Tobacco Necrosis Virus-A^C Single Coat Protein Amino Acid Substitutions Determine Host-Specific Systemic Infections of *Nicotiana benthamiana* and Soybean

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Plant viruses often infect several distinct host species. Sometimes, viruses can systemically infect a specific host whereas, in other cases, only local infections occur in other species. How viral and host factors interact to determine systemic infections among different hosts is largely unknown, particularly for icosahedral positive-stranded RNA viruses. The *Tobacco necrosis virus-A* Chinese isolate belongs to the genus *Alphacrovirus* in the family *Tombusviridae*. In this study, we investigated variations in systemic infections of tobacco necrosis virus-A^C (TNV-A^C) in *Nicotiana benthamiana* and *Glycine max* (soybean) by alanine-scanning mutagenesis of the viral coat protein (CP), which is essential for systemic movement of TNV-A^C. We found that three amino acids, R¹⁶⁹, K¹⁷⁷, and Q²³³, are key residues that mediate varying degrees of systemic infections of *N. benthamiana* and soybean. Further analysis revealed that variations in systemic trafficking of TNV-A^C CP mutants in *N. benthamiana* and soybean are associated with virion assembly and stability. The CP amino acids K¹⁷⁷ and Q²³³ are highly conserved among all TNV-A isolates and are replaced by Q and K in the TNV-D isolates. We demonstrated that systemic infectivity of either TNV-A^C K177A and Q233A or K177Q and Q233K mutants are correlated with the binding affinity of the mutated CPs to the host-specific Hsc70-2 protein. These results expand our understanding of host-dependent long-distance movement of icosahedral viruses in plants.

Keywords: coat protein, Hsc70-2, host-dependent systemic infection, site-directed mutagenesis, tobacco necrosis virus-A, virion assembly, virion stability

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The host range of a plant virus is determined by both viral and host factors, which requires a balance between viral systemic infection and suppression of host defense. Successful infection of a plant virus in a host depends on recognition between viral and host factors during the entire infection cycle, including replication, cell-to-cell movement, and long-distance movement (García and Pallás 2015). A plant virus failing to infect a certain species could be due to its incapability of completing the viral infection cycle or overcoming the plant defenses. Many viral proteins have been identified as host range determinants. For example, NIaPro of the papaya ringspot virus determines its ability to infect papaya (Chen et al. 2008), the P3 protein of the turnip mosaic virus plays a major role in the infection of cabbage and radish (Suehiro et al. 2004), the coat protein (CP) of the wheat streak mosaic virus is closely related to the establishment of infection in maize (Tatineni and French 2014), p33, p18, and p13 protein of the citrus tristeza virus are required for its systemic infection in some species of citrus (Tatineni et al. 2011). An interesting phenomenon is that the mutation of viral proteins results in varied effects among different hosts. For example, the substitution of the Asn²⁵ by Ile within the potato virus Y CP displays enhanced infection in tobacco but compromises virus infection in potato (Moury and Simon 2011). The rice yellow mottle virus encodes the genome-linked viral protein VPg. Mutation of VPg Arg⁴⁸ to I, G, or E increases the replication level of the virus in the rice of resistant genotype but impaired its replication in susceptible rice genotype (Poulicard et al. 2010); however, how these viral proteins determine host-dependent infection remains largely unknown.

For most icosahedral positive-stranded RNA viruses, their CP have three distinct structural domains, including the RNA binding domain (R) at the N-terminus, the shell domain (S) forming the shell of virus particles, and the C-terminal protruding domain (P), which is often related to receptor recognition in hosts and vector transmission (King et al. 2011; Ni and Cheng Kao 2013; Olson et al. 1983). R domains are often flexible, disordered, and responsible for binding to viral nucleic acids (King et al. 2011). Although the sequences of the S domain from different viral CPs have some similarity, their structures are highly analogous, forming a beta barrel structure consisting of eight parallel β -strands (Cheng and Brooks 2013; Rao 2006). More importantly, the encapsidation-competent CP is often required for the systemic trafficking of the icosahedral virus (Allison et al. 1990; Callaway et al. 2004; Sacher and Ahlquist 1989). Previous studies indicated that the S domain plays a major role in the long-distance movement of the virus

(Seo et al. 2013; Shin et al. 2013). For example, Hayden et al. (1998) tested more than one construct. They found that the replacement of amino acid (aa) residues within the conserved beta-strands region (most likely the S region) of the turnip yellow mosaic virus CP by the counterpart aa from the belladonna mottle virus CP affects the viral local and systemic infectivity to different extents. When the last 49 aa of the olive latent virus 1 CP were replaced by a shorter sequence of 39 aa in length, the virus failed to systemically infect *Nicotiana benthamiana*, although it retains the ability to form virions and move from cell to cell (Pantaleo et al. 2006). Salánki et al. (2011) identified three aa of the β B- β C loop of CP that were essential for the long-distance movement of cucumber mosaic virus (CMV) in cucumber. Despite the essential role of these key aa within the CP in maintaining the viral systemic trafficking capacity in a specific host, whether these aa have similar functions in other host plants remains to be explored.

Tobacco necrosis virus-A (TNV-A) is the type member of the genus *Alphanecrovirus* in the family *Tombusviridae*. Tobacco necrosis virus-A Chinese isolate (TNV-A^C), an isolate of TNV-A found in China, causes the necrosis and distortion of soybean (*Glycine max*) and has infected more than 29 plants belonging to eight families; however, systemic infection of TNV-A^C occurred only in soybean and *N. benthamiana* (Xi et al. 2008). The TNV-A^C genome consists of a positive-sense, single-stranded RNA that is 3,682 nt in length and shows 86.4% overall nucleotide sequence identity to TNV-A. The TNV-A^C contains five open reading frames (ORFs) and encodes five proteins, including the p8K, p6K, and p30K CP that are responsible for cell-to-cell and long-distance movement. Our previous studies indicated that the 9 nt downstream of the CP start codon are important for viral accumulation (Pu et al. 2013), and the intact TNV-A^C CP is not necessary for the local infection of *Chenopodium amaranticolor* but is essential for the systemic infection in *N. benthamiana* (Jiang et al. 2008); however, how the intact CP results in the systemic infection of TNV-A^C in *N. benthamiana* remains elusive.

In this study, a series of alanine scanning mutants of TNV-A^C CP was constructed. We examined the effects of these mutations on the systemic infection of TNV-A^C in *N. benthamiana* and soybean. We also tested the potential role of Hsc70-2 in affecting the systemic infection of TNV-A^C in different host plants and found that the binding affinity between host-specific Hsc70-2 and viral CP correlated to the systemic infection phenotype presented by the distinct virus-plant combinations.

RESULTS

Mapping the key aa residues that determine the systemic infection of TNV-A^C in *N. benthamiana* and soybean.

CPs of icosahedral viruses are usually folded into three structural domains, the R, S, and P domains, whereas TNV-A and TNV-D lack a discernible P domain (King et al. 2011). The aa sequence alignment shows that the S domain (aa 89 to 276 for TNV-A^C) of CP is conserved among different isolates of TNV-A and TNV-D, in contrast to the sequences of the R domain that diverge considerably (Supplementary Fig. S1). The S domain is important for the assembly and long-distance movement of the icosahedral virion (Pantaleo et al. 2006), and aa on the surface of the CP can more easily take part in the virion assembly and account for recognition and binding by host proteins. To identify the key aa that determine viral pathogenicity, we used the DNASTar software to predict aa in the TNV-A^C CP S domain that are possibly located on the surface of the virion (Supplementary Table S1). The higher the

predicted score, the greater the probability of surface location. We selected two or three adjacent aa with a predicted score above 1 as a cluster and mutated them into alanine simultaneously. In total, 18 TNV-A^C mutants were constructed by clustered alanine scanning mutagenesis within the S domain of TNV-A^C CP. To assess the infectivity of these mutants, two host plant species, *N. benthamiana* and *G. max*, were inoculated with different mutants and with the wild type (wt) TNV-A^C, which served as a control. Inoculated and systemic leaves were collected for Northern blot and Western blot analyses. Results showed that virus RNAs and CPs could be detected in the leaves of both *N. benthamiana* and soybean inoculated with all 18 alanine-scanning mutants (Fig. 1A) and that all mutants were comparable to the wild-type (wt) TNV-A^C in terms of replication and cell-to-cell movement. However, analysis of virus infection in uninoculated upper leaves showed that, except for two mutants, ²¹¹KLD²¹³→AAA and N194A, that induced systemic symptoms similar to that of the wt TNV-A^C (Fig. 1B, lanes 6 and 8), twelve mutants failed to systemically infect both *N. benthamiana* and soybean (Fig. 1B, lanes 4, 5, 7, 9, 11 to 13, 15 to 19). The remaining four TNV-A^C mutants retained their systemic infection capability for only one of the two host species tested. Among these four mutants, D²³²A/Q²³³A, ¹⁶¹RND¹⁶³→AAA, and ¹⁰⁸NTT¹¹⁰→AAA could systemically infect *N. benthamiana* but not soybean (Fig. 1B, lanes 3, 14, 20). In contrast, mutant ¹⁷⁵SYK¹⁷⁷→AAA induces systemic symptoms in soybean but not in *N. benthamiana* (Fig. 1B, lane 10). A summary of the infectivity of these 18 alanine-scanning mutants in *N. benthamiana* and soybean was listed in Table 1. Within the four groups of mutants listed, the type I, II, and III mutants were impaired for the systemic infectivity on at least one of the plant species tested.

Oda et al. (2000) reported the crystal structure of a TNV Japanese isolate (TNV-Toyama). TNV-Toyama capsids exhibit *T* = 3 icosahedral symmetry and are composed of 60 copies of the trimer with three conformationally distinct CP subunits. TNV-A^C CP shows a 50.36% aa sequence identity similarity to that of TNV-Toyama. To investigate the spatial distribution of the mutated aa clusters described above, a homology model of TNV-A^C trimeric CP was built, based on the reported TNV-Toyama CP structure (Fig. 1C to F). An analysis of the modeled structure showed that the clusters in type I and type III are mostly located on the inner surface of the virion or at the CP-CP interfaces (Fig. 1C and E), implying that these clusters may take part in RNA binding or the assembly of virions. Although the clusters in type IV are also located at the CP-CP interfaces (Fig. 1F), any mutations in them have no profound effect on the viral pathogenicity in *N. benthamiana* and soybean, suggesting that type IV clusters play a minor role in maintaining the structure of the TNV-A^C CP. The clusters of the type II group are predicted to be located within the structural pockets of TNV-A^C CP (Fig. 1D), which might be related to recognition or binding by the host factors.

R¹⁶⁹, K¹⁷⁷, and Q²³³ in the CP differentially contribute to the systemic trafficking of TNV-A^C in *N. benthamiana* and soybean.

To further determine one or more key aa in the alanine-scanned clusters described above, three mutants of R¹⁶⁹A/Q¹⁷¹A, D²³²A/Q²³³A, and ¹⁷⁵SYK¹⁷⁷→AAA, representing types I, II, and III, respectively, were selected for further analysis by site-directed mutagenesis. *N. benthamiana* and soybean were inoculated with the single-aa mutants followed by an analysis of the viral RNAs and CP in the local and systemic leaves (Fig. 2A to C). Northern blot and Western blot detection indicated that R169A mutation leads to the failure of systemic infection of TNV-A^C in both

N. benthamiana and soybean (Fig. 2A). The Q233A mutation disrupted the systemic trafficking of TNV-AC in soybean (Fig. 2B) but not in *N. benthamiana* (Fig. 1B). In contrast, the K177A mutation eliminated the long-distance movement of TNV-AC in

N. benthamiana but not in soybean (Fig. 2C). These results indicate that R¹⁶⁹, K¹⁷⁷, and Q²³³ are key aa within the clusters that affect virus ability to systematically infect one or both *N. benthamiana* and soybean.

Fig. 1. Infections of various tobacco necrosis virus-AC (TNV-AC) alanine scanning mutants in *Nicotiana benthamiana* and soybean. In vitro transcripts from 18 different mutants of TNV-AC coat protein (CP) shell domain (S) were mechanically inoculated onto *N. benthamiana* and soybean. Two individual plants were inoculated with each mutant. **A**, Northern and Western blot analyses of viral RNA and CP accumulation in the inoculated leaves at 3 days postinoculation (dpi). **B**, Northern and Western blot analyses of viral RNA and CP accumulation in the systemic leaves of *N. benthamiana* and soybean at 16 or 20 dpi, respectively. Viral RNAs were detected by Northern blot with a cDNA probe complementary to nt 3443-3682 at the 3' untranslated region of the TNV-AC genome. Total protein extracts were prepared and analyzed by Western blot with antibodies against TNV-AC CP. **C to F**, Three-dimensional model of trimeric CPs of TNV-AC. The three CP subunits are shown in light yellow, blue, and gray, respectively. Amino acid clusters mutated in type I are marked in red (**C**), type II in orange (**D**), type III in deep blue (**E**), and type IV in rose (**F**).

To better evaluate the impact of R169A, K177A, and Q233A on the systemic infection of TNV-A^C in *N. benthamiana* and *G. max*, three individual experiment repeats with at least four plants were conducted for inoculation with these three TNV-A^C mutants. The results showed that these three mutants induced similar symptoms on inoculated leaves of *N. benthamiana* and soybean, which was confirmed by the comparable CP accumulation (Fig. 2D, inoculated leaves) and was consistent with results shown in Figure 1A. At 16 days postinoculation (dpi), the stunted growth phenotype and necrosis appeared on newly emerging leaves of *N. benthamiana* plants inoculated with Q233A mutant, despite milder symptoms and a lower CP accumulation level than plants infected by wt TNV-A^C. No obvious symptoms were observed in the systemic leaves of *N. benthamiana* inoculated with either R169A or K177A mutants (Fig. 2D, left panels). The R169A mutant also failed to systemically infect soybean (Fig. 1B); however, the K177A mutant retained the ability to systemically infect soybean, although the efficiency was slightly impaired in comparison with the wt TNV-A^C. The Q233A mutation was found to significantly impact the systemic infection ability of TNV-A^C in soybean (Fig. 2D, right panels). Altogether, these results indicate that R¹⁶⁹ is indispensable for the ability of TNV-A^Cs to systematically infect both plants, whereas K¹⁷⁷ is essential for the TNV-A^C ability to systematically infect *N. benthamiana* but plays a minor role in the systemic infection of soybean. Conversely, the Q²³³ mutation drastically impairs the systemic infection of TNV-A^C in soybean but has little effect on the systemic infection of the virus in *N. benthamiana*.

Single-site mutations of R¹⁶⁹, K¹⁷⁷, and Q²³³ in the CP affect virion assembly and stability.

Virion assembly is essential for the long-distance movement of diverse icosahedral viruses (Niu et al. 2014; Pantaleo et al. 2006; Seo et al. 2013; Vaewhongs and Lommel 1995; Zhang et al. 2013). To investigate whether the failure of these TNV-A^C mutants to infect *N. benthamiana* or soybean was caused by the aberrant virion assembly in the specific host, *N. benthamiana* and soybean plants were inoculated with the Q233A, R169A, and K177A mutants, respectively. At 3 dpi, equal amounts of inoculated leaves were harvested for analysis of CP accumulation. The results showed similar CP accumulation levels in the total extracts derived from either *N. benthamiana* or

soybean infected by different mutants (Fig. 3A, upper panels). Equal amounts of the leaf samples were used for the partial purification of the virions (discussed below). We examined 10 µl of the partially purified virions, using Western blot with an antibody against TNV-A^C CP, and an additional 30 µl was used for RNA extraction followed by Northern blot analysis. We found that viral CPs could only be detected in *N. benthamiana* leaves inoculated with wt TNV-A^C and Q233A mutant but not from leaves inoculated with R169A and K177A mutants (Fig. 3A, bottom panels). In contrast, viral CPs were present in soybean leaves inoculated with either K177A or Q233A mutant, although the CP accumulation of the Q233A mutant was much lower than that of wt TNV-A^C. Northern blot analysis revealed the correlation of the viral RNA levels with the CP accumulations as shown above (Fig. 3A, bottom panels).

We performed a transmission electron microscopy (TEM) analysis to check the virions in the partially purified products. Virions were barely observed in the partially purified products from *N. benthamiana* inoculated with either the R169A or K177A mutant (Fig. 3B, top panel). In contrast, virions were readily observed from soybean inoculated with K177A, despite being less than that observed from the wt TNV-A^C. Partially purified products from soybean inoculated with the R169A and Q233A mutants consistently contained few, if any, virions (Fig. 3B, bottom panel). Given that the CP accumulations were similar in total extracts among wt TNV-A^C and its three variants, the difference shown in the TEM analyses of the partially purified products suggested that unassembled CP subunits or virions without sufficient stability were removed during the partial purification operation.

To further confirm the effects of these site mutations on virion assembly, the viral RNAs were tested by an endogenous RNase-sensitivity assay with virus-inoculated *N. benthamiana* leaf sap. We found that within the 10-min incubation, the viral RNAs of wt TNV-A^C were only slightly degraded (Fig. 3C and D). However, viral RNA from the R169A and K177A mutants were dramatically decreased after RNase treatment, in striking contrast to that of wt TNV-A^C (Fig. 3C and D). This is consistent with the failure of virion assembly for these two mutants in *N. benthamiana* (Fig. 3B). The viral RNAs from the Q233A mutant were moderately degraded in comparison with those of wt TNV-A^C (Fig. 3C and D). Altogether, these results indicated that the aa of R¹⁶⁹, K¹⁷⁷, and Q²³³ contribute to the assembly of

Table 1. Summary of the local and systemic infections of various tobacco necrosis virus-A^C mutants^a

Type	Alanine scanning mutants	<i>Nicotiana benthamiana</i>		<i>Glycine max</i>	
		I	S	I	S
I	217YPT ²¹⁹ →AAA	Y	N	Y	N
	214KPW ²¹⁶ →AAA	Y	N	Y	N
	208DIT ²¹⁰ →AAA	Y	N	Y	N
	182PPY ¹⁸⁴ →AAA	Y	N	Y	N
	172LSQ ¹⁷⁴ →A AA	Y	N	Y	N
	R ¹⁶⁹ A/Q ¹⁷¹ A	Y	N	Y	N
	166PTT ¹⁶⁸ →AAA	Y	N	Y	N
	158TYD ¹⁶⁰ →AAA	Y	N	Y	N
	C ¹⁴⁵ A/P ¹⁴⁶ A	Y	N	Y	N
	T ¹⁴⁸ A/T ¹⁴⁹ A	Y	N	Y	N
	131KYR ¹³³ →AA	Y	N	Y	N
	128LYS ¹³⁰ →AAA	Y	N	Y	N
	D ²³² A/Q ²³³ A	Y	Y	Y	N
	161RND ¹⁶³ →AAA	Y	Y	Y	N
	108NTT ¹¹⁰ →AAA	Y	Y	Y	N
III	175SYK ¹⁷⁷ →AAA	Y	N	Y	Y
IV	211KLD ²¹³ →AAA	Y	Y	Y	Y
	N ¹⁹⁴ A	Y	Y	Y	Y

^a I = Inoculated leaves, S = systemic leaves. Y indicates both viral coat protein (CP) and RNAs were detected; N indicates neither viral CP nor RNAs were detected.

Fig. 2. R¹⁶⁹, K¹⁷⁷, and Q²³³ are differentially required for the long-distance movement of tobacco necrosis virus-A^c (TNV-A^c) in *Nicotiana benthamiana* and soybean. **A**, Analysis of viral RNA of cluster R¹⁶⁹A/Q¹⁷¹A mutants in inoculated leaves or upper uninoculated leaves of *N. benthamiana* and soybean by Northern blot. **B**, Analysis of the viral RNA of cluster D²³²A/Q²³³A mutants in inoculated leaves or systemic leaves of soybean by Northern blot. **C**, Analysis of coat protein (CP) accumulation of cluster ¹⁷⁵SYK¹⁷⁷→AAA mutants in inoculated leaves or systemic leaves of *N. benthamiana* and soybean by Western blot. Coomassie brilliant blue (CBB)-stained large subunit of ribulose biphosphate carboxylase (RbcL) was used as the loading control. **D**, Symptom observation and Western blot analysis of CP accumulation in local and systemic leaves of plants inoculated with TNV-A^c containing different point mutations in the CP. The number of systemically infected plants among total inoculated plants obtained from three individual experiments, which are shown below the panel. Buffer-inoculated plants served as the control. The inoculated leaves were examined at 3 days postinoculation (dpi), and systemic leaves of *N. benthamiana* and soybean were examined at 16 and 20 dpi, respectively. Viral RNAs were detected with the cDNA probe complementary to the 3' untranslated region of TNV-A^c genome and the antibody against TNV-A^c CP was used for Western blot. CBB-stained RbcL served as the loading control.

Fig. 3. Mutation of R¹⁶⁹, K¹⁷⁷, and Q²³³ within the coat protein (CP) affects the virion assembly in a host-dependent manner. **A**, Analysis of the CPs and viral RNAs in local leaves inoculated with the various tobacco necrosis virus-A^C (TNV-A^C) mutants. The CPs in total protein extracts from *Nicotiana benthamiana* and soybean were detected by Western blot (upper panels). RbcL served as the loading control. Viral particles were further partially purified from the inoculated leaves, followed by RNA extraction, Western blot and Northern blot were then conducted to detect the purified particles and viral RNAs (lower panels). **B**, Transmission electron microscopy (TEM) analysis of partially purified viral particles from *N. benthamiana* and soybean. Scale bar = 100 nm. The number of particles was counted from 10 random fields of view and the average was calculated, which is shown on the right of the TEM images. Letters above the bars indicate statistically significant difference among different groups, as revealed by Tukey's multiple comparisons test ($P < 0.05$). **C**, Endogenous RNase sensitivity assay in extracts from *N. benthamiana* infected with wild-type TNV-A^C or its derivatives. Inoculated leaves were ground and incubated in piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer at 37°C at 5 and 10 min, to allow degradation of unprotected RNA by the endogenous RNase. The total RNAs were then extracted and analyzed by Northern blot. The ethidium bromide-stained gel of ribosomal RNA (rRNA) served as the loading control. **D**, Quantification of the degradation rate of the viral RNAs are shown in C. The relative viral RNA level at 0 min was set to 1.0. Data are shown from three independent repeats, error bars indicate standard deviation ($n = 3$).

TNV- A^C virion in *N. benthamiana* and soybean, resulting in a host-dependent long-distance movement of the virus.

Single-site mutations of R¹⁶⁹ or K¹⁷⁷ in the CP affect the stability or the self-interaction of CP in *N. benthamiana*.

To investigate whether R¹⁶⁹, K¹⁷⁷, or Q²³³ mutation affects the stability of CP, a semi-in vivo degradation assay was performed by the transient expression of CP subunits. We found that CP accumulation in *N. benthamiana* leaves expressing the wt CP remains nearly invariable at different timepoints in the presence of adenosine triphosphate (ATP). The CPs of three mutants in agroinfiltrated *N. benthamiana* leaves were degraded to different extents, especially the CP_{R169A}, whose accumulation was below the detectable level after 60 min of treatment (Fig. 4A and B), indicating that R¹⁶⁹ was a critical site for maintaining the stability of CP. Although both CP_{K177A} and CP_{Q233A} were degraded to some extent compared with CP_{wt}, both showed higher stability than that of CP_{R169A}. Despite CP_{K177A} having higher stability than that of CP_{Q233A}, the Q233A mutant of TNV- A^C is capable of systemically infecting *N. benthamiana* and assembling into viral particles, whereas the K177A mutant is not (Figs. 2D and 3). Considering that K¹⁷⁷ is located at the CP-CP interfaces (based on our 3D modeling of the trimeric CPs) (Fig. 1E), we presume that K¹⁷⁷ may be important for the self-interaction of CP. To test this speculation, a co-immunoprecipitation (Co-IP) assay was conducted to examine the effect of a mutation of K¹⁷⁷ on the self-interaction of

CP. We found that the self-interaction of CP_{K177A} proteins was severely impaired in comparison with that of CP_{wt} (Fig. 4C), suggesting that the systemic movement of TNV- A^C in *N. benthamiana* is coordinately regulated by the stability and self-interaction of CP.

Varied systemic infection of TNV- A^C mutants in *N. benthamiana* and soybean associates with the binding of their CPs to the host-specific Hsc70-2.

