INTRODUCTION

Newly synthesized membrane proteins undergo a strict quality-control checkpoint, and misfolded secretory proteins are targeted across the endoplasmic reticulum (ER) membrane back to the cytosol for proteasome degradation in a process known as ER-associated degradation (ERAD) (Bernasconi & Molinari, 2011; Ye et al., 2004). The ERAD machinery is quite complex and requires the synchronization of many different proteins, including B-cell receptor associated protein 31 (BAP31), Derlin-1, and members of the heat shock protein family.

A plant reovirus hijacks the DNAJB12–Hsc70 chaperone complex to promote viral spread in its planthopper vector

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Abstract

Many viruses usurp the functions of endoplasmic reticulum (ER) for virus-encoded membrane proteins proper functional folding or assembly to promote virus spread. Southern rice black-streaked dwarf virus (SRBSDV), a plant reovirus, exploits virus-containing tubules composed of nonstructural membrane protein P7-1 to spread in its planthopper vector Sogatella furcifera. Here, we report that two factors of the ER-associated degradation (ERAD) machinery, the ER chaperone DNAJB12 and its cytosolic co-chaperone Hsc70, are activated by SRBSDV to facilitate ER-to-cytosol export of P7-1 tubules in S. furcifera. Both P7-1 of SRBSDV and Hsc70 directly bind to the J-domain of DNAJB12. DNAJB12 overexpression induces ER retention of P7-1, but Hsc70 overexpression promotes the transport of P7-1 from the ER to the cytosol to initiate tubule assembly. Thus, P7-1 is initially retained in the ER by interaction with DNAJB12 and then delivered to Hsc70. Furthermore, the inhibitors of the ATPase activity of Hsc70 reduce P7-1 tubule assembly, suggesting that the proper folding and assembly of P7-1 tubules is dependent on the ATPase activity of Hsc70. The DNAJB12–Hsc70 chaperone complex is recruited to P7-1 tubules in virus-infected midgut epithelial cells in S. furcifera. The knockdown of DNAJB12 or Hsc70 strongly inhibits P7-1 tubule assembly in vivo, finally suppressing effective viral spread in S. furcifera. Taken together, our results indicate that the DNAJB12–Hsc70 chaperone complex in the ERAD machinery facilitates the ER-to-cytosol transport of P7-1 for proper assembly of tubules, enabling viral spread in insect vectors in a manner dependent on ATPase activity of Hsc70.

KEYWORDS
DNAJB12, Hsc70, P7-1, SRBSDV, tubule

1 | INTRODUCTION

Newly synthesized membrane proteins undergo a strict quality-control checkpoint, and misfolded secretory proteins are targeted across the endoplasmic reticulum (ER) membrane back to the cytosol for proteasome degradation in a process known as ER-associated degradation (ERAD) (Bernasconi & Molinari, 2011; Ye et al., 2004). The ERAD machinery is quite complex and requires the synchronization of many different proteins, including B-cell receptor associated protein 31 (BAP31), Derlin-1, and members of the heat shock protein family.
(Hsp) families (Geiger et al., 2011; Meusser et al., 2005; Sopha et al., 2012). BAP31 is a ubiquitous ER membrane protein that has been implicated in the ER retention of transmembrane proteins (Geiger et al., 2011; Yu et al., 2021). Hsp40/DNAJ family proteins contain a J-domain, a glycin/phenylalanine-rich region, and a single transmembrane (TM) domain (Grove et al., 2011; Sopha et al., 2017). DnaJb11 is an ER-targeted Hsp40 chaperone required for the proper folding, assembly, trafficking, and delivery of membrane proteins to immunoglobulin heavy chain binding protein in pre-B cells (Bip), the ER Hsp70 for ATP-dependent chaperoning (Jin et al., 2009; Tan et al., 2014). The heat shock cognate 70-kDa protein (Hsc70) is an important and general Hsp70 family member (Rosenzweig et al., 2019; Stricher et al., 2013). Another ER-targeted Hsp40 chaperone, DNAJB12, specifically interacts with Hsc70 to facilitate ER retention and delivery of membrane proteins for folding or degradation in the ERAD pathway (Grove et al., 2011). Hsc70 chaperone activities require the substrate targeting function of DNAJB12 (Alderson et al., 2016; Dupzyk et al., 2017; Walczak et al., 2014). DNAJB12 recruits specific substrates to contact Hsc70 and then stimulates its ATPase activity (Alderson et al., 2016; Mayer, 2013). The folding action of Hsc70 is related to its ATPase activity, and different binding modes in the ATP- and ADP-bound states of Hsc70 regulate substrate protein binding affinity (Kampinga & Craig, 2010). However, the mechanisms by which viruses activate the co-chaperones in the ERAD machinery remain elusive.

