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ORIGINAL PAPER

Analysis of bymovirus resistance genes on proximal barley chromosome 4HL provides the basis for precision breeding for BaMMV/BaYMV resistance

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Abstract

Key message **Unlocking allelic diversity of the bymovirus resistance gene *rym11* located on proximal barley chromosome 4HL and diagnostic markers provides the basis for precision breeding for BaMMV/BaYMV resistance.**

Abstract The recessive resistance gene *rym11* on barley chromosome 4HL confers broad-spectrum and complete resistance to all virulent European isolates of *Barley mild mosaic virus* and *Barley yellow mosaic virus* (BaMMV/BaYMV). As previously reported, *rym11*-based resistance is conferred by a series of alleles of naturally occurring deletions in the gene *HvPDIL5-1,* encoding a protein disulfide isomerase-like protein. Here, a novel resistance-conferring allele of *rym11* is reported that, in contrast to previously identified resistance-conferring variants of the gene *HvPDIL5-1*, carries a single non-synonymous amino acid substitution. Allelism was confirmed by crossing to genotypes carrying previously known *rym11* alleles. Crossing *rym11* genotypes with a cultivar carrying the recessive resistance gene *rym1*, which was reported to reside on the same chromosome arm 4HL like *rym11*, revealed allelism of both loci. This allelic state was confirmed by re-sequencing *HvPDIL5*-1 in the *rym1* genotype, detecting the haplotype of the *rym11-d* allele. Diagnostic PCR-based markers were established to differentiate all seven resistance-conferring alleles of the *rym11* locus providing precise tools for marker-assisted selection (MAS) of *rym11* in barley breeding.

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Introduction

The yellow mosaic virus disease of barley caused by *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) (*Bymovirus*, *Potyvirideae*) is one of the most important diseases of winter barley in Europe and East Asia (Kühne 2009). Infection typically causes mosaic symptoms (yellow discolorations on young leaves), stunted growth and delayed maturation of the plant. In Europe, infections may result in yield losses of up to 50 % of infected plants (Plumb et al. 1986), while more severe damage (i.e., complete losses of the crop) was observed in East Asia (Chen 2005; Kühne 2009). The bymovirus disease is soilborne and BaMMV/BaYMV is transmitted by the plasmodiophorid *Polymyxa graminis* (Adams et al. 1988) precluding agrochemistry-based plant protection (Kanyuka et al. 2003). Therefore, identification of naturally occurring resistances and their utilization in breeding is of high priority.

Over the last two decades, the monogenic recessive resistance gene *rym4*/*rym5* was the predominant source of resistance for most commercially registered barley varieties in Europe (Graner and Bauer 1993; Graner et al. 1999). Its introgression during the breeding process provided reliable protection for winter barley cultivars (Ordon et al. 2005). However, this resistance has been repeatedly overcome by different strains of

BaYMV/BaMMV in parts of Europe and Japan (Adams 1991; Habekuß et al. 2008; Hariri et al. 2003; Huth 1989; Kanyuka et al. 2004; Kashiwazaki et al. 1989; Steyer et al. 1995). Therefore, there is a constant need for novel resistance resources to ensure the sustainability of barley resistance breeding. To date, 18 resistance-conferring loci were reported for the primary and secondary gene pools of barley (Kai et al. 2012; Ordon et al. 2005). Phenotypic analysis based on testing with widely distributed (BaMMV, BaYMV and BaYMV-2) and newly emerging virulent isolates (BaMMV-Sil and BaMMV-Teik) of the BaYMV/BaMMV complex, indicated that genotypes carrying either of the resistance genes *rym1* or *rym11* exhibited broad-spectrum and complete resistance to all virulent European isolates (Habekuß et al. 2008; Kanyuka et al. 2004; Ordon et al. 2005). Both resistance genes were allocated to the same region on the proximal long arm of chromosome 4H (Ordon et al. 2005). Recently, a third recessive resistance gene, *rym18*, was mapped at a similar region of 4HL (Kai et al. 2012). The *rym18* carrying genotype, collected from Pakistan, was shown to be effective against all Japanese strains of BaMMV/BaYMV, whereas tests with European isolates are yet to be conducted. Furthermore, two resistance genes, *rym12* and *rym13*, whose donors of resistance confer the same phenotype are allocated to the distal long arm of chromosome 4H (Habekuß et al. 2008; Humbroich 2007; Humbroich et al. 2010). Due to their common allocation to the same chromosome arm and due to the identical resistance phenotype conferred by the five genes *rym1, rym11, rym12, rym13* and *rym18,* understanding the relationship between them is important to avoid redundant map-based cloning of allelic mutations of the same susceptibility factor, and the risk of exploiting redundant sources of resistance in barley breeding. As the donors of *rym8* and *rym9*, which are also located on the distal region of chromosome 4HL, are showing a different spectrum of resistance (Bauer et al. 1997; Werner et al. 2000), they were not included in this study.

