The Same Allele of Translation Initiation Factor 4E Mediates Resistance Against Two *Potyvirus* spp. in *Pisum sativum*

M. Bruun-Rasmussen, I. S. Møller, G. Tulinius, J. K. R. Hansen, O. S. Lund, and I. E. Johansen

Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, University of Aarhus, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

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Pathogenicity of two sequenced isolates of *Bean yellow mosaic virus* (BYMV) was established on genotypes of *Pisum sativum* L. reported to carry resistance genes to BYMV and other potyviruses. Resistance to the white lupin strain of BYMV (BYMV-W) is inherited as a recessive gene named *wlv* that maps to linkage group VI together with other *Potyvirus* resistances. One of these, *sbm1*, confers resistance to strains of *Pea seedborne mosaic virus* and previously has been identified as a mutant allele of the eukaryotic translation initiation factor 4E gene (*eIF4E*). Sequence comparison of *eIF4E* from BYMV-W-susceptible and -resistant *P. sativum* genotypes revealed a polymorphism correlating with the resistance profile. Expression of *eIF4E* from susceptible plants in resistant plants facilitated BYMV-W infection in inoculated leaves. When cDNA of BYMV-W was agroinoculated, resistance mediated by the *wlv* gene frequently was overcome, and virus from these plants had a codon change causing an Arg to His change at position 116 of the predicted viral genome-linked protein (VPg). Accordingly, plants carrying the *wlv* resistance gene were infected upon inoculation with BYMV-W derived from cDNA with a His codon at position 116 of the VPg coding region. These results suggested that VPg determined pathogenicity on plants carrying the *wlv* resistance gene and that *wlv* corresponded to the *sbm1* allele of *eIF4E*.

Additional keywords: *Pea early browning virus*.

Eukaryotic translation initiation factor 4E (*eIF4E*) and its isoform *eIF(iso)4E* play a key role during virus infection in plants (Robaglia and Caranta 2006). Most examples are known from the family *Potyviridae*; however, in the family *Bromoviridae*, *Cucumber mosaic virus* infection also has been demonstrated to rely on *eIF4E* as well as *eIF4G* (Yoshii et al. 2004). Accordingly, variants of *eIF4E* with a few codon differences

have been shown to confer resistance to infection, which is inherited as a recessive character (Gao et al. 2004b; Nicaise et al. 2003; Ruffel et al. 2002; Stein et al. 2005). Virus strains that can overcome recessive resistance genes have been identified in several *Potyvirus* spp. In most cases, breaking of resistance has been associated with mutations in the region encoding the genome-linked virus protein (VPg) (Ayme et al. 2006; Borgström and Johansen 2001; Moury et al. 2004; Nicolas et al. 1997; Schaad et al. 1997). In at least three examples where breaking of resistance is associated with VPg, the resistance gene was confirmed to be associated with mutations in *eIF4E*, namely the genes *sbm1* in *Pisum sativum* (Borgström and Johansen 2001; Gao et al. 2004b), *pvr2* in pepper (Moury et al. 2004; Ruffel et al. 2002), and *rym4* in barley (Kuhne et al. 2003; Stein et al. 2005). Therefore, it is likely that *Potyvirus* infection depends on interaction between host *eIF4E* and viral VPg, as was demonstrated recently between VPg precursors and *eIF(iso)4E* in planta (Beauchemin et al. 2007). Viruses with mutations in VPg often overcome resistance to virus accumulation (Nicaise et al. 2003), cell-to-cell movement (Gao et al. 2004b; Nicolas et al. 1997), or long-distance movement (Rajamäki and Valkonen 1999; Schaad et al. 1997), suggesting multiple functions of VPg. Though it is not known whether *eIF4E* plays a role in all these resistances, the interaction between VPg and *eIF4E* has been suggested to play a role in the early steps of infection during translation of virus proteins, viral replication, intracellular trafficking, and cell-to-cell movement (Robaglia and Caranta 2006).

In *P. sativum*, recessive resistance genes to several potyviruses have been mapped genetically to linkage groups II and VI (Provvidenti and Hampton 1991). The genes that map to linkage group II control resistance to *Bean common mosaic virus* (*bcm*), *Clover yellow vein virus* (CIYVV, *cyv1*), *Bean yellow mosaic virus* (BYMV, *mo*), *Watermelon mosaic virus 2* (*mo*), *pea mosaic virus* (*pmv*), and *Pea seedborne mosaic virus* (PSbMV, *sbm2*). The genes that map to linkage group VI show resistance to CIYVV (*cyv2*), PSbMV (*sbm1*), and white lupin mosaic virus (*wlv*) (Provvidenti and Hampton 1991). *Pea mosaic virus* and white lupin mosaic virus are now considered as strains of BYMV (Shukla et al. 1994). In this article, we will refer to white lupin mosaic virus as BYMV-W. The pathotype-specific resistance genes *sbm1* and *sbm2* that affect PSbMV have been studied in some detail. Gene *sbm1* is an allele of *eIF4E* (Gao et al. 2004b), whereas *sbm2* maps close to the isoform of *eIF4E* (*eIF(iso)4E*) (Gao et al. 2004a). The viral pathogenicity determinants toward *sbm1* and *sbm2* have been mapped to the regions encoding VPg and P3, respectively (Hjulsager et al. 2002). The identities of the other *Potyvirus* resistance genes

Corresponding author: I. E. Johansen; E-mail: e.johansen@dias.kvl.dk

Current address for I. S. Møller: Australian Centre for Plant Functional Genomics, Private Mail Bag 1, Glen Osmond, SA 5064, Australia.

