Development of CAPS Marker for Identification of *rym4* and *rym5* Alleles Conferring Resistance to the Barley Yellow Mosaic Virus Complex in Barley

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Abstract: In barley, soil-borne viruses of the barley mosaic virus complex (BaYMV, BaMMV, BaYMV-2) are of high importance due to their increased spread, yield losses, and the fact that protection with chemicals is not possible. Concerning resistance breeding, simple, cheap, and robust selection methods are required. Marker-assisted selection (MAS) has a great potential to meet this demand. A CAPS marker designed directly for SNPs causing mutations responsible for resistance of *rym4/rym5* alleles of the *Hv-eIF4E* gene was developed in this study. Results showed perfect correspondence between the CAPS marker and the allele identified indirectly on the basis of field tests.

Keywords: barley; marker-assisted breeding; mosaic virus complex; resistance; *rym* allele identification

The barley mosaic virus complex (BaYMV, BaMMV, BaYMV-2) is considered to be one of the most economically important diseases affecting winter barley in Western Europe. Due to transmission of the agents of barley yellow mosaic virus disease by the fungal vector *Polymyxa graminis* (Kanyuka *et al.* 2003), which has been detected viruliferous to a soil depth of about 60 cm, chemical measures against the disease are neither efficient nor economic; therefore, acquisition of resistance can be considered as the most cost-effective and environmentally friendly approach to plant protection that will ensure winter barley cultivation in the expanding area of fields infected by these viruses. Up to now, eight independent genetic loci distributed over the barley genome have been identified that confer mostly recessive resistance to either one or several strains of this virus complex (Werner *et al.* 2003). In European cultivars, resistance is based on recessive genes *rym4* (conferring resistance to BaMMV and BaYMV) and *rym5* (conferring resistance also to strain BaYMV-2). These genes are allelic forms of the *Hv-eIF4E* gene (Stein *et al.* 2005). Marker-assisted selection (MAS) has been proved as a valuable tool for breeding barley for resistance to the barley mosaic virus complex and many molecular markers for different resistance genes were developed (for a review see Ordon *et al.* 2004). For MAS of *rym4/rym5*, the molecular marker *Bmac29* linked to the *Hv-eIF4E* locus has been published (Graner *et al.* 1999). Unfortunately, the break in the linkage between *Bmac29* and *Hv-eIF4E* locus has already been observed (Kühne *et al.* 2003) and there is a need for a more precise marker, preferably one that is able to recognize the mutations in *Hv-eIF4E* gene, causing resistance of the *rym4/rym5* alleles.

Single nucleotide polymorphisms (SNPs) together with insertions and deletions (InDels) are the most common type of polymorphism in the genomes and in the case where SNPs result in a

change in amino acid codon of the active gene, a mutation could impact the change in phenotype (Chelkowski & Stephen 2001). SNPs can be recognized by specific restriction endonucleases and, in the case of polymorphic restriction patterns, they can be used to create cleaved amplified polymorphic sequence (CAPS) markers (Konieczny & Ausubel 1993). CAPS are codominant; they can be easily detected by agarose gel electrophoresis and in the case of recognizing SNP causing a change in the phenotype, they are treated as "perfect" markers. Development of such a perfect marker could have a great impact on barley breeding, because CAPS markers are simple, fast, and cheap, requiring only polymerase chain reaction (PCR) followed by restriction and agarose gel electrophoresis.

In this study, we describe the development of such a "perfect" CAPS marker, able to distinguish between *rym4* and *rym5* alleles exactly at the mutation points in the *Hv-eIF4E* gene.

MATERIAL AND METHODS

Plant material

A set of 20 barley lines was evaluated in field experiments in virus infested fields in Germany in comparison with standard varieties (Carola – resistant, *rym4*; Tokyo – resistant, *rym5*; Uschi – susceptible) within the cooperation between the Crop Research Institute, Prague-Ruzyně, and the Julius Kühn-Institute, Quedlinburg, Germany.

Resistance tests

Field resistance tests were carried out in locations infested with BaYMV, BaYMV-2, and BaMMV by growing at least 30 plants per line and location. Resistance was scored in 2004 and 2009 by visual assessment of disease symptoms. Resistance in the field infested with BaYMV and BaMMV and susceptibility in the field infested with BaYMV-2 indicated the presence of the *rym4* gene, while resistance in the fields infested with all three strains (BaYMV, BaMMV, and BaYMV-2) indicated the presence of the *rym5* gene.

