Nucleolin/Nsr1p binds to the 3’ noncoding region of the tombusvirus RNA and inhibits replication

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**A R T I C L E   I N F O**

Article history:
Received 14 May 2009
Returned to author for revision 13 June 2009
Accepted 3 October 2009
Available online 27 October 2009

Keywords:
Tomato bushy stunt virus
Yeast
nsr1
Nucleolin, *Nicotiana benthamiana*
Host factor

**A B S T R A C T**

Previous genome-wide screens identified >100 host genes affecting tombusvirus replication using yeast model host. One of those factors was Nsr1p (nucleolin), which is an abundant RNA-binding shuttle protein involved in RNA maturation and ribosome assembly. We find that overexpression of Nsr1p in yeast or in Nicotiana benthamiana inhibited the accumulation of tombusvirus RNA by ~10-fold. Regulated over-expression of Nsr1p revealed that Nsr1p should be present at the beginning of viral replication for efficient inhibition, suggesting that Nsr1p inhibits an early step in the replication process. In vitro experiments revealed that Nsr1p binds preferably to the 3’ UTR in the viral RNA. The purified recombinant Nsr1p inhibited the in vitro replication of the viral RNA in a yeast cell-free assay when preincubated with the viral RNA before the assay. These data support the model that Nsr1p/nucleolin inhibits tombusvirus replication by interfering with the recruitment of the viral RNA for replication.

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Introduction

RNA viruses, which have small genomes with limited coding potential, depend on recruited host factors during the replication process. Therefore, virus–host interaction is critical for successful viral infections as well as for triggering anti-viral responses in the host. Recent genome-wide screens with several RNA viruses revealed rather complex interactions involving several hundred host genes (Cherry et al., 2005; Hao et al., 2008; Jiang et al., 2006; Krishnan et al., 2008; Kushner et al., 2003; Panavas et al., 2005b; Serviene et al., 2006; Serviene et al., 2005). While many of the identified genes are important for RNA virus replication, other host genes were found inhibitory by reducing the accumulation of the viral RNA. The identified inhibitory genes could be part of the innate immune responses of the host.

*Tomato bushy stunt virus* (TBSV) has emerged as one of highly suitable model virus systems to study RNA virus replication and host–virus interaction due to the recent development of the highly tractable yeast as a model host (Nagy, 2008; Panavas and Nagy, 2003b) and cell-free approaches (Panaviene et al., 2004; Pogany and Nagy, 2008; Pogany et al., 2008). Replication of a short TBSV replicon (rep)RNA, which is a 621-nt defective interfering RNA carrying four noncontiguous regions from the genomic (g)RNA, in yeast requires the co-expression of the viral p33 and p92^pol^ replication proteins, which form the membrane-associated viral replicase (Panaviene, Panavas, and Nagy, 2005; Panaviene et al., 2004). Systematic, genome-wide, and proteomics approaches have led to the identification of more than 200 host proteins/genes affecting TBSV replication/recombination or interacting with the viral replication proteins/viral RNA (Jiang et al., 2006; Li et al., 2008; Li et al., 2009; Panavas et al., 2005b; Serva and Nagy, 2006; Serviene et al., 2006; Serviene et al., 2005). A rapidly progressing research area after the systematic genome-wide screens is the dissection of the functions of the identified host factors during virus replication. Five of the identified host factors are part of the viral replicase complex, facilitating the assembly of the replicase, regulating the ratio of plus- versus minus-strand RNA synthesis, enhancing the stability of the viral replication proteins or their intracellular transportations and insertions into subcellular membranes (Jonczyk et al., 2007; Li et al., 2008; Li et al., 2009; Pathak, Sasvari, and Nagy, 2008; Pogany et al., 2008; Serva and Nagy, 2006; Wang and Nagy, 2008; Wang, Stork, and Nagy, 2009). Other host proteins tested in more detail affected viral RNA degradation and viral recombination (Cheng et al., 2007; Cheng, Serviene, and Nagy, 2006; Jaag and Nagy, 2009) or had only indirect effect on TBSV repRNA accumulation (Jaag, Stork, and Nagy, 2007). Importantly, the relevance of several host genes identified in yeast has also been confirmed in the natural plant host (Jaag and Nagy, 2009; Wang and Nagy, 2008; Wang, Stork, and Nagy, 2009). These discoveries justify the use of yeast model host for replication studies with TBSV.

In this article, we further characterize the inhibitory role of the previously identified nucleolin (Nsr1p in yeast) in TBSV replication (Panavas et al., 2005b). Nucleolin/Nsr1p is an abundant, ubiquitously expressed protein, which is involved in ribosome biogenesis (Mongelard and Bouvet, 2007). Nucleolin also affects transcription of rRNA, processing and modification of rRNA and nuclear cytosolic transport of ribosomal protein and ribosomal subunits by shutting between the nucleus and the cytoplasm (Tuteja and Tuteja, 1998). Nucleolin is...
found in various cell compartments, and it is especially abundant in the nucleolus.

Nucleolin/Nsr1 has three well-defined domains; the N-terminal domain with alternating acidic and basic stretches is involved in RNA transcription by interacting with RNA repeats and histone H1 as well as in nuclear localization. The central portion is the RNA-binding domain carrying RRM (RNA recognition motif) repeats, whereas the C-terminal part contains the glycine–arginine-rich (GAR) domain. The GAR domain is involved in interaction with the ribosomal proteins, and it was suggested to affect ribosomal assembly and transport (Tuteja and Tuteja, 1998).