Current address for O. S. Lund: Department of Plant Biology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Nucleotide sequence data for the white lupin strain of *Bean yellow mosaic virus* and *Pisum sativum* *eIF4E* cDNAs are available in the GenBank database under accession numbers DQ641248, DQ641470, DQ641471, DQ641472, DQ641473, and DQ641474.

in *P. sativum* remain to be identified. It is still not known whether the closely linked resistances *cyv1*, *mo*, *pmv*, and *sbm2* in linkage group II, as well as *cyv2*, *sbm1*, and *wlv* in linkage group VI, are separate genes or the same gene with alleles of different specificity.

This study focuses on resistance to BYMV, which was first described by Yen and Fry (1956). The resistance gene *mo* was genetically characterized in *P. sativum* cv. Bonneville using an unspecified isolate of BYMV (Schroeder and Provvidenti 1971) and mapped to linkage group II (Marx and Provvidenti 1979). A second recessive resistance gene, *wlv*, conferring specific resistance to BYMV-W, was described in *P. sativum* plant introduction (PI) line PI193835 and mapped to linkage group VI (Provvidenti and Hampton 1993). BYMV-W was found to overcome resistance in Bonneville (Provvidenti and Hampton 1993) whereas resistance in PI193835 was overcome by an unspecified isolate of BYMV (Provvidenti and Hampton 1991).

Here, we describe the response of *P. sativum* genotypes to BYMV strains BYMV-S (Guyatt et al. 1996) and BYMV-W (Hampton et al. 1992) and analyze the role of *eIF4E* in resistance. Results imply that *wlv* gene-mediated resistance to BYMV-W and *sbm1* gene-mediated resistance to PSbMV is controlled by the same allele of *eIF4E* and that a mutation in VPg allows BYMV-W to overcome resistance.

RESULTS

BYMV isolates S and W display differential infection profiles on a panel of *P. sativum* genotypes.

The BYMV isolates BYMV-S (Randles et al. 1980) and BYMV-W (Hampton et al. 1992) were selected to initiate a study of BYMV resistance in *P. sativum*. BYMV-S originates from *Vicia faba* and was reported to be sensitive to an unspecified resistance in *P. sativum* (Randles et al. 1980), whereas BYMV-W originates from *Lupinus albus* and is sensitive to resistance mediated by the gene *wlv* (Provvidenti and Hampton 1993). The sequence of BYMV-W was determined in this study (DQ641248) and Guyatt and associates (1996) published the sequence of BYMV-S. Initially, BYMV-S and BYMV-W were screened on a panel of *P. sativum* genotypes with resis-

tance to potyviruses, and all have been tested previously for resistance to PSbMV (Gao et al. 2004b; Olsen and Johansen 2001). Virus isolates BYMV-S and BYMV-W were maintained in *P. sativum* cv. Brutus and sap of infected leaves was used to inoculate 2-week-old *P. sativum* plants. Virus accumulation in uninoculated upper leaves was determined by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) 3 weeks postinoculation (wpi) using antibodies that were raised against purified BYMV-S or BYMV-W particles. Plants were considered infected if DAS-ELISA absorbance at 405 nm (A_{405}) was at least five times higher than the mean A_{405} of uninoculated controls. Judged according to this criterion, four *P. sativum* lines displayed resistance to BYMV-S but were susceptible to BYMV-W. These lines include cv. Bonneville, cv. Dark-skinned perfection (DSP), PI347422, and PI347484. The opposite response was observed in lines PI193835, PI347464, PI347492, and JI1405 that all displayed resistance to BYMV-W but were susceptible to BYMV-S. The line PI269818, which has been reported to carry resistance to an unspecified isolate of BYMV (Provvidenti 1987), was susceptible to both BYMV-S and BYMV-W. Based on this screen, we selected the following genotypes for further analysis of BYMV resistance: Brutus and JI2009 (cv. Scout), which were susceptible to both BYMV-S and BYMV-W and to PSbMV (Olsen and Johansen 2001); cvs. Bonneville and DSP, which both displayed resistance to BYMV-S and were susceptible to BYMV-W; and PI193835 and PI347464, which both were susceptible to BYMV-S and displayed resistance to BYMV-W and to PSbMV (Olsen and Johansen 2001). Cv. Bonneville originally was used to determine inheritance of resistance gene *mo* (Schroeder and Provvidenti 1971). PI193835 was included, although some individuals of PI193835 displayed DAS-ELISA readings indicating that the plants were infected by BYMV-W, because PI193835 originally was used to determine inheritance of resistance gene *wlv* (Provvidenti and Hampton 1993). Pathogenicity profiles of BYMV and PSbMV isolates are summarized in Table 1.

Sequence differences in *eIF4E* correlate with the infection profile of BYMV-W.

Gene *sbm1*, mediating resistance against PSbMV, and gene *wlv*, mediating resistance against BYMV-W, both map to linkage group VI (Provvidenti and Hampton 1991). It has been shown previously that resistance to PSbMV in PI193835 is due to codon differences in *eIF4E* (Gao et al. 2004b). To determine whether a similar correlation existed between the *eIF4E* coding sequence and BYMV-W infection, *eIF4E* cDNAs from BYMV-W-susceptible Bonneville, DSP, PI269818, and Brutus and BYMV-W-resistant PI193835 and PI347464 were cloned and sequenced. An alignment of the predicted amino acid sequences with previously published sequences of JI2009 and JI1405 is shown in Figure 1. The alignment identified a correlation between Leu62, Asp73, Arg107, and Lys169 and resistance to BYMV-W. There was no correlation between BYMV-S resistance and specific amino acids in *eIF4E* of BYMV-S-resistant cvs. Bonneville and DSP. This observation agreed well with the assumption that resistance to BYMV-S is controlled by linkage group II gene *mo* in Bonneville and DSP (Provvidenti and Hampton 1991). However, sequencing *P. sativum* cDNA of *eIF(iso)4E* did not reveal differences correlating to BYMV-S resistance (DQ778076, DQ778077, DQ778078, and Bruun-Rasmussen; unpublished data).