DNA isolation and molecular analysis

Two pieces of leaf 3 cm long were collected from individual plants and dried at 40°C. DNA was extracted from these segments by the CTAB method (Keb-Llanes *et al.* 2002). PCR primers were designed from the NCBI GenBank® sequence AY661558 using FastPCR software (Kalendar 2008). SNP2CAPS software (Thiel *et al.* 2004) was used for finding a restriction endonuclease which is specific of the *rym4/rym5* mutation. PCR was carried out in a reaction volume of 10 µl consisting of 1 µl (25ng) genomic DNA, 5 µl of 2× PPP Master mix (Top-Bio, Czech Republic) containing 150mM Tris-HCl, pH 8.8; 40mM $(NH_4)_2SO_4$; 0.02% Tween 20; 5mM $MgCl_2$; 400µM dATP; 400µM dCTP; 400µM dGTP; 400µM dTTP; 100 U/ml Taq Purple DNA polymerase, monoclonal anti-Taq DNA polymerase (38nM), 0.1 µl (0.3µM) of each primer and 3.8 µl of PCR grade water. PCR was

Table1. Primer sequences designed for duplex PCR

Primer	Sequence	Expected length of PCR product (bp)
rym4F	5'- AGATCGCGGACGACGGAGAC	184
rym4R	5'- GGCTACCTCCAGAAGTCCTCGACG	
rym5F	5'- ATGTTGGAGCCGACTTCCAT	241
rym5R	5'- GTTCACCAATCAATGCCAGCAA	

Table 2. Predicted sizes of restriction fragments (bp); resolution of susceptible/*rym4/rym5* alleles

	32	75	77	82	107	159	241
Susceptible	×	×	×	×		×	
rym4			×	×	×	×	
rym5	×	×	×				×

run on the Biometra T-1 thermocycler (Biometra, Goettingen, Germany) using the following protocol: initial denaturation 95°C 5 min; denaturation 95°C 15 s, annealing 66°C 30 s, extension 72°C 45 s for 35 cycles; final extension 72°C 8 min. Restriction endonucleases AvaI and AvaII in FastDigest® format (Fermentas, St. Leon-Rot, Germany) were used for restriction analysis at 37°C for 5 min. PCR and restriction products were analyzed on 2% agarose gel and visualized by ethidium bromide under UV light (Vilber-Lourmat ETX-36.M, Marne-la-Valée, France).

RESULTS AND DISCUSSION

Stein *et al.* (2005) identified *Hv-EIF4e* gene mutations to be specific to *rym4/rym5* resistance. The *Hv-eIF4E* gene is of 4571 bp and the design of specific PCR primers for a product covering the whole gene could be a problem. A set of duplex PCRs covering exon 1 and exons 2 + 3 was therefore designed. Compatible primer pairs *rym4F*, *rym4R*, *rym5F*, and *rym5R* were selected from the output of FastPCR software (Kalendar 2008). Primer sequences are specified in Table 1. Mutation at 170 bp in exon 1 specific to the *rym4* allele and mutation at 478 bp in exon 3 specific to the *rym5* allele were selected as a target for restriction analysis. The restriction analysis was done by SNP2CAPS software (Thiel *et al.* 2004). Restriction endonucleases *AvaI* and *AvaII* with products specific to *rym4/rym5* alleles were selected from the software output. Predicted sizes of restriction fragments are summarized in Table 2. The annealing temperature was computed in

Table 3. Comparison of alleles identified indirectly by the resistance reaction in field test and by the duplex CAPS marker

