

Chapter 2

Exploration of Plant Virus Replication Inside a Surrogate Host, *Saccharomyces cerevisiae*, Elucidates Complex and Conserved Mechanisms

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Abstract Plant RNA viruses are intracellular infectious agents with limited coding capacity. Therefore, these viruses have developed sophisticated ways to co-opt numerous cellular factors to facilitate the viral infectious cycle. To understand virus-host interactions, it is necessary to identify all the host components that are co-opted for viral infections. Development of yeast (*Saccharomyces cerevisiae*) as a host greatly facilitated the progress in our understanding of plant virus, such as brome mosaic virus (BMV) and tomato bushy stunt virus (TBSV), interactions with the host cells. Systematic genome-wide screens using yeast genomic libraries have led to the identification of a large number of host factors affecting (+)RNA virus replication. In combination with proteomic approaches, both susceptibility and restriction factors for BMV and TBSV have been identified using yeast. More detailed biochemical and cellular studies then led to the dissection of molecular functions of many host factors that promote each step of the viral replication process. The development of *in vitro* systems with TBSV, such as yeast cell-free extract and purified active replicase assays, together with proteomics, lipidomics and artificial vesicle-based assays helped to comprehend the complex nature of virus replication. Subsequently, comparable pro- or antiviral functions of several of the characterized yeast host factors have been validated in plant hosts. Overall, yeast is an advanced model organism that has emerged as an attractive host to gain insights into the intricate interactions of plant viruses with host cells. This chapter describes our current understanding of virus-host interactions, based mostly on TBSV-yeast system. Many of the pioneering findings with TBSV are likely applicable to other plant and animal viruses and their interactions with their hosts. The gained knowledge on host factors could lead to novel specific or broad-range antiviral tools against viruses.

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2.1 Introduction

Viruses are the most abundant biological entities on Earth and they largely outnumber all other lifeforms. Regardless of their huge diversity in genome size, coding capacity, or the nature of their nucleic acids, single- or double-stranded, RNA or DNA, they are all molecular parasites that cannot multiply outside of their host cells. Their genomes are relatively small compared to their hosts' genomes. Among plant-infecting viruses, those with RNA genomes are the most widespread, usually coding only for a few conserved replication-associated proteins, coat proteins and plant virus-specific movement proteins and suppressors for gene silencing. Overall, plant viruses inevitably depend on the interactions between the viral components and the surrounding cellular proteins, lipids and metabolites that ensure successful viral multiplication. Accordingly, some cellular factors are essential for both cell propagation/survival and for virus multiplication to complete the infectious cycle. Yet, other host components can be modified, sequestered, retargeted and manipulated by viruses to create subcellular environment suitable for virus replication.

To explore how cellular processes are subverted by the virus after infection and how the viral replication proteins could change subcellular environment as well as how the cells fight back the infection requires systems level approaches. Virologists should identify all the molecular players both from the host and virus sides that participate in the infection process. The gained knowledge could be useful for developing novel anti-viral approaches or might be advantageous to optimize beneficial applications of viruses. We will also learn about the potential repertoire of cellular factors during normal and diseased states. The most feasible way to unveil all the interactions, or networks of interactions, is the utilization of genetically amenable model organisms, such as the baker yeast, *Saccharomyces cerevisiae*. The current chapter will shed light on the amazing complexity of positive-strand (+)RNA virus replication and its dependence on virus-host interactions. We will describe how the facile genetics of *S. cerevisiae* helps to unravel intricate molecular interactions based on molecular mimicry and how the relevance of the intriguing discoveries from yeast could provide deep insights into the natural host-virus interactions.

2.2 Overview of the Infectious Cycle of Positive-Sense RNA Viruses

Research during the last couple of decades established a trend that (+)RNA viruses, which form the largest group among viruses, share several common features in their replication strategies and their interactions with hosts. Briefly, the viral (+)RNA acts as mRNA that is used by the host ribosomes to produce

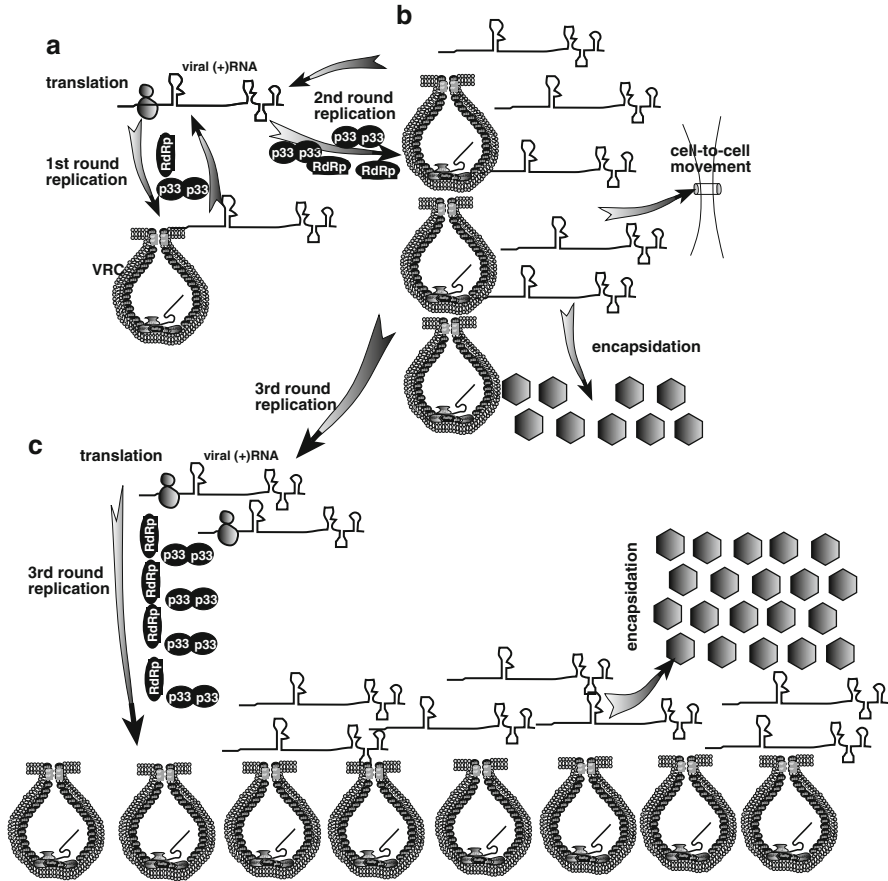


Fig. 2.1 The complex plant (+)RNA virus replication cycles includes the following steps: **(a)** After the initial translation of the invading TBSV (+)RNA by the cellular ribosomes, the freshly synthesized p33/p92 replication proteins recruit the viral (+)RNA for the assembly of the membrane-bound viral replicase (VRC, represented by a vesicle-like structure) and begins viral RNA replication (1st round). **(b)** Then, the newly made and released (+)RNA enters a new round of translation, followed by replication (2nd round). **(c)** The translation/replication cycle is repeated (3rd round). Note that a single infected cell likely perform ~20 sequential translation/replication cycles in 24–48 h that lead to the production of large amounts of viral (+)RNA progeny that participate in cell-to-cell movement and encapsidation

viral proteins at the early stage of infection (Fig. 2.1). This is followed by viral genome replication, then assembly of complete virus particles (virions), cell-to-cell and long-distance movement and spread to other plants. Interestingly, all these steps depend on the availability and functionality of many host factors (den Boon and Ahlquist 2010; Laliberté and Sanfaçon 2010; Nagy and Pogány 2012; Wang 2015).

2.2.1 *Genome Organization of Tomato Bushy Stunt Virus*

In this chapter, we will mainly focus on the advancement in plant virus replication made by utilization of yeast as a model host. A more general description of plant virus-plant host interactions could be found in several excellent recent reviews (Laliberte and Sanfacon 2010; Wang 2015). Studies on plant virus-host interactions have been pioneered using bromoviruses and tombusviruses in yeast (den Boon and Ahlquist 2010; Janda and Ahlquist 1993; Nagy 2008; Nagy and Pogany 2006; Nagy et al. 2014; Panavas and Nagy 2003). Here, we will mainly focus on tombusviruses, including Tomato bushy stunt virus (TBSV). TBSV has a small (+)RNA genome (4800 nucleotides), which rapidly multiplies in infected plants, and produces a huge amount of virions. In the last decade, it became clear that TBSV is an excellent model virus to study virus replication and virus-host interactions. The TBSV genome codes for two replication proteins, namely p33 replication cofactor and p92^{pol} RNA dependent RNA polymerase (RdRp), which is a readthrough product of p33 and is expressed at 5 % of p33 level (White and Nagy 2004). Ribosomal read-through of a translational stop codon is a widely utilized strategy for plant RNA viruses to control the expression of downstream open reading frame, which frequently include the viral RdRp or other replication proteins (Nicholson and White 2014). Three other TBSV proteins, which are expressed from two subgenomic RNAs made during TBSV replication, are the capsid protein (p41), the movement protein (p22) and p19 silencing suppressor (White and Nagy 2004). In addition to the protein coding sequences, the TBSV (+)RNA genome contains several regulatory elements, which are present in the 5' or 3' untranslated regions, and even in the coding regions (Nicholson and White 2014). These regulatory RNA elements drive different viral processes, including translation, replication and encapsidation. Interestingly, TBSV (+)RNA genome, which is not capped at 5' end and does not have a 3' poly(A) tail, carries noncanonical translation elements that facilitate efficient translation. For example, a cap independent translation enhancer (3' CITE) is located at the 3' untranslated region (UTR) of the TBSV RNA. The complex interactions between the 3' CITE and the 5' UTR along with another five long-range RNA-RNA interactions in the TBSV (+)RNA were identified (Nicholson and White 2014; Wu et al. 2013). Short and long-distance RNA-RNA interactions within the viral genome also bring *cis*-acting replication elements into close proximity to regulate replication and subgenomic RNA transcription (Nicholson and White 2014; Panavas and Nagy 2005; Pogany et al. 2003; Wu et al. 2009, 2013). Altogether, long-range interactions within the TBSV genome provide mechanisms to regulate a diverse array of viral functions (Nicholson and White 2014).