Expression of *eIF4E* from JI2009 leads to increased susceptibility to BYMV-W in plants carrying *wlv*.

To determine whether resistance gene *wlv* corresponds to *eIF4E*, we decided to test whether BYMV-W infection in re-

Table 1. Response of *Pisum sativum* genotypes to Bean yellow mosaic virus (BYMV) isolates W and S and Pea seedborne mosaic virus (PSbMV) isolates DPD1, L1, NEP1, and NY

Virus isolate	<i>P. sativum</i> genotype ^a				
	Bonneville DSP PI347422 PI347484 ^b	JI2009 Brutus	PI269818	PI193835 PI347464 JI1405 PI347492 ^c	
BYMV-W	S	S	S	R	
BYMV-S	R	S	S	S	
PSbMV-DPD1 ^d	S	S	R	R	
PSbMV-L1	R	S	R	R	
PSbMV-NEP1	R	S	S	R	
PSbMV-NY	S	S	S	R	

^a Evaluation of virus pathogenicity was performed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of virus coat protein in uninoculated upper leaves 3 weeks postinoculation. DSP = Dark-skinned perfection and PI = plant introduction. S = susceptible: DAS-ELISA absorbance at 405 nm (A_{405}) of 80 to 100% of the tested plants was at least five times higher than the mean A_{405} of uninoculated controls. R = resistant: A_{405} of 80 to 100% of the tested plants were less than five times higher than the mean A_{405} of uninoculated controls.

^b Resistance gene *mo* originally was characterized in *P. sativum* cv. Bonneville (Schroeder and Provvidenti 1971).

^c Resistance gene *wlv* originally was characterized in *P. sativum* PI193835 (Provvidenti and Hampton 1993).

^d Data on PSbMV (Hjulsager et al. 2002; Olsen and Johansen 2001).

sistant PI193835 could be complemented by coexpression of eIF4E from BYMV-susceptible *P. sativum* (S-eIF4E). To express the S-eIF4E in PI193835, we inserted the eIF4E coding region from *P. sativum* JI2009 into RNA2 of an expression vector based on *Pea early browning virus* (PEBV, genus *Tobravirus*) (Constantin et al. 2004). Two controls were included, one with an insertion of the sequence encoding eIF4E from PI193835 (R-eIF4E) and the other with the coding region of green fluorescent protein (GFP). The three recombinant PEBV RNA2 plasmids were named pCAPE2-S-eIF4E, pCAPE2-R-eIF4E, and pCAPE2-GFP. To allow agroinoculation of BYMV-W together with recombinant PEBV vectors, the BYMV-W sequence was cloned into a binary pCambia vector to generate pCABYMW-W.

For the complementation analysis, 2-week-old plants of BYMV-W-resistant PI193835 and BYMV-W-susceptible Bonneville were agroinoculated with pCABYMW-W, pCAPE1 that carries cDNA of PEBV RNA1, and pCAPE2-R-eIF4E, pCAPE2-S-eIF4E, or pCAPE2-GFP. Vectors pCABYMW-W and pCAPE1 were present in all agroinoculations and, therefore, only the pCAPE2 derivative will be mentioned in the following descriptions of experiments. In an initial experiment, infection by BYMV-W was determined by DAS-ELISA 5 days postinoculation (dpi) on an inoculated leaf of each plant. Samples were rated positive if the A_{405} reading was at least five times higher than the mean A_{405} of uninoculated controls. On PI193835 coinoculated with pCAPE2-S-eIF4E, 9 of 17 leaves tested positive, whereas 0 of 11 leaves and 2 of 18 leaves tested positive on plants coinoculated with pCAPE2-R-eIF4E

and pCAPE2-GFP, respectively. All Bonneville plants tested positive regardless of the coinoculated pCAPE2 vector. However, because the A_{405} readings generally were low, we decided to analyze inoculated leaves 8 dpi and also to analyze uninoculated leaves 3 wpi. Results of two further experiments (Fig. 2A) showed that 76% of leaves from PI193835 coinoculated with pCAPE2-S-eIF4E tested positive for BYMV-W, with an average A_{405} reading of 2.0. In leaves coinoculated with pCAPE2-GFP or pCAPE2-R-eIF4E, less than 10% of the samples tested positive for BYMV-W, with an average A_{405} reading of 0.36. A χ^2 test confirmed that coinoculation with pCAPE2-S-eIF4E resulted in significantly higher fraction of infected plants than coinoculation with pCAPE2-R-eIF4E or pCAPE2-GFP ($P < 0.001$). In Bonneville, DAS-ELISA tests showed that BYMV-W could be detected in inoculated leaves 8 dpi in all but one plant regardless of the coinfiltrated pCAPE2 vector. Thus, results of DAS-ELISA tests of inoculated leaves indicated that BYMV-W could be complemented in PI193835 by coinoculation with pCAPE2-S-eIF4E, but not by coinoculation with pCAPE2-GFP or pCAPE2-R-eIF4E.

