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Short communication

Influence of amino acid at position 132 in VPg on replication and systemic infection of *Barley yellow mosaic virus*

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ABSTRACT

A substitution of Lys with Asn or His at the amino-acid position 132 in VPg (VPg132) correlates with overcoming *rym4*-gene resistance by European strain 2 of *Barley yellow mosaic virus* (BaYMV-2). From the full-length cDNA clones for a Japanese BaYMV isolate JK05 (BaYMV-JK05) we generated virus mutants with Tyr, Lys, Asn, and Ala substituted for wild-type His at the VPg132. Only Tyr and Asn mutants replicated efficiently in protoplasts from barley varieties that are susceptible to wild-type virus. The Tyr mutant also infected susceptible barley plants systemically with the emergence of virus populations with partial or complete reversion to His, whereas the Asn mutant did not cause systemic infection. Thus, the VPg132 amino acid is essential for both efficient replication and systemic infection. Neither wild-type virus nor any of the mutants replicated in protoplasts from a *rym4* barley genotype. Therefore, substitution of the VPg132 amino acid alone cannot enable breaking *rym4*-mediated resistance in the BaYMV-JK05 background.

The viruses in the genus *Bymovirus* in the family *Potyviridae* infect small grain cereals and cause significant yield losses in global crop production (Kühne, 2009). These viruses are transmitted in soil by zoospores of *Polymyxa graminis*, a poorly understood root-inhabiting obligate organism which can retain the virus inside thick-walled resting spores in soil and root debris for decades (Kanyuka et al., 2003). All bymoviruses have a bipartite positive sense RNA genome, composed of RNA1 and RNA2. The 5′-ends of both genome segments are probably covalently linked to a viral genome-linked protein (VPg) and the 3′-ends are polyadenylated. The genomic organization of RNA1 shares great similarity with the RNA genome of well-characterized viruses in the monopartite genus *Potyvirus* in the family *Potyviridae*, hence also the biological properties of RNA1-encoded proteins are hypothesized to be similar with their potyviral counterparts (Adams et al., 2005; Urcuqui-Inchima et al., 2001).

Barley yellow mosaic virus (BaYMV) is the type species of the genus *Bymovirus* (Berger et al., 2005). The virus infects only winter barley plants, which develop a yellow mosaic disease, causing severe damage to barley cultivation in East Asia and Europe (Kühne, 2009). BaYMV RNA1 codes for a single polyprotein, which is cleaved by a serine proteinase activity residing in the C-terminal region of nuclear inclusion protein a (NIa) into eight proteins including the capsid protein (CP) (Fig. 1) (Berger et al., 2005). Internal cleavage of NIa produces the N-terminal VPg and the C-terminal proteinase (NIa-Pro). RNA2 also codes for a single polyprotein, which is cleaved into P1 and P2 by a cysteine proteinase activity in P1 (Fig. 1). P1 is required for systemic infection and P2 is a putative vector-transmission factor (You and Shirako, 2010).

To date, it is impossible to eliminate BaYMV by current agricultural practices, once viruliferous *P. graminis* was brought into a field (Kanyuka et al., 2003). Therefore, breeding barley cultivars that carry one or more *rym* (*r*esistance to *y*ellow *m*osaic) gene(s) is the only approach that has proved to be effective to control the yellow mosaic disease caused by BaYMV alone or in combination with *Barley mild mosaic virus* (BaMMV), another important bymovirus species (Ordon et al., 2005, 2009). However, none of these *rym* genes confer complete resistance to all BaYMV strains and resistance-breaking BaYMV variants are continuing to emerge (Kashiwazaki et al., 1989; Kühne et al., 2003; Nishigawa et al., 2008). The nature of resistance mediated by *rym* genes and of resistance breakage conferred by new variants is not yet understood. It was shown that *rym4* and *rym5* genes encode two mutant alleles of barley eukaryotic translation initiation factor 4E (eIF4E) conferring phenotypic resistance to common BaYMV and BaMMV strains (Kanyuka et al., 2005; Stein et al., 2005). In Europe, BaYMV strain 2 (BaYMV-2) was distinguished from the common BaYMV strain 1 (BaYMV-1), because BaYMV-2 could overcome *rym4*-mediated