2.2.2 Functions of *cis*-Acting Elements in the Genomic RNA During Replication of TBSV (+)RNA

The viral RNA is the master regulator of the replication process, as it serves multiple functions, including the template role, as an assembly platform for the replicase, and the RNA also organizes the replication proteins and host factors (Pathak et al. 2011; Pathak et al. 2012). These activities depend on various *cis*-acting replication elements within the genomic RNAs. Accordingly, the TBSV genomic RNA involves several *cis*-acting sequences that promote different steps of viral replication (Nicholson and White 2014; White and Nagy 2004). For example, the (+)RNA serves as a template for the synthesis of the complementary negative-strand (−)RNA, which then becomes the template for the synthesis of the (+)RNA progenies. Interestingly, (+)RNAs are produced in excess amounts, reaching up to 100 times more than (−)RNA. To tightly regulate this process, TBSV utilizes promoter elements and regulatory elements in both (+)- and (−)RNAs. The unrelated minus-strand and plus-strand initiation promoters are located at the 3' terminus of the (+) and the (−)RNAs, respectively. The former is called genomic promoter (gPR), while the latter is called the complementary promoter (cPR) and they are required for *de novo* (primer-independent) initiation of replication by the viral replicase complex (VRC). The VRC constitutes a membrane-bound large protein complex of p92^{pol} RdRp, p33 replication protein, the viral (+)- and (−) RNAs and over ten co-opted cellular factors (as discussed below). The main function of the gPR is to interact with and position the viral RdRp over the initiation sequence accurately to ensure the precise initiation of the (−)RNA synthesis. The activity of gPR is regulated by a replication silencer element (RSE), which participates in a five nt-long RNA-RNA interaction with the very 3' end sequence within the gPR. After the (−)RNA synthesis is finished, then the (+)RNA synthesis initiates from the cPR. Interestingly, the (+)RNA synthesis is enhanced by two replication enhancers (REs), one located close to the cPR (termed promoter proximal enhancer, PPE) and the other within the 5' end of the (−)RNA, called RIII(−) replication enhancer. The RIII(−) RE forms a long-range RNA-RNA interaction with the cPR at the 3' end (Panavas and Nagy 2005; Panavas et al. 2006). These viral RE elements ensure the production of excess amounts of infectious (+)RNAs. Overall, the viral RNAs are orchestrating viral replication proteins and a plethora of co-opted host factors to achieve robust and accurate replication.

Because viral replication is a step-wise process, below we will discuss the various steps as they occur in infected cells. Based on our current understanding, we can discriminate six main steps during TBSV replication inside the cell (Nagy and Pogany 2012). These steps are the following: (i) template selection for replication that results in a switch from translation to replication; (ii) recruitment of the RdRp/p33/viral (+)RNA complex to subcellular membrane surfaces; (iii) VRC assembly that also includes the activation of the membrane-bound p92^{pol} RdRp; (iv) (−)RNA synthesis that leads to the production of dsRNA replication intermediate; (v) (+)RNA

synthesis on the dsRNA template; and (vi) the release of (+)RNA progeny from the VRC into the cytosol to perform additional activities, including new translation/replication cycles, encapsidation or cell-to-cell movement.

2.2.3 (+)RNA Template Selection for Replication and a Switch from Translation to Replication

The genomes of (+)RNA viruses first serve as mRNAs for translation of viral proteins and then, the same viral (+)RNA molecules also act as templates in the subsequent replication process. Therefore, after the production of enough amounts of replication proteins – including the RdRp, the viral (+)RNA has to switch from the translation mode to execute the replication process. These two processes seem conflicting as during translation the ribosome moves from the 5'-to-3' direction on the (+)RNA, while the freshly expressed RdRp is destined to make (–)RNA on the same (+)RNA template, but progressing in 3'-to-5' direction. Although the detailed mechanism of the switch from translation to replication is not yet fully dissected for TBSV, the emerging picture is that multiple regulatory steps are in play at this step. For example, the p92^{pol} RdRp is initially inactive and requires an “activation” step that only takes place in a membrane-bound complex (Pathak et al. 2012; Pogany and Nagy 2012, 2015; Pogany et al. 2008). Therefore, it seems that the translation and the replication processes take place in different subcellular environment, possibly preventing the collision between the ribosomes and the viral RdRp on the same (+)RNA template.

Other (+)RNA viruses likely separate the two processes as well, as indicated for poliovirus, whose genome contains the replication element in close vicinity to the internal ribosome entry site. When cellular factors, namely the poly(C)- and poly(A)-binding proteins bind to the poliovirus (+)RNA, then translation is promoted. However, when the (+)RNA binds to the viral replication protein 3CD, then translation is repressed and replication is launched (Gamarnik and Andino 1998; Walter et al. 2002).

Unlike the cellular mRNAs, which are usually destined for degradation after translation, the viral (+)RNA is rescued by selective interaction with the viral replication protein(s). In case of TBSV, the specific viral (+)RNA template recognition within the heterogeneous pool of host RNAs, is performed preferably in *cis* by the dimerized p33 replication protein. The *cis*-recognition means that the replication protein readily binds to the very same viral (+)RNA that serves as a template for the translation of the viral p33 protein. The TBSV p33 and the p92^{pol} replication proteins interact with each other and they both contain an arginine-rich motif (RPR), that possesses selective viral (+)RNA binding capacity (Monkewich et al. 2005; Panavas et al. 2005a; Pogany et al. 2005; Rajendran and Nagy 2006). During template selection the abundant replication cofactor, p33 binds an internal recognition element (IRE) located within the coding region of the p92^{pol} open reading frame. The specific binding between p33 and the cognate (+)RNA depends on a conserved C-C mismatch present within an extensive RNA helix, called RII(+)-SL.

2.2.4 *Recruitment of the RdRp/Viral RNA Complex to Subcellular Membrane Surfaces*

The current model predicts that the viral (+)RNA is recruited to the site of replication as a (+)RNA-p33 complex (Monkewich et al. 2005; Pogany et al. 2005). TBSV, similar to other (+)RNA viruses, recruits components of the VRC (i.e., replication proteins, viral (+)RNA, co-opted host factors) from the cytosol to distinct membranous subcellular compartments. The recruitment of the VRC components either occurs into preexisting membranes or in extensively reorganized membranes, such as the TBSV-induced multivesicular bodies (Barajas et al. 2009a; Russo et al. 1994). TBSV facilitates this process by membrane targeting signals located in p92^{pol} and p33 proteins and by two transmembrane domains localized close to the N terminus of these proteins. The scope of the chosen subcellular membrane types is numerous, though mostly specific in case of most viruses. TBSV and the closely related tombusviruses, such as Cucumber necrosis virus (CNV) and Cymbidium ringspot virus, replicate on the cytosolic side of peroxisome membranes (McCartney et al. 2005; Navarro et al. 2006; Panavas et al. 2005a; Pathak et al. 2008), while another tombusvirus, Carnation Italian ringspot virus (CIRV) replicates on the outer mitochondrial membrane (Weber-Lotfi et al. 2002; Xu et al. 2012). Other plant viruses target various subcellular membranes, such as endoplasmic reticulum (ER), chloroplast, or vacuolar membranes for replication (Laliberte and Sanfacon 2010; Wang 2015).

2.2.5 *Assembly of the Active Viral Replicase Complex*

Recent discoveries using live yeast and yeast cell-free extract (CFE)-based assays revealed three major processes guiding the functional VRC assembly (Nagy and Pogany 2012; Xu and Nagy 2014). The first one utilizes the viral (+)RNA as an assembly platform that binds to p33 and p92^{pol} replication proteins and co-opted host factors. The second process is driven by interactions between p33 replication protein, membrane-bending proteins, such as the co-opted cellular ESCRT proteins, and particular phospholipids in subcellular membranes. These interactions lead to deformation of membranes around the replicase complex. The third process is the activation of the RdRp function of p92^{pol} replication protein within the membrane-bound VRC. *In vitro* experiments with TBSV revealed, that the activation of p92^{pol} replication protein requires two *cis*-acting elements in the TBSV (+)RNA, the p33 replication co-factor as well as cellular co-factors such as heat shock protein (Hsp70) and neutral lipids in the host cell membrane (Pogany and Nagy 2012; Pogany and Nagy 2015).

