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An RNA helicase-like protein is required for potyvirus infection

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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 Infection

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Footnotes:

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ABSTRACT

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 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 Prunus persica and 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 both AtRH8 and PpDDXL may be manipulated for the development of genetic resistance against potyvirus infections.
INTRODUCTION

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 downregulation 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 kilobases (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 (Urcuqui-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 kDa, which is cleaved into 10 mature proteins by viral proteases (Urcuqui-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 (CP) and the cylindrical inclusion protein (CI) (Oruetxebarria et al., 2001; Puustinen et al., 2002; Gabrenaite-Verkhovskaya et al., 2008). The non-structural protein is linked to the viral RNA by a phophodiester bond between the 5’ terminal uridine residue of the RNA and the O1-hydroxyl group of amino acid tyrosine (Murphy et al., 1996; Oruetxebarria et al., 2001; Puustinen et al., 2002). Mutation of the tyrosine residue that links VPg to the viral RNA abolishes virus infectivity completely (Murphy et al., 1996). In infected cells, VPg and its precursor NIa 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 m$^7$G 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 subsequently functional characterization of such interactions may advance knowledge in the intricate virus replication mechanisms and help develop novel antiviral strategies.

Previous studies have shown that VPg and its precursor NIa 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 eIF4E or its isoform eIF(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 eIF4E is an essential component for virus infectivity (Robaglia and Caranta, 2006). Mutations and knockout of eIF4E or eIF(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 eIF4E isoforms, depending on specific virus-host combinations (German-Retana et al., 2008). For instance, in Arabidopsis thaliana, eIF(iso)4E is required for infection by Turnip mosaic virus (TuMV), Plum pox virus (PPV), and Lettuce mosaic virus (LMV), while eIF4E is indispensable for infection by Clover yellow vein virus (CIYVV) (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, eIF4G. Analysis of A. thaliana knockout mutants for eIF4G or its isomers eIF(iso)4G1 and eIF(iso)4G2 has yielded results supporting the idea that the recruitment of eIF4G 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 NIa, a VPg precursor containing both VPg and
the proteinase NLa-Pro (Léonard et al., 2004). As the translation factors eIF(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 cysteine-rich plant protein,
potyvirus Vpg-interaction protein (PVIP), was also found to associate with Vpg
(Dunoyer et al., 2004). This plant-specific 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 A. thaliana DEAD-box RNA
helicase (DDX), AtRH8, and a 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 A. thaliana 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 P.
persica and A. thaliana

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 P. persica
leaf tissues in order to search Vpg-interacting host candidates in its natural host during
virus infection. A total of 1.3 x 10^6 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 ≤1x10^-10), a total of 85 P. persica
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% of
sequence similarity to a number of proteins including the ATP-dependent RNA helicase
and eIF4A that belong to the DDX family. Thus, this gene is designated *PpDDXL*
(*Prunus persica DDX-like*). Based on the multiple occurrences of *PpDDXL* in the screen,
the fact that RNA helicase is part of the eIF4F translation complex, and the assumption
that host RNA helicases may be involved in viral genome replication, *PpDDEL* was
chosen for further molecular and functional characterizations.

The full-length cDNA of *PpDDXL* was obtained using RACE PCR techniques
and deposited into GenBank (accession number GQ865547). The interactions between
the partial or full-length *PpDDXL* proteins with the PPV VPg were confirmed in yeast
(Fig. 1). The full-length cDNA of *PpDDXL* is 1692 bp with a 5´-UTR of 293 bp, an ORF
of 1242 bp, and a 3´-UTR of 157 bp (Supplemental Fig. S1). It encodes a polypeptide of
413 aa with a predicted molecular mass of 47 kDa and a pI of 5.48. The corresponding
 genomic DNA sequence of *PpDDXL* was obtained by PCR amplification of genomic
 DNA (GenBank accession number GQ865548). Alignment of the cDNA and genomic
 sequence of *PpDDXL* indicated *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 (aa 64 to 230) signature and
helicase conserved C-terminal (aa 298 to 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 *Prunus* sp., *A. thaliana* was selected as a model host
for exploring the roles of *PpDDXL* and related RNA helicase in potyvirus infections.
BLAST searches against the *A. thaliana* database revealed 10 *A. thaliana DDXs* that
shared high sequence similarity to *PpDDXL* (Supplemental Fig. S2A). Although the three
*A. thaliana eIF4As* and two putative *eIF4As* (AT3G13920, AT1G54270, AT1G72730,
AT3G1960 and AT1G51380, respectively) are most similar to *PpDDXL*, there were no
corresponding homozygous knockout T-DNA lines available. Extensive screening of
progeny plants from the eight heterozygous (HZ) *eIF4A* T-DNA lines (SALK_038072,
SALK_072655, SALK_107633, SALK_123728, SALK_135778, SALK_107633,
SAIL_755_B08, WiscDsLox254D02) failed to recover any homozygous plants. These
data suggest a possible detrimental effect to the plant when silencing these *eIF4As.*
Indeed, even the heterozygous T-DNA seedlings showed abnormal phenotypes in the
number and length of root hair (Supplemental Fig. 2B). Thus, AtRH8 (AT4G00660), the
next most related candidate to PpDDX1L, was selected for functional characterization. The
ORF of AtRH8 was obtained from A. thaliana wild-type Col-0 cDNA using RT-PCR
with gene specific primers. The ORF of AtRH8 consists of 1518 nucleotides (nt)
encoding a 505 aa protein. A yeast two-hybrid assay confirmed a positive interaction
between the PPV VPg and AtRH8 (Fig. 1).

