic disease. These viruses occur either separately or in mixed infections (Huth and Adams 1990) and cause severe yield losses due the increased sensitivity to cold stress and lower vigor of the infected plants (Huth 1984; Friedt et al. 1990). With respect to pathogenicity, in Japan seven strains of BaYMV and two of BaMMV have been distinguished (Nomura et al. 1996). In Europe, only two strains of BaYMV (BaYMV-1 and BaYMV-2) have been described (Huth 1989), and to date there has only been one strain of BaMMV reported in Germany (Huth and Adams 1990), although a second strain of BaMMV has recently been detected in France (Hariri et al. 2003).

In Germany, the resistance to barley yellow mosaic disease of commercial cultivars has been mainly based on the recessive resistance gene *rym4*, derived from the Dalmatian landrace Ragusa (Huth 1985). This gene has been assigned to the long arm of chromosome 3H using telotrisomic and restriction fragment length polymorphism (RFLP) analysis (Kaiser and Friedt 1989, 1992; Graner and Bauer 1993). The *rym4* gene confers resistance to both BaMMV and BaYMV-1 but is not effective against BaYMV-2 (Huth 1990). Therefore, *rym5*, detected in the Chinese landrace Mokusekko 3 (Konishi et al. 1997; Graner et al. 1999) and displaying resistance to all German yellow mosaic-inducing viruses, has been introduced into a number of barley cultivars (for example, Anastasia and Kamoto (Anonymous 2003). Because of the economic importance of *rym5*, an effort has been undertaken to isolate this gene via map-based cloning (Perovic et al. 2000b; Stein et al. 2004). At the present time *rym5* is the only resistance gene effective against all agents of the BaYMV complex in Germany, but it has already been overcome in France (Hariri et al. 2003).

In attempts to diversify the sources of resistance to barley yellow mosaic disease, extensive screening programs were carried out on barley germplasm. As a result of these, numerous recessive resistance loci were identified (Götz and Friedt 1993; Ordon et al. 1993; Ordon and Friedt 1993) and subjected to intensive molecular mapping (for overview, see Graner et al. 2000; Werner et al. 2003). Among the screened germplasm, the accession PI1963 was identified as being resistant to BaMMV, BaYMV-1 and BaYMV-2. The purpose of the project reported here was to map the resistance of PI1963, which in subsequent tests for allelism turned out to be different from *rym4* and *rym5*, and to develop closely linked markers suitable for marker-assisted selection.

Materials and methods

Plant material

Initial mapping was performed in a barley (*Hordeum vulgare*) population consisting of 57 F_1 anther-derived doubled haploid (DH) lines (W757) originating from a cross between cv. Marinka [susceptible (s)] and the accession PI1963 [resistant (r)]. In order to obtain a sufficient number of DH lines, we crossed four DH lines with contrasting resistance, which resulted in two F_1-derived DH populations, IPK1 [W757/112 (r) × W757/982 (s)] (191 DH lines)] and IPK2 [W757/924 (s) × W757/612 (r)] (161 DH lines). A total of 352 DH lines were thus available for mapping.

On the basis of the initial mapping results, we carried out tests for allelism on F_1 plants derived from crosses of resistant lines of the initial mapping population (W757) to resistant plants derived from the populations Russia 57 (*rym11*) × Magie and [Russia 57 (*rym11*) × Alraune] × Alraune originally used for mapping *rym11* (Bauer et al. 1997).

Disease assessment and DNA extraction

The 57 DH lines of the original W757 population have been tested for BaMMV by mechanical spray gun inoculation in the greenhouse (Ordon and Friedt 1993). In both 1998 and 1999, field tests (double rows, each row comprising 30 seeds) were performed in Höhefeld (Baden-Würtemberg, Germany) for resistance to BaMMV and BaYMV-1 and in Schladen (Lower Saxony, Germany) for resistance to BaYMV-2. Reaction to the different viruses was estimated by DAS-ELISA using antisera kindly provided by Dr. Frank Rabenstein, Federal Centre for Breeding Research on Cultivated Plants, Aschersleben, Germany.