Many (+)RNA viruses, similar to TBSV, induce membrane invaginations (called spherules) with narrow openings during VRC formation in given membranous subcellular compartments. Other (+)RNA viruses induce double-membrane vesicles or both single- and double-membrane vesicles (Romero-Brey and

Bartenschlager 2014; Wang 2015). TBSV and BMV induce ~70 nm diameter vesicular invaginations both in plant and yeast cells. Spherule induction most likely helps the virus evade from the cellular defense mechanism and protects the viral RNA from degradation. Altogether, the subcellular compartmentalization of the membrane-bound activated VRC prevents not only the collision between the ribosome and the RdRp, but this strategy also avoids viral RNA synthesis in the cytosol that would induce dsRNA-triggered antiviral defense mechanism of the host (Romero-Brey and Bartenschlager 2014; Wang 2015).

2.2.6 Viral (+)RNA Replication Leads to the Production of dsRNA Inside VRC

After the VRC assembly and activation of the p92^{pol} RdRp, (–)RNA synthesis starts from the 3' end of the genomic (+)RNA guided by the gPR promoter sequence. Because the VRC contains both the original (+)RNA and the newly synthesized (–)RNA, the question arises: What is the form of the replication intermediate? Is there any free (–)RNA that can be utilized for new (+)RNA synthesis? It has been shown with the help of *in vitro* experiments that naked (–)RNA does not seem to exist in the VRC at any time during replication. In stead, the (–)RNA is sequestered into double-stranded (ds)RNA, which appears before the robust production of (+)RNA progenies (Kovalev et al. 2014). Interestingly, the dsRNA is used by the RdRp via a strand-displacement mechanism, where the newly made (+)RNA replaces the previously synthesized (+)RNA in the dsRNA intermediate. This strategy ensures the temporal partition of the (–)RNA and (+)RNA synthesis within the VRC and likely provides the means to produce one (–)RNA per VRC and the generation of 20-to-100 (+)RNA progenies (Kovalev et al. 2014). Also, the dsRNA structure might control RdRp activities by supporting only new (+)RNA synthesis with the help of co-opted cellular helicases (Chuang et al. 2015).

2.2.7 Extensive (+)RNA Synthesis in VRCs

As during the (–)RNA synthesis, the viral RNA also regulates (+)RNA synthesis with the help of RNA structure and *cis*-acting elements that bind to protein co-factors. Briefly, the dsRNA structure of the replication intermediate represses the use of *cis*-acting elements on the (+)RNA part of the dsRNA template (Kovalev et al. 2014). However, the *cis*-acting elements in the (–)RNA portion of the dsRNA intermediate become accessible for the RdRp due to interaction with co-opted cellular helicases (Kovalev and Nagy 2014; Kovalev et al. 2012b). To initiate (+)RNA synthesis, the dsRNA intermediate structure must be opened within the cPR sequence. The role of different host factors involved in this process will be discussed below.

2.2.8 Release of (+)RNA Progeny from VRCs

Since (+)RNA virus replication occurs in membranous environment, while other viral processes with (+)RNA take place in the cytosol, there must be mechanism to release the viral (+)RNA from VRCs. Currently not much is known about the release of the viral (+)RNA from VRCs, or whether it is an active or passive mechanism, but it is assumed that VRCs with spherule structures likely use the narrow opening, called neck, to release the new (+)RNA progeny into the cytosol. The release of the (+)RNA through the neck provides a path for the newly synthesized (+)RNA to become encapsidated by the viral coat proteins in the vicinity of the spherules where the virion assembly takes place (Rao et al. 2014).

One full cycle of (+)RNA virus replication from template selection until the release of the new (+)RNA progeny is likely carried out in 2–3 h based on *in vitro* replicase assembly studies (Pogany and Nagy 2008; Pogany et al. 2008). A newly assembled VRC could start releasing new (+)RNA progeny in ~1 h. However, a fraction of the released viral (+)RNAs likely returns to a new round of translation/replication cycle in the infected cells that further enhance the amount of viral progeny. It is estimated that plant (+)RNA viruses might perform as many as twenty replication cycles in a sequential manner [i.e., the (+)RNA product of the previous replication cycle is the template for the new cycle] in single plant cells in ~48 h, resulting in the production of 100,000 to a million progeny (+)RNAs per cell (Miyashita et al. 2015). To achieve this massive production of progeny, many plant RNA viruses convert the host cells into viral replication factories, as explained in the following subchapters.

2.3 Yeast as a Model System to Study (+)RNA Virus Replication

(+)RNA viruses are intracellular infectious agents with limited coding capacity. Therefore, these viruses have developed sophisticated ways to co-opt numerous cellular factors to facilitate the viral infectious cycle. To understand virus-host interactions, it is necessary to identify all the host components that are subverted for viral infections. One major hurdle to implicitly dissect the interactions between a (+)RNA virus and its host is the still scarce availability of powerful experimental tools to manipulate the host's genome or proteome. Yeast with facile genetics is a model cellular eukaryotic organism, which possesses many archetypal aspects of fundamental cellular mechanisms. These include a whole set of eukaryotic chaperones, protein modifying factors, the ubiquitin/proteasome system, the vesicle trafficking and the secretory pathway, the components of mitochondrial and peroxisomal biology as well as the factors of lipid homeostasis and membranous structures. Another advantage is that these cellular processes and the players involved are the best characterized in yeast. Yeast was the first eukaryotic genome fully sequenced. The yeast genome codes for ~6000 genes and more than 75 % of

the genes have assigned functions (<http://www.yeastgenome.org/>). Besides the rapid growth and easy maintenance of the yeast cultures, the availability of wide collections of libraries, such as the gene deletion library, the essential gene knock-down library (Yeast tet promoter Hughes Collection), the GFP-tagged protein expression collection, the protein over-expression library, or the temperature-sensitive library of essential genes (Gelperin et al. 2005; Huh et al. 2003; Janke et al. 2004; Tong et al. 2001, 2004) render the yeast a very attractive model platform. Large-scale and high-throughput approaches and different molecular toolboxes have been developed to tag or delete genes and change promoters in the yeast genome (Hegemann and Heick 2011; Janke et al. 2004; Yofe et al. 2014). GFP and other fluorochrome tags fused to the yeast protein (either expressed from a plasmid or the chromosome) and to the viral proteins enable the simultaneous detection of the subcellular localization of the given proteins by confocal laser microscopy. The images are collected separately for each fluorochrome and then merged to detect whether the localizations of the proteins of interest overlap in the same subcellular compartment. Thus, the redistribution of host proteins due to virus infection or the altered localization of viral proteins in a mutant yeast background or the relative re-localizations of both viral and host proteins can be visualized in live cells. Yeast is a model system for the deduction of functional and mechanistic aspects of proteins, protein networks or lipid homeostasis shared by eukaryotes. Moreover, yeast is useful for the heterologous expression of human or plant proteins for assessment of their functions, which revealed enormous knowledge about various disease states. Examples are amongst defects in DNA mismatch repair (Gammie et al. 2007), pathogenic human mitochondrial gene mutations (Lasserre et al. 2015), defects in RNA processing (Sun et al. 2011) and even neurodegenerative diseases (Braun et al. 2010). The latter sounds surprising, however yeast shares many conserved pathways with higher eukaryotes that are known objects of susceptibility in neurodegenerative diseases.

2.3.1 Development of Viral Replication Systems in Yeast

A plant (+)RNA virus, namely BMV, was the first to be studied in yeast by the Ahlquist group (Janda and Ahlquist 1993; Price et al. 1996). In addition to BMV, the list of viruses studied in yeast includes TBSV and related tombusviruses, such as CIRV, CNV, and Cymbidium ringspot virus and members of alphanodaviruses (Flock house virus and Nodamuravirus) (Panavas and Nagy 2003; Pantaleo et al. 2003; Pogany et al. 2010; Rubino et al. 2007).

To achieve high level of TBSV (+)RNA accumulation in yeast, a small replicon (rep)RNA derived spontaneously from the full-length genomic RNA via multiple deletions was utilized (Panavas and Nagy 2003; White and Morris 1994). The short repRNA retains the collection of *cis*-acting elements essential for replication to ensure efficient multiplication. Interestingly, the repRNA does not code for proteins (also lack the expression of a selection protein), so its replication depends on the replication proteins provided by the helper virus or expressed from plasmids. Consequently, the repRNA is adapted to utilize replication components in *trans*.

When the repRNA accumulates, it slows down the replication of the helper virus as it competes for the same cellular resources as the helper virus. Hence the repRNA is also called defective interfering RNA (DI) (Pathak and Nagy 2009; White and Nagy 2004). When the replication proteins are ectopically expressed from plasmids or a yeast chromosome, then the repRNA replicates and depends on cellular resources in a largely similar manner to the viral genomic RNA as shown in several publications (Panavas and Nagy 2003; Nagy 2008; Nagy and Pogany 2010). Altogether, the use of repRNAs for TBSV or CIRV in replication studies is useful to dissect replication mechanisms, and to understand how these viruses exploit and reconstitute the cellular milieu the same way as it happens in the natural hosts.

