A Host RNA Helicase-Like Protein, AtRH8, Interacts with the Potyviral Genome-Linked Protein, VPg, Associates with the Virus Accumulation Complex, and Is Essential for Infection1[C][W][OA]

Tyng-Shyan Huang, Taiyun Wei, Jean-François Laliberté, and Aiming Wang*

Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, Ontario N5V 4T3, Canada (T.-S.H., T.W., A.W.); Department of Biology, University of Western Ontario, London, Ontario N6A 5B7, Canada (T.-S.H., T.W., A.W.); and Institut National de la Recherche Scientifique, Institut Armand-Frappier, Laval, Quebec H7V 1B7, Canada (J.-F.L.)

The viral genome-linked protein, VPg, of potyviruses is a multifunctional protein involved in viral genome translation and replication. Previous studies have shown that both eukaryotic translation initiation factor 4E (eIF4E) and eIF4G or their respective isoforms from the eIF4F complex, which modulates the initiation of protein translation, selectively interact with VPg and are required for potyvirus infection. Here, we report the identification of two DEAD-box RNA helicase-like proteins, PpDDXL and AtRH8 from peach (Prunus persica) and Arabidopsis (Arabidopsis thaliana), respectively, both interacting with VPg. We show that AtRH8 is dispensable for plant growth and development but necessary for potyvirus infection. In potyvirus-infected Nicotiana benthamiana leaf tissues, AtRH8 colocalizes with the chloroplast-bound virus accumulation vesicles, suggesting a possible role of AtRH8 in viral genome translation and replication. Deletion analyses of AtRH8 have identified the VPg-binding region. Comparison of this region and the corresponding region of PpDDXL suggests that they are highly conserved and share the same secondary structure. Moreover, overexpression of the VPg-binding region from either AtRH8 or PpDDXL suppresses potyvirus accumulation in infected N. benthamiana leaf tissues. Taken together, these data demonstrate that AtRH8, interacting with VPg, is a host factor required for the potyvirus infection process and that both AtRH8 and PpDDXL may be manipulated for the development of genetic resistance against potyvirus infections.

Plant viruses are obligate intracellular parasites that infect many agriculturally important crops and cause severe losses each year. One of the common characteristics of plant viruses is their relatively small genome that encodes a limited number of viral proteins, making them dependent on host factors to fulfill their infection cycles (Maule et al., 2002; Whitham and Wang, 2004; Nelson and Citovsky, 2005; Decroocq et al., 2006). In order to establish a successful infection, the invading virus must recruit an array of host proteins (host factors) to translate and replicate its genome and to move locally from cell to cell via the plasmodesmata and systemically via the vascular system. It has been suggested that down-regulation or mutation of some of the required host factors may result in recessively inherited resistance to viruses (Kang et al., 2005b).

Potyviruses, belonging to the genus Potyvirus in the family Potyviridae, constitute the largest group of plant viruses (Rajamäki et al., 2004). Potyviruses have a single positive-strand RNA genome approximately 10 kb in length, with a viral genome-linked protein (VPg) covalently attached to the 5′ end and a poly(A) tail at the 3′ end (Urcuiqui-Inchima et al., 2001; Rajamäki et al., 2004). The viral genome contains a single open reading frame (ORF) that translates into a polypeptide with a molecular mass of approximately 350 kD, which is cleaved into 10 mature proteins by viral proteases (Urcuiqui-Inchima et al., 2001). Recently, a novel viral protein resulting from a frameshift in the P3 cistron has been reported (Chung et al., 2008). Of the 11 viral proteins, VPg is a multifunctional protein and the only other viral protein present in the viral particles (virions) besides the coat protein and the cylindrical inclusion protein (CI; Orueixebraña et al., 2001; Puustinen et al., 2002; Gabrenaite-Verkhovskaya et al., 2008). The nonstructural protein is linked to the viral RNA by a phosphodiester bond

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* Corresponding author; e-mail aiming.wang@agr.gc.ca.

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between the 5′ terminal uridine residue of the RNA and the O²-hydroxyl group of amino acid Tyr (Murphy et al., 1996; Oruetebarria et al., 2001; Puustinen et al., 2002). Mutation of the Tyr residue that links VPg to the viral RNA abolishes virus infectivity completely (Murphy et al., 1996). In infected cells, VPg and its precursor Nla are present in the nucleus and in the membrane-associated virus replication vesicles in the cytoplasm (Carrington et al., 1993; Rajamäki and Valkonen, 2003; Cotton et al., 2009). As a component of the replication complex, VPg may serve as a primer for viral RNA replication (Puustinen and Mäkinen, 2004) and as an analog of the m⁷G cap of mRNAs for the viral genome to recruit the translation complex for translation (Michon et al., 2006; Beauchemin et al., 2007; Khan et al., 2008). Furthermore, VPg has been suggested to be an avirulence factor for recessive resistance genes in diverse plant species (Moury et al., 2004; Kang et al., 2005b; Bruun-Rasmussen et al., 2007). Thus, VPg plays a pivotal role in the virus infection process. The molecular identification of VPg-interacting host proteins and the subsequent functional characterization of such interactions may advance knowledge of the intricate virus replication mechanisms and help develop novel antiviral strategies.

Previous studies have shown that VPg and its precursor Nla interact with several host proteins, including three essential components of the host protein translation apparatus (Thivierge et al., 2008). The first protein is the cellular translation initiation factor elf4E or its isoform elf4E(iso)4E, identified through a yeast two-hybrid screen using VPg as a bait (Wittmann et al., 1997; Schaad et al., 2000). The protein complex of VPg and elf4E is an essential component for virus infectivity (Robaglia and Caranta, 2006). Mutations and knockout of elf4E or elf4E(iso)4E confer resistance to infection (Lellis et al., 2002; Ruffel et al., 2002; Nicaise et al., 2003; Gao et al., 2004; Kang et al., 2005a; Ruffel et al., 2005; Decroocq et al., 2006; Bruun-Rasmussen et al., 2007). It is well known that potyviruses recruit selectively one of the elf4E isoforms, depending on specific virus-host combinations (German-Retana et al., 2008). For instance, in Arabidopsis (Arabidopsis thaliana), elf4E(iso)4E is required for infection by Turnip mosaic virus (TuMV), Plum pox virus (PPV), and Lettuce mosaic virus, while elf4E is dispensable for infection by Clover yellow vein virus (Duprat et al., 2002; Lellis et al., 2002; Sato et al., 2005; Decroocq et al., 2006). The second cellular protein interacting with VPg is another translation initiation factor, elf4G. Analysis of Arabidopsis knockout mutants for elf4G or its isomers elf4E(iso)4G1 and elf4E(iso)4G2 has yielded results supporting the idea that the recruitment of elf4G for potyvirus infection is also isoform dependent (Nicaise et al., 2007). Recently, poly (A)-binding protein (PABP), the translation initiation factor that bridges the 5′ and 3′ termini of the mRNA into proximity, has been proposed to be essential for efficient multiplication of TuMV (Dufresne et al., 2008).

PABP was previously documented to interact with Nla, a VPg precursor containing both VPg and the proteinase Nla-Pro (Léonard et al., 2004). As the translation factors elf(iso)4E and PABP have been found to be internalized in virus-induced vesicles, it has been suggested that the interactions between VPg and these translation factors are crucial for viral RNA translation and/or replication (Beauchemin and Laliberté, 2007; Beauchemin et al., 2007; Cotton et al., 2009). Besides these three translation factors, a Cys-rich plant protein, potyvirus VPg-interaction protein, was also found to associate with VPg (Dunoyer et al., 2004). This plantspecific VPg-interacting host protein contains a PHD finger domain and acts as an ancillary factor to support potyvirus infection and movement (Dunoyer et al., 2004).

In this study, we describe the identification of an Arabidopsis DEAD-box RNA helicase (DDX), AtRH8, and a peach (Prunus persica) DDX-like protein, PpDDXL, both interacting with the potyviral VPg protein. Using the atrh8 mutant, we demonstrate that AtRH8 is not required for plant growth and development in Arabidopsis but is necessary for infection by two plant potyviruses, PPV and TuMV. Furthermore, we present evidence that AtRH8 colocalizes with the virus accumulation complex in potyvirus-infected leaf tissues, which reveals a possible role of AtRH8 in virus infection. Finally, we have identified the VPg-binding region (VPg-BR) of AtRH8 and PpDDX and show that overexpression of the VPg-BR either from AtRH8 or PpDDXL suppresses virus accumulation.

RESULTS

Identification of a VPg-Interacting DEAD Box RNA Helicase-Like Protein from Peach and Arabidopsis

To identify VPg-interacting host proteins in plants, a yeast two-hybrid CDNA library screen was carried out. The library was constructed from PPV-infected peach leaf tissues in order to search VPg-interacting host candidates in its natural host during virus infection. A total of $1.3 \times 10^9$ transformed cDNA clones were tested against the PPV VPg as bait. The resulting positive clones were rescued and isolated for sequencing. Based on the results of BLASTX searches (E value $\leq 1 \times 10^{-10}$), a total of 85 peach proteins were identified. Of these positive clones, five contained a stretch of the same cDNA sequence. The predicted peptide from the longest clone shares 96% to 98% sequence similarity to a number of proteins, including the ATP-dependent RNA helicase and elf4A, that belong to the DDX family. Thus, this gene is designated PpDDXL. Based on the multiple occurrences of PpDDXL in the screen, the fact that RNA helicase is part of the elf4E translation complex, and the assumption that host RNA helicases may be involved in viral genome replication, PpDDXL was chosen for further molecular and functional characterizations.

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The full-length cDNA of \textit{PpDDXL} was obtained using RACE PCR techniques and deposited into GenBank (accession no. GQ865547). The interactions between the partial or full-length \textit{PpDDXL} proteins and the PPV VPg were confirmed in yeast (Fig. 1). The full-length cDNA of \textit{PpDDXL} is 1,692 bp, with a 5’ untranslated region of 293 bp, an ORF of 1,242 bp, and a 3’ untranslated region of 157 bp (Supplemental Fig. S1). It encodes a polypeptide of 413 amino acids with a predicted molecular mass of 47 kD and a pI of 5.48. The corresponding genomic DNA sequence of \textit{PpDDXL} was obtained by PCR amplification of genomic DNA (GenBank accession no. GQ865548). Alignment of the cDNA and genomic sequence of \textit{PpDDXL} indicated that \textit{PpDDXL} contains three introns and four exons (Supplemental Fig. S1A). Domain analyses using the Pfam program (http://pfam.sanger.ac.uk/) identified the DEAD/DEAH box (amino acids 64–230) signature and helicase-conserved C-terminal (amino acids 298–374) domains (Supplemental Fig. S1, B and C).

Due to the unavailability of an efficient genetic transformation protocol for the characterization of gene functions in \textit{Prunus} species, Arabidopsis was selected as a model host for exploring the roles of \textit{PpDDXL} and related RNA helicases in potyvirus infections. BLAST searches against the Arabidopsis database revealed 10 Arabidopsis DDXs that shared high sequence similarity to \textit{PpDDXL} (Supplemental Fig. S2A). Although the three Arabidopsis \textit{elf4As} and two putative \textit{elf4As} (AT3G13920, AT1G54270, AT1G2730, AT3G1960, and AT1G51380, respectively) are most similar to \textit{PpDDXL}, there were no corresponding homoygous knockout T-DNA lines available. Extensive screening of progeny plants from the eight heterozygous \textit{elf4A} T-DNA lines (Supplemental Fig. S1, B and C).

To study if \textit{AtRH8} and VPg colocalize in planta, transient expression vectors encoding \textit{AtRH8}-cyano fluorescent protein (CFP) fusion (\textit{AtRH8}-CFP) and VPg-yellow fluorescent protein (YFP) fusion (VPg-YFP) were constructed. Transient expression of these chimeric genes was achieved through agroinfiltration. As a control, \textit{AtRH8} was expressed alone (Fig. 2A) or coexpressed with YFP (Fig. 2B). The distribution of \textit{AtRH8} was found in the cytoplasm (Fig. 2A, A and B), whereas YFP was in the cytoplasm and in the nucleus (due to diffusion; Fig. 2B). In addition, \textit{AtRH8} also formed punctate structures in the cytoplasm (Fig. 2A, A and B). In \textit{Nicotiana benthamiana} epidermal cells coexpressing \textit{AtRH8}-CFP and VPg-YFP, the two proteins colocalized in the nucleus and in the cytoplasm (Fig. 2C). Previously, VPg-YFP was reported to localize mainly in the nucleus when expressed alone (Wei and Wang, 2008). Thus, the VPg-YFP interfered in the distribution of \textit{AtRH8}-CFP. To further investigate the interaction between VPg and \textit{AtRH8} in planta, a bimolecular fluorescence complementation (BiFC) assay was carried out. Several BiFC negative control combinations were set up to ensure the validity of the BiFC results. These combinations included the N-terminal (YN) and C-terminal (YC) fragments of YFP, \textit{AtRH8}-YN and YC, YN and \textit{AtRH8}-YC, VPg-YN and YC, or coexpressed with YFP (Fig. 2B). The distribution of \textit{AtRH8} was found in the cytoplasm (Fig. 2A, A and B), whereas YFP was in the cytoplasm and in the nucleus (due to diffusion; Fig. 2B). Taken together, these data demonstrate a physical interaction between \textit{AtRH8} and VPg.

**Requirement of \textit{AtRH8} for Potyvirual Infection**

To investigate the functional role of \textit{AtRH8} in virus infection, a homozygous T-DNA line of \textit{AtRH8}, SALK\_016830, with a T-DNA insertion in the promoter region was acquired from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). The T-DNA PCR screen on genomic DNA as well as genetic analysis confirmed that there is only one T-DNA insertion (data not shown). RT-PCR analysis of total RNA isolated from leaf tissues of this mutant line and the wild type revealed no expression of \textit{AtRH8} in the homozygous T-DNA line (Supplemental Fig. S4). Thus, this line (\textit{atrh8}) is a true knockout mutant of \textit{AtRH8}.  

![Figure 1. Yeast two-hybrid assay of protein-protein interaction between the PPV VPg and \textit{PpDDXL} from peach or \textit{AtRH8} from Arabidopsis. Yeast cotransformants were grown on the selective medium SD/–Ade/–His/–Leu/–Trp plus X-a-Gal and incubated at 28°C for 4 d. [See online article for color version of this figure.]](image)
To test if AtRH8 is required for PPV infection, the atrh8 mutant and wild-type plants were mechanically inoculated with a Canadian PPV-D isolate. Total RNA was extracted from the upper newly emerged leaves 14 d post infection (dpi). RT-PCR assays were used to monitor the accumulation of the viral RNA. The PPV genomic RNA was detected only in the wild type (Fig. 3A). Mild disease symptoms such as slight growth retardation were found in the infected wild-type plants, consistent with our previous observation (Babu et al., 2008). In contrast, the atrh8 mutant plants inoculated with PPV did not show any disease symptoms, and no PPV was detectable in these inoculated mutant plants (Fig. 3A). Taken together, these data suggest that atrh8 mutants are resistant to PPV.

To test if AtRH8 is also needed by another potyvirus during infection, the wild-type and atrh8 plants were agroinfiltrated with a GFP-tagged TuMV infectious clone (TuMV-GFP). Diagnosis of these plants 14 d post agroinfiltration (dpa) with RT-PCR revealed the presence of the TuMV genomic RNA in the wild-type plants but not in the atrh8 mutants (Fig. 3B). Consistent with our PCR results, a strong emission of GFP fluorescence was observed in the newly emerged leaves of the infiltrated wild-type plants but not the atrh8 plants (Fig. 3C). Phenotypes of the wild-type plants and atrh8 mutants agroinfiltrated with TuMV-GFP or mock infiltrated were closely examined. Under the normal growth conditions without TuMV infiltration, atrh8 mutants showed no developmental differences from wild-type plants (see mock-inoculated wild type and atrh8; Fig. 3, D and E) and displayed normal vegetative growth, flowering development, and seed production. At 3 dpa, the wild-type plants agroinfiltrated with TuMV-GFP began exhibiting yellowing on the surface of the leaves and slight growth stunting. In contrast, no difference was observed between TuMV-infiltrated atrh8 mutants and mock-infiltrated wild-type or atrh8 plants (Fig. 3D). At the later infection stage (14 dpa), the infected wild-type plant displayed the full spectrum of disease symptoms, including mosaic and necrosis on leaves, severe growth retardation, reduced apical dominance, curled bolts, and the typical inflorescence stunting (Fig. 3E), similar to previous descriptions (Lellis et al., 2002). The TuMV-infiltrated atrh8 mutants, however, showed normal growth and fertility with no signs of infection symptoms. When the TuMV-infected plants were exposed under UV light at 19 dpa, the stunted wild-type plants exhibited bright green fluorescent emission from the tagged GFP, but no GFP was exhibited in TuMV-infiltrated atrh8 plants (Fig. 3F). Taken together, these results indicate that AtRH8 is required for both PPV and TuMV infections.

Colocalization of AtRH8 with Virus-Induced Replication-Associated Membranous Vesicles in Planta

To explore a possible role of AtRH8 in virus infection, the subcellular localization of AtRH8 was examined in planta in the presence of virus infection. Potyviral 6K2 protein is an integral membrane protein that induces the formation of the endoplasmic reticulum-derived vesicles (Schaad et al., 1997). Recently, we have found that these 6K2 vesicles originate at endoplasmic reticulum exit sites and target chloroplasts for virus replication (Fig. 4A; Wei and Wang, 2008; T. Wei and A. Wang, unpublished data). These 6K2 vesicles contain viral replication-associated proteins such as NiA and 6K2-NiA (two VPg precursors), NiB (viral RNA-dependent RNA polymerase), viral RNA (carrying VPg), and host factors such as elf(iso)4E, PABP2,
and eEF1A (Cotton et al., 2009). All of these components are essential for viral genome translation/replication. To visualize the subcellular localization of AtRH8 in virus-infected leaves, the AtRH8-CFP was transiently expressed in N. benthamiana infected with a TuMV infectious clone carrying an additional 6K2 tagged with YFP at the junction of P1 and HC-Pro (TuMV::6K-YFP). In contrast to the distribution of AtRH8 in the cytoplasm when expressed alone or coexpressed with a control protein (Fig. 2, A and B), AtRH8 was strongly localized with chloroplast-associated 6K2 vesicles during TuMV infection (Fig. 4B).

**Determination of VPg-BR in AtRH8 and PpDDXL**

To determine the VPg-BR of AtRH8, a series of deletions were conducted on AtRH8. Initially, the protein was truncated into two moieties, with the N-terminal portion containing 250 amino acids and the C-terminal portion containing 257 amino acids (Fig. 5, A and B). The truncated protein was fused into the pAD-GAL plasmid of the yeast two-hybrid system. The interaction assay was conducted using the PPV VPg as the interaction partner cloned into the pBD-GAL plasmid. Growth of the yeast transformants on selective medium showed that VPg positively interacted exclusively with the N-terminal fragment of AtRH8 and not the C-terminal portion, suggesting that the interaction site resides within the first 250 amino acids of AtRH8. The N-terminal 250 amino acids were subjected to additional sequential deletions (Fig. 5, A and B). Based on the deletion analyses, the VPg-BR of AtRH8 consists of 50 amino acids (amino acids 201–250; Fig. 5, A and B). Protein sequence comparison of the VPg-BR (amino acids 140–189) of PpDDXL and that of AtRH8 indicated a 74% similarity (Fig. 5C). The protein predictor SSpro version 4.5 program from the ExPasy Proteomics Server (http://www.expasy.ch) identified an α-helix in this region in both PpDDXL and AtRH8.

In order to verify the interaction between the PPV VPg and the VPg-BR in planta, BiFC assays between the PPV VPg and the VPg-BR of AtRH8 or that of PpDDXL were conducted in N. benthamiana plants. Three-week-old plants were agroinfiltrated to coexpress the BR-YN (the VPg-BR of AtRH8 or PpDDXL attached to the N-terminal fragment of YFP) and the VPg-YC (the PPV VPg fused to the C-terminal fragment of YFP) as well as the reverse combinations. Infiltrated leaf tissues were observed by confocal microscopy 2 dpa. A positive interaction was observed between the VPg-BR of AtRH8 and the PPV VPg mainly in the nucleus (Fig. 5D). The interaction of the VPg-BR of PpDDXL and the PPV VPg was found in the nucleus and in the cytoplasm (Fig. 5E). These data illustrate that the VPg-BR of AtRH8 and PpDDXL is

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**Figure 3.** Requirement of AtRH8 for potyvirus infection in Arabidopsis plants. A and B, Results from RT-PCR analysis of PPV and TuMV accumulation, respectively. Wild-type (WT) plants and ath8 mutants were mechanically inoculated and agroinfiltrated with PPV and TuMV, respectively. Total RNA extracted from upper newly emerged leaves 2 weeks post inoculation was used for cDNA synthesis. The cDNA was amplified using PPV Coat Protein (CP)-specific primers and TuMV HC-Pro-specific primers for the corresponding assay. Actin2 was selected as the endogenous reference gene to serve as an internal control of RT-PCR. C, Confocal imaging of newly emerged leaves of TuMV-infected wild-type and ath8 mutant plants. Three-week-old Arabidopsis plants were agroinfiltrated with a GFP-tagged TuMV infectious clone and observed 10 d post infiltration. Chl, Chloroplast autofluorescence. Bars = 48 μm. D and E, Phenotypes of wild-type and ath8 mutant plants 3 and 14 d post infiltration, respectively. Mock, Infiltrated with an empty vector; TuMV, infiltrated with the GFP-tagged TuMV infectious clone. F,
Suppression of Virus Infection by Transient Overexpression of the VPg-BR of AtRH8 and That of PpDDXL

As described above, potyvirus infection requires the presence of AtRH8. To determine if overexpression of AtRH8 affects potyvirus infection, N. benthamiana leaves were coinfiltred with the TuMV-GFP clone and an empty vector (as a control) or with TuMV-GFP and a plant AtRH8 expression vector. TuMV infection was assessed by real-time PCR analyses 2 dpa and visualized by confocal observation 3 dpa. In comparison with the control leaves (coinfiltrated with TuMV-GFP and an empty vector), leaves expressing AtRH8 and infected by TuMV-GFP displayed a much stronger green fluorescence intensity (Fig. 6A). Quantification of TuMV using real-time PCR revealed a 1.6-fold increase in virus accumulation in the leaves overexpressing AtRH8 (Fig. 6B).

To assess the effect of transient overexpression of the VPg-BR of AtRH8 on potyvirus infection, the TuMV-GFP infectious clone was coinfiltred into N. benthamiana with an expression plasmid containing the VPg-BR of AtRH8 and an empty vector. TuMV infection was assessed by real-time PCR analyses 2 dpa and visualized by confocal observation 3 dpa. In comparison with the control leaves (coinfiltrated with TuMV-GFP and an empty vector), leaves expressing AtRH8 and infected by TuMV-GFP displayed a much stronger green fluorescence intensity (Fig. 6A). Quantification of TuMV using real-time PCR revealed a 1.6-fold increase in virus accumulation in the leaves overexpressing AtRH8 (Fig. 6B).

DISCUSSION

In this study, we have reported the identification of two VPg-interacting plant DDX proteins, AtRH8 from Arabidopsis and PpDDXL from peach (Figs. 1 and 2). These DDX proteins share sequence homology with elf4A, a component of the elf4F multiprotein complex. We used the Arabidopsis AtRH8 homozygous T-DNA insertion lines to functionally characterize the requirement of AtRH8 in potyvirus infection. We found that AtRH8 knockout plants (atr8 mutants) grew and developed as the wild-type plants, indicating that AtRH8 is dispensable for the normal plant
growth and development (Fig. 3). But these mutants were unable to support PPV and TuMV infections, suggesting that AtRH8 is required for virus infections (Fig. 3). Therefore, AtRH8 is a host factor that plays an essential role in the virus infection cycle. To our knowledge, this report is the first showing that a plant DDX protein is required for virus infection in plants.

RNA helicases represent a large family of proteins implicated in almost every step of RNA metabolism (de la Cruz et al., 1999; Tanner and Linder, 2001; Figure 5.

