IDENTIFICATION AND CHARACTERIZATION OF HOST FACTORS INVOLVED IN TOMBUSVIRUS REPLICATION

Yi Jiang  
University of Kentucky, yjian2@uky.edu

Recommended Citation  
https://uknowledge.uky.edu/gradschool_diss/745
ABSTRACT OF DISSERTATION

Yi Jiang

The Graduate School
University of Kentucky
2009
IDENTIFICATION AND CHARACTERIZATION OF HOST FACTORS INVOLVED IN TOMBUSVIRUS REPLICATION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Yi Jiang
Lexington, Kentucky

Director: Dr. Peter Nagy, Professor
Department of Plant Pathology
Lexington, Kentucky
2009

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IDENTIFICATION AND CHARACTERIZATION OF HOST FACTORS INVOLVED IN TOMBUSVIRUS REPLICATION

Positive strand RNA viruses are intracellular parasites, and their genome replication and infection involves complex virus-host interactions. Therefore, identification of host factors and dissection of their functions during virus replication could facilitate our understanding of the mechanism of virus infection. Those host factors may also provide new targets for viral disease control. *Tomato bushy stunt virus* (TBSV) has recently become one of the model viruses to study positive strand RNA virus replication and host-virus interactions. To identify host factors involved in TBSV replication we used yeast as a model host. Co-expression of the replication proteins and a replicon RNA (DI RNA) via plasmids in yeast resulted in robust replication of the viral RNA. Previous work using a yeast single gene deletion library (YKO) revealed 96 yeast genes affecting virus replication. The essential yeast genes could not be deleted so we used the Yeast Tet Promoters Hughes Collection (yTHc) where the original promoter was replaced by Tetracyclin-titratable promoter. I tested the 800 essential host genes available in yTHc. In total, we found 30 new host genes whose down-regulated expression either increased or decreased the accumulation of a TBSV repRNA. The identified essential yeast genes fall into different categories on the basis of the cellular processes they are involved in, such as RNA transcription/metabolism, protein metabolism/transport etc. Detailed analysis of the effects of some of the identified yeast genes revealed that they might affect RNA replication by altering (i) the amounts of p33 and p92(pol) viral replication proteins, (ii) the activity of the tombusvirus replicase complex, and (iii) the ratio of plus- versus minus-stranded RNA replication products. Altogether, this and previous YKO screening of yeast led to the identification of 126 host genes (out of ~5,600 genes that represent ~95% of all the known and predicted yeast genes) that affected the accumulation of tombusvirus RNA.

In the YKO screening, we found *NSR1* (homologous to plant nucleolin) gene, whose deletion led to increased TBSV repRNA accumulation. Nucleolin is an abundant RNA binding protein, which shuttles between the nucleolus, the nucleoplasm and the cytoplasm. This protein is involved in rRNA maturation, ribosome assembly and regulation of cellular RNA metabolism. We found that over-expression of Nsr1p in yeast or nucleolin in *Nicotiana benthamiana* inhibited the accumulation of tombusvirus RNA.
by ~10-fold. Temporal regulation of Nsr1p over-expression revealed that the inhibitory effect of Nsr1p was more profound when it was expressed at early stages of viral replication. In vitro binding experiments showed that Nsr1p binds preferably to the RIII in the repRNA (which is derived from 3’ UTR of viral genome). Consistent with its RIII specific binding, over-expression of Nsr1p only reduced 40% of the accumulation of TBSVΔRIII repRNA in yeast. The purified recombinant Nsr1p inhibited the in vitro replication of the viral RNA in a yeast cell-free assay when pre-incubated with the viral RNA before the in vitro replication assay. Our data suggest that Nsr1p/nucleolin inhibits tombusvirus replication by interfering with the recruitment of the viral RNA for replication.

Key words: TBSV replication, host factors, Nsr1p/nucleolin, RNA binding, RNA recruitment

Yi Jiang
June, 2009
IDENTIFICATION AND CHARACTERIZATION OF HOST FACTORS INVOLVED IN TOMBUSVIRUS REPLICATION

By
Yi Jiang

Dr. Peter Nagy
Director of Dissertation

Dr. Lisa Vaillancourt
Director of Graduate Studies
June, 2009
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Dissertation

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ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me support and made this dissertation possible. First, I am deeply indebted to my supervisor Dr. Peter Nagy for his guidance, understanding and continuous support throughout my graduate study and thesis writing. His motivation and patience helped me overcome many crisis situations and complete this dissertation work. I would also like to thank my committee members, Dr. Chris Schardl, Dr. Goodin and Dr. Rebecca Dutch for their valuable suggestions, constructive criticisms and encouragements at different stages of my graduate study. I am also thankful to them for carefully reading, commenting and correcting grammar on my writings. I am especially thankful to Dr Goodin for kindly providing fibrillarin-RFP transgenic plants and for his great help in using confocal microscopy.

I would also like to specially thank Dr. Judit Pogany for giving me wise advices, helping me in various experiments during the past few years. I am obliged to Dr. Elena Serviene for providing me important guidance and getting my graduate career started productively. I would also like to gratefully and sincerely thank my friends and co-workers: Dr. Zhenghe Li, Kai Xu and Kunj Pathak for their stimulating discussion, encouragement and unselfish assistance in my research and for helping me get through the difficult times, and for all the emotional support, entertainment, and caring they provided. I would also acknowledge all my lab members for providing a stimulating and fun laboratory environment for working, especially Dr. Hannah Jaag, Dr. Daniel Barajas and Dr. Venugopal Mendu.

I would like to extend my heartfelt gratitude to Dr. David Smith and our DGS, Dr. Lisa vaillancourt, for their encouragement and support during my graduate study at the University of Kentucky. I also thank faculties, staffs and students in the Department of Plant Pathology for assisting me in many different ways. I am grateful to the Kentucky Opportunity Fellowship for their support for one year and also the graduate school for their financial support during my graduate career.

Especially, I would like to give my very special thanks to my husband Jiaqing Nong. His unwavering confidence in me and constant support allow me travel away from home to study in University of Kentucky. His support, encouragement, patience and love enabled
me to complete this work. My thanks also go to my mother Zhaohui Feng, father Guangqian Jiang and sisters Ying and Li for their love and support. Finally, I would like to take the opportunity to thank all my teachers, friends and anyone who helped me in my academic study and research.
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CHAPTER I

Introduction to Tombusvirus replication and host factors involved

Introduction

Plant viruses can cause huge economic losses in crop production. Viruses induce a series of physiological changes after invading the susceptible host plants which can lead to disease symptoms including systemic and local symptoms (Hull, 2002; Culver and Padmanabhan, 2007). The largest group of plant viruses is positive-strand RNA viruses, whose genomic RNA(s) is used as a messenger RNA to produce viral proteins (Hull, 2002; Noueiry and Ahlquist, 2003; Sanfacon, 2005).

Tombusviruses are one of the best studied plus-strand RNA virus groups and Tomato bushy stunt virus (TBSV) has recently become one of the model viruses to study plus-strand RNA virus replication and host-virus interactions. Indeed, more recent works on TBSV, Cucumber necrosis virus (CNV), Cymbidium ringspot virus (CymRSV) and Carnation Italian ringspot virus (CIRV) have led to a better understanding of the viral replication process and virus-host interactions (White and Nagy, 2004).

Overview of host-virus interactions, and mechanism of (+)RNA virus replication

Viruses, including (+)RNA viruses, are intracellular parasites and they complete their infectious cycle all within live cells from capsid disassembly, translation, genome replication to encapsidation. After entering the cell, viruses manipulate the host molecular machinery and convert the cell into “virus factories”. Given the limited coding ability of the (+)RNA viruses, their replication is dependent upon numerous host-coded proteins, host membranes and lipids. Several innate defense pathways of the host and the counteracting virus encoded suppressor(s) for gene silencing also play important roles in virus infection (Ahlquist, Noueiry et al., 2003; Sanfacon, 2005; Nagy, 2008). The central step in (+)RNA virus multiplication is the replication of the genomic RNA. All known (+)RNA viruses share several basic features in replication: the (+)RNA genome is replicated through a (-)RNA intermediate; (+)RNAs and (-)RNAs are synthesized asymmetrically, resulting in 20 to 100-fold more abundant (+) than (-) RNA; RNA
replication takes place on subcellular membranes (Ahlquist, Noueiry et al., 2003; Sanfacon, 2005; Denison, 2008; Nagy, 2008).

The process of (+)RNA replication can be divided into several steps, such as selection of the template RNA (switch from translation to replication), targeting of replication components to the subcellular membranes, replication complex (RC) assembly, viral RNA synthesis, (+) RNA release from RC and disassembly of the RC (Nagy and Pogany, 2006; Nagy, 2008). Roles of several host factors have been implicated in the above described various replication steps.

**TBSV replication--the roles of the viral components**

*Viral genome structure*

Tombusviruses belong to the family Tombusviridae, and these viruses contain a single-strand (+)RNA genome, which are about 4,800 nt long (Hull, 2002; White and Nagy, 2004). The genomic RNA is uncapped at the 5’ end, and no polyA tail is present at the 3’ end (Fig 1-1) (Hull, 2002; White and Nagy, 2004). The genomic RNA contains five open reading frames (ORFs), which encode 5 proteins, namely p33, p92, p41, p22 and p19. In addition to the coding region, there are also untranslated regions (UTRs) in the 5’ and 3’ ends that carry important cis-acting RNA elements for viral replication and translation (Hull, 2002; White and Nagy, 2004). The functions and importance of these 5 proteins have been well characterized (Hull, 2002; White and Nagy, 2004; Nagy and Pogany, 2006). The viral proteins are translated from the viral genomic and two subgenomic RNAs in a 5’ cap and 3’ poly(A) independent way. The 41kDa protein is the coat protein used to encapsidate the viral genome and assembles into icosahedral particles; p22 is the protein involved in cell to cell movement; whereas p19 is the gene-silencing suppressor. P33 and p92, translated directly from the genomic RNA via a readthrough mechanism, are the two viral proteins indispensable for virus RNA replication. P92 is the readthrough product of p33. During translation of the genomic RNA, the ribosomes occasionally insert a tyrosine residue at amber stop codon (UAG) of p33 ORF, followed by further translation of p92 ORF instead of termination of translation. The expression of the other 3 proteins occur from two subgenomic (sg) RNAs made during genome replication: the coat protein (p41) is translated from sgRNA1, whereas the movement protein (p22) and
the silencing suppressor (p19) are translated from sgRNA2. The latter proteins are expressed by ribosome leaky scanning.

**Defective Interfering RNAs**
Defective interfering (DI) RNAs are associated with tombusvirus infections (White and Morris, 1994; White and Morris, 1999). The tombusvirus DI RNA molecules are composed of noncontiguous RNA segments derived from the genomic RNA, and a prototypical TBSV DI RNA contains four noncontiguous segments (regions I to IV) (Wu and White, 1998; White and Nagy, 2004). RI is derived from the 5' untranslated region (UTR), RII is derived from an internal section within the p92 ORF, and RIII and RIV from the 3'-terminal portion of the viral genome. The DI RNAs do not code for proteins and are unable to replicate by themselves. The DI RNAs contain *cis*-acting RNA replication elements, which help them replicate efficiently when the viral replication proteins are provided in *trans* by the helper viruses (such as TBSV). In recent works, the DI RNAs, especially DI-72, have been frequently used as model templates to study tombusvirus replication focusing on *cis*-acting RNA elements, host-virus interaction, etc. (Panavas, Pogany et al., 2002; Panavas and Nagy, 2003; White and Nagy, 2004).

**Viral-coded replication proteins**
During genome replication, the viral encoded polymerase catalyzes RNA synthesis using the viral RNA as a template, which is different from the typical host cell mRNA synthesis that uses DNA as a template. Tombusvirus replication protein p92 contains the conserved RNA-dependent RNA polymerase (RdRp) motifs and can catalyze viral RNA synthesis as part of the replicase complex (O'Reilly and Kao, 1998; Panaviene, Panavas et al., 2004). Another essential replication protein, p33, does not have polymerase activity, but has many other functions and is involved in various steps of viral replication, such as template recruitment, membrane targeting and protein-protein interactions (Rajendran and Nagy, 2003; McCartney, Greenwood et al., 2005; Panavas, Hawkins et al., 2005; Nagy and Pogany, 2006).

**p33 replication protein**
p33 is a 33 kDa protein and is associated with peroxisomal membrane in the infected cells (McCartney, Greenwood et al., 2005; Panavas, Hawkins et al., 2005). There are two
hydrophobic putative trans-membrane domains (TMD) at the N-proximal part of TBSV p33 (amino acid stretches of 83-98 and 132-154), which enable the protein to be integrated into the membrane. In addition, the N-terminal half of p33 contains both peroxisomal and pER (peroxisomal endoplasmic reticulum) targeting signals (–K5R6–) (Fig. 1-2) (McCartney, Greenwood et al., 2005). The peroxisomal targeting signal consists of three distinct regions within the N-terminal half of the protein (i.e., -K11K12-, -K76R77R78R80-, and -R124K129K130-) which is necessary for p33 targeting from the site of synthesis in the cytosol to the peroxisomal membrane (Navarro, Rubino et al., 2004; McCartney, Greenwood et al., 2005).

A highly conserved arginine-proline rich motif (RPR), located in the C terminal part of TBSV p33 (located between positions 211-219), has been shown to be the core region for high affinity binding to tombusvirus RNA (Rajendran and Nagy, 2003). In addition to this core region, the p33:p33/p92 interaction domain, located downstream of the RPR motif, is also required for specific interaction with viral RNA RII internal replication element (IRE). The interaction of p33 with IRE is suggested to play a role in specific selection of viral RNA and its recruitment for replication (Pogany, White et al., 2005). This p33:p33/p92 interaction domain contains two sites (S1, S2), each consisting of 10 to 13 aa stretches at the C proximal part of p33 (Rajendran and Nagy, 2004). The p33:p33/p92 protein-protein interaction domain in p33 is also essential for virus replication and p92 peroxisomal localization (Panavas, Hawkins et al., 2005; Rajendran and Nagy, 2006).

In the infected or transfected cells, the p33 protein can be modified by ubiquitination (Barajas et al., submitted) or phosphorylation (Shapka, Stork et al., 2005). These modifications may have important roles in p33 function. Phosphorylation of threonine and serine residues close to the RPR domain in p33 reduces its viral RNA binding capacity and decreases replication (Shapka, Stork et al., 2005). The phosphorylation of p33 was proposed to help the viral RNA switch from replication to other processes during the infection cycle by promoting the release of the viral RNA from the replicase complex (Stork, Panaviene et al., 2005). Ubiquitination of the lysines in N terminal 1-76aa is needed for efficient replication and the host E2 ubiquitin-conjugating enzyme Cdc34p can ubiquitinate the p33 based on in vitro data (Li, Barajas et al., 2008). Thus, p33 is a
multifunctional viral replication protein that interacts with the viral RNA, the p92 replication protein, itself (homodimerization) and a group of host proteins. P33 plays critical roles in recruiting tombusvirus RNA for replication, assembly of the replication complex, intracellular localization of viral RNA and p92 and might also be implicated in regulation of different stages of viral life cycle for successful infection.

**p92 replication protein**

p92 contains the typical RdRp motifs in its C-terminal part and can initiate RNA synthesis de novo when part of the replicase complex (O'Reilly and Kao, 1998; Nagy and Pogany, 2000). The N terminal part of p92 shares the same sequence as p33; consequently, it also has all the domains in p33 in its N terminal part including TMD domain, RPR motif, S1, S2 protein interaction domain (Fig 1-2) (Rajendran and Nagy, 2003). In spite of the sequence identity, it is possible that the N-terminal segment of p92 plays different roles from p33. Indeed, the TMD domain in p92 is not essential for replication (Panavas, Hawkins et al., 2005), however the RPR domain in both p33 or p92 are essential for replication (Panaviene, Baker et al., 2003). Besides the RPR motif, additional RNA binding regions (RBR) in p92 also contributes to viral RNA binding: RBR2 region is located in close proximity of the RdRp signature motifs and RBR3 is present in the C-terminal segment of p92 (Rajendran and Nagy, 2003). In the infected plant and protoplast cells, the p33 and p92 are produced coordinately with p33 reaching 20-fold excess over p92 (Scholthof, Scholthof et al., 1995).

**TBSV cis-acting RNA replication elements**

For a positive strand RNA virus, the RNA genome serves multiple functions, such as a messenger RNA to produce viral proteins, template for complementary RNA synthesis and it is also involved in replicase assembly. In addition to the five ORFs, the genomic RNA also contains cis-acting elements for replication, including promoters, enhancers and a silencer (Panavas, Pogany et al., 2002; Fabian, Na et al., 2003; Panavas and Nagy, 2003; Pogany, Fabian et al., 2003).

The replication of TBSV RNA starts after the translation of replication proteins p33 and p92. The cellular messenger RNA has 5’ cap and 3’ polyA tail for its translation, whereas the TBSV genomic RNA does not have these structures. Instead, it contains a 3’ terminal
cap-independent translational enhancer (3’ CITE) and this 3’CITE interacts with 5’ sequences, which facilitates efficient translation (Fabian and White, 2004). The central domain of the 3’CITE is called region 3.5 (R3.5), which is located between RIII and RIV. The R3.5 is predicted to have a Y shaped secondary structure with a center junction and three helix stems: S-A, SL-B, SL-C. SL-B and SL-C are essential for the translation. The terminal loop of SL-B interacts with T-shaped domain (TSD) in the 5’ UTR by base pairing with the SL3 in the TSD. This long range 5’- 3’ interaction is critical for efficient viral protein translation (Fabian and White, 2004).

After the replication proteins have been translated, the viral RNA needs to be recognized and recruited into replication. The p33 recognition element (p33RE) was found within the p92 RdRp coding region of the TBSV genomic RNA (located in the RII of DI RNA), and this conserved internal replication element (IRE) can specifically interact with the RPR domain in p33. This IRE was showed to have an extended stem loop structure and a C•C mismatch inside the stem-loop, which is critical for specific p33 recognition and replication (Pogany, White et al., 2005). Interaction between the IRE and p33 RPR enables the tombusvirus RNA to be recruited to the site of replication (peroxisomal membrane) for RNA synthesis.

During viral RNA synthesis, the template RNA needs to provide promoter elements to direct the viral polymerase for initiation. The genomic RNA contains a genomic promoter (gPR) for (-) strand synthesis at the very 3’ end and the minus strand RNA contains a complementary promoter (cPR) for (+) strand synthesis (White and Nagy, 2004). The gPR is 19 nt long and located in the extreme 3’ end of genomic RNA. gPR forms a stem-loop structure with a single stranded CCC\textsubscript{OH} tail at the 3’ end (Fabian, Na et al., 2003). The cPR is 11nt at the 3’ terminus of minus strand RNA and it is used to initiate the plus strand synthesis. Based on in vitro data, the gPR is almost as active as cPR (Panavas, Pogany et al., 2002).

In the host cell, the positive strand RNA virus replicates asymmetrically (Panavas, Pogany et al., 2002). The asymmetric production of (+) and (-) RNA needs to be regulated by additional RNA elements. Silencer and enhancer RNA elements were found in TBSV RNA and were involved in the regulation of the asymmetrical synthesis of (+)
or (-) strand RNA (Fig 1-3). A replication silencer element (RSE) was found in the 3’ proximal region of the genomic RNA which modulates the synthesis of minus strand, eventually leading to seven times less minus strand synthesis in vitro (Pogany, Fabian et al., 2003). The silencer element contains 5 nucleotides which can base-pair with the 3’ terminal end of gPR promoter and specifically inhibits the minus-strand RNA synthesis in vitro. Importantly, the silencer and gPR elements are also required for the assembly of the functional viral replicase complex (Panaviene, Panavas et al., 2004). The 3’-terminal 130 nt sequence of tombusvirus is highly conserved and forms into 3 stem loop structures: named as SL1, SL2 and SL3 from 3’ to 5’ (Fabian, Na et al., 2003). SL1 together with the 3’ end single strand CCC OH is the genomic promoter. SL3 is a stem-loop structure including the silencer element within an extended internal loop. The formation and structure of these stem loop structures and direct interaction between silencer element and 3’ end of gPR is essential for viral RNA replication (Fabian, Na et al., 2003; Pogany, Fabian et al., 2003). The genomic RNA has even more complex RNA-RNA interactions, including a long-range interaction bridging ~3000 nt, which brings the IRE and the silencer/gPR elements into close proximity (Wu, Pogany et al., 2009).