2.3.2 Using Yeast to Obtain *In Vitro* Replication Systems

To dissect the mechanism of (+)RNA virus replication and characterize the functions of viral and co-opted host components, it is useful to develop *in vitro* approaches, which allow researchers to control components and conditions. Accordingly, two *in vitro* approaches based on yeast have been developed for TBSV. The first is based on the affinity purification of the assembled active replicase complex containing the viral replication proteins and several host factors after detergent-based solubilization of yeast membranes (Panaviene et al. 2004, 2005; Serva and Nagy 2006). Then, the purified replicase preparations could be tested *in vitro* for the efficiency to synthesize (+)RNA or (–)RNA depending on the external RNA template added. The advantage of using yeast, instead of TBSV-infected plant cells (Nagy and Pogany 2000) is that various yeast mutants can be used for preparation of the replicase, thus easily obtaining replicase preparations with altered/missing cellular components.

The second powerful approach to dissect the molecular mechanisms is based on yeast CFE. The CFE preparations can support one complete cycle of replication of the TBSV repRNA or the genomic RNA if the viral (+)RNA template, purified recombinant p33 and p92^{pol} replication proteins, and ribonucleotides are provided in the *in vitro* assay. The reconstituted CFE-based assay includes all the known replication steps (Pogany and Nagy 2008; Pogany et al. 2008, 2010). Therefore, the yeast CFE-based assay could be used to separately study the roles of membrane and lipid components as well as various host proteins required for RNA template recruitment, replicase assembly, RdRp activation, (–)RNA and (+)RNA synthesis. CFEs prepared from yeasts with different genetic background can help dissect the functions of not only individual components, but protein families, or even series of host factors that mediate a certain subcellular pathway or cellular networks. Importantly, the CFEs prepared from mutant yeast strains can be complemented with purified recombinant proteins or artificial lipids added back to the *in vitro* reaction. Yeast CFEs can also be used to test the antiviral effects of various chemicals or different conditions that may inhibit virus replication. The yeast CFEs can also be fractionated and subcellular organellar membranes, such as ER, mitochondria or peroxisomes and even artificial lipid vesicles in combination with soluble fraction

of yeast CFE could be used for *in vitro* replication studies with TBSV (Xu et al. 2012; Xu and Nagy 2015). Altogether, the combinations of live yeast and CFE-based *in vitro* approaches greatly facilitate the progress towards the complete understanding of a virus-host interaction system at the molecular and cellular levels.

2.4 Insights into the Intricate Virus-Host Interactions

A major advance made with yeast in plant virus-host interaction studies is the identification of host factors based on systematic genome-wide screens with yeast genomic libraries. Accordingly, the highthroughput screens were conducted with BMV and TBSV that led to the identification of over 100 yeast genes affecting either BMV or TBSV replication (Gancarz et al. 2011; Kushner et al. 2003; Panavas et al. 2005b; Serviène et al. 2005). Unfortunately, systematic genome-wide screens have not been conducted with plant RNA viruses in plant hosts.

Additional yeast-based screens with TBSV, including the yeast essential gene library, temperature-sensitive (ts) mutant library, and high-throughput over-expression of ~5,500 yeast genes in wt yeast contributed to the identification of ~250 additional host proteins that could affect TBSV replication (Jiang et al. 2006; Serviène et al. 2006; Shah Nawaz-UI-Rehman et al. 2012, 2013). Global proteomic-based screens with a yeast protein array carrying ~4,100 purified proteins that covers almost all soluble yeast proteins has led to the identification of 57 yeast proteins interacting with tombusvirus p33 replication protein, and 11 host proteins bound to the unique portion of tombusvirus p92^{pol} or to the TBSV repRNAs (Li et al. 2008, 2009). Moreover, yeast membrane-based two-hybrid assay (MYTH) with yeast cDNA libraries also led to the identification of novel set of host proteins interacting with p33 replication protein (Mendu et al. 2010). Altogether, four separate genomics and four proteomics screens with TBSV in yeast have led to the identification of ~500 yeast genes that could be involved in TBSV replication. These systems level approaches make the TBSV-yeast system one of the best characterized pathogen-host systems at the cellular level (Nagy 2011; Nagy and Pogany 2010). Exploiting the above invaluable data sets, detailed mechanistic studies with many of the identified host factors have led to a deeper understanding of plant virus- host interactions, as discussed below.

2.4.1 Membrane Rearrangements and Spherule Formation to Harbor the Viral Replicase Complex

Several plant (+)RNA viruses, including TBSV and BMV, induce the formation of numerous vesicle-like membranous structures that harbor the VRCs (den Boon and Ahlquist 2010; Wang 2015). Most of these virus-induced structures, called

spherules, contain narrow openings toward the cytosol to allow entry of metabolites/ribonucleotides and the escape of the produced viral (+)RNAs (Fig. 2.2a). But how are these intricate structures that are likely stable for several hours formed? Interestingly, genome-wide screens in yeast have identified that both TBSV and BMV subvert cellular membrane bending/remodeling proteins, including the so-called endosomal sorting complex required for transport (ESCRT) machinery (Barajas et al. 2009a, 2014a; Diaz et al. 2015). The ESCRT machinery is conserved across kingdoms of life and is required for the formation of intraluminal vesicles (ILVs) during the generation of multivesicular bodies (MVBs). The ESCRT complex plays a role in the sorting of membrane bound proteins into the MVB pathway to degrade cargo proteins and lipids in the vacuole (Hurley 2015). TBSV co-opts the ESCRT machinery via direct interactions between the viral replication proteins and Vps23 (ESCRT-I member) or Bro1 accessory ESCRT protein, leading to the relocalization of Vps23 and Bro1 to the peroxisome membrane, the site of TBSV replication. This is followed by the sequential recruitment of additional ESCRT proteins that bend the membrane away from the cytoplasm towards the lumen of membranous organelles due to the induction of negative curvatures in the membrane bilayer. Finally, TBSV recruits the ESCRT-associated Vps4 AAA+ ATPase and some auxiliary proteins, which would normally assist the disassembly of the ESCRT complex and leading to membrane scission to create ILVs (Hurley 2015). However, Vps4 function in membrane scission is likely blocked by interaction with p33 replication protein, thus stabilizing the spherule structure (Barajas et al. 2014a). When Vps4 is deleted in yeast, then the neck structure of the spherules remains wide and the replicase complex is no longer protected from the host defense surveillance system (Fig. 2.2b) (Barajas et al. 2014a).

The subversion of the ESCRT machinery by TBSV is critical for replication since in *vps23Δ* yeast, TBSV replication drops dramatically and the ribonuclease sensitivity of the viral (–)RNA is increased when compared to the wt yeast (Barajas et al. 2009a). Another tombusvirus, the mitochondria-based CIRV also recruits Vps23 via direct interaction with the replication protein (Richardson et al. 2014).

The replication of BMV RNA is also dependent on the membrane shaping function of the ESCRT complex in yeast (Diaz et al. 2015). BMV 1a replication protein binds to and recruits Snf7 (ESCRT-III member, also required for TBSV replication) to form spherules. The BMV replicase complex formation also depends on additional membrane shaping proteins, called reticulons, which seem to be dispensable for TBSV. The need of reticulons may seem surprising at first glance as they usually induce and stabilize positive membrane curvatures. BMV could still induce the formation of spherules in reticulon depleted cells, but the spherules are much smaller, ~30 nm compared to the original ~70 nm diameter (Diaz et al. 2010). Also protein 1a is not able to recruit the viral RNA template to the site of replication. It seems likely that reticulons are usurped inside the spherule to help expand the negative membrane curve via intercalating short opposing, positive curves from space to space. And also the co-opted reticulons may stabilize the