Six PI193835 plants coinoculated with pCAPE2-GFP or pCAPE2-R-eIF4E displayed DAS-ELISA readings more than five times those of the uninoculated controls, indicating that the plants were becoming infected. This was supported by DAS-ELISA data from uninoculated upper leaves sampled 3 wpi. At this time, 71% of the plants coinoculated with pCAPE2-S-eIF4E, 60% coinoculated with pCAPE2-GFP, and 40% coinoculated with pCAPE2-R-eIF4E were DAS-ELISA positive, all with comparable A_{405} readings. Though the percent-

Fig. 1. Alignment of predicted protein sequences of eIF4E from *Bean yellow mosaic virus* (BYMV)-W-susceptible Bonneville (Bon), DSP, JI2009, Brutus, and PI269818 and BYMV-W-resistant PI347464, PI193835, and JI1405. Hyphens mark amino acids that are conserved in *Pisum sativum* eIF4E, and open boxes indicate differences correlating with resistance to BYMV-W. The locations associated with resistance to *Lettuce mosaic virus* (Nicaise et al. 2003), *Potato virus Y* (Ruffel et al. 2002), and BaMMV (Stein et al. 2005) are indicated with triangles, circles, and squares, respectively.

age of DAS-ELISA-positive plants coinoculated with pCAPE2-S-eIF4E was higher than plants coinoculated with pCAPE2-GFP and pCAPE2-R-eIF4E, the difference was not significant ($0.1 > P > 0.05$).

The VPg sequence of virus isolated from PI193835.

We speculated that DAS-ELISA-positive samples of upper, uninoculated PI193835 leaves observed 3 wpi could be due to the appearance of BYMV-W variants that break resistance. In the initial analysis of BYMV-W, a few PI193835 plants displayed DAS-ELISA readings above the threshold set to five times the uninoculated controls. However, inoculation by agroinfiltration may increase the opportunity for mutants to arise during transcription from the binary vector. Because there are several examples that mutations in the VPg coding region are

associated with overcoming recessive resistance to potyviruses, we decided to analyze this region of the BYMV-W sequence. For each treatment (coinoculation with pCAPE2-GFP, pCAPE2-S-eIF4E, and pCAPE2-R-eIF4E), RNA was extracted from uninoculated leaves of two Bonneville and four PI193835 plants that tested positive in DAS-ELISA 3 wpi. The VPg coding region was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) and the resulting PCR products were purified and sequenced. The VPg sequences from Bonneville plants were identical to the VPg sequence in the cDNA clone, whereas a unique G to A change was found in all 12 samples from PI193835. The G to A change was found in virus from PI193835 regardless of the coinoculated pCAPE2 construct. The resulting CGC to CAC codon change resulted in an Arg to His change at position 116 in the predicted amino acid sequence of VPg. This suggested that BYMV-W had overcome resistance mediated by gene *wlv* by adaptation of VPg to PI193835.

BYMV-W with VPg codon 116 CGC accumulates in leaves coinoculated with pCAPE2-S-eIF4E.

The elevated A_{405} readings in leaves of PI193835 coinoculated with pCAPE2-S-eIF4E could be a result of local accumulation of the original BYMV-S with an unchanged CGC Arg codon at position 116 of VPg or accumulation of the CAC variant. In the VPg coding region of BYMV-W, the Arg codon 116 is part of an *HaeII* restriction site (PuGCGCPy, Arg codon in bold), which was eliminated when the Arg codon mutated to an His codon (AGCACT, His codon in bold). To determine which variant accumulated in PI193835 leaves coinoculated with pCAPE2-S-eIF4E, 2-week-old plants of BYMV-W-resistant PI193835 and BYMV-W-susceptible Bonneville were coinoculated with pCAPE2-S-eIF4E. Plants coinoculated with pCAPE2-R-eIF4E or with pCABYMW-W alone served as controls. At 8 dpi, inoculated leaves were homogenized and half of the sample was analyzed by DAS-ELISA (Fig 2B) and half was used to extract RNA. In DAS-ELISA, 15 of 24 samples tested positive for BYMV CP in plants coinoculated with pCAPE2-S-eIF4E, while none of the samples from plants coinoculated with pCAPE2-R-eIF4E or inoculated with pCABYMW-W alone were positive in DAS-ELISA. RNA samples were DNase treated to remove pCABYMW inoculum and RT-PCR was conducted using a primer set amplifying a 620-bp band of the VPg coding region. This region was modified in pCABYMW by insertion of a 189-bp intron, and any residual DNA from the inoculum would give rise to an 809-bp PCR fragment (Fig. 3A and B, lane 11 and 12). Digestion of the 620-bp PCR product of the BYMV-W VPg coding region by *HaeII* was expected to result in two fragments of 330 and 290 bp if the CGC Arg codon was still present (Fig. 3B, lane 11). RT-PCR resulted in amplification of the expected 620-bp fragment from RNA of PI193835 coinoculated with pCAPE2-S-eIF4E, but not from plants coinoculated with pCAPE2-R-eIF4E (Fig. 3B, lanes 3 to 6 and 9 and 10) or plants inoculated with pCABYMW alone (data not shown). RT-PCR resulted in amplification of the expected 620-bp fragment from Bonneville, regardless of the coinoculated pCAPE2 construct (Fig. 3B, lanes 1 and 2 and 7 and 8). When PCR fragments were digested with *HaeII*, the 620-bp band was cleaved into fragments corresponding in size to the expected 330 and 290 bp (Fig. 3C). Sequence analysis of the 620-bp PCR fragment amplified from PI193835 plants coinoculated with pCAPE2-S-eIF4E confirmed that the sequence was identical to the VPg coding sequence in the cDNA clone. This experiment demonstrated that BYMV-W with an unmodified VPg sequence was complemented specifically by S-eIF4E before it adapts to PI193835 by mutation of VPg codon 116 from CGC (Arg) to CAC (His).