Recently, we reported map-based cloning of the resistance gene *rym11* (Yang et al. 2014). Deletions in the underlying functional gene *HvPDIL5-1* were proven to confer resistance to *Bymovirus* infection. Use of these alleles in cultivars can enhance natural resistance to prevent yield losses caused by Bymoviruses. However, the lack of PCR-based allele-specific diagnostic markers is still limiting the direct selection of the corresponding genomic variations in barley breeding programmes.

The present study aimed at: (i) validating novel genomic variation conferring resistance at the *rym11* locus, (ii) revealing the relationship of *rym11* and other resistance loci *rym1*, *rym12*, *rym13* and *rym18*, and (iii) developing PCR-based diagnostic markers for the resistance alleles of *rym11*.

Materials and methods

Plant material and DNA extraction

Nine resistant genotypes W757/612 (*rym11-a*), Russia57 (*rym11-b*), HOR9483 (*rym11-c*), BCC468 (*rym11-d*), M_4 homozygous mutated lines 9699-1-1 (*rym11-9699*) and 10253-1-2 (*rym11-10253*), two Chinese landraces HOR11141 (locally called Mokusekko 3, *rym1* and *rym5*) (Konishi et al. 1997) and HOR11804, and DH45-10 (*rym18*) (Kai et al. 2012) (double-haploid progeny of PK23-2, kindly provided by Dr. Hiroomi Kai, FARC, Fukuoka, Japan), and three susceptible cultivars (cv.) Igri, Barke and Maris Otter were included in this study. Genomic DNA of the genotypes Muju covered 2 (*rym12*) and Taihoku A (*rym13*) was kindly provided by Dr. Dragan Perovic (JKI, Quedlinburg, Germany). DNA samples of 57 genotypes—a subset from a total of 1,816 barley accessions (Yang et al. 2014), were used for re-sequencing the gene *HvPDIL5-1*. Seedlings of the above-mentioned genotypes were cultivated under normal greenhouse conditions (16 h day/8 h night, 20 °C), and young leaves at three-leaf stage were sampled for DNA miniprep as described previously (Doyle and Doyle 1987; Stein et al. 2001).

Primer design and marker development

Oligonucleotides for Polymerase Chain Reaction (PCR) were designed based on the published sequences of the genes *HvPDIL5-1* (Yang et al. 2014) and *HvEIF4E* (Stein et al. 2005). PCR primers were picked using the online software Batch primer 3 (Untergasser et al. 2007). In case of allele-specific markers for detecting single nucleotide polymorphisms (SNP), the primer sequences of cleaved amplified polymorphic sequence (CAPS) markers and derived-CAPS (dCAPS) markers were generated using the software SNP2CAPS (Thiel et al. 2004) and dCAPS finder (Neff et al. 2002), respectively. PCR primers were ordered from the company Eurofins, Ebersberg, Germany (Table 1).

PCR amplification, restriction analysis and gel electrophoresis

PCR amplification of genomic DNAs was performed in a final volume of 20 µl, containing 20 ng genomic DNA, 0.1 µM forward and reverse primers, 0.5 U *Hot-star* polymerase (Qiagen, Germany), one-times concentrated (1×) PCR reaction buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs

Table 1 The PCR primers used in this study

Primers	Forward (5′ to 3′)	Reverse (5′ to 3′)	References
M_rym11_a	GGAGCTCCTACATGCTGCTC	TTGCAACTTCTTCGCCATC	C5B6coD1[a]
M_rym11_b	GGTCATCGAAGGTACAGATGAA	CCCAATGCAATAGATTCCAAC	This study
M_rym11_c	TTCCACTTACGGAAGGTTCG	AACAGCGTGGTTTTGGTTTC	PDI_487_1105[a]
M_rym11_d	TTCCACTTACGGAAGGTTCG	AACAGCGTGGTTTTGGTTTC	PDI_487_1105[a]
M_rym11_e	GGTTTTATTGATCATGAATGC	AGCATCCAAAGTTGCCTGAC	PDI_1501_2138[a]
M_rym11_9699	CACAGCAAGAGCCTAGGAACTCTCTG	CCCAATGCAATAGATTCCAAC	This study
M_rym11_10253	TTTGTGCAATTCTGCGTCCCATG	CAGCAAGCGGTCAAAACTCGCAT	This study
PDI_45_743	GTATCCGCCTTCTCCTCGTC	CGGTCAAAACTCGCATTGTA	PDI_45_743[a]
PDI_882_1580	TGCTCAAAGGTGGACATTCA	AATCCAACAGGGTGAGCAAC	PDI_882_1580[a]
EIFE_m56 s/309as	CGCCCGTCCGTCCTAGAAAAG	GGTAAGAGAGGAACGAATCGGAC	This study
EIF4E_1659 s/2394as	CACCGTGGCTTGTCTTCAGCAGG	CACAGTGAAGGGCTCACGCTCAG	This study
EIF4E_4857 s/5396as	ACCTGGGTAAATGCTATCACG	TTATGGAGTACAACGACGACAAACAC	Stein et al. (2005)

[a] These primers were described previously (Yang et al. 2014)

(Fermentas, Germany). Touch-down PCR amplification was generally performed as follows: 10 min at 94 °C for initial denaturation, followed by six cycles of 30 s at 94 °C, 30 s annealing (64 °C to 58 °C, −1 °C per cycle), 50 s at 72 °C for extension, and plus 35 cycles including 30 s at 94 °C, 30 s at 58 °C annealing, 50 s at 72 °C for extension, and finally PCR step by 5 min at 72 °C. The PCR extension was adjusted with a minor modification according to the size of PCR products (2 min for the marker M_rym11_a, 20 s for M_rym11_9699 and M_rym11_10253).

