Expression and Localization Patterns of a Small Heat Shock Protein that Interacts with the Helicase Domain of Cucumber Green Mottle Mosaic Virus

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ABSTRACT

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus* (family Virgaviridae), is an economically important virus that has detrimental effects on cucurbit crops worldwide. Understanding the interaction between host factors and CGMMV viral proteins will facilitate the design of new strategies for disease control. In this study, a yeast two-hybrid assay revealed that the CGMMV helicase (HEL) domain interacts with a *Citrullus lanatus* small heat shock protein (sHSP), and we verified this observation by performing in vitro GST pull-down and in vivo coimmunoprecipitation assays. Measurement of the levels of accumulated sHSP transcript revealed that sHSP is upregulated on initial CGMMV infection in both *Nicotiana benthamiana* and

C. lanatus plants, although not in the systemically infected leaves. We also found that the subcellular localization of the sHSP was altered after CGMMV infection. To further validate the role of sHSP in CGMMV infection, we produced and assayed *N. benthamiana* transgenic plants with up- and down-regulated sHSP expression. Overexpression of sHSP inhibited viral RNA accumulation and retarded disease development, whereas sHSP silencing had no marked effect on CGMMV infection. Therefore, we postulate that the identified sHSP may be one of the factors modulating host defense mechanisms in response to CGMMV infection and that the HEL domain interaction may inhibit this sHSP function to promote viral infection.

Cucumber green mottle mosaic virus (CGMMV) is an economically important virus that causes severe losses in cucurbit crops worldwide (Reingold et al. 2016). Cucurbit plants infected by CGMMV exhibit green mottling and mosaic on their leaves, and their fruits are unmarketable because of malformations, flesh sponginess, rotting, yellowing, and dirty red discoloration (Dombrovsky et al. 2017). Understanding the interaction between host factors and CGMMV viral proteins would contribute to the design of new strategies for the control of this disease.

As obligate parasites with very small genomes, plant pathogenic viruses have evolved complex interactive networks with their hosts to complete their lifecycles. For the virus to achieve successful infection, it is necessary that the interactions between viral and host factors proceed in a specific manner. This includes viral genome amplification, cell-to-cell and systemic movement, disease symptom development, and defense against host antiviral responses (García and Pallás 2015; Whitham and Wang 2004). Characterizing the specific interactions between viral and host proteins can shed light on how viruses utilize and alter cellular resources and how hosts respond to virus invasion.

CGMMV, a member of the genus *Tobamovirus* in the family Virgaviridae, possesses a 6,423-nucleotide single-stranded positive-sense RNA genome with four open reading frames (ORFs) encoding four proteins. The two coterminal 5' proximal ORFs encode the

129- and 186-kDa proteins required for efficient viral replication. Both replicase proteins possess an N-terminal methyltransferase (MT) domain and a 129-kDa C-terminal helicase (HEL) domain that are separated by a unique intervening region (IR) (Ishibashi and Ishikawa 2016; Rozanov et al. 1992). The longer 186-kDa replicase is expressed by suppression of a UAG termination codon and encodes an RNA-dependent RNA polymerase (RdRp) motif at its C-terminal domain (Beier et al. 1984). During replication, CGMMV produces two 3' coterminal subgenomic messenger RNAs (mRNAs) that are translated into two additional proteins, a cell-to-cell movement protein and a coat protein (Ugaki et al. 1991).

The CGMMV HEL domain is one of the domains shared by both the 129- and 186-kDa proteins. HEL domains play an important role in unwinding the double-stranded helical structure of nucleic acids by using the free energy of NTP hydrolysis. They are ubiquitous in many organisms, including viruses, and they are involved in all aspects of nucleic acid metabolism, including gene replication, transcription, translation, repair, and recombination (Abdelhaleem 2009). HEL domains have been classified into six superfamilies (SFs; SF1 to SF6) according to their motifs, amino acid sequence, and spacing (Gorbalenya and Koonin 1993; Singleton et al. 2007). The tobamovirus HEL domain belongs to the SF1 group and interacts with host proteins associated with host defense responses, viral symptom development, and replication (Goregaoker and Culver 2003; Ishibashi et al. 2010). Tobacco mosaic virus (TMV) suppresses salicylic acid (SA)-mediated host basal defenses through the interaction between its HEL domain and the host NAC domain transcription factor ATAF2 (Wang et al. 2009). The HEL domains of TMV and tobacco etch virus have been found to interact with a plant ortholog of P58^{IPK}, and this interaction has been proposed to activate the P58^{IPK} ortholog to enhance virus multiplication by preventing PKR-mediated translational suppression and apoptosis (Bilgin et al. 2003; Goodman et al. 2009). The TMV HEL domain has also been found to interact with the host AUX/IAA proteins, and it suppresses their function to avoid host age-related resistance and consequently, enhance TMV infection (Padmanabhan et al. 2008). Furthermore, by interacting with the N protein in an ATP-dependent manner, the TMV HEL domain also

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functions as an elicitor of N-mediated resistance (Les Erickson et al. 1999; Ueda et al. 2006).

