Heat shock protein 70 is necessary for *Rice stripe virus* infection in plants

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SUMMARY

Heat shock proteins 70 (HSP70s) are a highly conserved family of genes in eukaryotes, and are involved in a remarkable variety of cellular processes. In many plant positive-stranded RNA viruses, HSP70 participates in the construction of a viral replication complex and plays various roles during viral infection. Here, we found increased expression of HSP70 following infection by *Rice stripe virus* (RSV), a negative-stranded RNA virus, in both rice (the natural host) and *Nicotiana benthamiana* (an experimental host). Heat treatment of *N. benthamiana* (Nb) plants enhanced viral infection, whereas RSV infection was retarded and viral RNAs accumulated at a low level when HSP70 was silenced. In both bimolecular fluorescence complement and *in vitro* pull-down assays, the N-terminus of RSV RNA-dependent RNA polymerase (RdRp) interacted and co-localized with the HSP70s of both plants (OsHSP70 and NbHSP70). The localization of the N-terminus of RdRp when expressed alone was not obviously different from when it was co-expressed with OsHSP or NbHSP, and vice versa. RSV infection also had no effect on the localization of host HSP70. These results demonstrate that host HSP70 is necessary for RSV infection and probably plays a role in viral replication by interacting with viral RdRp, which provides the first evidence of an interacting host protein related to RSV replication, which has been little studied to date.

Keywords: HSP70, interaction, *Rice stripe virus*, viral replication.

INTRODUCTION

Heat shock proteins 70 (HSP70s) are a highly conserved family of cellular chaperones in eukaryotes, participating in a remarkable variety of cellular processes, including the folding of newly synthesized polypeptides, refolding of misfolded or aggregated proteins, translocation of proteins, protein complex assembly or

disassembly, protein degradation and receptor signalling (Mayer, 2005; Mayer and Bukau, 1998). During the viral life cycle, the expression of viral proteins depends on the host protein synthesis system, and therefore it is expected that viruses require Hsp70 chaperones to assist and maintain functional folding of the viral proteins. Correspondingly, viral infection frequently induces the up-regulated expression of HSP70 in plants and animals (Aranda *et al.*, 1996; Mayer, 2005). Research in the last 30 years has revealed that HSP70s are involved in all steps of the viral life cycle, including cell entry, virion disassembly, the transfer of the viral genome into the nucleus, replication of the viral genome, morphogenesis of the virion particles and transformation of the cell (Mayer, 2005; Nagy *et al.*, 2011).

The expression of HSP70 has been found to be induced by several plant positive-strand RNA viruses, including *Tobacco mosaic virus*, *Potato virus X* (PVX), *Cucumber mosaic virus* and *Watermelon mosaic virus* in *Nicotiana benthamiana*, and silencing of HSP70 compromises the infection of *Tobacco mosaic virus* (Chen *et al.*, 2008). The roles of HSP70 in plant virus replication have been particularly well studied using *Tomato bushy stunt virus* (TBSV), a model virus that can replicate a small replicon RNA in *Saccharomyces cerevisiae* and in plants. HSP70 is contained in the TBSV replication complex, and is necessary for viral replication, where it plays multiple roles affecting the subcellular localization and membrane insertion of the viral replication proteins, as well as the assembly of the viral replicase (Pogany *et al.*, 2008; Serva and Nagy, 2006; Wang *et al.*, 2009a, 2009b). The knocking down of cytosolic HSP70 levels by gene silencing or treatment with quercetin inhibitor reduced TBSV RNA accumulation dramatically in plants (Wang *et al.*, 2009a). HSP70 is also a core component of the replication complex of *Turnip mosaic virus* (TuMV), a virus belonging to the large genus *Potyvirus* (Dufresne *et al.*, 2008). HSP70-deficient *Arabidopsis* plants are more tolerant to infection by TuMV (Jungkunz *et al.*, 2011). The results indicate that HSP70 plays a role in the regulation of the functions of potyviral RNA-dependent RNA polymerase (RdRp). HSP70 may also regulate potyviral replication in a different manner, because it has been shown to act with its co-chaperone CIP1 to regulate the function of a potyvirus coat protein (CP), which, in turn, interferes with replication-associated translation (Hafren *et al.*, 2010).