Endonuclease digestion of PCR products was performed in a Thermocycler (Bio-Rad, München, Germany) with a final volume of 20 µl. The reagents included 10 µl PCR products, 0.5 U restriction endonucleases (NEB, England), 1 × digestion buffer, 1 × Bovine Serum Albumin (BSA, optional). The digestion reaction was initiated by adding ddH_2O to a final volume of 20 µl, followed by incubation for 4 h (can be extended overnight). The incubation temperature was selected on the basis of manufacturer's recommendations (NEB, England), and the cleaved products were separated by agarose gel electrophoresis (gel strength: 1.5−4 % agarose, Biozym, Germany).

PCR purification and Sanger sequencing

Purification and Sanger sequencing of PCR products were performed as previously described (Yang et al. 2013). In brief, PCR amplicons were purified using the NucleoFast 96 PCR Kit (Macherey–Nagel, Germany) according to the supplier's instructions. Two µl of purified amplicon each was subsequently checked by agarose gel electrophoresis to estimate PCR product concentrations. Normalized amounts of amplicon (10 ng per 100 bp) were used as template for cycle-sequencing (BigDye®Terminator v3.1, Applied Biosystems, Germany) using ABI-3730xl technology and the PCR oligonucleotides. Trimmed sequences were loaded into the software Sequencher 4.7 (Gene Codes, USA) for sequence assembly and detection of polymorphisms.

Resistance test

Resistance tests were performed under phytotron and field conditions (Habekuß et al. 2008; Yang et al. 2014). Plants were cultivated in a phytotron (Johnson Controls (York), Hannover, Germany) (16 h day/8 h night, 12 °C) until reaching the three-leaf stage (about 2 weeks), and then mechanically inoculated twice at an interval of 5–7 days using leaf sap of BaMMV-infected leaves of susceptible cv. Maris Otter mixed with K_2HPO_4 buffer (1:10; 0.1 M; pH 9.8) and silicon carbide (carborundum, mesh 400, 0.5 g/25 ml sap). Five weeks after the first BaMMV-inoculation, the number of infected plants with mosaic symptoms was scored, and DAS-ELISA was carried out according to the previously described protocols (Clark and Adams 1977) using BaMMV and BaYMV-specific antibodies, which were kindly provided by Dr. Frank Rabenstein (JKI, Institute for Epidemiology and Pathogen Diagnostics, Quedlinburg, Germany). In parallel, genotypes were cultivated under field conditions in Quedlinburg, Germany, under conditions of natural infection with BaMMV and BaYMV. The young leaves of approximately 15 plants per genotype were sampled and pooled for DAS-ELISA diagnostics. The presence of virus particles was determined by measuring absorbance at 405 nm using a Dynatech MR 5000 microtiter-plate reader (Dynatech, Rückersdorf, Germany). Plants with absorbance below a value of 0.1 (limit of detection sensitivity) were scored as being resistant (no virus particles detected).

Results

Identification of a novel resistance allele at the *rym11* locus

By re-sequencing of the gene *HvPDIL5-1* in a large geographically referenced barley collection (Yang et al. 2014), it was shown that the Chinese landrace HOR11804 (collection site, latitude: 29.646923, longitude: 91.117212) carried a single base-pair mutation (T392C, haplotype IX) of the coding sequence (base-pair position 1910 of the genomic sequence leading to a non-synonymous amino acid (AA) substitution (L131P) in proximity to the C'-terminus of the functional thioredoxin (TRX)-like domain (Yang et al. 2014). Genotype HOR11804 showed complete resistance upon mechanical inoculation with BaMMV as well as under natural inoculation conditions with BaMMV and BaYMV (Table 2). F_1 plants that were derived by a cross between HOR11804 and W757/612 (*rym11-a*) were resistant to BaMMV, while F_1 hybrids obtained by crossing HOR11804 with susceptible cv. Igri were susceptible (Table 2). In addition, F_2 progeny of HOR11804 × W757/612 was found to be resistant to mechanical inoculation with BaMMV (Table 2). Thus, a single substitution at AA position 131 (L > P) of the wild-type *HvPDIL5-1* protein conferred resistance to BaMMV and BaYMV. This new and confirmed resistance was therefore named *rym11-e*.

