Biochemical and Biophysical Research Communications 417 (2012) 451–456

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Interaction of the host protein NbDnaJ with *Potato virus X* minus-strand stem-loop 1 RNA and capsid protein affects viral replication and movement

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ARTICLE INFO

Article history:
Received 22 November 2011
Available online 7 December 2011

Keywords:
PVX
Stem-loop RNA
Interaction
NbDnaJ
Replication
Movement

ABSTRACT

Plant viruses must interact with host cellular components to replicate and move from cell to cell. In the case of *Potato virus X* (PVX), it carries stem-loop 1 (SL1) RNA essential for viral replication and movement. Using two-dimensional electrophoresis northwestern blot analysis, we previously identified several host proteins that bind to SL1 RNA. Of those, we further characterized a DnaJ-like protein from *Nicotiana benthamiana* named NbDnaJ. An electrophoretic mobility shift assay confirmed that NbDnaJ binds only to SL1 minus-strand RNA, and bimolecular fluorescence complementation (BiFC) indicated that NbDnaJ interacts with PVX capsid protein (CP). Using a series of deletion mutants, the C-terminal region of NbDnaJ was found to be essential for the interaction with PVX CP. The expression of *NbDnaJ* significantly changed upon infection with different plant viruses such as PVX, *Tobacco mosaic virus*, and *Cucumber mosaic virus*, but varied depending on the viral species. In transient experiments, both PVX replication and movement were inhibited in plants that over-expressed *NbDnaJ* but accelerated in plants in which *NbDnaJ* was silenced. In summary, we suggest that the newly identified NbDnaJ plays a role in PVX replication and movement by interacting with SL1(−) RNA and PVX CP.

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1. Introduction

Plant viruses require host components for their infection, replication, and spread within host cells [1,2]. Thus, information about plant-virus interactions is necessary for the control of viral diseases in many economically important plants [3]. To date, a broad range of host proteins that interact with plant viral factors such as proteins and RNA have been identified [4,5] using several approaches, including yeast-two hybrid screens, *in vitro* pull down assays, and co-immunoprecipitation [6–8]. Of the viral factors known, capsid proteins (CPs) and movement proteins (MPs) are widely used as a bait to identify host proteins due to their important functional roles in the viral life cycle [9,10].

Potato virus X (PVX) is among the top plant viruses in which molecular functions have been intensively studied [11]. PVX contains RNA-dependent RNA polymerase (RdRp), CP, and triple gene block proteins TGBp1, TGBp2, and TGBp3, as well as *cis*-acting elements (SL1 RNA), all of which are required for viral replication, movement, and assembly [12]. Compared to other plant viruses, few host proteins that interact with PVX have been identified. For example, a previous study reported a *Nicotiana benthamiana* protein named NbPCIP that interacts with PVX CP and is involved in viral replication [9]. In addition, a plastocyanin precursor protein has been found that interacts with PVX CP; it is a positive regulator of viral CP accumulation within the chloroplast, and affects symptom phenotypes in tobacco plants [13]. Recently, we performed two-dimensional electrophoresis (2DE) with northwestern blot analysis to identify host proteins interacting with SL1 RNA. Interestingly, the majority of identified proteins were homologous to known host factors that bind to many viral factors (unpublished data). To date, nothing is known about the molecular functions of host proteins that interact with the SL1 RNA of PVX. In this study, we further characterized a DnaJ-like protein from *N. benthamiana*, which was previously identified as a host factor that interacts with SL1 RNA. Using the electrophoretic mobility shift assay (EMSA) and bimolecular fluorescence complementation (BiFC), we demonstrated an interaction between this host protein and PVX viral factors. The functions of the DnaJ-like protein associated with PVX replication and movement were also characterized.

