Translation elongation factor 1B (eEF1B) is an essential host factor for Tobacco mosaic virus infection in plants

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A B S T R A C T

Identifying host factors provides an important clue to understand virus infection. We selected 10 host factor candidate genes and each gene was silenced in Nicotiana benthamiana (N. benthamiana) to investigate their roles in virus infection. The resulting plants were infected with Tobacco mosaic virus (TMV). The accumulation of viral coat protein and the spread of virus were greatly reduced in the plants that eukaryotic translation elongation factor 1A (eEF1A) or 1B (eEF1B) was silenced. These results suggest both eEF1A and eEF1B are required for TMV infection. We also tested for interactions between the eEFs and viral proteins of TMV. Both eEF1A and eEF1B proteins interacted directly with the methyltransferase (MT) domain of the TMV RNA-dependent RNA polymerase (RdRp). eEF1A and eEF1B also interacted with each other in vivo. Our data suggest that eEF1B may be a component of the TMV replication complex which interacts with MT domain of TMV RdRp and eEF1A.

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Introduction

Because viruses are simple and obligate intracellular parasites, they recruit host factors to complete their infection cycle (Kang et al., 2005a; Mazier et al., 2011; Nagy and Pogany, 2011). Most steps of viral infection involve interactions between viral components and host factors and the absence of appropriate host factors can confer resistance to the host (Ahlquist et al., 2003; Truniger and Aranda, 2009). A survey of natural virus resistance genes showed that more than half of the 200 known virus resistance genes in plants are recessively inherited, indicating that these resistance genes could have originated from a loss of function of host factors (Kang et al., 2005b). Some of these genes have been cloned from various crop species, and almost all of them were identified as members of the eukaryotic translation initiation factor 4E (eIF4E) or 4G (eIF4G) families (Diaz-Pendon et al., 2004; Kang et al., 2005b; Jenner et al., 2010; Wang and Krishnaswamy, 2012). A large number of natural resistance genes, however, have yet to be characterized, and many of them may not belong to the eIF4E or eIF4G families (Truniger and Aranda, 2009). Therefore, identifying new host factors and their roles in viral infection will help to broaden our understanding of plant virus infection, which in turn will promote the development of virus-resistant cultivars.

The eukaryotic elongation factor 1 (eEF1) family comprises eEF1A and eEF1B proteins in plant (Rodnina and Wintermeyer, 2009). The eukaryotic translation elongation factor eEF1A, one of the most abundant proteins in eukaryotic cells, has a role in delivering aminoacyl-tRNA (aa-tRNA) to the elongating ribosome in a GTP-dependent manner (Le Sourd et al., 2006). In addition to its role in peptide chain elongation, eEF1A has been reported to have many additional functions including in quality control of newly produced proteins, ubiquitin-dependent protein degradation, and organization of the actin cytoskeleton (Chuang et al., 2005; Gross and Kinzy, 2005). Numerous positive-strands of RNA viruses are known to utilize eEF1A in their replication. The first report showed that the host translation elongation factors EF-Tu and EF-Ts are components of the bacteriophage Qbeta (Qb) RNA replicase (Blumenthal and Carmichael, 1979). Interactions between viral genomic RNA and/or RdRp and eEF1A have been reported for several plant viruses including TMV (Zeenko et al., 2002), Brome mosaic virus (Bastin and Hall, 1976), Turnip mosaic virus (TuMV) (Dufresne et al., 2008; Thivierge et al., 2008) and Turnip yellow mosaic virus (TYMV) (Joshi et al., 1986; Matsuda and Dreher, 2004) and animal viruses including Bovine viral diarrhea virus (Johnson et al., 2001), poliovirus (Harris et al., 1994), West Nile virus (Blackwell and Brinton, 1997; Davis et al., 2007) and Dengue 4 virus (De Nova-Ocampo et al., 2002). Similar to its canonical role in translation, eEF1A has been found to bind to an aa-tRNA-like structure in the 3′-untranslated region of the TYMV and act as both a translational enhancer and a repressor of minus-strand RNA synthesis in vitro (Matsuda and Dreher, 2004;...
Matsuda et al., 2004). It was suggested that repression of minus-strand synthesis by eEF1A binding occurs at the early stage of infection to coordinate the competing translation and replication functions of the TYMV genomic RNA (Matsuda et al., 2004).