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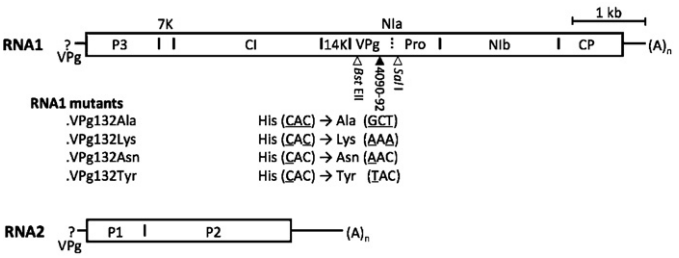

Fig. 1. The genome organizations of *Barley yellow mosaic virus* (BaYMV) RNA1, RNA1 mutants and RNA2. The upper rectangular box indicates a polyprotein translated from RNA1 open reading frame (ORF), which is cleaved into eight proteins by a nuclear inclusion protein a (NIa). Putative cleavage sites are shown by short vertical bars, and a dotted vertical bar in NIa indicates an internal cleavage to produce the N-terminal VPg and the C-terminal NIa-Pro. The lower rectangular box indicates a polyprotein translated from RNA2 ORF, which is cleaved into P1 and P2, by P1 proteinase. Four RNA1 mutant constructs were prepared from pBY1, and the derivative RNA1 mutants contain the replacement with Ala (VPg132Ala), Lys (VPg132Lys), Asn (VPg132Asn) or Tyr (VPg132Tyr) codons of the wild-type His codon (bases 4090–4092 in RNA1) for the VPg132 amino acid. The position of the mutation site is marked with a solid triangle, and the restriction enzyme cleavage sites, *Bst*EII (bases 3807/3808) and *Sal*I (bases 4295/4296), used for assembling RNA1 mutant constructs are indicated by open triangles.

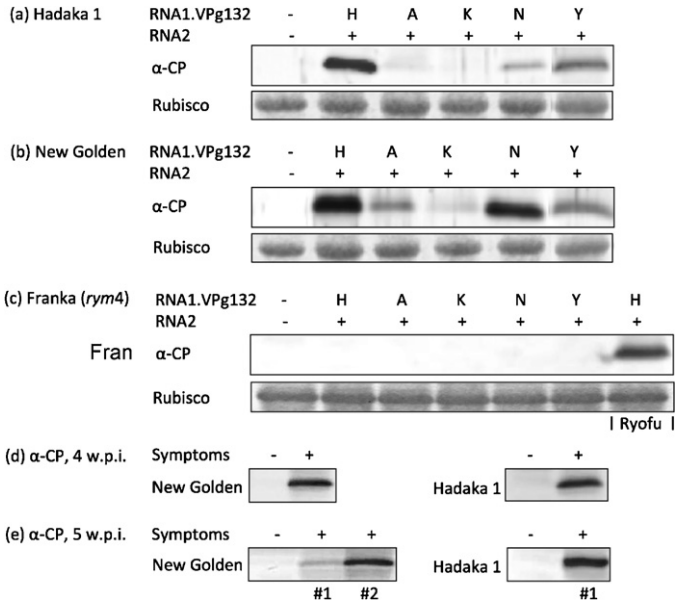

Fig. 2. Infectivity of wild-type (WT) RNA1 and RNA1 mutants in barley protoplasts and plants of cultivars Hadaka 1, New Golden, and Franka (*rym4*). Accumulation of CP in barley protoplasts of (a) Hadaka 1, (b) New Golden, and (c) Franka (*rym4*) transfected with WT or mutant RNA1 transcripts in combination with WT RNA2 transcripts were analyzed by western blotting using anti-CP serum. The transfected protoplasts were incubated at 15 °C for 60 h. Protoplasts transfected with water were used as negative control. Protoplasts from susceptible cultivar Ryofu were transfected with WT RNA1 and RNA2 transcripts and used as positive control in the experiment with Franka protoplasts. 'Rubisco' is the ribulose-1,5-bisphosphate carboxylase oxygenase large subunit stained with Coomassie Brilliant Blue G-250. (d) Western blot detection of CP from upper leaves of Hadaka 1 and New Golden plants, inoculated with WT RNA1 and WT RNA2, at the forth week. – indicates no symptoms; + indicates yellow mosaic symptoms. (e) Western blot detection of CP from upper leaves of Hadaka 1 and New Golden plants inoculated with VPg132Tyr RNA1 and WT RNA2, at the fifth week. – indicates no symptoms; + indicates WT-like symptoms, which was shown by two out of five (#1 and #2) New Golden plants and one out of six Hadaka 1 plants (#1).

resistance (Kühne et al., 2003). At the whole genomic scale, the two European strains differ significantly at the VPg132, where BaYMV-1 isolates have Lys and BaYMV-2 isolates have either Asn or His (supplementary Fig. S1). Compatible binding between the VPg of potyviruses and the eIF4E of the dicotyledonous host plants has been proved to occur during infection process (Charron et al., 2008). Therefore, it was hypothesized that an interaction between BaYMV-2 VPg and *rym4*-encoded eIF4E was responsible for infection success, as is the case of VPg/eIF4E interactions for potyvirus–host compatibilities (Kanyuka et al., 2005; Stein et al., 2005). However, this hypothesis could not be proved due to the absence of a reverse genetics system for bymoviruses.

Recently we developed a set of full-length cDNA clones for a Japanese BaYMV isolate JK05 (BaYMV-JK05), the first to be produced for any virus in the genus *Bymovirus* (You and Shirako, 2010), and showed that BaYMV-JK05 belongs to the Japanese pathotype II group (Kashiwazaki et al., 1989; You and Shirako, 2012). It is noteworthy that the BaYMV-JK05 does not break any resistance conferred by *rym1-6* genes. The in vitro transcripts of RNA1 and RNA2, derived from clones pBY1 and pBY2, respectively, were highly infectious to susceptible barley plants, and were used successfully in studying the functions of RNA2-encoded proteins in systemic infection (You and Shirako, 2010). At the VPg132, the BaYMV-JK05 isolate (Japanese pathotype II) has His as in the Japanese pathotype III, the European strain 2 and a Korean isolate, whereas other known BaYMV isolates have Lys as in the Japanese pathotype I, the European strain 1 and a Chinese isolate, Asn in the European strain 2 or Tyr in the Japanese pathotype IV (supplementary Fig. S1) (Chen et al., 1999; Lee et al., 2006; Kashiwazaki et al., 1990; Kühne et al., 2003; Nishigawa et al., 2008; Peerenboom et al., 1992; You and Shirako, 2010). To investigate the importance of the VPg132 amino acid in replication and systemic infection and its possible involvement in determining pathogenicity to *rym4* barley genotypes, mutant RNA1 constructs with Ala (GCT), Lys (AAA), Asn (AAC) or Tyr (TAC) codons for the WT His (CAC) codon for the VPg132 amino acid were prepared in the pBY1 background, using the sets of mutagenic primers (supplementary Table S1). The VPg132Ala, VPg132Lys, VPg132Asn or VPg132Tyr mutant RNA1 transcripts derived from their cDNA constructs contained substitutions of the VPg132 His codon (CAC) in wild-type (WT) RNA1 with Ala (GCU), Lys (AAA), Asn (AAC) or Tyr (UAC) codons, respectively (Fig. 1). The in vitro transcription reaction was performed as described previously for producing WT RNA1 transcripts (You and Shirako, 2010).

We first examined the requirement of the VPg132 amino acid for BaYMV pathogenicity in susceptible barley plants. Mesophyll protoplasts were prepared from primary leaves of 6-days-old seedlings of barley cultivars New Golden and Hadaka 1, as described by Ohsato et al. (2003). Approximately 0.5×10^5 protoplasts were transfected with WT or mutant RNA1 transcripts in combination with WT RNA2 transcripts using a polyethyleneglycol method (Ohsato et al., 2003). After incubation at 15 °C for 60 h, the conditions of the protoplasts from different barley cultivars looked the same under a light microscope. Replication efficiency of WT virus and the mutants in the transfected protoplasts, as showed by the levels of the accumulations of the capsid protein (CP), was examined by western blotting using primary antiserum against CP (You and Shirako, 2010). Rubisco proteins stained by Coomassie Brilliant Blue G-250 were used for normalization and loading controls (Fig. 2). As shown in Fig. 2(a) and (b), from the transfected Hadaka 1 and New Golden protoplasts, CP was detected abundantly with WT RNA1, to a lesser extend with VPg132Asn or VPg132Tyr RNA1, even weaker in the case of VPg132Ala RNA1, and almost undetectable with VPg132Lys RNA1. Therefore, the type of amino acid at the VPg132 is essential for efficient BaYMV replication at the cellular level.

In a second set of experiments Hadaka 1 and New Golden plants were mechanically inoculated with WT, VPg132Tyr or VPg132Asn RNA1 transcripts plus WT RNA2 transcripts, using the method as described previously (You and Shirako, 2010, 2012). After growth