The analysis of nucleolin functions is challenging due to the broad range of mechanisms performed by nucleolin, which affect DNA and RNA metabolism, and its presence in various subcellular locations (Mongelaud and Bouvet, 2007). In addition to binding to RNA/DNA and its role in proper folding of pre-rRNA, nucleolin also interacts with many proteins during ribosome assembly, and it is involved in regulating the RNA polymerase-I-based transcription. Arabidopsis has two nucleolin genes, but only AtNuc-L1 is expressed ubiquitously under normal growth conditions (Kojima et al., 2007; Pontvianne et al., 2007). The nucleolin gene from pea was able to complement nsr1Δ yeast by rescuing the reduced level of rRNA (Reichler et al., 2001), suggesting that the plant nucleolin has similar functions to the yeast NR1.

Here, we confirm that Nsr1p/nucleolin is an inhibitor of TBSV replication. Overexpression of the yeast Nsr1p in yeast or the Arabidopsis nucleolin in Nicotiana benthamiana reduced the accumulation of tombusvirus RNA and inhibited the in vitro activity of the tombusvirus replicase. We found that Nsr1p binds to the upstream portion of the 3′ UTR in (+)repRNA in vitro. Overall, these data suggest that Nsr1p could inhibit TBSV RNA replication by inhibiting the recruitment of the viral RNA for replication.

**Results**

**Overexpression of Nsr1p inhibits TBSV repRNA replication in yeast**

To test the effect of Nsr1p on TBSV repRNA accumulation in yeast, we overexpressed Nsr1p either as an N-terminal 6xHis-tagged Nsr1p or the C-terminal FLAG-tagged Nsr1p from a high copy number plasmid together with p33 and p92pol replication proteins and the TBSV repRNA (Fig. 1A). The accumulation of repRNA was measured via Northern blotting 24 h after induction of TBSV repRNA replication via the galactose-inducible GAL1 promoter. These experiments revealed that the C-terminal FLAG-tagged Nsr1p inhibited repRNA accumulation by 10-fold (Fig. 1A, lanes 4–6), while the inhibitory effect of 6xHis-tagged Nsr1p was less (by ~60%, lanes 1–3). Also, overexpression of the 6xHis-tagged Nsr1p inhibited repRNA accumulation in nsr1Δ yeast by ~3-fold (Fig. 1A, lanes 14–16), when compared with TBSV repRNA accumulation in nsr1Δ yeast. These experiments also confirmed that TBSV repRNA accumulation is 3-fold higher in nsr1Δ yeast (Fig. 1A, lanes 10–13) than in the parental BY4741 that expresses Nsr1p from the native promoter (lanes 7–9). Altogether, these data firmly established that Nsr1p is a potent inhibitor of TBSV repRNA accumulation in yeast.

Since Nsr1p is mostly a nuclear protein, it is possible that it could affect the plasmid-based transcription of the TBSV repRNA or the cleavage at the 3′ end by the ribozyme, which have been engineered to launch TBSV repRNA replication with the authentic 3′ end from the expression plasmid in the yeast model host (Panavas and Nagy, 2003b; Panaviene et al., 2004). Overexpression of Nsr1p, however, did not affect significantly the amount of repRNA transcripts made from the GAL1 promoter/expression plasmid in the absence of the viral replication proteins (Fig. 1B, lanes 2–4 versus 5–7). Also, the amount of p33 made in yeast overexpressing Nsr1p was comparable to that obtained in the parental yeast expressing native level of Nsr1p (not shown). These data suggest that overexpression of Nsr1p does not affect the amount of plasmid-borne repRNA, its processing by the ribozyme, or the expression of the viral replication proteins.

To test if Nsr1p can affect the activity of the tombusvirus replicase, we isolated membrane-bound replicase preparations from the above yeast strains, followed by in vitro replicase assay with the co-purified repRNA (Panavas, Panavas, and Nagy, 2005; Panaviene et al., 2004). As expected, we found that the tombusvirus replicase activity was ~3-fold lower when obtained from yeast overexpressing the FLAG-tagged Nsr1p (Fig. 1C, lanes 4–6) when compared with the preparation obtained from the parental BY4741 (lanes 1–3). On the contrary, the replicase preparation obtained from nsr1Δ yeast (lanes 7–9) was...
almost twice as active as the control preparation. Altogether, the in vitro data support the model that Nsr1p inhibits TBSV repRNA accumulation by inhibiting the viral replicase.