Replication enhancers have also been found in the minus strand RNA, which can stimulate the plus strand RNA synthesis in vitro and in vivo. One enhancer element is located within the complementary region III(-) and can upregulate the plus strand RNA synthesis by 10 to 20-fold based on in vitro and in vivo analysis (Panavas and Nagy, 2003; Ray and White, 2003). Without the region III, the DI RNA still can replicate but at a low level. This enhancer element consists of two stem-loop structures, namely SL1-III(-) and SL2III(-). The two hairpins play a redundant and interchangeable role in RNA synthesis. Either of the two stem-loops is capable of stimulating the plus strand RNA synthesis at similar levels. But duplication of the enhancer does not lead to higher accumulation of virus RNA and the reason is unclear (Panavas and Nagy, 2003).

In addition to the internal RIII enhancer, another enhancer element was found adjacent to the 3’ terminal of cPR in the minus strand RNA. This 3’ promoter proximal enhancer (PPE) can stimulate the plus strand synthesis by three-fold (Panavas, Panaviene et al., 2003). Deletion or modification of this PPE element led to reduced RNA accumulation in vivo. The sequence of PPE share high similarity with cPR (70% identity) and it was
suggested that the PPE element might have evolved from duplication of promoter sequence. In vitro work also showed that promoter duplication led to increased RNA synthesis.

In addition to the promoter sequence for plus strand synthesis (cPR in the minus strand RNA), the 5’ UTR of the genomic RNA contains two other important domains: the T-shaped domain (TSD) and the downstream domain (DSD). The TSD covering the 5’ terminal 78 nt has branched secondary structure, with 3-helix junction cores. Three stem-loop (S1, S2, S3) structures are important for efficient RNA replication. Deletion of the S1 sequence led to reduced DI RNA level up to 20-fold. The nucleotide residues in two base pairs of S4 are conserved and mutating them, even when mutations restored base-pairing, decreased DI RNA replication (Wu, Vanti et al., 2001; Ray, Na et al., 2004). Function of this TSD was suggested to protect the uncapped 5’ end of viral RNA (White and Nagy, 2004). The DSD contains complex helix and single strand regions and resides in the 3’ half of the 5’UTR following a stem-loop (SL5) structure. The TSD interacts with DSD through a pseudo-knot structure. Maintenance of the SL5 and DSD structure and TSD-DSD interaction are essential for efficient RNA accumulation (Ray, Wu et al., 2003).

**RNA as a platform for RdRp complex assembly**

Viral plus strand RNA plays a critical role in the assembly of active replicase. Indeed, co-expression of the viral RNA together with p33 and p92 is needed for obtaining active purified replicase (Panaviene, Panavas et al., 2004). The viral (+)RNA might serve as a platform for the assembly of the replicase complex (Nagy, 2008). Three elements, p33RE, RSE and gPR, in the TBSV RNA are critical for replicase assembly according to the in vitro replicase assay and the minimal template RNA containing these 3 elements can produce functional replicase (Panaviene, Panavas et al., 2005). It was suggested that the p33RE interaction with the p33/p92 complex, which enables the selection and recruitment of the RNA template into replication, is a critical step in the early stage of replicase assembly. The sequence of 3’ terminal gPR, internal loop sequences of the RSE and the interaction between SL3 and gPR are also required for functional replicase assembly (Panaviene, Panavas et al., 2005). These replicase assembly essential elements
are located distantly, p33RE in RII, RSE and gPR in the RIV. It has been shown that long-range RNA-RNA interactions bring these elements into proximity and facilitate the assembly of the viral replicase on the genomic RNA (Fig. 1-4) (Wu, Pogany et al., 2009).

The long-range RNA-RNA interaction consists of an 11 nt sequence long upstream linker (UL) in RII, which base-pairs with the downstream linker (DL) in the RIII enabling the communication between RII and RIV (Wu, Pogany et al., 2009). The UL is positioned 3’ proximal to the p33RE within the p92 ORF. This long distance RNA-RNA interaction might help to bring p33RE in RII in close proximity to RIV elements and facilitate the viral replicase assembly. Furthermore, the UL-DL interaction also brings replication elements in RII and RIV next to the Y shaped translational element R3.5, which contributes to forming a regulatory core for coordinating of translation and replication process (Fig. 1-4).

**The roles of host membranes in TBSV replication**

Host proteins, membranes and other cellular components are recruited to support various steps during the virus infection process, such as viral protein translation, cell-to-cell movement, sub-cellular localization and viral RNA synthesis. Replication complexes of positive strand RNA viruses also contain host components as well as viral -coded proteins and the viral RNA (Ahlquist, Noueiry et al., 2003; Sanfacon, 2005; Nagy and Pogany, 2006).

**Subcellular sites of (+)RNA virus replication**

Replication of (+)RNA viruses is always associated with various intracellular membranes. Their replication takes place on membranes including ER, mitochondria, chloroplast, peroxisome and tonoplast. (Salonen, Ahola et al., 2004; Mackenzie, 2005; Denison, 2008). Members of the tombusviruses replicate on membranes of different organelles such as peroxisomes or mitochondria (McCartney, Greenwood et al., 2005; Hwang, McCartney et al., 2008).

Many plant viruses replicate on ER membranes including members of bromoviruses, potyviruses, comoviruses etc (Sanfacon, 2005). Replication of BMV RNA takes place on the ER membrane (RestrepoHartwig and Ahlquist, 1996; Restrepo-Hartwig and Ahlquist,
BMV encodes two replication proteins, 2a is the RNA polymerase, 1a is a multifunctional protein with an N terminal RNA capping domain and a C terminal helicase domain which interacts with 2a and itself. The 1a protein, when expressed alone, is localized to the outer membranes of perinuclear ER (Chen and Ahlquist, 2000; den Boon, Chen et al., 2001; Schwartz, Chen et al., 2002). Other viral components of replication complexes, 2a and the RNA templates are recruited by 1a and targeted to the ER membrane (Chen and Ahlquist, 2000; Schwartz, Chen et al., 2002).

*Tobacco mosaic virus* (TMV) replication was also reported to take place on ER membranes or targeted to actin/ER network (Mas and Beachy, 1999; Christensen, Tilsner et al., 2009). But work on another tobamovirus, *Tomato mosaic virus* (ToMV) revealed that the ToMV replication complex mainly located on tonoplast membranes in plant cells (Hagiwara, Komoda et al., 2003). The tobamovirus genome encodes four proteins, two of which are required for viral replication: the 130 kDa protein and the 180 kDa protein (read-through product of 130K protein). The 180K protein is the RNA polymerase and the 130K protein contains a N-terminal methyltransferase domain and C-terminal helicase-like domain (Ishikawa and Okada, 2004). In addition to association with subcellular membranes, a significant amount of the ToMV replication proteins were found not associated with membranes. However only the membrane associated replication proteins were active in viral RNA synthesis (Hagiwara, Komoda et al., 2003; Nishikiori, Dohi et al., 2006).

In case of tombusviruses, TBSV replicates on peroxisomal membranes and (-)RNA intermediates, dsRNAs (possible replication intermediate) and replication proteins p33, p92 are localized on peroxisome membranes (McCartney, Greenwood et al., 2005; Panavas, Hawkins et al., 2005). When the peroxisome membranes are not available, TBSV can replicate on ER membranes and TBSV replicon (rep)RNA replicates with comparable kinetics to that in the wt strain. (Jonczyk, Pathak et al., 2007). Confocal laser microscopy revealed that the replication proteins were colocalized with ER marker protein in those *pex3* or *pex19* cells.

As for other members of tombusviruses: CymRV also replicates on the peroxisome membranes (Navarro, Rubino et al., 2004), while CIRV replicates on mitochondrial
membranes (Weber-Lotfi, Dietrich et al., 2002). The related Red clover necrotic mosaic virus (RCNMV) replicates on the ER membranes facilitated by the target signal in the N-terminal half of p27 and p88 replication proteins (Turner, Sit et al., 2004),

**Targeting of viral replication complex to subcellular membranes**

For (+)RNA virus replication, viral-encoded proteins have been shown to be involved in membrane targeting of the replication components. These replication proteins often contain membrane anchoring sequences. The viral protein would likely be delivered by host proteins and actin filaments or microtubules and recruit other components to the site of replication. For example, BMV 1a protein, Turnip yellow mosaic virus (TYMV) 140K protein and TBSV p33 are good examples of membrane targeting (Chen and Ahlquist, 2000; Jakubiec, Notaise et al., 2004; Panavas, Hawkins et al., 2005). Unidentified host proteins are likely involved in the membrane targeting.

For BMV replication, the ER membrane targeting and rearrangement of membranes depends on the protein 1a. 1a interacts and recruits the viral RNA template through its NTPase/helicase domain and 1a also recruits the 2a protein to the site of replication (Chen and Ahlquist, 2000; Wang, Lee et al., 2005). Expression of the 1a protein led to increased total membrane lipids and induced membrane invaginations in which RNA replication takes place (Schwartz, Chen et al., 2002). Expression of 2a alone did not induce membrane rearrangements, but when co-expressed with 1a, the increasing amount of 2a favored the formation of large multilayer of apprised double membrane and this structure supports BMV replication efficiently (Chen and Ahlquist, 2000; Schwartz, Chen et al., 2002; Schwartz, Chen et al., 2004). Protein 1a does not have a trans-membrane domain and does not protrude the ER membrane. Still, it is associated with the membrane with high affinity resistant to high salt and high pH treatments but susceptible to protease digestion (den Boon, Chen et al., 2001). The efficient ER association and normal perinuclear ER localization depends on an amphipathic α-helix, helix A, in the 1a protein (Liu, Westler et al., 2009). A predicted α-helix region which includes a 35 amino acid stretch in the 1a protein was found to be important for ER targeting. The helix region has a core 18 aa amphipathic helix A (aa 392-409), and this helix A could bind to hydrophobic membrane-mimicking micelles based on NMR analysis. The helix A is also
critically involved in 1a induced ER membrane rearrangement and functional replication complex assembly and is essential for virus replication in yeast and host plant.

Tombusvirus replication proteins, p33 and the N-terminal part of p92, contain peroxisome targeting sequences and facilitate the targeting of the replication proteins to the peroxisomal membranes in cells (Rubino and Russo, 1998; McCartney, Greenwood et al., 2005; Panavas, Hawkins et al., 2005). p33 expressed alone or co-expressed with p92 is localized on the peroxisomal membranes leading to peroxisomal membrane alterations and membrane proliferation. Three regions in the N-terminal part of p33 are essential for peroxisomal targeting, K5R6K11K12, KRRQR (76-80)- and-RPSVPKK (124-130). p33 also contains an ER targeting signal (-K5R6- aa) (McCartney, Greenwood et al., 2005). The peroxisomal localization of p33 also requires the host protein Pex19p for its intracellular transportation (Pathak, Sasvari et al., 2008).

In case of ToMV, the membrane association of the replication complex depends on interactions between the host membrane proteins TOM1/TOM3 and TOM2A and the 130K or 180K proteins of ToMV, since the replication proteins do not contain membrane-spanning regions (Yamanaka, Ohta et al., 2000; Yamanaka, Imai et al., 2002; Hagiwara, Komoda et al., 2003; Ishikawa and Okada, 2004).

**Membrane rearrangement during virus replication**

The (+)RNA viruses induce membrane alterations and membrane invaginations that lead to the formation of a membrane bound small compartment which contains the viral replication complex including the viral replication proteins and possibly host proteins and the viral RNA (Salonen, Ahola et al., 2004; Mackenzie, 2005; Denison, 2008).

Replication of BMV induces ER membrane invagination leading to spherule-like structure formations (50 to 60nm in diameter). These spherules have a narrow opening connected to the cytoplasm, which might allow the nucleotides, the viral RNA, or other materials to exchange between the spherule and cytoplasm (Restrepo-Hartwig and Ahlquist, 1999; Schwartz, Chen et al., 2002; Lee and Ahlquist, 2003). In addition to the formation of these spherules, BMV also induces another form of membrane rearrangement with stacked double ER membrane layers when excess amount of 2a protein is expressed (Schwartz, Chen et al., 2004).
Flock house virus (FHV) induces the invagination of the outer membranes of mitochondria to form ~50nm spherules located between inner and outer mitochondria membranes (Kopek, Perkins et al., 2007). The spherules are connected to the cytosol by a ~10nm neck channel. Viral RNA synthesis takes place within the spherules. The nucleotides and RNA products are supposedly exchanged through the spherule neck.

In TBSV infected plant cells, the peroxisome undergoes progressive change during the replication of the virus (McCartney, Greenwood et al., 2005). The peroxisomes get larger and fewer, becoming globular in shape when several peroxisomes coalesce. Later the pMVBs (peroxisome multivesicular bodies) appear and the pMVBs contain numerous vesicles 40 to 170nm in diameter. The vesicles are produced via peroxisomal membrane invagination and vesiculation.

**Host membrane functions**

The virus induced membrane invagination or spherule formation has been proposed to have important functions for efficient viral RNA replication (Schwartz, Chen et al., 2002; Salonen, Ahola et al., 2004). The membrane rearrangement would provide a small compartment, which confines the RNA synthesis to a specific location and protects the viral RNA especially the putative dsRNA intermediates from host defense recognition and responses. The compartmentalization also helps to isolate the viral RNA from the translation process and increase the local concentration of replication components. The membrane structures are also proposed to increase the surface area and behave as a scaffold for replicase assembly.

In BMV, the spherules induced by 1a provide a small compartment for RNA replication and protect the dsRNA intermediates from host RNA silencing. The RNA templates recruited by the 1a protein inside the spherules are resistant to nuclease digestion but susceptible to the detergent treatment. (Ahlquist, 2002; Schwartz, Chen et al., 2002).

Although spherule formation was not reported in ToMV replication, recent work suggests that the active replication proteins and (-)RNA are peripherally bound to membranes (Nishikiori, Dohi et al., 2006). The ToMV (-)RNA is synthesized in an isolated membrane compartment inaccessible to nuclease and the RNA is resistant to micrococcal
nuclease treatment. However, when treated with detergent, the RNA became susceptible to the nuclease.

The membrane compartment in which the TBSV replication takes place protects the replicase and RNA against proteases and RNase digestion (Pogany and Nagy, 2008). Based on the recent data from an in vitro cell free replicase system, the viral replicase complex is protected from the protease K treatment 20 to 40 min after the beginning of the assay containing recombinant viral proteins, the viral RNA and the yeast cell-free extract. The synthesized RNA is resistant to micrococcal nuclease at the 40 min time point, which suggested that the synthesized RNA is protected in a membrane bound compartment inaccessible to nuclease.

**Importance of membrane lipids**

Lipids are the main components of cellular membranes. Both lipid synthesis and composition are important for (+)RNA virus replication. BMV replication via 1a expression induces membrane proliferation and membrane lipid synthesis leading to increase total lipids by 25 to 33% in yeast (Lee and Ahlquist, 2003). The ratio of unsaturated fatty acids (UFAs) to saturated fatty acids (SFAs) influences the membrane fluidity and plasticity. Mutation of *OLE1* which encodes Delta9 fatty acid (FA) desaturase (converts saturated fatty acids to unsaturated forms) led to reduced UFAs level (UFAS/SFAs change from 2.5 to 1.5) (Lee and Ahlquist, 2003). In the *ole1* mutant yeast strain, BMV replication was severely inhibited prior to minus strand RNA synthesis, although the 1a protein induces membrane proliferation and spherule formation is not affected. Supplementation of media with UFAs to the yeast could rescue the defect (Lee, Ishikawa et al., 2001; Lee and Ahlquist, 2003).

In TBSV, recent unpublished data also suggested that the ergosterols and phospholipids are important for virus replication (Sharma and Nagy, unpublished). Deletion or inhibition of *ERG25* and other enzymes in ergosterol biosynthesis pathway led to reduced viral RNA accumulation. Deletion of *INO2* which encodes protein involved in phospholipids synthesis also decreased the virus replication (Panavas, Serviene et al., 2005).
The roles of host factors in virus replication

Host factors play important roles in various steps of (+)RNA virus replication, including recruitment of the viral RNA from translation to replication, transportation of replication components, spherule formation, replicase assembly, viral RNA synthesis and the release of (+)RNA progeny (Nagy and Pogany, 2006).

Host factors involved in recruiting RNA from translation to replication

The genome of (+)RNA viruses serves as both mRNA for viral protein translation and as the template for RNA replication. A key step in the replication of (+)RNA viruses is to recruit the viral RNA from the cellular translation machinery (in the cytoplasm) and then to recruit it to the viral replication complex (on the membrane surfaces of various organelles) (Ahlquist, 2002; Ahlquist, Noueiry et al., 2003; Salonen, Ahola et al., 2004; Panavas, Hawkins et al., 2005). The emerging picture is that one of the viral replication proteins can selectively bind to the viral RNA and recruit the (+)RNA from translation to replication. Examples include the 1a protein of BMV and p33 of TBSV as we discussed previously.

In addition to viral replication proteins, host proteins are also involved in the RNA recruitment step. The host Lsm1p-7p/Pat1p/Dhh1p decapping activator complex is involved in recruitment of BMV RNA out of translation and into the replication complex and deletion of components of the complex reduced BMV RNA accumulation. The Lsm1p-7p/Pat1p/Dhh1p complex functions in the deadenylation-dependent decapping of cellular mRNAs and transfer RNAs from active translation to the P body for degradation or other non translation process (Mas, Alves-Rodrigues et al., 2006; Beckham, Light et al., 2007). The decapping complex might facilitate the loss/release of ribosomes and translation factors from the BMV RNAs and recognition of the RNA by the 1a protein, resulting in switching viral RNA from translation to replication (Mas, Alves-Rodrigues et al., 2006). Lsm1-7p, Pat1p and Dhh1p are components of the cytoplasmic processing body (P body) and BMV RNAs were found to be accumulated in P bodies (Beckham, Light et al., 2007). The viral RdRp was also found to be partially colocalized with the P body component Lsm1p. The P body was suggested to facilitate BMV replication complex assembly. RNAs and replication proteins accumulation within the ER-associated
P bodies might be an important transition step during the assembly of replication complex (Beckham, Light et al., 2007). It is also proposed that the recruitment of RNA away from translation sites to replication sites would allow the assembly of the replication complex on viral RNA without interference by elongating ribosomes as well as could promote the escape of the viral (+)RNAs from degradation (Beckham, Light et al., 2007).

The host polypyrimidine-tract-binding protein (PTB) plays important roles in both translation and RNA synthesis of hepatitis C virus (HCV) (Aizaki, Choi et al., 2006; Chang and Luo, 2006). PTB specifically interacts with the 3’ UTR of HCV RNA and knock down of the PTB level by siRNAs led to reduced HCV proteins as well as RNA accumulation. PTB is also required for HCV RNA synthesis and was found to be a component of the HCV replication complex (Aizaki, Choi et al., 2006).

**Host factors involved in transportation of replication components and in spherule formation**

The replication of (+)RNA virus occurs on various cellular membranes and viral replication proteins of several plant viruses such as p33 of tombusvirus and 1a protein of BMV play critical roles in membrane targeting. Host factors interacting with viral RNA or viral replication proteins might facilitate the efficient transportation and localization of the essential components of replication machinery (Ahlquist, 2002; Ahlquist, Noueiry et al., 2003; Salonen, Ahola et al., 2004; Panavas, Hawkins et al., 2005).

Lsm1-7p, Pat1p and Dhh1p might also facilitate the localization of BMV RNA and replication proteins to the P body, which is the transition place before the viral components are transported to the ER (Beckham, Light et al., 2007).

The host membrane proteins TOM1/TOM3 and TOM2A play critical roles in the membrane association of the replication complex of ToMV (Yamanaka, Imai et al., 2002; Hagiwara, Komoda et al., 2003; Ishikawa and Okada, 2004). TOM1 and TOM2A are trans-membrane host proteins and TOM1 interacts with the TOM2A protein and with the helicase domain of ToMV replication proteins (Yamanaka, Ohta et al., 2000; Tsujimoto, Numaga et al., 2003). TOM1 and TOM2A, which are required for efficient ToMV replication, were suggested to be associated with the viral replication complex as well as
with the intracellular membrane during virus replication (Yamanaka, Ohta et al., 2000; Yamanaka, Imai et al., 2002; Tsujimoto, Numaga et al., 2003; Nishikiori, Dohi et al., 2006).

The host Pex19p protein was proposed to be a cellular transporter assisting tombusvirus p33 localization to the peroxisomal membranes (Pathak, Sasvari et al., 2008). Pex19p interacts with p33 and temporarily associates with the viral replicase based on a pull down assay and co-purification of Pex19p with p33. Mis-targeted Pex19p also led to redistribution of p33 and reduced TBSV RNA replication. The components of the cellular ESCRT (endosomal sorting complex required for transport) were found to be involved in TBSV replication proteins intracellular transportation, membrane invagination and viral RNA replication (Barajas, Jiang et al., submitted).