**Figure 5.** Identification of VPg-BR in AtRH8 and PpDDXL. A, Protein-protein interaction between the truncated AtRH8 and the PPV VPg in the yeast two-hybrid assay. Yeast cells were cotransformed with the VPg bait vector and prey vectors (AtRH8 deletions represented by diagram of bars in B), plated on the highly stringent selective medium SD/−Ade/−His/−Leu/−Trp plus X-α-Gal, and incubated at 28°C for 4 d. B, Summary of results from A. Clone names and schematic representation of various forms of the truncated AtRH8 are shown (corresponding amino acid positions are indicated). +, Positive interaction; −, negative interaction. C, Protein sequence alignment of the VPg-BR of AtRH8 and that of PpDDXL. The VPg-BR sequences were aligned using the ClustalW program. Amino acids within the binding region are colored in red and compared for sequence similarity. Asterisks indicate identical amino acids; colons indicate strongly related amino acids (belonging to the same group such as polar, nonpolar, basic, and acidic and having side chains sharing a similar chemical structure); and dots indicate weakly related amino acids (belonging to the same group such as polar, nonpolar, basic, and acidic and having side chains with different chemical structures). D, BiFC analysis of the interaction between the VPg-BR of AtRH8 and the PPV VPg 2 d post coinfiltration. Confocal microscopy imaging on the coexpression of AtRH8 VPg-BR fused with the N-terminal fragment of YFP (AtRH8 BR-YN) and the PPV VPg fused with the C-terminal fragment of YFP (VPg-YC) in 3-week-old N. benthamiana lower leaf epidermal cells is shown. Bars = 17 μm. E, BiFC analysis of the interaction between the VPg-BR of PpDDXL and the PPV VPg 2 d post coinfiltration. The VPg-BR was fused with the N-terminal fragment of YFP (PpDDXL BR-YN) and coexpressed with the PPV VPg fused with the C-terminal fragment of YFP (VPg-YC). Bars = 19 μm. Chl, Chloroplast autofluorescence.
Lorsch, 2002; Mohr et al., 2002). During the virus infection process, RNA helicases have been suggested to be involved in (1) translation of the viral RNA, (2) selection of the RNA template for translation or replication, (3) recruitment of the viral RNA for replication, (4) RNA synthesis, and/or (5) RNA stability (Li et al., 2009). Previously, it has been shown that in yeast cells, DED1, an eIF4A-like RNA helicase, is essential for the translation of Brome mosaic virus (BMV; bromovirus; Noueiry et al., 2000). A point mutation in DED1 does not affect yeast normal growth but results in the inhibition of BMV replication through selectively blocking the translation of the BMV RNA2 that encodes the viral RNA-dependent RNA polymerase 2a (Noueiry et al., 2000). A point mutation in DED1 does not affect yeast normal growth but results in the inhibition of BMV replication through selectively blocking the translation of the BMV RNA2 that encodes the viral RNA-dependent RNA polymerase 2a (Noueiry et al., 2000). By a yet unknown mechanism, this mutant also inhibits the replication of Tomato bushy stunt virus (TBSV), a tombusvirus (Jiang et al., 2006). In a recent yeast protein array using protein-RNA interactions, several other RNA helicases bound to BMV and TBSV genomic RNAs have been documented (Li et al., 2009). These results suggest that besides DED1, other RNA helicases may also participate in regulating the translation and/or replication of viral RNAs. In animal cells, it has been reported that Human immunodeficiency virus type 1 and Hepatitis C virus both recruit DEAD-box RNA helicase 3 (DDX3) for viral genome replication (Fang et al., 2004, 2005; Ariumi et al., 2007). It is assumed that DDX3 promotes the translocation of viral RNA through the nuclear pore complex by remodeling the virus replication complex (Yedavalli et al., 2004). Interestingly, DDX3 has also been shown to chaperone a type of mRNA granules for translation in the developing brain of rat embryos (Elvira et al., 2006). In this study, AtRH8 colocalized with the TuMV accumulation complex in virus-infected cells (Fig. 4). The potyvirus replication complex has been shown to contain viral replicase components (such as Nla, 6K2-Nla, and Nlb), viral genomic RNA (carrying Nla

Figure 6. Effect of transient overexpression of AtRH8 and its VPg-BR on TuMV accumulation in N. benthamiana plants. A, Ectopic expression of AtRH8 enhances TuMV infection in N. benthamiana. Confocal images of 3-week-old N. benthamiana leaves agroinfiltrated with different combinations of plasmids are shown. The photographs represent the infection pattern in N. benthamiana at 3 d post infiltration. Chl, Chloroplast autofluorescence; GFP, green fluorescence emission from a GFP-tagged TuMV infectious clone; TuMV, coinfiltration of a GFP-tagged TuMV clone with an empty vector; TuMV + AtRH8, coinfiltration of the TuMV-GFP clone and the AtRH8-expressing clone; TuMV + BR, coinfiltration of the TuMV-GFP clone and the AtRH8-BR-expressing clone. Bars = 300 μm. B, Real-time PCR quantification of TuMV accumulation in N. benthamiana at 2 d post infiltration. Three independent experiments were carried out for quantification analyses. In each experiment, three plants were used per treatment. The values represent means of fold change relative to the control (TuMV alone). The asterisks indicate that TuMV accumulation in N. benthamiana expressing AtRH8 (TuMV + AtRH8) or the AtRH8-BR (TuMV + BR) was significantly higher or lower than that in the control (TuMV; P < 0.01). Error bars represent SD.
or VPg), double-stranded RNA, and host translational proteins [such as eIF(iso)4E, PABP2, and eEF1A; Cotton et al., 2009]. It is possible that AtRH8 and PpDDXL both play a role in viral genome translation, as suggested for yeast DED1 and for human DDX3.

The presence of AtRH8 in the virus accumulation complex but not in the nucleus in infected cells (Fig. 4) is consistent with the recent finding that eIF(iso)4E, also a VPg-interacting translation initiation factor, is localized to the TuMV replication complex (Cotton et al., 2009). In infected cells, Na a is the major form of VPg, which, as a viral RNA genome-linked protein, is present in the cytoplasm or, as a nuclear localization signal-containing protein, is translocated into the nucleus (Restrepo-Hartwig and Carrington, 1992; Carrington et al., 1993; Rajamäki and Valkonen, 2003, 2009). It is puzzling that AtRH8 and eIF(iso)4E were mainly found in the cytoplasm but not in the nucleus. One possible explanation is that in the early infection stage, Na a or VPg is mainly intercepted by the virus replication complex for replication, with only a small amount of Na a or VPg transported to the nucleus. Indeed, large amounts of viral RNA (carrying Na a or VPg) are concentrated in the virus replication complex in the early infection stage (a few days after infection; Cotton et al., 2009; T. Wei and A. Wang, unpublished data), whereas high-level accumulation of Na a or VPg in the nucleus has only been shown in the later infection stage (i.e. 20 dpi; Rajamäki and Valkonen, 2003, 2009). Nuclear transport of Na a may be regulated by differential cleavage efficiency (Restrepo-Hartwig and Carrington, 1992). For instance, cleavage at the N terminus of 6K2 in the potyviral polyprotein occurs preferentially to the N terminus of Na a, leading to the transient accumulation of the 6K2-Na a precursor protein (Restrepo-Hartwig and Carrington, 1992). The cytoplasmic 6K2-Na a impedes nuclear localization of Na a (Restrepo-Hartwig and Carrington, 1992) and colocalizes with the TuMV replication complex (Cotton et al., 2009). In addition to different VPg precursors, several posttranslationally modified forms of VPg and Na a have also been found in infected plants (Hafren and Mäkinen, 2008). These modified forms of VPg and Na a may also be intracelullarly differentially distributed. It is possible that different VPg precursors or their modified forms have different binding abilities to AtRH8. Further determination of their subcellular localization over the infection course and analysis of their ability to interact with AtRH8 may help understand the mechanism underlying the recruitment of AtRH8 to the virus accumulation complex.

As discussed above, both PpDDXL and AtRH8 appear to be RNA helicases by sequence comparison (Supplemental Fig. S1). Interestingly, the potyviral CI also contains an RNA-binding domain and has ATPase and RNA helicase activities (Lain et al., 1990, 1991; Eagles et al., 1994). A genetic study on the CI using a TEV infectious clone revealed that CI plays essential dual roles in TEV replication and cell-to-cell movement (Carrington et al., 1998). In agreement with this finding, the potyviral CI has been shown to associate with the TuMV replication complex that contains host factors such as eIF(iso)4E (Cotton et al., 2009) and the pea seed-borne mosaic virus CI form plasmodesmata-associated cone-like structures that mediate the passage of virus into the adjacent cell (Roberts et al., 1998). In virus-infected plants, the potato virus A CI also binds to a subpopulation of viral particles through an interaction with the viral coat protein (Gabrenaite-Verkhovskaya et al., 2008). The importance of CI as a helicase is also implicated in several other studies as well (Jiménez et al., 2006; Abdul-Razzak et al., 2009; Shand et al., 2009). For instance, mutations in the C-terminal portion of the lettuce mosaic virus CI result in overcoming recessive resistance mediated by two eIF4E alleles (Abdul-Razzak et al., 2009), indicating the involvement of CI in the potyvirus-eIF4E interactome. This resistance breakage may be made by restoring the CI-interacting (directly or indirectly) protein network that includes the essential components of the eIF4F complex (Abdul-Razzak et al., 2009). Therefore, the potyviral CI may be directly involved in RNA synthesis through interacting with the nascent genome to regulate translocation and disassembly of the virion (Gabrenaite-Verkhovskaya et al., 2008). Since CI and AtRH8 both are physically present in close proximity to the virus accumulation complex and are functionally reciprocally irreplaceable in virus infection, they may coordinate to provide helicase activities required by different aspects of viral genome expression and replication.

The result in this study showing that atrh8 mutants were resistant to both PPV and TuMV (Fig. 3) is in concordance with the properties of recessive resistance. Recently, Kang et al. (2007) have reported that constitutive overexpression of a pepper (Capsicum annuum) recessive resistance gene, per1 (an eIF4E mutant), in tomato (Solanum lycopersicum) generates dominant resistance to TEV and other potyviruses, including Pepper mottle virus and Potato virus Y. In this report, overexpression of the VPg-BR of AtRH8 or that of PpDDXL significantly suppressed virus infection (Fig. 6; Supplemental Figs. S5 and S6). This resistance, resembling a dominant negative effect, is likely due to the interaction between VPg and the overexpressed VPg-BR that impairs the recruitment efficiency of functional AtRH8 or PpDDXL to the virus accumulation vesicles. This finding may open up a novel strategy in the development of genetic resistance against viruses in plants and other organisms. Both recessive and dominant negative approaches are superior to the pathogen-derived resistance strategy currently being widely used to engineer resistance to plant viruses. This strategy functions through RNA silencing directed targeting on the viral genome (Grumet et al., 1987; Baulcombe, 2004; Wang and Metzlafl, 2005; Wang et al., 2006). This type of viral resistance can be overcome in two scenarios: in mixed infections by a strong silencing suppressor from unrelated viruses (Mitter et al., 2001) and through random sequence
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mutation during virus replication that lacks a proof-reading mechanism (Kang et al., 2005b). Thus, our study also provides an immediate interest for agricultural studies and could potentially serve as a feasible solution to viral diseases in crops. However, the broader application of AtRH8 or PpDDXL for viral control will certainly depend on further elucidating the exact role of these DDX-like proteins in the translation, replication, and regulation of the virus infection cycle.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was conducted using the Matchmaker Library Construction and Screening Kits (Clontech) following the supplier’s instruction manual. The VPg of a PPV-D strain was cloned into the bait vector, pGBKTT, encoding the binding domain to generate plasmid pGBKTT-VPg. The peach (Prunus persica) cDNA library was prepared by inserting cDNA derived from PPV-infected peach leaf tissues into the prey vector pGADT7-rec, encoding the activation domain. Positive clones were isolated and transformed into the Escherichia coli DH5α strain for plasmid preparation and DNA sequencing.