Based on the results obtained on the initial 57 DH lines, there was no observable segregation of the resistance trait to the different viruses; i.e. all lines were completely resistant or completely susceptible to BaMMV, BaYMV-1 and BaYMV-2. Therefore, the progenies IPK1 and IPK2 were only tested for BaMMV resistance by mechanical inoculation in the greenhouse followed by DAS-ELISA (for details see Ordon and Friedt 1993). Each line was tested in two replications consisting of five plants each. Tests for allelism of BaMMV resistance were carried out in the same way by analysing at least ten F_1 seeds from the above-mentioned crosses in the greenhouse. DNA isolation was performed using the protocol described by Doyle and Doyle (1990).

RFLP analysis

RFLP analyses were performed according to Graner et al. (1990). In brief, 10 µg of genomic DNA was digested with either *Bam*HI (markers: MWG611, MWG880), *Dra*I (markers: MWG2036, MWG2134), *Eco*RI (markers: ABG3, MWG2246), *Hin*dIII (marker: MWG2299) or *Xba*I (markers: MWG948, MWG029, MWG057, MWG2180). Following electrophoresis on a 0.8% agarose gel, the DNA fragments were transferred (0.6 *M* NaCl, 0.4 *M* NaOH) to a nylon membrane (Pall, Dreieich). Upon completion of the transfer, the membranes were rinsed in 2× SSC (pH 7) and dried at 80°C.

For probe labelling, inserts were either excised from the plasmid vector or amplified using flanking primers and then purified by electrophoresis under standard conditions through 0.8 – 1.2% gels of low-melting-point agarose (SeaPlaque GTG, Biozym, Oldendorf, Germany). After visual quantification, the DNA was diluted to about 1 ng μl^{-1}. Aliquots were labeled with [^{32}P]-dCTP using random hexamer primers. Hybridisation was performed overnight in a solution containing 5× SSPE (pH 7.4), 5× Denhardt, 0.2% sodium dodecyl sulphate (SDS) and 250 µg ml^{-1} herring sperm DNA. Following purification from unincorporated dNTPs by chromatography through a Sepadex column, the labeled probe was added to yield a specific radioactivity of $1x10^6$ cpm ml^{-1} hybridisation solution. After two washing steps (30 min, 0.5× SSC, 0.1% SDS, 50°C;

10 min, 0.2× SSC, 0.1% SDS, 65°C) the DNA fragments were visualised by autoradiography (Hyperfilm MP, Amersham, Braunschweig; X-OMAT intensifying screens, Kodak, Rochester, N.Y.).

Bulked segregant analysis for identifying random amplified polymorphic DNA markers

In order to identify additional RAPD markers closely linked to the resistance locus, we carried out a bulked segregant analysis (BSA, see Michelmore et al. 1991) on pooled samples comprising seven resistant and seven susceptible DH lines selected from the W757 population. The number of lines included in the pools was limited to seven because W757 had already been pre-selected for resistance, thereby resulting in a loss of susceptible lines.

The RAPD analysis was carried out as described by Ordon et al. (1995) using primer sets from OP-AA to OP-AZ (Operon Technology, Alameda, Calif.).

Microsatellite analysis

As RAPD marker development was quite ineffective, a simple sequence repeat (SSR) analysis was carried out in the IPK1 and IPK2 populations. The resistance of PI1963 had been assigned to chromosome 4H based on the RFLP analysis (Fig. 1), therefore only SSRs located on this chromosome were included in the analysis. Twenty-four microsatellites (Ramsay et al. 2000; Thiel et al. 2003) were screened for polymorphism in the IPK1 and IPK2 parental lines (Table 1). The PCR analyses were as described by Ramsay et al. (2000) and Thiel et al. (2003), with SSR polymorphism detection carried out on denaturing 8% polyacrylamide gels run in a dual laser Li-Cor Model 4200 IR^2 Series DNA Sequencer (Lincoln, Neb.).