**Host factors inside the replicase complex**

Recent studies on (+)RNA virus unraveled that the active replicase complexes are membrane associated and composed of viral template RNA, viral replication proteins and cellular proteins which may be needed for replicase assembly (Noueiry and Ahlquist, 2003; Sanfacon, 2005; Nagy and Pogany, 2006; Nishikiori, Dohi et al., 2006).

The induction of heat shock protein expression is a common response to various viruses infection and the heat shock proteins including Hsp40, Hsp70, Hsp90 family members have also been found to play important roles in virus replication (Whitham, Yang et al., 2006). These protein chaperones are found to be components of the replicase or contribute to active replicase assembly for several plus strand RNA viruses such as BMV, TBSV, HCV and FHV (Tomita, Mizuno et al., 2003; Okamoto, Nishimura et al., 2006; Castorena, Weeks et al., 2007; Nakagawa, Umehara et al., 2007). A Hsp40 family chaperone Ydj1p is required for BMV active replicase assembly. Mutation of the *YDJ* gene led to inhibition of minus-strand synthesis without affecting the ER membrane localization of the 1a, 2a proteins and the viral RNA template (Tomita, Mizuno et al., 2003). The same mutation in the *YDJ* gene also caused aggregation of a fraction of 2a. Ydj1p was suggested to modulate the state of the 2a polymerase and assembly into an active replication complex. Another protein chaperone, Hsp70, was reported to be present within the tombusvirus replication complex and was also proposed to be important for the
proper folding or activation of the replication complex (Serva and Nagy, 2006). An in vitro replicase reconstitution assay using a yeast cell extract has indicated that the Hsp70 proteins Ssa1p and Ssa2p are critical for functional TBSV replicase assembly (Pogany, Stork et al., 2008). The replicase obtained from HSP70 mutant yeast was five times less active than that obtained from wild type yeast. The membrane fraction (without the soluble fraction of yeast extract) could not facilitate the functional replicase assembly even in the presence of p33, p92 and RNA template. Addition of functional Ssa1p to the membrane fraction from yeast assisted functional replicase assembly in vitro. However, addition of loss-of-function (deficient in ATP hydrolysis) Ssa1p did not help the assembly, which suggested that the Ssa1/2p might be involved in folding the viral replication proteins for the in vitro replication system. In addition to its role in replicase assembly, the Hsp70 chaperone was found to play important roles in subcellular localization and membrane association of replicase components (Wang, Stork et al., 2009). Based on confocal microscopy-based observations and cellular fractionation data in the ssa1ssa2 double mutant yeast, a large portion of p33 and p92 was localized in the cytosol instead of the peroxisomal membranes. In vitro membrane insertion assay also revealed that the Ssa1p helps p33 and p92 insertion into membrane and protects them from proteinase K digestion. HSP70 was also reported to associate with ToMV replication proteins (Nishikiori, Dohi et al., 2006).

The heat shock protein Ydj1p was also found to be important for replication complex assembly taking place on mitochondrial membranes in the case of Flock House Virus (FHV) (Weeks and Miller, 2008). Deletion of the YDJ1 gene led to dramatic reduction in FHV RNA replication partially due to the production of less viral RdRp. But when the replication location was retargeted to endoplasmic reticulum, the Ydj deficiency has no effect on FHV replication. The Hsp90 chaperone was found to facilitate FHV replication complex assembly in Drosophila S2 cells. Moreover, Hsp90 inhibition by using geldanamycin (Hsp90 inhibitor) or RNAi led to reduced FHV protein A accumulation and suppressed RNA replication without affecting the activity of assembled replication complexes (Kampmueller and Miller, 2005; Castorena, Weeks et al., 2007). Inhibition of Hsp90 activity selectively reduced protein A synthesis to ~ 20% of normal level, while the intracellular localization, degradation or membrane association of protein A were not
altered. The author proposed that Hsp90 is required for efficient synthesis of viral RdRp and facilitates replication complex assembly in *Drosophila* cells.

The Hsp90 protein is also involved in HCV replication. Inhibition or siRNA knockdown of Hsp90 resulted in reduced HCV RNA replication in HCV replicon infected cells (Nakagawa, Umehara et al., 2007). The non-structural protein NS3 of HCV was shown to directly interact with Hsp90. Inhibition of Hsp90 by 17-allylamino-geldanamycin caused the destabilization of NS3 and resulted in suppressed HCV replication (Ujino, Yamaguchi et al., 2009). There is also evidence showing that the Hsp90, NS5A and a host immunophilin FKBP8 form a complex, which might be involved in HCV RNA replication (Okamoto, Nishimura et al., 2006). RNAi knockdown of the gene decreased the HCV RNA more than 60%.

Other host proteins were also found to be involved in assembly of replicase complex. Cellular vesicle membrane transport proteins hVAP-A and B were revealed to be the components of HCV RNA replication complex (Evans, Rice et al., 2004; Gao, Aizaki et al., 2004). The CDC34 and translation elongation factor 1A (TEF1) have been implicated in TBSV replication (Li, Barajas et al., 2008; Li, Pogany et al., 2009). The cellular hVAP-A interacts with both HCV NS5A and NS5B and is critical for the viral replication complex formation and is associated with lipids rafts (Evans, Rice et al., 2004; Gao, Aizaki et al., 2004). The hVAP-A deficiency resulted in relocation of NS5B. The interaction between hVAP-A and NS5A is also required for efficient HCV RNA replication. HCV NS5A is a phosphoprotein and its phosphorylation is inversely correlated with its ability to interact with hVAP-A. Hyperphosphorylation of NS5A disrupted the interaction and strongly reduced HCV replication (Evans, Rice et al., 2004). Another vesicle-associated membrane protein (VAP) subtype B (VAP-B) was also revealed to be involved in the HCV replication. The hVAP-B interacts with NS5A and NS5B and colocalized with NS5A. Overexpression of VAP-B enhanced the expression of NS5A and NS5B and the replication of HCV RNA (Hamamoto, Nishimura et al., 2005).

The host E2 ubiquitin-conjugating enzyme Cdc34p and eukaryotic elongation factor 1A (eEF1A) are components of the tombusvirus replication complex (Li, Barajas et al., 2008; Li, Pogany et al., 2009). The host Cdc34p interacts with p33 and ubiquitinates the p33 (Li,
Barajas et al., 2008). The expression level of wt Cdc34p correlates with the accumulation of TBSV repRNA. The eEF1A interacts with both the viral protein p33 and a cis-acting element at the 3' end of RNA (Li, Pogany et al., 2009). eEF1A was suggested to play an important role in p33 stability and TBSV replication.

Host RNA-binding protein eEF1A was also found to play roles in flaviviruses and HCV replication (Kou, Chou et al., 2006; Davis, Blackwell et al., 2007). eEF1A binds to the 3’ terminal (+) stem-loop (SL) in WNV (west nile virus) RNA and other flavivirus (Davis, Blackwell et al., 2007). The interaction between eEF1A and the WNV 3’ SL facilitates viral minus-strand RNA synthesis. Coimmunoprecipitation also suggested that eEF1A colocalized with WNV replication complexes (RC) in infected cells. The eEF1A protein interacts with the HCV non structural proteins and plays critical role(s) in NS4A-mediated inhibition of protein synthesis (Kou, Chou et al., 2006). Host proteins eEF1A, TOM1 and TOM2A were co purified with the membrane associated 180k protein, which is active in RNA synthesis (Nishikiori, Dohi et al., 2006).

**Host factors involved viral RNA synthesis**

After the viral replication complex assembles on a membrane surface, the viral RdRp starts to synthesize the viral RNAs. The eukaryotic translation elongation factor eEF1a was found to interact with the 3’ UTR of WNV RNA and the interaction between eEF1a and RNA facilitates the minus-strand RNA synthesis (Davis, Blackwell et al., 2007). eEF1a mutations with decreased binding to the WNV RNA also decreased viral minus-strand RNA synthesis and mutations with increased efficiency of RNA binding also increased minus-strand RNA synthesis.

Asymmetric RNA synthesis is a hallmark feature of plus strand RNA virus replication. The host glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Tdh2/3) was showed to promote the asymmetric synthesis of TBSV RNA (Wang and Nagy, 2008). GAPDH is a cytosolic protein and it is re-localized to peroxisomal membrane during TBSV replication. Down regulation of GAPDH in yeast and plant decreased the TBSV replication and led to equivalent production of (+) and (-) viral RNA in yeast instead of the typical 10 to 100-fold more (+) RNA. GAPDH is present in the tombusvirus replication complex and interacts with the AU pentamer in the (-) TBSV RNA (Serva and Nagy, 2006; Wang and
Nagy, 2008). It was proposed that the GAPDH selectively keeps the (-) RNA in the RC and promotes the plus strand RNA synthesis.

Altogether, recent works have convincingly demonstrated that viral replication proteins in combination with selected host proteins play major roles in (+)RNA virus replication. Dissecting the functions of these viral and host factors during (+)RNA replication will help in designing new antiviral strategies and combat virus infections in humans, animals and plants.
**Figure 1-1. Genome organization of tombusviruses.** (A) The genomic RNA is uncapped at 5’ end and no poly A tail present at 3’ end. Coding regions are represented as boxes with approximate molecular masses (in kDa) prefixed with “p” and protein functions are indicated in parenthesis (Pol, polymerase; CP, coat; mov, movement; sup, suppressor of gene silencing). Coding regions in the genome translated to produce replication proteins p33 and p92. (B) Subgenomic (sg) mRNAs. Sg mRNAs 1 and 2 are transcribed during infections and mediate translation of CP and mov/sup, respectively.
Figure 1-2. Functional domains in the TBSV p33 and p92 replicase proteins. (A) Schematic representation of the known domains in p33 involved in peroxisome targeting, membranes association, interaction with the viral RNA [the arginine / proline-rich (RPR) motif], and with other p33 and the p92 RdRp proteins (S1 and S2). The phosphorylation sites which modulates p33 RNA-binding activity are also pointed out. (B) Schematic representation of the known p92 domains. Note that the sequence of the overlapping (prereadthrough) domain of p92 is identical with that of p33. p92 contains another two RNA-binding regions. Motifs A to E indicate the signature motifs of RNA polymerases.
Figure 1-3. The role of *cis*-acting RNA elements in regulation of viral RNA replication. (A) The genomic or DI RNAs contain the p33 recognition element (p33RE), gPR promoter and silencer. The activity of the gPR is downregulated by a replication silencer through RNA–RNA interaction between the 3′ terminal sequence of gPR and a 5-nt-long stretch in the replication silencer SL3 (B) The minus-stranded intermediates contain the cPR promoter at their 3′ ends. Plus-strand synthesis, which starts from cPR, is upregulated by two replication enhancers [PPE and RIII(−) enhancer. (C) The minimal template RNA containing 3 elements, p33RE, RSE and gPR can produce functional replicase.
Figure 1-4. Linear structure of DI-73 and schematic representation of long range RNA-RNA interaction. (A) Schematic linear representation of the TBSV RNA genome and the non-coding DI-73 RNA replicon. Regions in the TBSV genome that are present in DI-73 are delineated by thick horizontal lines under the genome. The regions of DI-73 that are derived from the TBSV genome are delineated by the dotted arrows with the corresponding genomic coordinates. The contiguous 3'-proximal segment is defined by three regions: RIII, R3.5, and RIV. (B) Secondary structure showing RII and RIII-R3.5-RIV and the UL–DL interaction.
CHAPTER II

Using model host systems and systematic biological approaches to study RNA virus replication

Introduction

RNA viruses are obligate intracellular pathogens with RNA genome encoding limited genetic information. Plus-stranded (+)RNA viruses, the largest, and the most economically important group among plant viruses, consist of massager-sense RNA genome that acts directly as mRNA for translation (Hull, 2002). Genome replication, the key step in the infection cycle of (+)RNA viruses, is a dynamic and complex process. Upon entry into host cell, viral RNA utilizes host translation machinery to produce viral proteins, including those required for virus replication (Replication proteins). Viral RNA also subsequently acts as a template for genome replication, being recruited to the site of replication possibly by replication proteins. Most (+)RNA viruses, if not all, replicate their genome on the subcellular membrane of host cell, for example, ER, peroxisome, chloroplast or mitochondrial membrane (Salonen, Ahola et al., 2004; Mackenzie, 2005; Miller and Krijnse-Locker, 2008). The replication process involves at least two steps: synthesis of minus (-)-strand replication intermediate and then the (-)RNA subsequently being used as a template to produce many (+) progeny RNA.

Plant (+)RNA viruses only encode 4-10 genes and among which, only few of them are required for functional replication (Hull, 2002). Apparently, viruses rely on host machinery to achieve robust replication, via numerous interactions with host proteins or membranes. The emerging picture is that, in additional to viral proteins, viruses also utilize, subvert, and co-opt a large number of host-encoded proteins or pathways for their own purpose (Ahlquist, Schwartz et al., 2005; Nagy and Pogany, 2006; Nagy, 2008). The interaction compatibility may define the host range of a given virus, the level of permissiveness for replication of a given cell type and the disease pathology as well as virus evolution (Barton, Black et al., 1995; Andino, Boddeker et al., 1999; Lellis, Kasschau et al., 2002; de Graaf, Herfst et al., 2008). Therefore, identification of host
factors and revealing their functions during virus replication could facilitate our understanding of the molecular mechanism of virus infection. Also, those host factors may represent novel strategies and provide new targets for viral disease control.

Despite their critical role in virus replication, our understanding of the cellular factors greatly lags behind that of the viral counterparts. This may partially due to the complexity of virus replication process and cellular pathway, as well as the lack of powerful model system and approach to study it. Recently, the budding yeast (*Saccharomyces cerevisiae*) has been developed as a model host to study plant (+)RNA viruses, *Brome mosaic virus* (BMV) and *Tomato bushy stunt virus* (TBSV) and related tombusivirus, as well as Nodavirus of animal (Janda and Ahlquist, 1993; Price, Rueckert et al., 1996; Panavas and Nagy, 2003; Pantaleo, Rubino et al., 2003). The genetic tractability of yeast with relatively small genome greatly facilitates the identification of host factors involved in viral replication (Kushner, Lindenbach et al., 2003; Panavas, Serviene et al., 2005). In recent years, with the emerging RNA interference (RNAi) technology, the large-scale loss-of-function screens also become possible in tissue culture cell (Echeverri and Perrimon, 2006; Boutros and Ahringer, 2008). The RNAi screens have led to the identification of a large number of host cofactors affecting the infection of Drosophila C virus (DCV), Hepatitis C virus (HCV), influenza virus, West Nile virus (WNV) and human dengue virus (DENV) (Cherry, Doukas et al., 2005; Ng, Mo et al., 2007; Randall, Panis et al., 2007; Hao, Sakurai et al., 2008; Krishnan, Ng et al., 2008; Supekova, Supek et al., 2008; Sessions, Barrows et al., 2009; Tai, Benita et al., 2009). The current chapter is aimed to summarize the use of model host systems and systematic approaches, such as genome-wide loss-of-function libraries and proteomic-wide screening, in studying virus-host interactions.

**Use yeast genome-wide screens to study BMV and TBSV replication**

Yeast is an attractive model system for studying many basic cellular processes due to its merits such as fast growing, tractable genetics and a relatively small and extensively annotated genome (~5,800 genes) (Bartlett and Nurse, 1990; Hohmann, 2005; Shinyashiki, Lopez et al., 2005; Mustacchi, Hohmann et al., 2006; Sanz, 2007). The pioneer work from Paul Ahlquist’s lab has developed an efficient system for studying
BMV viral RNA replication and subgenomic mRNA transcription in yeast (Janda and Ahlquist, 1993; Noueiry and Ahlquist, 2003). Random mutagenesis of yeast genome, via UV-irradiation or ethyl methanesulfonate treatment, identified a DEAD (Asp-Glu-Ala-Asp)-box RNA helicase (DED1), a component of mRNA deadenylation pathway (LSM1), OLE1 and YDJ1, required for efficient BMV replication and subgenomic mRNA synthesis (Ishikawa, Diez et al., 1997; Diez, Ishikawa et al., 2000; Noueiry, Chen et al., 2000; Lee, Ishikawa et al., 2001; Tomita, Mizuno et al., 2003). Further genomewide screening of 4,500 yeast single-gene knock-out (YKO) strains (about 80% of yeast genes) identified ~100 genes inhibiting or stimulating BMV RNA replication by 3- to 25-fold, including *LSM1* previously revealed by random mutagenesis and *LSM6* in the same mRNA deadenylation pathway (Kushner, Lindenbach et al., 2003). The yeast system was subsequently adapted for studying the replication of plant tombusviruses, i.e. TBSV and *Carnation Italian ringspot virus* (CIRV) (Panavas and Nagy, 2003; Pantaleo, Rubino et al., 2003). Genome-scale screening of YKO library revealed 96 host genes affecting the replication of TBSV DI-72 replicon RNA (Panavas, Serviene et al., 2005). Certainly, the host essential genes which are required for cell viability may also play essential roles during virus replication. For TBSV, this question was addressed by screening of Yeast Tet Promoters Hughes Collection (yTHc), in which the expression of given essential gene was control by a Tetracyclin-titratable promoter in the genome (Mnaimneh, Davierwala et al., 2004). Among 800 essential host genes present in this library (accounting for 70% of total 1,100 predicted essential yeast genes), 30 host genes were found to affect the accumulation of TBSV RNA when down-regulated (Jiang, Serviene et al., 2006). The YKO and yTHc libraries were also extended to study TBSV recombination, which led to identification of total 32 genes affecting TBSV evolution (Serviene, Shapka et al., 2005; Serviene, Jiang et al., 2006). All together, development of yeast as model system and the use of system biology approaches provide powerful tools to study the replication of these two plant viruses.

**Proteomics approaches revealed the complexity of virus-host interactions**

The virus replicase complex likely contains virus-encoded components, such as viral replication proteins and RNA, as well as host factors. Recently, Mass Spectrometry
(MS)-based approaches have become a routine to identify the unknown proteins present in highly purified replicase complex. Serva et al., expressed six-Histidine and Flag tagged *Cucumber necrosis virus* (CNV, a tombusvirus) replication proteins p33 and p92(pol) in yeast cells together with DI-72 replicon RNA, followed by Nickel metal-chromatography and anti-Flag immuno-affinity-chromatography purification of the replicase complex. The highly purified replicase preparations were subsequently separated by 2-Dimensional (2D) gel electrophoresis and Matrix-Assisted Laser Desorption/ionization (MALDI) Time-Of-Flight (TOF) MS analysis of proteins. The proteomics analysis identified at least three host proteins present in this complex, i.e. a member of the heat shock protein 70 (Hsp70) family (SSA1/2) chaperone, two metabolic enzymes: glyceraldehyde-3-phosphate dehydrogenase (TDH2/3) and a pyruvate decarboxylase (PDC1) (Serva and Nagy, 2006). Further proteome-wide screening based on yeast proteome microarray (protoarray), with 4080 purified yeast proteins fixed on the chip, identified 58 yeast proteins interacting with CNV p33 replication protein and 11 proteins interacting with the read-through portion of p92 (p92C). Among these proteins, the E2 ubiquitin-conjugating enzyme CDC34 could ubiquitinate p33 *in vitro* and affects virus replication in vivo (Li, Barajas et al., 2008). To identify host proteins that interact with viral RNA in a proteome-scale, Zhu et al. exploited a yeast protoarray and they were able to identify host proteins interacting with a small RNA hairpin in the 3’untranslated region of BMV which is required for the replication. They found 12 proteins specifically interacted with the functional RNA hairpin, including the pseudouridine synthase (PUS4) and actin patch protein 1 (APP1). Overexpression of Pus4p and App1p decreased BMV replication and dramatically inhibited virus systemic spread in plants, meanwhile Pus4p also prevented the encapsidation of BMV RNA, confirming the usefulness of global approaches in identification of specific viral RNA-binding proteins from the model host (Zhu, Gopinath et al., 2007). A similar approach also led to the discovery of 11 host proteins specifically bind to biotin-labeled TBSV DI-72 RNA probes, among which, translation elongation factor 1A (TEF1/2) has been shown to be a component of the tombusvirus replicase complex (Li, Pogany et al., 2009). These proteome-wide approaches revealed the complexity of virus-host interaction and provided useful additions to the large-scale genetic screening, whose functions can be compromised by the gene redundancy.
RNAi screening provides a powerful tool to study the replication of human and animal viruses

Double strand RNA (dsRNA) or synthetic small interfering (siRNAs) libraries offer researcher an exciting high-throughput tool to identify cellular factors implicated in virus infection (Cherry and Silverman, 2006; Echeverri and Perrimon, 2006). The model host of DCV, Drosophila, contains genome with only ~14,000 genes. A genome-wide RNAi screening based on dsRNA library covering 91% of the genes, led to identification of 112 host factors affecting DCV infection by >40% upon depletion. Surprisingly, 66 of the identified factors were ribosomal proteins, highlighting the sensitivity of DCV infection to the cellular ribosomal level (Cherry, Doukas et al., 2005). In an attempt to use the high-throughput genetic approaches, Hao and colleagues developed Drosophila D-Mel2 cells as a model host of Human influenza virus A/WSN/33 (WSN; H1N1). A genetically modified H1N1 virus, expressing vesicular stomatitis virus glycoprotein G on the virion envelopes, could enter *Drosophila melanogaster* (D-Mel2) cells and establish infection. By using a dsRNA library targeting 13,071 genes (90% of the Drosophila genome), more than 100 genes were found to significantly inhibit or stimulate the virus reporter gene expression. The human homologues of several identified genes also showed similar effects to the replication of influenza A virus, confirming the usefulness of genome-wide screening in model host (Hao, Sakurai et al., 2008). Similarly, Sessions et al. also adapted *Drosophila* D-Mel2 cells as a model host of DENV-2, an arthropod-borne virus, by serial passage. A genome-wide dsRNA silencing screening identified 116 unique factors affecting Dengue virus infectivity more than 1.5-fold based on measuring expression level of envelope protein (Sessions, Barrows et al., 2009). Several RNAi screens were also initiated to identify the cellular factors involved in the infection and replication of HCV in human hepatoma cells. Three limited RNAi screens which targeted ~4,000 genes (Ng et al., 2007), 510 human kinases (Supekova et al., 2008), and 62 genes previously identified host genes (Randall et al., 2007), founded 10, 3 and 26 host factors, respectively, decreasing HCV infection when silenced (Ng, Mo et al., 2007; Randall, Panis et al., 2007; Supekova, Supek et al., 2008). A more recent work using whole-genome siRNA library covering 21,094 genes, the entire human NCBI RefSeq transcript database, identified 96 host factors whose deletion decreased the HCV infection (Tai,
This genome-wide screening also revealed 13 factors inhibiting HCV replication (Tai, Benita et al., 2009). Surprisingly, limited overlap was found from the results of these screens, which can be explained by different siRNA sequences used for the same target gene, different HCV genotype or subgenotype replicon used, the way of siRNA being administrated and different levels of threshold for identifying positive hits (Tai, Benita et al., 2009). For another Flavivirus, WNV, a genome-scale siRNA screening covering 21,121 human genes in HeLa cells also found 305 cellular factors affecting the early stages of virus infection. Among those, 294 are novel factors, demonstrating the complexity of virus infection and the extent of their dependence on host functions (Krishnan, Ng et al., 2008).