Considering the difference in the systemic infection of the K177A mutant of TNV- A^C in *N. benthamiana* and soybean (Fig. 2C and D), we presume that specific host factors might play a role in determining the long-distance movement of TNV- A^C in different hosts. Previous studies indicated that Hsc70-2 was identified in the protein complex associated with the CP of several members in the family *Tombusviridae* (Alam and Rochon 2015, 2017; Wang et al. 2018) and plays an important role in virion assembly, a process that was indispensable for the systemic infection of the icosahedral virus, similar to the TNV- A^C . Therefore, *N. benthamiana* leaves systemically infected by wt TNV- A^C were subjected to partial purification of the virion followed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We found that NbHsc70-2 was identified in the partially purified products with a high score

Fig. 4. Mutation of R¹⁶⁹, K¹⁷⁷, and Q²³³ within the coat protein (CP) shows a varied effect on CP stability and self-interaction. **A**, Western blot analysis of CP protein levels at different timepoints. The plants were infiltrated with agrobacterium containing the wild-type (wt) tobacco necrosis virus- A^C (TNV- A^C) or its variants. Leaf samples were collected and treated with 100 mM cycloheximide in the presence of 10 mM adenosine triphosphate. At different timepoints, the samples were taken out for Western blot analysis with antibodies against TNV- A^C CP. **B**, Quantification of CP protein levels shown in A. The relative protein level at 0 min was normalized to rubisco, and this value was set to 1. Data are shown from three individual repeats, error bars indicate standard deviation ($n = 3$). **C**, Co-immunoprecipitation (Co-IP) assay to test the self-interaction of wt CP and CP_{K177A} mutant. Agrobacterium harboring the Flag-tagged CP or CP_{K177A} were coexpressed with the CP or CP_{K177A} in *Nicotiana benthamiana* leaves, followed by total protein extraction and IP with anti-FLAG agarose beads, the input and IP products were analyzed by Western blot with the anti-CP antibody. The ratio of CP to CP-Flag is shown below the upper panel. The target protein bands are indicated on the right of the panel.

Fig. 5. CP_{K177A} and CP_{Q233A} bind to the host-specific Hsc70-2 with different affinity. **A**, Co-immunoprecipitation (Co-IP) experiments to examine in vivo interactions between host-specific Hsc70-2 and different coat protein (CP) mutants. *Nicotiana benthamiana* leaf tissues agroinfiltrated with various combinations are indicated above the panel. Leaf samples were harvested at 3 days postinoculation. Total proteins from the harvested samples were subjected to IP with anti-FLAG agarose beads, and Western blot analysis was performed using anti-CP or anti-FLAG antibodies. The input panels (left) show protein input detected by anti-CP or anti-FLAG antibodies. Arrowheads indicate the target protein bands. **B**, A glutathione-S-transferase (GST) pull-down assay shows the interaction between host-specific Hsc70-2 and different CP mutants in vitro. Purified GST-GmHsc70-2 or GST-NbHsc70-2 was incubated with His-tagged CP or its mutants. After being immunoprecipitated with Glutathione-Sepharose beads, the proteins were analyzed by Western blot analysis with anti-His or anti-GST antibodies. GFP-His protein served as negative control. Experiments were repeated two times and representative results are shown.

Fig. 6. Analysis of the systemic infection of tobacco necrosis virus-A^C (TNV-A^C) coat protein (CP) K¹⁷⁷Q, Q²³³K single or double mutants in *Nicotiana benthamiana* and soybean. **A**, Comparison of the amino acids at sites 169, 177, and 233 within the CP of TNV-A with the counterpart in the CP of TNV-D. **B**, Symptom observation and Northern blot analysis of viral accumulation in local and systemic leaves of plants inoculated with TNV-A^C derivatives containing a single or double mutation at the K¹⁷⁷ and Q²³³ sites of the CP. In vitro transcripts from wild-type (wt) TNV-A^C and TNV-D^H were used as positive controls. Buffer-inoculated plants served as negative controls (Mock). The inoculated leaves were harvested at 3 days postinoculation (dpi) and systemic leaves of *N. benthamiana* and soybean were collected at 16 and 20 dpi, respectively. Viral RNAs were detected with the cDNA probe complementary to the 3' untranslated region of the TNV-A^C genome or CP open reading frame of the TNV-D^H genome. Ethidium bromide-stained ribosomal RNA (rRNA) served as the loading control. **C**, Co-immunoprecipitation (Co-IP) experiments to examine the in-vivo interactions between GmHsc70-2 and mutated TNV-A^C CP proteins as shown on the upper right. CP proteins from the wt TNV-A^C or TNV-D^H served as controls. Antibodies used for protein detection are shown on the left. Arrowheads indicate the target protein bands.

(Supplementary Table S1), suggesting an association of NbHsc70-2 with the TNV-A^C virion.

To examine the effects of the CP mutations on its interactions with NbHsc70-2, NbHsc70-2 together with its homolog in soybean, GmHsc70-2, were used for subsequent in-vivo and in-vitro interaction analysis. *Agrobacterium* containing the binary constructs of pMDC32-CP_{wt}, pMDC32-CP_{K177A}, or pMDC32-CP_{Q233A} were co-infiltrated with GmHsc70-2-Flag or NbHsc70-2-Flag, respectively, into *N. benthamiana* leaves, followed by a Co-IP assay. We found that CP_{K177A} was well coprecipitated with GmHsc70-2-Flag, which differs from the strong coprecipitation of CP_{wt} with GmHsc70-2-Flag or NbHsc70-2-Flag and is in contrast to that of the NbHsc70-2-Flag exhibiting a substantially lower efficiency of coprecipitation with the CP_{K177A}. Similarly, CP_{Q233A} showed a better affinity with the NbHsc70-2-Flag than that of the GmHsc70-2-Flag (Fig. 5A).

To further evaluate the binding ability between the CPs and host-specific Hsc70-2, an in-vitro glutathione-S-transferase (GST) pull-down assay was carried out using purified GST-NbHsc70-2, GST-GmHsc70-2, CP_{wt}-His, CP_{K177A}-His, and CP_{Q233A}-His (Fig. 5B). We found that GST-NbHsc70-2 could bind to CP_{Q233A}-His stronger than to CP_{K177A}-His (Fig. 5B). In contrast, GST-GmHsc70-2 could strongly bind to both CP_{wt}-His and CP_{K177A}-His in vitro and had a significantly lower binding affinity to CP_{Q233A}-His. These results are consistent with the performance of the corresponding TNV-A^C mutants in systemic infection of *N. benthamiana* or soybean, indicating that the recognition of TNV-A^C virions by host-specific Hsc70-2 is crucial for maintaining the systemic infection of TNV-A^C in different host plants.

It is worth noting that R¹⁶⁹ in the CP is conserved in all TNV-A and TNV-D isolates, whereas aa sites 177 and 233 were only identical within either the TNV-A or TNV-D isolates (Fig. 6A; Supplementary Fig. S1). To further determine the functional roles of these two sites on the systemic infection of TNV-A^C in different hosts, three TNV-A^C mutants, named K177Q, Q233K, and K¹⁷⁷Q/Q²³³K, were constructed. In-vitro transcripts of these three constructs were inoculated onto *N. benthamiana* and soybean as previously described. The wt TNV-A^C and TNV-D^H served as positive controls. We found that all three mutants can infect the local leaves of *N. benthamiana* and soybean, similar to that of wt TNV-A^C. Northern blot analysis confirmed the infection of these TNV-A^C mutants in the inoculated leaves. TNV-D^H showed more severe local necrotic lesions than that of TNV-A^C in *N. benthamiana* leaves (Fig. 6B, Ino. panels). All three mutants were able to systemically infect *N. benthamiana*, albeit showing milder symptoms and decreased viral accumulation compared with that of wt TNV-A^C. However, only the K177Q mutant retains the systemic infectivity in soybean, whereas the other two mutants, Q233K and Q¹⁷⁷K/K²³³Q, induced no symptoms in the systemic leaves of soybean similar to that of the TNV-D^H control, which cannot move systemically in soybean, as reported previously (Xi et al. 2007). Northern blot analysis showed viral RNA accumulation that was consistent with the observation of symptoms (Fig. 6B, Sys. panels). These data reinforce our conclusion that K¹⁷⁷ is not a determinant for TNV-A^C to systemically infect soybean but is crucial for the systemic viral infection in *N. benthamiana*. In contrast, Q²³³ is critical for the effective systemic infection of TNV-A^C in soybean but not for *N. benthamiana*. We also performed a Co-IP assay to compare the interaction between different CPs and GmHsc70-2. We found that only CP_{K177Q} was coprecipitated with GmHsc70-2-Flag, similar to CP_{wt}. In contrast, both CP_{Q233K} and CP_{K177Q/Q233K} as well as the CP_{TNV-DH} control showed a substantially lower binding affinity with GmHsc70-2-Flag than that of CP_{K177Q} and CP_{wt}. These results are consistent

with our hypothesis that Hsc70-2 might sustain the systemic infection TNV-A^C in different host plants.

DISCUSSION

The long-distance or vascular movement of a virus is a complex process and includes three steps: i) entry into the vascular system, ii) transport between phloem-associated cells, and- iii) exit into the mesophyll cells of the upper leaves (Séron and Haenni 1996). For most icosahedral RNA viruses, CP is required for long-distance transport, suggesting that these viruses systemically move in the form of intact virions. Our previous studies also proved that TNV-A^C CP is indispensable for the systemic infection of *N. benthamiana* (Jiang et al. 2008), but the formation of intact virions is not sufficient to enable the virus to systemically infect a host. Other components, in particular, the host proteins, were also required for fulfilling this process (Sit et al. 2001). Besides long-distance movement, many icosahedral RNA viruses, such as brome mosaic virus, hibiscus chlorotic ringspot virus, and CMV also require CP for cell-to-cell movement (Canto et al. 1997; Niu et al. 2014; Schmitz and Rao 1996). In contrast to these viruses, our previous studies showed that the deletion of the ORF, except for the 17 nt at the N terminus of CP, has a minor effect on virus replication and symptom induction in *Chenopodium amaranticolor* (Pu et al. 2013), which suggests that CP is not essential for the cell-to-cell movement of TNV-A^C. Alanine-scanning mutational analyses of TNV-A^C CP showed a comparable accumulation of viral RNAs among all kinds of CP mutants, consistent with that reported by Pu et al. (2013); however, in our study, several CP mutants affect the virion formation of TNV-A^C and failed to protect the viral RNA from degradation by endogenous RNase, resulting in the loss of viral systemic infectivity in *N. benthamiana* (Fig. 3). These results highlight the importance of virion formation in supporting the long-distance movement of icosahedral virus.

Homology modeling of the TNV-A^C CP structure shows that most of the basic residues in the R domain face the interior of the CP structure and very few of them are located on the exterior surface when assembled into virions, and it was presumed that these positively charged aa may contribute to the stabilization of the virion. It is worth noting that the three residues, R¹⁶⁹, K¹⁷⁷, and Q²³³ are positively charged, and computer-generated homotrimers of TNV-A^C CP suggests that they are spatially close to each other and may interact with specific host factors to regulate virus assembly and infection (Fig. 1C to F).

Both viral and host factors play a role in determining the host range of a virus (Callaway et al. 2001). Usually, it is relatively easy to identify the viral proteins that are responsible for host ranges by mutation analyses or recombination between different virus isolates. For example, residues at both positions 129 and 162 in the CP gene of the CMV M strain (M-CMV) were required to overcome the resistance against M-CMV in maize (Ryu et al. 1998); the mutation of aspartic acid residues at aa positions 289, 290, and 326 in wheat streak mosaic virus CP severely impaired virus systemic infection of maize but not of wheat (Tatineni and French 2014). Overall, although the key aa within the CP that function in host-dependent infection have been studied in many plant viruses, one or more molecular mechanisms for these effects remain to be investigated; very limited host factors involved in the systemic infection of the icosahedral virus have been identified to date. Our studies indicate that the assembly of some TNV-A^C CP mutants (K177A and Q233A) and their subsequent systemic infections are host-dependent, strongly suggesting that host factors are involved in the encapsidation and long-distance movement of TNV-A^C. By LC-MS/MS analysis of the partially purified TNV-A^C virions,

NbHsc70-2 was found to be associated with the virions (Supplementary Table S2).

Interestingly, K177Q recovered systemic infection in *N. benthamiana*, although with lower levels of viral RNA accumulation and mild symptoms, whereas K177A failed to systemically infect *N. benthamiana*. This differs from the results found for *N. benthamiana*, as neither A nor Q substitution at aa site 177 influences the systemic infection of TNV-A^C in soybean (Figs. 2C and 6B). Considering that Lys (K) is positively charged, Gln (Q) is polar without charge, and Ala is nonpolar, we presumed that the polarity of the aa at position 177 is important for maintaining the TNV-A^C systemic infectivity in *N. benthamiana* but has a minor effect on viral systemic infection in soybean. Both Q233K and Q233A mutants of TNV-A^C fail to systemically infect soybean, suggesting that Gln (Q) at residue 233 is strictly required for the long-distance movement of TNV-A^C in soybean but is replaceable without affecting viral systemic infection in *N. benthamiana*. These results support a scenario in which the TNV virus evolutionarily diverged in the CP to adapt to different hosts, thus ensuring better survival and subsequent spread of TNV virus among different plant hosts.

Heat shock protein 70 (Hsp70) chaperones, as central components of the cellular chaperone network, are involved in the folding process of nascent proteins by refolding misfolded and aggregated proteins (Mayer and Bukau 2005). Hsp70s are frequently recruited by diverse viral proteins and participate in multiple aspects of virus infection (Alam and Rochon 2015, 2017; Chromy et al. 2003, 2006; Hafren et al. 2010; Krenz et al. 2010; Mathioudakis et al. 2012; Wang et al. 2018). Alam and Rochon (2015, 2017) presented a paradigm by deciphering the functional role of Hsc70-2 in multiple aspects of cucumber necrosis virus infection, in particular, its roles in enhancing CP accumulation, solubilization, and virion assembly and disassembly. Our study is consistent with that of Alam and Rochon (2015, 2017), providing additional examples regarding the TNV-A^C CP-Hsc70-2 interaction. However, we extended the potential role of Hsc70-2 by demonstrating that Hsc70-2 not only plays a crucial role in sustaining the infection of an icosahedral virus in a specific host, but we also pinpointed the varied compatibility of orthologous Hsc70-2 from different hosts with the same viral CP, thus supporting a scenario in which Hsc70-2 could serve as a factor to affect the systemic infection phenotypes of a virus in distinct hosts.

The HSP70s are divided into an N-terminal ATPase domain and a C-terminal domain (Mayer and Bukau 2005). The N-terminal ATPase domain possesses an ATP or ADP binding property, and the C-terminal domain is responsible for binding target proteins (Mayer 2005). Sequence alignment showed that the ATPase domain identified in cellular HSP70s is similar to that encoded by closteroviral HSP70h proteins, whereas the C-terminal domain showed relatively low sequence identity between them (Agranovsky et al. 1991). Similarly, the sequences of Hsc70-2 from *N. benthamiana* and soybean are conserved in the ATPase domain but show divergence in the substrate binding domain (Supplementary Fig. S2), which may explain the difference of host-specific Hsc70-2 in binding to the same TNV-A^C CP. Interestingly, viruses in the family *Closteroviridae* could encode an Hsp70 homolog (HSP70h) itself to facilitate cell-to-cell movement and virion assembly (Peremyslov et al. 1999; Satyanarayana et al. 2000; Tatineni et al. 2010). These studies, together with the essential role of Hsc70 in the infection of a large number of phylogenetically distant or near viruses, suggested a coevolution mode between the viral proteins and Hsp70s during long-term virus-host interactions.

In summary, we determined the key aa within the TNV-A^C CP that differentially affect the systemic infection of virus in *N. benthamiana* and soybean, the discrepancy in the systemic

infection of different TNV-A^C CP variants associates with the efficiency of virion assembly, as well as CP stability in a specific host and found that the binding of host-specific Hsc70-2 to the virus CP also plays an important role in supporting the systemic infection of TNV-A^C in a specific host. Our results expand our understanding of the host-dependent long-distance movement of the icosahedral virus in different plant species.

MATERIALS AND METHODS

Plant growth conditions.

Nicotiana benthamiana plants and soybean (*Glycine max*) cv. Kenjian 23 were grown in a growth chamber at 23°C with a 14-h light and 10-h dark cycle.

Construction of various TNV-A^C derivatives.