Expression of the plant nucleolin inhibits TBSV replication in Nicotiana benthamiana host

To test if the plant nucleolin, the homolog of yeast Nsr1p, might have similar inhibitory function against TBSV, we expressed the Arabidopsis thaliana nucleolin (AtNuc-L1) tagged with GFP (Kojima et al., 2007) in N. benthamiana leaves via agroinfiltration (Jaag and Nagy, 2009). The genomic RNA of Cucumber necrosis virus (CNV), a very close relative of TBSV, was co-expressed with AtNuc-L1-GFP via agroinfiltration in the same leaves. Leaf samples taken 2.5 days later were analyzed via Northern blotting to estimate the level of CNV RNA accumulation (Fig. 2A). Interestingly, expression of AtNuc-L1-GFP in N. benthamiana leaves inhibited the accumulation of CNV RNA by ~10-fold when compared with the control that expressed GFP in

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Fig. 2. Inhibition of tombusvirus genomic RNA accumulation by transient expression of Arabidopsis nucleolin in N. benthamiana leaves. (A) Leaves of N. benthamiana were co-agroinfiltrated to express Nucleolin-GFP (AtNuc-L1-GFP) and CNV genomic RNA from expression plasmids. Total RNA samples were prepared from randomly chosen areas of the infiltrated leaves 2.5 days post-infiltration, followed by Northern blotting to detect the accumulation level of CNV gRNA. rRNA was used as a loading control. Agroinfiltrated leaves co-expressing CNV and GFP were used as controls. (B) Western blotting shows the accumulation level of AtNuc-L1-GFP in the agroinfiltrated N. benthamiana leaves 2.5 days post-infiltration in comparison with the accumulation level of GFP. (C) Transient expression of AtNuc-L1-GFP in agroinfiltrated N. benthamiana leaves did not cause significant growth inhibition 2.5 days post-infiltration in comparison with plant expressing GFP. (D) Leaves of N. benthamiana were agroinfiltrated to express AtNuc-L1-GFP or GFP, followed by inoculation with inoculum containing CNV (mutant 20KSTOP) virions. Total RNA samples were prepared from randomly chosen areas of the infiltrated leaves 4 days post-infiltration, followed by Northern blotting to detect the accumulation level of CNV gRNA. See panel A for details. Note that variation in CNV RNA levels in different samples within the same set of experiment is likely due to uneven distribution of “infection foci” (those areas in the leaf where the virus was able to start infection) and the random sampling approach. The experiments were repeated, and the averages were calculated based on 18–42 samples/experiment. Two panels are shown for CNV due to the low level accumulation. (E) Leaves of N. benthamiana were agroinfiltrated to express AtNuc-L1-GFP or GFP, followed by inoculation with inoculum containing TBSV virions. See panel D for details. (F) Leaves of N. benthamiana were co-agroinfiltrated to express AtNuc-L1-GFP and TRV genomic RNA1/2. See panel A for details.
leaves (Fig. 2A, lanes 1–12 versus 13–24). The agroinfiltrated leaves expressed the GFP control at a higher level than AtNuc-L1-GFP (Fig. 2B). Overexpression of AtNuc-L1-GFP had only mild effect on the uninoculated agroinfiltrated leaves during these experiments, reducing the overall size of the leaves and causing minor growth inhibition of the plants (Fig. 2C).

We found similar ~10-fold inhibition of CNV RNA accumulation when CNV replication was initiated by sap-inoculation with CNV virions, which represents one of the natural ways for CNV to spread, when CNV replication was initiated by sap-inoculation with CNV supergroup, we agroinfiltrated N. benthamiana leaves with CNV virions, which belongs to a different supergroup, we agroinfiltrated N. benthamiana leaves to co-express TRV RNAs and AtNuc-L1-GFP. Northern blot analysis of TRV RNA1 levels revealed the lack of inhibition of TRV accumulation by AtNuc-L1-GFP (Fig. 2F, lanes 1–12 versus 13–24). Thus, nucleolin has different effects on tombusviruses versus tobaviruses, which belong to different superfamilies of RNA viruses.

To test if AtNuc-L1 can also inhibit a distantly related plant RNA virus, namely, Tobacco rattle virus (TRV), which belongs to a different supergroup, we agroinfiltrated N. benthamiana leaves to co-express TRV RNAs and AtNuc-L1-GFP. Northern blot analysis of TRV RNA1 levels revealed the lack of inhibition of TRV accumulation by AtNuc-L1-GFP (Fig. 2F, lanes 1–12 versus 13–24). Thus, nucleolin has different effects on tombusviruses versus tobaviruses, which belong to different superfamilies of RNA viruses.

Fig. 3. The effect of time of expression of Nsr1p on inhibition of tombusvirus RNA accumulation in yeast. (A) A scheme showing the time of expression of 6xHis-Nsr1p in comparison with TBSV replication in yeast. 6xHis-Nsr1p was expressed form the GAL1 promoter, whereas repRNA replication was launched from the CUP1 promoter in the parental BY4741 yeast strain. (B–D) The yeast transformants were pre-grown in SC-UBH media with 2% glucose for 24 h at 28 °C, then transferred to a media with 2% galactose (starting OD600 was ~0.3) and further cultured at 29 °C. Copper sulfate (50 μM) was added at different time points, such as 20 or 6 h after or 6 h prior to the addition of galactose containing medium to initiate repRNA replication. The accumulation of repRNA was estimated using Northern blotting after 24 h of culturing of yeast in the presence of copper ions. See further details in the legend to Fig. 1A.

Nsr1p inhibits the early steps in TBSV replication

After confirming the relevance of nucleolin/Nsr1p in inhibition of tombusvirus RNA replication in yeast as well as in a plant host, our goal was to dissect what steps of TBSV replication could be inhibited by this host protein. Tombusvirus replication is a complex process that consists of at least six defined steps after translation of the viral RNA (Nagy and Pogany, 2006). The early steps include selection of the viral RNA by selective binding of the viral p33 to the p33RE cis-acting element in the (+)RNA (Monkewich et al., 2005; Pogany, White, and Nagy, 2005), followed by recruitment of the viral RNA/replication protein complex to the site of replication (peroxisomal or ER membranes), and the assembly of the viral replicase into special membranous spherules. This is followed by the late steps of replication, such as minus- and plus-strand synthesis, release of the newly synthesized (+)RNA progeny from the replicase, and the final disassembly of the replicase complex (Nagy and Pogany, 2006).