In plant, the lack of high-throughput method hindered the use of large-scale RNAi screening to study the host factors in virus infection. However, David C. Baulcombe’s lab used virus induced gene silencing (VIGS) by using potato virus X (PVX) vector to systematically screen the genes required for resistance (R) gene-mediated resistance, which could restrict the movement of virus carrying the corresponding avirulent (Avr) gene in inoculated leaves. Of the 4992 normalized Nicotiana benthamiana cDNA library, silencing N requirement gene 1 (NRG1) in N-transgenic plant allowed modified TMV expressing GFP protein escape from inoculated leaf to systemic leaf, indicating NRG1 is required for N-mediated resistance against TMV (Peart, Mestre et al., 2005). Silencing the expression of Heat shock protein 90 (HSP90) also caused loss of N-mediated resistance against TMV and Rx-mediated pot ato virus X resistance (Lu, Malcuit et al., 2003). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes were also found to be required for N-mediated resistance for TMV through the VIGS approach (Liu, Schiff et al., 2002). Recently, a high-throughput screening, employing PVX-based overexpression of N. benthamiana cDNA library, identified an ADP-ribosylation factor (ARF1) involved in non-host resistance and R gene-mediated resistance (Coemans, Takahashi et al., 2008).

Altogether, the using of model hosts and high throughput approached provide powerful tools to study the virus replication and virus-host interaction. In order to identify host factors involved in tombusviruses replication, we also performed high throughput screening of yeast (a model host) essential genes to test their effect on TBSV replication.
In addition to the yeast non-essential genes screening and proteomics approaches, we were able to find more than a hundred host proteins affecting tombusvirus replication. These genome-wide screenings greatly helped us in better understanding the molecular mechanism of tombusvirus replication.

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CHAPTER III

Identification of essential host factors involved in TBSV replication

(This article is published in Journal of Virology, 2006. Vol. 80 p. 7394-7404)
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(The author and Serviene E. cooperated in the high throughput screening and Panavas T. provided the important pGBK plasmid)

Introduction

Replication of plus-stranded RNA viruses requires many components of the host cells, including host proteins and intracellular membranes, which serve as sites of virus replication in infected cells (Buck, 1996; Ahlquist, 2002; Salonen, Ahola et al., 2004). Accordingly, the virus-specific replicase complex (RC) consists of virus- and host-coded proteins and the viral RNA, which assemble on intracellular membranes into functional complexes. In addition, the viral replication proteins and host factors likely play roles in template selection for replication and recruitment (intracellular transport/targeting) of the viral RNA into replication (Ahlquist, Noueiry et al., 2003; Nagy and Pogany, 2006). Host factors could also affect the stability/degradation of viral proteins and the viral RNA (Serviène, Shapka et al., 2005; Cheng, Serviene et al., 2006; Serviene, Jiang et al., 2006). Overall, viruses utilize/depend on many diverse resources of the host cells.

To identify the roles and/or effects of host genes on virus replication, systematic genome-wide screens were conducted in yeast, a model host, using the single-gene deletion library (YKO) with two distantly related plus-strand RNA viruses, namely Brome mosaic virus (BMV) and Tomato bushy stunt virus (TBSV) (Kushner, Lindenbach et al., 2003; Panavas, Serviene et al., 2005). These studies led to the identification of 100 host genes for each virus that either stimulated or inhibited virus replication. Interestingly, most of the identified genes had a virus-specific effect, whereas only a small number of genes affected the replication of both BMV and TBSV. These observations suggest that BMV and TBSV, belonging to different supergroups within plus-stranded RNA viruses, could use and/or be affected by mostly different host factors (Kushner, Lindenbach et al., 2003; Panavas, Serviene et al., 2005). Altogether, the above systematic screens tested only 80% of all the known genes, which are not essential for yeast growth, whereas the effect of essential yeast genes remained untested.
Tombusviruses, such as TBSV and *Cucumber necrosis virus* (CNV), are single-component RNA viruses of 4,800 nucleotides (nt). Among the five virus-coded proteins, only two, termed p33 and p92, are essential for TBSV replication (White and Nagy, 2004). p92 is the viral RNA-dependent RNA polymerase (RdRp), whereas p33 replication cofactor (which overlaps with the N-terminal pre-readthrough segment of p92) is an RNA-binding protein (Panaviene, Baker et al., 2003; Rajendran and Nagy, 2003; Pogany, White et al., 2005). Earlier work defined that p33 is involved in template selection and recruitment of viral RNA into replication (Monkewich, Lin et al., 2005; Panavas, Hawkins et al., 2005; Pogany, White et al., 2005). These proteins interact with each other, the viral RNA, and the host proteins in cells (Rajendran and Nagy, 2004; Panavas, Hawkins et al., 2005; Rajendran and Nagy, 2006; Serva and Nagy, 2006), which leads to the assembly of RC on peroxisomal membranes (Navarro, Rubino et al., 2004; Panavas, Hawkins et al., 2005). The CNV replication proteins can support the replication of TBSV defective interfering (DI) RNA, a small deletion derivative of the genomic RNA, as efficiently as TBSV replication proteins can (Oster, Wu et al., 1998; Nagy and Pogany, 2000). A recent genome-wide screen of the YKO library for tombusvirus replication led to the identification of 96 host genes, whose separate deletions affected replication of a TBSV replicon RNA (repRNA), which is based on a DI RNA, in yeast (Panavas, Serviene et al., 2005). Based on the large number of host genes identified, the emerging picture is that the host likely plays a complex role in virus replication (Panavas, Serviene et al., 2005; Nagy and Pogany, 2006).

In this paper, we extended the genome-wide screening to essential yeast genes to identify those affecting tombusvirus replication. Among the 800 essential host genes present in the yeast Tet promoters Hughes Collection (yTHC) (out of 1,100 predicted essential yeast genes) (Mnaimneh, Davierwala et al., 2004), we found that 30 genes (when down-regulated) affected the accumulation of tombusvirus RNA. These essential yeast genes, which either increased or decreased the accumulation of tombusvirus RNA, are involved in protein metabolism/transport, RNA transcription/metabolism, or other cellular processes. Detailed analysis of the effect of a selected group of yeast genes revealed that they could affect the amount of replication proteins or the initial level of RNA templates as well as the activity of the tombusvirus replicase. Overall, this and previous genome-
wide screening of 95% of all yeast genes defined that 2% of yeast genes affected the accumulation of TBSV RNA. In addition, documented interaction between the identified host proteins will facilitate future research aimed at dissecting their roles in tombusvirus replication.

Material and methods

Yeast strains and expression plasmids

The Tet promoter-based Hughes Collection (yTHC) of yeast strains were obtained from Open Biosystems. yTHC was provided in the haploid strain R1158 background (URA3::CMV-tTA MATa his3-1 leu2-0 met15-0). This strain was created by a one-step integration of the tTA transactivator, under the control of the cytomegalovirus (CMV) promoter, at the URA3 locus. The kanR-tetO7-TATA cassette was then integrated into the yeast genome, replacing the endogenous promoter for each gene (Mnaimneh, Davierwala et al., 2004).

The expression plasmids pH137-92 (containing CNV p92 gene and LEU2 marker) (Panaviene, Panavas et al., 2004) and pGBK-His33/DI-AU-FP (coexpressing p33 from the ADH1 promoter and DI-AU-FP RNA from the GAL1 promoter) have been previously described (Serviene, Jiang et al., 2006). Construction of dual expression plasmid pGBK-His33/DI-72 (coexpressing p33 from the ADH1 promoter and DI-72 RNA from the GAL1 promoter) was done by inserting DI-72 sequence including the ribozyme at the 3' end (Panavas and Nagy, 2003) together with the GAL1 promoter sequence between the ADH1 promoter and the F1 origin in pGBK-His33 plasmid (Panaviene, Panavas et al., 2004). These sequences were amplified by PCR using primers #1546 (CCGCGAATTCTACGGATTAGAAGCCGCCGAGCAGGGT) and #1069 (CCGGTCGAGCTCTACCAGGTAATATACCACAACGTGTGT) on pYC/DI-72 as a template. The 5' end of the ADH1 promoter from pGBK-His33 was amplified with primers #1543 (CCGCGAATTCTCGGTCGGGCCTCTCGCTATTACGCCA) and #1544 (GGAAGTCCATATTGTACCCGGAAACA). The two PCR products were ligated through the EcoRI site, and the full-length 5'P-ADH-P-Gal1-DI72 DNA was reamplified with primers #1069 and #1544. To obtain His3-F1ori DNA, the His3 gene together with F1 origin from pGBK-His33 was amplified with PCR using primers #1081
(CGGCCGTCCGGATAATTCCGTTTTAAGAGCTTGGT) and #1545 (CCGGTCGAGCTCATCGCCCTTCCCAACAGTTGCGCA) to introduce a SacI site. The obtained PCR products of 5'P-ADH-P-Gal1-DI72 and His3-F1ori were digested with Bsp1407I/SacI and BspEI/SacI, respectively, and cloned simultaneously into pGBK-His33 (Panaviene, Panavas et al., 2004) digested with BspEI/Bsp1407I.

**Yeast transformation and cultivation**

The parental strain (BY4741; Open Biosystems) and the strains in the yTHC collection were cotransformed with different combinations of plasmids using the LiAc-single-stranded DNA-polyethylene glycol method (Gietz and Woods, 2002), and transformants were selected by complementation of auxotrophic markers.

For separate analysis of DI-AU-FP RNA and DI-72 RNA accumulation, each transformed yTHC strain was inoculated into synthetic complete dropout medium lacking leucine and histidine (SC-LH– medium) containing 2% galactose and supplemented with Geneticin G418 (200 mg/liter) and cultured for 24 to 48 h at 29°C until an optical density at 600 nm of 0.8 to 1.0. For maximum level of essential gene expression, yeast was grown in the absence of doxycycline, whereas to reduce the expression levels of the essential genes, yeast was grown in the same medium in the presence of 10 mg/liter doxycycline (Mnaimneh, Davierwala et al., 2004). Our preliminary experiments showed that the use of 10 mg/liter doxycycline was as good as 25 or 50 mg/liter doxycycline and better than 3.3 mg/liter to affect TBSV replication (not shown). Therefore, we used 10 mg/liter doxycycline throughout the experiments to turn the particular gene off.

**RNA analysis**

Total RNA isolation and northern blot analysis were performed as described previously (Panavas and Nagy, 2003; Panaviene, Panavas et al., 2004). Briefly, for extraction of total RNA, yeast cells were broken by shaking for 1 to 2 min at room temperature with equal volumes of RNA extraction buffer (50 mM NaOAc, pH 5.2, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and water-saturated phenol and then incubated for 4 min at 65°C, followed by ethanol precipitation. The obtained RNA samples were separated on a 1.5% agarose gel and transferred to Hybond-XL membrane (Amersham) before hybridization with DI-72 RNA-specific probe. For detection of plus-strand repRNA, we
prepared 32P-labeled RIII/IV(−) probe with T7 transcription from PCR product obtained with primers #1165 (AGCGAGT AAGACAGACTCTTCA) and #22 (GTAATACGACTCACTATAGGGCTGCATTTCTGCAATGTTCC) on DI-72 templates.

**Protein analysis**

For protein analysis, yeast strains were cultivated as described above for RNA analysis. A total of 50 ml yeast culture was harvested, the pelleted cells were resuspended in 150 µl cold extraction buffer (200 mM sorbitol, 50 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mix; Sigma), and 250 µl of glass beads was added to each sample. The cells were broken with Genogrinder for 2 min at 1,500 rpm. Each sample was further mixed with 600 µl prechilled extraction buffer, and unbroken cells were removed by centrifugation at 100 x g for 5 min. The supernatant was mixed with 1/2 volume of 3x SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer followed by SDS-PAGE and western blot analysis as described previously (Panavas and Nagy, 2003; Panaviene, Panavas et al., 2004). The primary antibody was anti-His6 (Amersham), and the secondary antibody was alkaline-phosphatase-conjugated anti-mouse immunoglobulin G (Sigma).

**CNV replicase assays**

The "membrane-enriched" CNV replicase preparations, which are suitable to test the replicase activity on the endogenous templates present within the CNV replicase preparation, were obtained as previously described (Panaviene, Panavas et al., 2004). Briefly, frozen yeast cells were homogenized with Genogrinder for 2 min at 1,500 rpm in 150 µl cold extraction buffer (200 mM sorbitol, 50 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mix; Sigma) plus 250 µl of glass beads. Each sample was further mixed with 600 µl prechilled extraction buffer, and unbroken cells were removed by centrifugation at 100 x g for 5 min at 4°C (Panavas and Nagy, 2003). The supernatant was centrifuged for 10 min at 21,000 x g at 4°C, and then the pellet was resuspended and used in a standard CNV replicase assay (Panaviene, Panavas et al., 2004; Panaviene, Panavas et al., 2005). Because no template was added to the in vitro reaction, the replicase preparation could only use the
endogenous template present within the enriched membrane fraction. The replicase products were phenol-chloroform extracted, precipitated with isopropanol-ammonium acetate, and analyzed under denaturing conditions (i.e., 5% PAGE containing 8 M urea) (Pogany, Fabian et al., 2003).

To test the ratio of plus- versus minus-strand synthesis on the endogenous templates by the CNV replicase, we obtained the membrane-enriched fraction (see above), followed by standard replicase assay in the presence 32P-labeled UTP and the other three unlabeled rNTPs as described previously (Panaviene, Panavas et al., 2004). Equal amounts of unlabeled in vitro transcripts of plus-strand and minus-strand DI-72 RNAs (prepared by T7 RNA transcription in vitro) were denatured separately by heating for 5 min at 85°C in Tris-EDTA (TE) buffer and formamide (in a 1:1 ratio). Then the DI-72 plus-strand and minus-strand RNAs were separately blotted onto a Hybond XL membrane (Amersham) and cross-linked with UV light (GS Gene Linker; Bio-Rad). Hybridization with the heat-denatured 32P-labeled replicase products (see above) was done using ULTRAhyb solution (Ambion) at 68°C according to the supplier's instructions.

Results

Screening of the yTHC collection for essential host genes affecting tombusvirus replication

The yTHC collection contains 800 out of 1,100 essential yeast genes (Mnaimneh, Davierwala et al., 2004). In the yTHC collection, the expression of a given essential yeast gene is under the control of a Tet-titratable promoter in the genome (Open Biosystems). The expression of the essential gene can be turned off by the addition of doxycycline to the yeast growth medium (Mnaimneh, Davierwala et al., 2004). This approach allowed us to test tombusvirus RNA replication in the presence of the host protein (when yeast was grown without added doxycycline after the induction of tombusvirus repRNA replication) (Fig. 3-1A) or in its absence (when yeast was grown in the presence of doxycycline to turn off the expression of the particular gene) (Fig. 3-1B)

To identify essential host genes affecting tombusvirus replication, we transformed each of the yeast strains present in the yTHC collection with two plasmids (pGAD-His92 and pGBK-His33/DI-AU-FP, Fig. 3-1A and B). These plasmids expressed the p33 and p92
replication proteins of CNV, which support TBSV RNA replication as efficiently as TBSV replication proteins (Oster, Wu et al., 1998; Panavas and Nagy, 2003), from the constitutive ADH1 promoter and DI-AU-FP RNA replicon (Fig. 3-1A and B) (Shapka and Nagy, 2004) (from the galactose/glucose-inducible/repressible GAL1 promoter (Panavas and Nagy, 2003). Expression of p33, p92, and the DI-AU-FP repRNA in the parental yeast strain grown under standard growth conditions (see Materials and Methods) led to efficient replication of the 807-nt-long repRNA (20,000 copies per yeast cells). The addition of 10 mg/liter doxycycline to the growth medium did not alter the accumulation of the repRNA (Fig. 3-2, row A1) in the case of the parental yeast strain (which carries all yeast genes that are expressed from their natural promoters), suggesting that the presence of doxycycline did not affect replication of the DI-AU-FP repRNA in the parental yeast strain.

Identification of 30 essential host genes affecting tombusvirus replication
We performed high-throughput screening of the 800 strains present in the yTHC collection. Total RNA was extracted from samples (at least six samples per yeast strain, three each from yeast grown with or without doxycycline), electrophoresed in 1.5% agarose gel, stained with ethidium bromide, transferred to nylon membrane, and analyzed by northern blotting specific for the 3' end of DI-AU-FP (data for 15 selected strains are shown in Fig. 3-2, rows A1 and B1, and Fig. 3-3, row 1). We found that 9 of the yTHC strains were not transformable with the two plasmids and 35 strains grew too slowly to obtain enough viral and host RNAs for subsequent analysis (the list of genes omitted is shown in Table 3-5). Out of the remaining yeast strains, we found that 19 strains showed a 2- to-5-fold-decreased level and 11 strains showed between a 50 and 450% increased level of repRNA accumulation in the presence of doxycycline (Table 3-1). We scored only those strains that showed at least 50% or more change in repRNA accumulation (Panavas, Serviene et al., 2005). It is important to note that we compared the accumulation levels of repRNA for each yeast strain grown in the absence versus in the presence of doxycycline, instead of comparing with the repRNA accumulation in the parental yeast strain. This is because the expression level of the particular host protein from the TET promoter is expected to differ from its expression from the original promoter, which could be either higher or lower than that from the TET promoter.
We retested the above 30 yTHC yeast strain s with DI-72 repRNA, which is capable of 5-fold more robust replication (100,000 RNA copies per cell) than DI-AU-FP repRNA used above (Serviene, Shapka et al., 2005). DI-72 repRNA is the prototypical DI RNA carrying four noncontiguous regions (RI to RIV) from the TBSV genomic RNA, whereas DI-AU-FP repRNA contains an artificial AU-rich sequence between RI and RII that could promote recombination in yeast and in plants (Fig. 3-1) (Shapka and Nagy, 2004; Serviene, Shapka et al., 2005; Serviene, Jiang et al., 2006). Northern blot analysis of total RNA extracts obtained from the selected yeast strains coexpressing DI-72 repRNA, p33, and p92 revealed that DI-72 repRNA replication was also affected by the above identified 30 yeast genes (data for 15 selected strains are shown in Fig. 3-2, rows A2 and B2, and Fig. 3-3, row 2). Based on these screens, we conclude that the 30 identified host factors affected the accumulation of two different TBSV repRNAs.