Fig. 2. Complementation of *Bean yellow mosaic virus* (BYMV)-W by eIF4E expressed from a pea early browning virus (PEBV) vector. BYMV-W infection was assayed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of coat protein in inoculated leaves 8 days postinoculation. Columns present mean DAS-ELISA readings and bars standard error of mean. Numbers next to the columns indicate number of samples rated as positive with DAS-ELISA absorbance at 405 nm (A_{405}) $\geq 5 \times$ mean A_{405} of uninoculated controls (number of samples tested). **A**, Results of two independent experiments in which leaves of *Pisum sativum* Bonneville (susceptible to BYMV-W) and PI193835 (resistant to BYMV-W) were inoculated by agroinfiltration of pCABYMW-W together with pCAPE1 and pCAPE2-GFP (green fluorescent protein), pCAPE2-R-eIF4E (R-eIF4E), or pCAPE2-S-eIF4E (S-eIF4E). **B**, Results of an experiment in which leaves of *P. sativum* Bonneville and PI193835 were inoculated by agroinfiltration of pCABYMW-W alone (No PEBV), pCABYMW together with pCAPE1 and pCAPE2-R-eIF4E, or pCAPE2-S-eIF4E.

Mutation of VPg codon 116 in BYMV-W is sufficient to overcome *wlv* resistance.

To determine whether the single codon change at position 116 of the VPg coding region was responsible for breaking resistance in PI193835, we introduced this mutation to VPg of

Fig. 3. Analysis of reverse-transcriptase polymerase chain reaction (RT-PCR) products of the viral genome-linked protein (VPg) coding region from RNA extracted from leaves agroinfiltrated with pCABYMV together with pCAPE1 and pCAPE2-S-eIF4E or pCAPE2-R-eIF4E. **A**, T-DNA region of pCABYMV with an enlargement of the VPg coding region. Regions representing 5' and 3' untranslated regions are shown as horizontal lines, with the coding region as an open box with regions predicted to encode potyviral proteins P1pro, HCpro, P3, 6k1, CI, 6k2, Pro, RdRp, and CP (Revers et al. 1999). The intron (open triangle) that is absent in bean yellow mosaic virus (BYMV)-W RNA allowed PCR primers Fw-VPg and Rv-VPg (arrows) to discriminate between fragments amplified from viral RNA and DNA inoculum. The polymorphism at codon 116 is displayed with the BYMV-W Arg codon in the context of an *HaeII* restriction site above and the mutated His codon below the enlarged VPg coding region. **B**, Agarose gel displaying RT-PCR products amplified by Fw-VPg and Rv-VPg on RT reactions of RNA extracted from leaves 8 days post inoculation with pCABYMV, pCAPE1, and pCAPE2-S-eIF4E (lanes 1 to 6) or pCAPE2-R-eIF4E (lanes 7 to 10). Lanes 1, 2, 7, and 8: RNA from BYMV-W-susceptible *P. sativum* cv. Bonneville; lanes 3 to 6, 9, and 10: RNA from BYMV-W-resistant *P. sativum* PI193835. PCR products amplified from cDNA without (lane 11) and with (lane 12) an intron indicate the size of PCR products expected from RT-PCR of viral RNA and PCR of pCABYMV DNA inoculum, respectively. Lane M: 100-bp DNA ladder with 500-bp band marked by an arrowhead. PDS shows an RT-PCR amplification control corresponding to a 470-bp fragment of *P. sativum phytoene desaturase*. **C**, Agarose gel displaying *HaeII* digestion of RT-PCR products amplified by Fw-VPg and Rv-VPg. Lane numbering and markers are as in B.

BYMV-W, generating pBYMV-W^{H116}. To make a direct comparison of BYMV-W and BYMV-W^{H116} that was not influenced by the efficiency of inoculation of cDNA constructs, virus inocula were established by manual inoculation of susceptible cv. Brutus with plasmids pBYMV and pBYMV-W^{H116}. Sap from these plants was used to inoculate BYMV-W-resistant PI193835 and PI347464 and BYMV-W-susceptible Brutus. Pathogenicity was assayed by DAS-ELISA 3 wpi on leaf samples from uninoculated leaves (Fig. 4A). Of the plants inoculated with BYMV-W^{H116}, 97% of PI193835 and PI347464 were positive in DAS-ELISA compared with 6% of plants inoculated with BYMV-W (Fig. 4A), confirming that virus derived from a cDNA clone with this single codon change in VPg could overcome *wlv* resistance. In a second experiment, virus derived from pBYMV-W^{H116} also was inoculated to Bonneville and DSP (Fig. 4B). All DSP and Bonneville plants tested positive in DAS-ELISA, suggesting that the change of VPg codon 116 to His (CAC) did not have a major effect on pathogenicity on Bonneville and DSP plants.

DISCUSSION

We have established the infection profile of two sequenced BYMV isolates, BYMV-S and BYMV-W, on a panel of *P. sativum* lines with reported resistance to several potyviruses, including BYMV and PSbMV (Olsen and Johansen 2001; Provvidenti and Hampton 1993). Two of these lines, Bonneville and PI193835, were used originally to map the resistance genes *mo* (Schroeder and Provvidenti 1971) and *wlv* (Provvidenti and

Fig. 4. Pathogenicity of viral genome-linked protein (VPg) mutant Bean yellow mosaic virus (BYMV)-W^{H116} in *Pisum sativum*. Columns represent mean double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) absorbance at 405 nm (A_{405}) readings relative to the mean A_{405} reading of uninoculated controls and bars show standard error of mean. Numbers indicate samples rated as positive with DAS-ELISA $A_{405} \geq 5 \times$ mean A_{405} of uninoculated controls (number of samples tested). Samples were taken from uninoculated leaves 3 weeks post inoculation. **A**, Relative accumulation of BYMV coat protein in genotypes Brutus, PI193835, and PI347464 inoculated with BYMV-W (black bars) and BYMV-W^{H116} (hatched bars). **B**, Relative accumulation of BYMV coat protein in genotypes Brutus, Bonneville, DSP, PI193835, and PI347464 inoculated with BYMV-W^{H116}.

Hampton 1993) to linkage group II and VI, respectively. Genes mediating resistance to BYMV-W and PSbMV in linkage group VI originally were named *wlv* and *sbm*, respectively, and *sbm* was differentiated into *sbm1*, *sbm3*, and *sbm4* depending on the PSbMV pathotype (Provvidenti and Hampton 1991). Gao and associates (2004a) showed that *eIF4E* colocalized with *sbm* in linkage group VI and sequence analysis of *eIF4E* that identified three alleles of *eIF4E* in *P. sativum* lines JI2009, PI193835, and PI269818. In complementation experiments, it was found that expression of *eIF4E* from susceptible JI2009 supported infection of PSbMV in PI193835 and PI269818 (Gao et al. 2004b). This led to a revision of the nomenclature. Thus, *eIF4E* from PSbMV-susceptible JI2009 was designated *Sbm1*, *eIF4E* from PI193835 that is resistant to the four known pathotypes of PSbMV was designated *sbm1*, and *eIF4E* from PI269818 that is overcome by PSbMV pathotypes P3 and P4 was designated *sbm1*¹. The present study of resistance to BYMV-W in PI193835, which carries the gene traditionally known as *wlv* in linkage group VI (Provvidenti and Hampton 1993), demonstrated that BYMV-W infection was complemented by expression of *eIF4E* corresponding to *Sbm1* (Figs. 2 and 3). This suggested that *sbm1* and *wlv* correspond to the same allele of *eIF4E*. This parallels the situation in *Capsicum* spp., where resistances known as *pvr1*, *pvr2*¹, and *pvr2*² were found to be alleles of *eIF4E* conferring broad-spectrum or more specific resistance to viruses of the genus *Potyvirus* (Kang et al. 2005).

The *P. sativum* gene *sbm1*¹ is an allele of *eIF4E* that confers specific resistance to some pathotypes of PSbMV (Gao et al. 2004b). PSbMV pathogenicity on *P. sativum* that carries *sbm1*¹ was associated with differences in the VPg coding region (Borgström and Johansen 2001). In PSbMV, mutation of VPg codon 116, which is equivalent to codon 118 in BYMV-W, was sufficient to alter pathogenicity on plants carrying the *sbm1*¹ allele of *eIF4E*; however, it was not investigated whether these mutations affected pathogenicity on plants carrying the *sbm1/wlv* allele of *eIF4E* (Borgström and Johansen 2001). Similarly, other resistances mediated by alleles of *eIF4E* to other viruses in the family *Potyviridae* were overcome by virus isolates with mutations in the same part of the VPg coding region (Kuhne et al. 2003; Moury et al. 2004). In the present study, agroinoculation of BYMV-W from the binary plasmid pCABYMV resulted in the appearance of a resistance-breaking variant carrying a CGC (Arg) to CAC (His) codon change in the VPg coding region. This change was observed regardless of the coinoculated pCAPE2 construct, suggesting that, although S-eIF4E can complement BYMV-W in inoculated leaves (Fig. 3), this complementation was not sufficient to assist systemic infection by BYMV-W in competition with the resistance-breaking variant. It appeared that BYMV-W required only one nucleotide change to overcome resistance, because PI193835 and PI347464 plants inoculated with virus derived from pBYMV-W^{H116} displayed A₄₀₅ readings suggesting virus accumulation in uninoculated leaves (Fig. 4A). Of course, we cannot rule out the possibility that second-site compensatory mutations arose in the viruses during amplification in susceptible cv. Brutus. To minimize selective differentiation of BYMV-W and pBYMV-W^{H116}, both viruses were raised from cDNA clones and amplified in the same genetic background; however, it is possible that putative second-site mutations could be biased by the difference at VPg codon 116 in such a way that only virus derived from pBYMV-W^{H116} overcame resistance.

If breaking of resistance required only one nucleotide transition to change the Arg (CGC) codon to His (CAC), this may explain why the resistance-breaking isolate appeared frequently in plants infected by agroinoculation. Resistance breaking also was observed in plants inoculated by manual inoculation, but

at a much lower frequency. This raises the possibility that agroinoculation may be an efficient way to provoke amino acid changes to challenge resistance, thereby enabling predictions on resistance durability, as suggested by Ayme and associates (2006).

Although virus derived from BYMV-W^{H116} overcame resistance in PI193835 and PI347464, pathogenicity on Bonneville and DSP was not compromised (Fig. 4B). In contrast, BYMV-S, which has a His codon (CAC) at position 116 of VPg and was infectious on PI193835 and PI347464, did not infect Bonneville and DSP. This would suggest that the mechanism of resistance mediated by *sbm1/wlv* in PI193835 and PI347464 differed from *mo* resistance in Bonneville and DSP. Supporting this interpretation, the pathogenicity determinant in PSbMV toward *sbm2*, which is closely linked to *mo* (Provvidenti and Alconero 1988), was mapped to the P3 coding region (Johansen et al. 2001). The linkage between *eIF(iso)4E*, *sbm2*, and *mo* made *eIF(iso)4E* a likely candidate for the potyvirus resistance gene in linkage group II of *P. sativum* (Gao et al. 2004a). Therefore, it was surprising that we did not identify any coding differences in the cDNA of *P. sativum* *eIF(iso)4E* correlating to the presence of *mo* resistance (M. Bruun-Rasmussen, unpublished data). Other candidates may be found among the translation initiation factors (Albar et al. 2006; Ruffel et al. 2006). In conclusion, our experiments provide a link between classical genetic studies and virological experiments (Hampton et al. 1992; Provvidenti and Hampton 1991, 1993) to specific alleles of *eIF4E* and sequenced BYMV isolates. Our results suggest that, in *P. sativum*, resistances mediated by genes *sbm1* against PSbMV and *wlv* against BYMV-W are caused by the same allele of *eIF4E*. Further, we have shown that a single amino acid change in VPg of BYMV-W is sufficient to overcome resistance mediated by *wlv*, and that this mutation does not compromise BYMV-W infection on *P. sativum* with the resistance gene *mo*.