BSA for targeted AFLP identification

In order to achieve further marker saturation of this region, we carried out an amplified fragment length polymorphism (AFLP) analysis. Information on the genetic variability within IPK1 and IPK2 was obtained using ten AFLP primer combinations—E49M49, E56M32, E49m33, E59M41, E51M38, E57M40, E57M33, E54M35, E50M43 and E62M41—for analysing the parental lines. Two BSA assays were simultaneously conducted for the IPK1 and IPK2 populations. The resistant and susceptible bulks consisted of DNA aliquots from ten resistant and ten susceptible lines pooled for each population. A targeted AFLP marker identification was adopted as described by Lahaye et al. (1998). The bulked lines were selected based on the resistance phenotype and the genotypes, i.e. linked SSR and RAPD markers located outside of the targeted genomic window encompassing the *rym11* gene. Three genotypic classes were mixed in each bulk—plants showing no recombination, plants carrying recombinations at the proximal side of the target interval and

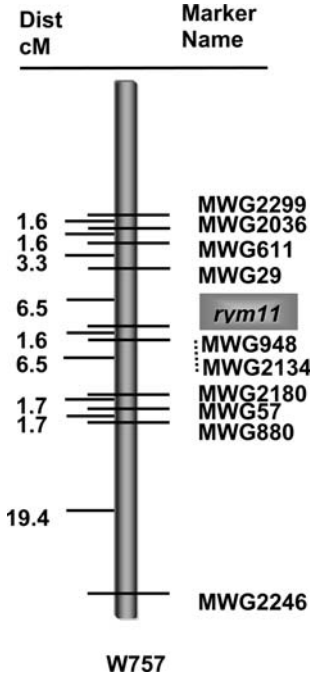

Fig. 1 Barley chromosome 4H partial RFLP map harbouring *rym11* [W757 population (Marinka × PI1963) comprising 57 DH lines]

Table 1 Polymorphism tests of the SSR markers on the IPK1 and IPK2 parental lines. For each SSR locus, the plus (+) and negative (−) signs indicate the presence or absence of polymorphism, respectively

SSR	IPK1	IPK2
HVM3	−	+
Bmag353	+	+
HVM68	+	−
Bmac181	+	−
Bmac30	+	+
Bmac557	−	−
HVM67	+	−
GBM1003	+	−
GBM1009	−	−
GBM1015	−	−
GBM1018	−	−
GBM1020	−	−
Bmac84	−	−
Bmag384	+	−
Bmac375	−	−
Bmac658	+	−
Ebmac701	−	−
Ebmac635	−	−
GBM1044	−	−
Bmac310	+	−
GBM1048	+	−
Ebmac679	−	−
Ebmac788	−	−
WMS6	−	−

plants with recombinations at the distal side of the target interval (Fig. 2). Such a strategy facilitates marker identification in a defined window instead of whole chromosomes (see Fig. 2).

The AFLP analysis [*Eco*RI + 3/*Mse*I + 3 (E02/M01)] was performed essentially as described by Zabeau and Vos (1993) with some modifications (Schiemann et al. 1999) using the AFLP Core Reagent kit (Gibco BRL/Life technologies, Karlsruhe, Germany). Polymorphisms were detected on 8% denaturing polyacrylamide gels (Long Ranger, FMC Biozym) according to Schiemann et al. (1999).

For mapping polymorphic AFLP markers developed by BSA, we defined a recombinant interval mapping population for each cross consisting solely of plants carrying recombinations within the target interval (Fig. 2) as only those are informative with respect to the mapping of more closely linked markers (Tanksley et al. 1995).

Data analysis

Genetic linkage analysis for RFLPs, RAPDs and SSRs was performed using MAPMAKER ver. 3.0 (Lander et al. 1987), with a LOD score of 3.0. Recombination frequencies were transformed to centiMorgans (cM) using the formula of Kosambi (1944). Polymorphic AFLPs were mapped by two-point analysis on a recombinant-interval mapping population defined for each cross.