Tomato Tm-1, which confers resistance to tobamoviruses, interacts with the HEL domain of tomato mosaic virus (ToMV), although not with that of a ToMV mutant that can overcome tomato resistance, suggesting that Tm-1 inhibits ToMV replication by binding to the viral HEL domain (Ishibashi et al. 2007). TOM1, which attaches to the tobamovirus replication complex by interacting with the viral HEL domain, facilitates the tethering of viral replication proteins to membranes. Knockout of TOM1 and its homolog TOM3 in *Arabidopsis thaliana* and *Nicotiana tabacum* inhibits tobamovirus multiplication, suggesting that TOM1 plays an important role in viral replication by interacting with the HEL domain (Asano et al. 2005; Fujisaki et al. 2006). The foregoing observations accordingly indicate that, by interacting with certain host factors, the HEL domain plays multiple roles in tobamovirus infection.

Plant heat shock proteins (HSPs) are ubiquitous proteins present in plant cells. They are stress-related proteins that participate in a broad range of cellular biological processes, including the modulation of cellular immune responses and the mediation of substrate–protein folding, refolding, oligomeric assembly, translocation, and degradation (Hartl et al. 2011). The small heat shock protein (sHSP) family is one of the most abundant and complex subsets among the five known classes of HSPs (HSPp100/ClpB, HSP90, HSP70/DnaK, HSP60/GroEL, and sHSP), and it is characterized by a conserved α -crystallin domain from amino acids 80 to 100 in its C-terminal region (Haslbeck et al. 2005; Wang et al. 2004). The monomeric size of sHSPs ranges between 12 and 42 kDa; however, in most cases, sHSPs form large oligomers containing 8 to 32 subunits, which show higher substrate affinity under stress and bind to nonnative proteins of similar weight (Basha et al. 2010; Tyedmers et al. 2010). The key function of sHSPs is to prevent nonnative protein aggregation, and in this regard, they can create a transient reservoir of substrates by forming a soluble complex with nonnative proteins for their subsequent refolding by ATP-dependent chaperone systems (Lee et al. 1997; Mchaourab et al. 2009). Furthermore, it is well known that sHSPs can stabilize several intracellular proteins, including defense-related signaling components, through their chaperone activity (Ahmed et al. 2015). In response to stress, the sHSPs interact with unfolded RNA binding proteins and their protected mRNAs, forming heat shock granules in the cell cytoplasm (Nover et al. 1989). sHSPs are commonly associated with abiotic stress conditions, such as heat, drought, cold, heavy metal, and oxidative stress; however, some plant sHSPs have been reported to play a role in defense against infection by fungi, bacteria, and nematodes (Ahmed et al. 2015; Escobar et al. 2003; Maimbo et al. 2007). Although a number of previous studies have reported on the involvement of plant HSP70s and HSP90s in viral infection (Mine et al. 2012), there have been very few such studies on sHSPs. sHSPs have, nevertheless, been shown to be induced in response to infection by diverse RNA viruses in susceptible *A. thaliana* plants (Whitham et al. 2003). Similarly, the interaction between rice stripe virus (RSV) Pc4 protein and a rice sHSP may be necessary for maintaining a denatured Pc4 competent for transport through the plasmodesmata (Lu et al. 2009). Furthermore, the RSV RdRp protein has been shown to interact with an sHSP, thereby altering the expression pattern and subcellular distribution of the sHSP (Li et al. 2015).

Although at present, the identity of the host factors that interact with CGMMV proteins remains unknown, in this study, we identified an interaction between the HEL domain of CGMMV and a host sHSP, and we found that expression of the sHSP is significantly upregulated on initial CGMMV infection. We also showed that overexpression of *Nicotiana benthamiana* small heat shock protein (NbsHSP) in transgenic *N. benthamiana* inhibits CGMMV accumulation and delays symptom development, which provides compelling evidence that the interaction between the

CGMMV HEL domain and the host sHSP may promote viral infection by inhibiting host sHSP function in defense responses.

MATERIALS AND METHODS

Plants and virus inoculation. All plants used in this study were grown in 10-cm pots filled with a mixture of meadow soil and vermiculite and maintained in growth chambers at 26°C under a 16/8-h light/dark regime and 70% RH. The CGMMV infectious clone C284R used in this study was constructed in our laboratory (Liu et al. 2017). *N. benthamiana* plants at the eight-leaf stage and *Citrullus lanatus* ‘Zhongke 6’ plants at the expanded cotyledon stage were infected with C284R via the genus *Agrobacterium*-mediated inoculation as described previously (Liu et al. 2017). The mock plants were similarly infected via the genus *Agrobacterium*-mediated inoculation using the empty vector used in constructing the infectious clone C284R. For mechanical inoculation, 1 g of fresh *N. benthamiana* leaves infected with CGMMV were ground in 10 ml of inoculation buffer. Approximately 4-week-old wild-type (WT) or transgenic *N. benthamiana* plants were inoculated mechanically and then maintained at 26°C. Mock plants were inoculated mechanically with a homogenate of healthy *N. benthamiana* leaves. At sampling time, leaves were collected from the plants, frozen, and stored at –80°C until use.