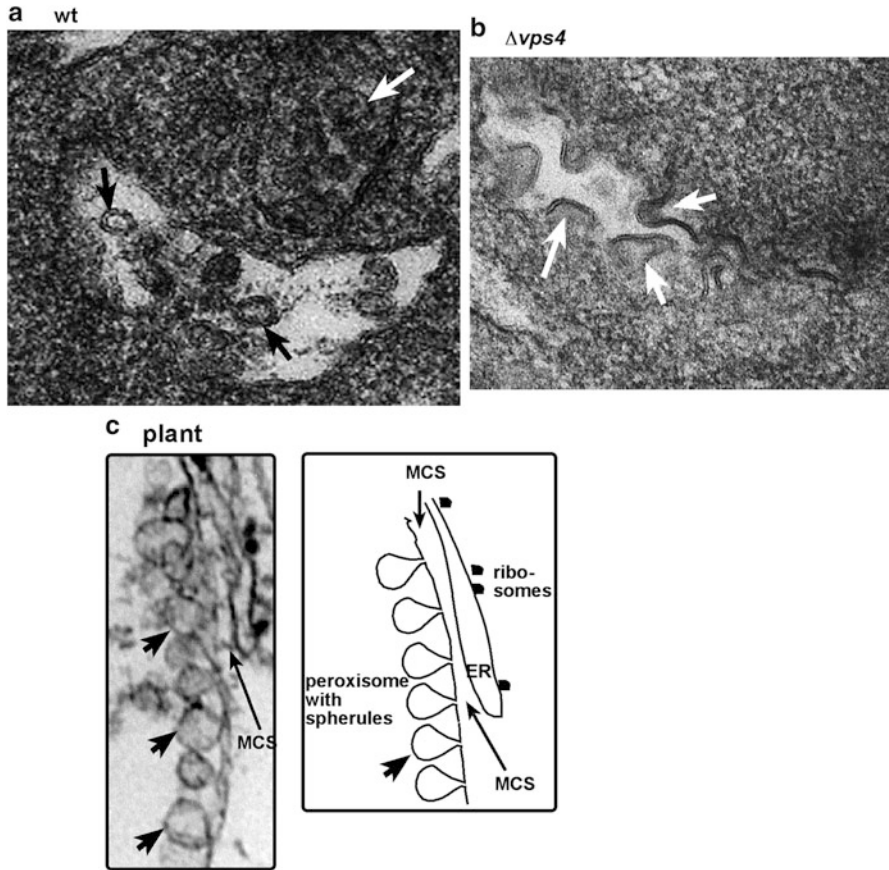


Fig. 2.2 The role of Vps4 ESCRT protein and membrane contact site (MCS) in the formation of spherule-like structures induced by tombusvirus replication proteins. (a) TEM of stained ultra-thin sections of wild type yeast cells replicating TBSV RNA with characteristic membranous compartments with tombusvirus-induced spherules. Arrows point to the spherules within the intracellular compartment. (b) *vps4Δ* yeast cells contain crescent-shaped membranes, which face the lumen of the compartment, but apparently fail to complete the spherule constriction since they have wide openings to the cytosol (white arrows). (c) The presence of MCS-like structures in the vicinity of tombusvirus-induced spherules in plant cells infected with CNV. Representative electron microscopic images of portion of a *N. benthamiana* cell. Several characteristic virus-induced spherules are marked with arrowheads and the MCS-like structures are indicated by arrows. These spherules are formed via membrane invagination into peroxisome-derived membranes

positively curved neck region in the spherule (Diaz et al. 2010). Importantly, the requirement for the co-opted cellular membrane-shaping ESCRT proteins has been confirmed in plants for both TBSV and BMV, further justifying the use of yeast as a model to dissect (+)RNA virus replication process (Barajas et al. 2009a, 2014a; Diaz et al. 2015).

2.4.2 (+)RNA Virus Replication Depends on Lipid Biosynthesis and Intracellular Lipid Transport

The genome-wide screens for host factors affecting TBSV and BMV replication also revealed roles for enzymes involved in lipid biosynthesis and intracellular transport (Kushner et al. 2003; Panavas et al. 2005b; Serviène et al. 2005). For example, deletion of yeast genes involved in sterol or phospholipid biosynthesis greatly hinders TBSV replication (Sharma et al. 2010, 2011). Interestingly, TBSV replication induces the upregulation of phospholipid synthesis, especially that of PE (phosphatidylethanolamine), which becomes highly enriched at the sites of TBSV or CIRV replication (Barajas et al. 2014c; Xu and Nagy 2015).

Why are lipids so important for (+)RNA virus replication? Cellular membranes are built from lipid bilayers that contain multitude of different lipids and proteins. Phospholipids, which are the major lipids in the membranes, contain a polar head group and a long hydrophobic chain that points towards each other in a membrane bilayer. The different charges of lipids modify the physical features of the membrane, and may block or promote the assembly and activity of the replicase. Indeed, while neutral lipids are advantageous, negatively charged lipids, such as phosphatidylglycerol (PG) has inhibitory effect on template recruitment and on tombusvirus RdRp activation (Pogany and Nagy 2015; Xu and Nagy 2015). In addition to the phospholipids, the cell membrane is tucked with sterols and covered with glycolipids. Lipids affect the fluidity and thickness of organellar membranes, and affect membrane curvature. Yeast with well-defined lipid metabolism could serve as an outstanding model to dissect the role of various lipids in plant (+)RNA virus replication. Accordingly, yeast and plant lipidomics corroborated that PE content is higher in hosts supporting TBSV replication than in the control, virus-free hosts (Xu and Nagy 2015). An interesting feature of PE is that PE promotes negative membrane curvature that could be beneficial during spherule formation. Hence it is possible that PE enrichment in membranous microdomains is used by other (+)RNA viruses to build spherules.

If lipids are so important for (+)RNA virus replication, then how can the virus subvert those lipids? The emerging picture about TBSV-yeast interaction is that TBSV channels sterols and possibly phospholipids to the site of replication by co-opting lipid-binding proteins. For example, the p33 replication protein binds oxysterol binding protein related proteins (ORPs) and VAP proteins in yeast and in plants and hijacks them to the membranous compartment where VRCs form (Barajas et al. 2014b). VAP proteins are present in all eukaryotes and are known to establish membrane contact sites (MCS), where subcellular membranes are juxtaposed and the microenvironment becomes suitable for sterol transfers (Fig. 2.2c) (Lahiri et al. 2015). Both p33 replication protein and the cellular VAPs bind ORPs and recruit them to MCSs. ORPs deliver sterols from the ER to the acceptor membranes at MCSs to increase sterol concentrations locally and to facilitate membrane bending during VRC formation. *In vitro* experiments with artificial vesicles demonstrated that the activity of the replicase was stimulated by the addition of sterols (Barajas et al. 2014b). The current model predicts that via recruiting VAPs and ORPs, TBSV facilitates the formation of MCSs and triggers

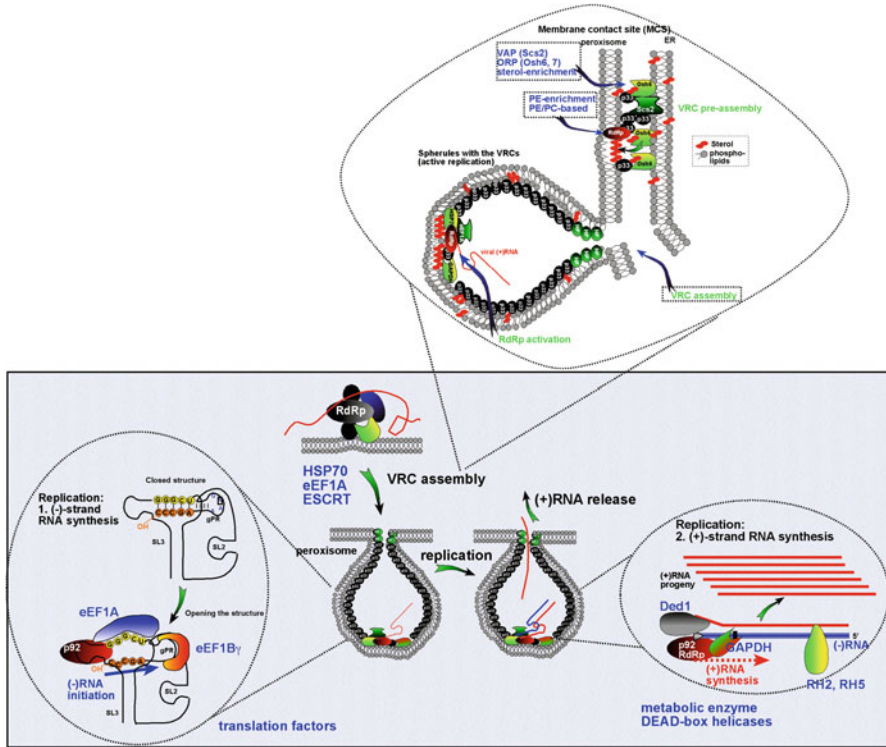


Fig. 2.3 Co-opted proviral host factors facilitate TBSV replication. The assembly of the membrane-bound tombusvirus VRCs is affected by the three shown yeast proteins or protein family (in blue). **TOP:** The VRC formation is facilitated by the stabilization of membrane contact site (MCS) between ER and peroxisome by p33 and the co-opted VAP (the yeast Scs2) and ORPs (members of the yeast Osh family). The function of MCS is to enrich sterols and possibly phospholipids (such as PE) at the viral replication sites (indicated by the vesicle-like spherule structure). **Left:** The minus-strand synthesis by the viral RdRp protein (p92, red oval) is regulated by two translation factors. **Right:** The synthesis of the more abundant (+)RNA (using the dsRNA replication intermediate) is assisted by subverted Ded1 and RH2/RH5 DEAD box helicases and GAPDH metabolic enzyme

sterol enrichment to aid the formation of spherules containing VRCs (Fig. 2.3) (Barajas et al. 2014b).