To study if AtRH8 and VPg colocalize in planta, transient expression vectors
encoding AtRH8-cyan fluorescent protein (CFP) fusion (AtRH8-CFP) and VPg-yellow
fluorescent protein (YFP) fusion (VPg-YFP) were constructed. Transient expression of
these chimeric genes was achieved through agro-infiltration. As a control, AtRH8 was
expressed alone (Fig. 2A) or coexpressed with YFP (Fig. 2B). The distribution of AtRH8
was found in the cytoplasm (Fig. 2 A and B), whereas YFP was in the cytoplasm and in
the nucleus (due to diffusion) (Fig. 2B). In addition, AtRH8 also formed some punctate
structures in the cytoplasm (Fig. 2 A and B). In N. benthamiana epidermal cells
coexpressing 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 AtRH8-CFP. To further investigate the interaction between VPg and
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, AtRH8-YN and YC, YN and AtRH8-YC, VPg-YN and YC, and YN
and VPg-YC (Supplemental Fig. S3). When AtRH8-YN was coexpressed with VPg-YC
in N. benthamiana plants, a strong emission of YFP fluorescence was observed in the
cytoplasm and in the nucleus as early as two days post agro-infiltration (Fig. 2D). Taken
together, these data demonstrate a physical interaction between AtRH8 and VPg.

Requirement of AtRH8 for potyviral infection

To investigate the functional role of AtRH8 in virus infection, a homozygous T-
DNA line of AtRH8, SALK_016830, with a T-DNA insertion in the promoter region was
acquired from Arabidopsis Biological Resource Center (ABRC, http://www.biosci.ohio-
The T-DNA PCR screen on genomic DNA as well as genetic analysis confirmed that there is only one T-DNA insertion (not shown). RT-PCR analysis of total RNA isolated from leaf tissues of this mutant line and 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}.

To test if \textit{AtRH8} is required for PPV infection, the \textit{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 days 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 \textit{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 \textit{atrh8} mutants are resistant to PPV.

To test if \textit{AtRH8} is also needed by another potyvirus during infection, the wild-type and \textit{atrh8} plants were agro-infiltrated with a green fluorescent protein (GFP)-tagged TuMV infectious clone (TuMV-GFP). Diagnosis of these plants 14 days post agro-infiltration (dpa) with RT-PCR revealed the presence of the TuMV genomic RNA in the wild-type plants but not in the \textit{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 \textit{atrh8} plants (Fig. 3C). Phenotypes of the wild-type plants and \textit{atrh8} mutants agro-infiltrated with TuMV-GFP or mock-infiltrated were closely examined. Under the normal growth conditions without TuMV infiltration, \textit{atrh8} mutants showed no developmental differences from wild-type plants (see mock-inoculated WT and \textit{atrh8}) (Fig. 3D and E), and displayed normal vegetative growth, flowering development and seed production. At 3 dpa, the wild-type plants agro-infiltrated 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 \textit{atrh8} mutants and mock-infiltrated wild-type or \textit{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 the ultraviolet light 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