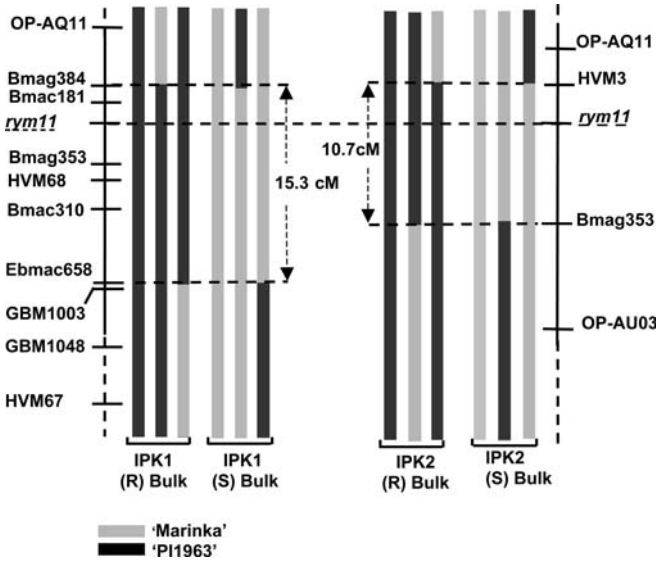

Fig. 2 Graphical illustration of the BSA for targeted AFLP identification. The resistant (*R*) and susceptible (*S*) pools each consist of ten lines representing three genotypic classes: (1) without recombination, (2) with proximal recombination relative to the target interval, (3) with distal recombination relative to the target interval carrying *rym11*. The map positions of the RAPDs and SSRs used to select suitable individuals are indicated on the *left* for IPK1 and on the *right* for IPK2

Results

RFLP analysis

Based on RFLP analysis of the 57 DH lines of the W757 population (Marinka × PI1963), the resistance locus of PI1963 being effective against BaMMV, BaYMV-1 and BaYMV-2 was located on chromosome 4HL with the most closely linked markers, MWG948 and MWG2134, at a distance of 1.6 cM (Fig. 1)—i.e. one recombination event. Both markers co-segregate with resistance gene *rym11* from Russia 57. In crosses of the resistant parental lines of IPK1 and IPK2 (PI1963 gene) to lines carrying Russia 57 in their pedigree, no susceptible F_1 plants to BaMMV could be detected. Therefore, we concluded that the BaMMV resistance gene of PI1963 is allelic to *rym11*.

RAPD analysis

A total of 576 arbitrary RAPD primers generated about 9,000 fragments that were analysed on the resistant and susceptible bulks. Of the 27 polymorphic RAPD fragments detected, six—OP-A17H700, OP-AQ11H250, OP-AT15H850, OP-AT20H350, OP-AU03H350, OP-H15H300—revealed linkage to *rym11*, with the closest markers, OP-AQ11H250 and OP-AU03H350, flanking *rym11* at a distance of 12.0 cM and 13.7 cM. The latter markers were also mapped on the progenies IPK1 and IPK2 (Fig. 3).

SSR analysis

Polymorphism rates of 40% and 12% were detected for IPK1 and IPK2 (Table 1). Only one polymorphic marker, Bmag353, is common to both populations.

Based on the varying number of polymorphic markers, maps of the resistance region covering 20.2 cM (GBM1048 and HVM67 not included) and 22.8 cM were obtained for IPK1 and IPK2. With respect to IPK1, the two closest SSRs, Bmac181 and Bmag353, flanked *rym11* at 2.1 cM and 2.7 cM. The only polymorphic SSRs in the case of IPK2, HVM3 and Bmag353, bridged the gene at genetic distances of 2.5 cM and 8.2 cM (Fig. 3).

AFLP analysis

For IPK1, 691 fragments were detected, of which 63 were polymorphic, corresponding to a polymorphism rate of 9%. Similar to the SSRs, the polymorphism rate was lower in IPK2; of 678 fragments, 39 were polymorphic, which is a polymorphism rate of 6%.