Hsp70 generally function as molecular chaperones that ensure proper folding and translocation of nascent membrane proteins (Rosenzweig et al., 2019). Virus-encoded membrane proteins, such as replication or movement proteins, are synthesized in the ER membrane and then transported into the cytosol for proper functional folding or assembly (Chen et al., 2019; Feng et al., 2016; Pogany & Nagy, 2015; Taguwa et al., 2015, 2019). Many viruses, including polyomaviruses, dengue virus, tomato bushy stunt virus, and tomato spotted wilt virus, have been reported to hijack host Hsps to assist in the synthesis, folding, and assembly of viral membrane proteins (Chen et al., 2019; Feng et al., 2016; Pogany & Nagy, 2015; Taguwa et al., 2015, 2019). Many viruses, including polyomaviruses, dengue virus, tomato bushy stunt virus, and tomato spotted wilt virus, have been reported to hijack host Hsps to assist in the synthesis, folding, and assembly of viral membrane proteins (Chen et al., 2019; Feng et al., 2016; Pogany & Nagy, 2015; Taguwa et al., 2015, 2019). In particular, the cytosolic chaperone Hsc70 could facilitate the proper folding of viral membrane proteins to play important roles in virus propagation, such as viral entry, intracellular trafficking, genome replication, and assembly (Dupzyk et al., 2017; Kaufer et al., 2012; Ravindran et al., 2015). The nonenveloped polyomavirus SV40 recruits the DNAJ proteins DNAJB11–14 to the foci structure on the ER membrane, leading to the recruitment of cytosolic Hsc70 to promote the ER-to-cytosol export of intact viral particles (Chen et al., 2019; Ravindran et al., 2015; Walczak et al., 2014). However, how viruses hijack the complex of ER membrane DNAJ proteins and their cytosolic chaperone Hsc70 to promote viral replication, spread, or release from infected cells is still poorly understood.

Southern rice black-streaked dwarf virus (SRBSDV) is an insect-borne plant reovirus transmitted by Sogatella furcifera (white-backed planthopper) in a persistent-propagative manner (Jia et al., 2014; Pu et al., 2012). SRBSDV encodes at least six putative structural proteins (P1, P2, P3, P4, P8, and P10) and six putative nonstructural proteins (P5, P6, P7-1, P7-2, P9-1, and P9-2) (Mao et al., 2013). Early in viral infection of insect vector cells, nonstructural proteins P5, P6, and P9-1 of SRBSDV associate together to form the initial viroplasm matrix, where viral replication and assembly of progeny virions occur (Mao et al., 2013). Then, tubular structures produced by nonstructural membrane protein P7-1 of SRBSDV package virions to facilitate efficient viral spread in the midgut epithelial cells of insect vectors (Jia et al., 2014; Mao et al., 2017; Wei & Li, 2016). Previously, we have determined that the positive-sense RNA of SRBSDV P7-1 synthesized within the viroplasm migrates to the adjacent ribosomes on the ER membrane to direct the synthesis of the tubular protein P7-1, which is then transported to the cytosol for assembly of virus-containing tubules (Jia et al., 2014; Mao et al., 2013; Yu et al., 2021). We have shown that the ER-associated proteins BAP31 and DNAJB11 can directly interact with P7-1, leading to the ER retention of P7-1, which further facilitates tubule formation in S. furcifera (Liu et al., 2011; Yu et al., 2021). Here, we further demonstrate that P7-1 of SRBSDV is retained by the ER-membrane protein DNAJB12 to the ER for protein synthesis and then is delivered to cytosolic Hsc70 via the DNAJB12 J-domain, which is essential for tubule assembly. Furthermore, DNAJB12–Hsc70 chaperone complex promotes P7-1 tubule formation in a manner dependent on the ATPase activity of Hsc70. More importantly, DNAJB12–Hsc70 chaperone complex is activated by SRBSDV infection to facilitate P7-1 tubule assembly and spread within viruliferous S. furcifera.

2 | RESULTS

2.1 | SRBSDV infection remodels the ER membrane in insect vectors

Electron microscopy showed that SRBSDV infection induced the formation of abundant virus-containing tubules in the cytoplasm of midgut epithelial cells of S. furcifera (Figure 1a). Approximately 30% of tubules observed were closely associated with the ER membrane (Figure 1a,b), while other tubules were accumulated in the cytoplasm as previously described (Jia et al., 2014). These results further confirmed that some tubular proteins were transported to the cytosol from the ER membrane for assembly of virus-containing tubules. Thus, SRBSDV infection may remodel the ER membrane to promote the assembly of P7-1 tubules in insect vectors.

