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Identification of Genes Conferring Resistance to Viral Diseases of Barley Using Multiplex PCR

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Abstract

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This paper describes a rapid and cost-effective assay for identifying economically important barley resistance genes based on multiplex PCR. A newly created reaction mixture is used for detecting the resistance genes *rym4*, *rym5*, *rym11-b* and *Ryd2* conferring resistance to barley yellow mosaic viruses and *Barley yellow dwarf virus* (BYDV). It is possible to identify all these genes using only a single PCR reaction without the necessity for subsequent cutting with restriction endonucleases. This is important for developing resistant barley genotypes inasmuch as it can increase work productivity and reduce costs.

Keywords: BaYMV; BaMMV; BaYMV-2; BYDV; *Hordeum*; resistance genes

Viral diseases are among the economically most important diseases attacking barley. The main group of viral diseases comprises the mosaic viruses *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV, BaYMV-2) and the *Barley yellow dwarf virus* (BYDV). When viral disease is spread via the soil fungus *Polymyxa graminis* (Kanyuka *et al*. 2003), which has been detected at depths of even 60 cm, there is no possible protection other than to grow resistant barley genotypes. BYDV is transmitted by aphids, and, although it is possible to provide protection by means of insecticide spraying and seed dressing, growing resistant genotypes of barley constitutes a less expensive and more environmentally friendly solution. To date, 18 resistance-conferring loci have been reported for the primary and secondary gene pools of barley (Ordon *et al.* 2005; Kai *et al*. 2012). In European varieties, resistance is known to be based predominantly on the resistance genes *rym4* and *rym5*, which are allelic forms of the *Hv-eIF4E* gene (Stein *et al.* 2005). However, this resistance has been repeatedly overcome by various strains of BaYMV/BaMMV in parts of Europe and Japan (Huth 1989; Kashiwazaki *et al.* 1989; Adams 1991; Steyer *et al.* 1995; Hariri *et al.* 2003; Kanyuka *et al.* 2004; Habekuss *et al.* 2008). The resistance gene *rym11* confers broad-spectrum resistance to all known European strains of BaMMV and BaYMV (Bauer *et al.* 1997; Kanyuka *et al.* 2004; Nissan-Azzouz *et al.* 2005; Habekuss *et al.* 2008). By surveying natural and induced diversity of the *HvPDIL5-1* gene, seven resistance-conferring alleles of the gene *rym11* were identified (Yang *et al.* 2014).

Marker-assisted selection is an effective breeding strategy for increasing resistance to viral diseases of barley. Breeding for complete and durable resistance may require the pyramiding of multiple naturally occurring resistance genes (for review, see Ordon *et al.* 2004), for example, by combining *rym5* and *rym11* in a single genotype (Yang *et al.* 2014).

Effective protection against BYDV has been provided so far by the *Ryd2* gene, which, unlike the resistance genes to BaMMV/BaYMV, is a semi-dominant gene (Rasmusson & Schaller 1959), and it can be presumed that its long-term effectiveness is given by a point mutation (Paltridge *et al.* 1998).

Several molecular markers enabling selection of barley genotypes bearing desirable genes have been

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published. For BaMMV/BaYMV, these are especially the microsatellite marker Bmac29 (Graner *et al.* 1999) and the duplex CAPS marker (Sedláček *et al.* 2010). A break in the linkage between the marker Bmac29 and the *Hv-eIF4E* locus has been observed already (Kühne *et al.* 2003), thus limiting the usefulness of this marker. The duplex CAPS marker is aimed directly at functional SNPs of the *Hv-eIF4E* gene and therefore preventing this breakage. Its use is also limited, however, because it is time-consuming and costly. New diagnostic PCR-based markers have been established to differentiate between all seven resistance-conferring alleles of the *rym11* locus, thereby providing precise tools for marker-assisted selection in barley breeding (Yang *et al.* 2014). The Ylp marker can similarly be used in selecting for the *Ryd2* gene (Ford *et al.* 1998).

This paper describes a rapid and cost-effective procedure which consists in using a reaction mixture for identifying the resistance genes *rym4*, *rym5*, *rym11-b* and *Ryd2* by means of multiplex PCR.