5′ RACE and 3′ RACE for PpDDXL Gene Cloning

To obtain the 5′ terminus of the PpDDXL gene, 5′ RACE was performed using the FirstChoice RLM-RACE kit (Ambion) following the manufacturer’s instructions. The 5′ RACE PpDDXL outer primer, 5′ RACE PpDDXL inner primer, 3′ RACE PpDDXL outer primer, and 3′ RACE PpDDXL inner primer (listed in Supplemental Table S1) were used to obtain the full-length PpDDXL cDNA. All PCRs were performed using the Phusion High-Fidelity DNA polymerase (New England Biolabs) at an annealing temperature of 60°C for 30 cycles. The PCR product was cloned with the Zero Blunt TOPO Cloning Kit (Invitrogen) and sequenced. Multiple sequence alignment to homolog proteins in different plant species was done using SeqMan from DNASTar version 6 and ClustalW alignment programs.

T-DNA Mutant Analysis

All the T-DNA insertion lines used in this study were obtained from the Arabidopsis Biological Resource Center. The T-DNA insertion site of the atrh8 mutant was confirmed by PCR using the T-DNA left border-specific primer (AtRH8-L) and AtRH8-specific primers (LP1,LP2). The position of the T-DNA insertion in the AtRH8 gene mutant allele was verified by DNA sequencing of the PCR products. The single T-DNA insertion was confirmed by DNA gel-blot analyses. The expression of AtRH8 was verified by RT-PCR and sequencing. The insert was subsequently cloned into the destination vector using LR Clonase II (Invitrogen) to generate plant expression vectors AtRH8-CFP, AtRH8-YN, AtRH8-YC, VPg-YFP, VPg-YN, VPg-YC, VPgBR-AtRH8-YN, VPgBR-AtRH8-YC, VPgBR-PpDDXL-YN, and VPgBR-PpDDXL-YC.

Agroinfiltration and Confocal Microscopy

The binary vectors were introduced into Agrobacterium tumefaciens strain GV3101 or EHA105 by electroporation. The Agrobacterium culture was prepared according to Sparkes et al. (2006). For the TuMV infection assay, 3-week-old Arabidopsis plants were agroinfiltrated with Agrobacterium containing the GFP-tagged TuMV plasmid as an optical density at 500 nm of 0.05. For BiFC and colocalization experiments, the 3-week-old Nicotiana benthamiana plants were agroinfiltrated with the mixture of Agrobacterium cultures (each at an optical density at 600 nm of 1.0) in 1:1 ratio. Confocal microscopy work was carried out following Wei and Wang (2008).

Virus Inoculation

Mechanical inoculation of Arabidopsis with PPV-D and TuMV-GFP was as described (Babu et al., 2008). For TEV inoculation, 3-week-old N. benthamiana plants were rubbed with the TEV inoculum prepared by grinding 1 g of TEV-infected N. benthamiana leaves with 5 mL of 1× phosphate-buffered saline buffer (pH 7.4).

RT-PCR and Real-Time RT-PCR

All RT-PCR and real-time PCR analyses were performed with three biological replicates. Total RNA was prepared following the instructions of the RNeasy Plant Mini Kit (Qiagen). The first-strand synthesis and subsequent PCR amplification of both the internal standard and target gene fragments were performed using the SuperScript two-step RT-PCR system (Invitrogen). For AtRH8 expression analysis, the Arabidopsis Actin2 gene was used as the internal control of RT-PCR using Actin2-specific primers At-actin-F and At-actin-R. For virus detection, newly emerged leaves of virus-inoculated plants were harvested at 14 dpi or 14 dpa. The infection of PPV and TuMV was diagnosed by RT-PCR with PPV-specific primers PPVcp-R and PPVcp-F and TuMV-specific primers HC-Pro-F and HC-Pro-R, respectively.

Real-time PCR preparations were carried out using the LightCycler480 Probes Master Kit (Roche) on a LightCycler480 real-time PCR system (Roche) following the manufacturer’s instructions. Three pairs of primers, TEVcp-F and TEVcp-R, TuHC-F and TuHC-R, and NbEF1a-F and NbEF1a-R, were used for quantification analyses of TEV, TuMV, and N. benthamiana elongation factor-1a (NbEF-1a), respectively. NbEF-1a served as the internal reference control. The hydrolysis probe designs were based on TaqMan Probe design tutorial guidelines by Beacon Designer and AlleleID. The corresponding hydrolysis probes to TEV, TuMV, and NbEF1a were listed in Supplemental Table S1. The standard curve of each sample was generated to achieve an efficiency of 2.0 prior to the relative quantification analysis. Each sample was assayed in triplicate 20-μL volumes, and data were analyzed using the LightCycler480 software SW1.5 (Roche). The RNA level was calculated using the mean threshold cycle value normalized to that of the reference gene, NbEF-1a.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GQ865547 and GQ865548.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of PpDDXL.

Supplemental Figure S2. Analysis of PpDDXL homologous genes in Arabidopsis.

Supplemental Figure S3. Negative controls for the BiFC assay of A1Ri8 and the PPV VPg in N. benthamiana.

Supplemental Figure S4. Expression analysis of the Arabidopsis A1Ri8 homologous T-DNA insertion line (SALK_016830).

Supplemental Figure S5. Effect of transient overexpression of the VPg:BR of A1Ri8 on TEV accumulation in N. benthamiana plants.

Supplemental Figure S6. Effect of transient overexpression of the VPg:BR of PpDDXL on TEV and TuMV accumulation in N. benthamiana plants.

Supplemental Table S1. Primers used in this study.

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