The 30 essential host genes identified in the above screen code for proteins with different molecular functions in various cellular processes (Yeast Genome Database, SGD; http://www.yeastgenome.org). These include RNA binding/processing (UTP9, UTP15, NAB2, PRP39, RNA14, MEX67, NOP4, RPL15A, and RRP9), RNA helicase/unwinding (DED1, PRP5, and SEN1), RNase (RRP42), or RNA polymerase/RNA transcription (RPO21, MED6, RPB11, TFA2, and ARP9) (Table 3-1). Others are involved in protein modification (SLN1, MOB1, and EPL1), protein synthesis (RPL17A), protein transport (COP1), or lipid biosynthesis (ERG25). LPD1 codes for a pyruvate dehydrogenase, RSC8 is involved in chromatin remodeling, and NOG1 and NOG2 code for putative GTPases. We also identified two genes with currently unknown functions (GRC3 and YDR327W) (Table 3-1). Altogether, the identified host factors could have either direct or indirect effects on tombusvirus replication (see below).

Effect of the identified host factors on transcription of viral RNA and on amounts of p33 and p92 replication proteins

To determine if the identified host factors affect the amount of viral RNA transcripts (the initial templates) generated from the yeast expression plasmid (pGBK-His33/DI-72), I performed northern blot analysis on nine selected strains, which decreased (Fig. 3-2A and B), and five strains that increased repRNA levels in the presence of doxycycline (Fig. 3-
The selected yeast strains expressed only DI-72 RNA and p33, but not p92, in these experiments to facilitate detection of the DI-72 RNA transcripts in the absence of replication. Comparison of the accumulation of DI-72 transcripts in nine of the yTHC strains that decreased replication of DI-72 RNA (Fig. 3-2, rows A5 and B5) in the presence of doxycycline revealed that the amount of DI-72 RNA transcripts decreased in six strains (DED1, COP1, SLN1, RPO21, NAB2, and MED6), increased in two strains (UTP15 and UTP9), and did not change in one strain (MOB1), whereas the transcripts increased slightly in the parental strain in the presence of doxycycline. Altogether, we did not find good correlation between altered DI-72 transcript levels and the alteration in replication of the repRNA.

Testing six of the yTHC strains that showed increased replication of DI-72 RNA (Fig. 3-3, row A5) in the presence of doxycycline, however, revealed a correlation between the elevated levels of DI-72 transcripts and increased levels of repRNA accumulation in five out of six strains (NOG1, NOP4, ARP9, GRC3, and RPL15A) in the presence of doxycycline. Therefore, it is possible that the initial amount of repRNA template could affect subsequent accumulation of repRNA in some yeast strains.

Since the amount of p33 replication cofactor could affect replication and p33 is the most abundant protein in the tombusvirus RC (Lai, 1992; Nagy and Simon, 1997), we tested the level of p33 present in total protein extracts obtained from 15 selected yTHC strains in comparison with the parental yeast using western blotting (Fig.3-2, rows A6 and B6, and Fig. 3-3, row 6) (Panaviene, Panavas et al., 2004). We also tested the membrane-enriched fraction (Panaviene, Panavas et al., 2004; Panaviene, Panavas et al., 2005), which contains the active tombusvirus replicase, for the levels of p33 (Fig. 3-2, rows A8 and B8, and Fig. 3-3, row 8) and the less abundant p92 (Fig. 3-2, rows A7 and B7, and Fig. 3-3, row 7).

These experiments revealed close correlation between level of p33 in total protein extracts and the level of p33 in the membrane-enriched fraction. This is not surprising since most p33 was localized to peroxisomal membranes in yeast (Panavas, Hawkins et al., 2005). We also found that three strains (DED1, SLN1, and RPO21) accumulated p33 (both in total and membrane-enriched fractions; Fig. 3-2, rows A6 and B6 and A8 and
B8) at a reduced level in the presence of doxycycline among the nine selected strains that showed reduced levels of DI-72 RNA accumulation. The remaining six strains (COP1, MOB1, UTP9, UTP15, NAB2, and MED6) showed comparable p33 levels when grown in the absence or presence of doxycycline (Table 3-2). The level of p92 was reduced in five strains (DED1, COP1, MOB1, NAB2, and RPO21; Fig. 3-2, rows A7 and B7), while it did not change in one strain (SLN1). An additional two strains (UTP9 and UTP15) contained small amount of p92 independent of doxycycline, whereas the MED6 strain showed a somewhat higher p92 level in the presence of doxycycline (Fig. 3-2, rows A7 and B7). Therefore, it is possible that the reduced amount of either p33 or p92 or both inhibits the replication of DI-72 repRNA in several, but not all, of the nine selected strains that supported reduced DI-72 RNA accumulation in the presence of doxycycline.

The picture of the possible role of alteration of p33/p92 levels on tombusvirus replication, however, is more complex for those strains that showed an increased level of DI-72 RNA accumulation in the presence of doxycycline (Fig. 3-3, row 8). For example, among the six selected strains, the level of p33 increased in the ARP9 strain and did not change in the NOG1, NOP4, GRC3, PRP5, and RPL15A strains in the presence of doxycycline (Fig. 3-3, row 8). In the presence of doxycycline, the level of p92 decreased in NOG1, NOP4, GRC3, and RPL15A strains and increased in the ARP9 strain, but it did not change in the PRP5 strain (Fig. 3-3, row 7; Table 3-2). Altogether, the changes in p33 and p92 levels do not show good correlation with replication levels (Table 3-2). Therefore, these data do not support the idea that all of the host genes identified in this work would affect replication of the repRNA by affecting p33 and p92 levels in host cells. However, it is possible that several genes, such as DED1, COP1, SLN1, MOB1, NAB2, RPO21, and ARP9, might affect repRNA accumulation via changing the accumulation levels of one or both of the p33 or p92 replication proteins (Table 3-2).

The tombusvirus RC contains 10- to 20-fold more p33 than p92 when purified from the parental yeast (Panaviene, Panavas et al., 2004; Rajendran and Nagy, 2006). Changing the above ratio between p33 and p92 might affect the efficiency of replication (Rajendran and Nagy, 2004; Rajendran and Nagy, 2006). Indeed, several of the identified yeast strains (COP1, MOB1, NOG1, NOP4, GRC3, and RPL15A; Fig. 3-2, rows A7 and -8 and
Fig. 3-3, rows 7 and 8) produced p92 at reduced level, whereas the levels of p33 remained comparable in the absence versus in the presence of doxycycline (Table 3-2). For example, down-regulation of\ COP1\ and\ MOB1\ reduced only the level of p92, but not that of p33 in the presence of doxycycline (Fig. 3-2, rows A7 and -8). Therefore, altering the ratio of p33 to p92 by the host factor might contribute to altered rate of tombusvirus replication in selected yeast strains.

**Reduced CNV replicase activity obtained from yeast strains that supported reduced DI-72 RNA accumulation in the presence of doxycycline**

To test if some of the host factors identified above could affect replication of the repRNA via altering the activity of the CNV RC, we performed functional assays with the tombusvirus replicase obtained from selected yeast strains. First, we prepared membrane-enriched fraction (Panaviene, Panavas et al., 2004; Panaviene, Panavas et al., 2005) from each selected yeast strain coexpressing p33, p92, and DI-72 repRNA. Subsequently, the obtained membrane-enriched preparations containing the CNV RC were tested in the presence of four ribonucleotides, including 32P-labeled UTP. Under these conditions, the CNV replicase completes RNA synthesis on the endogenous templates (i.e., on the viral RNA that was copurified with the RC, an in vitro reaction called "runoff synthesis"), which are part of the RC actively synthesizing viral RNA in the yeast cells at the time of extraction. The in vitro replicase runoff synthesis demonstrated that the CNV replicase synthesized 4- to 17-fold-decreased amounts of products in vitro when the replicase preparations were obtained from yeast strains with down-regulated expression of the \SLN1, MOB1, DED1, COP1, UTP15, UTP9, and NAB2\ genes, respectively, versus the replicase obtained from the parental yeast (Fig. 3-4A). Reduction of in vitro CNV replicase activity and the reduced in vivo accumulation of repRNA with the above strains in the presence of doxycycline were in good correlation (Table 3-2), suggesting that down-regulation of these genes might affect repRNA levels via inhibition of CNV replicase activity.

**Reduced ratio of plus- versus minus-strand synthesis by the CNV replicase obtained from selected strains**

One of the hallmark features of plus-stranded RNA viruses is asymmetrical strand
synthesis, which results in 10- to 100-fold more abundant plus strands than the minus-stranded intermediates (Buck, 1996; Ahlquist, 2002). To test if down-regulation of the expression levels of the \textit{SLN1}, \textit{MOB1}, \textit{DED1}, \textit{COP1}, \textit{UTP15}, \textit{UTP9}, and \textit{NAB2} genes could affect the ratio of plus- versus minus-strand RNA synthesis by the CNV replicase, we first performed replicase runoff experiments (see the above section) that produced 32P-labeled RNA based on the copurified repRNAs present in the replicase fraction, followed by using the labeled RNAs as probes in RNA blotting (Panaviene, Panavas et al., 2004). The target templates were the same amounts of denatured plus- and minus-stranded DI-72 repRNA fixed separately onto nylon membranes (Fig. 3-4B). After hybridization of the membranes with the 32P-labeled runoff products obtained from the in vitro replicase reactions, we measured the ratio of plus- versus minus-strand-specific signals on the RNA blots using a PhosphorImager (Nagy and Pogany, 2000; Panaviene, Panavas et al., 2004; Stork, Panaviene et al., 2005). These experiments demonstrated that the CNV replicase from \textit{SLN1}, \textit{MOB1}, \textit{DED1}, \textit{COP1}, \textit{UTP15}, \textit{UTP9}, and \textit{NAB2} yeast strains grown in the presence of doxycycline synthesized only 1- to 4-fold more plus strands than minus strands, whereas the CNV replicase from the same strains grown in the absence of doxycycline produced 7- to 11-fold more plus strands than minus strands (Fig. 3-4B). In comparison, the CNV replicase obtained from the parental strain produced 12- to 13-fold more plus strands than minus strands under both conditions (Fig. 3-4B). The reduction in ratio of plus-stranded versus minus-stranded repRNA was the largest for \textit{DED1} (11-fold) and intermediate for \textit{SLN1}, \textit{MOB1}, \textit{COP1}, \textit{UTP15}, \textit{UTP9}, and \textit{NAB2} (3- to 5-fold) (Fig. 3-4B). The altered ratio of plus- and minus-strand synthesis suggests that these host factors could affect the assembly and/or the functions of the replicase. Future experiments will be aimed at dissecting the role of these host factors in the asymmetrical RNA synthesis.

\textit{Down-regulation of NOG1, ARP9, and PRP5 expression alters the activity of the CNV replicase}

The in vitro replicase runoff synthesis performed with the membrane-enriched fraction showed two- and fivefold increases in the amount of in vitro RNA products when the replicase preparations were obtained from yeast strains with down-regulated expression of \textit{NOG1} and \textit{ARP9}, respectively, whereas the CNV replicase activity obtained from the
PRP5 strain was comparable to the replicase preparation obtained from the parental yeast in the absence or presence of doxycycline (Fig. 3-5A). The ratio of plus versus minus strand synthesized by the CNV replicase preparations obtained from NOG1, ARP9, and PRP5 yeast strains grown in the absence of doxycycline synthesized was rather variable with the ratio of plus strands and minus strands ranging from 3 to 16, whereas the CNV replicase from the same strains grown in the presence of doxycycline showed an increased ratio between plus strands and minus strands by 20 to 400% (Fig. 3-5B). The increase in ratio of plus-stranded versus minus-stranded repRNA was the largest for the ARP9 strain (fourfold), intermediate for the NOG1 strain (45%), and less pronounced in the PRP5 strain (20%) (Fig. 3-5B). In comparison, the CNV replicase obtained from the parental strain showed 15% reduction in the ratio of plus strands versus minus strands when grown in the presence of doxycycline (Fig. 3-5B).

The above data with the CNV replicase preparations obtained with yeast strains supporting increased DI-72 RNA accumulation in the presence of doxycycline suggest that down-regulation of ARP9 and, to a less extent, NOG1 expression facilitated the assembly of more tombusvirus RC or increased the activity of those complexes, whereas down-regulation of PRP5 had a somewhat smaller effect (Table 3-2). Moreover, the observations that the tombusvirus replicase was defective when obtained from the ARP9 yeast strain in the absence of doxycycline and only partly improved in the presence of doxycycline (Fig. 3-5, lanes 5 and 6) suggest that expression of ARP9 from the TET promoter has an inhibitory effect on replicase activity and on repRNA replication (Fig. 3-3, panels A1 and A2).

**Discussion**

*Two percent of all host genes affect tombusvirus replication*

Among the major ongoing research areas with plus-strand RNA viruses are (i) cataloging all the host genes affecting virus replication and (ii) definition of the roles of the identified factors in various steps of the replication process (Nagy and Pogany, 2006). Utilizing the yeast-based efficient replication of a tombusvirus repRNA and the available yeast genomic libraries, the effect of most of the host genes on tombusvirus replication has been studied with 4,800 nonessential single-gene deletions in the YKO library.
(Panavas, Serviene et al., 2005) and with 800 essential yeast genes in the yTHC library (this work). These genomic screens have led to the identification of 96 nonessential and 30 essential host genes, respectively, which affected the accumulation of the tombusvirus repRNA by 50% or more. Altogether, the two genetic screens combined have covered close to 95% of all the genes (estimated to be 5,800) present in the yeast genome. Therefore, the identified 126 host genes that affected repRNA accumulation by more than 50% when compared to the parental strain represent 2% of all the yeast genes tested. The identified host genes among the essential genes represent a higher ratio (3.75%) than among the nonessential genes (2%), suggesting that tombusviruses might have adapted to use and/or depend on essential genes to higher extent than nonessential genes. Altogether, this number of host genes is probably an underestimation, because genetic screens frequently overlook redundant genes (such as gene families) with similar functions whose deletion/down-regulation could be compensated for by other genes (Serva and Nagy, 2006). Nevertheless, the identified host genes represent a diverse set of host genes shown to affect viral RNA replication, and thus they should be valuable for studies on the mechanism of tombusvirus replication.

**Possible roles of the identified host factors in tombusvirus replication**

Measuring the effect of down-regulation of selected essential host genes on (i) the level of initial viral RNA transcripts (Fig. 3-2 and 3-3), (ii) the amount of p33 and p92 produced (Fig. 3-2 and 3-3), (iii) the activity of the viral replicase to synthesize viral RNA products, and (iv) the ratio of the plus versus minus strands (Fig. 3-4 and 3-5) revealed that many of the identified genes, such as \textit{DED1, SLN1, NAB2, RPO21,} and \textit{ARP9}, might affect repRNA accumulation via changing the accumulation levels of both p33 and p92 replication proteins (Table 3-2). On the other hand, down-regulation of \textit{COP1} and \textit{MOB1} reduced only the p92 level but had less of an effect on the amount of p33 in the presence of doxycycline (Fig. 3-2, rows A7 and -8) (Table 3-2). Therefore, either reducing the amount of p92 or altering the ratio of p33 to p92 by \textit{COP1} and \textit{MOB1} might contribute to the altered rate of tombusvirus replication in these yeast strains. The altered p33/p92 levels or the changes in p33/p92 ratio could have direct effects on the assembly of the tombusvirus replicase, as shown by us earlier (Rajendran and Nagy, 2006). This is supported by the correlation between the reduced activity of the CNV
replicase in vitro and the reduced in vivo accumulation of repRNA with the above strains in the presence of doxycycline. The above correlation is also valid for *ARP9* and, to a less extent, for *NOG1*, which enhanced CNV replicase activity and also increased DI-72 RNA accumulation in the presence of doxycycline. The correlation is weaker in case of *PRP5*, whose down-regulation had only small effect on CNV replicase activity yet led to 65% enhanced replication. It is possible that down-regulation of *PRP5* stimulates replication via enhancing the asymmetry of strand synthesis (Fig. 3-5), instead of affecting the total activity of the RC.

Roles of the identified host factors in tombusvirus replication might also be deducted from their known cellular functions. Therefore, below we will discuss briefly a group of identified host factors with known cellular functions in protein translation, modification, and stability. For example, *DED1* codes for an RNA helicase that is an essential translation factor (Linder, 2003; Cordin, Tanner et al., 2004; Yang and Jankowsky, 2005). It is likely that the reduced level of Ded1p in the presence of doxycycline directly inhibits p33 and p92 translation, which in turn could lead to a reduced level of RC assembly, resulting in reduction in repRNA replication. Interestingly, Ded1p was also found to affect BMV 2a replicase production and BMV replication in yeast (Noueiry, Chen et al., 2000), suggesting that Ded1p might be an important, widely used regulator of viral protein synthesis. The core protein of hepatitis C virus has been shown to bind to DBX, a DEAD-box RNA helicase, which can complement ded1p mutation in yeast (Mamiya and Worman, 1999). The HCV core protein-DBX interaction might explain how HCV infections could inhibit host cell translation. In addition, Ded1p was shown to bind to the particles of the yeast L-A virus, a double-stranded RNA virus, and promote the L-A virus negative-strand RNA synthesis in vitro (Chong, Chuang et al., 2004). It is important to point out that down-regulation of Ded1p likely affects translation of host genes too; therefore, further experiments will be needed to demonstrate if translation of the tombusvirus p33 and p92 is selectively inhibited in yeast with a reduced Ded1p level.

Reduced expression of *MOB1* selectively decreased the amount of p92, but not that of p33, in the presence of doxycycline (Fig. 3-4, lanes 5 and 6). The Mob1/phocein family of proteins, which are activating subunits of Dbf2-related protein serine/threonine
kinases, are essential in yeast, and they are also present in all eukaryotic cells (Devroe, Erdjument-Bromage et al., 2004). Therefore, it is possible that the reduced stability of p92 could be due to altered posttranslational modification of p92 when a reduced amount of Mob1p is present.

Down-regulation of $SLN1$, which codes for a membrane-bound histidine kinase, moderately decreased the level of p33 and had only a minor effect on p92 levels in the CNV replicase (Fig. 3-4, lanes 3 and 4), whereas replicase activity and repRNA accumulation were changed by 4- and 2.5-fold, respectively. It is possible that Sln1p might affect the activity/amount of p33, and thus replication of recRNA, directly. We cannot yet exclude, however, that the effect of Sln1p on repRNA replication is only indirect via affecting the activity of transcription factors, which could regulate the functions of many host proteins. Interestingly, however, Sln1p is known to interact with Cop1p, another host factor affecting repRNA accumulation (Table 3-1; also see below).

**Comparison of host factors affecting tombusvirus replication versus RNA recombination based on genome-wide screens**

One of the characteristic features of plus-stranded RNA viruses is high recombination frequency, which promotes rapid virus evolution, a major problem for the host antiviral defense and development of long-lasting antiviral strategies (Worobey and Holmes, 1999; Roossinck, 2003). The most frequent RNA recombination is based on template switching by the viral replicase during virus replication (Nagy and Simon, 1997; Worobey and Holmes, 1999). Thus, an intriguing question is whether host genes that affect virus replication could also affect RNA recombination. To answer this question, we recently performed genome-wide screens with the YKO (Serviene, Shapka et al., 2005) and yTHC collections (Serviene, Jiang et al., 2006) to identify host genes affecting tombusvirus RNA recombination. Comparison of the identified essential host genes in the yTHC collection revealed that down-regulation of four of the identified genes affected both RNA recombination (Serviene, Jiang et al., 2006) and tombusvirus replication (this work) (Table 3-3). Among these host genes identified, $COP1$ is the most interesting, because down-regulation of $COP1$ decreased repRNA accumulation, but increased the accumulation of recombinant RNAs (recRNAs) (Serviene, Jiang et al., 2006). Because
Cop1p is involved in intracellular protein transport, it is possible that it could affect the assembly of the viral RC and/or the intracellular targeting/localization of the viral RC. The altered RC then might be less efficient for replication, but more prone for recombination events. The possible role of Cop1p in tombusvirus replication has also been suggested by McCartney et al. (McCartney, Greenwood et al., 2005), who found that inhibition of vesicle formation at peroxisomes (which requires Cop1p activity) by expression of a dominant-negative mutant of ADP-ribosylation factor 1 affected intracellular sorting of p33 in plant cells.

