Influence of cytoplasmic heat shock protein 70 on viral infection of *Nicotiana benthamiana*

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SUMMARY

The accumulation of heat shock protein 70 (Hsp70) generally occurs in plants infected with viruses. However, the effect of Hsp70 accumulation on plant viral infection and pathogenesis remains elusive. In this study, the expression of six *Hsp70* genes was found to be induced by the four diverse RNA viruses, *Tobacco mosaic virus*, *Potato virus X* (PVX), *Cucumber mosaic virus* and *Watermelon mosaic virus*, in *Nicotiana benthamiana*. Heat treatment enhanced the accumulation and systemic infection of these viruses. Similar results were obtained for viral infection in plants heterologously expressing an *Arabidopsis* cytoplasmic Hsp70 through either a PVX vector or *Agrobacterium* infiltration. In contrast, viral infection was compromised in cytoplasmic *NbHsp70c-1* gene-silenced plants. These data demonstrate that the cytoplasmic Hsp70s can enhance the infection of *N. benthamiana* by diverse viruses.

INTRODUCTION

As obligate parasites, viruses are intimately associated with and completely dependent on host cellular resources, and modify host cell status to facilitate infections (Whitham and Wang, 2004). In susceptible hosts, these modifications include the induction of host factors required for viral replication, accumulation, intracellular and systemic movement, and suppression of host defence responses (Maule *et al.*, 2000; Senthil *et al.*, 2005; Whitham and Wang, 2004; Whitham *et al.*, 2003). Heat shock protein (*Hsp*) gene expression is frequently induced in response to both plant and animal viral infections (Aranda and Maule, 1998; Whitham *et al.*, 2003). Most Hsps function as molecular chaperones, i.e. proteins that assist the non-covalent folding/unfolding and

assembly/disassembly of other macromolecular structures. Five major families of Hsps have been recognized: Hsp70 (70-kDa Hsp) (DnaK) family, Hsp60 (chaperonin) family, Hsp90 family, Hsp100 (Clp) family, and the small Hsp (sHsp) family (Wang *et al.*, 2004). The induced Hsp70s may contribute to the folding and turnover of viral proteins, and may play a role in the development of plant disease (Aparicio *et al.*, 2005).

Hsp70s are probably the most conserved proteins in all organisms from eubacteria to plants and animals (Daugaard *et al.*, 2007). As a family of molecular chaperones, all known Hsp70 members display highly conserved amino acid sequences and domain structures. Hsp70s consist of a conserved N-terminal ATPase domain of 44 kDa and a C-terminal peptide domain of ~25 kDa containing a client protein-binding EEVD motif, which can also bind to a co-chaperone [non-client-binding partner that assists a chaperone (Hsp70 or Hsp90) in protein folding and other functions, e.g. DnaJ/Hsp40] or other Hsps. Furthermore, each Hsp70 member possesses a cellular localization signal that contributes to localize the protein to a specific compartment (Daugaard *et al.*, 2007; Mayer and Bukau, 2005; Wang *et al.*, 2004). Plant Hsp70s are encoded by a multiple-gene family and localize to one of the subcellular compartments: the cytoplasm, endoplasmic reticulum, plastids and mitochondria. Some family members of Hsp70 are constitutively expressed and are often referred to as Hsc70 (70-kDa heat shock cognate); other members are expressed only when the organism is challenged by abiotic stresses (Wang *et al.*, 2004). There are at least 18 genes encoding members of the Hsp70 family in the genome of *Arabidopsis thaliana*, and at least 12 Hsp70 members in the spinach genome (Sung *et al.*, 2001; Wang *et al.*, 2004). The *Hsp70* gene family in *Nicotiana benthamiana* includes at least six members: four (*NbHsp70c-1*, *NbHsp70c-2*, *NbHsp70c-3* and *NbHsp70c-4*) are predicted to localize in the cytoplasm, one (*NbHsp70er-1*) in the endoplasmic reticulum and one (*NbHsp70cp-1*) in the chloroplast (Kanzaki *et al.*, 2003).

Hsp70 family members have various functions in host growth and development under normal and stress conditions. Hsp70s

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with their co-chaperones and co-operating chaperones constitute a complex network of folding machines, and are involved in the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, and membrane translocation of the organellar and secretory proteins (Aoki *et al.*, 2002; Hartl, 1996; Mayer and Bukau, 2005). Cytoplasmic Hsp70s also play regulatory roles in stress-associated gene expression through interaction with heat shock factor (HSF), and subsequently participate in the modulation of signal transducers (Mayer and Bukau, 2005). Over-expression of Hsp70 in plants correlates positively with the acquisition of thermotolerance, but also results in enhanced tolerance to abiotic stress treatments, such as tunicamycin, salt and water (Su and Li, 2008; Wang *et al.*, 2004). In contrast, down-regulation of Hsp70s reduces tolerance to abiotic stresses (Lee and Schöffl, 1996; Su and Li, 2008). However, the maintenance of either elevated or reduced levels of Hsc70-1 in *Arabidopsis* appears to be deleterious to plant viability (Sung and Guy, 2003).