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Amongst the plant DNA viruses, it has been reported that the CP of *Tomato yellow leaf curl virus* (TYLCV) and the movement protein (MP) of *Abutilon mosaic virus* (AbMV) interact with host HSP70 (Gorovits *et al.*, 2013; Krenz *et al.*, 2010). HSP70 inactivation resulted in a decrease in TYLCV DNA levels, demonstrating the role of HSP70 in TYLCV multiplication *in planta* (Gorovits *et al.*, 2013), whereas silencing of the expression of the plastid HSP70 chaperone inhibited AbMV movement, but not viral DNA accumulation (Krenz *et al.*, 2010). The results indicate that the HSP70 chaperone is multifunctional in the life cycle of geminiviruses.

Rice stripe virus (RSV) is the type member of the genus *Tenuivirus*. Its genome consists of four negative single-stranded (ss) (–)RNA segments. RNA1 (–9 kb) contains a single open reading frame (ORF) encoding RdRp in the viral complementary strand (Barbier *et al.*, 1992). The other three segments (RNA2, 3.5 kb; RNA3, 2.5 kb; RNA4, 2.2 kb) have an ambisense coding strategy; each has two non-overlapping ORFs on opposite strands, separated by an intergenic region that plays a role in transcription termination (Hamamatsu *et al.*, 1993; Takahashi *et al.*, 1993; Wu *et al.*, 2013; Zhu *et al.*, 1991, 1992). RNA2 encodes two proteins, p2 and pc2. p2 exhibits RNA silencing suppressor activity, and pc2 is a non-structural protein with unknown function, but which has similarities to the glycoprotein precursor of phleboviruses, and is processed in insect cells into pc2-N and pc2-C (Du *et al.*, 2011; Zhao *et al.*, 2012). On RNA3, p3 and pc3 are the suppressor of RNA silencing and the nucleocapsid protein (Xiong *et al.*, 2009), respectively. RNA4 encodes a disease-specific protein p4 and the viral MP pc4 (Xiong *et al.*, 2008; Yuan *et al.*, 2011; Zhang *et al.*, 2012).

Because it is difficult to construct RSV infectious clones and inconvenient to inoculate rice with RSV, there have been few reports of the host factors that participate in the RSV life cycle. However, several host proteins have been identified to interact with RSV proteins, including a type I DnaJ protein and a small HSP of rice, which interact with RSV pc4, and OsSGS3, which interacts with p2 (Du *et al.*, 2011; Lu *et al.*, 2009). Recently, PsbP, a 23-kDa oxygen-evolving complex protein of plants, has been shown to interact with p4, and its silencing resulted in more severe symptoms and the accumulation of viral RNAs (Kong *et al.*, 2013).

Here, we demonstrate that host HSP70 is necessary for RSV infection. Moreover, HSP70 interacts with the N-terminus of RSV RdRp, indicating that HSP70 probably plays a role in viral replication by interacting with RdRp.

RESULTS

HSP70 transcripts were induced in RSV-infected host plants *Oryza sativa* *HSP70* (Os03g0821100, *OsHSP70*)

HSP70 has been reported to participate in multiple steps of the viral cycle of different viruses, including retroviruses, and those with genomes of double-stranded (ds)DNA, ssDNA, dsRNA and (+)-strand ssRNA (Mayer, 2005). During infection, host HSP70 levels increase, indicating that the induced expression of host HSP70 may be a common feature of viruses that need involvement of the host HSP70 during their infection (Nagy *et al.*, 2011). There are 32 sequences containing an HSP70 domain in the rice genome, 11 of which are predicted to be localized in the cytoplasm (Sarkar *et al.*, 2013). To determine whether RSV, a (–)RNA virus, would also induce the expression of host HSP70, we examined the change in expression of transcripts of these 11 cytoplasmic HSP70s in RSV-infected rice at 30 days post-inoculation (dpi). Quantitative real-time polymerase chain reaction (PCR) showed that five *HSP70*s were up-regulated and two exhibited no obvious change in expression in RSV-infected rice plants, whereas four others were undetectable in either RSV-infected or mock-infected plants, probably because their expression is induced only by abiotic stresses (Fig. 1A). Among the *HSP70*s with increased expression in RSV-infected plants, sequence Os03g0821100 (*OsHSP70*) transcripts in RSV-infected rice plants accumulated to at least twice the levels observed in non-infected plants (Fig. 1A). This sequence was selected for the following analysis to identify the possible function of HSP70 during RSV infection.

Nicotiana benthamiana can be infected by RSV through mechanical inoculation in the laboratory, and provides a simple viral inoculation system for the study of RSV–plant interactions (Xiong *et al.*, 2008; Zhang *et al.*, 2012). We detected the expression of a homologue of Os03g0821100 in *N. benthamiana*,

Fig. 1 Expression levels of heat shock protein 70 (HSP70) transcripts in *Rice stripe virus* (RSV)-infected rice (A) and *Nicotiana benthamiana* (B) systemic leaves at 30 and 15 days post-inoculation (dpi), respectively. Experiments were repeated three times. Bars represent the standard errors of the means. A two-sample unequal variance directional *t*-test was used to test the significance of the difference (* $P < 0.05$).

NbS00022307g0013.1 (*NbHSP70*), in RSV-infected plants, and found that it was also up-regulated at 15 dpi when systemic RSV symptoms appeared (Fig. 1B).

RSV RNAs accumulated in heat-treated *N. benthamiana*

In previous studies, heat treatment that induced the expression of HSP70 enhanced the accumulation and systemic infection of viruses (Chen *et al.*, 2008). We therefore investigated the effect of heat treatment on RSV infection. After exposure to high temperature (42 °C) for 30 min, expression of *NbHSP70* in plants was upregulated 2.5-fold compared with that in plants kept at 23 °C (Fig. 2A). Plants were then inoculated with RSV either before or after heat treatment, and grown at 23 °C. At 9 dpi, lesions appeared on both heat-treated and non-heat-treated inoculated leaves, but the numbers of lesions were greater on the heat-treated leaves (Fig. 2B). RSV RNAs accumulated to higher levels in heat-treated plants, irrespective of the timing of the inoculation (Fig. 2C). These results revealed that heat treatment enhanced RSV infection.

RSV RNAs were reduced in *HSP70*-silenced *N. benthamiana*

To understand the potential function of *HSP70* in RSV infection, we used the *Tobacco rattle virus* (TRV)-induced gene silencing system to investigate the effect of *NbHSP70* silencing on RSV infection. At 7 dpi of TRV:HSP70 to induce silencing of host HSP70, the systemic phenotype resulting from *NbHSP70* silencing appeared (Fig. 3A). Newly emerged leaves became small, thick and curved with necrosis (Fig. 3A). TRV had infected these leaves systemically (Fig. 3B), and the expression of *NbHSP70* was dramatically reduced, indicating the silencing of *NbHSP70* (Fig. 3C,D).

When *NbHSP70*-silencing phenotypes had become fully established in *N. benthamiana*, the plants were mechanically inoculated with RSV and monitored for symptom development (Fig. 4A). At 7 dpi of RSV, local lesions were observed on the inoculated leaves of both *NbHSP70*-silenced and non-silenced (treated with empty TRV vector, TRV:00) plants (Fig. 4B). Meanwhile, the numbers of infection foci were fewer on the inoculated leaves of *NbHSP70*-silenced plants than on the leaves of non-silenced plants (Fig. 4B). At 14 dpi of RSV, the typical symptoms of RSV appeared on the top leaves of non-silenced plants, where newly emerged leaves became crimped with severe mosaic (Fig. 4C). This symptom did not appear on most of the top leaves of *NbHSP70*-silenced plants, either at this time or later (Fig. 4C). However, typical symptoms of RSV did appear on some newly emerged leaves on a few shoots of RSV-inoculated *NbHSP70*-silenced plants (Fig. 4D). Real-time PCR confirmed that the expression of *NbHSP70* in leaves without RSV symptoms was at a low level, but that, in leaves with RSV

Fig. 2 Accumulation of *Rice stripe virus* (RSV) RNAs in heat-treated *Nicotiana benthamiana*. (A) Expression of *NbHSP70* was up-regulated in plants heat treated at 42 °C for 30 min. (B) When plants were heat treated either before (+RSV) or after (RSV+) inoculation of RSV, the number of infection foci on the inoculated leaves increased at 9 days post-inoculation (dpi) with RSV. (C) RSV RNAs accumulated much more in the inoculated leaves of heat-treated plants at 9 dpi with RSV. CP, coat protein. Three repeat experiments were performed. Bars represent the standard errors of the means. A two-sample unequal variance directional *t*-test was used to test the significance of the difference (**P* < 0.05).

Fig. 3 Effects of *Tobacco rattle virus* (TRV)-induced *NbHSP70* silencing on *Nicotiana benthamiana* plants at 7 days post-inoculation (dpi). (A) *NbHSP70* silencing caused the newly emerged leaves to be small, thick and curved with necrosis. (B) TRV coat protein (CP) was detected by real-time polymerase chain reaction (RT-PCR) showing systemic infection by TRV. (C, D) Expression of *NbHSP70* transcripts (C) and proteins (D) was down-regulated in TRV:HSP70-treated plants. Three repeat experiments were performed. Bars represent the standard errors of the means. A two-sample unequal variance directional *t*-test was used to test the significance of the difference (* $P < 0.05$).

symptoms, it was at a high level (Fig. 4D). These results indicate the recovery of *NbHSP70* silencing in those leaves with RSV symptoms on RSV-inoculated *NbHSP70*-silenced plants.

Northern blotting was used to investigate the accumulation of RSV RNAs in *NbHSP70*-silenced plants. RSV RNAs accumulated at a low level in *NbHSP70*-silenced plants (either inoculated leaves at 7 dpi or upper leaves at 14 dpi of RSV) compared with non-silenced plants (Fig. 5A,B). There was no accumulation of RSV RNAs in *NbHSP70*-silenced top leaves, but their accumulation in *NbHSP70*-recovered top leaves was at a similar level to that in

Fig. 4 Effect of *NbHSP70* silencing on *Rice stripe virus* (RSV) infection. (A) Flow chart showing RSV inoculation at 7 days after TRV:HSP70 treatment, and the different leaf samples (I, U and T) collected for analysis. 'I' are the inoculated leaves; 'U' are the leaves immediately above the RSV-inoculated leaves; 'T' are the top leaves. (B) At 7 days post-inoculation (dpi) of RSV, local lesions were observed on the inoculated leaves of both *NbHSP70*-silenced and non-silenced (treated with empty *Tobacco rattle virus* (TRV) vector; TRV:00) plants, and the number of infection foci was reduced on inoculated leaves of *NbHSP70*-silenced plants. (C) Fourteen days after RSV inoculation, RSV symptoms appeared on the top leaves of non-silenced plants, whereas there were no obvious RSV symptoms on the top leaves of *NbHSP70*-silenced plants. (D) In several *NbHSP70*-silenced plants, typical symptoms of RSV appeared on some newly emerged leaves (R) from a few shoots. Expression levels of *NbHSP70* transcripts in R leaves were similar to those in non-silenced plants, indicating that the silencing of *NbHSP70* was recovered in the R leaves. Three repeat experiments were performed. Bars represent the standard errors of the means. A two-sample unequal variance directional *t*-test was used to test the significance of the difference (* $P < 0.05$; ** $P < 0.01$).

Fig. 5 Accumulation of *Rice stripe virus* (RSV) RNAs in *NbHSP70*-silenced plants. (A) Accumulation of RSV RNAs was reduced in the inoculated leaves at 7 days post-inoculation (dpi) of RSV when *NbHSP70* was silenced (three repeats are shown). 'S' and 'NS' represent *NbHSP70*-silenced and non-silenced plants, respectively. (B) Accumulation of RSV RNAs was also reduced in the leaves immediately above the inoculated ones ('U' leaves in Fig. 4A) at 14 dpi of RSV when *NbHSP70* was silenced (three repeats are shown). (C) At 14 dpi of RSV, RSV RNAs were undetectable in the top leaves without symptoms (T) on the *NbHSP70*-silenced plants, whereas from the top leaves with viral symptoms (R) on the *NbHSP70*-silenced plants, RSV RNAs could be detected. Four repeats are shown, and two (3 and 4) had R leaves. CP, coat protein.

non-silenced plants at 14 dpi of RSV (Fig. 5C). These results indicate that *NbHSP70* is necessary for RSV infection.

To exclude any effect of TRV on RSV infection in *N. benthamiana* plants, we compared RSV infection on TRV-infected plants with that on mock-inoculated plants. RSV was inoculated at 7 dpi with TRV. Lesion numbers, RSV RNA accumulation on inoculated leaves and viral systemic symptoms on RSV and TRV double-infected plants at 14 dpi with RSV were recorded and compared with those on RSV-infected plants. There was no

obvious change in lesion numbers and accumulation of RSV RNAs in the inoculated leaves, and the viral systemic symptoms were similar to the control, indicating that TRV infection has no obvious effect on the following RSV infection (Fig. S1, see Supporting Information).

We also used the chemical inhibitor of HSP70, quercetin, to silence the expression of *NbHSP70*, and detected its effect on RSV infection. Results showed that treatment with quercetin reduced the accumulation of *NbHSP70* transcripts (Fig. 6A) and RSV RNAs

Fig. 6 Accumulation of Rice stripe virus (RSV) RNAs in *Nicotiana benthamiana* quercetin-treated leaves. (A) Quercetin (QT) treatment reduced the expression of *NbHSP70* compared with the control [dimethylsulphoxide (DMSO) treatment]. (B) Accumulation of RSV RNAs was reduced in the inoculated quercetin-pretreated leaves at 7 days post-inoculation (dpi) of RSV (three repeats are shown). CP, coat protein. Bars represent the standard errors of the means. A two-sample unequal variance directional *t*-test was used to test the significance of the difference (* $P < 0.05$).

compared with the control leaves treated only with dimethylsulphoxide (DMSO). The results further support the finding that *NbHSP70* is necessary for RSV infection (Fig. 6B).

Both OsHSP70 and NbHSP70 interacted with the N-terminus of RSV RdRp

HSP70 usually has its main effect on viral infection by interacting with viral RdRp and functioning as a component of the replication complex. We therefore examined the interaction between *NbHSP70* and RSV RdRp by bimolecular fluorescence complement (BiFC) assay. Because the RdRp protein is huge (337 kDa) and inconvenient for analysis, we divided it into five peptides based around its conserved domains (Fig. 7A). The first fragment (R1) containing the N-terminus of RSV RdRp interacted with both *NbHSP70* and *OsHSP70*, but the other fragments did not (Fig. 7A,B). In other experiments, no interactions were detected between HSP70 and the RSV MP (pc4) or CP (pc3) (data not shown). In the *in vitro* pull-down assay, the specific interaction between R1 and both *NbHSP70* and *OsHSP70* was further confirmed (Fig. 7C).

Localization of OsHSP70 or NbHSP70 was not affected by expression of the N-terminus of RSV RdRp or by RSV infection

The co-localization of R1 and HSP70 was investigated. Green fluorescent protein (GFP)-fused R1 and red fluorescent protein (RFP)-fused *OsHSP70* or *NbHSP70* were co-expressed in epidermal cells of *N. benthamiana* leaves using *Agrobacterium*. At 4 dpi, GFP-fused R1 co-localized with RFP-fused *OsHSP70* (Fig. 8B) or RFP-fused *NbHSP70* (Fig. 8C) in the cytoplasm and nucleus, which further indicates that R1 interacts with both *OsHSP70* and *NbHSP70*. We also noticed that the localization of *OsHSP70* or *NbHSP70* was not obviously different from that when co-expressed with R1, and vice versa (Fig. 8A). In further experiments, the localization of GFP-fused *OsHSP70* or GFP-fused

NbHSP70 did not appear to be affected by RSV infection (Fig. 8D), and fluorescence was still observed in both the cytoplasm and nucleus. These results indicate that the localization of host HSP70 is not changed by R1 expression or RSV infection, suggesting that the function of host HSP70 might not be dramatically changed by R1 expression or RSV infection.

DISCUSSION

As a chaperone, HSP70 is one of the best studied host factors related to virus infection, and is known to be involved in replication, movement and other steps of the viral life cycle (Mayer, 2005; Nagy *et al.*, 2011). In our experiments, silencing of *NbHSP70* reduced the accumulation of RSV RNAs in the inoculated leaves and in upper leaves, whereas RSV RNAs could not be detected in the uppermost newly emerged leaves, although they were found in those leaves that had recovered from *NbHSP70* silencing. These results indicate that HSP70 is necessary for RSV infection, showing that RSV, a plant (–)RNA virus, resembles DNA viruses and (+)RNA viruses in requiring the involvement of host HSP70 during infection. Moreover, HSP70 interacted with the N-terminus of RSV RdRp, suggesting that HSP70 might function at the RSV replication step. Because RSV RNAs accumulated at a low level in the inoculated leaves of silenced plants, it is possible that silencing of *NbHSP70* may reduce the efficiency of RSV replication. Amongst the plant (+)RNA viruses, it has been reported that HSP70 participates in the assembly of the replicase complexes of TBSV, TuMV and Red clover necrotic mosaic virus (RCNMV) (Dufresne *et al.*, 2008; Mine *et al.*, 2012; Pogany *et al.*, 2008). Until now, there have been no reports identifying the components of the replicase complex of a plant (–)RNA virus, but our results provide the first evidence indicating that *NbHSP70* is involved.

RSV is transmitted by the small brown planthopper (*Laodelphax striatellus*, SBPH) in a persistent manner. In the laboratory, inoculation of RSV to rice plants relies on the use of viruliferous SBPH, which is one of the inconvenient factors limiting research on this

Fig. 7 Both OsHSP70 and NbHSP70 interact with the N-terminus of *Rice stripe virus* (RSV) RNA-dependent RNA polymerase (RdRp). (A) Five peptides were selected to cover the whole RSV RdRp based around the conserved domains (OUT, DUF3778 and Bunya-RdRp domain) of the protein. Sites for division are indicated. The table on the right shows that the N-terminus of RSV RdRp interacts with both NbHSP70 and OsHSP70, but the other fragments do not. (B) Fluorescence was visible in bimolecular fluorescence complement (BiFC) analysis, indicating that both OsHSP70 and NbHSP70 interact with the N-terminus of RSV RdRp. YFP, yellow fluorescent protein. (C) *In vitro* pull-down assay of the N-terminus of RSV RdRp (R1) with NbHSP70 and OsHSP70. Immunoprecipitation (IP) was performed with anti-His or anti-Flag. Precipitates were detected by streptavidin alkaline phosphatase. p53 protein was used as a negative control. R1 without His mixed with HSP70s was also used as a control. Fluorescence photographs were taken at 4 days post-inoculation (dpi). Scale bar: 25 μ m.

virus. However, the recent report that the experimental plant *N. benthamiana* can be infected by RSV through mechanical inoculation provides a simple viral inoculation system for RSV–plant interaction research (Xiong *et al.*, 2008; Zhang *et al.*, 2012).

NbHSP70 and *OsHSP70* have a high level of nucleotide and amino acid identity to each other, and both proteins interact with the N-terminus of RSV RdRp, and co-localize with it, suggesting that HSP70 functions in a similar way in both host plants.

Fig. 8 Localization analyses of OsHSP70, NbHSP70 and the N-terminus of *Rice stripe virus* (RSV) RNA-dependent RNA polymerase (RdRp). (A) OsHSP70, NbHSP70 and the N-terminus of RSV RdRp (R1) all localized in both the cytoplasm and nucleus when they were expressed in leaf epidermal cells alone. (B) OsHSP70 and the N-terminus of RSV RdRp co-localized when the two proteins were co-expressed. (C) NbHSP70 and the N-terminus of RSV RdRp co-localized when the two proteins were co-expressed. (D) RSV infection had no effect on the localization of OsHSP70 or NbHSP70. Fluorescence photographs were taken at 4 days post-inoculation (dpi). GFP, green fluorescent protein. Scale bar: 25 μm.

It has been reported previously that host HSP70 can also interact with viral MPs and CPs. Co-immunoprecipitation (Co-IP) assays have shown that TYLCV CP interacts with tomato HSP70 (Gorovits *et al.*, 2013). By yeast two-hybrid assays and BiFC, *Arabidopsis thaliana* heat shock cognate 70-kDa protein (cpHSC70-1) was shown to interact with AbMV MP (Krenz *et al.*, 2010). A tomato heat shock protein cognate 70 was identified to interact with *Pepino mosaic virus* capsid protein (Mathioudakis *et al.*, 2012). We found no interaction between NbHSP70 and the MP or CP of RSV, suggesting that NbHSP70 might not be directly involved in RSV movement, particle assembly or other specific functions of the MP and CP.

Although several host proteins have been identified to interact with RSV proteins (Du *et al.*, 2011; Lu *et al.*, 2009), our studies with HSP70 provide the first evidence of an interacting host

protein related to RSV replication. The demonstration that HSP70 interacts with the RSV RdRp also provides a starting point for research on RSV replication, which has been little studied to date.

EXPERIMENTAL PROCEDURES

Plants and virus inoculation

RSV-infected *O. sativa* was prepared as described by Yan *et al.* (2010). Briefly, viruliferous *L. striatellus* (carrying the RSV-Zhejiang isolate) was transferred onto healthy rice seedlings (*O. sativa* L. spp. *japonica* cv. Nipponbare) at the three-leaf stage. After 72 h, the insects were removed. *Nicotiana benthamiana* plants at the six-leaf stage were mechanically inoculated with crude extracts from RSV-infected *O. sativa* leaves, as described previously (Wu *et al.*, 2013). Systemic infection was confirmed

by real-time PCR 10 days after inoculation. All *N. benthamiana* plants were grown in a glasshouse at 23–25 °C, with a 12-h day/night light cycle under well-watered conditions. Leaves were collected from the infected plants, frozen and stored at –80 °C until use.

For heat treatment, approximately 3-week-old *N. benthamiana* plants were subjected to heat stress by exposure to high temperature (42 °C) for 30 min and then maintained at 23 °C. Plants were inoculated with RSV either before or after the heat stress treatment. Control plants were kept at 23 °C and leaves in the same position as the heat-stressed plants were inoculated with an equivalent amount of RSV. The experiments were repeated three times.

Virus-induced gene silencing

TRV vectors, pYL196 and pTRV were kindly provided by Dr Yule Liu, Tsinghua University, Beijing, China (Liu *et al.*, 2002). pTRV containing TRV RNA2 was used to express the partial sequence of NbHSP70 amplified with primers (f, 5'-TCTAGAGTACAAGGCAGAAGACGAGGAT-3'; r, 5'-GGT ACCCATCATCATCCATAGGTGCACC-3') to silence the internal expression of NbHSP70. The sequence was ligated into pTRV with *Xba*I and *Kpn*I, producing vector TRV:HSP70. The empty vector, named TRV:00, was used for the control treatment. Viral infection by *Agrobacterium* infiltration was performed as described by Peng *et al.* (2011). The leaves above the agroinfiltrated leaves were inoculated with RSV at 7 dpi.

RNA analysis

Total RNA was isolated from RSV-infected plants with Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. For Northern blot analyses, a DNA probe targeting RSV CP was synthesized with primers (f, 5'-CTCCTGGTCATCATGCAAG-3'; r, 5'-GCCAGCGCATCG AAGATTGTG-3') and labelled with digoxin (DIG) according to the manufacturer's protocol (DIG Oligonucleotide 3'-End Labelling Kit, Roche, Basel, Switzerland). Pre-hybridization, hybridization and signal detection were performed according to the protocol of the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche).

HSP70 expression was detected by real-time PCR using the primers (f, 5'-GATGTACACCTCTGTCTTG-3'; r, 5'-GGTCTTGTCTCGGCTGATACG-3') according to the previous report (He *et al.*, 2012). *Nicotiana benthamiana* Ubiquitin C (*UBC*) gene (Accession Number: AB026056.1) was used as the internal reference gene for analysis (primers: 5'-GAGGAAGAGACTGGTGAGGGAT-3' and 5'-CACAGAGCAAAGACTGGAT TGA-3'). A Roche LightCycler®480 Real-Time PCR System was used for the reaction and the results were analysed by the $\Delta\Delta C_T$ method.

To confirm the systemic infection of TRV, viral RNAs in a new leaf were amplified by real-time PCR with primers designed from the TRV CP sequence (f, 5'-TCATTGACAAGTCGGGC-3'; r, 5'-TGTGTTGGATTCG CAGG-3').

Quercetin treatment

Quercetin (Sigma-Aldrich, St. Louis, Missouri, USA) was used to inhibit *N. benthamiana* HSP70 gene expression. Quercetin was dissolved in DMSO to prepare a 1 M stock solution and diluted to 1 mM concentration in lysis buffer (10 mM NaCO₃, pH 9.6) for infiltration (Hafren *et al.*, 2010;

Wang *et al.*, 2009a). Plants were inoculated with RSV followed by infiltration with quercetin. The corresponding amount of DMSO in the same buffer was used in control infiltrations. RSV infection assays were initiated 1 h after infiltration with quercetin or DMSO.

In vitro pull-down assay

The *in vitro* pull-down assay was performed using a TNT Coupled Wheat Germ Extract System following the manufacturer's recommendations (Promega, Madison, Wisconsin, USA). pCMV:His-RdRp1, pCMV:Flag-OsHSP70 and pCMV:Flag-NbHSP70 were produced with the full-length coding regions of OsHSP70 and NbHSP70 and the fragments of RSV RdRp-1 ligated into pCMVTNT expression vectors (Promega). The plasmid for the generation of mouse p53 protein was used as a negative control in the experiments. *In vitro* translated His-RdRp1 (15 µL) and OsHSP70 (15 µL) or NbHSP70 (15 µL) were incubated with the appropriate 10 µL Flag-antibody or His-antibody (Transgen Biotech, Beijing, China) at 4 °C overnight. Fifty microlitres of Protein G-agarose beads (Merck Millipore, Billerica, Massachusetts, USA) were added and incubated for another 3 h. The beads were washed (4 × 1 min) with washing buffer (0.05 M Tris-HCl, 0.15 M NaCl) and the bound complexes were eluted by boiling with protein loading buffer for 5 min. Co-immunoprecipitates were then run on a 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and detected by streptavidin alkaline phosphatase.

Western blotting

Total proteins of plant samples were extracted with lysis buffer (100 mM Tris-HCl, pH 8.8, 60% SDS, 2% β-mercaptoethanol). Proteins were separated in a 12% SDS-PAGE gel, transferred onto nitrocellulose (Amersham, Sweden) by wet electroblotting and detected with anti-HSP70 (Agrisera, Vännäs, Sweden) primary antibody and anti-rabbit (Sigma-Aldrich, St. Louis, Missouri, USA) secondary antibody. The antigen-antibody complexes were visualized using nitroterazolium blue chloride/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) buffer (Sigma-Aldrich, St. Louis, Missouri, USA) under standard conditions.

BiFC and co-localization assay

The plant binary vectors used in BiFC and co-localization assay experiments have been described previously (Lu *et al.*, 2011; Yan *et al.*, 2012). *Escherichia coli* strain TG1 was used for transformation. The full-length coding regions of OsHSP70 and NbHSP70 were cloned from uninfected plants and the RSV RdRp was cloned from RSV-infected rice. The sequence of RSV RdRp was divided into five fragments and amplified using the primers listed in Table 1.

Binary plasmids carrying the full-length coding regions of OsHSP70 and NbHSP70 or RSV RdRp fragments fused either to the N-terminal or C-terminal parts of yellow fluorescent protein (YFP) were introduced into *Agrobacterium tumefaciens* strain EHA105 and agroinfiltrated in pairwise combinations at a ratio of 1:1 into fully expanded leaves of 3-week-old *N. benthamiana*. The full-length coding regions of OsHSP70, NbHSP70 and the fragments of RSV RdRp were ligated into pCV:GFP or pCV:RFP with *Bam*HI and *Kpn*I. The co-localization assays were performed as described in the BiFC assay (Lu *et al.*, 2011).

Table 1 Sequences of the primers used (the italic type is restriction endonuclease).

OsHSP70-f	5'-GGTACCATGGCGCCGACCAAGGGG-3'
OsHSP70-r	5'-GAGCTCTTAGTCGACCTCCTCAATCTTA-3'
NbHSP70-f	5'-GGGTACCATGGCGGAAAGGTGAAGG-3'
NbHSP70-r	5'-GGGATCCTTAATCCACCTCCTCAATCTTAG-3'
RdRp-1f	5'-GGTACCATGACGACACCCTCTCG-3'
RdRp-1r	5'-GAGCTCTTACTCATAGATCTCAGCATTTGG-3'
RdRp-2f	5'-GGTACCGAGATCTATGAGGCTGTTGAT-3'
RdRp-2r	5'-GAGCTCTTAGCTCGGTGGCAAGATCAG-3'
RdRp-3f	5'-GGTACCTCTGATCTGCCACCGAGC-3'
RdRp-3r	5'-GAGCTCTTACGTACAGGCAATCCAAACATT-3'
RdRp-4f	5'-GGTACCGTTTGGATTGCTGTACGAC-3'
RdRp-4r	5'-GGATCCTTAGTCTCTTCATTAGCTGCTC-3'
RdRp-5f	5'-GGTACCCAGCTAATGAAGGAGAACACT-3'
RdRp-5r	5'-GAGCTCTCAGAAATCGAAGTATGGTCT-3'
Os01g62290f	5'-GCTGGGGCTCGAGACAGCA-3'
Os01g62290r	5'-GCGTCTCGTCCATGCCAG-3'
Os03g16880f	5'-CGTAAGCAGGCAGAAAATGTACA-3'
Os03g16880r	5'-CTCTTGATGGTGATGCTGT GC-3'
Os03g0821100f	5'-CCTGGTGTGTTGATTGAGTC-3'
Os03g0821100r	5'-CTGGATCGCTGCTCGACC-3'
Os05g38530f	5'-TACGAGGGCGAGCGGCCA-3'
Os05g38530r	5'-CTCTTCTTGTCTCGGCGGC-3'
Os11g08445f	5'-GGTGTGAGATCTATGATGAAAAT-3'
Os11g08445r	5'-ATCGATACGG GCTACGCCGG-3'
Os11g08460f	5'-TTTATGTGGTGGAACCGATGATA-3'
Os11g08460r	5'-TGTCGAAAGT GACATCGATA GAT-3'
Os11g08470f	5'-GCAGCAGCAACAGCGACAATG-3'
Os11g08470r	5'-CGGCGCTCGGC GTTGTCGGAG TC-3'

Microscopy

A Leica TCS SP5 (Leica Microsystems, Bannockburn, IL, USA) confocal laser scanning microscope system was used to examine the fluorescence of GFP, RFP and YFP. Fluorescence was visualized between 2 and 4 days post-infiltration by confocal microscopy. All images were processed using Adobe Photoshop version 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 *Tobacco rattle virus* (TRV) infection has no effect on *Rice stripe virus* (RSV) infection. (A) RSV infection on a TRV-infected plant and a mock-inoculated plant. (B) Accumulation of RSV RNAs in the inoculated leaves of a TRV-infected plant and a mock-inoculated plant.