We next tested whether viral infection affected the expression levels of ER-associated proteins from S. furcifera, such as chaperones calnexin (CANX), protein disulphide isomerase (PDI), Bip, DNAJB12, and the DNAJB12 co-chaperone Hsc70. Reverse transcription quantitative PCR (RT-qPCR) showed that the mRNA transcript levels of Bip, PDI, DNAJB12, and Hsc70 were significantly increased, while that of CANX did not significantly change during viral infection in S. furcifera (Figure 1c). We thus prepared antibodies against Bip, PDI, DNAJB12, Hsc70, and CANX. Western blot assay confirmed that Bip, PDI, DNAJB12, and Hsc70 were sustainably increased at the
Southern rice black-streaked dwarf virus (SRBSDV) infection remodels the endoplasmic reticulum (ER) membrane in *Sogatella furcifera*. (a) The association of virus-containing tubules with ER membranes in the cytoplasm of viruliferous *S. furcifera* midgut, as examined by transmission electron microscopy. Virus-free midgut from nonviruliferous *S. furcifera* served as the control. Bars, 100 nm. (b) Percentage of the number of ER-associated or cytosol-present tubules as measured from more than 30 midgut epithelial cells. ***p < 0.001. (c) At 6 days post-first access of insects to diseased plants (padp), mRNA transcript levels of Bip, PDI, DNAJB12, CANX, and Hsc70 in nonviruliferous and viruliferous *S. furcifera* were measured by reverse transcription quantitative PCR (RT-qPCR) assay. Means (± SEM) from three biological replicates are shown (n ≥ 30). The significance of any observed differences was tested using an independent t test (*p < 0.05; ns, not significant). (d) At 6 days padp, protein accumulation levels of P7-1 and P10 of SRBSDV and ER-associated proteins, including Bip, DNAJB12, PDI, CANX, and cytosolic Hsc70 in nonviruliferous and viruliferous *S. furcifera*, were detected by western blot assay by using P7-1-, P10-, Bip-, DNAJB12-, PDI-, CANX-, and Hsc70-specific IgG. Insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with GAPDH-specific IgG as an internal control. Relative protein levels were determined using ImageJ and the accumulation of Bip, DNAJB12, PDI, CANX, and Hsc70 protein levels in nonviruliferous insects were normalized to 1. V-, nonviruliferous; V+, viruliferous.
protein levels during viral infection (Figure 1d). These results indicate that SRBSDV infection can remodel ER membranes to facilitate SRBSDV infection in insect vectors.

2.2 | The DNAJB12–Hsc70 chaperone complex interacts with tubular protein P7-1

We next investigated whether P7-1 of SRBSDV directly interacted with the ER-associated proteins of S. furcifera. Yeast two-hybrid assay showed that P7-1 interacted with DNAJB12 and Hsc70, but not with Bip, PDI, and CANX (Figure 2a). As expected, DNAJB12 also directly interacted with its co-chaperone Hsc70 (Figure 2b). DNAJB12 is an ER-associated Hsp40 family protein containing a cytosolic J-domain to contact cytosolic Hsc70 for facilitating the folding and/or degradation of membrane proteins (Grove et al., 2011; Younger et al., 2004). DNAJB12 from S. furcifera shared 41.6% amino acid identity with human DNAJB12, which also contained a conserved J-domain, a Gly/Phe-rich region, and a single TM domain (Figure 2c and S1).

Yeast two-hybrid assay further revealed that both P7-1 of SRBSDV and Hsc70 interacted with DNAJB12 via the J-domain, but not the other domains (Figure 2c–e). Cytosolic Hsc70 contained one ATPase domain (ATP), one substrate-binding domain (SBD), a short linker between the ATP and SBD regions, and a C-terminus domain (CTD) (Figure 2c). Yeast two-hybrid assay further indicated that P7-1 of SRBSDV and DNAJB12 interacted with Hsc70 via its SBD and CTD domains, respectively (Figure 2f,g). Thus, the biogenesis of P7-1 tubule formation may be mediated by the DNAJB12–Hsc70 chaperone complex.

2.3 | The DNAJB12–Hsc70 chaperone complex facilitates P7-1 tubule formation

The expression of the membrane protein P7-1 of SRBSDV alone induced the formation of tubules in S. frugiperda (S9) cells (Liu et al., 2011). We initially investigated P7-1 subcellular localization in S9 cells by using the recombinant baculovirus expression system. Immunofluorescence microscopy revealed that P7-1 was initially observed at 18 hours postinfection (hpi) and then aggregated to form tubular structures in the cytoplasm at 36 hpi (Figure S2). At 24 hpi, P7-1 of SRBSDV was detected in a pattern that overlapped with Bip, an ER marker, but the assembling tubules had formed and released from the ER membrane at 48 hpi (Figure 3a). These findings further confirmed that P7-1 was initially synthesized in the ER and then exported from the ER to the cytosol for tubule assembly.

Based on the interaction among P7-1, DNAJB12, and Hsc70, we next co-infected S9 cells with recombinant baculoviruses containing DNAJB12, Hsc70, or P7-1 to examine their relationship by immunofluorescence microscopy. When expressed alone, DNAJB12 and Hsc70 appeared to be distributed in the ER and cytosol, respectively (Figure 3b). The co-infection of DNAJB12 and Hsc70 led to the redistribution of Hsc70 into the ER-like structure of DNAJB12 (Figure 3c).