rym1 and *rym11* are allelic mutations of *HvPDIL5-1*

Based on previous studies, the recessive resistance loci *rym1*, *rym11* and *rym18* were allocated to the proximal long arm of chromosome 4H (Kai et al. 2012; Konishi et al. 1997; Lüpken et al. 2013; Nissan-Azzouz et al. 2005). Two additional resistance genes, *rym12* and *rym13*, were reported to reside on the same chromosome arm, however, at a more distal position (Humbroich 2007; Humbroich et al. 2010). The full-length coding region (1,974-bp, from the starting codon ATG to the stop codon TAG) of the gene *HvPDIL5-1,* underlying *rym11*-based resistance, was re-sequenced in a number of genotypes reported to carry one of either of the resistance loci *rym1*, *rym12*, *rym13* or *rym18* (Table 3), respectively. The genotypes Muju covered 2 (*rym12*), Taihoku A (*rym13*) and DH45-10 (*rym18*) displayed sequence identity to the haplotype I, which is conferring susceptibility to the bymovirus disease (Yang et al. 2014). Therefore, based on analysis of coding sequence only, the genes *rym12*, *rym13* and *rym18* are not containing allelic mutations of *HvPDIL5-1*. Further analysis of the expression of the gene *HvPDIL5-1* of these genotypes or more comprehensive tests of

Table 2 Identification of allele *rym11-e*—a novel resistance allele at the *rym11* locus

Genotypes	Generation	Field test		Phytotron test by BaMMV-inoculation[a]	
		BaMMV	BaYMV	No. of tested plants	No. of infected plants
HOR11804	Parent	R	R	16	0
W757/612	Parent	R	R	10	0
Igri	Parent	S	S	16	11
HOR11804 × W757/612	F_1	nd	nd	20	0
HOR11804 × Igri	F_1	nd	nd	10	4
HOR11804 × W757/612	F_2	nd	nd	69	0
Maris Otter	Susceptible control	S	S	16	14

nd Not determined

[a] Mechanical inoculation with BaMMV usually leads to infection rates below 100 % in susceptible genotypes (Kaiser and Friedt 1992; Goetz and Friedt 1993)

Table 3 Re-sequencing of the genes *HvPDIL5-1* and *HvEIF4E* in genotypes with reported resistance genes on barley chromosome 4HL

Sources	*HvPDIL5-1*[a]	*HvEIF4E*[a]	References
Mokusekko 3 (*rym1*, *rym5*)	VII, *rym11-d*	*rym5*	Konishi et al. (1997)
BCC468	VII, *rym11-d*	*rym5*	Yang et al. (2014)
W757/612 (*rym11-a*)	XXVIII, *rym11-a*	no *rym4*/*rym5*	Yang et al. (2014)
Muju covered 2 (*rym12*)	I	no *rym4*/*rym5*	Humbroich (2007)
Taihoku A (*rym13*)	I	no *rym4*/*rym5*	Werner et al. (2003)
DH45-10 (*rym18*)	I	no *rym4*/*rym5*	Kai et al. (2012)
Igri (Susceptible)	I	no *rym4*/*rym5*	Yang et al. (2014)
Barke (Susceptible)	I	no *rym4*/*rym5*	Stein et al. (2005)

[a] Haplotypes of *HvPDIL5-1* and *HvEIF4E* are named according to Yang et al. (2014) and Stein et al. (2005), respectively

Table 4 Sequence variations and PCR-based diagnostic markers for independent resistance-conferring alleles of *rym11*

Alleles	Genomic variations	Diagnostic markers	Product size (bp)	
			Resistance	Susceptibility
rym11-a	−567′ to 789′, 1,375-bp deletion, no transcript	M_rym11_a/PDI_45_743	541/No product	1916/699
rym11-b	802′ to 818′, 17-bp deletion, frameshift	M_rym11_b	223	240
rym11-c	570′, 1-bp deletion, frameshift	M_rym11_c + *Mse*I	435, 81, 58, 44	517, 58, 41
rym11-d	567′, G > A, pre-stop	M_rym11_d + *Dde*I	441, 178	619
rym11-e	1910′, T > C, amino acid substitution	M_rym11_e + *Sml*I	638	509, 129
rym11-9699	703′, G > A, pre-stop	M_rym11_9699 + *Dde*I	257, 24	281
rym11-10253	568′, G > A, pre-stop	M_rym11_10253 + *Nco*I	106	88, 18

allelism to *rym11*-containing genotypes will be required to exclude allelism between *rym11* and the loci *rym12*, *rym13*, and *rym18*, respectively. The Chinese landrace Mokusekko 3 (HOR11141) was reported to carry two resistance genes *rym1* on 4HL and *rym5* on 3HL (Konishi et al. 1997), respectively. Re-sequencing of the genes *HvPDIL5-1* and *HvEIF4E* of Mokusekko 3 and a second Chinese landrace BCC468 revealed that both accessions contained the resistance genes *rym11-d* (Genomic sequence, G567A, pre-stop) and *rym5* (Table 3). Thus, the resistance gene *rym1* reported from Mokusekko 3 is in fact representing the *d* allele at the *rym11* locus. F_1 hybrids of W757/612 (*rym11-a*, no *rym4/5*) × BCC468 (*rym1*/*rym11-d*, *rym5*) were found to confer resistance upon mechanical inoculation with BaMMV (Yang et al. 2014). Therefore, *rym11-a* and *rym1 (rym11-d)* represent alleles of the same resistance gene.