In addition to the above selective enrichment of sterols at replication sites, TBSV also induces membrane proliferation via generation of new membranes. This is achieved in yeast via interaction of p33 replication protein with the yeast Opi1 and Scs2 (a VAP) proteins, which are phospholipid sensors and Opi1 represses the transcription of phospholipid biosynthesis genes (Barajas et al. 2014c). When p33 binds Scs2 and Opi1 in the ER, then the suppression of phospholipid genes (such as *INO1*, *OPI3* and *CHO1*) is relieved and phospholipids are increasingly synthesized (Barajas et al. 2014c). This observation suggests that TBSV can also utilize *de novo* synthesized phospholipids. Accordingly, deletion of

OP11 repressor increases TBSV RNA accumulation in yeast and stimulates the activity of the replicase in the CFE assay (Barajas et al. 2014c).

2.4.3 Subcellular Locations for (+)RNA Virus Replication

One of the intriguing aspects of (+)RNA virus replication is the variation of the sites for VRC assembly in spite of the common dependence of these viruses on subcellular membranes. Why does one (+)RNA virus favor a particular subcellular location over the other locations, while another related or unrelated (+)RNA virus prefers a different location? For example, TBSV favors the peroxisomal membrane for VRC assembly in yeast and plants (McCartney et al. 2005; Panavas et al. 2005a), while the closely related CIRV selects the outer mitochondrial membrane (Weber-Lotfi et al. 2002). Yeast and CFE-based studies also help to gain insights into this question. Elimination of peroxisomes via deletion of peroxisome membrane-biogenesis genes, such as *PEX3* or *PEX19*, in yeast has not inhibited TBSV replication, which “switched” to the ER membranes for VRC assembly (Jonczyk et al. 2007). Also CFE-based work with isolated ER or mitochondria from yeast revealed that TBSV could efficiently replicate in the ER membrane and to a lesser extent in the mitochondrial membrane *in vitro* (Xu et al. 2012). Similarly, the insect virus FHV replication can be retargeted from the mitochondrial membrane to the ER without adverse effects at the cellular level (Miller et al. 2003). Thus, (+)RNA viruses seem to be flexible to some extent in their abilities to exploit various subcellular membranes.

However, our understanding of the roles of various organellar membranes in plant (+)RNA virus replication is far from complete. For example, down-regulation of ER resident secretory proteins that play essential role in peroxisome biogenesis affected TBSV replication negatively (Sasvari et al. 2013a). This suggests that the early steps in peroxisome membrane formation are important for TBSV to replicate. Thus, even if the presence of fully matured peroxisome is dispensable and TBSV can assemble VRCs in the ER, it is still important to initiate peroxisome-like membranes for TBSV. It is possible that proximity of various organelles is important for TBSV to reorganize subcellular membranes- accordingly, peroxisome and mitochondria are, in general, in close vicinity to the ER membranes and they regularly transport/exchange metabolic compounds, sterols and lipids (Lahiri et al. 2015).

2.4.4 Co-opted Heat Shock Proteins and Activation of the Viral RdRp

The sophisticated nature of plant (+)RNA viruses is obvious in many subchapters described here, yet one of the unexpected faces of virus replication is the dependence on cellular “house keeping” proteins. A fascinating example is the discovery

of the virus replication-associated role of heat shock protein 70 (Hsp70), which is a molecular chaperone involved in refolding of misfolded cellular proteins. There are three groups of cytosolic Hsp70 chaperones coded in the yeast genome. One group is termed Ssa1-4. Ssa1 and Ssa2 are constitutively expressed and 98 % identical, while Ssa3 and Ssa4 are stress-inducible and 80 % identical to Ssa1/2. Other Hsp70 chaperones in yeast are the ribosome associated Ssb1-4 group, and also the Sse1-4 group. Interestingly, the purified TBSV replicase contained the yeast Ssa1/Ssa2, as determined by 2D-gel electrophoresis and mass-spectrometry analysis (Serva and Nagy 2006). Hsp70 is a highly conserved protein family and it is involved in folding of newly synthesized and refolding of misfolded/aggregated proteins; protein degradation; protein translocation across, or insertion into the membrane; protein complex assembly and disassembly and receptor signaling (Daugaard et al. 2007; Qu et al. 2015). Contributions of Hsp70s to various virus infections were reported, however, characterization of the specific role of Hsp70s in virus replication is far from being straightforward. It is widely observed that at early time of infection, Hsp70 level goes up in response to the affliction of the cell. In general, Hsp70s are mostly involved in co- or posttranslational folding of the viral proteins; however they may also play specialized roles in (+)RNA virus replication (Nagy et al. 2011). Accordingly, specialized pro-viral role of Hsp70s has been discovered in case of TBSV replication. Ssa1-4 are interchangeable for TBSV replication, hence to dissect the mechanism behind the involvement of Hsp70 in the replicase complex, double or triple mutant yeast strains had to be used. It was found that *ssa1 Δ ssa2 Δ* double mutant yeast supported TBSV replication only marginally, which observation was validated in plants by applying Hsp70 inhibitors to the leaves (Serva and Nagy 2006; Wang et al. 2009a). Further analysis revealed that Ssa1/2 is diverted from its cytosolic distribution to the peroxisome membrane by p33 and p92^{pol} replication proteins. If all the four SSA genes were deleted, yeast cannot grow. However, the simplicity of yeast reverse genetics allows the combinations of diverse mutations. Thus, using a yeast strain harboring ts mutant Ssa1 and lacking SSA2-4, the pro-viral function of Ssa1^{ts} can be debilitated or partially debilitated at elevated temperature. Under these circumstances functional VRC could not assemble (Wang et al. 2009b). CFE-based TBSV replication assay also corroborated that Ssa1 (in the absence of the other Ssa members) is essential for VRC assembly and activation of the RdRp function of p92^{pol} (Pogany and Nagy 2015; Pogany et al. 2008). Taken together, using the yeast model platform, distinct functions of Hsp70 chaperones in TBSV replication could be determined. Ssa1/2 proteins are essential for the early steps of TBSV replication: for the recruitment of p33 and p92^{pol} to the membrane, membrane insertion of the replication proteins, VRC assembly and activation of p92^{pol}, while Hsp70s are dispensable for subsequent minus- and plus-strand synthesis.

Besides Hsp70 chaperones, members of the Hsp90 and the J-domain-containing Hsp40 families are often utilized by viruses (Nagy et al. 2011). For example, Ydj1 Hsp40 co-chaperone, which regulates Hsp70 and Hsp90 functions, affected FHV accumulation on the mitochondrial membrane (the native site of replication for FHV) in yeast. Moreover, *in vitro* experiments revealed that Ydj1

is required for the assembly of the FHV replicase complex and for the stability of FHV RdRp. However lack of Ydj1 had little if any effect when FHV replication was retargeted to the ER. This result demonstrates that cellular chaperones may have subcellular membrane-specific differences (Weeks and Miller 2008; Weeks et al. 2010). Ydj1 is also required for BMV RNA replication, though BMV replicates on the ER membrane in yeast. Ydj1 maintains the cytosolic solubility of the BMV 2a polymerase prior to membrane integration but does not affect the recruitment of 1a and 2a proteins to the ER. Despite the correct integration of the BMV replication proteins into the membrane, (–)RNA synthesis is hindered when Ydj1 is mutated in yeast. This suggests that Ydj1 might be needed for the activation of the BMV replicase complex (Tomita et al. 2003) and that Ydj1 has somewhat similar functions in the replication of FHV and BMV. Overall, the above studies on the role of heat shock proteins and their associated J-domain co-chaperones have been greatly facilitated by the facile genetics of yeast, indicating that the challenges with multimember protein families could be overcome in yeast cells.

2.4.5 Complex Roles of Co-opted Host Proteins During Viral RNA Synthesis

The central process in (+)RNA virus replication is RNA synthesis, which generates the new infectious progeny (+)RNAs. This process is driven by the viral-coded RdRp, but co-opted host proteins likely affect RNA synthesis. Accordingly, proteomic-based screens led to the identification of eukaryotic translation elongation factor 1A (eEF1A) as a component of the purified tombusvirus replicase and an interactor with the viral replication proteins as well as the viral RNA (Li et al. 2009, 2010, 2014). eEF1A bears multiple cellular functions, including its canonical role to deliver aminoacyl tRNA to the ribosome. However, other cellular functions, such as quality control of newly produced proteins, ubiquitin-dependent protein degradation, and organization of the actin cytoskeleton were also assigned to this highly abundant protein (Mateyak and Kinzy 2010). Interestingly, eEF1A selectively stimulates TBSV (–)RNA synthesis by acting as a “matchmaker”, via facilitating the interaction between p92^{pol} and the gPR promoter at the 3' end of (+)RNA (Fig. 2.3) (Li et al. 2010). However, eEF1A does not function alone, but acts synergistically together with another translation factor, called eEF1Bγ, in the TBSV replicase complex. eEF1Bγ binds to the stem-loop structure of gPR that leads to the opening up the RNA-RNA interaction between gPR and the RSE (Sasvari et al. 2011). This open configuration of gPR and RSE facilitates the binding of eEF1A and p92^{pol} to the 3' end, and ultimately promotes (–)RNA synthesis (Fig. 2.3) (Sasvari et al. 2011). Thus, the interplay among co-opted cellular translation factors, the TBSV RdRp and the template (+)RNA regulates (–)RNA synthesis within the membrane-bound VRCs.