Another interesting gene is \textit{ARP9}, which increased repRNA accumulation (Table 3-1), but decreased the ratio of recRNA versus repRNA (Serviene, Jiang et al., 2006) when down-regulated. Thus, a small amount of Arp9p might somehow increase the fidelity of virus replication, albeit the process could be indirect via an additional host factor.

The other two common host genes (\textit{RPB11} and \textit{RRP9}) reduced the accumulation of both repRNA and recRNAs in the presence of doxycycline (Table 3-3). The availability of less viral RNA templates due to reduced replication could also lead to reduced recombination (albeit recombination was affected to a higher extent than replication), suggesting that these factors might have an indirect effect on RNA recombination. Nevertheless, these studies should open new ways to study the involvement of host genes in virus replication/recombination and they will be useful in understanding the mechanism of virus replication/recombination.

\textit{Interactions among host factors that might influence tombusvirus replication}

Host proteins do not function alone in the host cells, but interact with many other proteins forming dynamic complexes or protein networks that perform various functions (Cusick, Klitgord et al., 2005; Dunker, Cortese et al., 2005; Rual, Venkatesan et al., 2005). Therefore, it is possible that viruses take advantage of some of these complexes (such as the ribosome for translation) during virus replication. In an opposite scenario, viruses might have to compete for selected host proteins with other host proteins in order to recruit the selected host proteins away from regular cellular processes (such as intracellular transport or those taking place in the nucleus). The advantage of genome-wide screens is that they could possibly identify host factors involved in either of the
above scenarios. Therefore, we searched the SGD database for documented host protein interactions (Table 3-4).

The most intriguing interaction found is between Sln1p and Cop1p, both of which down-regulates repRNA accumulation in the presence of doxycycline (Table 3-4). It is possible that Sln1p kinase affects the intracellular transport activity of Cop1p, or vice versa, Cop1p might affect the intracellular location of Sln1p kinase, which then could affect the assembly of the functional tombusvirus RC.

An example for competition between host proteins and tombusvirus replication could involve Nog1p and Nop4p. Down-regulation of either of these proteins, which interact with each other (Table 3-4) with doxycycline, led to increased accumulation of repRNA. We speculate that, to perform a cellular function, Nog1p and Nop4p might associate with a common host factor, which is also needed for tombusvirus replication. When either Nog1p or Nop4p is present only in a reduced amount, then the tombusvirus replicase proteins or RNA might gain easier access to this putative common host factor.

Interestingly, we found host factors that affected replication and interacted with other host proteins that also influenced tombusvirus recombination. For example, Lpd1p interacts with Dci1p, which is involved in fatty acid metabolism and localized to the peroxisome—where tombusvirus replication takes place (Navarro, Rubino et al., 2004; Panavas, Hawkins et al., 2005). Absence of Dci1p was found to reduce the accumulation of tombusvirus recombinants (Serviene, Shapka et al., 2005). Therefore, it is possible that interaction between host factors could influence not only tombusvirus replication, but RNA recombination as well.

Interactions among other host proteins might also help group host factors affecting tombusvirus replication only indirectly. For example, Med6p, Rsc8p, and Arp9p are known to interact with Rox3p, Sin3p, and Rsc8p, respectively, which could affect transcription in the host cells, possibly altering the amounts of other host factors necessary for virus replication and/or the amounts of viral proteins and viral RNA transcripts.
Conclusions

The identified host genes in the previous (Panavas, Serviene et al., 2005) and current (Table 1) works reveal a complex picture for the host in tombusvirus replication. For example, host proteins in rather diverse groups, such as (i) RNA-binding proteins, ribonucleases, helicases; (ii) intracellular transport proteins; (iii) kinases and phosphatases; (iv) protease, protein reductase, and endopeptidase; (v) transcription factors; (vi) DNA replication factors; and (vii) proteins with unknown functions, were implicated. In addition, some of the above proteins stimulated, while others inhibited, tombusvirus replication. Overall, the systematic genome-wide screens likely identified proteins playing direct or indirect functions in tombusvirus replication. Further detailed studies will establish the functional roles of many of the identified proteins in tombusvirus replication.
Figure 3-1. Schematic representation of launching replication of TBSV DI RNA replicon (repRNA) in yeast strains based on the yTHC collection. Tombusvirus p33 and p92 replication proteins are expressed constitutively from the ADH1 promoter, whereas DI-AU-FP repRNA (or the wild-type DI-72 RNA; not shown) is expressed from the regulatable GAL1 promoter. Replication of repRNA takes place in the cytoplasm (on peroxisomal membrane surfaces). The expression of a particular host gene occurs in the absence of doxycycline (−Dox) (as shown in panel A), whereas the expression of the particular host gene is switched off in the presence of doxycycline (+Dox) (as shown in panel B). Replication of DI-AU-FP repRNA with four noncontiguous regions (RI to RIV, also present in DI-72 RNA [not shown]) derived from TBSV genomic RNA and the artificial AU-FP region is shown via a minus-stranded intermediate RNA (Shapka and Nagy, 2004).
Figure 3-2. Representative group of yTHC yeast strains supporting a decreased level of TBSV repRNA accumulation in the presence of doxycycline. Panels A and B include different sets of host genes as shown. Rows A1 and B1 show northern blot analysis of total RNA extracts from the shown yeast strains was performed with a radiolabeled RNA complementary to RIII/IV. Four independent samples are shown for each strain; two samples were grown without (−Dox) and two with (+Dox) doxycycline to illustrate the reproducibility of repRNA accumulation. An arrow points at the DI-AU-FP replicon RNA. Rows A2 and B2 show northern blot analysis of DI-72 repRNA accumulation. The probe was complementary to RIII/IV. Rows A3 and B3 show ethidium bromide-stained agarose gel to demonstrate DI-72 repRNA accumulation in selected yeast strains. Rows A4 and B4 show ethidium bromide-stained agarose gel with 18s rRNA as a control for yeast growth. Rows A5 and B5 show northern blot analysis of
DI-72 RNA transcripts in the absence of replication from total RNA extracts obtained from selected yTHC yeast strains coexpressing p33, but lacking p92. Rows A6 and B6 show western analysis of p33 replication protein in total protein samples using anti-His-tagged antibody. Rows A7 and B7 show western analysis of p92 and rows A8 and B8 show western analysis of p33 replication proteins in membrane-enriched fractions.
Figure 3-3. Identification of yTHC yeast strains supporting increased level of repRNA accumulation in the presence of doxycycline. See further details in the legend to Fig. 2.
Table 3-1. Name and functions of the essential host genes affecting repRNA accumulation

<table>
<thead>
<tr>
<th>Gene name</th>
<th>%Rep⁴</th>
<th>Molecular function²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Decreased accumulation of repRNA:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COP1</td>
<td>29</td>
<td>protein transporter, ER to Golgi and retrograde transport</td>
</tr>
<tr>
<td>DED1</td>
<td>43</td>
<td>DEAD-box RNA helicase, translation</td>
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<tr>
<td>EPL1</td>
<td>46</td>
<td>histone acetyltransferase activity</td>
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<tr>
<td>ERG25</td>
<td>43</td>
<td>ergosterol biosynthesis</td>
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<tr>
<td>LPD1</td>
<td>50</td>
<td>dihydrolipoyl dehydrogenase activity/ pyruvate dehydrogenase</td>
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<td>MED6</td>
<td>35</td>
<td>RNA polymerase II transcription mediator activity</td>
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<tr>
<td>MEX67</td>
<td>17</td>
<td>Poly(A)RNA binding protein</td>
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<tr>
<td>MOB1</td>
<td>18</td>
<td>protein amino acid phosphorylation</td>
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<tr>
<td>NAB2</td>
<td>35</td>
<td>Nuclear polyadenylated RNA-binding protein; related to human hnRNPs</td>
</tr>
<tr>
<td>PRP39</td>
<td>36</td>
<td>RNA binding, nuclear mRNA splicing</td>
</tr>
<tr>
<td>RNA14</td>
<td>48</td>
<td>RNA binding/ mRNA cleavage</td>
</tr>
<tr>
<td>RPB11</td>
<td>34</td>
<td>RNA polymerase</td>
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<td>RPO21</td>
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<td>RNA polymerase</td>
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<td>RRP42</td>
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<td>3'-5'-exoribonuclease activity</td>
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<td>TFA2</td>
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<td>general RNA polymerase II transcription factor activity</td>
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<td>UTP15</td>
<td>43</td>
<td>snoRNA binding, interacts with UTP9</td>
</tr>
<tr>
<td>UTP9</td>
<td>28</td>
<td>snoRNA binding, interacts with UTP15</td>
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### Table 3-1. Name and functions of the essential host genes affecting repRNA accumulation (continued)

**Increased accumulation of repRNA:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Value</th>
<th>Function</th>
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</thead>
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<tr>
<td><em>ARP9</em></td>
<td>450</td>
<td>Actin-related protein involved in transcriptional regulation</td>
</tr>
<tr>
<td><em>GRC3</em></td>
<td>200</td>
<td>Unknown, possibly involved in rRNA processing</td>
</tr>
<tr>
<td><em>NOG1</em></td>
<td>211</td>
<td>Putative GTPase</td>
</tr>
<tr>
<td><em>NOG2</em></td>
<td>175</td>
<td>Putative GTPase</td>
</tr>
<tr>
<td><em>NOP4</em></td>
<td>184</td>
<td>RNA binding, ribosomal RNA processing</td>
</tr>
<tr>
<td><em>PRP5</em></td>
<td>164</td>
<td>RNA helicase in the DEAD-box family</td>
</tr>
<tr>
<td><em>RPL15A</em></td>
<td>290</td>
<td>Binds to 5.8 S rRNA</td>
</tr>
<tr>
<td><em>RPL17A</em></td>
<td>200</td>
<td>Structural constituent of ribosome</td>
</tr>
<tr>
<td><em>RRP9</em></td>
<td>306</td>
<td>RNA binding/processing</td>
</tr>
<tr>
<td><em>SEN1</em></td>
<td>200</td>
<td>Putative helicase required for processing of tRNAs, rRNAs, and snoRNAs</td>
</tr>
<tr>
<td><em>YDR327W</em></td>
<td>159</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

---

1. Percentage of repRNA accumulation in the particular yeast strain in the presence of doxycycline, compared to in the absence of doxycycline (representing 100%).
2. Based on the *Saccharomyces* Genome Database, SGD, at http://www.yeastgenome.org
Table 3-2. Effect of down-regulation of selected host genes on level of initial viral RNA, p33, and p92 replication proteins and on activity of the tombusvirus replicase

<table>
<thead>
<tr>
<th>Gene</th>
<th>% DI-72 repRNA accumulation$^b$</th>
<th>% Initial viral transcript level$^c$</th>
<th>% Replicase protein$^d$</th>
<th>% Replicase activity in vitro$^e$</th>
<th>Plus/ minus-strand ratio$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental</td>
<td>118</td>
<td>98.1</td>
<td>101</td>
<td>92</td>
<td>100.0</td>
</tr>
<tr>
<td>ARP9</td>
<td>450</td>
<td>343.0</td>
<td>212</td>
<td>325</td>
<td>517.0</td>
</tr>
<tr>
<td>COP1</td>
<td>29</td>
<td>43.5</td>
<td>95</td>
<td>&gt;1</td>
<td>35.6</td>
</tr>
<tr>
<td>DEDI</td>
<td>43</td>
<td>40.9</td>
<td>51</td>
<td>8</td>
<td>13.6</td>
</tr>
<tr>
<td>GRC3</td>
<td>200</td>
<td>300.0</td>
<td>102</td>
<td>50</td>
<td>NT</td>
</tr>
<tr>
<td>MED6</td>
<td>35</td>
<td>42.1</td>
<td>102</td>
<td>113</td>
<td>NT</td>
</tr>
<tr>
<td>MOBI</td>
<td>18</td>
<td>93.0</td>
<td>120</td>
<td>37</td>
<td>5.8</td>
</tr>
<tr>
<td>NAB2</td>
<td>35</td>
<td>44.3</td>
<td>85</td>
<td>&gt;1</td>
<td>19.4</td>
</tr>
<tr>
<td>NOG1</td>
<td>211</td>
<td>259.0</td>
<td>102</td>
<td>57</td>
<td>214.0</td>
</tr>
<tr>
<td>NOP4</td>
<td>184</td>
<td>307.0</td>
<td>88</td>
<td>59</td>
<td>NT</td>
</tr>
<tr>
<td>PRP5</td>
<td>164</td>
<td>94.6</td>
<td>120</td>
<td>128</td>
<td>104.0</td>
</tr>
<tr>
<td>RPL15</td>
<td>4290</td>
<td>342.0</td>
<td>83</td>
<td>27</td>
<td>NT</td>
</tr>
<tr>
<td>RPO21</td>
<td>27</td>
<td>25.5</td>
<td>69</td>
<td>43</td>
<td>NT</td>
</tr>
<tr>
<td>SLN1</td>
<td>41</td>
<td>12.5</td>
<td>59</td>
<td>78</td>
<td>25.0</td>
</tr>
<tr>
<td>UTP9</td>
<td>28</td>
<td>232.0</td>
<td>109</td>
<td>76</td>
<td>15.6</td>
</tr>
<tr>
<td>UTP15</td>
<td>43</td>
<td>154.0</td>
<td>113</td>
<td>61</td>
<td>16.3</td>
</tr>
</tbody>
</table>

$^a$ The above values reflect results from yeast grown in the presence of doxycycline in comparison with yeast grown in the absence of doxycycline (which represents 100% for each strain).

$^b$ Percentage of DI-72 repRNA accumulation in the particular yeast strain in the presence of doxycycline compared to the yeast strain grown in the absence of doxycycline (which represents 100% for each strain) (Table 3-1).

$^c$ Percentage of repRNA transcripts in the absence of p92 (Fig. 3-2 and 3-3, panels A5 and B5).

$^d$ Accumulation levels of p33 and p92 replication proteins (Fig. 3-2 and 3-3, panels A7 and -8 and B7 and -8).

$^e$ In vitro activity of CNV replicase preparations in the enriched membrane fractions (Fig. 3-4 and 3-5A).

$^f$ Ratio of plus- versus minus-strand synthesis based on in vitro activity of CNV replicase preparations on endogenous RNA templates (Fig. 3-4 and 3-5B).

$^g$ NT, not tested.
Figure 3-4. Down-regulation of expression of selected essential yeast genes inhibits the activity of CNV replicase in vitro. (A) (Top row) In vitro activity of CNV replicase present in membrane-enriched preparations. Each replicase preparation, obtained from the yeast shown coexpressing p33, p92, and DI-72 (+)RNA, was tested with the copurified endogenous template. Strains were grown in the absence (–DOX) or presence (+DOX) of doxycycline, as indicated. 32P-labeled RNA products from the above preparations were analyzed on denaturing 5% PAGE-8 M urea gels. For quantification, we measured the intensity of 32P-labeled RNA products by using a PhosphorImager. Activity of the CNV replicase obtained from the parental yeast strain in the absence of doxycycline corresponds to 100%. (Bottom row) A western blot shows the accumulation level of p33 in the membrane-enriched preparations. (B) Effect of down-regulation of selected essential genes on asymmetrical RNA synthesis by the CNV replicase obtained from the shown yeast strains. Unlabeled T7 RNA polymerase transcripts of DI-72 (+)RNA (marked as "+" or "||") and DI-72 (–)RNA (marked as "=") (400 ng each), respectively, were blotted on the membrane. The blotted RNAs were then hybridized
with denatured 32P-labeled RNA probes, which were generated by the CNV replicase in vitro in a runoff experiment on the endogenous templates present in the enriched membrane fractions obtained from selected yTHC strain grown in the absence (left row) or presence (right row) of doxycycline. The ratio between plus- and minus-stranded RNAs in the in vitro replicase assay was calculated based on PhosphorImager analyses from three separate experiments. For example, the value of 13.7 means that the CNV replicase produced 13.7-fold more plus-stranded RNA than minus-stranded RNA in vitro using the endogenous template.
Figure 3-5. Down-regulation of *NOG1*, *ARP9*, and *PRP5* expression alters the in vitro activity of the CNV replicase. See further details in the legend to Fig. 3-4.
Table 3-3. Host factors affecting both replication and RNA recombination

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Effect on Replication$^1$</th>
<th>Effect on Recombination$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>COP1</td>
<td>decrease</td>
<td>increase</td>
</tr>
<tr>
<td>RPB11</td>
<td>decrease</td>
<td>decrease</td>
</tr>
<tr>
<td>ARP9</td>
<td>increase</td>
<td>decrease</td>
</tr>
<tr>
<td>RRP9</td>
<td>decrease</td>
<td>decrease</td>
</tr>
</tbody>
</table>

$^1$Based on Table 1.
$^2$Based on Serviene et al., submitted.

Table 3-4. Interaction among the host proteins affecting tombusvirus replication or recombination

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein: function (effect on replication or recombination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ded1p</td>
<td>Xrn1p: 5’-3’ exoribonuclease (replication - down/recombination - up)</td>
</tr>
<tr>
<td>Ded1p</td>
<td>Nsr1p: RNA binding/rRNA processing (replication - up)</td>
</tr>
<tr>
<td>Cop1p</td>
<td>Sln1p: protein histidine kinase activity (replication - down)</td>
</tr>
<tr>
<td>Sln1p</td>
<td>Cop1p: protein transporter (replication - down)</td>
</tr>
<tr>
<td>Mob1p</td>
<td>Mps1p: protein threonine/tyrosine kinase (recombination - down)</td>
</tr>
<tr>
<td>Utp15p</td>
<td>Utp9: snoRNA binding (replication - down)</td>
</tr>
<tr>
<td>Utp9p</td>
<td>Utp15p: snoRNA binding (replication - down)</td>
</tr>
<tr>
<td>Utp9p</td>
<td>Gph1p: glycogen phosphorylase (replication - down)</td>
</tr>
<tr>
<td>Lpd1p</td>
<td>Dci1p: peroxisomal delta(3,5)-delta(2,4)-dienoyl-CoA isomerase, involved in fatty acid metabolism; (recombination - down)</td>
</tr>
<tr>
<td>Med6p</td>
<td>Rox3p: RNA polymerase II transcription mediator (replication - down)</td>
</tr>
<tr>
<td>Rsc8p</td>
<td>Sin3p: histone deacetylase (replication - down)</td>
</tr>
<tr>
<td>Rsc8p</td>
<td>Arp9p: Actin-related protein involved in transcriptional regulation; subunit of the chromatin remodeling Snf/Swi complex (replication - up, recombination - down)</td>
</tr>
<tr>
<td>Nog1p</td>
<td>Nop4p: RNA-bonding (replication - up)</td>
</tr>
<tr>
<td>Nop4p</td>
<td>Nog1p: Putative GTPase (replication - up)</td>
</tr>
<tr>
<td>Arp9p</td>
<td>Rsc8p: chromatin remodeling (replication - down)</td>
</tr>
</tbody>
</table>

$^a$ Based on references 26, 40, and 41.
### Table 3-5. The list of yeast strains from the yTHC library that were not tested

<table>
<thead>
<tr>
<th>Slow growing strains:</th>
<th>Strains missing from the original plates:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ORF name</strong></td>
<td><strong>gene name</strong></td>
</tr>
<tr>
<td>YDR168W</td>
<td>CDC37</td>
</tr>
<tr>
<td>YKL018W</td>
<td>SWD2</td>
</tr>
<tr>
<td>YLR106C</td>
<td>MDN1</td>
</tr>
<tr>
<td>YBR192W</td>
<td>RIM2</td>
</tr>
<tr>
<td>YDR437C</td>
<td>PRP3</td>
</tr>
<tr>
<td>YER006W</td>
<td>NUG1</td>
</tr>
<tr>
<td>YJR002W</td>
<td>MPP10</td>
</tr>
<tr>
<td>YKL203C</td>
<td>TOR2</td>
</tr>
<tr>
<td>YLR005W</td>
<td>SSL1</td>
</tr>
<tr>
<td>YLR145W</td>
<td>YLR145w</td>
</tr>
<tr>
<td>YLR223C</td>
<td>IFH1</td>
</tr>
<tr>
<td>YOR168W</td>
<td>GLN4</td>
</tr>
<tr>
<td>YNL310C</td>
<td>YNL310c</td>
</tr>
<tr>
<td>YPR019W</td>
<td>CDC54</td>
</tr>
<tr>
<td>YPR180W</td>
<td>AOS1</td>
</tr>
<tr>
<td>YPL010W</td>
<td>RET3</td>
</tr>
<tr>
<td>YOR146W</td>
<td>YOR146w</td>
</tr>
<tr>
<td>YOR169C</td>
<td>YOR169c</td>
</tr>
<tr>
<td>YBL034C</td>
<td>STU1</td>
</tr>
<tr>
<td>YNL150W</td>
<td>YNL150w</td>
</tr>
<tr>
<td>YNL256W</td>
<td>FOL1</td>
</tr>
<tr>
<td>YNL272C</td>
<td>SEC2</td>
</tr>
<tr>
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<td>ARP7</td>
</tr>
<tr>
<td>YOL120C</td>
<td>RPL18A</td>
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<td>YLL050C</td>
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</tr>
<tr>
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</tr>
<tr>
<td>YML077W</td>
<td>BET5</td>
</tr>
<tr>
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<td>CDC5</td>
</tr>
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<td>YNL061w</td>
</tr>
<tr>
<td>YDR498C</td>
<td>SEC20</td>
</tr>
<tr>
<td>YPL082C</td>
<td>MOT1</td>
</tr>
<tr>
<td>YLR323C</td>
<td>CWC24</td>
</tr>
<tr>
<td>YBL030C</td>
<td>PET9</td>
</tr>
<tr>
<td>YER165W</td>
<td>PAB1</td>
</tr>
<tr>
<td>YMR309C</td>
<td>TAD3</td>
</tr>
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</table>

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CHAPTER IV

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

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 (Kushner, Lindenbach et al., 2003; Cherry, Doukas et al., 2005; Panavas, Serviene et al., 2005; Serviene, Shapka et al., 2005; Jiang, Serviene et al., 2006; Serviene, Jiang et al., 2006; Hao, Sakurai et al., 2008; Krishnan, Ng et al., 2008; Tai, Benita et al., 2009). 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 responses of the host.