To test if Nsr1p could inhibit early or late steps of TBSV replication, we started the overexpression of Nsr1p from the galactose-inducible GAL1 promoter at various time points when compared with initiating TBSV replication from the copper-inducible CUP1 promoter (chosen as 0-h time point, Fig. 3A). Overexpression of Nsr1p starting from 20 or 6

Fig. 4. In vitro binding of recombinant Nsr1p to the (+)repRNA. (A, top) UV-cross-linking assay with 2 μg of purified recombinant GST-ΔRBD (Nsr1p missing the central RNA-binding domains), GST-Nsr1, or GST and 50 μM 32P-labeled DI-72 (+)repRNA. (A, bottom) Coomassie blue staining of the SDS-PAGE shown in the top panel, showing the purified recombinant GST-ΔRBD, GST-Nsr1, and GST proteins from E. coli. The fusion proteins were purified using GST affinity chromatography. (B) A gel mobility shift assay showing interactions between the recombinant GST-Nsr1 and 32P-labeled TBSV DI-72 (+)repRNA. The in vitro binding was analyzed in 4% non-denaturing polyacrylamide gel. The unbound, free RNA probe and the shifted (bound) RNA/protein complexes are marked on the right. GST-Nsr1 and GST were used in increasing amounts (400, 800, and 1600 ng protein/per lane).
h prior to launching TBSV replication resulted in ~10- to 15-fold inhibition of TBSV repRNA accumulation (Figs. 3B, C). This level of inhibition is higher than that obtained when Nsr1p was overexpressed from the 0-h time point (Fig. 1A, lanes 4–6). However, overexpression of Nsr1p 6 h after launching TBSV replication (Fig. 3D) resulted in only a moderate level (~30%) inhibition of TBSV repRNA accumulation. Altogether, these data support the model that Nsr1p inhibits TBSV replication most efficiently at the early time points.

Nsr1p binds to the 3′ UTR of the TBSV (+)RNA in vitro

To identify the target of Nsr1p during TBSV repRNA replication that leads to inhibition of replication, we tested if the purified recombinant Nsr1p could bind to p33 and p92pro repRNA replication proteins and/or the viral (+)RNA. Although we could not detect interaction between Nsr1p and the viral replication proteins in vitro (not shown), we observed that Nsr1p bound to the 32P-labeled DI-72 (+) repRNA in a UV-cross-linking assay (Fig. 4A, lane 2). Deletion of the known RNA-binding domain in the recombinant Nsr1p (mutant GST-ARBD, lane 1) (Bouvet et al., 2001) abolished the ability of Nsr1p to bind to the repRNA. The purified GST was incapable of binding to the repRNA under the conditions used, suggesting that the recombinant GST-Nsr1 was responsible for RNA binding.

To confirm the results from the above UV-cross-linking experiments, we performed gel mobility-shift experiments with purified recombinant GST-Nsr1 and 32P-labeled DI-72 (+) repRNA. This experiment revealed that Nsr1p bound to the viral RNA (Fig. 4B). Since the extent of the band shift increased with increasing amounts of GST-Nsr1, it is likely that more than one Nsr1p molecule can bind to the same viral RNA molecule in vitro.

To test if there is a specific binding site for Nsr1p in DI-72(+) repRNA, we separately used the four segments of DI-72(+), known as RI-RIV (Fig. 5A) (White and Morris, 1994), as 32P-labeled probes in UV cross-linking experiments. This analysis revealed that Nsr1p bound preferably to RIII(+), moderately to RIV(+), and to a lesser extent to RI(+) and RI(+) (Fig. 5B). Gel mobility-shift assays confirmed that Nsr1p binding to RIII(+), was the most efficient (Fig. 5C, lanes 8–9). However, RI(+) and RIV(+) also bound to Nsr1p (lanes 6 and 12), while binding of RI(+) was the least efficient (lane 3).

Template-competition experiments with cold RIII(+) confirmed that the cold template competed the least efficiently against the 32P-labeled RIII(+) probe (Fig. 5D, lanes 11–12), while it competed efficiently against the RI(+) probe (lanes 2 and 4). Again, RII(+) and RIV(+) showed moderate level of competition based on the amount of released probes when excess amount of cold competitor was used.

Finally, we used the cold RI(+), RII(+), RIII(+), and RIV(+) competitors separately against the 32P-labeled RIII(+) RNA probe in a gel mobility-shift assay (Fig. 5E). Only RII(+) competed efficiently with the labeled DI-72(+) RNA for binding to Nsr1p (Fig. 5E, lane 10), confirming that RII(+) is the preferred site for Nsr1p binding. RIII(+) does not contain the previously identified nucleolin recognition element, which is a stem-loop structure with the loop containing UCCGA sequence (Bouvet et al., 2001). Thus, it is likely that RII(+) contains a not yet defined sequence/structure recognized by Nsr1p.