Whether at 24 or 48 hpi, the co-infection of P7-1 and DNAJB12 showed that P7-1 was always retained in the ER-like structure of DNAJB12 (Figure 3d). However, the co-infection of P7-1 and Hsc70 showed that P7-1 was colocalized with Hsc70 at 24 hpi and then the P7-1 tubules were formed at 48 hpi (Figure 3e). Thus, the overexpression of DNAJB12 retained P7-1 in the ER, while Hsc70 was involved in the assembly of P7-1 tubules. Interestingly, at 48 hpi, the S9 cells triple infected with DNAJB12, Hsc70, and P7-1 exhibited the clear formation of P7-1 tubules in the cytoplasm (Figure 3f). We then calculated the percentage of tubule-forming cells in a total of 100 S9 cells, which were singly infected with P7-1, co-infected with DNAJB12 and P7-1, co-infected with Hsc70 and P7-1, or triply infected with DNAJB12, Hsc70, and P7-1, respectively. These results clearly showed that overexpression of Hsc70 and DNAJB12 promoted and inhibited the assembly of P7-1 tubules, respectively (Figure 3g).

To confirm the interaction among DNAJB12, Hsc70, and P7-1, S9 cells triply infected with DNAJB12, Hsc70, and P7-1 were subjected to immunoprecipitation with DNAJB12 antibody. This resulted in the co-immunoprecipitation of Hsc70 and P7-1 by DNAJB12 antibody (Figure 3h). Furthermore, S9 cells co-infected with Hsc70 and P7-1 were immunoprecipitated with Hsc70 antibody, and P7-1 was also co-immunoprecipitated with Hsc70 (Figure 3i). Thus, P7-1 of SRBSDV formed complexes with Hsc70 and DNAJB12.

As Hsc70 was recruited to the ER membrane and important in P7-1 tubule formation, we hypothesized that it might promote the extraction of P7-1 tubules into the cytosol from the ER membrane. Western blot assay showed that P7-1 was mostly retained in the insoluble portion of the total extracted proteins from S9 cells co-infected with P7-1 and DNAJB12 (Figure 4a,b), while P7-1 accumulated in the soluble portion of S9 cells co-infected with P7-1 and Hsc70 (Figure 4c). Furthermore, a semipermeabilized cytosol arrival assay was used to monitor the transport of P7-1 into the cytosol from the ER membrane.

Western blot assay showed that P7-1 was mostly retained in the insoluble portion of the total extracted proteins from S9 cells singly infected with P7-1 and DNAJB12 (Figure 4a,b), while P7-1 accumulated in the soluble portion of S9 cells co-infected with P7-1 and Hsc70 (Figure 4c). Western blot assay indicated that DNAJB12 overexpression strongly retained P7-1 in the ER, as detected by CANX antibody, while the overexpression of Hsc70 increased the P7-1 levels in the cytosol (Figure 4d). These results suggest that DNAJB12 as a sorting factor may transiently retain the newly synthesized P7-1 in the ER and then deliver it to the Hsc70-containing chaperoning complex in the cytosol to ensure the proper assembly of P7-1 tubules.

2.4 | The assembly of P7-1 tubules is dependent on ATPase activity of Hsc70

To examine whether the assembly of P7-1 tubules was dependent on the ATPase activity of Hsc70 chaperone, different concentrations
Interactions among P7-1 of SRBSDV, DNAJB12, and Hsc70. (a) Interactions between P7-1 of SRBSDV with Bip, PDI, CANX, DNAJB12, and Hsc70 of Sogatella furcifera were detected by the DUALhunter system yeast two-hybrid assay. For the yeast two-hybrid assay, yeast transformants were assayed for growth on nonselective plates (SD/−Leu/−Trp, DDO) and selective plates (SD/−Leu/−Trp/−His/−Ade with 5 mM 3-AT, QDO/5 mM 3-AT). Transformants on plates were labelled as follows: BD, pDHB1; AD, pPR3-N; positive, positive control (pLargeT/p53); negative, negative control (pDHB1/pPR3-N). Serially diluted yeast cultures were grown on DDO and QDO/5 mM 3-AT plates. (b) Interactions between DNAJB12 and Hsc70 were detected by yeast two-hybrid assay as described above. (c) Schematic of DNAJB12 and Hsc70 domains. The domains were labelled as follows: JPD, J-domain protein (amino acids 101–163); Gly/Phe, glycine/phenylalanine-rich region; TM, transmembrane domain (amino acids 240–262); ATPase, ATPase domain (amino acids 1–378); SBD, substrate-binding domain (amino acids 385–518); CTD, C-terminus domain (amino acids 519–656). (d–g) Interactions between P7-1 and DNAJB12 domains (d), between Hsc70 and DNAJB12 domains (e), between DNAJB12 and Hsc70 domains (f), and between P7-1 and Hsc70 domains (g) were detected by yeast two-hybrid assay as described above.
of ATP were added to the cellular medium after a 3-h infection of SF9 cells with recombinant baculovirus containing P7-1 of SRBSDV. Treatment with 1.25 mM ATP effectively inhibited P7-1 tubule formation in SF9 cells (Figure 5a,b). An allosteric inhibitor of VER155008, which competed with ATP for Hsc70/Hsp70 binding, was used to inhibit the folding function of Hsc70 in SF9 cells. The 20 μM VER155008 treatment showed low toxicity to SF9 cells (Figure S3), but effectively inhibited P7-1 tubule formation in SF9 cells (Figure 5c,d). Immunoelectron microscopy confirmed that the formation of tubular structures (of about 85 nm in diameter) that specifically reacted with P7-1 antibody in the cytoplasm of SF9 cells was strongly inhibited by the treatment of 1.25 mM ATP or 10 μM VER155008 (Figure 5e). Thus, Hsc70 inhibitors significantly suppressed the ability for correct folding and assembly of P7-1 tubules in SF9 cells, suggesting that P7-1 tubule biogenesis was dependent on the ATPase activity of Hsc70.
DNAJB12–Hsc70 chaperone complex is essential for P7-1 tubules assembly in vivo

The observations above led us to examine whether the co-chaperones DNAJB12 and Hsc70 facilitated P7-1 tubule formation during viral infection of S. furcifera. RT-qPCR and western blot assays showed that viral infection up-regulated the expression of DNAJB12 and Hsc70 at the mRNA and protein levels (Figure 1c,d). In virus-free midgut epithelial cells of nonviruliferous S. furcifera, immunofluorescence microscopy showed that DNAJB12 and Hsc70 were localized to small discrete puncta throughout the cytoplasm (Figure 6a). However, at 6 days post-first access of insects to diseased plants (padp), in the virus-infected midgut epithelial cells of viruliferous S. furcifera, immunofluorescence microscopy showed that DNAJB12 and Hsc70 were localized to small discrete puncta throughout the cytoplasm (Figure 6a).

3 | DISCUSSION

The ERAD system contains several ER-related proteins, such as BAP31, Derlin1, Derlin2, the DNAJ family proteins DNAJB11 and DNAJB12, and the Hsp70 family proteins Bip and Hsc70 (Baaklini et al., 2020; Geiger et al., 2011; Grove et al., 2011; Jin et al., 2009; Oda et al., 2006; Tan et al., 2014; Yu et al., 2021). SRBSDV P7-1 is initially retained in the ER, and then secreted into the cytosol for tubule assembly and further packaging of viral particles to be spread within the bodies of insect vectors (Yu et al., 2021). How P7-1 crosses the ER membrane to the cytosol for tubule assembly has been an unexplained step. Previously, we have shown that the ER-membrane protein BAP31 binds to the N-terminus of P7-1, which can mediate the direct ER retention (Yu et al., 2021). Furthermore, the ER-targeted DNAJB11 competes with BAP31 to bind P7-1, ultimately ensuring
the proper assembly of P7-1 tubules (Yu et al., 2021). DNAJB11, an ER-targeted Hsp40 chaperone, generally binds to membrane proteins and delivers them to Hsp70 Bip in the ER membrane for ATP-dependent chaperoning (Jin et al., 2009); however, we have confirmed that S. furcifera Bip did not directly interact with SRBSDV P7-1. Thus, P7-1 can bind directly to DNAJB11 in the ER without being delivered to Bip for ATP-dependent chaperoning. Here, we further reveal the DNAJB12–Hsc70 chaperone complex facilitates the ER-to-cytosol transport of P7-1 of SRBSDV for tubule assembly in a manner that depends on the ATPase activity of Hsc70 (Figure 7).

DNAJB12 is an ER-associated Hsp40 chaperone containing a J-domain facing the cytosol that directs cytosolic Hsp70 to function on the ER surface in facilitating the folding and/or degradation of membrane proteins (Choi et al., 2014; Walczak et al., 2014; Ye et al., 2004). In the present study, we show that DNAJB12 binds to the membrane protein P7-1 via its J-domain, which can mediate the
direct ER retention of P7-1. The expression of DNAJB12 leads to the retention of P7-1 in the ER to form an insoluble DNAJB12–P7-1 complex. However, co-expression of DNAJB12 and Hsc70 leads to the transport of P7-1 from the ER to the cytosol for tubule assembly, thus making P7-1 soluble. Thus, DNAJB12 can act as an ER retention “receptor” for P7-1 by forming a complex with P7-1. Both Hsc70 and P7-1 interact with DNAJB12 at its J-domain facing the cytosol, while P7-1 interacts with cytosolic Hsc70 at the SBD domain. Thus, the co-expression of DNAJB12 and Hsc70 ultimately leads to the delivery of P7-1 from the DNAJB12–P7-1 complex in the ER to Hsc70 in the cytosol. We thus deduce that DNAJB12 as a sorting factor may transiently retain the newly synthesized P7-1 in the ER and then deliver it with the J-domain to the Hsc70-containing co-chaperoning complex in the cytosol, ultimately ensuring the proper folding and assembly of P7-1 tubules (Figure 7). Similarly, DNAJB12 has been reported to deliver the newly synthesized membrane protein cystic fibrosis transmembrane conductance regulator to a specific ER membrane complex, the Derlin-1 complex (Grove et al., 2011; Okiyoneda et al., 2010). Indeed, the J-domain of DNAJ proteins contacts Hsc70 to stimulate the ATPase activity of the Hsc70 chaperone to promote substrate–Hsc70 interaction (Grove et al., 2011; Sopha et al., 2017).

We further show that the inhibitors of Hsc70 ATPase activity effectively reduced P7-1 tubule assembly ability, suggesting the proper folding and assembly of P7-1 tubules is dependent on the ATPase activity of Hsc70. Thus, the P7-1-associated DNAJB12 potentially directly contacts Hsc70 to stimulate ATP hydrolysis. Furthermore, in its ADP-bound state, Hsc70 may tightly bind P7-1 in the cytosol, thus dissociating DNAJB12 and ultimately ensuring the proper assembly of P7-1 tubules. Based on these discussions, we deduce that DNAJB12–Hsc70 chaperone complex in the ERAD machinery facilitates ER-to-cytosol transport of P7-1 for proper assembly of tubules.

Recently, Zhang et al. (2021) reported that viral particles of SRBSDV could be enclosed in the intracellular vesicles in midgut epithelial cells of viruliferous S. furcifera through direct interaction of the viral major outer capsid protein P10 and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Furthermore, down-regulation of the expression of the SNARE complex would significantly reduce viral transport from the midgut epithelium into the haemolymph (Zhang et al., 2021). Thus, it seems that SRBSDV could hijack both P7-1 tubules and SNARE complex-associated vesicles to overcome the midgut membrane barrier. The transport of the membrane protein P7-1 of SRBSDV from the ER membrane to the cytosol for tubule assembly is essential for efficient viral spread in viruliferous S. furcifera (Jia et al., 2014; Yu et al., 2021). We have determined that the components in the ERAD machinery, including BAP31, DNAJB11, DNAJB12, and Hsc70, are involved in P7-1 tubule assembly, thereby enhancing viral transmission by S. furcifera. Similarly, nonenveloped SV40 reaching the cytosol from the ER also co-opts ERAD components, such as BAP31, DNAJB11–14, and Hsc70 (Dudzik & Tsai, 2016; Ravindran et al., 2015). SRBSDV infection activates and up-regulates the expression of DNAJB12 and Hsc70. Furthermore, the DNAJB12–Hsp70 chaperone complex is reorganized from discrete foci in virus-free cells to P7-1 tubules in virus-infected midgut epithelial cells in viruliferous S. furcifera. The knockdown of DNAJB12 or Hsc70 expression strongly inhibited P7-1 tubule assembly in vivo, ultimately suppressing the effective viral spread in the bodies of insect vectors. Thus, the expression of DNAJB12–Hsp70 chaperone complex is induced to remodel the ER membrane for tubule assembly. Collectively, our results show that virus-mediated regulation of DNAJB12–Hsp70 chaperone complex has been hijacked by SRBSDV to ensure the proper assembly of virus-induced tubules to support viral propagation and transmission by insect vectors.

4 | EXPERIMENTAL PROCEDURES

4.1 | Insects, cells, virus, and reagents

Nonviruliferous individuals of the white-backed planthopper S. furcifera were collected from Fujian Province in southern China and reared in cages with rice seedlings in a greenhouse at 28°C with 16 h light and 8 h dark. Sf9 cells were grown at 27°C in SF900 III medium with 5% foetal bovine serum (Gibco). Rice plants infected with SRBSDV were initially collected from Fujian Province. Polyclonal antibody against major outer capsid protein P10 peptide CTWKVKTKAKDEQD was prepared in rabbit by the Genscript USA Innovation Company, with approval from the Science Technology Department of Jiangsu Province, and polyclonal antibody against nonstructural protein P7-1 was prepared as previously described (Jia et al., 2014). The antibodies against CANX, GAPDH, and PDI were purchased from Sangon Biotech. The StrepMAB- Classics was purchased from IBA Lifesciences. The anti-6x His tag antibody was obtained from Abcam. IgGs were purified from specific polyclonal antisera and conjugated to fluorescein isothiocyanate (FITC), rhodamine, or Alexa Fluor 647 according to the manufacturer’s instructions (Thermo Fisher Scientific). Anti-rabbit IgG (whole molecule)-gold antibody produced in goat was purchased from Sigma-Aldrich. ATP was obtained from Sigma-Aldrich. The inhibitor VER155008 was obtained from MedChemExpress. Polyclonal antisera against Bip, DNAJB12, and Hsc70 of S. furcifera were prepared as described previously (Moriyasu et al., 2000; Wei et al., 2009). Briefly, the complete open reading frames (ORFs) of Bip, DNAJB12, and Hsc70 were amplified by RT-PCR and inserted into the pEASY-Blunt E1 expression vector (TransGen Biotech). The resulting plasmids E1-Bip, E1-DNAJB12, and E1-Hsc70 were transformed into Escherichia coli Rosetta for expression by adding isopropyl-β-D-thiogalactopyranoside (IPTG). Bip, DNAJB12, and Hsc70 were purified using a Ni-NTA resin column.

4.2 | RT-qPCR

Third-instar nymphs of S. furcifera fed on SRBSDV-infected rice plants for 2 days were then transferred to healthy rice seedlings for 6 days. Total insect RNAs were isolated from 30 nonviruliferous
or viruliferous *S. furcifera* adults following the standard TRizol reagent protocol (Invitrogen). The relative quantification of *Bip*, *PDI*, *DNAJB12*, *CANX*, and *Hsc70* mRNA transcript levels was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The expression levels of these genes were normalized with that of the *S. furcifera* *actin5C* gene. All RT-qPCRs were conducted in triplicate for each sample, and three biological replicates were maintained.

### 4.3 Yeast two-hybrid assay

The DUALhunter (Dualsystems Biotech) yeast two-hybrid system was used for protein interaction screening. Briefly, the coding sequence of each tested gene was amplified from *S. furcifera* cDNA. *P7-1* and *Hsc70* were cloned into pDH81 as the bait vectors, respectively, and *Bip*, *PDI*, *CANX*, *DNAJB12*, and *Hsc70* were cloned into
pPR3-N as the prey vectors. The pairs of yeast two-hybrid plasmids were co-transformed into the yeast strain NMY51 following the standard transformation protocol (Dualsystems Biotech). Moreover, a pair of plasmids, pLargeT and p53, also were transformed into NMY51 yeast to serve as the unrelated positive control, while the empty pDHB1/pRR3N served as the negative control. The yeast colonies were grown on SD/-Trp/-Leu (double dropout, DDO) non-selective plates and SD/-Trp/-Leu/-His/-Ade (quadruple dropout, QDO) selective medium plates with 5 mM 3-amino-1,2,4-triazole (3-AT). The colonies were diluted in sterilized water for colour formation in a β-galactosidase assay. Furthermore, to examine the DNAJB12 and Hsc70 interaction domains, Hsc70 WT, Hsc70ΔATP, Hsc70ΔSBD, Hsc70ΔCTD, DNAJB12 WT, DNAJB12ΔJ, DNAJB12ΔTM, or DNAJB12ΔGTM were cloned into pDH1 or pPR3-N as the bait or prey vectors, respectively. The transformants were screened using selective medium as described above.

4.4 | Recombinant baculovirus expression of SRBSDV P7-1, DNAJB12, and Hsc70 in Sf9 cells

Sf9 cells infected with recombinant baculovirus vectors have been previously described (Liu et al., 2011). In brief, P7-1 of SRBSDV, Bip-His, DNAJB12-His, Hsc70-His, and Hsc70-Strep were cloned into the vector pFastBac1 (Thermo Fisher Scientific). Then, the recombinant baculovirus vectors were introduced into Escherichia coli...
DH10Bac (Thermo Fisher) to generate recombinant bacmids. The recombinant bacmids were isolated to transfect SF9 cells in the presence of Cellfectin II (Thermo Fisher) according to the manufacturer’s instructions. After high-titre baculoviral stocks were harvested, an appropriate volume was used for cellular infection on coverslips or in flasks. SF9 cells infected with recombinant baculovirus were fixed in 4% paraformaldehyde, permeabilized in 2% (vol/vol) Triton X-100, and then processed for immunofluorescence microscopy, as described previously (Yu et al., 2021). SF9 cells, cultured in flasks and infected with recombinant bacmids, were harvested for either western blot or co-immunoprecipitation assays at 48 hpi.

4.5 | Co-immunoprecipitation assay

A co-immunoprecipitation assay was performed using the Pierce Co-Immunoprecipitation (Co-IP) Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, DNAJB12 antibody and pre-immune IgG were added to resin at room temperature for a 2-h immobilization. At 2 days postinfection, SF9 cells co-expressing DNAJB12, Hsc70, and P7-1 were lyzed by lysis buffer. The supernatants were incubated with the purified DNAJB12-immobilized resin at 4°C for 1 h. The Co-IP fraction was eluted and collected for subsequent SDS-PAGE and western blot analysis. Hsc70 antibody was added to the resin at room temperature for a 2-h immobilization. At 2 days postinfection, SF9 cells expressing P7-1 or co-expressing P7-1 and Hsc70 were lyzed by lysis buffer. The supernatants were then incubated with the purified Hsc70-immobilized resin at 4°C for 1 h. The Co-IP fraction was eluted and collected for subsequent SDS-PAGE and western blot analysis. Bands were visualized using specific antibodies.

4.6 | Semipermeabilized cytosol arrival assay

The crude subcellular fractions of recombinant baculovirus-infected SF9 cells were separated and tested by the semipermeabilized cytosol arrival method (Ravindran et al., 2015). In brief, at 2 days after infection with the recombinant baculoviruses SF9 cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and lyzed in extraction buffer containing 0.1% Triton X-100 on ice for 10 min, following separation into supernatant (regarded as cytosol) and pellet (regarded as membrane) fractions by centrifugation at 16,500 × g at 4°C for 10 min. To isolate ER-localized P7-1, the pellet fraction was treated with extraction buffer containing 1% Triton X-100 on ice for 10 min and centrifuged at 16,500 × g at 4°C for 10 min. Then, the samples were dissolved in 1× SDS sample buffer and analysed by western blot assay.

4.7 | Effects of Hsc70 inhibitor treatment on the formation of P7-1 tubules in SF9 cells

SF9 cells infected with recombinant baculovirus containing SRBSDV P7-1 were incubated with the appropriate concentrations of ATP (0.5, 1.25, and 2.5 mM) and VER155008 (5, 10, 20, and 40 μM). DMSO treatment served as a control. After 48 hpi, the SF9 cells were fixed, permeabilized, immunolabelled with P7-1-specific IgG conjugated to rhodamine (P7-1-rhodamine) and examined by immunofluorescence microscopy.

4.8 | Effects of knockdown of in vivo expression of DNAJB12 or Hsc70 on P7-1 tubule formation in S. furcifera

At 6 days padp, 30 nonviruliferous or viruliferous S. furcifera individuals were dissected, fixed, permeabilized, and immunolabelled with P7-1-, DNAJB12-, or Hsc70-specific antibodies conjugated to FITC or rhodamine (P7-1-FITC, DNAJB12-rhodamine or Hsc70-rhodamine) and actin dye phallolidin-Alexa Fluor 647, and then examined by immunofluorescence microscopy.

RNA interference (RNAi) was performed to knock down the expression of DNAJB12 and Hsc70. The double-stranded RNAs of DNAJB12 (dsDNAJB12), Hsc70 (dsHsc70), and GFP (dsGFP) were synthesized in vitro using the T7 RiboMAX Express RNAi System, according to the manufacturer’s protocol (Promega). Then, 200 third-instar nymphs of S. furcifera, after being fed on diseased rice plants for 2 days, were microinjected with 55 nl of 0.3 μg/μl dsRNAs, followed by feeding on healthy rice seedlings for 6 days. The interference efficiency of dsRNAs targeting DNAJB12 and Hsc70 were measured by RT-qPCR and western blot assays, respectively. Total RNAs from 30 treated insects were extracted and detected by RT-qPCR as described above. The entire experiment was repeated three times for each group. Relative mRNA transcript levels were normalized to the housekeeping gene actin5C and estimated using the 2-ΔΔCt method (Livak & Schmittgen, 2001). Total proteins were extracted from 30 dsRNA-treated insects. The protein levels of DNAJB12, Hsc70, and P7-1 were analysed by western blot assay with DNAJB12-, Hsc70-, and P7-1-specific IgGs, respectively, and each normalized to the reference protein GAPDH. At the same time, the midguts of 30 viruliferous adults of S. furcifera from each treatment group were dissected, fixed, permeabilized, and immunolabelled with P7-1-FITC, P10-specific IgG conjugated to rhodamine (P10-rhodamine) and actin dye phallolidin-Alexa Fluor 647, and then examined by immunofluorescence microscopy.

4.9 | Electron microscopy

To observe the subcellular formation process of virus-induced tubules, the midguts from viruliferous and nonviruliferous S. furcifera, or from dsRNAs-treated viruliferous S. furcifera were fixed, dehydrated, and embedded, and ultrathin sections were cut as previously described (Mao et al., 2017). Ultrathin sections were examined with an H-7650 transmission electron microscope (Hitachi). For immunoelectron microscopy, the SF9 cells on coverslips were immunolabelled with SRBSDV P7-1 antibody as the primary antibody, followed by treatment with goat
anti-rabbit IgG conjugated with 10-nm gold particles as the secondary antibody (Sigma-Aldrich), as described previously (Jia et al., 2014).

4.10 Statistical analyses

All data were analysed with SPSS software v. 17.0. Multiple comparisons of the means were conducted using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test at a $\alpha = 0.05$ significance level. Comparisons between two means were conducted using an independent t test. The data were back-transformed after analysis for presentation in the text and figures.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Protein sequences data are available in the protein database at www.ncbi.nlm.nih.gov with accession numbers MZ322899 and MZ322900. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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