Development of diagnostic markers for *rym11*

The resistance gene *rym11* confers broad-spectrum resistance to all known European strains of BaMMV and BaYMV (Bauer et al. 1997; Habekuß et al. 2008; Kanyuka et al. 2004; Nissan-Azzouz et al. 2005). By surveying natural and induced diversity of the gene *HvPDIL5-1*, seven resistance-conferring alleles of *rym11* were identified in the present and in previous studies. This included complete loss-of-function (*rym11-a*), frame-shift (*rym11-b* and *-c*), pre-mature STOP-codon (*rym11-d*, *-9699* and *-10253*) or amino acid substitution mutations (*rym11-e*), respectively (Table 4). To facilitate the utilization of these sources of resistance for efficient breeding, allele-specific diagnostic markers were developed relying on sequence variations of the different *rym11* alleles and susceptible haplotypes (Table 4, Fig. 1). Insertion–deletion polymorphism (Indel) markers were produced to detect the resistance alleles *rym11-a* and *rym11-b*, CAPS markers for *rym11-c*, *-d* and *-e*, as well as dCAPS markers for chemically induced alleles *rym11-9699* and *-10253* (Table 4). These diagnostic markers performed well at homozygous and simulated heterozygous state of the analyzed samples (Fig. 1). Subsequently, these marker sets were tested in seven genotypes carrying the different *rym11* alleles (*rym11-a*, *-b*, *-c*, *-d*, *-e*, *-9699* and *-10253*) (Fig. 2). These could pinpoint the resistance *rym11* allele, therefore confirming the allele specificity of these markers even among different *rym11* resistance alleles. Screening using the marker M_rym11_9699, which is proposed to be specific for the chemically induced allele *rym11-9699*, could generate 281-bp, 264-bp and 257-bp fragments (by sequencing, cannot separate on 4 % agarose gel) in genotypes carrying either of wild-type *HvPDIL5-1*, *rym11-b* and *rym11-9699*, respectively, therefore revealing the genotypes carrying *rym11-b*. In case of the allele *rym11-a*, which is characterized by a large deletion in the 5′region of the gene (Yang et al. 2014), a two-step detection may be required to reliably distinguish heterozygous from homozygous state. The marker M_rym11_a preferentially amplifies the 541-bp rather than the 1,916-bp fragments in heterozygous genotypes as could be shown under simulated heterozygous conditions using a mixed *rym11-a*/wild-type DNA-pool (Fig. 1a, left panel). PCR with a second marker PDI_45_743 will generate PCR products only in genotypes with a wild-type (or non-deleted) allele, e.g., in heterozygous or non-*rym11-a* genotypes (Fig. 1a, right panel). A combination of two sets of primers was used for the precise identification of the *rym11-a* allele even in simulated heterozygous state. The design of a multiplex assay with primer binding sites inside of the deletion to allow simultaneous, co-dominant amplification of similar sized *rym11-a* and wild-type *HvPDIL5-1* fragments may be feasible but was not followed further within this study. As a result, these PCR-based allele-specific markers are tools for marker-assisted selection of the resistance resources carrying *rym11*.

The diagnostic value of the established markers was subsequently surveyed in 57 barley accessions which were randomly selected as a subset of 1,816 naturally collected geographically referenced barley accessions (Yang et al. 2014). The resistance allele *rym11-b* was shared in 10 accessions and *rym11-d* was found in a single accession HOR11141. None of the 57 genotypes was identified to carry either of the naturally occurring resistance alleles *rym11-a*, *-c*, *-e,* respectively, and none of the tested genotypes was detected