Tomato bushy stunt virus (TBSV) has emerged as one of the 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 (Panavas and Nagy, 2003; Nagy, 2008) and cell-free approaches (Panaviene, Panavas et al., 2004; Pogany and Nagy, 2008; Pogany, Stork et al., 2008). Replication of a short TBSV replicon (rep)RNA in yeast requires the co-expression of the viral p33 and p92 replication proteins, which form the membrane-associated viral replicase (Panaviene, Panavas et al., 2004; Panaviene, Panavas et al., 2005). 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 (Panavas, Serviene et al., 2005; Serviene, Shapka et al., 2005; Jiang, Serviene et al., 2006; Serva and Nagy, 2006; Serviene, Jiang et al., 2006; Li, Barajas et al., 2008; Li, Pogany et al., 2009). 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 (Serva and Nagy, 2006; Jonczyk, Pathak et al., 2007; Li, Barajas et al., 2008; Pathak, Sasvari et al., 2008; Pogany, Stork et al., 2008; Wang and Nagy, 2008; Li, Pogany et al., 2009; Wang, Stork et al., 2009). Other host proteins tested in more detail affected viral RNA degradation and viral recombination (Cheng, Serviene et al., 2006; Cheng, Jaag et al., 2007; Jaag and Nagy, 2009) or had only indirect effect on TBSV repRNA accumulation (Jaag, Stork et al., 2007). Importantly, the relevance of several host genes identified in yeast has also been confirmed in the natural plant host as well (Wang and Nagy, 2008; Jaag and Nagy, 2009; Wang, Stork et al., 2009). These discoveries justify the use of yeast model host for replication studies with TBSV.

In this paper, we further characterize the inhibitory role of the previously identified nucleolin (Nsr1p in yeast) in TBSV replication (Panavas, Serviene et al., 2005). Nucleolin/Nsr1p is an abundant, ubiquitously expressed protein, which is involved in ribosome biogenesis (Mongelard and Bouvet, 2007). Nucleolin also affects transcription of rDNA, processing and modification of rRNA and nuclear - cytosolic transport of ribosomal protein and ribosomal subunits by shuttling 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 rDNA transcription by interacting with rDNA 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 functions performed by nucleolin, which affect DNA and RNA metabolism, and its presence in
various subcellular locations (Mongelard 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, Suzuki et al., 2007; Pontvianne, Matia et al., 2007). The nucleolin gene from pea was able to complement *nsr1D* yeast by rescuing the reduced level of rRNA (Reichler, Balk et al., 2001), suggesting that the plant nucleolin has similar functions to the yeast *NSR1*.

Here, we confirm that Nsr1p/nucleolin is an inhibitor of TBSV replication. Overexpression 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.

**Materials and methods**

**Yeast and Escherichia coli plasmids**

To study the effect of over-expression 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, Serviene et al., 2006), pGAD-His92-CUP1 (containing the CNV p92 gene behind the *CUP1* promoter) (Li, Barajas 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 (CGCGGGATCCATGGCTAAGACTACTAAAG) and #1948 (CGCGCTCGAGTCAATCAAATGTTTTCTTTGAACC) from a yeast genomic DNA preparation. The PCR product was treated with BamHI 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 (CgcgAAGCTTACCATGGCTAAGACTACTAAAG) and #2832 (CGACCTCGAGTCACTTATCGTGTCATCCTTGAATCATCAAATGTTTTCTTTGAAAC-C) and the 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 35S promoter was the generous gift of Dr. K. Nakamura (Kojima, Suzuki et al., 2007). pGDG plasmid which can express autofluorescent proteins GFP was used as a control (Goodin, Dietzgen et al., 2002). The CNV expression plasmid pGD-CNv and pGD-p19 were described (Cheng, Jaag et al., 2007; Jaag and Nagy, 2009). The TRV plasmids pTRV1 and pTRV2 were described (Liu, Schiff et al., 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 plasmids at the BamHI and EcoRI sites by using PCR and primers #1972 (CgcgGGATCCGACTTCTCTTCTTTCTTTGAACC) and #2040 (CGCGGAATTCGTCGACTTCTCTTCTTTCTTTGAACC). Then, to obtain pGEX-NSR1, the full-length sequence of NSR1 (primers #1947 and #1948) was inserted between the BamHI and XhoI sites of modified pGEX-2T plasmid. Plasmid pGEX-NSR1ΔRBD was obtained by ligating together the PslI-treated DNA sequence representing the N-terminal part of NSR1 gene generated by PCR using primers #1947 and #1975 (CgcgCTGCAGAGTAGCTGGTTCTTC) and the C-terminal part with primers #1978 (CgcgCTGCAGACTTCTTTCTTTCTTCTTTGAACC) and #1948. The ligated PCR products were then inserted between the BamHI and XhoI 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 uracine, leucine and histidine as described before (Panaviene, Panavas et al., 2004). The transformed yeast cells were grown at 29°C for 24 hours 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-NSR1∆RBD 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 was resuspended in 1x 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, Panavas 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, Pogany 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, Panavas et al., 2004). Briefly, the yeast pellets were resuspended in 0.1M NaOH, followed by vortexing for 30s and shaking for another 10 min. Then, the samples were centrifuged at 15,000 x g for 5 min at 4 ºC and the pellet was resuspend in 1x SDS-PAGE buffer. The protein samples were electrophoresed 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 phosphotase (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 (50mM Tris-HCl, 10mM KCl, 15mM MgCl2, 2mM EDTA, 20% Glycerol), followed by centrifugation at 400 x g for 5 min at 4ºC. The supernatant was mixed with 0.5 volume of 3x SDS loading buffer and heated at 85ºC 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, Jaag et al., 2007). Briefly, expression plasmids pGWB5, pGDG and pGD-p19 and pGD-CNv or pGD-TRV1 / pGD-TRV2 were transformed into *Agrobacterium* C58C1. Tranformants were selected in LB medium containing 50μg/ml kanamycin, 100μg/ml rifampicin and 5μg/ml tetracycline. The transformed agrobacteria were grown in LB media containing the antibiotics and 20 μM acetosyringone at 29ºC 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 acetosyringone] and incubated for 2-4 hrs on the bench. We used the obtained Agrobacteria culture of 0.8~1.0 OD600 for agro-infiltration. Agrobacteria carrying pGWB5 (or pGD-GFP as a control), pGD-P19 and pGD-CNv were mixed in a ratio of 5:5:1 prior to infiltration to *N. benthamiana* leaves. For the TRV experiment, the
For silencing nucleolin, a segment of *N. benthamiana* nucleolin gene was cloned into virus-induced gene silencing (VIGS) vectors pTRV1 and pTRV2 (Dinesh-Kumar, Anandalakshmi et al., 2003). The sequence of the *N. benthamiana* nucleolin gene was not available but *N. tabaccum* was derived via a blast search based on the known *A. thaliana* nucleolin gene using the Solanaceae Genomics Resource (www.tigr.org) from J. Craig Venter Institute database. The Nb nucleolin segment was RT-PCR amplified from a total RNA extract of *N. benthamiana* using the following pair of primers: #2855 (CGACTCTAGACTGATGTAGAAATGGTTGATGC) and #2856 (CGACGGATCCAAACTCAATATAAGCCATCCC). The primes were designed based on *N. tabaccum* nucleolin sequences. To generate pTRV2-Nuc, a 500-bp cDNA fragment of *Nb* nucleolin The RT-PCR product was digested with XbaI–BamHI and cloned into XbaI–BamHI-digested pTRV2. The VIGS assay was described previously (Wang and Nagy, 2008). Nine days after agroinfiltration, the systemic silenced leaves were inoculated with the sap containing TBSV virions prepared from TBSV infected *N. benthamiana* leaves.

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, Jaag 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 nucleolin on CNV infections started via rub-inoculation, agrobacterium strains carrying pGWB5 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 et al., 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-72SXP (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 $^{32}$P-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 crosslinking assay was performed according to (Hirose and Harada, 2008). The reaction mixture was 12 µl containing 2 µg purified GST-Nsr1 protein, 10 ng (about 5nM) $^{32}$P-UTP labeled RNA probe, 10mM Hepes, pH 7.9; 100 mM KCl; 1mM MgCl$_2$; 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 50nM or 500nM amount. The reaction mixtures were incubated at room temperature for ~20 min to allow the formation of RNA-protein complexes. To crosslink 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 digested by 1mg/ml RNase A for 10 min at 37ºC. Samples were mixed with 0.5 volume 3x SDS loading dye and boiled for 10 min. Analysis was performed using SDS-PAGE and phosphoimaging.

For the band shift (gel mobility shift) assays, the reaction mixtures were set up as described above for UV crosslinking, except that the $^{32}$P-UTP labeled RNA probes were diluted ~50 times and 10U of RNase inhibitor was also included. Analysis was performed using 4% nondenaturing 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.
**In vitro replicase assays**

One of the replicase assays was based on the membrane-enriched fraction of yeast as described earlier (Panaviene, Panavas et al., 2004). Yeast co-transformed with pGAD-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 hr, then switched to 2% galactose for 4-5 hr before adding 50 µM copper sulfate to the media. After culturing for 22 hrs in the presence of copper sulfate, the yeast cells were harvest by centrifugation (Panaviene, Panavas 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 x g for 5 min at 4°C to remove cell debris. Then, the enriched membrane fraction was obtained by centrifugation at 21,000 x g for 10 min. Before the replicase reaction, we performed western blotting for estimating p33 levels in order to normalize the amount of p33 in each sample. The in vitro replicase 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 replicase, a yeast cell free extract was prepared as described previously (Pogany and Nagy, 2008). Briefly, yeast cells expressing p33/p92 from plasmids pGAD-His92 (Panaviene, Panavas et al., 2004) and pHisGBK-His33 (Panavas, Hawkins et al., 2005) were cultured in LH media containing glucose for 24 hr, followed by pelleting and resuspension in buffer A. After breaking the cells gently by glass beads, the cell debris was removed by centrifugation at 500 x g to obtain the cell free extract. The in vitro replication assays were also performed as described (Pogany and Nagy, 2008). The replication mixture (total of 20 µl) contained 1 µl cell free extract, 50 mM HEPES-KOH, pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate, 0.2 M sorbitol, and 0.4 µl actinomycin D (5 mg/ml), 2 µl of 150 mM creatine phosphate; 2 µl of 10 mM ATP, CTP, and GTP and 0.25 mM UTP; 0.3 µl of 32P-UTP, 0.2 µl of 10-mg/ml creatine kinase, 0.2 µl of RNase inhibitor, 0.2 µl of 1 M dithiothreitol, and 0.2 µg DI-72 (+)RNA transcript. The reaction mixture was incubated at 25°C for 3 hr and terminated by adding 110 µl stop buffer (1%
SDS and 0.05 M EDTA, pH 8.0), followed by phenol-chloroform extraction, isopropanol-ammonium acetate precipitation, and analysis with electrophoresis on 5% PAGE containing 8 M urea and phosphoimaging.

Confocal microscopy-based observation of nucleolin localization
Transgenic *N. benthamiana* expressing fibrillarin-RFP was kindly provided by Dr. Goodin (Goodin, Chakrabarty et al., 2007). Transient expression of pGWB5 (to express GFP tagged nucleolin), pGD-P19 and pGD-CNV via agroinfiltration was done as described above. The confocal laser microscopy was performed on an Olympus FV1000 (Olympus America Inc., Melville, NY). The images were acquired using sequential line-by-line mode in order to reduce excitation and emission crosstalk (Wang and Nagy, 2008). The primary objective used was water-immersion PLAPO60XWLSM (Olympus). Image acquisition was conducted at a resolution of 512 × 512 pixels and a scan-rate of 10μs/pixel. Image acquisition was performed by using Olympus Fluoview software version 1.5.

Results

Over-expression of Nsr1p inhibits TBSV repRNA replication in yeast
To test the effect of Nsr1p on TBSV repRNA accumulation in yeast, we over-expressed 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 p92 replication proteins and the TBSV repRNA (Fig. 4-1A). The accumulation of repRNA was measured via northern blotting 24 hours 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. 4-1A, lanes 4-6), while the inhibitory effect of 6xHis-tagged Nsr1p was less (by ~60%, lanes 1-3). Also, over-expression of the 6xHis-tagged Nsr1p inhibited repRNA accumulation in *nsr1D* yeast by ~3-fold (Fig. 4-1A, lanes 14-16), when compared with TBSV repRNA accumulation in *nsr1D* yeast. These experiments also confirmed that TBSV repRNA accumulation is 3-fold higher in *nsr1D* yeast (Fig. 4-1A, 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, 2003; Panaviene, Panavas et al., 2004). Over-expression 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. 4-1B, lanes 2-4 versus 5-7). Also, the amount of p33 made in yeast over-expressing Nsr1p was comparable to that obtained in the parental yeast expressing native level of Nsr1p (not shown). These data suggest that over-expression of Nsr1p does not affect the amount of plasmid-born repRNA, its processing 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 (Panaviene, Panavas et al., 2004; Panaviene, Panavas et al., 2005). As expected, we found that the tombusvirus replicase activity was ~3-fold lower when obtained from yeast over-expressing the FLAG-tagged Nsr1p (Fig. 4-1C, lanes 4-6) when compared with the preparation obtained from the parental BY4741 (lanes 1-3). On the contrary, the replicase preparation obtained from nsr1D 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.

Silencing nucleolin led to moderately increased accumulation of TBSV genomic RNA in plant

To test the effect of the Nb nucleolin on the accumulation of the TBSV gRNA, we took plant samples for RNA analysis 3 days after TBSV sap-incoculation of N. benthamiana leaves after knockdown of Nb nucleolin. RNA analysis (Fig 4-2 B) revealed that samples obtained from the nucleolin-silenced plants showed only 50% increased accumulation level of the TBSV gRNA compared with the non-silenced plants.
Expression of the plant nucleolin inhibits TBSV replication in Nicotiana benthamiana host cells

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, Suzuki 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 latter were analyzed via northern blotting to estimate the level of CNV RNA accumulation (Fig. 4-3A). 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 leaves (Fig. 4-3A, lanes 1-12 versus 13-24). The agroinfiltrated leaves expressed the GFP control at a higher level than AtNuc-L1-GFP (Fig. 4-3B). Over-expression of AtNuc-L1-GFP had only mild effect on the agro-infiltrated leaves during these experiments (Fig. 4-3C).

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, in *N. benthamiana* leaves agroinfiltrated 2 days earlier with a DNA construct expressing AtNuc-L1-GFP (Fig. 4-3D). The inhibitory effect of AtNuc-L1-GFP was less pronounced against infections started with TBSV virions (Fig. 4-3E). It is possible that higher level expression of AtNuc-L1-GFP is required against TBSV than CNV infection. Overall, these data demonstrate that the plant nucleolin can inhibit the accumulation of tombusvirus genomic RNAs in an experimental host, even when the infection is initiated with the highly infectious virions.

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. 4-3F, lanes 1-12 versus 13-24). Thus, nucleolin has different effects on tombus- versus tobraviruses, which belong to different supergroups of RNA viruses.
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, Lin et al., 2005; Pogany, White et al., 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 over-expression of Nsr1p from the galactose-inducible \textit{GAL1} promoter at various time points when compared with initiating TBSV replication from the copper-inducible \textit{CUP1} promoter (chosen as 0 hr time point, Fig. 4-4A). Over-expression of Nsr1p starting from 20 hr or 6 hr prior to launching TBSV replication resulted in ~10-15-fold inhibition of TBSV repRNA accumulation (Fig. 4-4B-C). This level of inhibition is higher than that obtained when Nsr1p was over-expressed from 0 hr time point (Fig. 4-1A, lanes 4-6). However, over-expression of Nsr1p 6 hours after launching TBSV replication (Fig. 4-4D) resulted in only a moderate level (by ~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 p92 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. 4-5A, lane 2). Deletion of the known RNA-binding domain in the recombinant Nsr1p (mutant GST-DRBD, lane 1) (Bouvet, Allain 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. 4-5C). Since the extent of the band shift increased with increasing amounts of GST-Nsr1, it is likely that more than one Nsr1p molecules 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. 4-6A) (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 RII(+) (Fig. 4-6B). Gel mobility-shift assays confirmed that Nsr1p binding to RIII(+) was the most efficient (Fig. 4-6C, lanes 8-9). However, RII(+) 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. 4-6D, lane 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 $^{32}$P-labeled full-length DI-72(+) RNA probe in a gel mobility-shift assay (Fig. 4-6E). Only RIII(+) competed efficiently with the labeled DI-72(+) RNA for binding to Nsr1p (Fig. 4-6E, lane 10), confirming that RIII(+) 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 UCCCGA sequence (Bouvet, Allain et al., 2001). Thus, it is likely that RIII(+) 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 (Panavas and Nagy, 2003; 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 DRIII repRNA, lacking RIII, was inhibited only slightly by the over-expression of Nsr1p (Fig. 4-7, lane 6-13), which inhibits DI-72 repRNA accumulation by 10-fold (Fig. 4-1A, 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 pre-incubated with GST-Nsr1 prior to adding to the cell-free extract (Fig. 4-8, 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 fibrillarin-RFP, a nucleolar marker protein (Kanneganti, Bai et al., 2007). Confocal laser microscopy revealed mostly nucleolar localization of AtNuc-L1 in both CNV infected and control plant cells (Fig. 4-9). 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 nucleus-to-cytosol re-distribution of nucleolin.

**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 ligase, which are part of the viral replicase complex together with p33 and p92 replication proteins. These host factors have been shown to regulate the ratio of plus- versus minus-strand synthesis; participate in the assembly of the replicase; promote insertion of viral proteins into subcellular membranes; enhance the stability of the viral replication proteins or affect their intracellular transportations (Serva and Nagy, 2006; Li, Barajas et al., 2008; Pathak, Sasvari et al., 2008; Pogany, Stork et al., 2008; Wang and Nagy, 2008; Li, Pogany et al., 2009; Wang, Stork et al., 2009). The detailed functions of additional host factors identified during previous genome-wide screens (Panavas, Serviene et al., 2005; Serviene, Shapka et al., 2005; Jiang, Serviene et al., 2006; Serviene, Jiang et al., 2006) are not yet known.
The second group of host proteins inhibits tombusvirus replication and they might be components of the host innate immunity. The best-characterized example is Xrn1p 5’-3’ exoribonuclease (Xrn4p in plants/mammals), which is involved in degradation of tombusvirus RNA, including partially degraded viral RNAs generated by endoribonucleases (Cheng, Serviene et al., 2006; Cheng, Jaag et al., 2007; 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 member of this group of inhibitory host factors is Nsr1p/nucleolin characterized in this work, which has been identified during the genome-wide screen of yeast strains for affecting TBSV repRNA accumulation (Panavas, Serviene et al., 2005). Deletion of NSR1 increased TBSV RNA accumulation by 2-fold, suggesting that this host protein has anti-TBSV activity as confirmed in this paper. Accordingly, over-expression of Nsr1p in yeast led to ~10-fold inhibition of repRNA accumulation (Fig. 4-1A). Interestingly, the over-expression was the most effective when it occurred before or at the beginning of TBSV repRNA replication, suggesting that Nsr1p could inhibit an early step in the viral replication process. This step could be the viral RNA recruitment step, since preincubation of repRNA and the purified recombinant Nsr1p inhibited the activity of the in vitro assembled tombusvirus replicase more efficiently than adding Nsr1p directly to the cell-free extract. Indeed, we found strong interaction between the RNA-binding domain of Nsr1p and repRNA, while we could not demonstrate direct interaction between Nsr1p and p33 or p92 replication proteins in this work (not shown) or during previous proteomics screens (Li, Barajas et al., 2008).