In TMV infection, it was demonstrated that eEF1A is a component of TMV replication complex. Down-regulation of eEF1A using virus-induced gene silencing (VIGS) in N. benthamiana inhibited the accumulation of TMV RNA and spreading (Yamaji et al., 2010). The eEF1A proteins from wheat germ and N. benthamiana interact with the pseudoknot (PK) structure upstream of the tRNA-like structure (TLS) in the 3'-UTR of the TMV genome (Zeenko et al., 2002). In addition, the methyltransferase (MT) domain of TMV RdRp interacts with eEF1A (Yamaji et al., 2006).

The eEF1B protein, a guanine nucleotide exchange factor, binds to GDP-bound eEF1A. There are different subunits constituting eEF1B, depending on the species. The yeast eEF1B is made of two subunits, a guanine nucleotide exchange protein (eEF1Bx) and a structural protein (eEF1Br). In plants, eEF1B is composed of a structural protein (eEF1Bγ) and two nucleotide exchange subunits (eEF1Bβ and eEF1Bδ), whereas the metazoan complex is a heteromer of at least four subunits: a structural protein (eEF1Bγ), two exchange factors (eEF1Bβ and eEF1Bδ), and a unique tRNA synthetase, the valine-tRNA synthetase (Le Sourd et al., 2006; Rodnina and Wintermeyer, 2009). The eEF1Bβ protein is a plant-specific nucleotide exchange protein and the C-terminal domain of both eEF1Bx and eEF1Bβ proteins is known to be the binding site for eEF1A (Le Sourd et al., 2006). Recently, it has been reported that eEF1Bγ binds to the 3' end of the Tomato bushy stunt virus (TBSV) RNA for stimulation of minus-strand synthesis (Sasvari et al., 2011).

Identification of host factors and elucidation of their participation in virus susceptibility can lead to the development of powerful strategies against plant viruses. Genetic screens in the model plant Arabidopsis thaliana have provided clues about new host factors involved in viral pathogenicity. Chili pepper (Capsicum annuum L.) is an important vegetable crop used as a spice and a source of red pigment. Moreover, a large number of actin.

RNA extraction and RT-PCR analysis

Total RNA was extracted from leaves of N. benthamiana and C. annuum ‘Early Carl Wonder’ plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using 2 μg total RNA, oligo dT primers and M-MLV reverse transcriptase (Promega, Mannheim, Germany) according to the manufacturer’s protocol. The expression levels of target genes were monitored by semi-quantitative RT-PCR using gene-specific primers that anneal outside the region targeted for silencing (Table S1). For quantitation, gels were scanned, and the pixel intensity for each band was determined using the ImageJ program (NIH Image, Bethesda, MD) and normalized to the amount of actin.

Virus infection and evaluation of resistance

TMV-GFP inoculum was prepared from leaves of N. benthamiana and A. tumefaciens strain GV3101 carrying the infectious TMV-GFP genome. Plants were inoculated at the four-to-six-leaf stage. The two oldest leaves received a light application of carborundum followed by rub-inoculation with inoculums produced by grinding systemically infected N. benthamiana tissue in 100 mM phosphate buffer, pH 7.0 (1 g tissue: 10 ml buffer). Mock-inoculated and non-inoculated controls were routinely included. After inoculation, the plants were monitored daily for the appearance of symptoms. Leaf tissue was tested for the presence of virus using DAS-ELISA according to the manufacturer’s instructions (Agdia, Inc., USA). Virus accumulation was tested at 7 days post inoculation (dpi) for inoculated leaves and upper non-inoculated leaves. GFP was visualized with a MultiDoc-it digital imaging system using a SYBR Green filter (bandpass 515–570 nm; UVP) or a GFP filter (excitation 470/40 nm; emission 525/50 nm) (UVP, http://www.uvp.com) and confocal scanning microscopy (LSM 510; Carl Zeiss, Jena, Germany).

Yeast two-hybrid analysis

Yeast transformation and analyses were performed using the ProQuest two-Hybrid System™ with Gateway Technology™ (Invitrogen, http://www.invitrogen.com). The eEF1A and eEF1B genes

**Materials and methods**

**Plasmid construction for virus-induced gene silencing (VIGS)**

DNA sequences of N. benthamiana homologs of C. annuum genes were amplified using ExTaq DNA polymerase (TaKaRa, Shiga, Japan) and oligonucleotide primers (Table S1). We used modified ligation-independent cloning for high-throughput cloning into the TRV VIGS vector (Dong et al., 2007). All PCR products were purified using DNA clean & Concentrator™-100 (Zymo Research, Orange, CA, USA). A total of 15 fmol of purified PCR products were treated with T4 DNA polymerase, LIC qualified (Novagen, San Diego, CA, USA) in 10X reaction buffer containing 5 mM dATP at 22 °C for 30 min, followed by inactivation of the T4 DNA polymerase for 20 min at 70 °C. The TRV2-LIC vector was then digested with PstI and treated with T4 DNA polymerase using TdT. A total of 22.5 fmol of treated PCR product and TRV2-LIC vector were mixed and incubated at 65 °C for 2 min and then at 22 °C for 10 min. Subsequently, 3 μl of the mixture was transformed into E. coli DH10B competent cells. Transformants were tested by PCR amplification using primers based on TRV2-LIC vector sequences: 5'-TGTACTCAAGGAGCCAGTGAC-3' and 5'-CAGGCCAGCAGTTCTATTTAAGAACATG-3'. Plasmids from positive clones were purified and then sequenced at NICEM (http://nicem.snu.ac.kr).

**Plant materials and Agrobacterium infiltration**

N. benthamiana and C. annuum ‘Early Carl Wonder’ plants were grown at 25 °C in a growth chamber with a 16 h light/8 h dark cycle. For the VIGS experiment, the TRV VIGS system was used (Dinesh-Kumar et al., 2003; Lu et al., 2003). Briefly, pTRV1 and pTRV2 or its derivatives were introduced into cells of Agrobacterium tumefaciens strain GV2260. Overnight Agrobacterium cultures were grown at 28 °C in LB medium containing antibiotics (50 mg/L kanamycin and 50 mg/L rifampicin). Agrobacterium cells were pelleted and resuspended in infiltration medium (10 mM MgCl2, 10 mM MES, 200 μM acetosyringone), adjusted to 0.4 OD600, and incubated at room temperature for 3–4 h. Agrobacterium carrying pTRV1 was mixed in a 1:1 ratio with pTRV2 or its derivatives and infiltrated into leaves of N. benthamiana and C. annuum.

**Plant materials and Agrobacterium infiltration**

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**Yeast two-hybrid analysis**

Yeast transformation and analyses were performed using the ProQuest two-Hybrid System™ with Gateway Technology™ (Invitrogen, http://www.invitrogen.com). The eEF1A and eEF1B genes
were amplified by PCR from *C. annuum* ‘Early Cal Wonder’ and the MT domain of RdRp and the movement protein of TMV were amplified from TMV (P1, 2)-infected *N. benthamiana*. ProQuest yeast two-hybrid vectors pDEST22, containing *Capsicum* eEF1A or eEF1B, and pDEST32, containing individual viral components were transformed into yeast strain MaV203 (ProQuest; Invitrogen, http://www.invitrogen.com). Yeast transformants were plated in synthetic complete medium (SC) lacking leucine (−Leu) and tryptophan (−Try). After 72 h, large colonies were picked and cultured in liquid medium. One day later, 10 μl drops of cultured cells were applied to selection plates to screen for expression of reporter genes. Interactions were assessed by growth on SC lacking leucine (−Leu), tryptophan (−Try), and histidine (−His) with 10 mM 3-amino-1, 2, 4-triazole (3AT).

**Co-immunoprecipitation (co-IP) assay**

Proteins were transiently co-expressed by *Agrobacterium* infiltration in leaves of *N. benthamiana*. HA-tagged *C. annuum* eEF1A and eEF1B were expressed using the pEarlyGate (pEG) 201 vector (Earley et al., 2006) and FLAG-tagged MT domain of TMV RdRp was expressed using the pEG202 vector (Earley et al., 2006). Co-IP assays were performed as described previously (Oh and Martin, 2011). Briefly, the leaves were harvested for 2 days after infiltration, and total protein was extracted with extraction buffer (GTEN) (10% glycerol, 25 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl), 10 mM DTT, 0.1% Triton X-100, 1X plant protease inhibitor (Sigma-Aldrich), 1X phosphatase inhibitor (Sigma), and 2% w/v polyvinylpolypyrrolidone. Protein extracts were incubated with HA-tag antibody-agarose beads for IP 2 h to overnight. Finally, beads were collected and washed six times with the IP buffer (GTEN, 0.15% Nonidet P-40, 1 mM DTT). The proteins were eluted with 5x sample buffer and denatured by boiling at 95 °C for 5 min. The proteins were separated via 12% SDS-PAGE gels and immunoblotted with anti-HA antibody (Sigma) and anti-FLAG antibody (Sigma).

**Results**

**Screening of candidate genes in *N. benthamiana***

To identify host factors required for virus infection, 10 candidate genes encoding translation initiation, elongation, and release factors were selected in *C. annuum* (Table 1). The *C. annuum* genes were subcloned into a virus-induced gene silencing (VIGS) vector and VIGS was performed in *N. benthamiana*. *N. benthamiana* was infected with large number of diverse plant viruses and also

### Table 1

List of candidate host factors used in this study.

<table>
<thead>
<tr>
<th>Candidate host factor</th>
<th>Description</th>
<th>Locus in Arabidopsis</th>
<th><em>C. annuum</em> Unigenes (e-value)</th>
<th><em>N. benthamiana</em> Unigenes (e-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eukaryotic elongation factor 1A (eEF1A)</td>
<td>GTP binding, GTPase activity, translation elongation factor activity</td>
<td>AT1G07940, AT1G07920, AT1G07930, AT5G60390</td>
<td>SGN-U196116, SGN-U196120, SGN-U196119 (0)</td>
<td>SGN-US13228, SGN-US13229 (0)</td>
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<tr>
<td>Eukaryotic elongation factor 1B (eEF1Bα/β)</td>
<td>Translation elongation factor activity</td>
<td>AT5G12110, AT5G19510, AT2G18110, AT1G30230</td>
<td>SGN-U196232, SGN-U196232 (0)</td>
<td>SGN-US10074, SGN-US10074 (0)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 1A (eIF1A)</td>
<td>RNA binding, translation initiation factor activity</td>
<td>AT2G04520, AT5G35680</td>
<td>SGN-US195181 (8e-47)</td>
<td>SGN-US195181 (8e-47)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 2A (eIF2A)</td>
<td>RNA binding, translation initiation factor activity</td>
<td>AT5G05470, AT2G40290</td>
<td>SGN-US198813 (0)</td>
<td>SGN-US198813 (0)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4A (eIF4A)</td>
<td>ATP-dependent RNA helicase</td>
<td>AT3G13920, AT1G27230, AT1G45270, AT3G19760</td>
<td>SGN-US196814, SGN-US199030 (0)</td>
<td>SGN-US16932, SGN-US16932 (0)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4E (eIF4E)</td>
<td>RNA cap binding, protein binding, translation initiation factor activity</td>
<td>AT4G18040, AT1G29550, AT1G29550</td>
<td>SGN-US197400, SGN-US197400 (0)</td>
<td>SGN-US209001, SGN-US209001 (3e-85)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 5A (eIF5A)</td>
<td>Translation initiation factor activity</td>
<td>AT1G13950, AT1G69410, AT1G26630</td>
<td>SGN-US197940, SGN-US197940 (0)</td>
<td>SGN-US15723, SGN-US15723 (0)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 6 (eIF6)</td>
<td>Protein binding, ribosome binding, translation initiation factor activity</td>
<td>AT2G39820</td>
<td>SGN-US204121 (0)</td>
<td>SGN-US204121 (0)</td>
</tr>
<tr>
<td>Novel cap-binding protein (nCBP)</td>
<td>RNA binding, RNA cap binding</td>
<td>AT5G18110</td>
<td>SGN-US200956 (0)</td>
<td>SGN-US200956 (5e-8)</td>
</tr>
<tr>
<td>Poly(A)-binding protein (PABP)</td>
<td>RNA binding, poly(A) binding, translation initiation factor activity</td>
<td>AT1G49760, AT4G34110, AT2G23350, AT1G22760, AT1G17170, AT2G36660, AT1G34140, AT1G16380</td>
<td>SGN-US196404, SGN-US196404 (0)</td>
<td>SGN-US111094, SGN-US111094 (0)</td>
</tr>
</tbody>
</table>

The sequences for silencing constructs are shown boldface.

* b blast searches in *S. lycopersicum* Unigenes database (http://solgenomics.net).

* c blast search in *C. annuum* and *N. benthamiana* Unigenes databases (http://solgenomics.net).

* d blast search in *N. tabacum* EST sequences (http://solgenomics.net).
amenable to facile method for VIGS. Besides, *Capsicum* and *Nicotiana* both are members of the Solanaceae family and there is usually high degree of similarity in gene coding sequences. The degree of silencing of each gene was determined by semi-quantitative RT-PCR at 13 dpi using primers that anneal outside of the silencing sequence (Table S1). In three independent repeats, each with three plants, the mRNA expression level of the target genes was significantly reduced when compared to TRV::00 plants (Fig. 1B). These results indicate that *Capsicum* gene fragments could successfully silence homologous genes in *N. benthamiana*.

*N. benthamiana* plants in which *eEF1A*, *eEF1B*, *eIF1A*, *eIF2A* or *eIF4A* was silenced were characterized by arrested growth (Fig. 1A), and *eEF1A*-silenced plants also showed chlorotic leaves and spontaneous necrosis at 20 dpi (data not shown). These phenotypes were in contrast to infection with the TRV empty vector (TRV::00), which causes mild symptoms and reduced growth from which the plants recover to resemble non-silenced control plants. The *eIF4E*-*, *eIF6*- or *nCBP*-silenced *N. benthamiana* plants were similar to TRV::00 control plants and the *PABP*-silenced plants displayed curved leaves (Fig. 1A).

To investigate the influence of TMV infection on the silencing of a target gene, *N. benthamiana* plants were co-infected with TRV::PDS and TMV-GFP or infected with TRV::PDS alone as a control. TRV2::PDS-infected plants showed a typical photo-bleaching phenotype at 10–12 dpi (Fig. 2A). Co-infection with TMV-GFP did not alter this silencing phenotype, indicating that TMV-GFP does not interfere with TRV-induced gene silencing.

To determine the effect of silencing host genes on viral amplification, TMV-GFP was inoculated on the upper two leaves of silenced plants. The accumulation of viral coat protein in inoculated and non-inoculated upper leaves was confirmed by enzyme-linked immunosorbent (ELISA) assays at 7 dpi. The accumulation level of TMV-GFP was abundant in the non-silenced and TRV::00 plants (Fig. 2B). Similar high levels were detected in the *eEF1A*-, *eIF2A*-, *eIF4A*-, *eIF4E*-, *eIF5A*-, *eIF6*-, *nCBP*-, or *PABP*-silenced plants. Significant reduction of virus accumulation was detected only in *eEF1A*- or *eEF1B*-silenced plants compared to TRV::00 infected plants (Fig. 2B). In conclusion, of the 10 candidate genes tested, only *eEF1A* and *eEF1B* had significant effects on TMV-GFP accumulation. Since translation elongation in
eukaryotes requires a set of elongation factors including eEF1A and eEF1B, it is possible that the expression of each gene is coordinately regulated. We confirmed that silencing of eEF1A or eEF1B gene does not affect the expression of other gene (Fig. 2C).

Silencing effects of the eEF1B genes on TMV infection

Because it has already been reported that eEF1A is involved in TMV replication (Zeenko et al., 2002; Yamaji et al., 2006, 2010), we focused on detailed analysis of the role of eEF1B in TMV infection. Under UV light, TMV-GFP infection foci were abundant in the inoculated leaves and non-inoculated upper leaves of non-silenced and TRV::00 plants at 5 dpi (Fig. 3A, panels f, g, and h). However, only a few infection foci were detected in the inoculated leaves, and no infection foci were detected in the upper leaves of eEF1B-silenced plants (Fig. 3A, panels i and j). To confirm the effects of eEF1B on TMV multiplication, we monitored virus spread at the cellular level using confocal microscopy. Following TMV-GFP infection, the inoculated leaves of TRV::00 control plants showed GFP fluorescence at 2 dpi, and strong fluorescence was detected at 4 dpi (Fig. 3B, panels a–c). Systemic infection was also detected in the uninoculated upper leaves at 7 dpi (Fig. 3B, panel d). By contrast, only limited fluorescence was detected in the inoculated leaves of eEF1B-silenced plants at 4 dpi (Fig. 3B, panels e–g), and no fluorescence was detected in the uninoculated upper leaves (Fig. 3B, panel h). These results suggest that eEF1B is necessary for successful amplification of TMV. In Fig. 2B, no significant difference in TMV accumulation was observed in the inoculated leaves of eEF1B-silenced plants although a slight difference was detected. The difference in virus accumulation detected by ELISA (Fig. 2B) and GFP observation (Fig. 3A and B) could be explained by the speed of virus accumulation and the sampling time after inoculation.

The experiments above used infectious leaf sap containing virions as the inoculum for TMV-GFP. Since co-translational disassembly of virions could be inhibited in eEF1B-silenced plants (Yang et al., 2009), we tested the effects of eEF1B silencing on TMV replication using infectious viral RNA as the inoculum source to overcome this potential complication. N. benthamiana plants were first inoculated with TRV::00 or TRV::eEF1B and then we inoculated TMV-GFP by Agrobacterium infiltration at 12 dpi. Agrobacterium infiltration results in the production of infectious RNA transcripts in the recipient cells (Yang et al., 2009). In agreement with the sap inoculation results, TMV-GFP fluorescence was dramatically inhibited in the eEF1B-silenced plants at 5 dpi (Fig. 3C).

Silencing of eEF1B in C. annuum

We also investigated the effects of eEF1B silencing on the accumulation of TMV in pepper. The newly emerging cotyledons of germinating C. annuum ‘ECW’ plants were infiltrated with Agrobacterium culture containing pTRV2::PDS, pTRV2::eEF1B or pTRV2 empty vector together with pTRV1. Plants infiltrated with TRV::PDS were taken as a positive control in the VIGS experiment. Silencing was monitored in the upper leaves of the plants, and the silencing phenotype appeared 2 weeks after Agro-infiltration. At that point of time, eEF1B-silenced pepper plants showed specific silencing phenotypes such as curved leaves and arrested growth similar to eEF1B-silenced N. benthamiana (Fig. 4A). The mRNA expression level of the eEF1B gene in the silenced plants was greatly reduced at 25 dpi (Fig. 4B). TMV-GFP was applied to
the upper two leaves of TRV::00 control plants and eEF1B-silenced plants at 26 dpi. TMV-GFP infection foci were monitored up to 7 dpi for signs of systemic infection characterized by GFP fluorescence in systemic tissue. After 7 days, intense GFP fluorescence was detected in the inoculated leaves of TRV::00 plants, whereas much weaker fluorescence was observed in the inoculated leaves of eEF1B-silenced plants (Fig. 4C). These results are consistent with the observed spreading of GFP fluorescence in N. benthamiana. Taken together, eEF1B appears to be an essential factor for TMV infection.

**Interaction between eEF1B and TMV RdRp**

Previously, it was reported that eEF1A interacts with the MT domain of TMV RdRp (Yamaji et al., 2006). To investigate whether eEF1B also could interact with viral proteins of TMV, we
performed yeast two-hybrid assays using the MT domain of TMV RdRp (MT) and the movement protein (MP) that facilitates cell-to-cell transport of TMV genomic RNA through plasmodesmata. As shown in Fig. 5A, both eEF1A and eEF1B showed weak interactions with TMV RdRp (MT), whereas TMV MP did not interact with either eEF1A or eEF1B. To confirm these interactions in plant cells, co-IP assays were performed. For co-IP, HA-tagged eEF1A and eEF1B were co-expressed with FLAG-tagged TMV RdRp (MT). As shown in Fig. 5B, both eEF1A and eEF1B co-immunoprecipitated with TMV RdRp (MT). A negative control, GFP-FLAG, did not co-immunoprecipitate with eEF1A or eEF1B protein (Fig. 5B). These results demonstrate that eEF1B directly interacts with TMV RdRp (MT) and could be involved in TMV replication.

eEF1A and eEF1B interaction

In the QB virus, the viral RdRp forms a replicative complex with the host translation factors EF-Tu (the prokaryotic counterpart of eEF1A) and EF-Ts (the prokaryotic counterpart of eEF1B), while EF-Tu and EF-Ts tightly interact with each other in the QB virus replicase (Kidmose et al., 2010; Takeshita and Tomita, 2010). To test the interaction between Capsicum eEF1A and eEF1B, yeast two-hybrid and co-IP assays were performed. Yeast cells cotransformed with Capsicum eEF1A and eEF1B were able to grow on SC-Leu–Try–His+3AT (Fig. 5A), indicating that Capsicum eEF1A and eEF1B interacted with each other. To confirm this interaction in vivo, FLAG-tagged eEF1A and HA-tagged eEF1B were transiently co-expressed in N. benthamiana leaves and co-IP was performed using anti-HA agarose beads. As shown in Fig. 5B, FLAG-tagged eEF1A was co-immunoprecipitated with HA-tagged eEF1B. These results indicate that eEF1A and eEF1B directly interact with each other in plants. Taken together, it is possible that eEF1A and eEF1B could form a viral replicative complex via interacting with the viral RdRp during the TMV infection process.

Discussion

Identification of eEF1B as a new host factor for TMV infection

Here, we discovered that eEF1B is a novel host factor required for TMV infection. Of the host factors tested, silencing of only eEF1A or eEF1B significantly reduced TMV accumulation in Solanaceous plants. The effect of silencing eEF1A on TMV replication in N. benthamiana is consistent with the previous studies (Zeenko et al., 2002; Yamaji et al., 2006, 2010). In this study, we demonstrated that silencing of eEF1B greatly reduced TMV-GFP accumulation in N. benthamiana plants. Furthermore, we showed that...
silencing of the eEF1B gene affected TMV infection in Capsicum plants. This is the first report showing that eEF1B is an essential host factor for TMV infection in plants.

Previous studies have shown that TRV-mediated VIGS is a useful tool for studying TMV (Liu et al., 2004) and geminivirus infections in N. benthamiana (Lozan-Duran et al., 2011). Before testing the silencing effect of candidate genes, we tested whether double infection of TMV and TRV could affect accumulation of TMV and confirmed that double infection of TMV and TRV does not alter TMV accumulation and, thus, that any reduction of TMV accumulation was solely due to silencing of the target genes.

Silencing of eEFIA or eEF1B led to the developmental defects because EFs are ubiquitous and essential factors for protein synthesis and cell viability. Developmental defects have frequently been observed in other studies when essential genes for plant survival were silenced (Yang et al., 2009; Xu et al., 2012). However, it appears that the reduction of virus accumulation in eEFIA- or eEF1B-silenced plants may not be due to such developmental defects. The silencing of eEF1A led to more severe developmental defects than that of eEFIA or eEF1B, but viral accumulation was similar to that in TRV:00 control plants. In addition, the eIF2A- or eIF4A-silenced plants also showed inhibition of plant growth similar to eEF1B-silenced plants, but TMV accumulation was not decreased in those lines. There are six distinct genes (eEFIA-a1, eEFIA-a2, eEFIA-a3, and eEFIA-a4 and eEFIA-b1 and eEFIA-b2) coding for eEFIA in N. benthamiana and their coding regions are almost identical. Nonspecific silencing of the eEFIA gene family members causes severe stunting of plants and a significant reduction of TMV accumulation (Yamaji et al., 2010). However, it was shown that the decreased TMV accumulation was not due to growth retardation because partial silencing of eEFIA (by silencing only eEFIA-a or eEFIA-b) has little effect on the growth, but still reduced TMV accumulation (Yamaji et al., 2010). Together these results demonstrate that eEFIA plays a critical role in TMV infection. The eEF1B protein in plants contains three subunits, a structural protein (eEF1Bγ) and two nucleotide exchange subunits (eEF1Bζ and eEF1Bβ) (Le Sourd et al., 2006). The eEF1Bζ subunit is highly conserved throughout the eukaryotic kingdom. In this study, eEF1Bζ, a plant-specific nucleotide exchange protein, was used for silencing construct and protein interaction studies. However, the eEF1Bζ silencing constructs may have caused silencing of both eEF1Bζ and eEF1Bβ because eEF1Bζ shares 94% nucleotide similarity with eEF1Bβ in Capsicum (data not shown) while there is no significant similarity between eEF1Bζ/β and eEF1Bγ. Further work is required to reveal the specific effects of each subunit of eEF1B on plant growth and TMV infection.

Significance of the interaction between eEF1B and TMV RdRp

It is known that naturally occurring mutations in eIF4E determine the infectivity of several RNA virus families, including bymoviruses, carmoviruses, and potyviruses (Gao et al., 2004; Kang et al., 2005a; Kanyuka et al., 2004; Ling et al., 2009; Nicaise et al., 2003; Nieto et al., 2007; Ruffell et al., 2005, 2006; Stein et al., 2005; Truniger and Aranda, 2009). The best known example demonstrating interaction of host factors and viral components is potyvirus VPg and eukaryotic translation initiation factor 4E (eIF4E). eIF4E encoded by resistance alleles (pvr1, pvr11, and pvr12) fails to interact with the Tobacco etch virus (TEV) VPg, whereas eIF4E from the susceptible allele Pvr1 interacts strongly (Kang et al., 2005a). Similarly, the interaction between TuMV VPG and A. thaliana elFiso4E was shown using the yeast two-hybrid system and this interaction is needed for virus infection (Leonard et al., 2000). Recently, it has been demonstrated that eIF4E-mediated natural recessive resistances against potyviruses result from non-synonymous mutations in an elf4E protein, which impair its direct interaction with the potyviral protein VPg (Mazier et al., 2011; Ashby et al., 2011). In addition, the multifunctional helper component proteinase (HCpro) of Potato virus A (PVA) interacts with both elf4E and elfIso4E (Ala-Poikela et al., 2011). Point mutations introduced into a 4E binding motif identified in the C-terminal region of HCpro decrease the interactions with translation initiation factors and are detrimental to the virulence of PVA (Ala-Poikela et al., 2011).