Complementary DNA library construction. A *C. lanatus* leaf-specific complementary DNA (cDNA) library was constructed as pray from *C. lanatus* leaves infected with CGMMV following the procedures outlined in the Make Your Own “Mate & Plate” Library System User Manual (Clontech). A CGMMV cDNA fragment (nucleotides 2,635 to 3,399) encoding the full-length HEL domain was amplified using 2× Fast Pfu Master Mix (Novoprotein) and then inserted into the *Sma*I site of a yeast GAL4 binding domain pGBKT7 (BD) vector using an In-Fusion HD Cloning Kit (Takara) to create the recombinant bait plasmid BD-HEL. The plasmids BD-129, BD-MT, and BD-IR were constructed using the same procedure as used for BD-HEL. The coding sequences of *ClisHSP* and *NbsHSP* were amplified from *C. lanatus* and *N. benthamiana* cDNA using the primer pairs AD-*ClisHSP*-F/AD-*ClisHSP*-R and AD-*NbsHSP*-F/AD-*NbsHSP*-R, respectively, and then, they were inserted into the *Sma*I site of a yeast GAL4 transcriptional activation domain (AD) pGADT7 vector using the In-Fusion HD Cloning Kit to create the recombinant pray plasmids AD-*ClisHSP* and AD-*NbsHSP*.

Yeast transformation and library screening. Yeast transformation and library screening were conducted in accordance with the Yeastmaker Yeast Transformation System 2 and Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech). The positive candidate plasmids containing the *C. lanatus* cDNAs were isolated and sequenced. To confirm the protein–protein interactions in yeast, the bait and pray plasmids were cotransformed into *Saccharomyces cerevisiae* strain Y2HGOLD (Gietz and Woods 2002). Cotransformants were initially plated on synthetic defined medium lacking leucine and tryptophan with 40 µg/ml X- α -Gal (SD/-Leu/-Trp/X- α -Gal) medium, and positive yeast colonies were subsequently transferred onto higher-stringency SD/-Ade/-His/-Leu/-Trp/X- α -Gal medium. Meanwhile, AD-T and BD-53 were cotransformed as a positive control, and AD-T and BD-Lam were cotransformed as a negative control. Three independent experiments were performed to confirm these results.

In vitro pull-down assay. For the in vitro pull-down assay, the coding sequence of the HEL domain was amplified with the primer pair PD-HEL-F/PD-HEL-R using the plasmid BD-HEL as a template and then inserted into the *Sma*I site of a pGEX-6p-1 vector using the In-Fusion HD Cloning Kit to create the recombinant plasmid pGEX-6p-HEL. Similarly, the coding sequences of *ClisHSP* and *NbsHSP* were amplified with the primer pairs PD-*ClisHSP*-F/PD-*ClisHSP*-R or PD-*NbsHSP*-F/PD-*NbsHSP*-R using the plasmid AD-*ClisHSP* or AD-*NbsHSP* as a template, respectively,

and then, they were inserted into the *NcoI/XhoI* sites of a pET-28a vector using the In-Fusion HD Cloning Kit to create the recombinant plasmids pET-28a-ClhHSP and pET-28a-NbsHSP. The recombinant plasmids were transformed into chemically competent *Escherichia coli* *Transtetta* (DE3) cells (Transgen). The target proteins GST-HEL, His-ClhHSP, and His-NbsHSP and the control proteins GST and His were expressed after induction by adding 0.5 mmol/liter isopropyl- β -D-thiogalactoside (IPTG) to the medium, and then, they were purified using GST Sefinose Resin and an Ni-NTA-Sefinose Column (Sangon), respectively. Equal amounts of purified protein (e.g., GST and His-NbsHSP, GST-HEL and His-NbsHSP, GST-HEL and His, GST and His-ClhHSP, GST-HEL and His-ClhHSP, and GST and His) were mixed and incubated for 2 h at 4°C in a glutathione sefinose resin prepacked column. The resin was washed three times with 5 ml binding/wash buffer, and the GST-tagged proteins were eluted with 2 ml elution buffer. The eluted proteins were analyzed by western blotting as described previously (Jiang et al. 2014). Briefly, protein samples were separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred onto 0.22- μ m Immobilon-NC Transfer Membranes (Solarbio) by wet electroblotting, and detected with anti-GST or anti-6X His tag antibodies (Abcam) and horseradish peroxidase (HRP)-goat antirabbit immunoglobulin G secondary antibody (EarthOx). The antigen-antibody complexes were flashed using an EasySee Western Blot Kit (Transgen) in accordance with the manufacturer's instructions and imaged using a Chemiluminescence Imaging System (Tanon).

In vivo coimmunoprecipitation assay. For the in vivo coimmunoprecipitation (Co-IP) assay, the fusion fragments FLAG-HEL, cMYC-NbsHSP, and cMYC-ClhHSP were obtained after two PCR amplifications with primer pairs CI-HEL-F/CI-HEL-R and CI-FLAG-F/CI-HEL-R, CI-NbsHSP-F/CI-NbsHSP-R and CI-cMYC-F/CI-NbsHSP-R, and CI-ClhHSP-F/CI-ClhHSP-R and CI-cMYC-F/CI-ClhHSP-R, respectively. Thereafter, fragments were inserted into the *NcoI/BstEII* sites of a pCambia1302 vector using the In-Fusion HD Cloning Kit to create the recombinant plasmids pCambia1302-FLAG-HEL, pCambia1302-cMYC-NbsHSP, and pCambia1302-cMYC-ClhHSP. These recombinant plasmids were transformed into *A. tumefaciens* strain GV3101. GV3101 strains carrying pCambia1302-FLAG-HEL, pCambia1302-cMYC-NbsHSP, pCambia1302-cMYC-ClhHSP, pCambia1302-FLAG-HEL/pCambia1302-cMYC-NbsHSP, and pCambia1302-FLAG-HEL/pCambia1302-cMYC-ClhHSP mixture were infiltrated into *N. benthamiana* leaves. After transient expression for 72 h, the infiltrated leaves were harvested and used for total protein extraction. The in vivo Co-IP assay was performed using a Pierce Immunoprecipitation (Co-IP) Kit following the manufacturer's recommendations (Thermo). Briefly, anti-FLAG and anti-cMYC antibodies (Abcam) were immobilized onto Aminolink Plus coupling resin prepacked in a Pierce spin column. Extracted total protein was added to the appropriate resin and incubated overnight at 4°C with gentle mixing. The following day, the resin was washed three times with 200 μ l of immunoprecipitation (IP) lysis/wash buffer and eluted with 10 μ l of elution buffer. Eluted samples were analyzed by western blotting using anti-cMYC and anti-FLAG antibodies.