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 (ER)-derived vesicles (Schaad et al., 1997). Recently we have found that these 6K2 vesicles originate at ER 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 Nla, 6K2-Nla (two VPg precursors) and Nlb (viral RNA-dependent RNA polymerase), viral RNA (carrying VPg), and host factors, i.e., eIF(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 the VPg-binding region (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 aa and the C-terminal portion containing 257 aa (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 media showed VPg positively interacted exclusively with the N-terminal fragment of AtRH8 and not the C-terminal portion, suggesting the interaction site resides within the first 250 aa of AtRH8. The N-terminal 250 aa was subjected to additional sequential deletions (Fig. 5 A and B). Based on the deletion analyses, the VPg-BR of AtRH8 consists of 50 aa (from aa 201 to 250) (Fig. 5 A and B). Protein sequence comparison of the VPg-BR (aa 140 to 189) of PpDDXL and that of AtRH8 indicated a 74% similarity (Fig. 5C). The protein predictor SSpro v4.5 program from ExPasy Proteomics Server (http://www.expasy.ch) identified an alpha 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 agro-infiltrated 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 combination. Infiltrated leaf tissues were observed under 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 responsible for protein-protein interaction with the PPV VPg.

**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 coinfiltrated 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 to
the control leaves (infiltrated 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 coinfiltrated into
*N. benthamiana* with an expression plasmid containing the VPg-BR of AtRH8.
Coexpression of the VPg-BR led to a reduction of the virus accumulation relative to the
control (coinfiltration of TuMV-GFP clone with an empty vector) (Fig. 6A). Quantitative
analysis of the viral RNA indicated that viral RNA in the leaves expressing the VPg-BR
of AtRH8 accumulated about 31% of that in the control (Fig. 6B). Furthermore, we tested
the effect of the VPg-BR of AtRH8 on the infection of *Tobacco etch virus* (TEV),
another member of the *Potyvirus* genus. Since the GFP-tagged TEV infectious clone was
incompatible with the agro-infiltration system (Schaad et al., 1997), mechanical
inoculation of TEV was used for infection. The accumulation of TEV in the leaves
expressing the VPg-BR of AtRH8 was approximately 34% of that in the control
(Supplemental Fig. S5). Confocal visualization of the inoculated tissues revealed a
consistent reduction of green fluorescence emission as quantified by real-time PCR
results (Supplemental Fig. S5). Similarly, real-time PCR analyses of TEV and TuMV
infections in the presence of the VPg-BR of PpDDXL revealed a decrease of virus
accumulation by approximately 5- and 3-fold in comparison with the respective controls,
(Supplemental Fig. S6). These data suggest that overexpression of the VPg-BR of
AtRH8 or that of PpDDXL exerts a suppression effect on potyvirus infections.

**DISCUSSION**

In this study, we have reported the identification of two VPg-interacting plant
DDX proteins, i.e., AtRH8 from *A. thaliana* and PpDDXL from *P. persica* (Figs. 1 and
2). These DDX proteins share sequence homology with eIF4A, a component of the eIF4F
multiprotein complex. We used the *A. thaliana* AtRH8 homozygous T-DNA insertion
lines to functionally characterize the requirement of AtRH8 in potyvirus infection. We
found that AtRH8 knockout plants (atrh8 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 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 was the first showing a plant DDX protein is required for virus infection in plants.