To date, more than 10 host factors have been identified to interact with the replication proteins of tobanovirus including ATAF2, eEF1A, GCD10, PS8PR, TOM1/TOM3 and 14-3-3 proteins (reviewed in Ishibashi et al., 2010). The full content and function of these replication complexes, however, remains unknown. In this study, we showed that both eEF1A and eEF1B proteins interact with TMV RdRp (MT) and that they interact with each other. It is possible that the eEF1B is one of the components of the TMV replication complex together with eEF1A and that the interaction between eEF1A and eEF1B is needed for efficient binding to viral proteins.

Possible roles of eEF1B in TMV multiplication

There are at least two possible roles of eEF1B in TMV infection. First, it could be involved in TMV replication by interacting with TMV RdRp. Since positive-strand RNA viruses replicate their genomes in intracellular membranes, extensive membrane rearrangements leading to cytoplasmic membranous structure production are observed during the infection cycle of these viruses (Miller and Krijnse-Locker, 2008; Cotton et al., 2009). These virus-induced structures are known to shelter the virus replication complex, which carries out viral RNA synthesis. Irregularly shaped cytoplasmic inclusion bodies, virus replication complexes, are observed in TMV-infected plant cells. These replication complexes contain ribosomes, tubulin-like structures, endoplasmic reticulum, viral replication proteins (126/183K), MP, and viral RNAs (Saito et al., 1987; MÁS and Beachy, 1999; Asumendi et al., 2004; Kawakami et al., 2004; Liu et al., 2005). It appears that eEF1A and eEF1B may form part of the TMV replication complex by interacting with TMV RdRp.

Second, the interaction between eEF1B and TMV RdRp (MT) could have an effect on viral cell-to-cell movement. Although TMV cell-to-cell transport controlled by the 30 kDa movement protein (MP) has been intensively studied, it was demonstrated that the 126/183 kDa replica proteins are also involved in cell-to-cell movement (Knapp et al., 2005). TMV encodes two replication proteins (126 and 183 kDa), and they are components of the virus replication complex (Saito et al., 1987; MÁS and Beachy, 1999; Liu et al., 2005). Both replication proteins contain MT and helicase (Hel) domains. The 183 kDa protein (183K) contains another conserved domain, the polymerase domain, in addition (Ishikawa et al., 1986; Watanabe et al., 1999). When 126K protein was mutated in the MT domain, defective RNAs (dRNAs) did not replicate (Lewandowski and Dawson, 2000) and failed to move or moved poorly (Knapp et al., 2005). Knapp and co-workers demonstrated that expression of at least approximately 50% of the MT domain was required for efficient dRNA movement in N. benthamiana (Knapp et al., 2005). In this study, we showed that limited GFP fluorescence was detected in the TMV-GFP inoculated leaves of eEF1B-silenced plants and no infection foci were observed in the systemic leaves. Although we have not determined the precise role of eEF1B in TMV infection, it is possible that the interaction between eEF1B and TMV RdRp (MT) could participate in TMV replication and cell-to-cell movement.
Engineering TMV-resistant plants

Further study to elucidate the roles of eEF1B in RNA virus infection are required, for instance to identify the specific domain of eEF1B responsible for binding to TMV RdRp. Mutations in this region of eEF1B, which should disrupt interaction with TMV RdRp, might confer TMV resistance. Indeed, virus resistance could be mediated by disruption or impairment of direct interaction between host factors and viral proteins. Amino acid substitutions G107R (pvr1) and V67E (pvr2) in eIF4E have been shown to prevent interactions with Vpg from TEV and Potato virus Y (PVY), respectively, and confer resistance in pepper (Charron et al., 2008; Nieto et al., 2007). TILLING (targeting induced local lesions in genomes) approaches could be applied to isolate eIF4E mutants efficiently. Recently, TILLING of eEF1B was successfully exploited to engineer virus resistance in melon and tomato (Nieto et al., 2007; Piron et al., 2010). Any genes encoding susceptibility factors could be potential targets for mutation breeding and genome-wide association breeding (Nieto et al., 2007; Piron et al., 2010). Our study demonstrates that eEF1B could be a strong potential target for mutation breeding for virus resistance.

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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.virology.2013.02.004.

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