Subcellular localization assay. The plant expression vectors pBWA-mKATE and pBI121-GFP (green fluorescent protein) were stored in our laboratory. The coding sequences of *ClhHSP* and *NbsHSP* were amplified using primer pairs Sub-ClhHSP-F/Sub-ClhHSP-R and Sub-NbsHSP-F/Sub-NbsHSP-R. Thereafter, the fragments were inserted into the *SmaI* site of a pBWA-mKATE vector using the In-Fusion HD Cloning Kit to create the recombinant plasmids pBWA-ClhHSP-mKATE and pBWA-NbsHSP-mKATE. The coding sequence of the HEL domain was amplified with the primer pair Sub-HEL-F/Sub-HEL-R and then, inserted into the *XbaI* site of a pBI121-GFP vector using the In-Fusion HD Cloning Kit to create the recombinant plasmid pBI121-HEL-GFP. The aforementioned recombinant plasmids were transformed into *A. tumefaciens* strain GV3101, and GV3101 strains carrying

pBWA-ClhHSP-mKATE or pBWA-NbsHSP-mKATE were infiltrated into healthy and CGMMV-infected *N. benthamiana* leaves. pBI121-HEL-GFP and the mixture containing pBWA-ClhHSP-mKATE/pBI121-HEL-GFP or pBWA-NbsHSP-mKATE/pBI121-HEL-GFP were infiltrated into healthy *N. benthamiana* leaves. After infiltration for 72 h, the fluorescence patterns in infiltrated leaves were analyzed using a Leica confocal laser-scanning microscope (Leica). The excitation wavelengths used for mKATE and GFP were 543 and 488 nm, respectively, and the emitted light from mKATE and GFP was captured between 520 and 580 nm, respectively, and 500 and 550 nm, respectively.

Stable plant transformation. To construct an NbsHSP overexpressing vector, the coding sequence of *NbsHSP* was amplified with the primer pair Overex-NbsHSP-F/Overex-NbsHSP-R using the plasmid AD-NbsHSP as a template, and the amplified fragment was then cloned into a pCambia1300 vector to generate pCambia-Overex-NbsHSP. An NbsHSP hairpin structure consisting of two reversed partial sequences of NbsHSP (corresponding to the amino acid sequence from residue 38 to 110) and a noncoding intron were cloned into the pCambia1300 vector to construct an NbsHSP silencing vector (pCambia-RNAi-NbsHSP). All constructs were introduced into *A. tumefaciens* strain EHA105. The genus *Agrobacterium*-mediated transformation of *N. benthamiana* was performed by following a previously published protocol (Horsch et al. 1985). Transgenic *N. benthamiana* seedlings were initially selected on Murashige and Skoog medium supplemented with 50 μ g/ml kanamycin. Two weeks thereafter, kanamycin-resistant seedlings were transferred to 10-cm pots filled with a mixture of meadow soil and vermiculite and grown to maturity in a greenhouse. T₁ plants containing overexpressed or silenced *NbsHSP*, as confirmed by quantitative reverse transcription PCR (qRT-PCR), were used for CGMMV inoculations.

RNA analysis. Total RNA was extracted from *N. benthamiana* and *C. lanatus* leaves using RNAiso Plus (Takara) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) analyses for confirmation of CGMMV systemic infection were performed after the synthesis of first-strand cDNA using HEL-R in accordance with the protocol of a Fastking RT Kit (Tiangen). PCR was performed using the primer pair HEL-F/HEL-R to amplify a 765-bp fragment.

For northern blot analyses, the DNA probe used for detecting CGMMV RNA was synthesized as described previously (Liu et al. 2017). The procedures were performed using a DIG Northern Starter Kit according to the manufacturer's instructions (Roche). Hybridization was assessed using the Chemiluminescence Imaging System (Tanon).

The expression of sHSPs was detected by real-time qRT-PCR using the primer pairs RT-ClhHSP40-F/RT-ClhHSP40-R, RT-ClhHSP65-F/RT-ClhHSP65-R, RT-ClhHSP12-F/RT-ClhHSP12-R, RT-ClhHSP97-F/RT-ClhHSP97-R, RT-ClhHSP45-F/RT-ClhHSP45-R, and RT-NbsHSP-F/RT-NbsHSP-R. The internal reference primers CICAC-F/CICAC-R (*C. lanatus Actin*) and NbUBC-F/NbUBC-R (*N. benthamiana Ubiquitin Conjugating Enzyme*) were designed based on previous studies (Kong et al. 2014; Schmidt and Delaney 2010) (Supplementary Table S1). The qRT-PCRs were performed using a Roche LightCycler 480II Real-Time PCR system (Roche), and the amplified products were analyzed using the $\Delta\Delta C_T$ method.