To detect *rym4*/*rym5* alleles, a SNP of the *Hv-elF4E* gene (Stein *et al.* 2005) was used. In contrast to the previously used CAPS marker (Sedláček *et al.* 2010), allele-specific PCR was utilized.

To detect *rym11*-type resistance, the *rym11-b* allele was selected, because it can be easily scored on the basis of standard PCR and agarose gel electrophoresis in codominant form and it is the most frequent allele within the donors (Yang *et al.* 2014). To detect the *rym11-b* allele, a 17 bp deletion in the *HVPDIL5-1* gene (Yang *et al.* 2014) was used. An SNP used in application of the Ylp (CAPS) marker (Ford *et al.* 1998) was used to detect the *Ryd2* gene on the basis of an allele-specific PCR. Primers for the targeted multiplex PCR were designed *de novo* in such a way as to have the same annealing temperature and to be easily detectable by agarose gel electrophoresis. An online SMS toolkit (Stothard 2000) was used for design and *in silico* testing of primers. The following NCBI codes of sequences were used to generate PCR primers:

rym4/5	AY661558.1
rym11	HG793095.1
Ryd2	AK369569.1

Five barley genotypes – Carola (*rym4*), Tokyo (*rym5*), Wysor (*Ryd2*), Russia57 (*rym11-b*), and Uschi (susceptible) – were exploited for development and testing of the reaction mixture. DNA was isolated from dried leaf tissue using the CTAB method (Lu 2011). The reaction mixture (10 µl) contained 1 µl DNA of each cultivar together with 5 µl of the agent 2× PPP Master mix (Top-Bio, Prague, Czech Republic) containing 150 mM Tris-HCl (pH 8.8), 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/ml Taq Purple DNA polymerase, 38 nM monoclonal antibody anti-Taq DNA polymerase, 3.8 µl of PCR-quality H_2O, plus 2.5 pmol primer rym5AS_F, 2.5 pmol primer rym5AS_R, 2.5 pmol primer rym4AS_F, 2.5 pmol primer rym4AS_R, 2.5 pmol primer Yd2AS2_F, 2.5 pmol primer Yd2AS2_R, 0.75 pmol primer rym11_F, and 0.75 pmol primer rym11_R (Generi-Biotech, Hradec Králové, Czech Republic) with the following sequences:

rym5AS_F	TAACAAGTTGAGTGGTATTTCAG
rym5AS_R	CCTTTGCCACAACTGACGC
rym4AS_F	GACAACCCGCAGGGCAATTT
rym4AS_R	TCACGAACTAGAGTAGCTAGGA
YD2AS2_F	ATCAGACAAGAATATGAGCGGAAAG
YD2AS2_R	GGTCAACTAGTATCTCTGGCTTAG
rym11_F	TCAAGAGTATCGAACCCAATGCA
rym11_R	TCGGAAAAGTTGATTGCGGTGC

These sequences are proposed for point mutations/deletion of the gene responsible for phenotypic manifestation of resistance. It is also advantageous that this analysis can be carried out across a wide range of plant development stages. Evaluation was made at emergence, heading, and medium milk stages. PCR was carried out in a thermal cycler (T1 Thermocycler, Biometra, Göttingen, Germany) with the following cyclingconditions:

(1) 94°C/5 min
(2) 94°C/15 s
(3) 59°C/30 s
(4) 72°C/1 min
(5) Steps 2 to 4, 34×
(6) 72°C/5 min
(7) cooling to 4°C

PCR products were separated on a 3% agarose gel with ethidium bromide in Tris-borate-EDTA buffer (running time 1 h, 10 V/cm). Figure 1 shows the fragment pattern of the PCR products for the five reference varieties (fragment sizesUschi: 225 bp, Carola: 410 + 225 bp, Tokyo: 161 + 225 bp, Russia 57: 208 bp, Wysor: 1205 + 225 bp).