In addition to the above described role in (–)RNA synthesis, eEF1A plays additional roles in TBSV replication, including enhancing the stability of p33 replication protein and promoting VRC assembly (Li et al. 2010). Therefore, eEF1A is an elegant example that (+)RNA viruses could co-opt cellular protein (s) to perform multiple pro-viral functions. Accordingly, the replications of numerous plant and animal (+)RNA viruses are affected by eEF1A (Mateyak and Kinzy 2010; Thivierge et al. 2008). The detailed role of eEF1A in (–)RNA synthesis was also highlighted in case of West Nile virus (Brinton 2014).

For a long time it was an open question if the same proteins are involved in (–)RNA synthesis as in (+)RNA synthesis. This is because the promoter sequences and enhancer/silencer *cis*-acting elements are different both in sequences and structures in the (+)RNA versus the (–)RNA. How can the same RdRp recognize all these elements and perform the asymmetrical RNA synthesis leading to excess amount of (+)RNA over the (–)RNA during the course of replication? Answers to these questions start to emerge for TBSV based on yeast and CFE replication assays. The high-throughput screens helped identify the essential DEAD-box RNA helicase, Ded1, which selectively affects TBSV (+)RNA level (Kovalev et al. 2012b). Another co-opted cellular protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase coded by Tdh2 and Tdh3 in yeast), which was identified via a proteomic approach, is also sequestered to the TBSV replicase complex and affect (+)RNA level (Wang and Nagy 2008). The identification of these cellular proteins in the tombusvirus VRCs and their effects mostly on (+)RNA levels strongly suggested that host proteins involved in (–)RNA and (+)RNA synthesis are not the same.

Although many (+)RNA viruses code for helicases that likely facilitate unwinding of RNA structures or remodeling protein-RNA complexes, small (+)RNA viruses, like TBSV, do not code for helicases. However, the emerging picture is that TBSV recruits several cellular helicases to facilitate (+)RNA synthesis. The first subverted helicases characterized were Ded1 and Dbp2, which have partially redundant functions during TBSV replication. Both Ded1 and Dbp2 bind to the 3' end of the (–)RNA and, in an ATP-dependent manner, and facilitate (+)RNA synthesis (Kovalev et al. 2012a, b). The major function of Ded1/Dbp2 is to open up the dsRNA intermediate only at one of the ends, which harbors the cPR [i.e., 3' end of the (–)RNA]. This then allows the loading of the p92 RdRp onto the 3'-end of the (–)RNA, followed by initiation of (+)RNA synthesis guided by the cPR sequence (Fig. 2.3). Interestingly, Ded1 also facilitates the release of the p92 RdRp from the (+)RNA when the RdRp is paused (usually at the end of the template when complementary RNA synthesis is accomplished) (Chuang et al. 2015). Therefore, these functions of co-opted Ded1 help the RdRp switch from (–)RNA to (+)RNA synthesis. The Arabidopsis homolog of Ded1/Dbp2, called AtRH20, also promotes (+)RNA synthesis in a yeast CFE-based assay, suggesting that plant helicases with corresponding functions are present in plant hosts (Kovalev et al. 2012a).

Although the formation of dsRNA intermediate during (–)RNA synthesis (Kovalev et al. 2014) prevents new (–)RNA synthesis due to “burying” the gPR and other *cis*-acting sequences within the dsRNA structure, while allowing (+)RNA

synthesis with the help of Ded1/Dbp2, it seems that this strategy is not robust enough to guarantee 20-to-100-fold excess of (+)RNA synthesis over (−)RNA synthesis. Indeed, TBSV recruits a second group of cellular helicases, which consist of Fal1 and Dbp3 in yeast and AtRH2 and AtRH5 in plant, to “boost” (+)RNA synthesis (Fig. 2.3). The members of this group of helicases have redundant functions and they open up the dsRNA intermediate within the RIII(−) RE sequence located close to the 5′ end of (−)RNA. Since the opening of dsRNA only takes place locally within the RE sequence, the actual 5′ end of (−)RNA and thus the 3′-end of (+)RNA carrying the gPR are still buried in dsRNA form. Interestingly, opening of RIII(−) RE brings the 5′- and the 3′-ends of (−)RNA into proximity via long range base-pairing and enhances multiple rounds of (+) RNA synthesis via repeatedly “recycling” the RdRp from termination to new round of (+)RNA initiation from the cPR sequence. Thus, the current model predicts that the coordinated actions of these co-opted cellular helicases are needed for the asymmetric accumulation of (+)RNA (Fig. 2.3) (Kovalev and Nagy 2014). Ded1 helicase was also shown to play a role in BMV replication in yeast, albeit in a different role. Ded1 was shown to selectively inhibit the translation of the viral 2a RdRp to down-regulate 2a protein level compared with the 1a replication protein (Noueiry et al. 2000).

Another RNA-binding cellular protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is also sequestered to the TBSV replicase complex (Serva and Nagy 2006). This protein has ample functions unrelated to its well-known glycolytic function (Sirover 2014). Yeast has two copies of GAPDH, Tdh2 and Tdh3, and at least one must be functional for viability. The replicase complex was purified from a wild type and from a mutant strain (*tdh2Δ* and down-regulated TDH3) and it was found that in the absence of sufficient amount of GAPDH, the asymmetric nature of TBSV replication was abolished, the synthesis of (+)RNA has dramatically dropped (Huang and Nagy 2011; Wang and Nagy 2008). Down-regulation of GAPDH in plant also decreased TBSV replication. Hence it seems that a very neat choreography involving the viral RNA, p92 RdRp and p33 RNA chaperone in concert with co-opted cellular helicases and a metabolic enzyme is at work to maintain the required over-production of viral (+)RNAs during infections.

2.4.6 Discovery of Cell-Intrinsic Viral Restriction Factors in Yeast

The cells are not passive “hosts” of viruses, but recognize viral components or the damage caused by the viral infection and launch various cellular responses. Moreover, cells likely have antiviral factors that guard against viruses and limit the infection process. These cellular factors are termed cell-intrinsic restriction factors (CIRFs) (Diamond and Gale 2012; Sasvari et al. 2014). The yeast-based genome-wide screens and proteomics approaches can also lead to identification of CIRFs, as

demonstrated for TBSV. For example, certain members of the Cyp40 cyclophilin family, which are peptidyl-prolyl-*cis-trans*-isomerases, strongly inhibit TBSV replication in yeast. Cyclophilins work by binding to client proteins and performing isomerisation of peptidyl-prolyl bonds. Interestingly, the yeast Cpr1 (the human orthologue is called CypA) and Cpr7 (Cyp40-like) bind to the RNA binding motif (RPR) of p33 replication protein (Kovalev and Nagy 2013; Lin et al. 2012; Mendu et al. 2010). This interaction leads to inhibition of p33-driven (+)RNA template selection and viral (+)RNA recruitment to the replicase complex (Fig. 2.4). The corresponding cyclophilins from *Arabidopsis* are the strongest inhibitors by reducing TBSV genomic RNA accumulation by 90 %. This result verified the anti-viral effect of Cyp40-like cyclophilins in plants.

In addition to the antiviral cyclophilins, ~70 other CIRFs were also identified by yeast library screens that impede TBSV replication. These include the WW domain proteins carrying a highly conserved structure responsible for protein-protein interactions. For example, the yeast NEDD40-like Rsp5 E3 ubiquitin ligase possesses WW domain and was identified as a very potent inhibitor of TBSV replication in yeast (Barajas et al. 2009b). Several plant derived WW domain proteins also had strong negative regulatory effect on tombusvirus genomic RNA accumulation. Interestingly, replication of FHV and NoV are also refrained by certain WW proteins in yeast (Barajas et al. 2015; Qin et al. 2012). Over-expression of certain WW-domain proteins in yeast also reduces the quantity of several host-factors co-opted in the VRCs. The amount of subverted cellular ESCRT proteins, eEF1A, GAPDH and Pex19 were found the most reduced. The current model predicts that certain proteins with WW domain prevents new VRC assembly when the availability of pro-viral proteins becomes limited (Fig. 2.4). This late-stage regulation of replication may trigger the switch from progeny RNA synthesis to virion assembly (Barajas et al. 2015).

CIRFs will likely have multiple functions and they may interact with tens or hundreds of other proteins probably manifesting diverse roles in seemingly unrelated pathways. Moreover, even if physical interaction cannot be detected between given proteins, these proteins can be genetically connected. These interactions usually are visualized as a network. The main nodes in the network, the Hub genes, have an extraordinary number of connections that interact with many unrelated pathways. To gain insight into the function of the identified CIRFs of TBSV replication, a protein network, including the identified restriction factors, has been built based on the yeast interaction map (SGD database, <http://www.yeastgenome.org>). Three Hub proteins were unveiled, Xrn1p 5'-3' exoribonuclease (Fig. 2.4), Act1p actin protein and Cse4p centromere protein (Sasvari et al. 2014). Protein network analysis of orthologous plant genes revealed three strongly connected groups, similar to those found in the yeast network. In summary, CIRFs seem to function as either direct antagonists of viral components through binding and blocking viral functions, or they may inhibit the pro-viral functions of other co-opted host proteins. Others, like cyclophilins may also act as 'guardians' by protecting cellular chaperones, like Hsp70 through inhibiting their subversion by the virus (Sasvari et al. 2014).

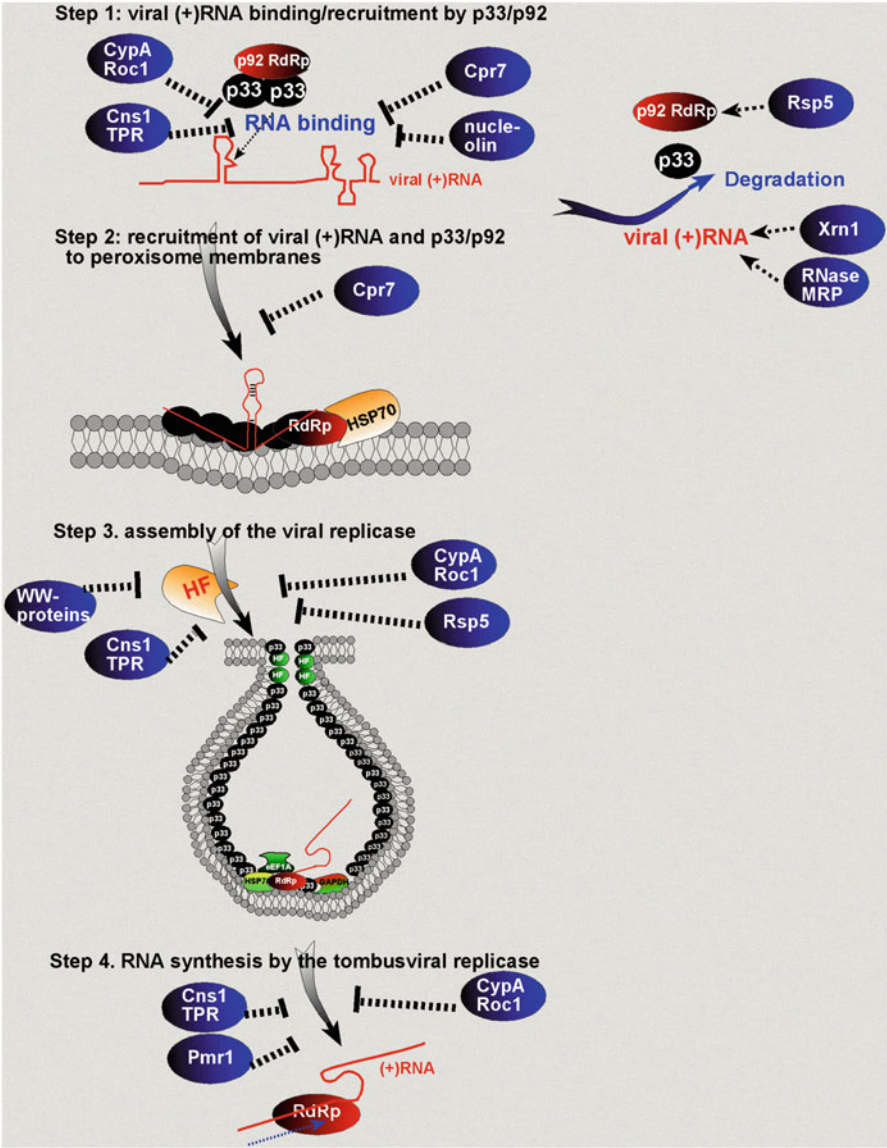


Fig. 2.4 Targets and antiviral functions of CIRFs in tombusvirus replication. The sequential TBSV replication steps and degradation of viral components (p33/p92 and the viral RNA) are shown. “HF” indicates pro-viral host factors co-opted by TBSV. The virus induced spherule (vesicle-like structure) harboring the membrane-bound VRC is shown. The detailed functions of CIRFs are described in Sasvari et al. (2014)

2.4.7 Additional Aspects of Viral Processes Dissected in Yeast: Viral Sensing of the Subcellular Environment

Overall, yeast provides a powerful platform to identify and dissect the molecular functions of cellular factors exploited by viruses throughout their infectious cycles. Because host factors are recruited to assist every step during replication and several of the host factors are kept permanently in the VRCs, therefore (+)RNA viruses should likely “sense” the molecular environment, especially the availability/accessibility of host factors as virus replication progresses with incredible speed and efficiency. If host factors became scarce/limited due to their robust exploitation during previous rounds of VRC assembly, then (+)RNA viruses will likely halt new VRC assembly. Accordingly, based on TBSV and yeast, it has been shown that the availability of several pro-viral host proteins versus the regulatory WW-domain proteins determine if new VRC assembly continues or halt (Barajas et al. 2015). The pro-viral host factors bind with higher affinity to p33 and these pro-viral host proteins will be sequestered first for VRC assembly. Then, when the pro-viral host factors become limited, the yeast WW-domain proteins, which bind to p33 with lower affinity, could bind to the TBSV p33/p92^{pol} proteins, resulting in a complex that hinders the assembly of new VRCs, blocking p33-viral (+)RNA interactions and promoting the degradation of p92^{pol} (Barajas et al. 2015). TBSV might also be able to “sense” the availability of suitable membranes for new VRC assembly through p33/p92^{pol} binding to PE versus PG phospholipids in the subcellular membranes. Binding to PE is more favoured due to its higher amount and the induction of PE synthesis and membrane proliferation by TBSV (Xu and Nagy 2015), leading to the activation of p92 RdRp and VRC assembly. However, at the late stage of infection, the availability of “free” PE might be limited, and p33/p92^{pol} could bind to the accessible PG in membranes that would block new VRC assembly and inactivate p92 RdRp (Pogany and Nagy 2015). These interactions would free the new viral (+)RNA from replication cycle to facilitate robust encapsidation.

2.4.8 A Major Effect of Cellular ion Homeostasis on TBSV Replication in Yeast

An unexpected outcome of global screens is the identification of cellular factors involved in maintaining ion homeostasis in cells. The lipid bilayer in the subcellular membranes is impermeable for ions and polar molecules. Permeability is conferred by ion pump- or channel-proteins embedded in various subcellular membranes. Interestingly, the inactivation of PMR1, which codes for a $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump, greatly increases TBSV replication and also viral RNA recombination in yeast (Jaag et al. 2010). This surprising effect by Pmr1 is due to regulation of Mn^{2+} concentration in the cytosol, which increases when pmr1 is deleted, leading to the

utilization of Mn^{2+} ions instead of Mg^{2+} by the viral replicase, which renders the replicase more active, but error prone (Jaag et al. 2010).

The deletion of another ion transporter, Gef1, strongly inhibits TBSV replication in yeast (Sasvari et al. 2013b). Gef1 is the only proton-chloride exchanger in yeast and it is responsible for the maintenance of cytosolic and organelle pH. Deletion of GEF1 transporter affects Cu^{2+} homeostasis and Cu^{2+} may replace the Mg^{2+} in the active center of viral RdRp, rendering the RdRp inactive. Indeed, deletion of Ccc2 copper ion pump also changes Cu^{2+} concentration and hampers TBSV replication in yeast (Sasvari et al. 2013b). The above discoveries show that the yeast-based TBSV replication system is highly suitable to explore the effect of ion homeostasis on (+)RNA virus replication, further contributing to our growing understanding of cellular factors affecting virus-host interactions at the cellular level.

2.5 Conclusions and Prospects

Development of yeast as a host greatly facilitated the progress in our understanding of TBSV and BMV plant viruses' interactions with the host cells. Systematic genome-wide screens using yeast genomic libraries have led to the identification of a large number of host factors affecting (+)RNA virus replication. More detailed biochemical and cellular studies then led to the dissection of molecular functions of many host factors that promote each step of the viral replication process. The development of *in vitro* systems with TBSV, such as yeast CFE and purified active replicase assays, together with proteomics, lipidomics and artificial vesicle-based assays helped to comprehend the complex nature of virus replication. Despite of the rapidly emerging details on host-virus interactions, our knowledge is far from complete. Dissection of the molecular features of viral components and their interrelationship with cellular factors may reveal non-canonical roles of host components or new features of these molecules that are only "invented" by viruses.

In a nutshell, using yeast platform can bring various cellular conditions on the same page and give an opportunity to compare the effects of viral infection-caused cellular perturbations, genetic variations, genetic disorders, protein malfunctions, and environmental factors at the systems and molecular levels. Once the processes have been characterized in yeast, then the discoveries can be further explored and applied to native organisms. Detailed knowledge on interactions from the highest resolution to the most complex systems facilitates targeted anti-viral drug design in many ways. This is because host components are less prone to genetic variations than viruses, thus drugs that block pro-viral functions of host factors are less sensitive to the threat of drug resistance. Furthermore the gained knowledge may advance virus-mediated strategies to combat debilitating genetic diseases in various organisms.

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