RESULTS

The CGMMV HEL domain interacts with a host sHSP in yeast. To identify the host factor(s) interacting with the CGMMV HEL domain during viral infection, a *C. lanatus* leaf-specific cDNA library was constructed from watermelon seedlings infected with CGMMV. The library titer and recombination rate were 5.4×10^8 CFU/ml and 100%, respectively. The average length of the inserted fragments was ~ 1 kbp. These results indicated that we had constructed a high-quality library that could be used for screening.

We then inserted the CGMMV HEL coding region into the vector pGBKT7 (pGBKT-HEL) for expression as a GAL4 fusion and used this as the bait to screen the watermelon cDNA library. We accordingly succeeded in identifying a single positive clone. The nucleotide sequence of this clone was blasted in the cucurbit genomic database and showed 100% homology with a watermelon gene (Cla017945) that presumably encodes an sHSP, which we designated ClsHSP (GenBank accession number HQ681888). We cloned the coding region of ClsHSP and that of the closest homolog of this sequence in *N. benthamiana* (designated *NbsHSP*; XM_016604898.1). Using a yeast two-hybrid assay, we demonstrated that NbsHSP interacts with the viral HEL domain, and we similarly confirmed positive interaction between the full-length ClsHSP and the HEL domain (Fig. 1A). Because the HEL domain is one of the domains of the 129-kDa protein, we further examined the interaction between the sHSP and the 129-kDa protein, MT domain, or IR region. The results showed that, whereas sHSP interacts with the HEL domain and the 129-kDa protein, no interaction could be detected between this sHSP and either the MT domain or the

IR region, which indicates the specificity of the interaction between the sHSP and the HEL domain during CGMMV infection (Fig. 1B).

GST pull-down and Co-IP analyses of the interaction between the CGMMV HEL domain and sHSP. The interaction between the CGMMV HEL domain and the sHSP was further confirmed based on an in vitro GST pull-down assay. The GST-tagged HEL (GST-HEL) domain and the His-tagged sHSP (His-ClhHSP or His-NbsHSP) were expressed in *Transtetta* (DE3) cells. Equal amounts of purified GST-HEL and His-sHSP were mixed and then pulled down with GST binding beads followed by western blotting using anti-GST or anti-His antibodies. In these experiments, the combinations of GST/His-ClhHSP, GST/His-NbsHSP, GST-HEL/His, and GST/His were used as controls. The results of these experiments showed that only GST-HEL could pull down His-sHSP (Fig. 2A), thereby demonstrating the in vitro interaction between the CGMMV HEL domain and the sHSP.

Subsequently, we used a Co-IP assay to examine the in vivo interaction between the two proteins. Constructs designed to express FLAG-HEL, cMYC-ClhHSP, cMYC-NbsHSP, FLAG-HEL/

Fig. 1. Interactions of a *Citrullus lanatus* small heat shock protein (ClsHSP) or a *Nicotiana benthamiana* small heat shock protein (NbsHSP) and cucumber green mottle mosaic virus (CGMMV) 129-kDa protein (129) or its different domains in cotransformed yeast cells. **A**, Autoactivation of ClsHSP and NbsHSP and interaction between ClsHSP or NbsHSP and the CGMMV helicase (HEL) domain. **B**, Interaction between ClsHSP or NbsHSP and 129 or its different domains. *Saccharomyces cerevisiae* strain Y2HGOLD was cotransformed with the plasmids shown in the right panel and grown on synthetic defined medium lacking adenine, histidine, leucine, and tryptophan with 40 µg/ml X-α-Gal (SD/-Ade/-His/-Leu/-Trp/X-α-Gal) at three different concentrations.

cMYC-ClHSP, and FLAG-HEL/cMYC-NbsHSP were the genus *Agrobacterium* infiltrated into *N. benthamiana* leaves, and total proteins were extracted from *N. benthamiana* leaves and subsequently used in Co-IP assays at 72 h postinfiltration. As shown in Figure 2B, FLAG-HEL coimmunoprecipitated with either cMYC-ClHSP or cMYC-NbsHSP. The interactions were confirmed by performing the reciprocal experiment, which duly indicated that cMYC-ClHSP or cMYC-NbsHSP could coimmunoprecipitate with FLAG-HEL. These results, therefore, provide convincing evidence that CGMMV HEL also interacts with ClHSP or NbsHSP in vivo, further supporting a biologically relevant interaction between the virus and plant proteins.

sHSPs are induced in the area of initial CGMMV infection. The *C. lanatus* genome contains 20 putative genes that harbor an sHSP domain, 11 of which are class I sHSPs. We used RT-PCR to analyze the expression of these 11 genes in healthy and CGMMV-infected plants, and we reliably detected transcripts for five of the 11 class I sHSPs, whereas the other six were undetectable in either CGMMV- or mock-infected watermelon plants, which can probably be attributed to their specific induction under different conditions and/or in other tissues. To confirm whether CGMMV infection can induce the expression of host sHSPs, we used qRT-PCR to examine changes in the expression patterns of these five endogenous sHSPs in CGMMV-inoculated *C. lanatus* leaves at 2 days postinoculation (dpi). The results indicated that three sHSPs were

upregulated, whereas the remaining two exhibited no obvious change in expression. Among the three sHSPs with increased expression, ClsHSP (Cla017945) showed the highest expression, with a sixfold change compared with the mock-inoculated plants. However, at 14 dpi, when CGMMV symptoms initially appeared on systemically infected *C. lanatus* plants, the three sHSPs exhibited no obvious change in expression (Fig. 3A). We also examined the expression level of NbsHSP in CGMMV-inoculated *N. benthamiana* leaves at 2 dpi and systemically leaves at 7 dpi, when CGMMV symptoms initially appeared on the latter leaves, and we found that NbsHSP expression was highly consistent with that of ClsHSP (Fig. 3B). These results accordingly indicate that sHSPs are transcriptionally induced in the area of initial CGMMV infection but not in systemically infected leaves.

The CGMMV HEL domain affects sHSP subcellular localization through interaction. To assay the subcellular localization of ClsHSP and NbsHSP, ClsHSP or NbsHSP fused to mKATE at their C termini was transiently expressed in healthy or CGMMV-infected *N. benthamiana* epidermal cells. Observation of mKATE fluorescence revealed that both proteins formed numerous granules in the cytoplasm of the healthy cells, whereas we were unable to detect these granules in CGMMV-infected cells (Fig. 4A). We also inserted the cDNA sequence of the HEL domain into the pBI121-GFP vector, and after transient expression in *N. benthamiana* epidermal cells, we observed that GFP fluorescence was localized in

Fig. 2. GST pull-down and coimmunoprecipitation (Co-IP) analyses of the interaction between cucumber green mottle mosaic virus (CGMMV) helicase (HEL) domain and a small heat shock protein (sHSP). **A**, GST pull-down analysis of the interactions between the CGMMV HEL domain and *Citrullus lanatus* sHSP (ClsHSP) or *Nicotiana benthamiana* sHSP (NbsHSP). A GST-HEL fusion protein or GST was expressed in *Escherichia coli*, and after extraction, it was mixed with equal amounts of purified His-ClHSP, NbsHSP, or His and then, pulled down with GST binding beads followed by western blotting using anti-GST (upper panel) or anti-His (lower panel) antibodies. **B**, Co-IP analysis of the interactions between the CGMMV HEL domain and ClsHSP or NbsHSP. Total protein was extracted from *N. benthamiana* leaves transiently expressing FLAG-HEL, cMYC-ClHSP, cMYC-NbsHSP, FLAG-HEL/cMYC-ClHSP, or FLAG-HEL/cMYC-NbsHSP and immunoprecipitated with anti-FLAG or anti-cMYC antibodies followed by western blotting using anti-FLAG (upper panels) or anti-cMYC (lower panels) antibodies. IB, immunoblot; IP, immunoprecipitation.

both the nucleus and cytoplasm (Fig. 4B). To further establish whether the change in sHSP distribution is related to an interaction with the HEL domain, the latter was coexpressed with ClsHSP or NbsHSP in *N. benthamiana* epidermal cells, and we found that the distributions of ClsHSP and NbsHSP were consistent with their distribution in the CGMMV-infected *N. benthamiana* epidermal cells (Fig. 4C). Whereas the HEL domain and sHSP were colocalized in the cytoplasm, coexpression had no influence on the nuclear localization of the HEL domain, suggesting that the interaction occurs specifically in the cytoplasm. These results accordingly indicate that, by interacting with a host sHSP, the CGMMV HEL domain can affect the subcellular localization of this sHSP.

Alteration of NbsHSP expression affects CGMMV symptom development and RNA accumulation. *N. benthamiana* is an excellent experimental host for CGMMV, providing a simple genetic transformation system for studying the function of proteins interacting with CGMMV. To confirm the function of sHSPs during CGMMV infection, we generated NbsHSP-silenced transgenic *N. benthamiana* (RNAi-NbsHSP) plants and NbsHSP overexpressing transgenic *N. benthamiana* (35S-NbsHSP) plants. Under the growth chamber conditions used in this study, RNAi-NbsHSP and 35S-NbsHSP transgenic *N. benthamiana* plants showed normal growth comparable with that of WT plants. The results of qRT-PCR indicated that the expression level of NbsHSP in RNAi-NbsHSP *N. benthamiana* plants was significantly suppressed, whereas it increased significantly in 35S-NbsHSP *N. benthamiana* plants relative to that in the WT plants (Fig. 5A). For the purposes of subsequent research, we selected a single line for which the sHSP silencing efficiency was highest among the 12 RNAi-NbsHSP lines and one line for which sHSP expression was highest among the 15 35S-NbsHSP lines.

To analyze the role of the sHSP during CGMMV infection, we inoculated WT, RNAi-NbsHSP, and 35S-NbsHSP transgenic *N. benthamiana* plants via mechanical inoculation or the genus *Agrobacterium* infiltration of an infectious clone. We found that symptoms of CGMMV appeared on systemically infected leaves of RNAi-NbsHSP transgenic and WT *N. benthamiana* plants at 6 dpi.