MATERIALS AND METHODS

P. sativum genotypes and BYMV isolates.

The following *P. sativum* genotypes were used in this study: cvs. Bonneville (by courtesy of K. E. Keller, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Corvallis, OR, U.S.A.), JI1405 and JI2009 (by courtesy of A. J. Maule, John Innes Centre, England), DSP (Dæhnfeldt, Odense, Denmark), and Brutus (DLF-Trifolium, St. Heddinge, Denmark); and PI lines PI193586, PI193835, PI193836, PI269774, PI269818, PI347492, PI347329, PI347422, PI347464, and PI347484 (Western Regional Plant Introduction Station, Pullman, WA, U.S.A.).

The BYMV-S isolate (Guyatt et al. 1996) was provided by A. Davidson, Monash University, Australia. BYMV-W (Hampton et al. 1992) was provided by K. E. Keller, USDA-ARS. BYMV-S and BYMV-W particles were purified as described by (Olsen and Johansen 2001) from *P. sativum* Brutus and DSP, respectively, and used to raise polyclonal antibodies in rabbits. Antibodies were used in DAS-ELISA to assay plant tissue samples of 0.25 g for the presence of virus coat protein (Kohnen et al. 1995). In all experiments, at least four uninoculated *P. sativum* plants were included to determine the mean A₄₀₅ reading of uninfected plant tissue.

Cloning and sequencing of BYMV-W.

Standard cloning procedures were employed (Sambrook et al. 1989). Restriction and modifying enzymes (Roche Diagnostics, Mannheim, Germany and New England Biolabs, Ipswich, MA, U.S.A.), *Taq* DNA polymerase (GibcoBRL Roskilde, Denmark), *Pfu* DNA polymerase (Stratagene, La

Jolla, CA, U.S.A.), and M-MLV reverse transcriptase (Invitrogen, Taastrup, Denmark) were used according to the manufacturer's instructions. PCR products were cloned using TOPO-technology (Invitrogen). Oligonucleotide primers and sequencing analyses were delivered by MWG (Ebersberg, Germany).

RNA was extracted from virus particles and used as template for oligo(dT) primed cDNA synthesis by M-MLV reverse transcriptase. The cDNA was amplified by PCR using *Taq* DNA polymerase using a series of degenerate primers, which were designed on the basis of the BYMV-S sequence (U47033). PCR products were cloned, and sequences obtained from these clones covered nucleotides 26 to 2,400, 4,250 to 5,750, and 6,100 to 8,200. Based on these three sequences, primers were designed to obtain sequence of the 5' terminus by 5' rapid amplification of cDNA ends (RACE) (Clontech, Mountain View, CA, U.S.A.) and amplify cDNA to obtain a complete sequence. The 5' RACE gave sequences up to nucleotide 21 as predicted by comparison with BYMV-S (U47033) and BYMV-MB4 (NC 003492).

For assembly of a full-length clone of BYMV-W, the complete genome was amplified by PCR using *Pfu* DNA polymerase generating overlapping cDNA fragments using (forward/reverse) primer pairs 5' GTCTAGAAAAATATAAAACCAATCAAGAC/5' TGGTGTCTATTCTGATTCGGCTAC for nucleotides 1 to 2,453, 5' CCATCAGCGCAGGGTACCATG/5' GTCTGCAGCATCTCTGAACCTC for nucleotides 2,310 to 5,731, 5' CCTGAGTGTGTTGCACAGTGCAC/5' ATTTGGC GGTATGGAGGTAAACAC for nucleotides 5,404 to 6,808, and 5' CTTTGTGAAGGGTGCAGGGC/5' GCGTCTAGATTTTT TTTTTTTTTTTTTT for nucleotides 6,058 to poly(A). Sequences were obtained from at least two independent clones of these fragments and the BYMV-W sequence was submitted to the National Center for Biotechnology Information (NCBI) Nucleotide Sequence database, accession number DQ641248. Putative coding regions were predicted by comparison with BYMV-S and BYMV-MB4 (Guyatt et al. 1996; Nakamura et al. 1996) and the VPg coding region assigned to nucleotides 5,661 to 6,233.

Construction of infectious clones pBYMV-W^{H116} and pCABYMV-W.

The complete cDNA sequence of BYMV-W was assembled between the 35S promoter and nopaline synthase (NOS) terminator in pAGUS1 in a series of standard cloning steps essentially as described by Johansen (1996). Nonviral nucleotides after the transcription start site were eliminated by mung bean nuclease (NEB, Ipswich, MA, U.S.A.) treatment. To stabilize the plasmid in *Escherichia coli*, intron IV from *Solanum tuberosum* gene ST-LS1 was inserted after nucleotide 5,729 to generate pBYMV-W. Virus derived from pBYMV-W has the same infection profile on *P. sativum* genotypes as native BYMV-W (data not shown).

pBYMV-W^{H116} was constructed by mutating GC at nucleotide 6,027 to 6,028 to AT, thus changing the predicted VPg codon 116 CGC (Arg) to CAT (His). Plasmids pBYMV and pBYMV-W^{H116} (0.5 µg/µl in H₂O) were inoculated by mechanical inoculation by hand to *P. sativum* Brutus, and sap (leaf tissue homogenized in 0.03 M phosphate buffer, pH 7.6, with 4% polyethyleneglycol) from these plants was used to inoculate test plants.