All TNV-A^C derivatives that contain alanine substitution at different positions within the CP were constructed based on the infectious complementary DNA (cDNA) clone of TNV-A^C (pMTC27) (Xi et al. 2008). To construct the TNV-A^C derivative harboring the double D²³²A/Q²³³A mutations within the CP, two fragments were amplified, using primer pairs TN-132/TN-158 and TN-157/TN-64, respectively. The resulting two PCR fragments were joint by overlap PCR with primer pair TN-132/TN-64 to generate the fragment containing the target mutations within the CP. The resultant fragment was digested by *DraIII/EcoRV* and was cloned into the similarly digested pMTC27 plasmid. Other alanine scanning clusters were amplified with the same strategy, using the corresponding primer pairs listed in Supplementary Table S2. The single aa mutation within the TNV-A^C CP was constructed using the QuickChange site-directed mutagenesis kit (Agilent Technologies) according to manufacturer instructions.

For transient expression of wt CP or its various mutants in plants, CP fragments were amplified from unmodified infectious clones of TNV-A^C or clones containing the R169A, K177A, and Q233A mutation within the CP ORF, respectively, and were recombined into the pMDC32 at the *KpnI* and *SpeI* sites (Curtis and Grossniklaus 2003), using the Seamless assembly cloning kit (Clone Smarter), resulting in the plasmids pMDC32-CP_{wt}, pMDC32-CP_{R169A}, pMDC32-CP_{K177A}, and pMDC32-CP_{Q233A}.

For the co-IP assay, the DNA fragment encoding three linked glycine residues and three tandem repeats of Flag epitopes (3xFlag) was amplified and was engineered downstream of the multiple cloning site at the *SpeI* and *SacI* restriction sites of the vector pMDC32, to yield the plasmid pMDC32-3xFlag. The DNA fragment encoding GmHsc70-2 was amplified from the cDNA of *G. max* and was cloned into pMDC32-3xFlag using the Seamless assembly cloning kit (Clone Smarter). CP_{TNV-DH}-Myc, CP_{wt}-Myc, CP_{K177Q}-Myc, CP_{Q233K}-Myc, and CP_{K177Q/Q233K}-Myc fragments were amplified from unmodified infectious clones of TNV-D^H or TNV-A^C or that containing a K177Q, Q233K, and K¹⁷⁷Q/Q²³³K mutation within the CP ORF, respectively, and were recombined into the SPDK3167 plasmid at the *StuI* and *AvrII* sites (Zhang et al. 2019). The other plasmids used for expressing NbHsc70-2-Flag and GFP-Flag were described previously (Wang et al. 2018).

For prokaryotic expression of the target protein, GmHsc70-2 was amplified and fused to the C-terminus of the GST at the *BamHI* and *XhoI* restriction sites of the vector pGEX-KG, to generate the plasmid GST-GmHsc70-2. CP_{wt}-His, CP_{K177A}-His, and CP_{Q233A}-His fragments were amplified from the unmodified infectious clones of TNV-A^C or those containing K177A and Q233A mutations within the CP and were combined into pET30a (+) vector. Other plasmids used for

expressing GST-NbHsc70-2 and GFP-His proteins were described previously (Wang et al. 2018).

The primers used for constructing these plasmids are listed in Supplementary Table S2 and sequence analyses were performed to authenticate all plasmids.

In vitro RNA transcription and mechanical inoculation.

The *Sma*I-linearized plasmid (200 ng) were used as templates for in-vitro transcription, using T7 RNA polymerase (Promega). Freshly prepared in-vitro transcripts were analyzed by 1% (wt/vol) agarose gel electrophoresis to evaluate the quality of the RNA transcripts. The RNA transcripts were then mixed with an equal volume of FES inoculation buffer (0.1 M glycine, 0.06 M dipotassium phosphate, 1% sodium pyrophosphate decahydrate, 1% bentonite, 1% celite, pH 8.5), followed by mechanical inoculation onto two-week-old *N. benthamiana* or soybean. Plants were incubated in a growth chamber at 23°C for symptom induction and observation.

Northern blot.

Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies) and was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific). Total RNA (4 µg) was separated on a 1.2% agarose gel under denaturing conditions and was then transferred onto Hybond-N⁺ nylon membranes (0.45 µm; GE Healthcare), followed by hybridization with a cDNA probe complementary to nt 3443-3682 at the 3' untranslated region of TNV-A^C genome or a cDNA probe complementary to nt 2651-2890 at the CP ORF of TNV-D^H genome.

Western blot analysis.

Leaves were ground to a fine powder in liquid nitrogen and total proteins were extracted by adding sodium dodecyl sulfate (SDS) extraction buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, and 5% β-mercaptoethanol) followed by thorough vortexing and centrifugation. Total proteins were then electrophoresed through 12.5% SDS polyacrylamide gel electrophoresis, were blotted onto nitrocellulose membranes (0.45 µm; Bio-Rad), and were probed with specific antibodies against the target protein. Band signals were detected with an enhanced chemiluminescence detection kit (GE Healthcare) and were visualized under a ChemiDoc touch imaging system (Bio-Rad). Band signal intensity was quantified using ImageJ software (Schneider et al. 2012).

Homology structure modeling.

The TNV-A^C CP homology model was built by using the online homology modeling server SwissModel. The crystal structure of TNV-Toyama (Protein Data Bank ID number 1C8N) was chosen as the template (aa identity = 50.36%). The constructed model was assessed by GMQE (Benkert et al. 2011), QMEAN (Benkert et al. 2008), and Verify-3D (Eisenberg et al. 1997). The structure was visualized using the PyMOL program 2.3 (Schrödinger, NY, U.S.A.).

Partial purification of virions.

Partial purification of virus particles was carried out as described previously (Alam and Rochon 2015), with minor modifications. Briefly, inoculated leaves were collected at 3 dpi and were ground in liquid nitrogen and 1 g of leaf powder was vortexed in 2 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 5% β-mercaptoethanol. After incubation on ice for 1 h, the homogenate was centrifuged at 8,000 × g for 30 min at 4°C to remove insoluble debris. The supernatant PEG₆₀₀₀ was added to a final concentration of 8% and the homogenate was gently rotated at 4°C for 3 h. After centrifugation at 13,000 × g

for 30 min at 4°C, the pellets containing viral particles were resuspended in 100 µl of 10 mM sodium acetate buffer (pH 5.0). The suspension was gently rotated at 4°C overnight, and the crude extracts containing virions were obtained by centrifugation at 8,000 × g for 20 min at 4°C.

TEM.

TEM analysis was performed as described previously (Li et al. 2020). Partially purified virions were dropped onto the parafilm and a 200-mesh copper grid was floated on top of the drop for 2 min. Samples were washed with deionized sterile water and were then stained with 2% uranyl acetate. Observations of the virions were performed under a JEM-1230 (JEOL Co. Ltd.) transmission electron microscope operated at 80 kV, with iTEM to capture the images.

RNase sensitivity assay.

The RNase sensitivity assay was performed according to previously reported methods with minor modifications (Zhao et al. 2015). Briefly, 0.1 g of in vitro transcripts-inoculated leaves were ground in liquid nitrogen and were incubated in 100 µl of piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (50 mM PIPES, pH 6.7, 0.1% Tween 20) at 37°C. Total RNA was extracted at different timepoints and was analyzed by Northern blot as described above.

Semi-in vivo degradation assay.

A semi-in vivo degradation assay was performed as previously described (Fu et al. 2018). Briefly, *Agrobacterium tumefaciens* harboring pMDC32-CP_{wt}, pMDC32-CP_{R169A}, pMDC32-CP_{K177A}, or pMDC32-CP_{Q233A} were infiltrated into different regions of the same leaf of *N. benthamiana*. Different infiltrated regions were excised from the leaves at 3 dpi and were respectively ground to a fine powder in liquid nitrogen. Total proteins were extracted by adding the native extraction buffer (50 mM Tris-MES [pH 8.0], 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM dithiothreitol [DTT], and 1× protease inhibitor cocktail) to the leaf powder. For analysis of CP degradation, the indicated concentrations of ATP and cycloheximide were added to the protein extracts. The mixtures were incubated at room temperature and were analyzed at 0, 30, 60, and 90 min. Reactions were stopped by the addition of SDS sample buffer and were boiled for 5 min before Western blot analyses.

Reverse transcription-quantitative real-time PCR (RT-qPCR).

RT-qPCR was performed as previously reported (Liu et al. 2012). Briefly, 6 µg of DNase-treated total RNA was reverse-transcribed into cDNA using an oligo(dT) and M-MLV reverse transcription (Promega). The gene fragments were amplified using 2× SsoFast EvaGreen Supermix (Bio-Rad). *Elongation factor 1α* (*EF-1α*) gene was used as the internal control, and the data were analyzed using a CFX Manager (Bio-Rad). All primer pairs used for RT-qPCR analyses are listed in Supplementary Table S2.

Mass spectrometry.

The partially purified preparations of TNV-A^C described above were digested with trypsin. The digested peptides were separated by nanoscale C18 reverse-phase liquid chromatography (Waters) and were then electro-sprayed into a Q-Exactive mass spectrometer (Thermo Fisher Scientific) at the Mass Spectrometry Facility of China Agricultural University.

Co-IP assay.

Co-IP was performed according to previously described methods with minor modifications (Yang et al. 2018). *Agrobacterium*

containing plasmid NbHsc70-2-Flag or GmHsc70-2-Flag, together with agrobacterium carrying the plasmid of pMDC32-CPs or CPs-Myc were mixed at the final concentration of OD₆₀₀ = 0.5 and were then infiltrated into *N. benthamiana* leaves. At 3 dpi, 4 g of infiltrated leaves were ground in liquid nitrogen, followed by the addition of two volumes of freshly prepared protein extraction buffer to the leaf powder (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% [vol/vol] glycerol, 2% [wt/vol] polyvinylpyrrolidone, 10 mM DTT, 1× protease inhibitor cocktail, and 0.1% [vol/vol] Tween 20) and incubation on ice for 40 min. After centrifugation, the supernatant was incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) at 4°C for 4 h. The resin was washed three times with IP buffer containing GTEN and 0.1% (vol/vol) Tween 20 and was then analyzed by Western blot, using antibodies against the FLAG epitope, TNV-A^C CP, or Myc epitope.

Expression and purification of recombinant proteins.

All plasmids were transformed into *E. coli* strains BL21 or Rosetta. The bacteria containing plasmids were incubated at 37°C until it reached an OD₆₀₀ = 0.8. The target protein expression was then induced by the addition of 0.2 mM isopropyl-β-D-thiogalactoside to the medium and incubation at 18°C for 18 h in a shaker. Recombinant proteins were purified from bacteria, using Glutathione Sepharose 4 Fast Flow (GE Healthcare) or Ni-NTA agarose (Qiagen) according to manufacturer instructions.

GST pull-down assay.

A GST pull-down assay was conducted as described previously, with minor modifications (Wang et al. 2018). About 10 µg of purified GST-NbHsc70-2 or GST-GmHsc70-2 proteins were preincubated with 15 µl of Glutathione Sepharose 4 Fast Flow (GE Healthcare) for 1.5 h at room temperature in 1 ml binding buffer (50 mM Tris-HCl [pH 7.5] containing 250 mM NaCl, 5 mM DTT, 0.2% [vol/vol] glycerol, and 0.6% [vol/vol] Triton X-100). After centrifugation, the supernatant was removed and 10 µg of His-tagged proteins were incubated with the GST beads at room temperature for 2 h. The GST beads were washed six times with washing buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.2% [vol/vol] glycerol, and 0.6% [vol/vol] Triton X-100) prior to Western blot analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

SwissModel protein structure server: <https://swissmodel.expasy.org>

LITERATURE CITED

Agranovsky, A. A., Boyko, V. P., Karasev, A. V., Koonin, E. V., and Dolja, V. V. 1991. Putative 65 kDa protein of beet yellows closterovirus is a homologue of HSP70 heat shock proteins. *J. Mol. Biol.* 217:603-610.

Alam, S. B., and Rochon, D. 2015. Cucumber necrosis virus recruits cellular heat shock protein 70 homologs at several stages of infection. *J. Virol.* 90:3302-3317.

Alam, S. B., and Rochon, D. 2017. Evidence that Hsc70 is associated with Cucumber necrosis virus particles and plays a role in particle disassembly. *J. Virol.* 91:e01555-16.

Allison, R., Thompson, C., and Ahlquist, P. 1990. Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. *Proc. Natl. Acad. Sci. U.S.A.* 87: 1820-1824.

Benkert, P., Biasini, M., and Schwede, T. 2011. Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics* 27:343-350.

Benkert, P., Tosatto, S. C., and Schomburg, D. 2008. QMEAN: A comprehensive scoring function for model quality assessment. *Proteins* 71:261-277.

Callaway, A., Giesman-Cookmeyer, D., Gillock, E. T., Sit, T. L., and Lommel, S. A. 2001. The multifunctional capsid proteins of plant RNA viruses. *Annu. Rev. Phytopathol.* 39:419-460.

Callaway, A. S., George, C. G., and Lommel, S. A. 2004. A sobemovirus coat protein gene complements long-distance movement of a coat protein-null Dianthovirus. *Virology* 330:186-195.

Canto, T., Prior, D. A. M., Hellwald, K.-H., Oparka, K. J., and Palukaitis, P. 1997. Characterization of cucumber mosaic virus. *Virology* 237:237-248.

Chen, K. C., Chiang, C. H., Raja, J. A., Liu, F. L., Tai, C. H., and Yeh, S. D. 2008. A single amino acid of niapro of papaya ringspot virus determines host specificity for infection of papaya. *Mol. Plant-Microbe Interact.* 21: 1046-1057.

Cheng, S., and Brooks, C. L., 3rd. 2013. Viral capsid proteins are segregated in structural fold space. *PLoS Comput. Biol.* 9:e1002905.

Chromy, L. R., Oltman, A., Estes, P. A., and Garcea, R. L. 2006. Chaperone-mediated in vitro disassembly of polyoma- and papilloma-viruses. *J. Virol.* 80:5086-5091.

Chromy, L. R., Pipas, J. M., and Garcea, R. L. 2003. Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc. Natl. Acad. Sci. U.S.A.* 100:10477-10482.

Curtis, M. D., and Grossniklaus, U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133:462-469.

Eisenberg, D., Lüthy, R., and Bowie, J. U. 1997. VERIFY3D: Assessment of protein models with three-dimensional profiles. Pages 396-404 in: *Methods in Enzymology*, Academic Press, Cambridge, MA, U.S.A.

Fu, S., Xu, Y., Li, C., Li, Y., Wu, J., and Zhou, X. 2018. Rice stripe virus interferes with S-acylation of remorin and induces its autophagic degradation to facilitate virus infection. *Mol. Plant* 11:269-287.

García, J. A., and Pallás, V. 2015. Viral factors involved in plant pathogenesis. *Curr. Opin. Virol.* 11:21-30.

Hafren, A., Hofius, D., Rönholm, G., Sonnwald, U., and Mäkinen, K. 2010. HSP70 and its cochaperone CIP promote potyvirus infection in *Nicotiana benthamiana* by regulating viral coat protein functions. *Plant Cell* 22:523-535.

Hayden, C. M., Mackenzie, A. M., Skotnicki, M. L., and Gibbs, A. 1998. Turnip yellow mosaic virus isolates with experimentally produced recombinant virion proteins. *J. Gen. Virol.* 79:395-403.

Jiang, L., Min, L., Cui, L., Yang, G., DaWei, L., ChengGui, H., and JiaLin, Y. 2008. Effects on the local symptoms of subgenomic RNAs expressions and their translational products of *Tobacco necrosis virus* A Chinese isolate. *Sci. Bull. (Beijing)* 53:1682-1690.

King, A. M. Q., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J. 2011. *Virus taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier, San Diego, CA, U.S.A.

Krenz, B., Windeisen, V., Wege, C., Jeske, H., and Kleinow, T. 2010. A plastid-targeted heat shock cognate 70kDa protein interacts with the Abutilon mosaic virus movement protein. *Virology* 401:6-17.

Li, Z., Jiang, Z., Yang, X., Yue, N., Wang, X., Zhang, K., Jackson, A. O., Li, D., and Zhang, Y. 2020. Construction of an infectious poa semilatifolius virus cDNA clone and comparisons of hordevirus cytopathology and pathogenicity. *Phytopathology* 110:215-227.

Liu, D., Shi, L., Han, C., Yu, J., Li, D., and Zhang, Y. 2012. Validation of reference genes for gene expression studies in virus-infected *Nicotiana benthamiana* using quantitative real-time PCR. *PLoS One* 7:e46451.

Mathioudakis, M. M., Veiga, R., Ghita, M., Tsikou, D., Medina, V., Canto, T., Makris, A. M., and Livieratos, I. C. 2012. *Pepino mosaic virus* capsid protein interacts with a tomato heat shock protein cognate 70. *Virus Res.* 163:28-39.

Mayer, M. P. 2005. Recruitment of Hsp70 chaperones: A crucial part of viral survival strategies. Pages 1-46 in: *Reviews of Physiology, Biochemistry and Pharmacology*, Springer, Berlin.

Mayer, M. P., and Bukau, B. 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* 62:670-684.

Moury, B., and Simon, V. 2011. dN/dS-based methods detect positive selection linked to trade-offs between different fitness traits in the coat protein of potato virus Y. *Mol. Biol. Evol.* 28:2707-2717.

Ni, P., and Cheng Kao, C. 2013. Non-encapsidation activities of the capsid proteins of positive-strand RNA viruses. *Virology* 446:123-132.

Niu, S., Gil-Salas, F. M., Tewary, S. K., Samales, A. K., Johnson, J., Swaminathan, K., and Wong, S. M. 2014. *Hibiscus chlorotic ringspot*

- virus* coat protein is essential for cell-to-cell and long-distance movement but not for viral RNA replication. *PLoS One* 9:e113347.
- Oda, Y., Saeki, K., Takahashi, Y., Maeda, T., Naitow, H., Tsukihara, T., and Fukuyama, K. 2000. Crystal structure of tobacco necrosis virus at 2.25 Å resolution. *J. Mol. Biol.* 300:153-169.
- Olson, A. J., Bricogne, G., and Harrison, S. C. 1983. Structure of tomato bushy stunt virus IV. The virus particle at 2.9 Å resolution. *J. Mol. Biol.* 171:61-93.
- Pantaleo, V., Grieco, F., Di Franco, A., and Martelli, G. P. 2006. The role of the C-terminal region of olive latent virus 1 coat protein in host systemic infection. *Arch. Virol.* 151:1973-1983.
- Peremyslov, V. V., Hagiwara, Y., and Dolja, V. V. 1999. HSP70 homolog functions in cell-to-cell movement of a plant virus. *Proc. Natl. Acad. Sci. U.S.A.* 96:14771-14776.
- Poulicard, N., Pinel-Galzi, A., Hebrard, E., and Fargette, D. 2010. Why rice yellow mottle virus, a rapidly evolving RNA plant virus, is not efficient at breaking rymv1-2 resistance. *Mol. Plant Pathol.* 11:145-154.
- Pu, H., Li, J., Li, D., Han, C., and Yu, J. 2013. Identification of an internal RNA element essential for replication and translational enhancement of tobacco necrosis virus A(C). *PLoS One* 8:e57938.
- Rao, A. L. 2006. Genome packaging by spherical plant RNA viruses. *Annu. Rev. Phytopathol.* 44:61-87.
- Ryu, K. H., Kim, C. H., and Palukaitis, P. 1998. The coat protein of cucumber mosaic virus is a host range determinant for infection of maize. *Mol. Plant-Microbe Interact.* 11:351-357.
- Sacher, R., and Ahlquist, P. 1989. Effects of deletions in the N-terminal basic arm of brome mosaic virus coat protein on RNA packaging and systemic infection. *J. Virol.* 63:4545-4552.
- Salánki, K., Kiss, L., Gellért, A., and Balázs, E. 2011. Identification a coat protein region of cucumber mosaic virus (CMV) essential for long-distance movement in cucumber. *Arch. Virol.* 156:2279-2283.
- Satyanarayana, T., Gowda, S., Mawassi, M., Albiach-Martí, M. R., Ayllón, M. A., Robertson, C., Garnsey, S. M., and Dawson, W. O. 2000. Closterovirus encoded Hsp70 homolog and p61 in addition to both coat proteins function in efficient virion assembly. *Virology* 278:253-265.
- Schmitz, I., and Rao, A. L. N. 1996. Molecular studies on bromovirus capsid protein. I. Characterization of cell-to-cell movement-defective RNA3 variants of brome mosaic virus. *Virology* 226:281-293.
- Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9:671-675.
- Seo, J. K., Vo Phan, M. S., Kang, S. H., Choi, H. S., and Kim, K. H. 2013. The charged residues in the surface-exposed C-terminus of the Soybean mosaic virus coat protein are critical for cell-to-cell movement. *Virology* 446:95-101.
- Séron, K., and Haenni, A. L. 1996. Vascular movement of plant viruses. *Mol. Plant-Microbe Interact.* 9:435-442.
- Shin, H. I., Chae, K. H., and Cho, T. J. 2013. Modification of turnip yellow mosaic virus coat protein and its effect on virion assembly. *BMB Rep.* 46:495-500.
- Sit, T. L., Haikal, P. R., Callaway, A. S., and Lommel, S. A. 2001. A single amino acid mutation in the carnation ringspot virus capsid protein allows virion formation but prevents systemic infection. *J. Virol.* 75:9538-9542.
- Suehiro, N., Natsuaki, T., Watanabe, T., and Okuda, S. 2004. An important determinant of the ability of turnip mosaic virus to infect *Brassica* spp. and/or *Raphanus sativus* is in its P3 protein. *J. Gen. Virol.* 85:2087-2098.
- Tatineni, S., and French, R. 2014. The C-terminus of *Wheat streak mosaic virus* coat protein is involved in differential infection of wheat and maize through host-specific long-distance transport. *Mol. Plant-Microbe Interact.* 27:150-162.
- Tatineni, S., Gowda, S., and Dawson, W. O. 2010. Heterologous minor coat proteins of citrus tristeza virus strains affect encapsidation, but the coexpression of HSP70h and p61 restores encapsidation to wild-type levels. *Virology* 402:262-270.
- Tatineni, S., Robertson, C. J., Garnsey, S. M., and Dawson, W. O. 2011. A plant virus evolved by acquiring multiple nonconserved genes to extend its host range. *Proc. Natl. Acad. Sci. U.S.A.* 108:17366-17371.
- Vaewhongs, A. A., and Lommel, S. A. 1995. Virion formation is required for the long-distance movement of red clover necrotic mosaic virus in movement protein transgenic plants. *Virology* 212:607-613.
- Wang, X., Cao, X., Liu, M., Zhang, R., Zhang, X., Gao, Z., Zhao, X., Xu, K., Li, D., and Zhang, Y. 2018. Hsc70-2 is required for beet black scorch virus infection through interaction with replication and capsid proteins. *Sci. Rep.* 8:4526.
- Xi, D., Li, J., Cao, C., Han, C., Li, D., Yu, J., and Zhou, X. 2007. Characterization of *Tobacco necrosis virus A*, an isolate infected soybean (*Glycine max*). *Acta Phytopathol. Sin.* 37:595-603.
- Xi, D., Li, J., Han, C., Li, D., Yu, J., and Zhou, X. 2008. Complete nucleotide sequence of a new strain of *Tobacco necrosis virus A* infecting soybean in China and infectivity of its full-length cDNA clone. *Virus Genes* 36:259-266.
- Yang, M., Zhang, Y., Xie, X., Yue, N., Li, J., Wang, X. B., Han, C., Yu, J., Liu, Y., and Li, D. 2018. Barley stripe mosaic virus γb protein subverts autophagy to promote viral infection by disrupting the ATG7-ATG8 interaction. *Plant Cell* 30:1582-1595.
- Zhang, X., Zhao, X., Zhang, Y., Niu, S., Qu, F., Zhang, Y., Han, C., Yu, J., and Li, D. 2013. N-terminal basic amino acid residues of *Beet black scorch virus* capsid protein play a critical role in virion assembly and systemic movement. *Virol. J.* 10:200.
- Zhang, Y., Song, G., Lal, N. K., Nagalakshmi, U., Li, Y., Zheng, W., Huang, P. J., Branon, T. C., Ting, A. Y., Walley, J. W., and Dinesh-Kumar, S. P. 2019. TurboID-based proximity labeling reveals that UBR7 is a regulator of N NLR immune receptor-mediated immunity. *Nat. Commun.* 10:3252.
- Zhao, X., Wang, X., Dong, K., Zhang, Y., Hu, Y., Zhang, X., Chen, Y., Wang, X., Han, C., Yu, J., and Li, D. 2015. Phosphorylation of *Beet black scorch virus* coat protein by PKA is required for assembly and stability of virus particles. *Sci. Rep.* 5:11585.