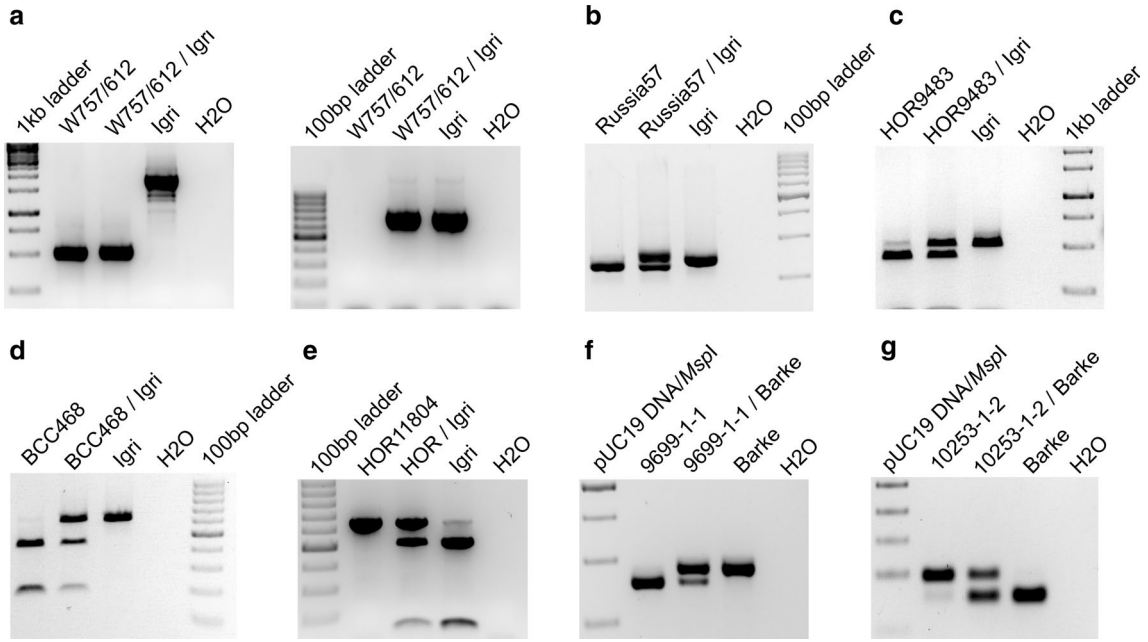

Fig. 1 Diagnostic markers for differentiation of resistance-conferring alleles of *rym11*. The resistance allele *rym11-a* **a**, left/right panel), *rym11-b* **b**, *rym11-c* **c**, *rym11-d* **d**, *rym11-e* **e**, *rym11-9699* **f** and *rym11-10253* **g** were analyzed by the markers M_rym11_a/PDI_45_743 (**a**, left/right panel), M_rym11_b, M_rym11_c, M_rym11_d, M_rym11_e, M_rym11_9699 and M_rym11_10253, respectively. PCR amplification was conducted in resistant homozygote, simulated heterozygote (equimolar mixtures of resistance allele and wild-type genotype DNAs) and susceptible wild type. As examples, PCR amplification of the marker M_rym11_b may generate 240-bp and 223-bp fragments in susceptible and *rym11-b* carrying genotypes, respectively **b** PCR products of the marker M_rym11_d combined with *Dde*I-digestion generated a 619-bp fragment in susceptible genotypes, but 441-bp and 178-bp fragments in *rym11-d* containing genotypes **d**

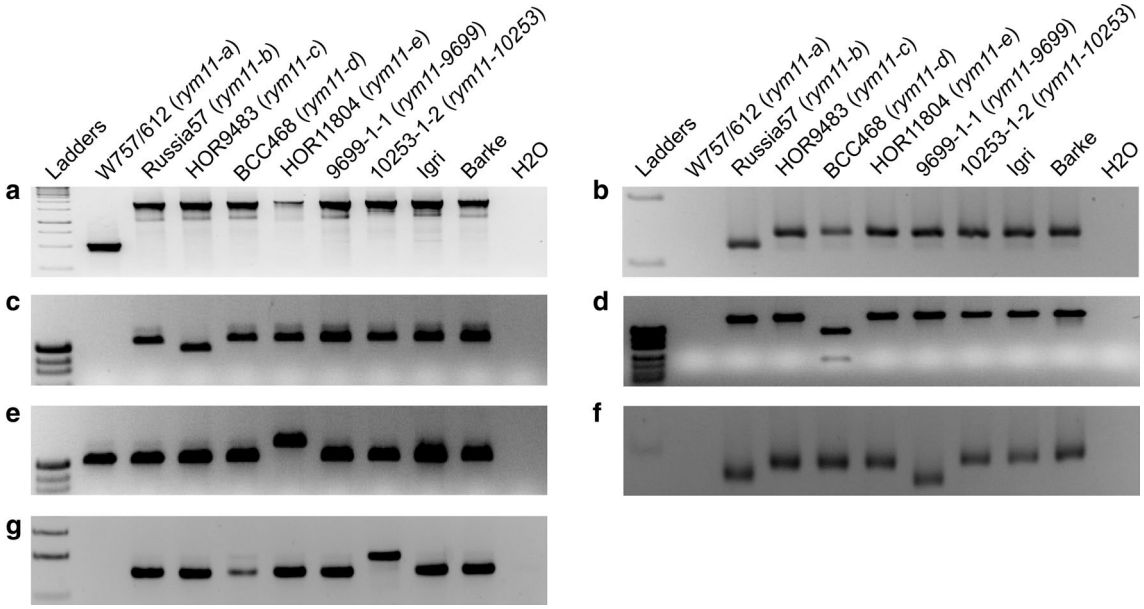

Fig. 2 Examination of diagnostic markers among different resistance *rym11* alleles. PCR amplification and gel separation with markers M_rym11_a **a**, M_rym11_b **b**, M_rym11_c **c**, M_rym11_d **d**, M_rym11_e **e**, M_rym11_9699 **f** and M_rym11_10253 **g**. The cultivars Igri and Barke are susceptible controls