The Hsp70s induced by animal viruses have been shown to be involved in viral infection by increasing virion assembly (Chromy *et al.*, 2003), enhancing viral replication (Glotzer *et al.*, 2000; Oglesbee *et al.*, 1996) and/or participating in virus entry as a cell receptor (Reyes-del Valle *et al.*, 2005). Studies in yeast have demonstrated that Hsp70 is associated with the replicase of bromoviruses or tombusviruses and enhanced plant viral RNA replication (Serva and Nagy, 2006; Tomita *et al.*, 2003). In plants, infection by a diverse range of viruses can induce Hsp70s (Aparicio *et al.*, 2005; Jockusch *et al.*, 2001), and the induction of Hsp70s is spatially and temporally controlled by viral infections (Aparicio *et al.*, 2005; Aranda and Maule, 1998; Aranda *et al.*, 1996; Escaler *et al.*, 2000; Whitham and Wang, 2004). For plant viruses, Hsp70s are expected to play a role in viral protein maturation and turnover during virus multiplication and/or movement (Aparicio *et al.*, 2005). The closteroviruses encode an Hsp70 homologue (Hsp70h) that plays a role in virion assembly and stability and in viral movement through plasmodesmata (PD) (Alzhanova *et al.*, 2001; Prokhnevsky *et al.*, 2002). However, little is known about the effect of Hsp70 induction on infection by plant viruses.

In this study, we detected the induction of six *Hsp70* genes by heat treatment and by infection with four diverse RNA viruses. To gain an insight into the involvement of Hsp70 during pathogenesis, virus accumulation and movement were assessed in *N. benthamiana* plants in which the level of Hsp70 was either up-regulated by transient expression of an *Arabidopsis* cytoplasmic Hsp70 (*AtHsc70-3*) or down-regulated by virus-induced gene silencing (VIGS) of the cytoplasmic *Hsp70* (*NbHsp70c-1*) gene. The results suggest that Hsp70 can influence viral infection by facilitating virus accumulation and movement.

RESULTS

Induction of *Hsp70* gene expression in *N. benthamiana*

Initially, we analysed the expression of *Hsp70* genes after heat treatment and infection by one of the four compatible viruses: *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), *Cucumber mosaic virus* (CMV) and *Watermelon mosaic virus* (WMV). Total RNA was extracted from the leaves which had been treated at 42 °C for 2 h or infected systemically by each of the four viruses. The specificity of cDNA amplification of each *Hsp70* RNA was confirmed by sequencing the reverse transcriptase-polymerase chain reaction (RT-PCR) products. Semi-quantitative RT-PCR showed that the expression levels of the cytoplasmic *Hsp70* genes (*NbHsp70c-1* to *NbHsp70c-4*), *NbHsp70cp-1* and *NbHsp70er-1* were enhanced to different extents by heat treatment and viral infection (Fig. 1). Both heat treatment and viral infection were equally efficient in inducing *NbHsp70c-1*, *NbHsp70cp-1* and *NbHsp70c-3*. However, *NbHsp70c-2* and *NbHsp70c-4* accumulated to higher levels in heat-treated leaves, whereas the expression of *NbHsp70er-1* was more obviously triggered by viral infection (Fig. 1).

Heat treatment facilitates the infection of plants with different viruses

To determine the role of Hsp70s in establishing viral infection, the effect of heat treatment on the accumulation of plant viruses was

Fig. 1 Induction of heat shock protein 70 (*Hsp70*) gene expression in *Nicotiana benthamiana*. Total RNAs were isolated from leaves pre-treated by heat (42 °C, 2 h) or inoculated with viruses [*Potato virus X* (PVX), *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV) and PVX-Nb70i] at 10 days post-inoculation (dpi). Non-heat-treated and virus-free leaves were used as negative controls. Reverse transcriptase-polymerase chain reaction (RT-PCR) products were separated by a 2% agarose gel as indicated, and the *NbRbcS* gene (bottom) was used as an internal and loading control.

Fig. 2 Effect of heat treatment on infection of four different viruses in *Nicotiana benthamiana*. (A) Comparison of viral accumulation in inoculated leaves of heat-treated (42 °C, 2 h) plants and non-heat-treated plants (control) at 2 and 3 days post-inoculation (dpi) using antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA). All data are the mean value $\pm$ standard deviation (SD) of eight samples, normalized to that of non-heat-treated samples (negative controls). (B) Comparison of the percentages of viral systemic infection in heat-treated (42 °C) plants and non-heat-treated plants (control) at 4 and 5 dpi using ACP-ELISA. For each treatment, at least seven samples were tested. CMV, Cucumber mosaic virus; PVX, Potato virus X; TMV, Tobacco mosaic virus; WMV, Watermelon mosaic virus.

evaluated. The plants were exposed to 42 °C for 2 h, followed by inoculation with TMV, PVX, CMV and WMV. The plants were then grown in normal conditions. Inoculated leaves were collected at 2 and 3 days post-inoculation (dpi) and systemic leaves were collected at 4 and 5 dpi for analysis of viral accumulation. As determined by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA), the viral accumulation level in inoculated leaves was higher in heat-pre-treated plants than in control plants without heat treatment (Fig. 2A). At 4 and 5 dpi, the proportions of heat-treated plants systemically infected by TMV, PVX, CMV and WMV were also higher than those of non-heat-treated control plants (Fig. 2B). For instance, the TMV infection rates at 4 and 5 dpi were 60% (number of symptomatic plants/number of inoculated plants, 6/10) and 88.3% (10/12), respectively, for heat-treated plants, but only 20% (2/10) and 42.9% (3/7), respectively, for non-heat-treated control plants. These results indicate that both viral accumulation and systemic infection in *N. benthamiana* are enhanced significantly after heat pre-treatment.

Heterologous expression of AtHsc70-3 enhances viral infection in *N. benthamiana*

To elucidate the possible role of Hsp70s in plant viral infection, the *AtHsc70-3* gene (At3g12580, accession no. AJ002551) was cloned into pSPYNE-35S to produce p35S-At70. *Agrobacterium* harbouring p35S-At70 was infiltrated into the entire lamina of fully expanded leaves of *N. benthamiana*. MMA buffer [10 mmol/L MgCl₂, 10 mmol/L 2-(*N*-morpholino)ethanesulphonic acid (MES) (pH 5.6) and 100 μ mol/L acetosyringone] or *Agrobacterium* carrying the empty vector pSPYNE-35S was used as control. At 24 h after agro-infiltration, TMV-green fluorescent protein (GFP) was inoculated within an encircled area (5 mm in diameter) on these leaves. TMV-GFP induced the development of green fluorescent foci at 3 dpi, which were clearly visible under long-wavelength UV light (data not shown). At 5 dpi, the largest radius of fluorescent foci induced in leaves expressing AtHsc70-3 from p35S-At70 ($n = 3$) was 8.27 ± 1.14 mm, which was about

Fig. 3 Heterologous expression of AtHsc70-3 facilitated viral infection in *Nicotiana benthamiana*. (A) Representative examples of the fluorescent foci of *Tobacco mosaic virus*-green fluorescent protein (TMV-GFP) at 5 days post-inoculation (dpi) in leaves infiltrated with MMA buffer, pSPYNE-35S and p35S-At70. The dark lesion in the centre of fluorescent foci was induced by mechanical inoculation. The distance between the edge of the inoculated area and the outside edge of fluorescent foci was measured. Scale bar, 5 mm. (B) Analysis of AtHsc70-3 expression by Western blotting with anti-c-Myc antibody as indicated. The Coomassie brilliant blue-stained 8% sodium dodecylsulphate-polyacrylamide gel (bottom) for different samples was used as loading control. (C) Expression of AtHsc70-3 in leaves inoculated with buffer (mock inoculation), *Potato virus X* (PVX) or PVX-At70 was analysed by Western blotting using anti-Hsp70 antibody, as indicated. The Coomassie brilliant blue-stained 8% sodium dodecylsulphate-polyacrylamide gel (bottom) for different samples was used as loading control. (D) Analysis of TMV-GFP accumulation at 4 dpi in inoculated leaves that had been pre-inoculated with PVX-At70, as well as PVX and buffer (mock inoculation). Reverse transcriptase-polymerase chain reaction (RT-PCR) products of the TMV-GFP CP gene were separated by a 1% agarose gel as indicated. Signal values obtained from each gene were normalized to the *NbRbcS* (internal control) signal value, and the resulting mean values of three independent experiments were presented as relative units. Error bars indicate $\pm$ standard deviation of the mean value.

1.94-fold larger than that in leaves infiltrated with pSPYNE-35S ($n = 3$) (4.27 ± 0.75 mm) and smaller than that in leaves infiltrated with MMA buffer ($n = 3$) (9.37 ± 1.33 mm) (Fig. 3A). The timing of expression is similar to that in a previous report, where the transient expression of *AtHsc70-3* introduced by *Agrobacterium* infiltration reached a maximum within 4–5 days and then decreased gradually (Wroblewski *et al.*, 2005). Expression of *AtHsc70-3* occurred only in leaf tissues infiltrated with *Agrobacterium* carrying p35S-At70, as evidenced by the positive detection of AtHsc70-3-Myc using an anti-c-Myc antibody (Fig. 3B).

In another assay, transient over-expression of *AtHsc70-3* was achieved using the PVX vector. The transcripts of PVX-At70 were inoculated onto the leaves of 3-week-old *N. benthamiana*, with empty PVX vector and buffer used as controls. Western blot analysis demonstrated that, at 7 dpi, the level of Hsp70 in PVX-At70-infected plants was much higher than that in control plants, most probably because of the transient expression of *AtHsc70-3* from PVX-At70 (Fig. 3C). The pre- or mock-inoculated

leaves were then challenge inoculated with TMV-GFP. Four days after TMV-GFP inoculation, RT-PCR with primers for the TMV-GFP CP gene showed that the relative level of TMV RNA normalized to *NbRbcS* RNA (internal control) in plants inoculated with PVX-At70 was approximately 20% higher than that in those inoculated with PVX or those mock inoculated with buffer (Fig. 3D).

These results indicate that heterologous expression of *AtHsc70-3* through either agro-infiltration or virus vector can enhance viral infection in *N. benthamiana*.

Silencing of *NbHsp70c-1* in *N. benthamiana* impairs viral multiplication and movement

The *NbHsp70c-1* gene was silenced by VIGS in *N. benthamiana* plants inoculated with PVX-Nb70i. At 4 dpi, typical mosaic symptoms were observed in the upper non-inoculated leaves and, at 10 dpi, PVX-Nb70i-infected plants started to exhibit a stunted phenotype and the upper leaves showed the distinctive

Fig. 4 Silencing of *NbHsp70c-1* in *Nicotiana benthamiana* impaired viral infection. (A) Stunting phenotype caused by silencing of *NbHsp70c-1* in *N. benthamiana*. (B) Tobacco mosaic virus-green fluorescent protein (TMV-GFP) infection foci in *NbHsp70c-1*-silenced and control plants. At 2 days post-inoculation (dpi), the number and size of TMV-GFP infection foci in the plants inoculated with PVX-Nb70i were less than in control plants (a, d). At 4 dpi, systemic infection by TMV-GFP was observed in controls (b) but not in *NbHsp70c-1*-silenced plants (e). Systemic infection by TMV-GFP occurred in *NbHsp70c-1*-silenced plants until 8 dpi (f), which is inefficient compared with controls (c). The red arrows indicate the inoculated leaves. (C) The expression of *NbHsp70c-1* mRNA in the uppermost leaves of the systemically infected plants shown in (B) (b, c, e and f) was analysed. Reverse transcriptase-polymerase chain reaction (RT-PCR) products of *NbHsp70c-1* mRNA were separated by agarose gel, as indicated. The *NbRbcS* gene (bottom) was used as an internal and loading control. (D) Heterologous expression of AtHsc70-3 in *NbHsp70c-1*-silenced leaves by agro-infiltration-facilitated TMV-GFP infection. At 24 h after infiltration, TMV-GFP was inoculated and the accumulation of mRNAs was analysed by RT-PCR at 5 dpi, with total RNAs isolated from the inoculated leaves infiltrated with MMA buffer, pSPYNE-35S and p35S-At70. Signal values obtained from each gene were normalized to the *NbRbcS* signal value, and the resulting mean values of three independent experiments were presented as relative units. Error bars indicate $\pm$ standard deviation of the mean value.

abnormality of underdeveloped veins (Fig. 4A). Later, these plants became severely stunted relative to PVX vector-inoculated plants because of the death of both the meristem and young leaves (data not shown). At 10 dpi, three to four leaves above that inoculated were used to confirm the gene silencing of *NbHsp70c-1*. RT-PCR results showed that the level of *NbHsp70* mRNA was reduced in silenced plants relative to PVX vector-inoculated plants, indicating that silencing of the *NbHsp70c-1* gene had occurred. To address the specificity of gene silencing, we also detected the expression levels of five other *NbHsp70* genes; the results demonstrated that *NbHsp70c-1* silencing affected the

expression of other tested *NbHsp70* genes to varying degrees (Fig. 1).

Leaves of *NbHsp70c-1*-silenced and non-silenced control plants were inoculated with TMV-GFP. The number and size of TMV-GFP infection foci in the plants inoculated with PVX-Nb70i were less than those in control plants (Fig. 4B). Systemic infection by TMV-GFP occurred in *NbHsp70c-1*-silenced plants at 7–8 dpi, in contrast with 3–4 dpi in the controls. RT-PCR assay did not reveal any detectable level of TMV RNA in the upper non-inoculated leaves of *NbHsp70c-1*-silenced plants at 4 dpi (data not shown). In *NbHsp70c-1*-silenced plants, TMV-GFP moved out

of the inoculated leaves and translocated to the uppermost parts at 8 dpi. Semi-quantitative RT-PCR results showed that *NbHsp70c-1* mRNA expression in *NbHsp70c-1*-silenced plants was approximately the same as that in control plants in the uppermost leaves, with similar levels of GFP fluorescence (Fig. 4C). Hence, viral accumulation and movement were compromised in *NbHsp70c-1*-silenced plants.

To further assess the effect of Hsp70 induction on viral infection, *AtHsc70-3* was expressed in *NbHsp70c-1*-silenced leaves by agro-infiltration, and TMV-GFP was inoculated 24 h later. At 5 dpi, the inoculated leaves were collected to analyse the accumulation of TMV-GFP. RT-PCR results showed that the amount of relative units of TMV RNA and *NbRbcS* in the plants infiltrated with p35S-*At70* was about 30% higher than in control plants infiltrated with pSPYNE-35S (Fig. 4D), suggesting that TMV shows a relatively high level of replication in leaves expressing *AtHsc70-3*.

DISCUSSION

In this study, we have demonstrated that expression of the six *N. benthamiana* Hsp70 genes is induced by heat treatment and by four different RNA viruses (PVX, TMV, CMV and WMV), indicating that the induction of Hsp70 gene expression may be a general response to viral infection (Fig. 1). As the whole family of Hsp70s has not been identified in *N. benthamiana*, comprehensive analysis of Hsp70 induction by viruses is currently not possible. Based on the differential induction of individual Hsp70s by viral infection and heat shock, we speculate that a specific mechanism may contribute to regulate the expression of each individual Hsp70 protein, and that their biological roles may be determined by their subcellular location and/or interaction with particular Hsp70-associated proteins (Wang *et al.*, 2004). We also found that heat treatment enhanced viral infection (Fig. 2). It is known that Hsp70s are typical proteins induced by heat shock; thus, we presume that the effect of heat treatment on viral infection should be at least partially a result of the induction of Hsp70 genes.

To address this, we employed PVX- and *Agrobacterium*-mediated transient gene expression systems to express *Arabidopsis AtHsc70-3*, and anticipated that *AtHsc70-3* may play a similar role to *NbHsp70c-1* in *N. benthamiana*. Indeed, the accumulation of TMV-GFP was enhanced in leaves expressing *AtHsc70-3* (Fig. 3). Interestingly, we also observed that the fluorescent foci in the leaves infiltrated with MMA buffer were larger than those in agro-infiltrated leaves. This may result from the virulence of *Agrobacterium* on the cellular tissue, which affects virus accumulation, although the precise mechanism remains to be revealed.

Using PVX-based VIGS, we provided further evidence that *NbHsp70c-1* plays a critical role in TMV-GFP infection of *N. benthamiana*. Silencing of *NbHsp70c-1* caused plant stunting

and abnormal leaf development, similar to the phenotypes reported previously (Kanzaki *et al.*, 2003; Senthil-Kumar *et al.*, 2007), except that the silencing of *NbHsp70c-1*, PVX accumulation and/or other unknown co-silenced Hsp70 genes may also be involved in the formation of the phenotype. In some cases, constitutive knockout of Hsp70 genes was lethal or resulted in malformed leaves and growth retardation at permissive temperatures (Su and Li, 2008; Sung and Guy, 2003). Intriguingly, in these *NbHsp70c-1*-silenced plants, viral infection was compromised, and systemic movement of TMV-GFP was severely inhibited (Fig. 4A), although we cannot exclude the possibility that the influence of Hsp70 on viral infection may be the consequence of abnormal plant development. However, TMV-GFP infection was restored in *NbHsp70c-1*-silenced leaves expressing *AtHsc70-3*, which complemented the function of *NbHsp70c-1*, supporting the concept of the involvement of Hsp70 in viral infection.

Some studies have suggested that Hsp70 family members may play various roles in plant RNA virus multiplication, such as viral protein folding, virion assembly and protein expression. In yeast, the molecular chaperones Ssa1/2p (yeast homologues of Hsp70 proteins) probably play a role in the assembly of the *Cucumber necrosis virus* replicase and in enhancing viral RNA replication (Serva and Nagy, 2006). *Arabidopsis* Hsc70-3 and poly(A)-binding proteins have been identified as interactors of *Turnip mosaic virus* RNA-dependent RNA polymerase, implying their potential role in viral replication (Dufresne *et al.*, 2008). Interaction *in vivo* between the *Potato virus Y* coat protein and a host protein of the DnaJ family suggests the involvement of Hsp70-related mechanisms in viral infection (Hofius *et al.*, 2007). However, a recent study has demonstrated that Hsp70 negatively controls animal rotaviral protein bioavailability in intestinal epithelial Caco-2 cells (Broquet *et al.*, 2007). It is noteworthy that this study may reflect a relatively specific effect of Hsp70s on virus morphogenesis in an animal host, which may be different from the situations in plants.

For plant viruses, the expression of Hsp70s may play an important role in virus movement. *Tomato spotted-wilt virus* (TSWV) movement protein (NSm) interacts with DnaJ-like proteins from plants, suggesting that the recruitment of Hsp70-related chaperones is likely to be needed in TSWV movement (von Bargon *et al.*, 2001). Hsp70 class proteins are capable of trafficking through PD; therefore, these proteins may have the ability to actively translocate viral movement complexes through PD pores (Boevink and Oparka, 2005). Functional analysis of Hsp70h encoded by closteroviruses has demonstrated that Hsp70h is associated with PD and functions as one of the closteroviral movement proteins (Medina *et al.*, 1999; Peremyslov *et al.*, 1999), suggesting a direct role of molecular chaperones in virus movement. Furthermore, Hsp70h also possesses an activity that links the local and systemic spread of a plant virus by docking a long-distance transport factor to virions (Prokhnevsky *et al.*, 2002). Consistent with these

findings, our results showed that cytoplasmic Hsp70 is probably involved in facilitating virus movement.

In addition, Hsp70s have been shown to play an important role in the signal transduction of the hypersensitive response-mediated defence mechanism. In *N. benthamiana*, cytoplasmic Hsp90 and Hsp70 are essential components of the hypersensitive response and non-host resistance (Kanzaki *et al.*, 2003). In support of this, *Arabidopsis* cytosolic Hsc70 interacts with SGT1 (an Hsp90 co-chaperone), and the SGT1–Hsc70 association is important for the regulation of plant response to stresses (Noël *et al.*, 2007).

Taken together, our data indicate that the induction of differentially located Hsp70s is a general plant response to RNA viral infection, and that cytoplasmic Hsp70 plays a positive role in virus accumulation and/or movement. A better understanding of the specific roles of a given Hsp70 will be conducive to elucidating the functions of these proteins in virus–plant interactions.

EXPERIMENTAL PROCEDURES

Plant materials and virus inoculation

Nicotiana benthamiana plants were grown at 25 °C with a photoperiod of 16 h light/8 h dark for 3 weeks. Heat treatment was performed by exposing plants to 42 °C for 2 h. After heat treatment, aerial parts of the plants were collected, frozen in liquid nitrogen immediately and kept at –80 °C.

TMV, PVX, CMV and WMV inocula were prepared separately from infected *N. benthamiana*. Sap inoculum of TMV tagged with GFP (TMV-GFP) was prepared from *N. benthamiana* leaves that became infected after infiltration with *Agrobacterium tumefaciens* strain EHA105 harbouring an infectious cDNA clone p35S-30B::GFP (Jia *et al.*, 2003).

Semi-quantitative RT-PCR

To analyse transcription, semi-quantitative RT-PCR was performed using specific primers to amplify the partial fragments of Hsp70 RNAs according to Kanzaki *et al.* (2003). The gene-specific primers for the six *Hsp70s* examined in this study are listed in Table 1. One microgram of total RNA was used as template for first-strand cDNA synthesis by oligo (dT) priming using ReverTra Ace (Toyobo). The *NbRbcS* gene was used as an internal constitutively expressed control. The amplicons were separated in 2% agarose gel and the gene specificity was confirmed by directly sequencing all RT-PCR products.

The mRNA level for the TMV-GFP *CP* gene was semi-quantified using RT-PCR. Three rounds of RT-PCR were conducted with three independently isolated total RNA samples. Twenty microlitres from each PCR were fractionated by a 1% (w/v) agarose gel in tris(hydroxymethyl)aminomethane (Tris)-acetate ethylenediami-

Table 1 Oligonucleotide primers used in reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Primer*	Sequence
<i>Hsp70c-1</i>	Hsp70c-1F	5'-AAGCTTGTGCGACGAATTCAGATTGC-3'
	Hsp70c-1R	5'-GCTTGCTGCTGCGGTTCAAC-3'
	Nb70c-1 <i>Clal</i> F	5'-GGATCGATGCATCAACCTGATGAGG-3'
	Nb70c-1 <i>SaI</i> R	5'-GTGCGACGATGGCTTCTCAATGGCAT-3'
<i>Hsp70c-2</i>	Hsp70c-2F	5'-TACCCGGGATCCGATTGAGAAG-3'
	Hsp70c-2R	5'-CGCTTAATCAACCTCTCAATC-3'
<i>Hsp70c-3</i>	Hsp70c-3F	5'-TGGTTTGAGAAGATTGCATCAAGC-3'
	Hsp70c-3R	5'-CGACGGCCAGTGAATTCGAGC-3'
<i>Hsp70c-4</i>	Hsp70c-4F	5'-GGATGATAAGATTAGTTCTAAGC-3'
	Hsp70c-4R	5'-CACACTTAATCGACCTCC-3'
<i>Hsp70er-1</i>	Hsp70er-1F	5'-GTCAAGGCTAATTACATTTC-3'
	Hsp70er-1R	5'-GCAGCAAGTTCTTATGTCTG-3'
<i>Hsp70cp-1</i>	Hsp70cp-1F	5'-GTGGAGTCATGACCAAAATTATC-3'
	Hsp70cp-1R	5'-GAAGCTGCATCGATAAC-3'
<i>NbRbcS</i>	NbRbcSF	5'-CCTCTGCAGTTGCCACC-3'
	NbRbcSR	5'-CCTGTGGGTATGCCTTCTTC-3'

*F, forward primer; R, reverse primer.

netetraacetic acid (EDTA) buffer, and stained with 0.5% (w/v) ethidium bromide. The stained gels were digitally photographed with an Alphamager imaging system (Alpha Innotech Corp.), and the Alphamager v 4.1.0 program was used to quantify the intensity of the DNA bands from the negative image of the gel.

Over-expression of *AtHsc70-3* and VIGS of *NbHsp70c-1* in *N. benthamiana*

Over-expression and VIGS were performed using a modified PVX vector (Chapman *et al.*, 1992; van Wezel *et al.*, 2001) containing the full-length open reading frame (ORF) of *A. thaliana* (Col-0) *AtHsc70-3* and a 650-bp fragment of the *N. benthamiana* *NbHsp70c-1* gene amplified with specific primers (Table 1). The corresponding PCR products were inserted into the *Clal/SaI* site of the PVX vector to generate pPVX-At70 and pPVX-Nb70i, respectively. Plasmids of pPVX-At70 and pPVX-Nb70i were *SpeI* linearized and used for *in vitro* transcription. The transcripts were inoculated onto the leaves of *N. benthamiana*.

Agrobacterium-mediated transient expression of *AtHsc70-3*

The *AtHsc70-3* gene was amplified by PCR and cloned into the pSPYNE-35S vector to generate p35S-At70 (Walter *et al.*, 2004), which expressed the *AtHsc70*-Myc fusion protein. p35S-At70 was introduced into *A. tumefaciens* strain GV3101 and *A. tumefaciens* cells were cultured, set to an optical density at 600 nm of 1.0 in MMA buffer, and used to infiltrate the leaves of 3-week-old *N. benthamiana* plants, essentially as described previously (Jia *et al.*, 2003).

Western blotting assay

Total soluble proteins were extracted with buffer (220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM MgCl₂, 50 mM KCl) containing β -mercaptoethanol (10 mM) and phenylmethylsulphonylfluoride (100 μ M). The protein content was measured by the Bradford method (Bradford, 1976). Ten micrograms of crude extract were loaded and separated by 8% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted onto nitrocellulose membranes, and immunodetection was performed using antiserum against a bacterial-expressed AtHsc70-3 or anti-c-Myc antibody (Tiangen Biotech, China), followed by alkaline phosphatase-conjugated protein A, and visualized by nitroblue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate staining.

Assays of viral infection

To assess viral movement, the fluorescence of GFP in plants was visualized using a 100-W hand-held long-wave ultraviolet lamp (UV Products, model B 100AP), and photographed by a digital camera (Sony DSC-H5, high ISO mode).

ACP-ELISA was performed to determine the levels of virus accumulation, as described previously (Koenig, 1981). The plates were coated with plant extracts in carbonate buffer (pH 9.6). The polyclonal antibodies against different viruses were diluted 1 : 500 and applied, and alkaline phosphatase-conjugated protein A was used at 1 : 3000 dilution. The absorption was determined at A_{405 nm}, and leaf extract from uninfected plants and phosphate buffer were used as negative control and blank control, respectively.

Unless stated otherwise, all the experiments described in this research were repeated at least three times.

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REFERENCES

- Alzhanova, D.V., Napuli, A.J., Creamer, R. and Dolja, V.V. (2001) Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. *EMBO J.* **20**, 6997–7007.
- Aoki, K., Kragler, F., Xoconostle-Cázares, B. and Lucas, W.J. (2002) A

- subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. *Proc. Natl. Acad. Sci. USA*, **99**, 16 342–16 347.
- Aparicio, F., Thomas, C.L., Lederer, C., Niu, Y., Wang, D.W. and Maule, A.J. (2005) Virus induction of heat shock protein 70 reflects a general response to protein accumulation in the plant cytosol. *Plant Physiol.* **138**, 529–536.
- Aranda, M. and Maule, A. (1998) Virus-induced host gene shut off in animals and plants. *Virology*, **243**, 261–267.
- Aranda, M.A., Escaler, M., Wang, D. and Maule, A.J. (1996) Induction of HSP70 and polyubiquitin expression associated with plant virus replication. *Proc. Natl. Acad. Sci. USA*, **93**, 15 289–15 293.
- von Barga, S., Salchert, K., Paape, M., Piechulla, B. and Kellmann, J.W. (2001) Interactions between the tomato spotted wilt virus movement protein and plant proteins showing homologies to myosin, kinesin and DnaJ-like chaperones. *Plant Physiol. Biochem.* **39**, 1083–1093.
- Boevink, P. and Oparka, K.J. (2005) Virus–host interactions during movement processes. *Plant Physiol.* **138**, 1815–1821.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
- Broquet, A.H., Lenoir, C., Gardet, A., Sapin, C., Chwetzoff, S., Jouniaux, A.-M., Lopez, S., Trugnan, G., Bachelet, M. and Thomas, G. (2007) Hsp70 negatively controls rotavirus protein bioavailability in Caco-2 cells infected by the rotavirus RF strain. *J. Virol.* **81**, 1297–1304.
- Chapman, S., Kavanagh, T. and Baulcombe, D. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2**, 549–557.
- Chromy, L.R., Pipas, J.M. and Garcea, R.L. (2003) Chaperone-mediated in vitro assembly of polyomavirus capsids. *Proc. Natl. Acad. Sci. USA*, **100**, 10 477–10 482.
- Daugaard, M., Rohde, M. and Jäättelä, M. (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. *FEBS Lett.* **581**, 3702–3710.
- Dufresne, P.J., Thivierge, K., Cotton, S., Beauchemin, C., Ide, C., Ubalijoro, E., Laliberté, J.-F. and Fortin, M.G. (2008) Heat shock 70 protein interaction with *Turnip mosaic virus* RNA-dependent RNA polymerase within virus-induced membrane vesicles. *Virology*, **374**, 217–227.
- Escaler, M., Aranda, M.A., Thomas, C.L. and Maule, A.J. (2000) Pea embryonic tissues show common responses to the replication of a wide range of viruses. *Virology*, **267**, 318–325.
- Glotzer, J.B., Saltik, M., Chiocca, S., Michou, A.I., Moseley, P. and Cotten, M. (2000) Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature*, **407**, 207–211.
- Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature*, **381**, 571–579.
- Hofius, D., Maier, A.T., Dietrich, C., Jungkunz, I., Börnke, F., Maiss, E. and Sonnewald, U. (2007) Capsid protein-mediated recruitment of host DnaJ-like proteins is required for *Potato virus Y* infection in tobacco plants. *J. Virol.* **81**, 11 870–11 880.
- Jia, H.G., Pang, Y.Q. and Fang, R.X. (2003) Agroinoculation as a simple way to deliver a *Tobacco mosaic virus*-based expression vector. *Acta Bot. Sin.* **45**, 770–773.
- Jockusch, H., Wiegand, C., Mersch, B. and Rajes, D. (2001) Mutants of *Tobacco mosaic virus* with temperature-sensitive coat proteins induce heat shock response in tobacco leaves. *Mol. Plant–Microbe Interact.* **14**, 914–917.
- Kanzaki, H., Saitoh, H., Ito, A., Fujisawa, S., Kamoun, S., Katou, S., Yoshioka, H. and Terauchi, R. (2003) Cytosolic HSP90 and HSP70 are

- essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*. *Mol. Plant Pathol.* **4**, 383–391.
- Koenig, R. (1981) Indirect ELISA methods for the broad specificity detection of plant viruses. *J. Gen. Virol.* **55**, 53–62.
- Lee, J.H. and Schöffl, F. (1996) An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*. *Mol. Gen. Genet.* **252**, 11–19.
- Maule, A.J., Escaler, M. and Aranda, M.A. (2000) Programmed responses to virus replication in plants. *Mol. Plant Pathol.* **1**, 9–15.
- Mayer, M.P. and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* **62**, 670–684.
- Medina, V., Peremyslov, V.V., Hagiwara, Y. and Dolja, V.V. (1999) Subcellular localization of the HSP70-homolog encoded by Beet yellows closterovirus. *Virology*, **260**, 173–181.
- Noël, L.D., Cagna, G., Stuttmann, J., Wirthmüller, L., Betsuyaku, S., Witte, C.-P., Bhat, R., Pochon, N., Colby, T. and Parker, J.E. (2007) Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates *Arabidopsis* immune responses. *Plant Cell*, **19**, 4061–4076.
- Oglesbee, M.J., Liu, Z., Kenney, H. and Brooks, C.L. (1996) The highly inducible member of the 70 kDa family of heat shock proteins increases canine distemper virus polymerase activity. *J. Gen. Virol.* **77**, 2125–2135.
- 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. USA*, **96**, 14 771–14 776.
- Prokhnovsky, A.I., Peremyslov, V.V., Napuli, A.J. and Dolja, V.V. (2002) Interaction between long-distance transport factor and Hsp70-related movement protein of Beet yellows virus. *J. Virol.* **76**, 11 003–11 011.
- Reyes-del Valle, J., Chávez-Salinas, S., Medina, F. and del Angel, R.M. (2005) Heat shock protein 90 and heat shock protein 70 are components of Dengue virus receptor complex in human cells. *J. Virol.* **79**, 4557–4567.
- Senthil, G., Liu, H., Puram, V.G., Clark, A., Stromberg, A. and Goodin, M.M. (2005) Specific and common changes in *Nicotiana benthamiana* gene expression in response to infection by enveloped viruses. *J. Gen. Virol.* **86**, 2615–2625.
- Senthil-Kumar, M., Govind, G., Kang, L., Mysore, K.S. and Udayakumar, M. (2007) Functional characterization of *Nicotiana benthamiana* homologs of peanut water deficit-induced genes by virus-induced gene silencing. *Planta*, **225**, 523–539.
- Serva, S. and Nagy, P.D. (2006) Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. *J. Virol.* **80**, 2162–2169.
- Su, P.H. and Li, H.M. (2008) *Arabidopsis* stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiol.* **146**, 1231–1241.
- Sung, D.Y. and Guy, C.L. (2003) Physiological and molecular assessment of altered expression of Hsc70-1 in *Arabidopsis*. Evidence for pleiotropic consequences. *Plant Physiol.* **132**, 979–987.
- Sung, D.Y., Vierling, E. and Guy, C.L. (2001) Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family. *Plant Physiol.* **126**, 789–800.
- Tomita, Y., Mizuno, T., Díez, J., Naito, S., Ahlquist, P. and Ishikawa, M. (2003) Mutation of host dnaJ homolog inhibits Brome mosaic virus negative-strand RNA synthesis. *J. Virol.* **77**, 2990–2997.
- Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näke, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K. and Kudla, J. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**, 428–438.
- Wang, W., Vinocur, B., Shoseyov, O. and Altman, A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.* **9**, 244–252.
- van Wezel, R., Liu, H., Tien, P., Stanley, J. and Hong, Y. (2001) Gene C2 of the monopartite geminivirus Tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. *Mol. Plant–Microbe Interact.* **14**, 1125–1128.
- Whitham, S.A. and Wang, Y.Z. (2004) Roles for host factors in plant viral pathogenicity. *Curr. Opin. Plant Biol.* **7**, 365–371.
- Whitham, S.A., Quan, S., Chang, H.S., Cooper, B., Estes, B., Zhu, T., Wang, X. and Hou, Y.M. (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant J.* **33**, 271–283.
- Wroblewski, T., Tomczak, A. and Micheltore, R. (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnol. J.* **3**, 259–273.