RNA helicases (RH) represent a large family of proteins implicated in almost every step of RNA metabolism (de la Cruz et al., 1999; Tanner and Linder, 2001; Lorsch, 2002; Mohr et al., 2002). During the virus infection process, RHs have been suggested to be involved in (i) translation of the viral RNA, (ii) selection of the RNA template for translation or replication, (iii) recruitment of the viral RNA for replication, (iv) RNA synthesis, and/or (v) 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). 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 RHs bound to BMV and TBSV genomic RNAs have been documented (Li et al., 2009). These results suggest that besides DED1, other RHs 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 (HIV-1) and Hepatitis C virus (HCV) 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 (NPC) by remodeling the virus replication complex (Yedavalli et al., 2004). Interestingly, DDX3 has also been shown to chaperon a type of mRNA granules for translation in the developing brain of rat embryos (Elvira et al., 2006). These DDX3-containing granules contain the full protein translation apparatus including both the small and large ribosomal subunits as well as mRNA. 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 NIa, 6K2-NIa and NIb), viral genomic RNA (carrying NIa 
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 an 
VPg-interacting translation initiation factor, is localized to the TuMV replication 
complex (Cotton et al., 2009). In infected cells, NIa 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, NIa or VPg is 
mainly intercepted by the virus replication complex for replication with only a small 
amount of NIa or VPg transported to the nucleus. Indeed, large amounts of viral RNA 
(carrying NIa or VPg) are concentrated in the virus replication complex in the early 
infection stage (a few days post infection) (Cotton et al., 2009; Wei and Wang, 
unpublished data), whereas high-level accumulation of NIa 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 NIa 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 NIa, leading to 
the transient accumulation of the 6K2-NIa precursor protein (Restrepo-Hartwig and 
Carrington, 1992). The cytoplasmic 6K2-NIa impedes nuclear localization of NIa 
(Restrepo-Hartwig and Carrington, 1992) and colocalizes with the TuMV replication 
complex (Cotton et al., 2009). In addition to different VPg precursors, several post-
translationally modified forms of VPg and NIa have also been found in infected plants 
(Hafrán and Mäkinen, 2008). These modified forms of VPg and NIa may also be 
intracellularly differentially distributed. It is possible 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 (Laín 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., 1989). 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 (CP) (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
LMV 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 via 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
genoexpression 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 recessive resistance gene \textit{pvr1} (an \textit{eIF4E} mutant) in tomato generates dominant resistance to TEV and other potyviruses including \textit{Pepper mottle virus} and \textit{Potato virus Y}. In the present 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 direct targeting on the viral genome (Grumet et al., 1987; Baulcombe, 2004; Wang and Metzlaff, 2005; Wang et al., 2006). This type of viral resistance can be overcome in two scenarios, i.e., in mixed infections by a strong silencing suppressor from unrelated viruses (Mitter et al., 2001), and through the event of random sequence mutation during virus replication where 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 the 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 & Screening Kits (Clontech) following the supplier’s instruction manual. The VPg of a PPV-D strain was cloned into the bait vector, pGBKT7 encoding the binding domain (BD) to generate plasmid pGBKT7-VPg. The peach cDNA library was prepared by inserting cDNA derived from PPV-infected \textit{P. persica} leaf tissues into the prey vector pGADT7-rec encoding the activation domain (AD). Positive clones were
isolated and transformed into the *E. 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, the 5´ rapid amplification of cDNA ends (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 Tab. S1) were used to obtain the full-length *PpDDXL* cDNA. All PCR reactions were performed using the PhusionTM 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 protein in different plant species was done using SeqMan from DNASTar v6 and CLUSTALW alignment programs.

**T-DNA mutant analysis**

All the T-DNA insertion lines used in this study were obtained from ABRC. The T-DNA insertion site of the *atrh8* mutant was confirmed by PCR, using the T-DNA left border-specific primer, LBa1, and *AtRH8*-specific primers (LP16830 and RP16830). 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 with *AtRH8*-specific primers, *AtRH8*-F and *AtRH8*-R to confirm the T-DNA line as a true knockout mutant.

**Cloning AtRH8, and deletion analysis**

cDNA encoding the full-lenth of *AtRH8* was amplified by PCR with primers *AtRH8*-F, and *AtRH8*-BamHI-R using cDNA derived from the wild-type *A. thaliana* plants. The PCR products were inserted into the pGADT7 vector to generate plasmid pGAD-T7-*AtRH8*. *AtRH8* deletion analysis fragments were obtained using primers listed in Supplemental Tab. S1. All constructs were confirmed by sequencing.
Plasmid construction for expression in plants

To construct the TuMV::6K-YFP infectious clone, the infectious clone TuMV::6K-GFP (or pCambiaTunos/6KGFP) (Cotton et al., 2009) was digested with SmaI and KpnI and the resulting GFP-containing DNA fragment was cloned into the pBluescript vector (Stratagene) to obtain plasmid pBlue-6K2-GFP-HCPro. This plasmid was subjected to three steps of subcloning to replace GFP with YFP that was retrieved from a plasmid previously constructed to express YFP (Wei and Wang, 2008). The resulting plasmid pBlue-6K2-YFP-HCPro clone, upon sequencing confirmation, was cleaved with SmaI and KpnI and the 6K2-YFP-HCPro fragment was re-cloned into the corresponding sites of pCambiaTunos/6KGFP to create the TuMV::6K-YFP construct.

Gateway technology (Invitorgen) was used to generate plasmids for expression in plants as described previously (Wei and Wang, 2008; Lu et al., 2009). PCR-amplified DNA segments including AtRH8 (with primers AtRH8-Gate-F and AtRH8-Gate-R), VPg (with primers VPg-Gate-F and VPg-Gate-R) and VPg-BR from AtRH8 and PpDDXL (with primers VPgBR-AtRH8Gate-F, VPgBR-AtRH8Gate-R, VPgBR-PpDDXLGate-F, and VPgBR-PpDDXLGate-R) were transferred by recombination into the entry vector pDONR201 (Invitrogen) using BP clonase II (Invitrogen) following the manufacturer's protocol. The insert of the resulting pDONR clone was verified by 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.

Agro-infiltration 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 A. thaliana plants were agro-infiltrated with the Agrobacterium containing the GFP-tagged TuMV plasmid at an OD500 of 0.05. For BiFC and colocalization experiments, the 3-week-old N. benthamiana plants were ago-infiltrated with the mixture of Agrobacterium cultures (each at an OD600 of 1.0) in 1:1 ratio. Confocal microscopy work was carried out.
following Wei and Wang (2008).

**Virus inoculation**

Mechanical inoculation of *A. thaliana* 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 mls of 1X PBS 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 *A. thaliana* 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 14 dpi or dpa. The infection of PPV and TuMV was diagnosed by RT-PCR with PPV-specific primers PPVcp-F and PPVcp-R, and TuMV-specific primers HC-Pro-F and HC-Pro-R, respectively.

Real-time PCR reaction preparations were carried out using the LightCycler®480 Probes Master kit (Roche) on a LightCycler®480 real-time PCR system (Roche) following the manufacturer’s instructions. Three pairs of primers, i.e., TEVcp-F and TEVcp-R, TuHC-F and TuHC-R, and NbEF-1α-F and NbEF-1α-R were used for quantification analyses of TEV, TuMV, and *N. benthamiana elongation factor-1α* (*NbEF-1α*), respectively. NbEF-1α served as the internal reference control. The hydrolysis probe designs were based on TaqMan® Probe design tutorial guidelines by Beacon DesignerTM & AlleleID®. The corresponding hydrolysis probes to TEV, TuMV and NbEF1α are listed in Supplemental Tab. S1. 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 LightCycler®480 software SW1.5 (Roche). The RNA level was calculated using the
mean threshold cycle (Ct) value normalized to that of the reference gene, *NbEF-1a*.

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Figure legend

**Figure 1.** Yeast two-hybrid assay of protein-protein interaction between the PPV VPg and PpDDXL from peach (*P. persica*) or AtRH8 from *A. thaliana*. Yeast co-transformants were grown on the selective medium SD/-Ade/-His/-Leu/-Trp plus X-α-Gal and incubated at 28ºC for four days.

**Figure 2.** *In planta* interaction between AtRH8 and the PPV VPg. (A) Confocal microscopy imaging of AtRH8-CFP expressed in *N. benthamiana* leaf. Bars, 14 μm. (B) Coexpression of YFP and AtRH8 fused with CFP (AtRH8-CFP) in 3-week-old *N. benthamiana* plants. *Agrobacterium* EHA105 strain containing YFP and AtRH8-CFP expression plasmids were coinfiltrated into the lower epidermal leaf surface. AtRH8-CFP distributed in the cytoplasm, and YFP labeled both the cytoplasm and the nucleus (due to diffusion). Bars, 16 μm. (C) Coexpression of AtRH8-CFP and VPg fused with YFP (VPg-YFP). VPg-YFP was localized to the cytoplasm as well as the nucleus. AtRH8-CFP colocalized with VPg-YFP. Bars, 16 μm. (D) Bimolecular fluorescence complementation (BiFC) analysis of AtRH8 and VPg. AtRH8 was fused with the N-terminal fragment of YFP (AtRH8-YN) and coexpressed with the fusion of VPg with the C-terminal fragment of YFP (VPg-YC). Bars, 17 μm. All the confocal images were taken two days post infiltration. Chl, chloroplast autofluorescence.

**Figure 3.** Requirement of *AtRH8* for potyvirus infection in *A. thaliana* plants. (A) and (B) represent results from RT-PCR analysis of PPV and TuMV accumulation, respectively. Wild type (WT) plants and *atrh8* mutants were mechanically inoculated and agro-infiltrated with PPV and TuMV, respectively. Total RNA extracted from upper newly emerged leaves two 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-infiltrated WT and *atrh8* mutant plants. Bars, 48 μm. Three-week-old *A. thaliana* plants were agro-infiltrated with a GFP-tagged TuMV infectious clone and observed ten days post infiltration. (D) and (E) are the phenotype of WT and *atrh8* mutant plants 3 and 14 days post infiltration, respectively. Mock, infiltrated with an
empty vector; TuMV, infiltrated with the GFP-tagged TuMV infectious clone. (F)
Photographs of WT and arth8 mutant plants inoculated with the GFP-tagged TuMV infectious clone under UV lights (19 days post agro-infiltration).

Figure 4. Colocalization of AtRH8 with the virus accumulation complex in TuMV-infected N. benthamiana epidermal cells. (A) Confocal microscopy imaging of N. benthamiana epidermal cells that had been pre-infected with a TuMV infectious clone tagged with a 6K2-YFP fusion (TuMV::6K-YFP) for four days and then agro-infiltrated with an empty vector. In plant cells infected by a similar infectious clone (TuMV::6K-GFP), 6K2 vesicles contain viral replication-associated proteins such as NIa, 6K2-NIa (two VPg precursors) and NIb (viral RNA-dependent RNA polymerase), viral RNA (carrying VPg), and host factors, i.e., eIF(iso)4E, PABP2 and eEF1A (Cotton et al., 2009). These 6K2 vesicles originate at ER exit sites (Wei and Wang, 2008) and target chloroplasts for virus replication (T. Wei and A. Wang, unpublished data). Bars, 10 μm.

(B) AtRH8-CFP was transiently expressed in N. benthamiana leaves that had been pre-infected with the TuMV::6K-YFP infectious clone for four days. AtRH8-CFP colocalized with the chloroplast-bound 6K2-YFP vesicles. Bars, 10 μm. All the confocal images were taken two days post agro-infiltration with the empty vector or the AtRH8 expression vector. Chl, chloroplast autofluorescence.

Figure 5. Identification of the VPg-binding region (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 BD-VPg vector (bait) and prey vectors (AtRH8 deletions represented by diagram of bars on the left), plated on the highly stringent selective medium SD/-Ade/-His/-Leu/-Trp+X-α-gal and incubated at 28ºC for four days. (B) Summary of results from (A). Clone names and schematic representation of various forms of the truncated AtRH8 (corresponding amino acid positions 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 Clustal W program. Amino acids within the binding region are colored in red and compared for sequence similarity. *, identical; :, strongly related (belonging to the same group such as polar, non-polar, basic, and acidic, and having side chains sharing...
a similar chemical structure); ·, weakly related (belonging to the same group such as polar, non-polar, 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 two days 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. Bars, 17 μm. (E) BiFC analysis of the interaction between the VPg-BR of PpDDXL and the PPV VPg two days 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.

**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 agro-infiltrated with different combinations of plasmids. The photographs represent the infection pattern in *N. benthamiana* three days post infiltration. Bars, 300 μm. 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; GFP, green fluorescence emission from a GFP-tagged TuMV infectious clone; Chl, chloroplast autofluorescence. (B) Realtime PCR quantification of TuMV accumulation in *N. benthamiana* two days 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 changes 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), respectively. The error bars represent standard deviations.
A List of Supplemental Data

Supplemental Figure S1. Analysis of \textit{PpDDXL}. (A) Distribution of exons and introns in the \textit{PpDDXL} genomic sequence. The genomic sequence of \textit{PpDDXL} consists of 2147 nucleotides (nt). 5’ UTR (untranslational region), nt 1 to 293; Intron 1, nt 362 to 447; Intron 2, nt 556 to 813; Intron 3, nt 1243 to 1353; 3 UTR, nt 1991 to 2147. (B) Functional domain organization of the \textit{PpDDXL} protein. The amino acid positions are indicated. (C) Comparison of the conserved motifs of \textit{PpDDXL} in consensus with other DEAD-box RNA helicase proteins. (D) Multi-sequence alignment of the deduced amino acid sequence of \textit{PpDDXL} with DEAD-box RNA helicases from \textit{Pisum sativum}, \textit{Medicago truncatula}, \textit{Helianthus annuus}, \textit{Vitis vinifera}, \textit{Populus trichocarpa}, \textit{Oryza sativa}, \textit{Triticum aestivum}, \textit{Zea mays}, \textit{Pennisetum glaucum}, \textit{Nicotiana tabacum}, and \textit{Arabidopsis thaliana}. Multiple alignment was done using the CLUSTAL W program. All the conserved helicase domains (Q, I, Ia, Ib, II, III, IV, V, VI) are shown in boxes. The accession numbers of the aligned protein sequences are \textit{Pisum} (AAN74635), \textit{Medicago} (ABN09109), \textit{Helianthus} (AAR23806), \textit{Vitis} (CAO65326), \textit{Populus} (ABK94270), \textit{Oryza} (NP_001058481), \textit{Triticum} (P41378), \textit{Zea} (NM_001111926), \textit{Pennisetum} (AAY33860), \textit{Nicotiana} (Q40465), and \textit{Arabidopsis} (NM_112246).

Supplemental Figure S2. Analysis of \textit{PpDDXL} homologous genes in \textit{A. thaliana}. (A) Phylogenetic analysis of top 10 closest \textit{A. thaliana} DDXs to \textit{PpDDXL} including AT3G13920, \textit{eIF4A-1} or \textit{AtRH4}; AT1G54270, \textit{eIF4A-2} or \textit{AtRH19}; AT1G72730, \textit{eIF4A-like} or \textit{AtRH23}; AT3G197560, \textit{eIF4A-like} or \textit{AtRH2}; AT1G51380, \textit{eIF4A-like} or \textit{AtRH34}; AT4G0060, \textit{AtRH8}; AT3G61240, \textit{AtRH12}; AT2G45810, \textit{AtRH6}; AT5G1120, \textit{AtRH15-1}; AT5G11170, \textit{AtRH15-2}. (B) Root hair phenotypes of \textit{A. thaliana} wild-type (WT) and \textit{eIF4A-1} (AT3G13920) heterozygous (HZ) seedlings. The dissection microscope pictures were taken on 4-day-old seedlings. Bars, 750 μm.

Supplemental Figure S3. Negative controls for the BiFC assay of \textit{AtRH8} and the PPV VPg in \textit{N. benthamiana}. Confocal images were taken two days post agro-infiltration. YC, the C-terminal moiety of YFP; YN, the N-terminal moiety of YFP; \textit{AtRH8-YC}, \textit{AtRH8} fused to the C-terminal moiety of YFP; \textit{AtRH8-YN}, \textit{AtRH8} fused to the N-terminal moiety of YFP; \textit{VPg-YC}, \textit{VPg} fused to the C-terminal moiety of YFP; \textit{VPg-YN}, \textit{VPg} fused to the N-terminal moiety of YFP. Bars, 47 μm. YFP, YFP fluorescence; Chl.
chloroplast autofluorescence;

Supplemental Figure S4. Expression analysis of A. thaliana AtRH8 homozygous T-DNA insertion line (SALK_016830). RT-PCR was conducted using cDNA derived from A. thaliana wild-type plants (WT) and atrh8 mutants with AtRH8 gene-specific primers. The internal control used for the RT-PCR reaction is the Actin2 gene.

Supplemental Figure S5. Effect of transient overexpression of the VPg-BR of AtRH8 on TEV accumulation in N. benthamiana plants. (A) Transient expression of the VPg-BR reduces TEV infection in N. benthamiana. Confocal images of 3-week-old N. benthamiana leaves mechanically inoculated with TEV. The photographs showed the infection pattern three days post infection. TEV, infiltrated with an empty vector and then inoculated with a recombinant TEV tagged with GFP (control); TEV + BR, infiltrated with VPg-BR and then inoculated with the recombinant TEV tagged with GFP (one day post infiltration of VPg-BR). Bars, 300 μm. GFP, green fluorescence emission from the recombinant TEV; Chl, chloroplast autofluorescence. (B) Realtime PCR analysis of TEV accumulation in N. benthamiana two days post inoculation. Three independent experiments were carried out for quantification analyses. In each experiment, three plants were used per treatment. The values represent means of fold changes relative to the control (TEV alone). The asterisks indicate that TEV accumulation in N. benthamiana expressing the AtRH8 BR (TEV + BR) was significantly lower than that in the control (TEV) (P < 0.01). The error bars represent standard deviations.

Supplemental Figure S6. Effect of transient overexpression of the VPg-BR of PpDDXL on TEV and TuMV accumulation in N. benthamiana plants. Realtime PCR analysis of virus accumulation in N. benthamiana two days post inoculation. Three independent experiments were carried out for quantification analyses. In each experiment, three plants were used per treatment. The values represent means of fold changes relative to the control (TEV or TuMV with an empty vector). The asterisks indicate that TEV or TuMV accumulation in N. benthamiana expressing the PpDDXL VPg-BR was significantly lower than that in the control (TEV or TuMV) (P < 0.01). The error bars represent standard deviations.

Supplemental Table S1. Primers used in this study
Figure 1. Yeast two-hybrid assay of protein-protein interaction between the PPV VPg and PpDDXL from peach (P. persica) or AtRH8 from A. thaliana. Yeast co-transformants were grown on the selective medium SD/-Ade/-His/-Leu/-Trp plus X-α-Gal and incubated at 28°C for four days.
Figure 2. *In planta* interaction between AtRH8 and the PPV VPg. (A) Confocal microscopy imaging of AtRH8-CFP expressed in *N. benthamiana* leaf. Bars, 14 μm. (B) Coexpression of YFP and AtRH8 fused with CFP (AtRH8-CFP) in 3-week-old *N. benthamiana* plants. *Agrobacterium* EHA105 strain containing YFP and AtRH8-CFP expression plasmids were coinfiltrated into the lower epidermal leaf surface. AtRH8-CFP distributed in the cytoplasm, and YFP labeled both the cytoplasm and the nucleus (due to diffusion). Bars, 16 μm. (C) Coexpression of AtRH8-CFP and VPg fused with YFP (VPg-YFP). VPg-YFP was localized to the cytoplasm as well as the nucleus. AtRH8-CFP colocalized with VPg-YFP. Bars, 16 μm. (D) Bimolecular fluorescence complementation (BiFC) analysis of AtRH8 and VPg. AtRH8 was fused with the N-terminal fragment of YFP (AtRH8-YN) and coexpressed with the fusion of VPg with the C-terminal fragment of YFP (VPg-YC). Bars, 17 μm. All the confocal images were taken two days post infiltration. Chl, chloroplast autofluorescence.
**Figure 3.** Requirement of AtRH8 for potyvirus infection in A. thaliana plants. (A) and (B) represent results from RT-PCR analysis of PPV and TuMV accumulation, respectively. Wild-type (WT) plants and atrh8 mutants were mechanically inoculated and agro-infiltrated with PPV and TuMV, respectively. Total RNA extracted from upper newly emerged leaves two 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-infiltrated WT and atrh8 mutant plants. Bars, 48 μm. Three-week-old A. thaliana plants were agro-infiltrated with a GFP-tagged TuMV infectious clone and observed ten days post infiltration. (D) and (E) are the phenotype of WT and atrh8 mutant plants 3 and 14 days post infiltration, respectively. Mock, infiltrated with an empty vector; TuMV, infiltrated with the GFP-tagged TuMV infectious clone. (F) Photographs of WT and atrh8 mutant plants inoculated with the GFP-tagged TuMV infectious clone under UV lights (19 days post agro-infiltration).
Figure 4. Colocalization of AtRH8 with the virus accumulation complex in TuMV-infected *N. benthamiana* epidermal cells. (A) Confocal microscopy imaging of *N. benthamiana* epidermal cells that had been pre-infected with a TuMV infectious clone tagged with a 6K2-YFP fusion (TuMV::6K-YFP) for four days and then agro-infiltrated with an empty vector. In plant cells infected by a similar infectious clone (TuMV::6K-GFP), 6K2 vesicles contain viral replication-associated proteins such as N1a, 6K2-N1a (two VPg precursors) and N1b (viral RNA-dependent RNA polymerase), viral RNA (carrying VPg), and host factors, i.e., eIF(iso)4E, PABP2 and eEF1A (Cotton et al., 2009). These 6K2 vesicles originate at ER exit sites (Wei and Wang, 2008) and target chloroplasts for virus replication (T. Wei and A. Wang, unpublished data). Bars, 10 μm. (B) AtRH8-CFP was transiently expressed in *N. benthamiana* leaves that had been pre-infected with TuMV::6K-YFP for four days. AtRH8-CFP colocalized with the chloroplast-bound 6K-YFP vesicles. Bars, 10 μm. All the confocal images were taken two days post agro-infiltration with the empty vector or the AtRH8 expression vector. Chl, chloroplast autofluorescence.
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