Table 5 Tests of diagnostic markers in 57 natural barley accessions

No.	Accessions	Diagnostic markers (M_rym11)							Sequencing[b]	FAO countries
		_a	_b	_c	_d	_e	_9699	_10253[a]		
1	HOR976	wt	wt	wt	wt	wt	wt	wt	wt	FRA
2	HOR2363	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	CHN
3	HOR3130	wt	wt	wt	wt	wt	wt	wt	wt	KOR
4	HOR3296	wt	wt	wt	wt	wt	wt	wt	wt	RUS
5	HOR3298	wt	wt	wt	wt	wt	wt	wt	wt	IRN
6	HOR3299	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	CHN
7	HOR3500	wt	wt	wt	wt	wt	wt	wt	wt	BGR
8	HOR4219	wt	wt	wt	wt	wt	wt	wt	wt	AFG
9	HOR4224	wt	wt	wt	wt	wt	wt	wt	wt	JPN
10	HOR5734	wt	wt	wt	wt	wt	wt	wt	wt	ETH
11	HOR7259	wt	wt	wt	wt	wt	wt	wt	wt	AFG
12	HOR9288	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	CHN
13	HOR11024	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	PRK
14	HOR11027	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	PRK
15	HOR11078	wt	wt	wt	wt	wt	wt	wt	wt	PRK
16	HOR11079	wt	wt	wt	wt	wt	wt	wt	wt	PRK
17	HOR11088	wt	wt	wt	wt	wt	wt	wt	wt	PRK
18	HOR11091	wt	wt	wt	wt	wt	wt	wt	wt	PRK
19	HOR11096	wt	wt	wt	wt	wt	wt	wt	wt	PRK
20	HOR11098	wt	wt	wt	wt	wt	wt	wt	wt	PRK
21	HOR11099	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	PRK
22	HOR11132	wt	wt	wt	wt	wt	wt	wt	wt	JPN
23	HOR11139	wt	wt	wt	wt	wt	wt	wt	wt	Unknown
24	HOR11140	wt	wt	wt	wt	wt	wt	wt	wt	Unknown
25	HOR11141	wt	wt	wt	*rym11-d*	wt	wt	wt	*rym11-d*	CHN
26	HOR11143	wt	wt	wt	wt	wt	wt	wt	wt	JPN
27	HOR11524	wt	wt	wt	wt	wt	wt	wt	wt	CHN
28	HOR11525	wt	wt	wt	wt	wt	wt	wt	wt	CHN
29	HOR11527	wt	wt	wt	wt	wt	wt	wt	wt	JPN
30	HOR11529	wt	wt	wt	wt	wt	wt	wt	wt	JPN
31	HOR11540	wt	wt	wt	wt	wt	wt	wt	wt	CHN
32	HOR11543	wt	wt	wt	wt	wt	wt	wt	wt	CHN
33	HOR11545	wt	wt	wt	wt	wt	wt	wt	wt	CHN
34	HOR11546	wt	wt	wt	wt	wt	wt	wt	wt	CHN
35	HOR11551	wt	wt	wt	wt	wt	wt	wt	wt	CHN
36	HOR11564	wt	wt	wt	wt	wt	wt	wt	wt	CHN
37	HOR11568	wt	wt	wt	wt	wt	wt	wt	wt	CHN
38	HOR11586	wt	wt	wt	wt	wt	wt	wt	wt	KOR
39	HOR11590	wt	wt	wt	wt	wt	wt	wt	wt	KOR
40	HOR11591	wt	wt	wt	wt	wt	wt	wt	wt	KOR
41	HOR11594	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	KOR
42	HOR11600	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	KOR
43	HOR11602	wt	wt	wt	wt	wt	wt	wt	wt	KOR
44	HOR11604	wt	wt	wt	wt	wt	wt	wt	wt	KOR
45	HOR11610	wt	wt	wt	wt	wt	wt	wt	wt	KOR
46	HOR11611	wt	wt	wt	wt	wt	wt	wt	wt	KOR
47	HOR11614	wt	wt	wt	wt	wt	wt	wt	wt	KOR

Table 5 continued

No.	Accessions	Diagnostic markers (M_rym11)							Sequencing[b]	FAO countries
		_a	_b	_c	_d	_e	_9699	_10253[a]		
48	HOR11641	wt	wt	wt	wt	wt	wt	wt	wt	KOR
49	HOR11670	wt	wt	wt	wt	wt	wt	wt	wt	JPN
50	HOR11681	wt	wt	wt	wt	wt	wt	wt	wt	JPN
51	HOR11706	wt	wt	wt	wt	wt	wt	wt	wt	JPN
52	HOR11711	wt	wt	wt	wt	wt	wt	wt	wt	KOR
53	HOR11851	wt	wt	wt	wt	wt	wt	wt	wt	JPN
54	HOR11861	wt	wt	wt	wt	wt	wt	wt	wt	JPN
55	HOR11862	wt	wt	wt	wt	wt	wt	wt	wt	JPN
56	HOR11597	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	KOR
57	HOR11598	wt	*rym11-b*	wt	wt	wt	*rym11-b*	wt	*rym11-b*	KOR
Reliability of diagnostic markers		100 %	100 %	100 %	100 %	100 %	100 %	100 %		

[a] 281-bp, 264-bp and 257-bp for the genotypes carrying wild type, *rym11-b* and *rym11-9699*, respectively

[b] Deletions/mutations in introns of *HvPDIL5-1* were clarified as wild type (wt)

to carry any of the chemically induced mutant alleles *rym11-9699* and *-10253* (Table 5). Thus, re-sequencing the gene *HvPDIL5-1* in 57 barley accessions revealed the perfect consistency between screening by the diagnostic markers and by Sanger sequencing (Table 5), therefore proving the reliability for precisely and efficiently selecting the resistance alleles of *rym11* in natural collections of barley germplasm.