Moreover, we detected similar levels of viral RNA accumulation in these plants. In contrast, 35S-NbsHSP transgenic plants were symptomless until 11 dpi, at which time the symptoms in RNAi-NbsHSP transgenic and WT plants were already severe (Fig. 5B). The results of a northern blot assay were consistent with the development of disease symptoms. At 11 dpi, when the 35S-NbsHSP transgenic plants showed the initial symptoms of disease, CGMMV RNAs were detected in the 35S-NbsHSP transgenic plants, although their accumulation was lower than that in WT and RNAi-NbsHSP transgenic plants (Fig. 5C). These results were reproducible using either mechanical inoculation or *Agrobacterium tumefaciens* mediated inoculation, implying either that any effects of *Agrobacterium tumefaciens* did not affect the findings or that no such effect occurred. Collectively, our results clearly indicate that sHSP overexpression affects the accumulation of CGMMV RNA and the subsequent development of disease symptom in infected plants.

DISCUSSION

During pathogens invasion, plants utilize a wide range of responses as protective measures against infection. Expression of HSPs is one of the effective responses that enable plant cells to adapt to environmental changes and survive adverse conditions (Gupta et al. 2010). To establish successful infection, pathogens use a series of countermeasures to suppress host defenses. Characteristically, bacteria and fungi introduce effector molecules into plant cells to modulate cellular functions and thereby, disrupt host defense responses (Ahmed et al. 2016). However, viruses, which are obligate parasites, will also adopt a variety of strategies to promote their multiplication and dispersal. In this study, using yeast two-hybrid assays, we detected a host sHSP that interacts with the HEL domain of CGMMV. This interaction was further verified both in vitro and in vivo. On the basis of these observations, we subsequently performed a number of experiments to elucidate the role of this sHSP during CGMMV infection. We accordingly demonstrated that changes in the distribution pattern of the sHSP were closely associated with expression of the viral HEL domain,

Fig. 3. Relative expression levels of small heat shock protein (sHSP) transcripts in the inoculated and systemically infected leaves of *Citrullus lanatus* and *Nicotiana benthamiana* plants infected with cucumber green mottle mosaic virus (CGMMV). **A**, Relative transcript accumulation levels of five sHSPs in the inoculated and systemically infected leaves of CGMMV-infected *C. lanatus* at 2 and 14 days postinoculation (dpi). **B**, *N. benthamiana* NbsHSP expression level in the inoculated and systemically infected leaves of CGMMV-infected *N. benthamiana* at 2 and 7 dpi. Experiments were repeated three times.

which indicates that, as a new countermeasure against host defense response, CGMMV alters host protein biological functions by disturbing their subcellular distribution. To the best of our knowledge, there have been no previous descriptions of this type of host modulation with respect to tobamovirus infection.

HSPs occur ubiquitously in plant and animal cells and are essential components involved in the control of cellular homeostasis under both optimal and adverse growth conditions. They function in protein folding, assembly, translocation, and degradation during normal cellular growth and development, and they are also responsible for protein stabilization and refolding in cells under stress (Lindquist and Craig 1988; Wang et al. 2004; Whitley et al. 1999). sHSPs are generally considered to be stress-related defense proteins that respond to a range of abiotic or biotic stress conditions (Efeoglu

2009). They are known to stabilize several intracellular proteins, including defense-related signaling components, through their chaperone activity (Ahmed et al. 2015). sHSPs are also referred to as “holdases,” the characteristic features of which include acting as ATP-independent molecular chaperones that bind to denatured proteins or newly synthesized proteins to prevent them from forming irreversible aggregations under stress conditions (Zhang et al. 2015).

Although under normal growth conditions, sHSPs are generally undetectable in vegetative tissues, they can be significantly induced by environmental stresses or developmental stimuli (Sun et al. 2002). Previously, it has been shown that, in *Oryza sativa* and *A. thaliana*, host sHSP transcript levels increase after infection by different viruses, indicating that the induction of host sHSP expression

Fig. 4. Subcellular localization of *Citrullus lanatus* small heat shock protein (ClshHSP), *Nicotiana benthamiana* small heat shock protein (NbsHSP), and cucumber green mottle mosaic virus (CGMMV) helicase (HEL) domain. **A**, Subcellular localization of ClshHSP and NbsHSP in healthy or CGMMV-infected *N. benthamiana* epidermal cells. **B**, Subcellular localization of the HEL domain in *N. benthamiana* epidermal cells. **C**, Subcellular localization of ClshHSP and NbsHSP coexpressed with the HEL domain.

might be a common response during viral infection (Li et al. 2015; Whitham et al. 2003). sHSPs also play a fundamental role in protecting animal cells during viral infection (Piotrowska et al. 2010). In this study, we found that host plant sHSPs are upregulated in the area of initial CGMMV infection, which is consistent with the findings of previous studies that they have demonstrated the upregulation of these proteins in response to viral infection in *O. sativa* and *A. thaliana*. Nevertheless, we found that, in systemically infected leaves, sHSP expression returns to normal levels concomitant with development of the initial disease symptoms, which indicates that sHSPs may play roles associated with the host basal defense response to CGMMV infection.