RIII is mostly derived from the 3′ UTR of the TBSV genome, but its function is not essential and the currently known role is on the minus-strand serving as a replication enhancer (Pogany and Nagy, 2003a; Ray and White, 2003). Accordingly, deletion of RIII in DI-72 leads to vastly reduced replication (Ray and White, 2003). Since Nsr1p binds preferably to RIII(+), we reasoned that deletion of RIII should make the repRNA insensitive to expression of excess amount of Nsr1p. Indeed, the low level accumulation of ∆RIII repRNA, lacking RIII, was inhibited only slightly by the overexpression of Nsr1p (Fig. 6, lanes 6–13), which inhibits DI-72 repRNA accumulation by 10-fold (Fig. 1A, lanes 4–6). This further supports that RIII in the tombusvirus genome is the main target of Nsr1p.

The recombinant Nsr1p inhibits the tombusvirus replicase in vitro

To test if the purified recombinant GST-Nsr1p can inhibit the tombusvirus replicase, we used a tombusvirus replicase assay based on a yeast cell-free extract containing subcellular membranes. This extract is capable of supporting authentic TBSV replication in vitro (Pogany and Nagy, 2008), due to the requirement of viral RNA recruitment and replicase assembly in the membranous fraction in vitro. Programming the cell-free extract with DI-72(+) repRNA leads to asymmetrical replication, resulting in small amount of (−)RNA intermediate and abundant (+)RNA progeny (Pogany and Nagy, 2008). Interestingly, addition of increasing amounts of purified GST-Nsr1 to the cell-free extract led to ~90% inhibition of TBSV repRNA replication when the (+)repRNA was preincubated with GST-Nsr1 prior to adding to the cell-free extract (Fig. 7, lanes 5–7). On the other hand, adding GST-Nsr1 and DI-72(+) repRNA simultaneously to the cell-free extract resulted in less inhibition (by 60%, lanes 16–18) when compared with the GST control (lanes 13–15). These results suggest that Nsr1p inhibits an early step in TBSV replication, likely the recruitment of the (+)repRNA into replication (see Discussion).

Lack of changes in subcellular localization of nucleolin during tombusvirus replication in plants

To test if the subcellular localization of nucleolin changes during replication of tombusviruses, we transiently expressed the AtNuc-L1-GFP fusion protein via agroinfiltration in transgenic plants expressing brillarin-RFP, a nucleolar marker protein (Kanneganti et al., 2007). Confocal laser microscopy revealed mostly nucleolar localization of AtNuc-L1 in both CNV-infected and control plant cells (Fig. 8). In addition, we also observed a small portion of AtNuc-L1 in the nucleus in both experiments. Overall, the subcellular distribution of AtNuc-L1-GFP was comparable in CNV-infected and control plant cells, suggesting that tombusvirus replication did not lead to nucleolus-to-cytosol re-distribution of nucleolin. Additional detailed experiments will address if fraction of nucleolin might be redistributed at early time points of CNV infection to the cytosol or if the viral RNA might be redirected to the nucleus.
Discussion

Host proteins could affect viral replication in various ways. Those host proteins, which facilitate or regulate virus replication, are called host factors. Previous works have identified several host factors for TBSV, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP70 heat shock protein, eEF1A translation elongation factor, and Cdc34 E2 ubiquitin conjugating enzyme, which are part of the viral replicase complex together with p33 and p92pol replication proteins. These host factors have been shown to regulate the ratio of viral replicase proteins in this work (not shown) or during previous proteomics screens (Li et al., 2008).

The best-characterized example is Xrn1p 5′-3′ exoribonuclease (Xrn4p in plants), which is involved in degradation of tombusvirus RNA, including partially degraded viral RNAs generated by endoribonucleases (Cheng et al., 2007; Cheng, Serviene, and Nagy, 2006; Jaag and Nagy, 2009). In the absence of Xrn1p/Xrn4p, tombusvirus RNA accumulation increased several fold and novel viral recombinant RNAs or variants emerged rapidly in yeast and in plants. Thus, in addition to inhibiting tombusvirus RNA accumulation, Xrn1p also affects the rate of virus evolution, suggesting complex interactions between host proteins and plant viruses (Nagy, 2008).

Another piece of evidence for the role of Xrn1p in inhibition of repRNA recruitment is the ability of Xrn1p to bind to RII(+) (+) in the repRNA region. This is not known to play a role in the assembly of the tombusvirus replicase (Panaviene, Panavas, and Nagy, 2005). It is more likely that binding of Xrn1p to RII(+) could lead to sequestration of the viral RNA, inhibiting its recruitment by the p33 replication protein, which binds to RII(+) (Pogany, White, and Nagy, 2005). Also, overexpression of Xrn1p prior to the viral repRNA in yeast was the most effective in inhibiting repRNA accumulation. This fits well with the model that high concentration of nucleolin could sequester the TBSV repRNA, especially at the early stage of infection when the viral RNA is present in limiting amounts.

In addition to the broad range of activities of nucleolin in the host cell, it is also involved in replication/pathogenesis of various RNA and DNA viruses. Similar to the findings in this article that Xrn1p/nucleolin can be inhibitory to tombusvirus replication, nucleolin has also been found to act as an inhibitor of DNA replication of simian virus 40 (SV40) virus. It has been shown that nucleolin inhibited the unwinding of SV40 origin (Daniely and Borowiec, 2000). However, in several other cases, nucleolin has been shown to stimulate viral
translation of the poliovirus RNA both in vivo and in vitro (Izumi et al., 2001). The NS5B RdRp protein of hepatitis C virus interacts with nucleolin, which could be relevant for virus replication (Kusakawa et al., 2007). The NS1 protein of influenza A virus, a negative-strand RNA virus, binds to nucleolin and colocalizes with nucleolin in the nucleolus, possibly affecting cellular events, such as shut down of host protein synthesis (Murayama et al., 2007). Herpes simplex virus 1 affects the subcellular localization of nucleolin in order to regulate rRNA levels and ultimately to alter cellular metabolism (Bertrand and Pearson, 2008). Nucleolin is also involved in the budding of retrovirus virions from the infected cells by interacting with the gag protein and the RNA packaging signal (Ueno et al., 2004). Overexpression of the C-terminal portion of nucleolin inhibited the assembly of retrovirus virions, suggesting that nucleolin–gag interaction is critical during the virion assembly process (Bacharach et al., 2000).

Based on data presented here, it seems that the yeast Nsr1p and the Arabidopsis nucleolin play comparable inhibitory roles in tombusvirus replication, thus adding another example that host factors affecting TBSV repRNA accumulation in yeast are also effective against the fully infectious tombusvirus genomic RNA in plants. Further experiments will be conducted to see if nucleolin/Nsr1p acts alone against tombusviruses or it is part of a larger innate immunity system of the host.

Materials and methods

Yeast and Escherichia coli plasmids

To study the effect of overexpression of Nsr1p protein on viral RNA replication, we transformed Saccharomyces cerevisiae parental strain (BY4741) or nsr1Δ strain from the YKO library (Open Biosystems) with three plasmids: pHisGBK-His33/DI-72 (co-expressing CNV p33 from the ADH1 promoter and DI-72 (+) RNA from the GAL1 promoter) (Jiang et al., 2006), pGAD-His92-CUP1 (containing the CNV p92pol gene behind the CUP1 promoter) (Li et al., 2008), and pYES-C-FLAG-NSR1 (expressing C terminal FLAG-tagged NSR1) or pYES-Nsr1 or empty plasmid pYES-NT-C (Invitrogen) as a control.

To study the effect of Nsr1p expression at different time points on tombusvirus RNA replication, we transformed the S. cerevisiae parental strain (BY4741) with three plasmids: pHisGBK-His33/DI72-CUP1 (co-expressing CNV p33 from the ADH1 promoter and DI-72 (+) RNA from the CUP1 promoter), pGAD-His92-CUP1 (Li et al., 2008) and pYES-Nsr1.

To obtain pYES-NSR1, the full-length NSR1 sequence was amplified by PCR with primers #1947 (CCGGGATCCATGGCTAAGACTAC-TAAAG) and #1948 (CCGGCTCGAGTCACTTATCGTCGT-GACTCTAAAG) from a yeast genomic DNA preparation. The PCR product was treated with BanHI and XhoI and ligated to pYES-NT-C, which was also treated with the same enzymes. The expression plasmid pYES-C-FLAG-NSR1 was prepared by PCR using primers #1951 (CggaAAGCTTACATGGCTAAGACTCTAAAG) and #2832 (CGACCTCGAGTCACTTATCGTCGTC-TTCTTTGAACC-C) from a yeast genomic DNA as template. The PCR product was inserted between HindIII and XhoI sites (engineered in the Nagy lab) in pYES-NT-C (Invitrogen).

Plasmid pGWB5 expressing the Arabidopsis nucleolin (AtNuc-L1p) from the 3SS promoter was the generous gift of Dr. K. Nakamura (Kojima et al., 2007). GFP expression plasmid pGD was used as a control (Goodin et al., 2002). The CNV expression plasmids pGD-CNV and pGD-p19 were described (Cheng et al., 2007; Jaag and Nagy, 2009). The TRV plasmids pTRV1 and pTRV2 were described (Liu, Schiff, and Dinesh-Kumar, 2002).

To generate the E. coli expression plasmids for Nsr1p and its deletion derivative NSR1ΔRBD lacking the central RNA-binding domain with the two RBD repeats, we introduced the C-terminal portion of Nsr1 and an extra XhoI restriction site into pGEX-2T

infections. For example, nucleolin has been shown to interact with the 5′ UTR of poliovirus (PV) and stimulate an early step of PV replication in vitro (Waggoner and Sarnow, 1998). Nucleolin was also shown to relocalize from the nucleolus to the cytoplasm in PV-infected cells, suggesting the existence of virus-induced mechanism to redistribute certain nuclear proteins in infected cells. Interestingly, the 5′ UTR of PV also binds to nucleolin, and this interaction affects the IRES-mediated
plasmids at the BamHI and EcoRI sites by using PCR and primers #1972 (CggcGGATCCTGATCCTCCTTCAAGACC) and #2040 (CggcGGATCTGGATTCACTGGAACTTCTCTTCCAAGACC). Then, to obtain pGEX-NSR1, the full-length sequence of NSR1 (primers #1947 and #1948) was inserted between the BamHI and Xhol sites of modified pGEX-2T plasmid. Plasmid pGEX-NSR1ARBD was obtained by ligating together the PsrI-treated DNA sequence representing the N-terminal part of NSR1 gene generated by PCR using primers #1947 and #1975 (CggcCTGCAGAGTAGCTGGTTCTTCGG) and the C-terminal part with primers #1978 (CggcCTGCAGACCTCCTCCTTCCAAGACC) and #1948. The ligated PCR products were then inserted between the BamHI and Xhol sites of modified pGEX-2T plasmid.

Yeast transformation and culturing

Yeast transformation was done by using the standard lithium acetate-single-stranded DNA-polyethylene glycol method, and transformants were selected by complementation of auxotrophic markers, ULH′ media lacking uracil, leucine, and histidine as described before (Panaviene et al., 2004). The transformed yeast cells were grown at 29 °C for 24 h in SC media (synthetic media, SC-ULH′) and 2% galactose as the carbon source and 50 μM copper sulfate to express p92 and DI-72 RNA.

Expression and purification of recombinant Nsr1p protein

We used pGEX-NSR1 and pGEX-NSR1ΔRBD plasmids to express the GST tagged protein in E. coli. Purification of recombinant NSR1 protein was performed as described with slight modification (Rajendran and Nagy, 2006). Briefly, E. coli Epicurion BL21-CodonPlus RIL (Stratagene) cells were pelleted from 25 ml culture media, resuspended in 1× PBS buffer (with 0.7% beta-mercaptoethanol) and sonicated and centrifuged to remove cell debris. The supernatant was loaded on GST resin column in PBS buffer, and then the GST fusion protein was eluted in 0.32% glutathione in PBS. Similarly expressed and purified GST protein from pGEX-2T plasmid was used as a control in the RNA-binding assay.

RNA analysis and Northern blotting

Total RNA isolation and Northern blot analysis were done as described (Panaviene et al., 2004). Briefly, pelleted yeast cells were resuspended in RNA extraction buffer [50 mM sodium acetate, pH 5.2, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)] and the same volume of phenol. Samples were vortexed for ~1 min at room temperature, followed by incubation for 4 min at 65 °C and on ice for ~1 min. Then, the total RNA was precipitated with ethanol. The obtained total RNA samples were separated by 1.5% agarose gel electrophoresis and were transferred to a Hybond-XL membrane (GE Healthcare). Northern blotting was done as described (Li et al., 2009). Briefly, the blotted total RNA samples fixed on the membrane were hybridized with a mixture of two 32P-labeled probes to detect DI-72 (+)RNA and the 18S rRNA. Hybridization signals were detected using a Typhoon 9400 imaging scanner (GE Healthcare) and quantified by ImageQuant software.

Protein extraction and Western blotting

Total protein extraction from yeast and Western blot were performed as described previously (Panaviene et al., 2004). Briefly, the yeast pellets were resuspended in 0.1 M NaOH, followed by vortexing for 30 s and shaking for another 10 min. Then, the samples were centrifuged at 15,000×g for 5 min at 4 °C and the pellet was resuspended in 1× SDS-PAGE buffer. The protein samples were electro-phoresed in 0.1% SDS–8% PAGE gel, and transferred to a PVDF membrane (Bio-Rad). Nonspecific binding was blocked with 5% nonfat dry milk solution. The primary antibody was anti-His antibody (GE Healthcare), and the secondary antibody was anti-mouse IgG alkaline phosphatase (Sigma).

Total protein from plant leaf samples was extracted from 30 mg plant leaf tissue. The plant tissue was grinded with a pestle in a microcentrifuge tube in 30 μl buffer A (50 mM Tris–HCl, 10 mM KCl, 15 mM MgCl2, 2 mM EDTA, 20% glycerol), followed by centrifugation at 400×g for 5 min at 4°C. The supernatant was mixed with 0.5 volume of 3× SDS loading buffer and heated at 85 °C for 15 min, followed by electrophoresis in 0.1% SDS–9% PAGE. Western blot analysis was done using anti-GFP as the primary antibody and anti-chicken as the secondary antibody.

Transformation of agrobacterium, agroinfiltration, and inoculation of plants

The procedure used was as described (Cheng et al., 2007). Briefly, expression plasmids pGWBS, pGD-GFP and pGD-p19 and pGD-CN1 or pGD-TRV1/pGD-TRV2 were transformed into agrobacterium C58C1. Transformants were selected in LB medium containing 50 μg/mL kanamycin, 100 μg/mL rifampicin, and 5 μg/mL tetracycline. The transformed agrobacteria were grown in LB media containing the antibiotics and 20 μM acetylsyringone at 29 °C until the OD600 reached 1.0. The bacterial cells were pelleted and resuspended in MMA media [10 mM MES (pH 5.6), 10 mM MgCl2, 200 μM acetyl-syringone] and incubated for 2–4 h on the bench. We used the obtained Agrobacteria culture of 0.8–1.0 OD600 for agroinfiltration. Agrobacteria carrying pGWBS (or pGD-GFP) as a control, pGD-P19, and pGD-CN1 were mixed in a ratio of 5:5:1 prior to infiltration to N. benthamiana leaves. For the TRV experiment, the agrobacteria cultures containing pGWBS (or pGD-GFP), pGD-p19 and pGD-TRV1/pGD-TRV2 were mixed in a ratio of 5:5:1:1 before infiltration.

For the analysis of agroinfiltrated leaf tissues, we randomly chose the same-sized leaf areas and excised 30 mg of leaf tissue to extract total RNA (Cheng et al., 2007; Jaag and Nagy, 2009). Then, the leaf samples were grinded in liquid nitrogen, followed by shaking for 5 min at room temperature in 200 μl of RNA extraction buffer [50 mM NaOAc (pH5.2), 10 mM EDTA, 1% SDS] and 200 μl water-saturated phenol and then additional incubation for 4 min at 65 °C. The RNA was precipitated by ethanol. The obtained total RNA samples were analyzed by Northern blotting as described (Jaag and Nagy, 2009).

To test the effect of nuclease on CNV infections started via rub inoculation, agrobacterium strains carrying pGWBS and pGD-p19 were mixed in a ratio of 1:1 prior to agroinfiltration into N. benthamiana leaves. Two days after agroinfiltration, the infiltrated leaves were inoculated with sap preparation containing CNV virions. The plant sap preparation was obtained from CNV/20KSTOP gRNA transcript-inoculated N. benthamiana plants in 0.02 M sodium-acetate pH 5.3 as described (Cheng, Serviene, and Nagy, 2006). The infiltrated leaves were also inoculated with the sap containing TBSV virions prepared from TBSV infected N. benthamiana leaves.

RNA probes and competitors used for RNA–protein interactions

To study the binding of Nsr1p to the full-length DI-72 (+)RNA and its four different regions, we PCR amplified DI-725XP (White and Morris, 1994) or its portions using primers described in Rajendran and Nagy (2003). The RNA transcripts were synthesized on the PCR templates using T7-based transcription in the presence or absence of 32P-UTP to generate labeled probes or cold transcripts, which were used as competitors during RNA–protein interactions. The amounts of transcripts were quantified by UV spectrophotometer (Beckman).

Nsr1p–viral RNA interactions in vitro

The UV-cross-linking assay was performed according to Hirose and Harada (2008). The reaction mixture was 12 μl containing 2 μg RNA samples loaded on GST resin column in PBS buffer, and then the GST fusion protein was performed as described with slight modification.
purified GST-Nsr1 protein, 10 ng (about 5nM) \^32P-UTP-labeled RNA probe, 10 mM HEPES, pH 7.9; 100 mM KCl; 1mM MgCl2; 10% glycerol; 0.5% NP40; 2 μg tRNA; and 0.2 μg heparin. In the competition assay, we used cold RNA transcripts as competitors in 5 or 50 nM amounts. The reaction mixtures were incubated at room temperature for ~20 min to allow the formation of RNA–protein complexes. To cross-link RNA and protein, we transferred the reaction mixture to a 96-well plate on ice, then irradiation was done at 254 nm for 10 min using an UV Stratalinker 1800 (Stratagene). Then, the unprotected RNAs were harvested by centrifugation at 21,000×g at 37 °C. Samples were mixed with 0.5 volume 3× SDS loading dye and boiled for 10 min. Analysis was performed using SDS-PAGE and phosphoimaging. For the template competition assay, we used the cold RNA transcripts as competitors in 0, 0.05, and 0.5 μM concentration.

\textbf{In vitro replica assays}

One of the replica assays was based on the membrane-enriched fraction of yeast as described earlier (Panaviene et al., 2004). Yeast co-transformed with pCAD-His92-CUP1/PHisGBK-His33/DI-72 and one of the following: pYES-NSR1, pYES-C-FLAG-NSR1, or empty plasmid pYES-NT-C (used as control) was pre-grown in Sc-ULH media containing 2% glucose at 29 °C for 24 h, then switched to 2% galactose for 4–5 h before adding 50 μM copper sulfate to the media. After culturing for 22 h in the presence of copper sulfate, the yeast cells were harvested by centrifugation (Panaviene et al., 2004). The membrane-enriched fraction for each strain was prepared by disrupting the cells in an ice cold extraction buffer (200 mM sorbitol, 50 mM Tris–HCl (pH 7.5), 15 mM MgCl2, 10 mM KCl, 10 mM β-mercaptoethanol, 1% yeast protease inhibitor mix; Sigma), followed by centrifugation 100 × g for 5 min at 4 °C to remove cell debris. Then, the enriched membrane fraction was obtained by centrifugation at 21,000 × g for 10 min. Before the replica reaction, we performed Western blotting for estimating p33 levels in order to normalize the amount of p33 in each sample. The in vitro replica reactions were set up according to Panaviene et al. (2004). The RdRp products were analyzed by electrophoresis on 5% PAGE containing 8 M urea and phosphoimaging.

To test the effect of Nsr1p on the activity of the in vitro assembled tombusvirus replica, a yeast cell-free extract was prepared as described previously (Pogany and Nagy, 2008). Note that this essay is distinguishable from the previous one. Note that this essay is distinguishable from the previous one. To test the effect of Nsr1p on the activity of the in vitro assembled tombusvirus replica, a yeast cell-free extract was prepared as described previously (Pogany and Nagy, 2008). Note that this essay is distinguishable from the previous one. To test the effect of Nsr1p on the activity of the in vitro assembled tombusvirus replica, a yeast cell-free extract was prepared as described previously (Pogany and Nagy, 2008). Note that this essay is distinguishable from the previous one. To test the effect of Nsr1p on the activity of the in vitro assembled tombusvirus replica, a yeast cell-free extract was prepared as described previously (Pogany and Nagy, 2008). Note that this essay is distinguishable from the previous one.