Discussion

A single amino acid substitution of HvPDIL5-1 is sufficient to confer BaMMV/BaYMV resistance in barley.

Our previous work identified six mutant alleles at the *rym11* locus conferring resistance to the BaMMV/BaYMV complex. These alleles represented either knock-out (no transcript, *rym11-a*), pre-mature stop codons (*rym11-d*, *-9699* and *-10253*) or frame-shift mutations (*rym11-b* and *-c*) of *HvPDIL5-1* (Yang et al. 2014) leading in all cases to loss of function of the causal gene. In the present work, we report a novel resistance-conferring allele of *HvPDIL5-1* found in a Chinese landrace HOR11804. F_1 plants and F_2 segregating progeny resulting of a cross between a *rym11-a* containing genotype W757/612 and the accession HOR11804 were completely resistant upon mechanical inoculation with BaMMV proving its allelic state to *rym11*. This novel resistance allele (denominated *rym11-e*) falls in a different category compared to the other previously found resistance-conferring variations of *rym11* (*-a*, *-b*, *-c*, *-d*, *-9699* and *-10253*). The resistance is conferred here by a single non-synonymous mutation at AA 131 in the C'-terminal part of the conserved TRX domain of the protein. The genotype HOR11804 showed resistance to virulent isolates BaMMV and BaYMV of the bymovirus complex. It remains to be determined whether this variant can terminate the infection caused by virulent isolates BaMMV-SIL, BaMMV-Teik and BaYMV-2 similar to the previously identified mutant alleles that are conferring loss of function. It may be speculated that non-synonymous substitution of an amino acid in the newly characterized allele of *rym11* interferes with a putative interaction of the host protein and yet to be determined virus components just in analogy to the model proposed for the interaction eIF4E and potyviral genome-linked protein (VPg) (Charron et al. 2008; Robaglia and Caranta 2006). As a consequence, viral replication may be blocked then resulting in termination of the virus proliferation cycle.

Marker-assisted breeding for complete and durable bymovirus resistance in barley

It was shown that the resistance loci *rym1* and *rym11* conferred complete resistance to all virulent European isolates of the BaMMV/BaYMV complex (Bauer et al. 1997; Habekuß et al. 2008; Kanyuka et al. 2004) and both loci were reported to be allocated close to each other at the proximal long arm of chromosome 4H (Konishi et al. 1997; Lüpken et al. 2013; Nissan-Azzouz et al. 2005). Here, we could demonstrate that the loci *rym1* and *rym11* represent different alleles of the same gene *HvPDIL5-1* (*rym1* is the *d* allele of *rym11*). The resistance locus *rym1* was firstly reported about 40 years ago in a Chinese landrace Mokusekko 3 (originally named '*Ym1*') (Takahashi et al. 1973). It is still providing complete and reliable protection against most of the Japanese virulent isolates (BaYMV-I, BaYMV-II, BaMMV-Ka1 and BaMMV-Na1)

(Konishi and Kaiser-Alexnat 2000; Konishi et al. 2002; Okada et al. 2003). However, at least one Japanese isolate, BaYMV-III, was reported to have overcome *rym1*-based resistance (Okada et al. 2003). To be substantiated again, such findings may need further testing since the gene underlying *rym11*-based resistance has been cloned (Yang et al. 2014) and can be traced now with diagnostic markers. However, the observation of *rym11*-based resistance breaking isolates may indicate that breeding for resistance on the basis of *rym11* only is not sufficient for achieving durable protection to bymovirus strains, especially in East Asia. Breeding for complete and durable resistance still may require pyramiding of multiple naturally occurring resistance genes (for review, see Ordon et al. 2004), e.g., combining *rym5* and *rym11* in a single genotype. Here, we developed diagnostic markers differentiating between wild-type susceptible and all the so far identified resistance-conferring *rym11* alleles. They represent tools for easy and precise selection of *rym11*-based resistance in barley breeding programmes. These markers reveal diagnostic size differences to the wild-type susceptible genotype due to the deletions found in the respective *rym11* alleles. In case of the naturally occurring and the induced resistance alleles *rym11-d*, *-e*, *-9699* and *-10253*, however, the diagnostic differences to susceptible genotypes may be further used to develop markers suited for any of the various SNP genotyping platforms (i.e., KASPar and SNaPshot), which would allow for rapid and high-throughput screening.

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Conflict of interest The authors declare no conflict of interest.

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