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

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**Abstract**


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
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-elF4E 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-elF4E 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), Russia 57 (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 (NH₄)₂SO₄, 0.02% Tween 20, 5 mM MgCl₂, 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 H₂O, 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: TAAACAGTTGACGTTATTTCAG
- rym5AS_R: CCTTTGCAACACTGACGC
- rym4AS_F: GACAACCGAGGCAGTTTT
- rym4AS_R: TCACGAACAGTACGCTGAGGA
- YD2AS2_F: ATCAGAAGAAATGTACGGGAAAG
- YD2AS2_R: GGCTCAACTGATCTCTGGCTTAG
- rym11_F: TCAAGAGTTGATTGCCTGA
- rym11_R: TCGGAAAGTTTGGTCGCTG

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 cycling conditions:

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 sizes: Uschi: 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
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


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