Previously, there have been a few studies that have examined the interaction of sHSPs with plant viruses. An sHSP interacts with the RSV Pc4 protein, and this interaction may contribute to maintaining the denatured Pc4 competent for transport through plasmodesmata. Moreover, the presence of the sHSP would facilitate the re-naturation of viral material on entering an adjacent cell (Lu et al. 2009). The interaction between a host sHSP and the RSV RdRp has also been found to alter the subcellular localization of the sHSP, and it suppresses the formation of stress-related granules in the cytoplasm, which may represent a mechanism whereby the virus disrupts host antiviral responses (Li et al. 2015). Consistent with the findings of Li et al. (2015), a subcellular localization assay performed in this study revealed that sHSPs formed granules in healthy cells and that the distribution was altered in response to

expression of the HEL domain within these cells. In response to stress, sHSPs form granules by interacting with their protected proteins and mRNAs to maintain plant fitness. It can accordingly be hypothesized that, during CGMMV infection, inhibition of granule formation may be an effective mechanism whereby the virus antagonizes host defense responses; however, whether the host proteins protected by the sHSP are exploited for the benefit of viral infection must await further clarification.

We also demonstrated that sHSP silencing in NbsHSP transgenic *N. benthamiana* plants had no significant effect on CGMMV infection, with transgenic plants showing symptoms and viral RNA accumulation similar to those observed in WT plants. It is conceivable that sHSP functional redundancy may underlie this phenotype, because there are numerous sHSP homologs that may perform similar functions. However, compared with WT plants inoculated with CGMMV, those plants overexpressing NbsHSP showed delayed systemic symptoms, and northern blot results revealed that CGMMV RNA accumulation was negatively correlated with sHSP expression. Thus, upregulation of plant sHSP expression may be an effective plant defense strategy deployed in response to CGMMV infection.

Additional roles of sHSPs in host defense responses have been suggested based on the findings of studies showing that sHSPs can activate autophagy in mammalian cells (Liang et al. 2018), which is known to contribute to innate immune responses through the elimination of intracellular pathogens, including viruses. Previous

Fig. 5. Cucurbit green mottle virus (CGMMV) symptom development and RNA accumulation in transgenic and wild-type (WT) *Nicotiana benthamiana* plants. **A**, Relative expression levels of *N. benthamiana* small heat shock protein (NbsHSP) transcripts in RNAi-NbsHSP, WT, and 35S-NbsHSP *N. benthamiana* plants. **B**, Symptoms in systemically infected leaves of 35S-NbsHSP, WT, and RNAi-NbsHSP *N. benthamiana* plants inoculated with CGMMV at 11 days postinoculation (dpi). **C**, Northern blot analysis of CGMMV RNA accumulation in the systemically infected leaves of 35S-NbsHSP, WT, and RNAi-NbsHSP *N. benthamiana* plants inoculated with CGMMV at 11 dpi.

studies have shown that autophagic mechanisms are antagonized or even exploited by a number of animal viruses (Dong and Levine 2013), and autophagy has begun to be studied with respect to plant-virus interactions (Cheng and Wand 2016; Hafren et al. 2017, 2018). sHSPs can induce the catalytic activity of the 26S proteasome, which degrades ubiquitinated proteins under stress conditions (Lanneau et al. 2010), and in yeast, the 26S proteasome has been found to be degraded by autophagy-related protein 8-mediated autophagy (Marshall et al. 2016). Accordingly, the possibility that the sHSP interactions identified for CGMMV in this study are indirectly related to host autophagy via mediation of 26S proteasome catalytic activity is an attractive hypothesis worthy of additional investigation. Thus, although the interaction between the host sHSP and the viral protein has been convincingly established, the specific biological function of the sHSP during CGMMV infection needs to be further examined.

RNA virus HEL domains are generally considered to be motor proteins involved in the unwinding of double-stranded RNA (dsRNA) replication intermediates and destabilization of strong secondary structures via NTP hydrolysis during virus replication (Caruthers and McKay 2002). The CGMMV HEL domain is one of the domains present in both 129- and 186-kDa replicases that are generated via direct translation of viral genomic RNAs (gRNAs) during early infection. As a critical regulator of viral infection, the HEL domain in tobamovirus is implicated in the regulation of several processes via its interaction with host factors during viral infection, including viral multiplication, symptom development, host defense responses, and *N* gene resistance (Padmanabhan et al. 2005; Ueda et al. 2006; Wang et al. 2009; Yamanaka et al. 2002). The interaction between the CGMMV HEL domain and host plant sHSP may be related to the disruption of host plant basal defense responses to facilitate viral infection. The multifunctional nature of the HEL domain suggests that it participates in several host biological processes to accelerate viral infection. Given the functional versatility of the HEL domain in the viral lifecycle, it has been proposed as an ideal single-hit target to inhibit coinfection of the human immunodeficiency virus and hepatitis C virus (Anwar et al. 2018). Similarly, the HEL domain may be an important target for future plant antiviral strategies.

Although host factors are acknowledged to be key components in viral infection, there have been no reports of host factors that interact with CGMMV proteins. In this study, we identified a host sHSP that interacts with the CGMMV HEL domain. This interaction was closely associated with the subcellular relocalization of the sHSP, the accumulation of CGMMV RNA, and the development of disease symptoms. We suspect that the identified sHSP may form part of a defense line that is deployed against CGMMV during the initial stages of viral infection. Thus, our study may provide a new theoretical basis for the breeding of CGMMV-resistant plants.

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