Another piece of evidence for the role of Nsr1p in inhibition of repRNA recruitment is the ability of Nsr1p to bind to RIII(+) sequence in the repRNA. This region is not known to play a role in the assembly of the tombusvirus replicase (Panaviene, Panavas et al., 2005). It is more likely that binding of Nsr1p to RIII(+) could lead to sequestration of the viral RNA, inhibiting its recruitment by the p33 replication protein, which binds to RII(+)
(Pogany, White et al., 2005). Also, over-expression of Nsr1p 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 paper that Nsr1p/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 infections. For example, nucleolin has been shown to interact with the 3’ 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 translation of the poliovirus RNA both in vivo and in vitro (Izumi, Valdez et al., 2001). The NS5B RdRp protein of hepatitis C virus interacts with nucleolin, which could be relevant for virus replication (Kusakawa, Shimakami 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, Harada 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, Tokunaga et al., 2004). Over expression 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, Gonsky 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.
Figure 4-1. Inhibition of tombusvirus RNA accumulation by over-expression of Nsr1p in yeast. (A) Nsr1p as a FLAG or 6xHis fusion protein and the TBSV DI-72 repRNA were expressed from the GAL1 promoter, whereas 6xHis-p33 and 6xHis-p92 were expressed from the ADH1 and CUP1 promoters, respectively, by simultaneous induction with galactose and copper ions in the parental BY4741 or nsrc1D yeast strains. The total RNA samples were obtained after 24 hour culturing at 29 °C. The accumulation of repRNA was estimated using northern blotting. The 18S rRNA was used as a loading control. Each experiment was repeated twice. (B) The level of transcription of TBSV DI-
72 repRNA from the \textit{GAL1} promoter in BY4741 strain expressing Nsr1-FLAG or a short peptide (pYES), but not expressing p92, was estimated by northern blotting. The two bands represent the ribozyme cleaved and uncleaved TBSV repRNA transcripts. Note that the replicating TBSV repRNA in yeast co-expressing p33/p92 was used as a size marker. The replicating repRNA reaches more than 1,000-fold higher accumulation level.

(C) Decreased replicase activity from yeast over-expressing Nsr1p. The upper panel shows the replicase activity assay with membrane-enriched preparations obtained from yeast expressing a high level of Nsr1p in the parental BY4741 or \textit{nsr1}Δ yeast strains. The membrane-enriched fraction contains the endogenous repRNA template that is used during the in vitro replicase assay in the presence of \textsuperscript{32}P-UTP and the other unlabeled rNTPs. Note that the in vitro activities of the tombusviral replicase were normalized based on p33 levels (see bottom panel). The lower panel also shows the level of FLAG or 6xHis-tagged Nsr1p expression based on western blotting with the mixture of anti-FLAG and anti-6xHis antibodies.
Figure 4-2. Silencing of nucleolin in *N. benthamiana* led to moderately increased TBSV gRNA accumulation. (A) Sequences similarity between *N. benthamiana* nucleolin and *N. tobaccum* (only showed the segment which used in silencing) (B) The TBSV gRNA accumulation increased 50% in nucleolin silenced plants compared to the control plants.
Figure 4-3. 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 AtNuc-L1-GFP and CNV genomic RNA. 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 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. (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.
Figure 4-4. 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 from the *GAL1* promoter, whereas repRNA replication was launched from the *CUP1* promoter in the parental BY4741 yeast strain. (B-C-D) The yeast transformants were pre-grown in SC-ULH medium with 2% glucose for 24 hr at 29°C, then transferred to a medium with 2% galactose (starting OD<sub>600</sub> was ~0.3) and further cultured at 29°C. Copper sulfate (50 mM) was added at different time points, such as 20 hr or 6 hr after or 6 hr prior to the addition of galactose containing medium to initiate repRNA replication. The accumulation of repRNA was estimated using northern blotting after 24 hours of culturing of yeast in the presence of copper ions. See further details in the legend to Fig. 4-1A.
Figure 4-5. In vitro binding of recombinant Nsr1p to the (+)repRNA. (A) UV-cross-linking assay with 2 mg of purified recombinant GST-DRBD (Nsr1p missing the central RNA binding domains), GST-Nsr1 or GST and ~5 nM $^{32}$P-labeled DI-72 (+)repRNA. (B) Coomassie blue staining of the SDS-PAGE shown in panel A, showing the purified recombinant GST-DRBD, GST-Nsr1 and GST proteins from *E. coli*. The fusion proteins were purified using GST affinity chromatography. (C) A gel mobility shift assay showing interactions between the recombinant GST-Nsr1 and $^{32}$P-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 (from right to left) amounts (400, 800 and 1600 ng protein/per lane).
Figure 4-6. Nsr1p binds to RIII in (+)repRNA in vitro. (A) Schematic representation of the various regions in DI-72(+)-repRNA. The 169 nt long RI(+) represents the 5' UTR; the 239 nt long RII(+) is derived from the p92 ORF, whereas the 82nt long RIII(+) represents a short segment of the very 3' end of p19/p22 ORF and the 5' portion of 3' UTR and the 131 nt long RIV(+) is from the very 3' end of the genomic RNA. RII(+) contains the p33 recognition element (p33RE), which is a stem-loop structure with a C•C mismatch, required for RNA recruitment, while RIV(+) contains the replication silencer element and the genomic promoter (circled), required together with p33RE for the assembly of the viral replicase. (B) UV-cross-linking assay with 2 mg of purified GST-Nsr1 and ~5 nM 32P-labeled RI(+), RII(+), RIII(+) or RIV(+) of DI-72 repRNA. (C) A gel mobility shift assay showing interactions between the recombinant GST-Nsr1 and 32P-labeled RI(+), RII(+), RIII(+) or RIV(+). GST-Nsr1 was used in increasing amounts (0, 800 and 1600 ng protein/per lane). (D) Band shift experiments with cold competitor RIII(+) RNA. The gel mobility shift assay was performed with 32P-labeled RI(+), RII(+), RIII(+) or RIV(+) and recombinant GST-Nsr1 (2 mg / per lane) in the absence or presence of 50 and 500 nM cold competitor RIII(+) RNA. We quantified the unbound RNA and the values show the % of the control samples (no GST-Nsr1 and competitor RNA, such as lanes 1, 5, 9 and 13). (E) Gel mobility shift assay with cold competitor RI, RII, RIII and RIV RNAs. The assay was performed with 32P-labeled DI-72 (+)RNA and recombinant GST-Nsr1 (2 mg / per lane) in the absence or presence of 50 and 500 nM cold competitor RI(+), RII(+), RIII(+) or RIV(+) RNAs. We quantified the bound RNA and the values show the % of the control samples.
Figure 4-7. Accumulation of tombusvirus RNA lacking RIII sequence is only moderately inhibited by over-expression of Nsr1p in yeast. (A) Schematic representation of the repRNA used and the model for Nsr1p-based inhibition of repRNA accumulation. (B) To study the effect of over-expression of Nsr1p on the accumulation of DI-ΔRIII RNA, BY4741 yeast were transformed with three plasmids, such as pHisGBK-His33/DI-ΔRIII-Gal [co-expressing CNV p33 from the *ADHI* promoter and DI-ΔRIII RNA from the *GAL1* promoter], pGAD-His92-CUP1 (containing the CNV p92 gene behind the *CUP1* promoter), and pYES-NSR1 (expressing N-terminally 6xHis-tagged Nsr1p from the *GAL1* promoter) (lanes 10-13). Alternatively, we also used pYES-C-FLAG-NSR1 (expressing C-terminal FLAG-tagged Nsr1p from the *GAL1* promoter) (lanes 6-9) or the empty plasmid pYES-NT-C expressing a short peptide as a control. The accumulation of repRNA was estimated using northern blotting. See Fig. 4-1A for further details.
**Figure 4-8. Inhibition of replication of the TBSV repRNA in the cell-free yeast extract.** The panel shows a denaturing PAGE analysis of the $^{32}$P-labeled RNA products obtained when the 621 nt DI-72(+) repRNA added to the cell-free extract in the absence of GST-Nsr1 (lanes 1, 11, 12) or in the presence of the purified recombinant GST-Nsr1 or GST as shown. The cell-free extract was obtained from yeast expressing p33 and p92 replication proteins. The amount of RNA synthesis by the replicase assembled in the yeast cell-free extract was compared to control samples, which did not contain recombinant GST-Nsr1 or GST proteins. The 200 ng repRNA and the recombinant proteins (400, 800 and 1600 ng per sample) were pre-incubated for 15 min (lanes 2-10) or without pre-incubation (lanes 13-18), and then mixed with the replicase mixture to perform in vitro TBSV replication.
A. Nucleolin

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B. Nucleolin + CNV infection

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Figure 4-9. Nucleosomal and nucleolar localization of nucleolin in *N. benthamiana* cells expressing AtNuc-L1-GFP and infected with CNV. (A) Nucleosomal and nucleolar localization of nucleolin in the absence of CNV replication. The *A. thaliana* fibrillarin1-RFP expressed transgenically was used as a nucleolar marker. Each experiment was repeated and 20 or more cells were analyzed. (B) Similar localization of AtNuc-L1-GFP in transgenic fibrillarin1-RFP *N. benthamiana* cells infected with CNV.
CHAPTER V
Summary and discussion

The replication of (+)RNA viruses involves interactions with host cells in various steps from viral protein translation to viral progeny RNA synthesis. In the early stage, viral RNA interacts with the host translation machinery and out-competes cellular mRNAs to produce the viral replication proteins. Host proteins, membranes, and lipids are also involved in the later stages of virus replication, such as during intracellular transport of replication components, assembly of membrane associated replicase complex, and production of replication intermediate (-)RNA and (+)RNA progeny, etc. Evidence from recent studies indicates the host-virus interactions form a complicated network of protein-protein, protein-membrane or protein-RNA interactions. Thus, identifying host factors involved in virus replication and dissecting their functions in virus replication and their interactions with viral proteins or viral RNA is important for better understanding of viral replication and the biological bases of viral induced disease.

Lots of effort has been devoted to characterizing the host factors affecting virus replication. In recent years, powerful genetic and biochemical tools have made the high throughput and systematic approaches available, which promoted the identification of more than a hundred host factors. The genome-wide screenings, based on RNAi, single gene knock-outs or gene down-regulation, have been used to identify host components involved in the replication of (+)RNA viruses. Due to its small and well annotated genome, less genomic redundancy and the ability to grow fast, yeast has been developed as a powerful model host to systematically identify host factors for plant viruses. Especially, for TBSV and BMV, the genome-wide screening led to the discovery of more than 100 host genes for each of them. Moreover, proteomics approaches facilitated isolation of additional host proteins interacting with viral proteins or viral RNA for both TBSV and BMV.

Tombusviruses are a group of plant (+)RNA viruses with small genomes and can frequently generate DI RNAs during replication. To identify the host factors affecting TBSV replication, we used yeast as a model host. Co-expression of the replication proteins, p33 and p92, together with a repRNA (DI RNA) via plasmids resulted in robust
DI RNA replication in yeast. Previous work by Panavas et al. using a yeast single gene deletion library (YKO), which contains ~4800 yeast genes and covers 80% of the genome, revealed 96 yeast genes which increase or decrease TBSV RNA accumulation. The above study tested the nonessential genes, while the essential host genes, which are required for cell viability, were tested in this work, since they also likely play important roles in TBSV replication. To identify the host essential genes for TBSV, we extended the genome-wide screening to 800 essential host genes available in Yeast Tet Promoters Hughes Collection (yTHc), in which the original promoter was changed to Tetracyclin-titratable promoter. We performed high throughput screening of the yTHC and found that down-regulation of 19 yeast genes decreased TBSV repRNA levels by 2- to 5-fold and down-regulation of 11 genes increased repRNA accumulation. Genomic screens in yeast including this work and the previous work on YKO library have covered ~95% of the host genome and about 2 percent (126 genes) of the tested genes were identified to affect the viral repRNA accumulation.

The identified 30 essential host genes have different functions in various cellular processes including RNA binding/processing, RNA transcription, protein modification, transportation or lipid biosynthesis etc. They are possibly involved in different steps of viral replication. We tested the effects of the identified genes on the amounts of p33 (both total and membrane associated fraction) and p92 replication proteins from 15 selected strains. We found that six of the nine selected strains with reduced repRNA levels, such as DED1, COPI, etc, produced lower levels of p33 or p92 proteins. In the case of strains with higher repRNA level, the changes in p33 and p92 levels do not have good correlation with replication levels. One of the possibilities that host genes might affect viral repRNA accumulation via changing the amount of p33 or p92 replication proteins. Roles of the identified yeast genes in viral replication might be also related to their cellular functions. For example, the ERG25 gene encodes an important catalytic enzyme in the host lipid biosynthesis pathway and therefore might affect the function of membrane associated viral replicase complex; while the COPI gene encodes a protein transporter from ER to Golgi and for retrograde transportation, suggesting its involvement in cellular transportation of viral replication components.
We further tested the effects of a selected group of host genes on viral replicase function. The \textit{in vitro} replicase activity assay and plus-/minus-strand ratio assay demonstrated that selected host genes could alter the tombusvirus RdRp activity and change the ratio of plus- and minus-strand RNA synthesis. The mechanisms of their effects on viral replicase and asymmetrical RNA synthesis need to be addressed in further work. The genome-screening work also showed that interactions exist among the identified host factors, which is part of the complex interaction network among host and virus components.

The systematic genome-wide screenings identified more than 100 host genes with diverse cellular functions, which provide valuable players for further studies on host-TBSV interactions. Due to genetic or functional redundancy and high level of threshold for identifying positive hits, these screens have likely missed the identification of some host factors. Indeed, additional proteomics-based approaches have led to the identification of host components that are part of the viral replicase, such as Tdh1/2p, Ssa1/2p, and Cdc34p. Additional approaches, like yeast two-hybrid screens and deletion of more than one gene in the gene families could identify additional potential host factors involved in virus replication.

The identified host factors can be divided into two groups based on their implication in virus multiplication: one group includes factors that facilitate virus replication directly or indirectly; another group contains factors with inhibitory effect on the virus replication. In the YKO screening, we found a possible inhibitor \textit{NSR1} (plant nucleolin) gene, whose deletion led to increased TBSV repRNA accumulation. Nucleolin/Nsr1p is a multifunctional protein and involved in several steps of ribosome biogenesis and regulates cellular DNA and mRNA metabolism. Nucleolin shuttles between the nucleolus, the nucleoplasm and the cytoplasm, and is involved in nuclear - cytosolic transport of ribosomal proteins and ribosomal subunits. Nucleolin is composed of an N-terminal domain rich in acidic residues, a central domain containing two RNA recognition motifs and a C-terminal GAR (glycine/arginine-rich) domain. The central two RBDDs (RNA binding domains) specifically interact with a short stem-loop structure containing UCCCGA sequences within the loop called NRE (nucleolin-recognition element) in pre-rRNA. Nucleolin also can interact with other sequences, such AU rich elements.
To test the effect of nucleolin on TBSV repRNA accumulation, we over-expressed nucleolin in yeast. Over-expression of Nsr1p led to up to 90% reduction in viral RNA accumulation, however the transcription level of TBSV repRNA and amount of p33 replication protein are not affected by the Nsr1p over-expression. The in vitro RdRp assay revealed that Nsr1p over-expression also led to reduced viral replicase activity and deletion of NSR1 led to increased activity compared with the parental strain. Based on these in vitro data, the decrease in TBSV repRNA accumulation caused by Nsr1p might due to the direct inhibitory effect of Nsr1p on the viral replicase activity.

To analyze if nucleolin in a host plant could also inhibit TBSV genome replication, we transiently over-expressed the *A. thaliana* nucleolin in *N. benthamiana* leaves via agroinfiltration and tested its effect on TBSV genomic RNA accumulation. Tombusvirus infection was initiated via agro-infiltration or sap-inoculation with virions in this work. Importantly, we observed that over-expression of *A. thaliana* nucleolin inhibited the tombusvirus genomic RNA replication in both cases. However, the inhibition was greater when viral infection was initiated via agro-infiltration. We also found that nucleolin is not a universal inhibitor for plant RNA viruses since over-expression of nucleolin had no effect on infection by TRV, a distantly related virus. Altogether, we confirmed that the yeast nucleolin and its plant homolog inhibit TBSV replication in both yeast and plant.

We also tested what steps of TBSV replication were inhibited by nucleolin. As mentioned above, nucleolin does not affect translation or transcription (if plasmids were used to initiate infections) of viral proteins or RNA. We found that Nsr1p efficiently inhibited viral RNA synthesis at early time points during virus replication, while it was less effective when expressed at latter time points. In addition to the in vivo experiment, we also performed in vitro replicase assays by using a recently developed yeast cell-free extract containing subcellular membranes, which is capable of supporting authentic TBSV replication, to test the effect of purified Nsr1p on TBSV replication. We found that TBSV replication was inhibited by the purified recombinant Nsr1p and the inhibition was more pronounced when Nsr1p was pre-incubated with the TBSV template RNA before adding to the cell-free extract. These in vivo and in vitro data suggest that Nsr1p inhibits TBSV replication at an early step other than translation, likely the recruitment of the TBSV (+)repRNA into replication.
Subsequently, we searched for the potential viral targets of nucleolin by performing in vitro interaction experiments. Attempts to detect interaction between Nsr1p and p33 or p92 replication proteins have been unsuccessful; however, we found strong binding between the central RBD domains of Nsr1p and TBSV (+)repRNA by UV-cross-linking and gel-mobility shift assays. To analyze if the binding of Nsr1p involves a specific site in TBSV DI-72(+) RNA, we tested four defined segments in DI-72(+) RNA. We observed that Nsr1p binds most efficiently to RIII(+) in both UV-cross-linking and gel-mobility shift experiments. The template competition experiments also confirmed that Nsr1p binds preferably to RIII(+). Consistent with the specific binding to RIII(+), over-expression of Nsr1p slightly reduced (40%) the accumulation of TBSVΔRIII repRNA in yeast, in contrast to the 90% reductions with DI-72(+) repRNA. These data suggest that Nsr1p interacts with TBSV (+)RNA during replication and RIII(+) in the genomic RNA is the main target of Nsr1p.

RIII(+) is derived from the 3’UTR of the TBSV genome and RIII(-) serves as a replication enhancer for plus-strand synthesis. But the function of RIII(+) is not yet defined. Fluorescence microscopy analysis showed that the presence of tombusvirus proteins/RNA did not change the localization of nucleolin, and vice versa, the over-expression of nucleolin does not interfere with the sub-cellular transportation and peroxisomal targeting of viral replication protein. Based on the available data, we suggest that excess nucleolin might bind to the viral RNA and interferes with the recruitment of TBSV RNA via interacting with the RIII(+) portion of the 3’UTR within the TBSV genome that leads to inhibition of TBSV replication.

Further work needs to be done to address the following questions: what sequences or structures within RIII(+) is recognized by Nsr1p and how the interaction with Nsr1p could interfere with the recruitment of the viral RNA and/or the assembly of the viral replicase. RIII(+) does not contain the previously characterized NRE sequence known to be recognized by Nsr1p, thus it is likely that Nsr1p may recognize a novel feature/sequence motif present in the TBSV RNA. It would also be important to test if binding of the viral RNA by Nsr1p could change the localization of (+)repRNA or change the stability of repRNA.
Altogether, we have discovered a new function for nucleolin during TBSV infections. This discovery opened the possibility that nucleolin is part of the innate immune system of plants against selected plant viruses.
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VITA

Name: Yi Jiang

Date of Birth: April 10th, 1975

Place of Birth: Xinjiang, China

Education:

Major: Public health
Beijing Medical University, Beijing, China

Major: Microbiology
Chinese Academy of Sciences, Beijing, China

Research Experience:

2000-2004: Research associate:
Institute of Microbiology, Chinese Academy of Sciences
Beijing, China.

Honors:

2008-2009 Kentucky Opportunity Fellowship, University of Kentucky
2007-2008 Graduate school fellowship, University of Kentucky
2006-2007 Graduate school fellowship, University of Kentucky
2005-2006 Graduate school fellowship, University of Kentucky
2006 Graduate travel grant from American Society for Virology

Publications:

