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Association of VPg and eIF4E in the host tropism at the cellular level of *Barley yellow mosaic virus* and *Wheat yellow mosaic virus* in the genus *Bymovirus*

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ABSTRACT

Barley yellow mosaic virus (BaYMV) and *Wheat yellow mosaic virus* (WYMV) are separate species in the genus *Bymovirus* with bipartite plus-sense RNA genomes. In fields, BaYMV infects only barley and WYMV infects only wheat. Here, we studied the replicative capability of the two viruses in barley and wheat mesophyll protoplasts. BaYMV replicated in both barley and wheat protoplasts, but WYMV replicated only in wheat protoplasts. The expression of wheat translation initiation factor 4E (eIF4E), a common host factor for potyviruses, from the WYMV genome enabled WYMV replication in barley protoplasts. Replacing the BaYMV VPg gene with that of WYMV abolished BaYMV replication in barley protoplasts, whereas the additional expression of wheat eIF4E from BaYMV genome restored the replication of the BaYMV mutant in barley protoplasts. These results indicate that both VPg and the host eIF4E are involved in the host tropism of BaYMV and WYMV at the replication level.

Introduction

The genus *Bymovirus* in the family *Potyviridae* consists of viruses with bipartite plus-sense RNA genomes, with 7.3–7.6 kb RNA1 and 3.5–3.7 kb RNA2. Each RNA is encapsidated by the capsid protein (CP) to form filamentous virions averaging 250–300 nm and 500–600 nm in length, respectively, and 13 nm in diameter. Both RNAs have viral genome-linked proteins (VPg) covalently bound to the 5′ end and are polyadenylated at the 3′ end (Adams et al., 2012). RNA1 encodes one large polyprotein that is cleaved by a nuclear inclusion protein a-proteinase (NIa-Pro) into eight mature proteins: from the N terminus, the gene order is P3, 6K1, cylindrical inclusion protein (CI), 6K2, VPg, NIa-Pro, nuclear inclusion protein b (NIb) and CP (Adams et al., 2005). An additional overlapping open reading frame termed '*pipo*' has been predicted to be expressed as a fusion protein with the N terminal region of P3 by ribosomal frameshifting (Chung et al., 2008). RNA1 replicates autonomously in transfected cells (You and Shirako, 2010). The gene organization of bymovirus RNA1 is homologous to that of monopartite virus RNA in other genera in the family *Potyviridae*, expect for the lack of P1 and helper component-protease (HC-Pro) genes in the 5′ terminal region. Instead, RNA2 encodes a polyprotein that is cleaved into P1 and P2 (Adams et al., 2005). P1 is a cysteine autoproteinase that facilitates the accumulation of CP, whereas P2 is required for efficient systemic spread in the host plant (You and Shirako, 2010). Neither P1 nor P2 has significant amino-acid sequence similarity with the P1 and HC-Pro proteins of other viruses in the family *Potyviridae*.

The genus *Bymovirus* contains six known species: *Barley yellow mosaic virus* (BaYMV) as the type species, *Wheat yellow mosaic virus* (WYMV), *Barley mild mosaic virus*, *Oat mosaic virus*, *Rice necrosis mosaic virus* and *Wheat spindle streak mosaic virus* (Adams et al., 2012). BaYMV causes a yellow mosaic disease in winter barley that was first reported in 1940 in Japan (Ikata and Kawai, 1940). The disease has resulted in serious yield reductions in Europe and East Asia (Chen et al., 1999; Huth and Adams, 1990; Lee et al., 2006; Nishigawa et al., 2008). WYMV is another agriculturally important species in the genus *Bymovirus* and causes a similar yellow mosaic disease in wheat. WYMV first appeared in 1927 in Japan (Sawada, 1927) and has continually spread in Japan and China (Han et al., 2000; Namba et al., 1998; Ohki et al., 2014). These yellow mosaic diseases in barley and wheat caused by bymoviruses occur in fields under low-temperature conditions (Kusaba et al., 1971; Ohto et al., 1999). The optimal temperature for BaYMV infection is 15 °C (You and Shirako, 2010), and that for WYMV infection is 12 °C (this study), under laboratory conditions.

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Bymoviruses have relatively narrow host ranges, and each species can infect one single type of host. The only known natural host of BaYMV is barley, and that of WYMV is wheat. For systemic infection in plants, viruses must accomplish several distinct steps: virus entry, replication, cell-to-cell movement, long-distance movement (Narayanasamy, 2008), and transmission. Among the 11 viral proteins, NIb (Li et al., 1997), VPg (Puustinen and Mäkinen, 2004) and 6K2 (Restrepo-Hartwig and Carrington, 1994) are predicted to have a function in replication, and CP (Dolja et al., 1995), P3N-PIPO (Vijayapalani et al., 2012; Wei et al., 2010; Wen and Hajimorad, 2010) and CI (Carrington et al., 1998) may be involved in cell-to-cell movement. These predictions are based on amino-acid sequence similarities with proteins of viruses in the genus *Potyvirus*. Furthermore, P2 is not required for replication but facilitates efficient systemic infection (You and Shirako, 2010) and is predicted to be involved in vector transmission (Dessens and Meyer, 1996). For each step, plant viruses may also utilize additional host proteins to accomplish these functions. For example, host translation initiation factor 4E (eIF4E) and its isoform eIF(iso)4E interact with viral VPg (Léonard et al., 2000; Roudet-Tavert et al., 2007; Schaad et al., 2000; Wittmann et al., 1997). Systemic infection can be disturbed at the entry, replication and movement steps in host plants. The blocked steps that result in the host restriction of BaYMV and WYMV are unknown.

As the first step in understanding the mechanisms determining the host range of BaYMV and WYMV in fields, we examined their replication at the cellular level using infectious *in vitro* transcripts in isolated barley and wheat protoplasts. We also delivered barley or wheat eIF4E, a common host factor for potyviruses (Charron et al., 2008), to the reciprocal host and examined the effect of exchanging VPg genes between BaYMV and WYMV on their replication using *in vitro* transcripts from mutant cDNA clones. Our results demonstrate that both host eIF4E and viral VPg are involved in the host tropism of BaYMV and WYMV at the replication level.

Results

Comparison between BaYMV and WYMV genomes

As members of the same genus *Bymovirus* in the family *Potyviridae*, BaYMV (isolate JK-05, Japanese Pathotype II; GenBank: AB500948 for RNA1 and AB500949 for RNA2) and WYMV (isolate Nagano-B, Japan; GenBank: AB948222 for RNA1 and AB948223 for RNA2) share amino acid sequence identities between 56.0% within the 6K2 protein and 87.9% within the 6K1 protein among all 11 mature proteins (Supplementary Table S1).

BaYMV replication in barley and wheat protoplasts

We first examined the ability of BaYMV to replicate in barley and wheat mesophyll protoplasts. Protoplasts transfected with *in vitro* transcripts from pBY1 for RNA1 (Fig. 1A) and from pBY2 for RNA2 (Fig. 1B) (You and Shirako, 2010) were incubated at 15 °C for 66 h. After incubation, total protoplast extracts were subjected to Western blot analysis. Detection of a 32-kDa CP band was used as a criterion for RNA1 replication and quantitative analysis. In our previous study, BaYMV RNA1, which encodes a set of replication proteins, autonomously replicated in barley protoplasts. The additional presence of RNA2, replication of which relies on RNA1, resulted in a high level of CP accumulation by a function of the expressed P1 protein (You and Shirako, 2010); the mechanism is not known at this moment. As shown here again, a very low level of BaYMV CP accumulation was detected in barley protoplasts transfected with only BaYMV RNA1 transcript in the absence of RNA2, and a high level of BaYMV CP accumulation was detected in the presence of RNA2 (Fig. 2A lanes 1–3 and B bars 1–3). We further examined the transfection of barley protoplasts with *in vitro* transcripts from pBY1 and pWY2, the latter of which is a full-length cDNA clone of WYMV RNA2 (Fig. 1B). The result showed a high level of BaYMV CP accumulation in the barley protoplasts, although the accumulation was slightly reduced compared to transfection with BaYMV RNA1 and BaYMV RNA2 (Fig. 2A lane 4 and B bar 4).

To examine the replicative ability of BaYMV in wheat protoplasts, we transfected them with BaYMV RNA1 and BaYMV RNA2 transcripts. A reduced BaYMV CP accumulation was observed in wheat protoplasts compared to in barley protoplasts (Fig. 2A lane 5 and B bar 5). Next, wheat protoplasts were transfected with BaYMV RNA1 and WYMV RNA2 transcripts. The result showed that CP was detected at a similar level as observed after BaYMV RNA1 and BaYMV RNA2 transfection in wheat protoplasts (Fig. 2A lane 6 and B bar 6). These results indicated that both barley and wheat protoplasts supported replication of BaYMV RNA1 together with BaYMV or

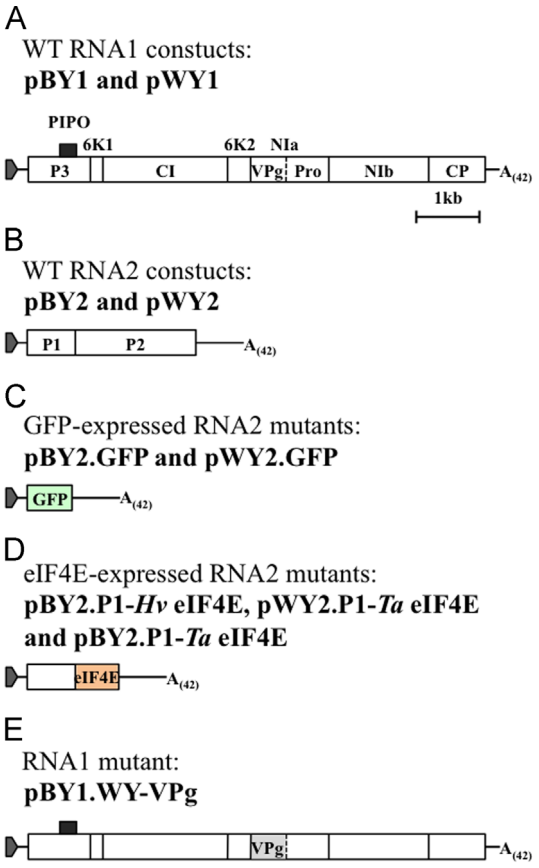

Fig. 1. Schematic representation of wild-type (WT) and mutant RNA1 and RNA2 constructs of BaYMV and WYMV. (A) pBY1 and pWY1: the infectious full-length cDNA clone of WT RNA1 of BaYMV or WYMV. The large box represents the long open reading frame (ORF) of RNA1 and mature proteins (P3/6K1/CI/6K2/VPg/NIa-Pro/NIb/CP) processed from the proteolytic cleavage of the large polyprotein on RNA1 are indicated in boxes. A small box above the long ORF indicates the *pipo* ORF. (B) pBY2 and pWY2: the infectious full-length cDNA clone of WT RNA2 of BaYMV or WYMV. The large box represents the polyprotein ORF of RNA2 and mature proteins (P1/P2) processed from the proteolytic cleavage of the large polyprotein on RNA2 are indicated in boxes. (C) pBY2.GFP and pWY2.GFP: pBY2- or pWY2-derivative mutants with a GFP gene in the place of the P1/P2 polyprotein gene. (D) pBY2.P1-*Hv* eIF4E, pWY2.P1-*Ta* eIF4E and pBY2.P1-*Ta* eIF4E: pBY2- or pWY2-derivative mutants with the eIF4E gene from barley (*Hv* eIF4E) or wheat (*Ta* eIF4E) in the place of the BaYMV P2 or WYMV P2 gene. (E) pBY1.WY-VPg: A chimeric construct derived from pBY1 by replacing the BaYMV VPg gene with the WYMV VPg gene. The rightward filled pentagons at the 5′ end indicate the T7 or SP6 promoter. Map scale, 1 kilobase (kb).

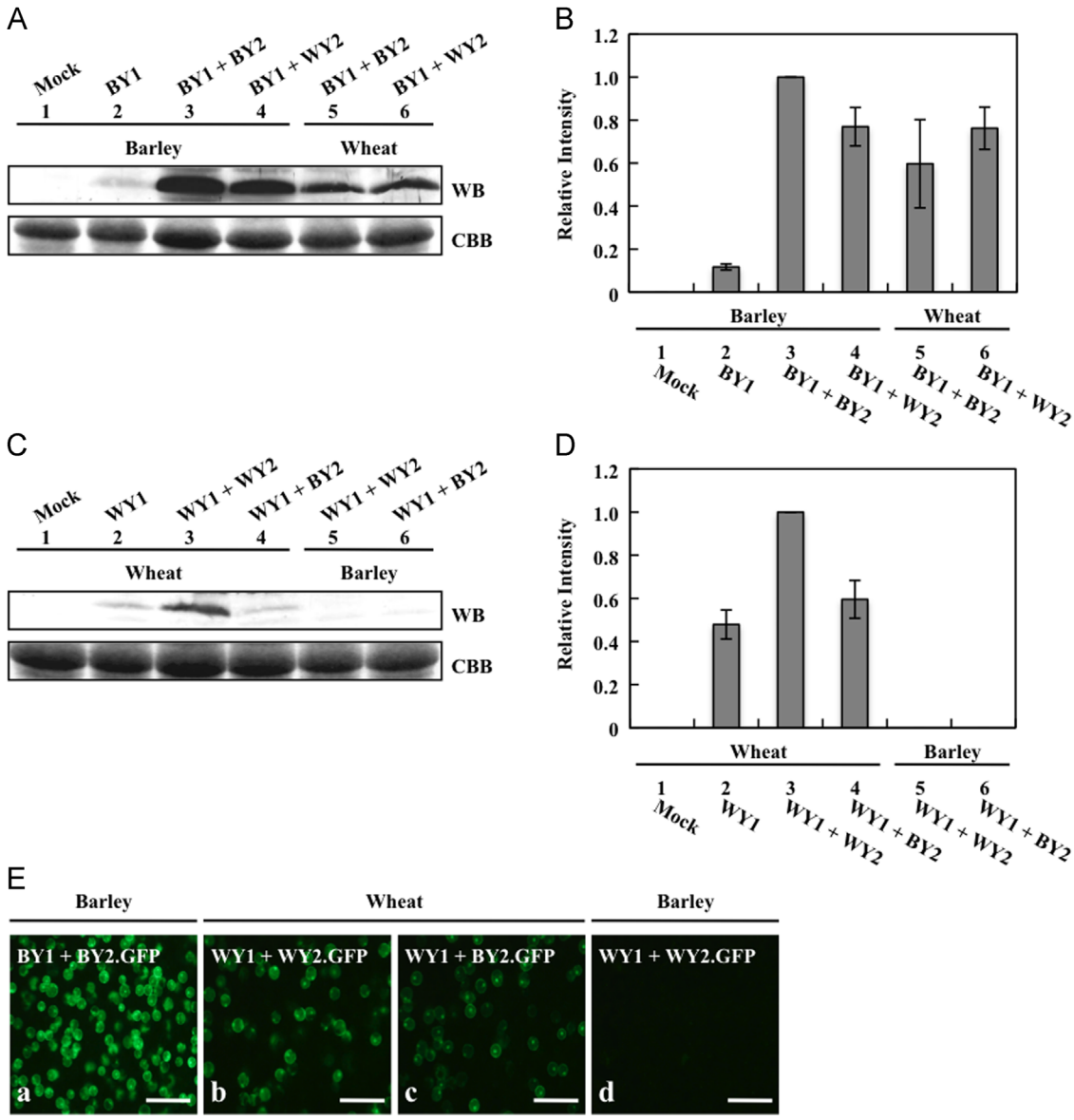

Fig. 2. Infectivity assay of BaYMV and WYMV in mesophyll protoplasts. Western blotting analyses showed (A) BaYMV CP and (C) WYMV CP accumulation in protoplasts transfected with *in vitro* transcripts. WB: Total proteins subjected to Western blotting analysis using anti-BaYMV CP (α-BaYMV CP) in (A) or anti-WYMV CP (α-WYMV CP) in (C). CBB: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. Relative intensities were estimated according to (B) BaYMV CP and (D) WYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1.0 for the analysis of BaYMV CP accumulation. WYMV CP accumulation in wheat protoplasts transfected with WYMV RNA1 and RNA2 transcripts was also used as a control and set as 1.0 for the analysis of WYMV CP accumulation. (E) GFP fluorescence observed in protoplasts transfected with RNA1 and GFP RNA2 transcripts. Bars, 200 μm. The protoplasts transfected with BaYMV RNA1 were incubated at 15 °C for 66 h, and those transfected with WYMV RNA1 were incubated at 12 °C for 90 h.

WYMV RNA2, although in wheat protoplasts BaYMV replication efficiency was reduced than in barley protoplasts.

Mechanical inoculation of BaYMV to barley and wheat plants

We further examined systemic infectivity using the *in vitro* transcripts (6 to 8 plants at a time, repeated three times). Inoculation with BaYMV RNA1 and BaYMV RNA2 transcripts to barley plants resulted in systemic infection as shown previously (You and Shirako, 2010). On the contrary, inoculation with BaYMV RNA1 and WYMV RNA2 transcripts to barley plants, BaYMV RNA1 and BaYMV RNA2 transcripts to wheat plants, and BaYMV RNA1 and WYMV RNA2 transcripts to wheat plants resulted in no infection as confirmed with Western blot analysis of upper uninoculated leaf extracts using anti-BaYMV CP antiserum (data not shown).

WYMV replicated in wheat protoplasts but not in barley protoplasts

Next, we examined the replicative ability of WYMV in barley and wheat mesophyll protoplasts. Because this is the first study to use a set of WYMV infectious *in vitro* transcripts from pWY1, a full-length cDNA clone of WYMV RNA1 (Fig. 1A), and pWY2 (Fig. 1B) to wheat protoplasts, we first determined the optimal incubation temperature and time length after transfection. Incubation of transfected wheat protoplasts at 12 °C for 90 h resulted in the highest level of CP accumulation (data not shown); thus, we chose this condition for

transfection experiments using WYMV RNA1 transcript. Transfection of wheat protoplasts with WYMV RNA1 transcript resulted in a low level of WYMV CP accumulation in the absence of RNA2 transcript and a higher level of WYMV CP accumulation in the presence of WYMV RNA2 transcript (Fig. 2C lanes 1–3 and D bars 1–3), indicating that WYMV RNA1 replicated autonomously in wheat protoplasts and that a high level of WYMV CP accumulation was achieved when transfected in combination with the WYMV RNA2 transcript similar to the result with BaYMV (You and Shirako, 2010). We further examined the transfection of wheat protoplasts with a heterologous combination of WYMV RNA1 and BaYMV RNA2 transcripts. In contrast to the results using BaYMV RNA1 in barley protoplasts (Fig. 2A lanes 2 and 4 and B bars 2 and 4), BaYMV RNA2 enhanced WYMV CP accumulation in wheat protoplasts only slightly (Fig. 2C lane 4 and D bar 4) compared to WYMV RNA1 only (Fig. 2C lane 2 and D bar 4). To examine whether WYMV RNA1 supported the replication of BaYMV RNA2 in wheat cells, we transfected wheat protoplasts with WYMV RNA1 transcript and BaYMV GFP (green fluorescence protein) RNA2 transcript from pBY2.GFP (Fig. 1C) (You and Shirako, 2010), using WYMV GFP RNA2 transcript from pWY2.GFP (Fig. 1C) as a control. In this assay, GFP was expressed from replicating RNA2 using active replication proteins from the wild-type RNA1. Positive control experiments using BaYMV RNA1 and BaYMV GFP RNA2 transcripts in susceptible barley protoplasts always gave more than 50% in transfection efficiency with strong GFP fluorescence (Fig. 2E-a). Mock-inoculated negative controls gave no fluorescence, making this assay a quite sensitive and confirmative method over RNA1-mediated CP detection on Western blots. Wheat protoplasts transfected with WYMV RNA1 and WYMV GFP RNA2 transcripts emitted GFP strongly (Fig. 2E-b). Strong GFP fluorescence was also detected in wheat protoplasts transfected with WYMV RNA1 and BaYMV GFP RNA2 transcripts (Fig. 2E-c), indicating that BaYMV RNA2 replicated with WYMV RNA1 in wheat protoplasts although an elevated level of CP accumulation was not detected (see the Discussion section).

When barley protoplasts were transfected with WYMV RNA1 and RNA2 transcripts, no WYMV CP band was detectable (Fig. 2C lane 5 and D bar 5) on Western blots. The same results were obtained in barley protoplasts transfected with WYMV RNA1 and BaYMV RNA2 transcripts. No WYMV CP accumulation was observed in these barley protoplasts (Fig. 2C lane 6 and D bar 6). Therefore, we also examined GFP fluorescence microcopy, which is more sensitive than CP band detection on Western blots as mentioned above. No GFP fluorescence was detected in barley protoplasts transfected with WYMV RNA1 and WYMV GFP RNA2 transcripts (Fig. 2E-d). No GFP fluorescence was detected in barley protoplasts transfected with WYMV RNA1 and BaYMV GFP RNA2 either (data not shown), indicating that WYMV RNA1 can not replicate in barley protoplasts.

Barley eIF4E enhanced BaYMV replication in wheat protoplasts, and wheat eIF4E enabled WYMV to replicate in barley protoplasts

eIF4E and *eIF(iso)4E* are well-identified recessive resistance genes against virulent strains of many virus species in the genus *Potyvirus*. Here, we examined whether eIF4E could be involved in the host tropism of BaYMV and WYMV at the replication level. For this purpose, we constructed two derivative mutant constructs pBY2.P1-*Hv* eIF4E and pWY2.P1-*Ta* eIF4E from pBY2 and pWY2, respectively (Fig. 1D). In pBY2.P1-*Hv* eIF4E, the BaYMV P2 gene whose product is not required for replication (You and Shirako, 2010) was replaced with a barley eIF4E (*Hv* eIF4E) gene; that is, a serine codon was placed after the P1 gene, followed by the full-length barley eIF4E gene, so that the translated P1-*Hv* eIF4E polyprotein would be cleaved into the mature P1 autoproteinase and the barley eIF4E protein with an additional serine at the N terminus. Similarly, in pWY2.P1-*Ta* eIF4E, the WYMV P2 gene was replaced with a wheat eIF4E (*Ta* eIF4E) gene. Using *in vitro* transcripts from these mutant RNA2 constructs, barley and wheat eIF4E could be expressed in wheat and barley protoplasts. There are 16 amino acid differences within the 215 amino acids between the two eIF4E proteins (Fig. 3A).

Barley protoplasts transfected with BaYMV RNA1 and BaYMV P1-*Hv* eIF4E RNA2 transcripts resulted in the same level of BaYMV CP accumulation as with the BaYMV RNA1 and BaYMV RNA2 transcripts (Fig. 3B lanes 1 and 2 and C bars 1 and 2). Thus, exogenous delivery of the barley eIF4E gene and its expression in transfected barley protoplasts (confirmed by Western blot analysis using P1 and eIF4E antisera, data not shown) did not appear to have an additional effect on BaYMV replication in barley cells using the intrinsic host eIF4E function. In wheat protoplasts, transfection with BaYMV RNA1 and BaYMV P1-*Hv* eIF4E RNA2 transcripts resulted in higher BaYMV CP accumulation than with the BaYMV RNA1 and BaYMV RNA2 transcripts (Fig. 3B lanes 3 and 4 and C bars 3 and 4). This result indicated that the exogenous barley eIF4E protein expressed from the transfected mutant BaYMV RNA2 transcript enhanced BaYMV CP accumulation in wheat cells.

Wheat protoplasts transfected with WYMV RNA1 and WYMV P1-*Ta* eIF4E RNA2 transcripts showed a slightly higher level of CP accumulation than cells transfected with the WYMV RNA1 and WYMV RNA2 transcripts (Fig. 3D lanes 1 and 2 and E bars 1 and 2). Barley protoplasts did not support WYMV replication, as was previously shown (Fig. 3D lane 3 and E bar 3; Fig. 2C lane 5 and D bar 5). However, in barley protoplasts transfected with WYMV RNA1 and WYMV P1-*Ta* eIF4E RNA2 transcripts, a highly elevated level of WYMV CP accumulation was detected (Fig. 3D lane 4 and E bar 4). This result indicated that the exogenous wheat eIF4E expressed from the transfected mutant WY2 RNA2 was functional for WYMV RNA replication in barley cells.

The combination of WYMV VPg and wheat eIF4E enables the replication of a BaYMV mutant in barley protoplasts

Potyvirus VPg is a virulence factor that breaks down eIF4E-mediated recessive resistance, mostly caused by mutations in the VPg proteins (Bruun-Rasmussen et al., 2007; Kanyuka et al., 2004; Léonard et al., 2000; Ruffel et al., 2002; Schaad et al., 2000). Therefore, we also examined the role of VPg in the replication of BaYMV and WYMV RNA in barley and wheat protoplasts. BaYMV VPg and WYMV VPg differ at 51 out of 187 amino acid positions throughout the proteins (Fig. 4A).

For this purpose, we constructed a BaYMV RNA1-derived chimeric mutant cDNA clone with the BaYMV VPg gene replaced by the WYMV VPg gene (pBY1.WY-VPg) (Fig. 1E). Transfection of barley protoplasts with BaYMV WY-VPg RNA1 and BaYMV RNA2 transcripts resulted in no BaYMV CP accumulation (Fig. 4B lane 2 and C bar 2) compared to a high level of BaYMV CP accumulation after BaYMV RNA1 and BaYMV RNA2 transfection (Fig. 4B lane 1 and C bar 1; Fig. 2A lane 3 and B bar 3; Fig. 3B lane 1 and C bar 1). Transfection of barley protoplasts with BaYMV WY-VPg RNA1 and BaYMV GFP RNA2 transcripts also resulted in no GFP fluorescence (Fig. 4D-a), indicating that BaYMV WY-VPg RNA1 did not replicate in barley protoplasts at all.

Next we tested the replication of BaYMV WY-VPg RNA1 in wheat protoplasts. Wheat protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV RNA2 transcripts demonstrated a high level of BaYMV CP accumulation (Fig. 4B lane 3 and C bar 3). Wheat protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV GFP RNA2 transcripts also showed strong GFP fluorescence (Fig. 4D-b), indicating that BaYMV WY-VPg RNA1 replicated in wheat protoplasts, although this mutant RNA1 did not replicate in barley protoplasts.

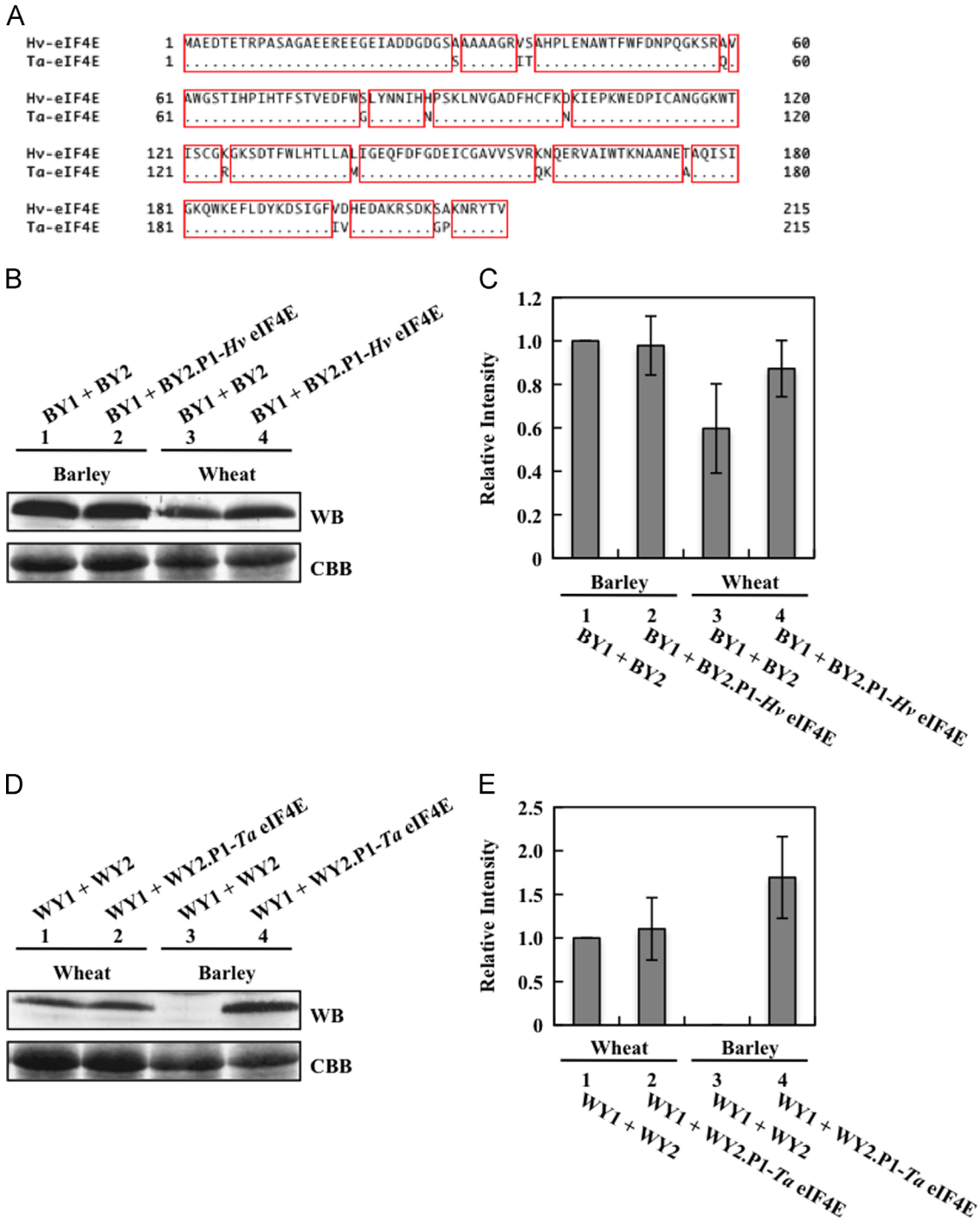

Fig. 3. Infectivity assay of BaYMV and WYMV co-expressed with eIF4E in barley and wheat mesophyll protoplasts. (A) Alignments of amino acid sequences of eIF4Es isolated from barley (cv. KoA) and wheat (cv. Shiranekomugi) using GENETYX-MAC software (Genetyx, Japan). Western blotting analyses showed (B) BaYMV CP and (D) WYMV CP accumulation in protoplasts transfected with RNA1 and RNA2 or eIF4E-expressed RNA2 transcripts. WB: Total proteins subjected to Western blotting analysis using anti-BaYMV CP (α-BaYMV CP) in (B) or anti-WYMV CP (α-WYMV CP) in (D). CBB: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. Relative intensity was estimated according to (C) BaYMV CP and (E) WYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1.0 for the analyses of BaYMV CP accumulation. WYMV CP accumulation in wheat protoplasts transfected with WYMV RNA1 and RNA2 transcripts was also used as a control and set as 1.0 for the analysis of WYMV CP accumulation. The protoplasts transfected with BaYMV RNA1 were incubated at 15 °C for 66 h, and those with WYMV RNA1 were incubated at 12 °C for 90 h.

Together with the previous result that WYMV RNA1 and WYMV P1-*Ta* eIF4E RNA2 replicated well in barley protoplasts (Fig. 3D lane 4, and E bar 4), we hypothesized that a pair of WYMV VPg and wheat eIF4E may form a part of functional replication complex for BaYMV-derivative RNA in barley cells. To test this hypothesis, we constructed a pBY2 mutant (pBY2.P1-*Ta* eIF4E) in which the dispensable P2 gene was replaced by a wheat eIF4E gene (Fig. 1D) in pBY2 background. Barley protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV P1-*Ta* eIF4E RNA2 transcripts exhibited a high level of BaYMV CP accumulation (Fig. 4B lane 4 and C bar 4), indicating that the replication of BaYMV WY-VPg RNA1 occurred in barley cells in the presence of RNA2-derived wheat eIF4E.

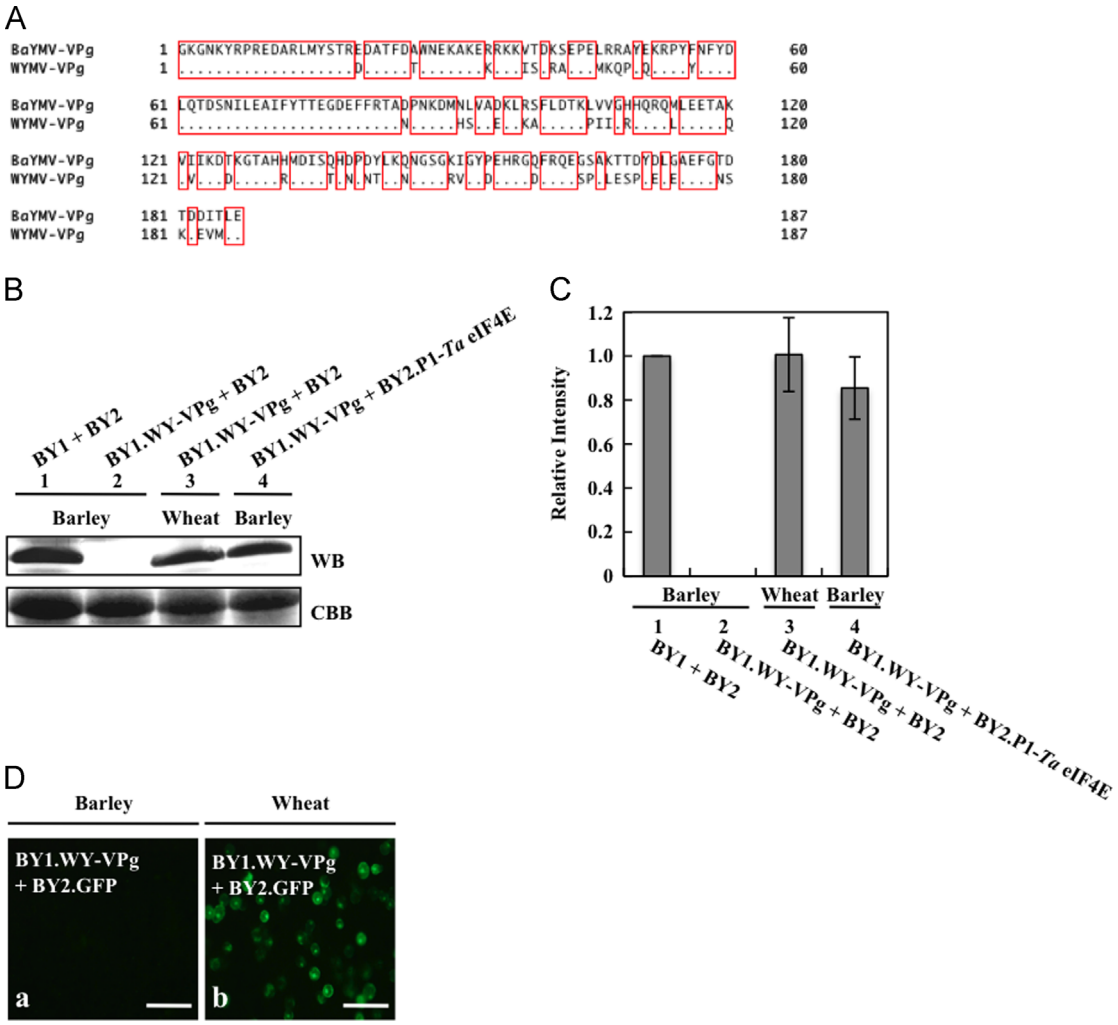

Fig. 4. Infectivity assay of WYMV VPg-expressed BaYMV mutant in barley and wheat mesophyll protoplasts. (A) Alignments of amino acid sequences of VPgs from BaYMV and WYMV using GENETYX-MAC software. (B) Western blotting analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. WB: Total proteins subjected to Western blotting analysis using anti-BaYMV CP (α-BaYMV CP). CBB: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. (C) Relative intensity of BaYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1. (D) GFP fluorescence observed in transfected protoplasts with BaYMV WY-VPg RNA1 and GFP RNA2 transcripts. Bars, 200 μm. The protoplasts transfected with BaYMV WT and mutant RNA1 transcripts were incubated at 15 °C for 66 h.

Discussion

Agriculturally, BaYMV and WYMV cause similar yellow mosaic diseases in barley and wheat, respectively. However, the hosts of these two bymoviruses are distinct in fields. BaYMV is detected in barley plants, whereas WYMV is detected only in wheat plants. The objective of this study was to examine the host tropism of BaYMV and WYMV at the cellular level.

BaYMV replicated not only in barley protoplasts but also in wheat protoplasts albeit less efficiently. In contrast, WYMV replicated only in wheat protoplasts but not in barley protoplasts, as determined by CP band appearance on Western blots and GFP fluorescence. Same results were obtained regardless of the source of RNA2 (either BaYMV or WYMV), indicating that the replication efficiency and the host tropism at the cellar level are determined by factors residing on RNA1 rather than on RNA2. On the other hand, mechanical inoculation to wheat plants with BaYMV RNA1 transcript together with BaYMV or WYMV RNA2 transcripts did not caused systemic infection (repeated 3 times), indicating that a lack of field wheat infection by BaYMV is determined at the movement level as well. Previously BaYMV P2 was shown to be required for systemic infection of BaYMV in barley plants (You and Shirako, 2010). In addition to BaYMV P2, P3N-PIPO (Vijayapalani et al., 2012; Wei et al., 2010; Wen and Hajimorad, 2010), CI (Carrington et al., 1998) and CP (Dolja et al., 1995) may also be functional in systemic infection of BaYMV in wheat plants as in the cases of viruses in the genus *Potyvirus*. Further studies on the host tropism at the whole plant level are required.

In this study to detect replication of BaYMV or WYMV in transfected protoplasts, we used two criteria, CP detection on Western blots and GFP fluorescence. Intensities of GFP fluorescence directly reflected efficiency of RNA2 replication. On the other hand, intensities of CP bands on Western blots were significantly reduced in the absence of RNA2-expressed P1 protein (this study; You and Shirako, 2010). A question is whether P1 facilitated efficient replication of RNA1 to elevate CP expression level or P1 stabilized CP and protected it from degradation. With our additional mutant construct with a GFP gene inserted between the NIb gene and the CP gene in pBY1, similar intensities of GFP fluorescence were observed in the presence and absence of BaYMV RNA2 transcript in barley protoplasts (data not shown), suggesting that P1 stabilized expressed CP rather than that P1 enhanced RNA1 replication. Recently an interaction between P1 and CP has been also shown by a bimolecular fluorescence complementation assay (Sun et al., 2014).

To date, approximately 200 resistance genes against virus infections have been reported in plants, and half of them are recessively inherited (Kang et al., 2005; Truniger and Aranda, 2009). All the recessive resistance genes against viruses in the family *Potyviridae* encode translation initiation factors - either eIF4E (Charron et al., 2008; Kanyuka et al., 2005; Stein et al., 2005), eIF4G (Lee et al., 2010), or their isoforms (Albar et al., 2006; Gallois et al., 2010; Hébrard et al., 2008). Because BaYMV and WYMV are members in the family *Potyviridae*, we investigated whether eIF4E is also involved in their host tropism at the cellular level. Co-expression of wheat eIF4E enabled WYMV to replicate in barely protoplasts, and co-expression of barley eIF4E increased BaYMV replication in wheat mesophyll protoplasts. These results suggest that eIF4E is also an important cellular factor that determines the host range of BaYMV and WYMV at the cellular level.

Virus counterparts that interact with eIF4E family proteins to induce resistance include VPg or P1 of *Clover yellow vein virus* (Nakahara et al., 2010), HC-Pro of *Potato virus A*, *Potato virus Y* and *Tobacco each virus* (Ala-Poikela et al., 2011) and CI of *Lettuce mosaic virus* (Abdul-Razzak et al., 2009). Here, we also investigated whether VPg is involved in host range determination at the cellular level. A BaYMV RNA1 mutant with a WYMV VPg gene did not replicate in barley protoplasts in the presence of BaYMV RNA2 expressing the P1 and P2 proteins. However, transfection together with a BaYMV RNA2 mutant expressing P1 and wheat eIF4E allowed the BaYMV RNA1 mutant with the WYMV VPg gene to replicate efficiently in barley cells. This result clearly indicates that the WYMV VPg located in the BaYMV RNA1 mutant was associated with wheat eIF4E expressed from the BaYMV RNA2 mutant, forming a functional replication complex with other essential viral proteins such as the NIb RNA polymerase and unidentified host factors.

It should be emphasized that even if there is no sign of replication as judged by the absence of a CP band by Western blot and a lack of GFP fluorescence proteins encoded on the input transcripts should be translated using the host translational machinery and exist for a certain period of time depending on the stability of individual proteins. BaYMV WYMV-VPg RNA1 in barley cells was one case and did not replicate unless wheat eIF4E was initially provided from the RNA2 transcript. BaYMV WYMV-VPg RNA1 in wheat cells could replicate by itself, using host wheat eIF4E, not only as in the translation machinery but also for BaYMV WYMV-VPg RNA1 replication. WYMV RNA1 in barley cells was another case and could replicate only when wheat eIF4E was initially expressed from the RNA2 transcript.

It has been hypothesized that host eIF4E might play a role in potyvirus genome expression and replication as well as in virus cell-to-cell movement (Gao et al., 2004; Robaglia and Caranta, 2006). As discussed above, BaYMV host range determination is apparently based at both the replication and movement levels, whereas that of WYMV is based primarily at the replication level. Host eIF4E involvement in bymovirus cell-to-cell movement and systemic infection requires further study.

In this study, an association between VPg and eIF4E proteins for replication was clearly shown, but a direct or indirect interaction of the two proteins has not been observed by a yeast two-hybrid assay, as was shown with potyviruses (Léonard et al., 2000; Schaad et al., 2000), or by a co-immunoprecipitation assay (data not shown). A low temperature requirement for replication or the presence of other intermediate factors (Abdul-Razzak et al., 2009; Ala-Poikela et al., 2011; Nakahara et al., 2010) may be the reasons for negative results by yeast two hybrid assays. In the present study, we found that wheat eIF4E is quite unstable in cells and has a short half-life (data not shown) (Monzingo et al., 2007), making detection of its possible interaction with VPg even more difficult by a co-immunoprecipitation assay.

There are 16 amino acid differences within the 215 amino acids throughout the proteins between BaYMV and WYMV VPgs, and 51 amino acid differences within the 187 amino acids throughout the proteins between barley and wheat eIF4Es, as shown in Figs. 3A and 4A. Further studies should focus on the precise determination of specific region(s) or amino acid(s) of VPgs and eIF4Es involved in the host tropism of BaYMV and WYMV.

Materials and methods

Plants and viruses

Barley (*Hordeum vulgare*, cv. Ryofu) and wheat (*Triticum aestivum*, cv. Shiranekomugi) were used as plant materials in this study. BaYMV was isolated from a barley field in Kurashiki in 2005, and WYMV was isolated from a wheat field in Nagano in 2009.

Infectious full-length cDNA clones of BaYMV and WYMV

The infectious full-length cDNA clones of BaYMV RNA1 and RNA2 were constructed and referred to as pBY1 and pBY2 (You and Shirako, 2010), respectively. Similarly infectious full-length cDNA clones of WYMV RNA1 and RNA2 were constructed and designated as pWY1 and pWY2, respectively. An SP6 promoter was used in both pWY1 and pWY2 for *in vitro* transcription.

cDNA isolation of Hv eIF4E and Ta eIF4E genes

The *Hv* eIF4E gene was isolated from barley (susceptible cv. KoA, You and Shirako, 2013), and the *Ta* eIF4E gene was isolated from wheat (cv. Shiranekomugi). For cDNA cloning of the eIF4E gene, total RNA was extracted from the leaves of barley or wheat using QuickGene-800 (Fuji Film, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA using PrimeScript™ Reverse Transcriptase (Takara Bio, Japan), and PCR was performed using PrimeSTAR® HS DNA Polymerase (Takara Bio, Japan). PCR fragments were purified using a FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Japan) and directly submitted for sequencing. The following GenBank nucleotide sequences were used as references: AJ699059 for barley cv. Morex, AB592973 for barley cv. KoA and Z12616 for wheat cv. unknown.

RNA1 and RNA2 mutant cDNA constructs of BaYMV and WYMV

Three types of mutant cDNA constructs were prepared from pBY1, pWY1, pBY2 and pWY2: (i) GFP-expressed mutant RNA2 constructs pBY2.GFP and pWY2.GFP. The mutant construct pBY2.GFP was modified from pBY2 with a GFP gene replacement of the P1 and P2 genes between the 5′- and 3′-untranslated regions (UTRs) of RNA2 (You and Shirako, 2010). The pWY2.GFP plasmid was constructed from pWY2 in the same manner. (ii) eIF4E-expressed mutant RNA2 constructs pBY2.P1-*Hv* eIF4E, pWY2.P1-*Ta* eIF4E and pBY2.P1-*Ta* eIF4E. These three mutant constructs were modified from RNA2 constructs of pBY2 or pWY2 by an insertion of the eIF4E gene to replace the P2 gene. To generate pBY2.P1-*Hv* eIF4E, PCRs were performed in the presence of pBY2 and the *Hv* eIF4E gene as separate templates. The resultant PCR products were purified and served as a mixed template to generate the P1-*Hv* eIF4E fragment. Subsequently, the fragment was digested and ligated into pBY2 using *Mlu*I and *Spe*I sites. To generate pWY2.P1-*Ta* eIF4E, PCRs were performed in the presence pWY2 and the *Ta* eIF4E gene as the templates. The resultant PCR products were purified and served as a mixed template to generate the fragment P1-*Ta* eIF4E. The final PCR product was digested with *Nde*I and *Spe*I and ligated into pWY2. To generate pBY2.P1-*Ta* eIF4E, PCRs were performed in the presence of pBY2 and the *Ta* eIF4E gene as separate templates. The resultant PCR products were purified and served as a mixed template to generate

BaYMV P1-*Ta* eIF4E. The final amplified fragments were purified and cloned into pBY2 with *Nsi*I and *Eco*RI sites using an In-Fusion HD Cloning System CE kit (Clontech Laboratories, USA). (iii) WYMV VPg-expressed chimera BaYMV RNA1 construct pBY1.WY-VPg. The construct was modified from pBY1 by a replacement of the BaYMV VPg gene with the WYMV VPg gene using pBY1 and pWY1 as the templates.

All the recombinant DNA procedures were performed according to standard methods using *Escherichia coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts. The primers used for the construction of these mutants are listed in the Supplementary Table S2.

In vitro transcription

Infectious cDNA clones were linearized and subsequently used as templates for *in vitro* transcription. BaYMV RNA1 (pBY1) and its derivative (pBY1.WY-VPg) were linearized using *Xho*I and *Bam*HI, respectively, and BaYMV RNA2 (pBY2), WYMV RNA1 (pWY1), WYMV RNA2 (pWY2) and their derivatives (pBY2.GFP, pBY2.P1-*Hv* eIF4E, pBY2.P1-*Ta* eIF4E, pWY2.GFP and pWY2.P1-*Ta* eIF4E) were linearized using *Spe*I. *In vitro* transcription reactions were performed with the T7 RNA polymerase for the pBY1, pBY2, pBY2.GFP, pBY2.P1-*Hv* eIF4E, pBY2.P1-*Ta* eIF4E, pBY1.WY-VPg, and pWY2.GFP constructs or SP6 RNA polymerase for the pWY1, pWY2 and pWY2.P1-*Ta* eIF4E constructs. T7 transcription reactions were performed using an mMESSAGE mMACHINE® T7 Kit (Ambion, USA). A 10 μL reaction mixture contained 2 μL of RNase-free water, 5 μL of 2× NTP/CAP, 1 μL of 10 × Reaction Buffer, 1 μL of Enzyme Mix and 100 ng of linearized DNA as the template. SP6 transcription reactions were performed as described previously (Yamamiya and Shirako, 2000) in a 10 μL reaction mixture containing 40 mM of Tris–HCl (pH 8.0), 8 mM of $MgCl_2$, 2 mM of spermidine, 1 mM of rNTP Mix, 2.5 mM of DTT, 20 units of RNase inhibitor (Takara Bio, Japan), 0.5 mM of m7G(5′)ppp(5′)G RNA Cap Structure Analog (New England Biolabs, USA), 10 units of SP6 RNA polymerase (New England Biolabs, USA) and 100 ng of linearized DNA as the template. Both T7 and SP6 transcription reactions were performed at 37 °C for 1 h.

Mesophyll protoplast preparation and RNA transfection

Barley and wheat seeds were sown in plastic pots containing a mixture of peat moss and vermiculite at a 1:1 ratio, and the pots were placed in a growth cabinet maintained at 22 °C under a 16-h light:8-h dark. The seedlings were fertilized with half-strength Hoagland solution (PhytoTechnology Laboratories, USA). Barley and wheat seedlings at the 6–7-day-old stage were harvested for protoplast preparation according to the procedure described previously (Ohsato et al., 2003), except that 0.2% Cellulase Onozuka RS (Yakult Pharmaceutical, Japan) was used instead of 2% Cellulase Onozuka R-10. Approximately 5×10^5 cells were transfected with capped *in vitro* transcripts of WT or mutant RNA1 and RNA2 (approximately 2 μg transcript for each) using a PEG method and incubated for 66 h at 15 °C for BaYMV and 90 h at 12 °C for WYMV in the dark.

Viral infection to plants using in vitro transcripts

Inoculums applied to plant seedings was prepared by mixing 5 μg of *in vitro* transcripts for each with 500 μL of inoculation buffer containing 50 mM glycine, 30 mM K_2HPO_4 (pH 9.2). Each plant at 2–3-seedings stage was first dusted with carborundum powder and then mechanically inoculated with 100 μL of this mixture by an electric toothbrush (Omron, Japan). Inoculated plants wrapped with a plastic bag were placed in dark box for 2 days at 15 °C (in case of BaYMV RNA1) or 12 °C (in case of WYMV RNA1), and then kept in a growth cabinet at 15 °C or 12 °C with 16 h of illumination.

Western blotting analysis

Transfected protoplast cells and plants were harvested for Western blotting analysis. Extracted samples were suspended in 100 μL 1 × Sample Buffer containing 100 mM Tris–HCl (pH 9.0), 4% SDS, 30% sucrose and 5% 3-Mercapto-1,2-propanediol. The proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis and then electroblotted onto 0.45 μm nitrocellulose membranes (Protran BA85, Whatman, Germany) with a Trans-Blot® Turbo™ Transfer system (Bio-Rad, USA). Western blotting analysis was performed with polyclonal antibodies against BaYMV CP (diluted 1:5000) (You and Shirako, 2010) and against WYMV WYCP (diluted 1:1000) (this study) as the primary antibody. Antibody binding was visualized by alkaline phosphatase-conjugated affinipure goat anti-rabbit IgG secondary antibody (Jackson Immuno Research, USA). The specific blots were detected using a NBT/BCIP method. Images were taken with a scanner, and ImageJ software (Wayne Rasband National Institutes of Health, USA) was used to measure the strength of CP bands (You and Shirako, 2013).

Fluorescent light microscopy

After incubation in the dark, protoplasts transfected with GFP RNA2 transcripts were monitored for the detection of GFP fluorescence under a fluorescent light microscope. Images were obtained with a VB-7010 cooled CCD camera (Keyence, Japan) equipped with an IX70 fluorescence microscope (Olympus, Japan) using the following settings: one second exposure time and 200 ISO film.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.010.

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