To allow agroinoculation of BYMV-W cDNA, the plasmid pCABYMV-W was generated by inserting the expression cassette from pBYMV-W, including 35S promoter, BYMV-W cDNA, and NOS terminator, into the binary vector pCambia-1300 (Cambia, Canberra, Australia), which was modified by deletion of the hygromycin resistance gene (Constantin et al. 2004).

Sequencing of *eIF4E* cDNA from *P. sativum* genotypes.

To obtain sequence information on *eIF4E* from *P. sativum*, primers AD-FW: 5' CCCAAAAAAGAGATCGAATTAGGA and AD-RV: 5' CTCTGCAGTAATACGACTCACTAT (flanking the inserts of λ-phage library vector pAD-Gal4-2-1 inserts) (Stratagene) and eIF-FW: 5' GAGTTTGGAGCATTTACAA TAAC and eIF-RV: 5' CACAACAGCTCC(AG)CATATTTTCAT (designed on the basis on predicted *eIF4E* EST sequences from legumes) were used to amplify DNA fragments from a cDNA library of cv. Scout provided by A. J. Maule John Innes Centre, U.K. The two fragments amplified with primer pairs AD-FW: eIF-RV and eIF-FW:AD:RV were cloned using TOPO TA cloning kit (Invitrogen) and inserts were sequenced. The sequence differed at a single nucleotide at the fifth codon, which was GAC (Asp) in contrast to GAA (Glu) from the published sequence (ARR04332). Oligonucleotide 5' CAGTCAAAAGA TTATCCGCACACA annealing at the 3' untranslated region (UTR) of *eIF4E* was used to prime cDNA synthesis on RNA extracted from Brutus, Bonneville, DSP, PI269818, PI193835, and PI347464 using RNAeasy kit (Qiagen, Hilden, Germany). Two primers that annealed to the 5' (5' GGCACGAGGAGAG AGCAAAAATG) and 3' (5' CGGAGAGGCATCTCATGCAA) UTR were used to PCR amplify the region coding *eIF4E* by *Taq* DNA polymerase. The PCR fragments were TOPO cloned and sequenced. The sequence of eIF4E from PI269818 (AY611425) and PI193835 (AY611423) were published by Gao and associates (2004b); however, we consistently found the fifth codon of the open reading frame to be GAC. The sequences of eIF4E from Brutus, Bonneville, DSP, PI347464, and PI193835 were submitted to the NCBI Nucleotide Sequence database and assigned accession numbers DQ641470, DQ641471, DQ641472, DQ641473, and DQ641474.

Construction of pCAPE2-R-eIF4E and pCAPE2-S-eIF4E.

The PEBV vector is composed of two binary plasmids, pCAPE1 and pCAPE2, carrying cDNA of PEBV RNA1 and a modified RNA2 for insertion of heterologous fragments (Constantin et al. 2004). The 684-nucleotide coding region of eIF4E from BYMV-W-susceptible (JI2009) and -resistant (PI193835) *P. sativum* genotypes were amplified by PCR using *Pfu* DNA polymerase with primers 5' GCCCATGGTTGTAGA AGACACCCCCAAATCC and 5' GTTACACAACATATTTGT TTTTAGCATTTTC). The fragments were TOPO cloned and sequenced. Binary vectors pCAPE2-R-eIF4E and pCAPE2-S-eIF4E were constructed by replacing the GFP coding region of pCAPE2-GFP (Constantin et al. 2004) with the coding region of *eIF4E* from PI193835 and JI2009, respectively.

Functional complementation assay by PEBV-vectored eIF4E.

Agrobacterium sp. strain GV3101 was transformed with pCABYMV-W, pCAPE2-R-eIF4E, pCAPE2-S-eIF4E, and cultures for agroinoculation were grown as described in Constantin and associates (2004). Optical density at 550 nm (OD₅₅₀) was determined on overnight cultures, and bacteria were harvested by centrifugation and resuspended in 100 µl of infiltration medium (10 mM NaCl, 1.75 mM CaCl₂, 100 µM acetosyringone) per milliliter of culture per 1.0 unit at OD₅₅₀. For inoculation, infiltration cultures of pCABYMV-W, pCAPE1, and pCAPE2-derivative (GFP or R-eIF4E or S-eIF4E) were mixed 1:1:1 and infiltrated to four leaves of BYMV-W-susceptible (Bonneville) and -resistant (PI193835) *P. sativum* plants. The experiment was repeated three times and, in each experiment, at least six plants were used per treatment. In the first experiment, inoculated leaves were assayed by DAS-ELISA for the presence of BYMV coat protein 5 dpi. In the second, third, and fourth experiments, inoculated leaves

were assayed for the presence of virus coat protein at 8 dpi and uninoculated leaves at 3 wpi.

Sequence analysis of the VPg coding region.

To determine the sequence of the VPg coding region of virus in infected plants, RNA was extracted from leaf tissue and the VPg coding region was amplified by RT-PCR and sequenced as described by Borgström and Johansen (2001) using primers Fw-VPg (5' GCGTACGAAACAGAACTGAGGTTTCAGAG) and Rv-VPg (5' TCTCGAGTAAGCAAACATTTGAAGC). For analysis of the VPg coding region in leaves agroinoculated with pCABYMV, RNA was treated with RQ1 RNase-free DNase (Promega Corps., Madison, WI, U.S.A.) to degrade DNA inoculum. RT-PCR products amplified by *Taq* DNA polymerase were analyzed by agarose gel electrophoresis either undigested or after digestion with restriction enzyme *Hae*II (Roche Diagnostics). As a control of RNA extractions, a fragment of *P. sativum* *PDS* was amplified by RT-PCR using primers 5' ATGGGCGGTGAAGTCCATCTTAATTC and 5' GATCTGCAGAAATTCATCAGGG.

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