The aforementioned reaction mixture has been successfully tested by the authors at the Research Centre SELTON, Ltd. The evaluation of 1900 plants (lines, breeding materials) was carried out using the newly developed markers and 7600 data points (*rym4*, *rym5*, *rym11-b*, *Ryd2*) were generated. Pyramiding of

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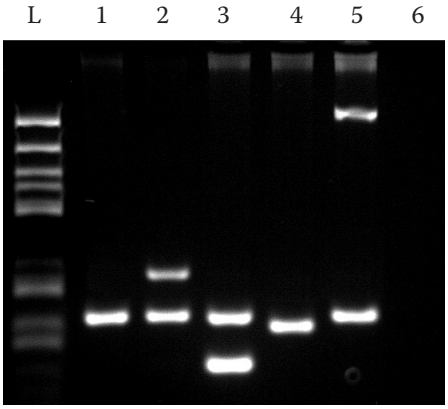

Figure 1. Electrophoreogram of a molecular size standard (L; 970, 750, 595, 544, 447, 305, 239, 194 bp – Top Bio, CZ) and PCR products of barley varieties with verified resistance genes; 1 – Uschi (susceptible), 2 – Carola (*rym4*), 3 – Tokyo (*rym5*), 4 – Russia57 (*rym11-b*), 5 – Wysor (*Ryd2*), and 6 – negative control (H_2O)

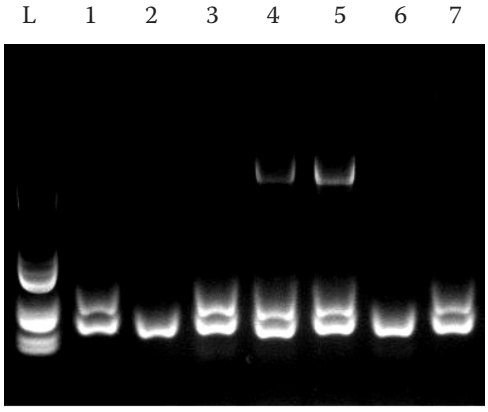

Figure 2. Evaluation of breeding lines (L; 970, 750, 595, 544, 447, 305, 239, 194 bp); 1 – *rym4*, 2 – susceptible, 3 – *rym4*, 4 – *rym4* + *Ryd2*, 5 – *rym4* + *Ryd2*, 6 – susceptible, 7 – *rym4*

resistance genes was detected in many cases (e.g. 276 *rym4* + *Ryd2, 5 rym5* + *Ryd2)* (Figure 2). For *rym11-b*, no resistance source except Russia 57 was detected.

The reaction mixture makes it possible to identify all economically important resistance genes to viral diseases of barley in just a single PCR reaction without the necessity for subsequent restriction by means of restriction endonucleases. This is fundamentally important for breeding and cultivating resistant barley genotypes, inasmuch as it can increase work productivity several times over even as it reduces costs.

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References

Adams M.J. (1991): The distribution of barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) in UK winter barley samples, 1987–1990. Plant Pathology, 40: 53–58.

Bauer E., Weyen J., Schiemann A., Graner A., Ordon F. (1997): Molecular mapping of novel resistance genes against Barley Mild Mosaic Virus (BaMMV). Theoretical and Applied Genetics, 95: 1263–1269.

Ford C.M., Paltridge N.G., Rathjen J.P., Moritz R.L., Simpson R.J., Symons R.H. (1998): Rapid and informative assays for *Yd2*, the barley yellow dwarf virus resistance gene, based on the nucleotide sequence of a closely linked gene. Molecular Breeding, 4: 23–31.

Graner A., Streng S., Kellermann A., Schiemann A., Bauer E., Waugh R., Pellio B., Ordon F. (1999): Molecular mapping and genetic fine-structure of the *rym5* locus encoding resistance to different strains of the barley yellow mosaic virus complex. Theoretical and Applied Genetics, 98: 285–290.

Habekuss A., Kuhne T., Kramer I., Rabenstein F., Ehrig F., Ruge-Wehling B., Huth W., Ordon F. (2008): Identification of *Barley mild mosaic virus* isolates in Germany breaking *rym5* resistance. Journal of Phytopathology, 156: 36–41.