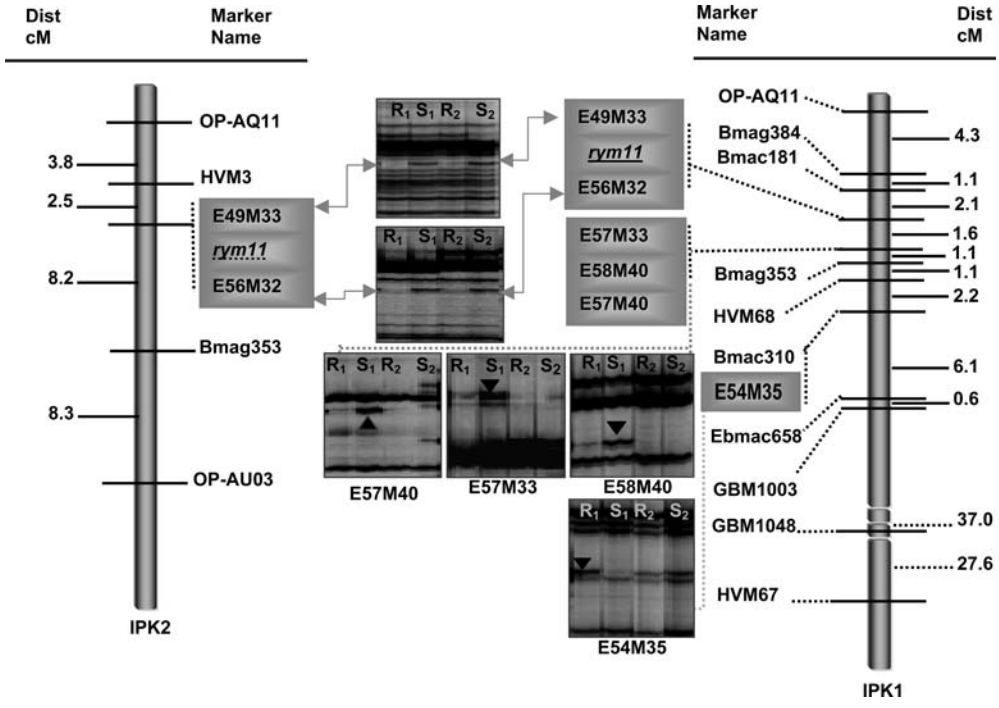

Fig. 3 Genetic maps of the IPK1 and IPK2 progenies representing the *Rym11* locus together with linked RAPD, SSR and AFLP markers. AFLP signals in the resistant (*R1*, *R2*) and susceptible (*S1*, *S2*) pools of IPK1 and IPK2 are illustrated

Based on these results, 193 E02M01 + 3 selective primer combinations were screened on the IPK1 or IPK2 bulks. To efficiently identify tightly linked markers, we designed bulks encompassing a window of 15.3 cM between Bmag384 and Ebmac658 (IPK1) and a window of 10.7 cM between HVM3 and Bmag353 (IPK2). Two combinations, E56M32 and E49M33, were polymorphic on the bulks of both populations, and primer combinations E58M40, E57M40, E57M33 and E54M35 were polymorphic only on IPK1. Of these, E54M35 generated an additional fragment on lines carrying the resistance-encoding allele, while E49M33, E56M32, E57M33, E57M40 and E58M40 generated an additional fragment on lines carrying the susceptibility-encoding allele. In order to map the polymorphic AFLP fragments, we defined a recombinant-interval mapping population for each cross, i.e. the respective AFLPs were analysed only on those individuals carrying a recombination in the target interval (see Fig. 2). These subpopulations comprised 21 and 14 DH lines for IPK1 and IPK2, respectively.

Mapping at a resolution of 0.523 cM for IPK1 and of 0.621 cM for IPK2, the AFLP markers were integrated based on the recombination frequencies between pairs of markers. On the basis of this approach, E54M35 turned out to be co-segregating, with Bmac310 mapping at 6.0 cM from *rym11*. The co-segregating AFLPs, E57M33, E57M40, and E58M40, were located about 1.6 cM (exactly 1.56 cM) distal to *rym11*, and E49M33 and E56M32 turned out to be co-segregating with *rym11*. Both markers also co-segregated with *rym11* in IPK2 (Fig. 3). By applying this iterative approach, we have been able to develop closely linked SSR and AFLP markers for *rym11*.