2. Materials and methods

2.1. Isolation of cDNA encoding a DnaJ-like protein from N. benthamiana

A DnaJ-like protein was identified by 2DE, using S100 protein extracts prepared from tobacco BY-2 suspension cells. Northwestern

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doi:10.1016/j.bbrc.2011.11.137

blot analysis was performed with a PVX SL1(−) RNA probe (unpublished data). To isolate the full-length cDNA for the DnaJ-like protein, total RNA was extracted from *N. benthamiana* leaves and reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously [14] using gene-specific primers including NtDnaJ 5′ NTR_F (5′-TATTGAAGGAAAACCAAATAGGTCA-3′) and NtDnaJ 3′ NTR_R (5′-ACACATCACAACAAACCAGCTTTAA-3′). The primers were designed based on a known *Nicotiana tabacum* DnaJ-like protein gene sequence (GenBank ID: BAH70368). The identified nucleotide sequence of NbDnaJ was deposited at GenBank in National Center for Biotechnology Information (NCBI).

2.2. EMSA

The SL1 RNA fragments were amplified by PCR and were transcribed *in vitro* by the T7 promoter [15]. The radioactivity of each labeled probe was measured using a liquid scintillation counter, and only RNA probes showing 20,000 counts per minute (cpm) were used for EMSA. To produce maltose binding protein (MBP) fusion protein, the *Eco*RI-flanked NbDnaJ cDNA was cloned into the pMAL-c2X vector (New England Biolabs, USA) and expressed in *Escherichia coli* BL21 cells. NbDnaJ fused to MBP was purified by affinity purification according to the manufacturer's instructions (NEB, USA). EMSA was performed as described previously [16]. Proteins were loaded on 5% non-denaturing polyacrylamide gels in 0.5× TBE buffer. After hybridization, autoradiography results were obtained using Fujifilm Bio-Imaging Analyzer Systems (BAS-2500, Fujifilm, Japan).

2.3. Quantitative real-time RT-PCR (qRT-PCR)

The following primers were used for qRT-PCR analysis: PVX 54_F (5′-ACACACCCGCTTGAAAAAGC-3′), PVX 111_R (5′-TTGGTAAACCTC GCGCACTT-3′), CMV CP (Fny) 147_F (5′-TCGTCCAACTATTAACCACC-CAACC-3′), CMV CP (Fny) 252_R (5′-AGACCCACGGTCTATTTTTGGTG GC-3′), TMV 135_F (5′-AGTTTCAAACACAACAAGCGCGAAC-3′), TMV 240_R (5′-ACCTTAAAGTCACTGTCAGGGAACC-3′), NbDnaJ 914_F, (5′-TTCCAAAAGACCCGACAAAGA-3′), and NbDnaJ977_R (5′-CGAA-CAGGGAACTTGATGTCAAA-3′). The ABI PRISM 7500 Sequence Detection System was used, with SYBR Green PCR Master Mix according to the standard protocol from Applied Biosystems (Applied Biosystems, USA). The expression level of the *NbDnaJ* gene was obtained after normalization with the expression levels of *ubiquitin 3* (*ubi3*) and *elongation factor 1B* (*EF-1B*) [17].

2.4. Generation of over-expression and silencing constructs for NbDnaJ

The amplified full-length cDNA for NbDnaJ was cloned into pPZP212 modified for over-expression [9]. To make a silencing construct, the partial sequence of *NbDnaJ* was amplified by PCR using the primers NbDnaJ 101 *Mlu*I_F (5′-CGACGCGT TCCTGATAA-GAACCC-3′) and NbDnaJ 300 *Mlu*I_R (5′-CGACGCGT GGGCCTTCC CCGGTA-3′). The three copies of the partial sequences were cloned into the modified pPZP212 vector. Two of the constructs generated were transformed into *Agrobacterium tumefaciens* strain GV2260. For transient overexpression, pPZP-NbDnaJ and p19 of *Tomato bushy stunt virus* (pTBSV-p19) [9] were co-infiltrated.

2.5. Confocal laser scanning microscopy

The LSM510 confocal laser scanning microscope system (LSM 510, Carl Zeiss, Germany) was used to examine the fluorescence of YFP. Excitation and emission wavelengths for YFP accumulation were 488/510–540 nm.

2.6. Bimolecular fluorescence complementation analysis

The *Stu*I-flanked full-length or deletion mutants for NbDnaJ were cloned into the BiFC vector containing the N-terminal region of yellow fluorescent protein (YFP^N) [9]. Genes encoding four PVX proteins (CP, TGB1, TGB2, and TGB3) and NbDnaJ were cloned into the vector containing the C-terminal region of YFP (YFP^C) [9]. The interaction between YFP^N-NbPCIP and YFP^C-PVX CP was used as a positive control [9]. All constructs were transformed into *A. tumefaciens* strain GV2260. Fluorescent signals were detected by CLSM 3 days after agro-infiltration into *N. benthamiana* leaves.

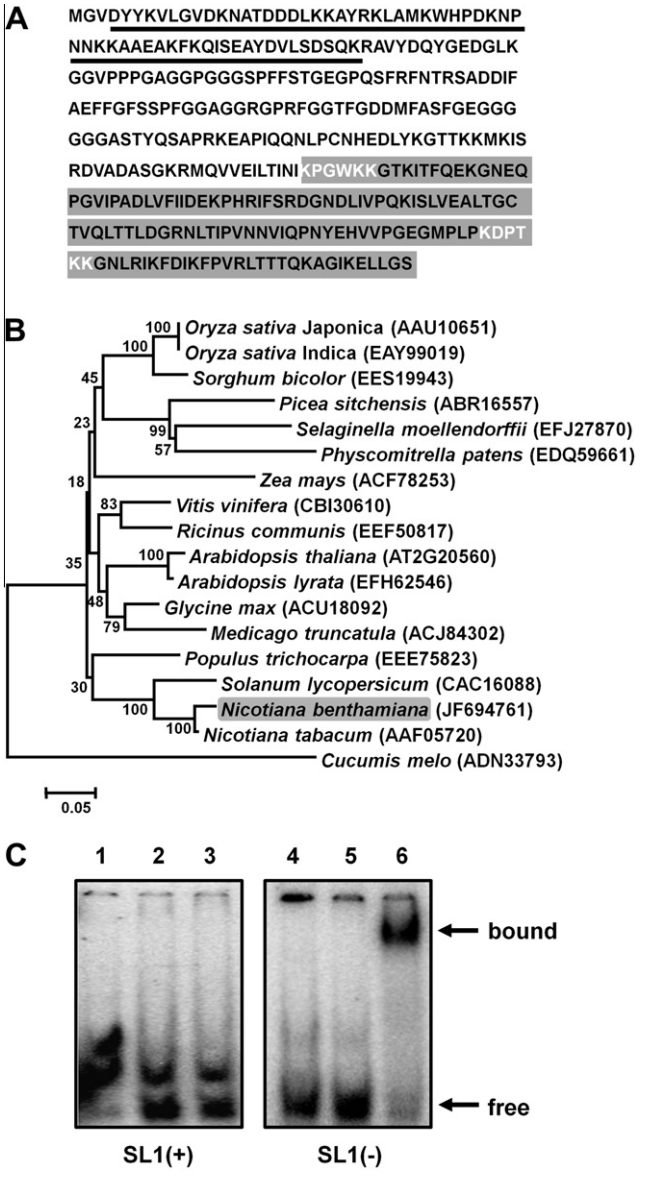

Fig. 1. Isolation of NbDnaJ protein, which interacts with the minus strand of SL1 RNA. (A) Amino acid sequence of the NbDnaJ gene. Underlined and gray colored amino acids indicate the J domain and the C-terminal region, respectively. The two white colored sequences are lysine-enriched motifs. (B) A phylogenetic tree of sequences from 17 plant species homologous to NbDnaJ. Due to the redundancy of DnaJ-like proteins, only the best-matched protein from each plant species was used to make the phylogenetic tree, which was constructed using MEGA 4.1 [29]. Bootstrap values derived from 1000 replications are indicated at each branch. (C) EMSA to confirm the interaction between NbDnaJ and SL1 RNA. ^{32}P-labeled SL1(+) (lanes 1–3) and SL1(−) (lanes 4–6) transcripts were mixed with buffer (lanes 1 and 4), maltose binding protein (MBP, lanes 2 and 5), and NbDnaJ fused to MBP (lanes 3 and 6). The top arrow indicates RNA–protein complexes.

2.7. PVX-sGFP movement assay

pPZP-NbDnaJ (over-expression), pPZP-NbDJ3 (silencing), and pPZP (empty vector) constructs were agro-infiltrated into *N. benthamiana* leaves. Three and ten days after agroinfiltration, pSPVX-sGFP [9] was challenge-inoculated into the upper leaves. Ten days later, the leaves were observed under the Dark Reader HL28T UV lamp to detect green fluorescence (Clare Chemical Research, USA).

3. Results

3.1. NbDnaJ binding to the minus strand of SL1 RNA

We previously performed 2DE with northwestern blot analysis to identify host factors that interact with SL1 RNA of PVX (unpublished). DnaJ-like protein, one of the identified host proteins, which binds to the minus strand of SL1 RNA (SL1−), was further characterized here. To isolate full-length cDNA for the DnaJ-like protein gene from *N. benthamiana*, RT-PCR was performed using gene-specific primers based on *N. tabacum* DnaJ-like protein gene sequences. We named the product *N. benthamiana* DnaJ-like protein (NbDnaJ; GenBank ID: JF694761). The sequence for NbDnaJ consisted of 343 amino acids (aa), with a J domain (3–62 aa) and a conserved C terminal region (211–340 aa) (Fig. 1A). A BlastP search against the NCBI protein database revealed several protein sequences with homology to NbDnaJ, represented by 17 different sequences from various plant species. A phylogenetic tree constructed with these sequences showed that NbDnaJ is most closely related to NtDnaJ followed by a DnaJ-like protein from tomato (*Solanum lycopersicum*) (Fig. 1B).

To confirm the interaction of NbDnaJ with minus-strand SL RNA, we performed EMSA. The results showed that ^{32}P-labeled SL1(−) RNA and NbDnaJ formed a complex with delayed migration compared to that of ^{32}P-labeled SL1(+) RNA, indicating that the purified NbDnaJ interacts with only SL1(−) RNA *in vitro* (Fig. 1C). The lanes containing only MBP or buffers showed no interaction with SL1 RNAs, as indicated by bands migrating to the bottom of the gel.

3.2. NbDnaJ expression is induced upon virus infection

To examine the mRNA levels of *NbDnaJ* after viral infection, we inoculated the leaves of *N. benthamiana* with purified PVX. First, we analyzed the levels of PVX RNA at different time points after viral infection. RT-PCR results showed that the levels of PVX RNA increased dramatically from 24 to 60 h post inoculation (hpi) (Fig. 2A). In parallel, we examined the expression level of *NbDnaJ* and found that transcripts of *NbDnaJ* started to accumulate 12 h, reached a maximal level 24 h and normalized 36 h after PVX infection (Fig. 2B). These results indicate that the expression of *NbDnaJ* was temporarily induced at the start of PVX RNA accumulation. To determine if the induction of *NbDnaJ* expression occurred in a virus-dependent manner, we inoculated the leaves of *N. benthamiana* with purified *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV). The accumulation of TMV and CMV RNA dramatically increased 24 h after viral inoculation. For TMV infection, the expression of *NbDnaJ* was strongly induced between 24 and 60 h (Fig. 2C). In contrast, the level of *NbDnaJ* was not significantly changed by CMV infection (Fig. 2D).

3.3. Interaction between NbDnaJ and PVX proteins

PVX SL1 RNA binds to PVX CP, and this interaction is required for PVX replication and movement [12]. Therefore, we examined the interaction between NbDnaJ and PVX proteins using a BiFC assay. After co-agroinfiltrating different combinations of YFP^N and YFP^C fusion constructs, we observed yellow fluorescence by CLSM. As a positive control, we used NbPCIP and PVX CP (Fig. 3B). Yellow fluorescence was observed with NbDnaJ in combination with PVX CP (Fig. 3C), however, no fluorescence was observed with any other combination (Fig. 3A and D–F). These results indicate that NbDnaJ interacts only with PVX CP. To identify an essential domain for the interaction with PVX CP, we made three deletion constructs by deleting the J region and the C terminal domain (Fig. 3J). The BiFC results showed that PVX CP interacts with the ΔJ domain, in which the J domain was deleted, however, PVX CP did not interact with

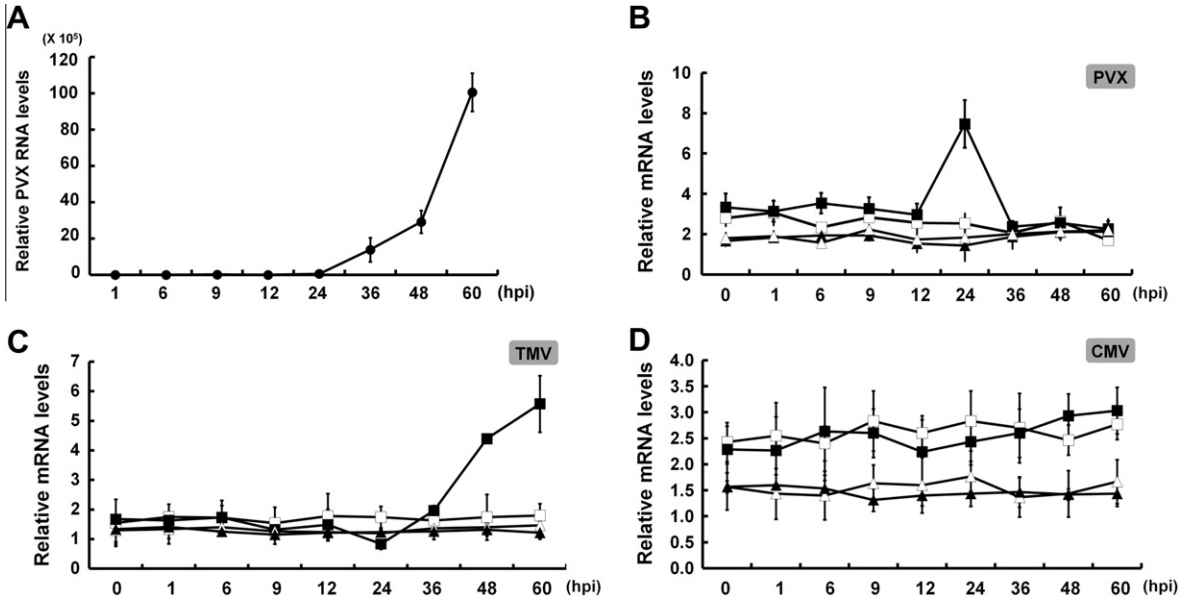

Fig. 2. Quantitative RT-PCR analysis of the levels of *NbDnaJ* and viral RNA after viral infection. Infection of *N. benthamiana* leaves with purified PVX (A and B), TMV (C), and CMV (D) was followed by viral RNA accumulations at different time points from 0 to 60 h, detected by qRT-PCR. The levels of *NbDnaJ* were examined by qRT-PCR using gene-specific primers for *NbDnaJ* (square) and *elongation factor-1 α* (triangle). After PVX, TMV, and CMV infection of *N. benthamiana* leaves, total RNA was prepared from PVX-infected (closed square and triangle) and healthy *N. benthamiana* (opened square and triangle). Error bars indicate standard deviations from triplicate experiments.

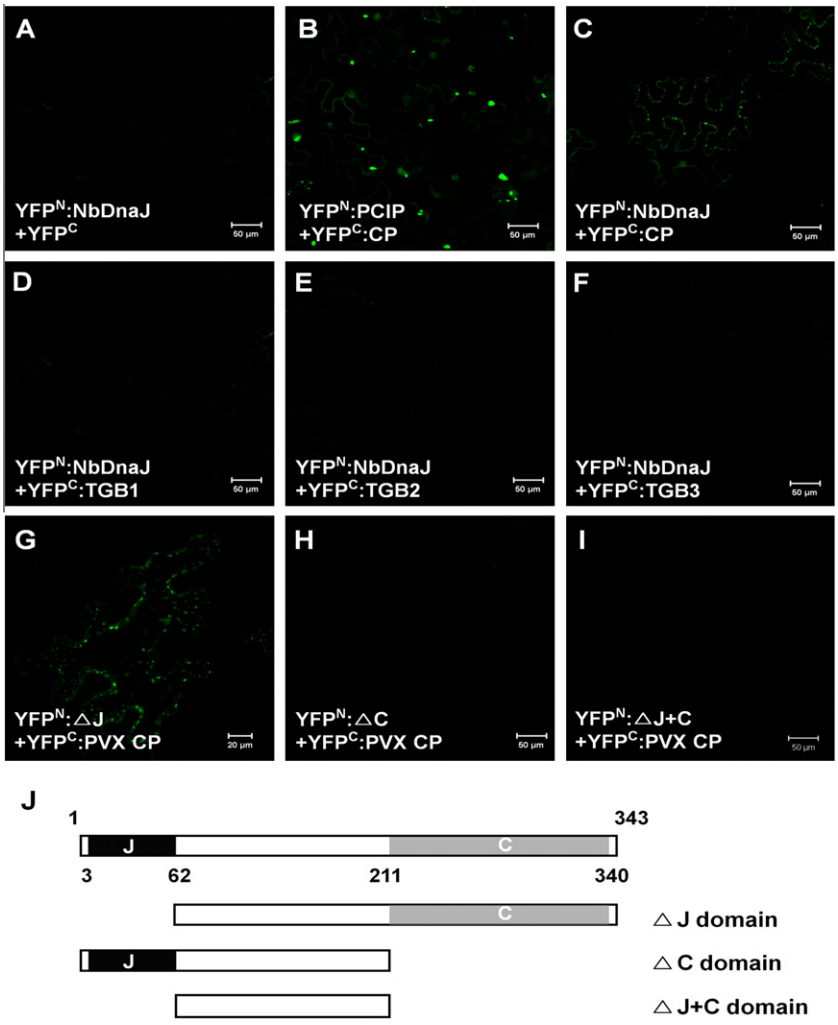

Fig. 3. Interaction between NbDnaJ and PVX proteins measured by BiFC. The interaction between NbDnaJ and PVX proteins was investigated with co-expressed BiFC constructs (described in each image). Images (A) and (B) are the negative and positive control, respectively. (C–F) Interaction between NbDnaJ and four PVX proteins. (G–I) Interaction between NbDnaJ deletion mutants and PVX CP. (J) Schematic illustration of NbDnaJ deletion mutants. Scale bars represent 10 μm.

ΔC or ΔJ + C (Fig. 3G–I). These data suggest that the C-terminal region of NbDnaJ is required for its interaction with PVX CP.

3.4. Involvement of NbDnaJ in PVX replication and movement

DnaJ-like proteins are involved in viral replication and movement [18,19]. To determine the functions of NbDnaJ, we constructed over-expression and silencing vectors for the NbDnaJ gene. Using these constructs and the agroinfiltration method, we produced *N. benthamiana* plants that transiently over- or under-express NbDnaJ. The qRT-PCR results showed that transcript levels for NbDnaJ were dramatically higher in NbDnaJ over-expressing plants (Fig. 4A), but three times lower in under-expressing plants than in wild type plants (Fig. 4B). To examine PVX replication, we prepared protoplasts from over-expressing, silenced, and wild type plants. After infection with pSPVX, the level of PVX RNA was analyzed by qRT-PCR and was found to be reduced in the over-expressing plants and slightly increased in the silenced plants compared to the wild type plants (Fig. 4C). These results indicate that NbDnaJ inhibits the accumulation of PVX RNA.

The strong interaction between NbDnaJ and PVX CP suggests that NbDnaJ might affect PVX movement. To test the functional role of NbDnaJ in PVX movement, we challenge-inoculated NbDnaJ over-expressing, silenced, and wild type plants using a construct of PVX fused to sGFP. In the over-expressing plants, movement of PVX-sGFP was inhibited compared to control plants (Fig. 4D). In contrast, movement of PVX-sGFP was increased for silenced plants (Fig. 4E). This suggests that NbDnaJ may be a negative regulator of PVX movement. Overall, NbDnaJ plays an important role in PVX replication and movement.

4. Discussion

SL structures in many plant viruses play important roles in the virus infection cycle including replication, translation, and movement [20]. For example, the 5′ SL structure of *Brome mosaic virus* (BMV) is required for positive strand RNA production [21] and SL1 RNA of PVX is involved in viral RNA accumulation, infectivity, and movement [12,22]. Therefore, it is very important to find host factors that interact with viral SL RNA and to determine the functions of host proteins associated with viral infection. To that end, we recently identified several host proteins that bind to PVX SL RNA. We isolated and characterized a host protein called NbDnaJ that was initially identified as one of the proteins that interacted with SL1 RNA.

Although numerous host factors interact with viral proteins, the number of host proteins known to interact with viral RNA is very limited. This study provides the initial steps needed to further research the interactions between viral RNA and host proteins. We

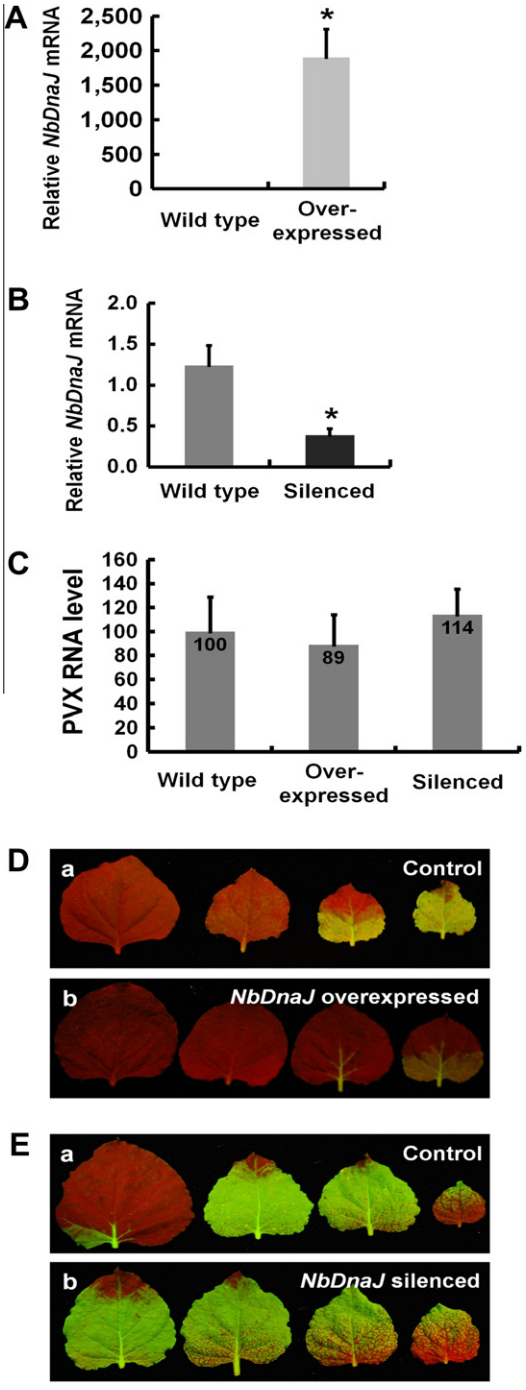

Fig. 4. PVX replication and movement in plants transiently over- and under-expressing NbDnaJ. Over-expression (pPZP-NbDnaJ) (A) and silencing (pPZP-NbDJ3) (B) constructs for *NbDnaJ*, as well as a control vector (pPZP), were agroinfiltrated into the leaves of *N. benthamiana*. To check the transcript amount of *NbDnaJ* in each infiltrated plant, qRT-PCR was used. (C) Accumulation of PVX RNA was examined using protoplasts isolated from transient over-expressing, silenced, and wild type plants. The error bars mean SD, and significance is assessed by a Student's *t* test ($^*P < 0.05$). (D and E) To examine PVX movement, plants expressing pPZP (a in panels D and E), pPZP-NbDnaJ (b in panel D), or pPZP-Nb2P3 (b in panel E) were challenge-inoculated with pSPVX-sGFP. Ten days later, movement of pSPVX-sGFP was observed with a UV hand lamp.

provide strong evidence that viral RNA, like the SL1 region, is bound by host proteins, possibly through binding with viral proteins. NbDnaJ may interact not only with SL1 RNA but also with PVX CP because this viral protein binds to plus and minus strands of SL1 RNA (unpublished data). In particular, we confirmed that NbDnaJ interacts only with the minus strand of SL1 RNA, and confirmed this by EMSA. This suggests that the binding affinity is different for plus and minus strands of PVX SL1 RNA, and may provide host protein binding specificity for SL1 RNA. In addition, we showed that the host protein NbDnaJ bound to SL1(−) RNA can interact with other viral proteins. This idea is also supported by previous 2-DE analyses with Northwestern blot, which identified 24 host proteins that interact with SL1 RNA (unpublished data). Interestingly, they are homologous to known host proteins, which bind to various viral proteins.

Interaction of NbDnaJ with the minus strand of SL1 RNA and PVX CP could be related to functions of NbDnaJ upon PVX infection. Loss of *NbDnaJ* function led to increased PVX-sGFP movement while overexpression of *NbDnaJ* caused decreased movement. These results indicate a role for NbDnaJ in the inhibition of PVX movement. A previous study showed that the yeast protein Ydj1p is involved in the replication of BMV [18]. Similarly, we suspect that NbDnaJ is a negative regulator of PVX replication because a PVX replication assay using isolated protoplasts showed inhibited replication of the virus consistent with previous reports that SL1 RNA is required for viral replication and movement [12]. Moreover, three partial sequences of NbDnaJ were very successful in silencing the expression of the DnaJ gene family, which is redundantly present in these plants.

The heat shock proteins (Hsps), also known as molecular chaperons, are involved in a wide range of cellular processes such as protein folding, translocation of proteins, and degradation of misfolded proteins [23]. In plants, Hsp families can be divided into six major groups, of which Hsp70 and DnaJ (Hsp40) are the most abundant. For example, the *Arabidopsis thaliana* genome contains at least 17 and 89 proteins for Hsp70 and DnaJ, respectively [24]. The interaction of these two protein families is required for their efficient function as molecular chaperons. Members of host Hsp protein families are frequently found to interact with various viral replicases and MPs. For example, CP of *Potato virus Y* interacts with two DnaJ-like proteins (NtCPIP1 and NtCPIP2a), which are negative effectors during PVY infection [19]. In the case of *Tomato spotted wilt virus* (TSWV), NSm protein is required for cell-to-cell movement of TSWV and interacts with two DnaJ proteins from *N. tabacum* and *A. thaliana* [25]. The MP of *Rice stripe virus* is reported to interact with two rice proteins including DnaJ small Hsp20 [26]. Similarly, NbDnaJ interacts with PVX CP and SL1 RNA. In a deletion assay, we revealed that the conserved C-terminal region of NbDnaJ is important for the interaction with PVX CP. This result is highly consistent with previous reports that have shown that the C-terminal domain of a tobacco DnaJ-like protein is important for the interaction with TSWV NSm and TMV MP [25]. However, we do not know whether this C-terminal region is required for SL1 RNA binding. Taken together, our findings and previous reports suggest that NbDnaJ might be a universal host protein that interacts with many host and viral proteins.

In general, viral infection induces the expression of many heat shock proteins including Hsp70 and Hsp40 (DnaJ-like protein) [27,28]. Interestingly, PVX infection increased the transcript level of *NbDnaJ* temporally, indicating that NbDnaJ might be required in the early stages of PVX infection. However, we do not know whether the expression patterns of other *DnaJ* genes are similar to that of *NbDnaJ*. NbDnaJ has a similar expression pattern to many transcription factors that regulate gene expression at early time points of various environmental stimuli. Moreover, TMV and CMV infection caused very different expression patterns for *NbDnaJ*. These data suggest that the functional role of NbDnaJ is related to viral attack and is virus species-specific.

In summary, we propose that the newly identified NbDnaJ is a negative regulator of PVX replication and movement in the early stages of viral infection and that it interacts with PVX SL RNA and CP.

Acknowledgments

This research was supported in part by Grants from the National Research Foundation (Grant No. 20110012328) funded by the Ministry of Education, Science, and Technology (MEST), the Vegetable Breeding Research Center (No. 710001-03) through the Agriculture Research Center program from the Ministry for Food, Agriculture, Forestry and Fisheries; and the Next-Generation BioGreen 21 Program of Rural Development Administration (No. PJ00819801). W.K.C. was supported by a post-graduate research fellowship from the Brain Korea 21 Project.

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