Variety/breeding line		Field test				Resistance gene identified by CAPS marker
		reaction in infected field			declared resistance gene	
ID	name	BaYMV	BaYMV-2	BaMMV		
1	Carola	r	s	r	*rym4*	*rym4*
2	Tokyo	r	r	r	*rym5*	*rym5*
3	Uschi	s	s	s	none	none
4	SG-L 00/115/A/07	r	s	r	*rym4*	*rym4*
5	SG-L 00/132/A/05	s	s	s	none	none
6	SG-L 00/132/A/07	r	s	r	*rym4*	*rym4*
7	SG-L 3412/06	s	s	s	none	none
8	SG-L 3423/B/07/II	r	s	r	*rym4*	*rym4*
9	SG-L 3423/D/07	s	s	s	none	none
10	SG-L 3423/I/05	r	s	r	*rym4*	*rym4*
11	SG-L 5002/06	r	s	r	*rym4*	*rym4*
12	SG-L 5003/06	r	s	r	*rym4*	rym4
13	SG-L 96/080/06	s	s	s	none	none
14	SG-L 98/010/07	s	s	s	none	none
15	SG-L 99/014DH/A/07	s	s	s	none	none
16	SG-L 00/114/07	s	s	s	none	none
17	SG-L 00/132/B/07	r	s	r	*rym4*	*rym4*
18	SG-L 01/072DH/A/08	r	s	r	*rym4*	*rym4*
19	SG-L 01/131DH/A/08	s	s	s	none	none
20	SG-L 01/131DH/B/08	r	s	r	*rym4*	*rym4*
21	SG-L 3404/A/07	r	r	r	*rym5*	*rym5*
22	SG-L 99/032/08	r	s	r	*rym4*	*rym4*
23	SG-L 99/114/08	s	s	s	none	none

r – resistant; s – susceptible

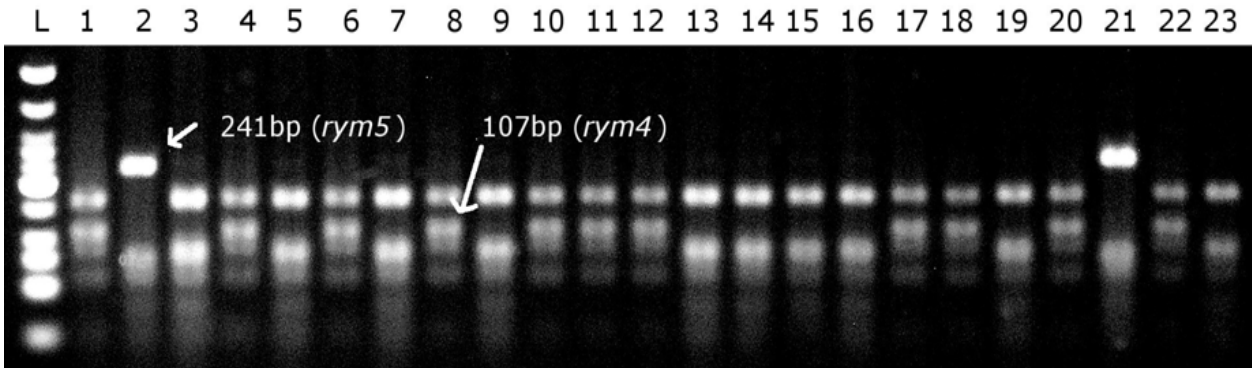

Figure 1. Electrophoresis of the duplex CAPS marker on a set of winter barley lines (L = DNA ladder NEB N3233L; 1–23 = samples according to Table 3)

FastPCR software (Kalendar 2008) and verified experimentally; the best results were observed at 66°C. Observed PCR product sizes were 241 bp and 184 bp, which is in agreement with the expected sizes. Restriction of PCR products by *AvaI* and *AvaII* resulted in fragment sizes of 32, 75, 77, 82, and 159 bp for the susceptible allele; 77, 82, 107, and 159 bp for the *rym4* allele; 32, 75, 77, and 241 bp for the *rym5* allele, which is all in agreement with the predicted sizes of restriction fragments; therefore, the presence of a 241 bp fragment after restriction is specific to the *rym5* allele and the presence of a 107 bp fragment after restriction is specific to the *rym4* allele (Figure 1). A designed duplex CAPS marker was verified on a set of 3 standard varieties and 20 winter barley lines with identified resistance alleles. Results are summarized in Table 3. Results showed perfect correspondence between the identification of resistance gene by the CAPS marker and the resistance reaction in field tests.

It can be concluded that the developed duplex CAPS marker was found to be able to distinguish between *rym4* and *rym5* alleles exactly at the mutation points in the *Hv-eIF4E* gene. The identified alleles fully correspond to the results of field tests. It makes this marker suitable for exploitation in breeding barley for resistance to the BaYMV complex.

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