Hariri D., Meyer M., Prud'homme H. (2003): Characterization of a new barley mild mosaic virus pathotype in France. European Journal of Plant Pathology, 109: 921–928.

Huth W. (1989): Ein weiterer Stamm des Barley yellow mosaic virus gefunden. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes: 41: 6–7.

Kai H., Takata K., Tsukazaki M., Furusho M., Baba T. (2012): Molecular mapping of *Rym17*, a dominant and *rym18* a recessive barley yellow mosaic virus (BaYMV) resistance genes derived from *Hordeum vulgare* L. Theoretical and Applied Genetics, 124: 577–583.

Kanyuka K., Ward E., Adams M.J. (2003): *Polymyxa graminis* and the cereal viruses it transmits: a research challenge. Molecular Plant Pathology, 4: 393–406.

Kanyuka K., Mc Grann G., Alhudaib K., Hariri D., Adams M.J. (2004): Biological and sequence analysis of a novel European isolate of *Barley mild mosaic virus* that overcomes the barley *rym5* resistance gene. Archives of Virology, 149: 1469–1480.

Kashiwazaki S., Ogawa K., Usugi T., Omura T., Tsuchizaki T. (1989). Characterization of several strains of barley yellow mosaic virus. Annals of the Phytopathological Society of Japan, 55: 16–25.

Kühne T., Shi N., Proeseler G., Adams M.J.,Kanyuka K. (2003): The ability of a bymovirus to overcome the *rym4*

doi: 10.17221/171/2015-CJGPB

– mediated resistance in barley correlates with a codon change in the VPg coding region on RNA 1. Journal of General Virology, 84: 2853–2859.

Lu Y. (2011): DNA extraction from dried plant tissues using 96-well format (CTAB method). Bio-protocol, Bio101: e89. Available at http://www.bio-protocol.org/e89

Nissan-Azzouz F., Graner A., Friedt W., Ordon F. (2005): Fine-mapping of the BaMMV, BaYMV-1 and BaYMV-2 resistance of barley (*Hordeum vulgare*) accession PI1963. Theoretical and Applied Genetics, 110: 212–218.

Ordon F., Friedt W., Scheurer K., Pellio B., Werner K., Neuhaus G., Huth W., Habekuss A., Graner A. (2004): Molecular markers in breeding for virus resistance in barley. Journal of Applied Genetics, 45: 145–159.

Ordon F., Ahlemeyer J., Werner K., Kohler W., Friedt W. (2005): Molecular assessment of genetic diversity in winter barley and its use in breeding. Euphytica, 146: 21–28.

Paltridge N.G., Collins N.C., Bendahmane A., Symons R.H. (1998): Development of YLM, a codominant PCR marker closely linked to the *Yd2* gene for resistance to barley yellow dwarf disease. Theoretical and Applied Genetics, 96: 1170–1177.

Rasmusson D.C., Schaller C.W. (1959): The inheritance of resistence in barely to the yellow dwarf virus. In: Burnett P.A. (ed.): World Perspectives on Barley Yellow Dwarf. Mexico, CIMMYT: 368–382.

Sedláček T., Mařík P., Chrpová J. (2010): Development of CAPS marker for identification of *rym4* and *rym5* alleles conferring resistance to the barley yellow mosaic virus complex in barley. Czech Journal of Genetic and Plant Breeding, 46: 159–163.

Stein N., Perovic D., Kumlehn J., Pellio B., Stracke S., Streng S., Ordon F., Graner A. (2005): The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in *Hordeum vulgare* (L.). The Plant Journal, 42: 912–922.

Steyer S., Kummert J., Froidmont F. (1995): Characterization of a resistance-breaking BaYMV isolate from Belgium. Agronomie, 15: 433–438.

Stothard P. (2000): The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques, 28: 1102–1104.

Yang P., Lüpken T., Habekuss A., Hensel G., Steuernagel B., Kilian B., Ariyadasa R., Himmelbach A., Kumlehn J., Scholz U., Ordon F., Stein N. (2014): Protein disulfide isomerase leke 5-1 is a susceptibility factor to plant viruses. Proceedings of the National Academy of Sciences of USA, 111: 2104–2109.

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