Discussion

The purpose of the present study was to develop closely linked markers for the resistance of PI1963, which is effective against all of the strains of BaYMV and BaMMV occurring in Germany. The gene is allelic to the previously described resistance gene *rym11* (Bauer et al. 1997). In both the IPK1 and IPK2 populations, we mapped microsatellite markers tightly linked to *rym11*—Bmac181 and HVM3 at 2.1 cM and 2.5 cM, respectively—and developed AFLP markers E49M33 and E56M32 that co-segregated with *rym11*.

BSA in conjunction with AFLPs has been successfully used for marker development and marker saturation in several studies (for example, Simons et al. 1997). In the present investigation this approach showed a high efficiency with respect to the number of markers detected and their degree of linkage to the target locus *rym11*. For IPK1, six AFLP markers were identified in the target interval, with two combinations—E49M33 and E56M32—showing co-segregation with *rym11*. In the case of IPK2, which in general revealed a lower level of polymorphism, only two but also co-segregating markers were identified. These results demonstrate the usefulness of targeted BSA and the recombination interval mapping approach, but also imply that the success of AFLP-based marker saturation is to some extent based on the parental combination chosen to build up a mapping population.

As in other studies for mapping resistance genes against the barley yellow mosaic virus complex (Werner et al. 2003; Le Gouis et al. 2004), SSRs turned out to be very efficient tools. In the present assessment, SSR markers belonging to barley chromosome 4H (Ramsay

et al. 2000; Thiel et al. 2003) were selected for mapping. In most cases, the order given by Ramsay et al. (2000) could be found in both populations. The SSR markers showing incongruent positions were compared to the updated Igri × Franka map (Perovic et al. 2000a), which carries five SSR loci (HVM3, Bmac181, HVM68, Bmac310 and HVM67) common to the map obtained in this investigation. For all of the common markers the same linkage positions were obtained in the IPK1, IPK2 and Igri × Franka maps.

Closely linked markers for *rym11* are of special importance given that this locus comprises resistance to BaMMV, BaYMV-1 and BaYMV-2, as seen in the W757 population, while previous studies were based exclusively on resistance to BaMMV (Bauer et al. 1997). Therefore, *rym11* would be an interesting source for broadening the genetic basis of resistance to all members of the barley yellow mosaic virus complex, which in European barley breeding programmes is entirely based on *rym5* at the present time (Friedt et al. 2000; Anonymous 2003).

The closely linked markers developed in this study can, on the one hand, be the starting point for a map-based cloning approach (Ordon et al. 2000, 2003) and, on the other hand, facilitate efficient marker-assisted selection procedures. Based on their close linkage to *rym11*, the co-segregating AFLPs appear to be highly suitable for integration in marker-assisted selection (MAS) programmes, especially following their conversion into sequence-tagged sites (Paran and Michelmore 1993; Pellio et al. 2004).

MAS has emerged as a promising strategy by which to increase the genetic gain of phenotypic selection (Peleman and van der Voort 2003), and marker-assisted backcross is especially suited for the incorporation of recessive genes (Young 1996; Ordon et al. 1999). To facilitate a reliable selection for resistance to the barley yellow mosaic virus complex in early generations, plants have to be mechanically inoculated with BaMMV in the greenhouse or to be tested in infested fields for BaYMV-1 and BaYMV-2 reactions. Both procedures are time-consuming, which is a major constraint. Moreover, in the field, symptom expression is strongly dependent on the environmental conditions, thus making a reliable selection not feasible each year (Ordon et al. 2003). Given all these facts, molecular markers may be considered to be efficient tools in breeding for this resistance. In this respect, the closely linked markers developed in this study facilitate the efficient and rapid introgression of the *Rym11* locus into adapted breeding lines. In addition to using co-segregating AFLPs that are difficult to handle in applied breeding programmes, correct assessment of the genotype at the *Rym11* locus may be achieved in more than 99.94% of assays using the flanking markers Bmac181 and Bmag353. Furthermore, these markers provide the opportunity to use *rym11* efficiently in programmes aimed at pyramiding resistance genes (Werner et al. 2000; Wang et al. 2001; Datta et al. 2002).

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