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Yeast as a model host to dissect functions of viral and host factors in tombusvirus replication

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Abstract

RNA replication is the central process during the infectious cycles of plus-stranded RNA viruses. Development of yeast as a model host and powerful in vitro assays with purified replicase complexes, together with reverse genetic approaches make tombusviruses, small plant RNA viruses, excellent systems to study fundamental aspects of viral RNA replication. Accordingly, in vitro approaches have led to the identification of protein–RNA interactions that are essential for template selection for replication and assembly of the functional viral replicase complexes. Moreover, genome-wide approaches and proteomics analyses have identified a new set of host proteins that affected tombusvirus replication. Overall, rapid progress in tombusvirus replication has revealed intriguing and complex nature of virus–host interactions, which make robust replication of tombusviruses possible. The knowledge obtained will likely stimulate development of new antiviral methods as well as other approaches that could make tombusviruses useful tools in biotechnological applications.
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Keyword: Replication

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Plant viruses with plus-stranded RNA genomes are not only important pathogens of crop plants, but they also serve as excellent model systems to study fundamental aspects of virus replication with useful implications for animal and

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human RNA viruses. The recently emerging picture is that in spite of the diverse genome organization and gene expression strategies, the mechanism of genome replication and the functions of viral and host factors might be analogous to some extent among various plus-stranded RNA viruses. Indeed, all plus-stranded RNA viruses replicate their genomes through minus-stranded replication intermediates, which are less abundant than the new plus-stranded progeny (called asymmetrical replication). Moreover, all known plus-stranded RNA viruses assemble their own replicase complexes, likely containing both viral- and host-coded proteins (Ahlquist, 2002; Ahlquist et al., 2003; Buck, 1996; Noueiry and Ahlquist, 2003). In addition, replication takes place in membranous compartments derived from intracellular organelles, such as endoplasmatic reticulum, mitochondrium, and peroxisome. Therefore, identification of the roles of various replication-associated or replication-modulating viral and host factors represents one of the major frontiers in current virus research. This review focuses on tombusvirus replication and tombusvirus–yeast interactions with the goal of providing an overview of our current understanding of virus replication and the usefulness of yeast as a model host system.

Tombusviruses as useful model systems for replication studies

Tombusviruses are a group of single-component RNA viruses of plants within the large Tombusviridae family. Among the five viral-coded proteins, only p33 and p92 are essential replication proteins (Fig. 1A) (Oster et al., 1998; Panaviene et al., 2003; Rajendran and Nagy, 2004; Scholthof et al., 1995; White and Nagy, 2004). The sequence of p33 overlaps with the N-terminal region of p92, yet the functions of these regions are different in the two proteins. p33 is a replication cofactor, which is involved in binding to the viral RNA via its RNA-binding region (termed RPR, Fig. 1A) (Rajendran and Nagy, 2003). The RPR domain is essential for the function of p33, whereas it plays a lesser role in p92, which functions as the RNA-dependent RNA polymerase (Panavas et al., 2003, 2005a). p33 also includes two essential membrane-spanning domains (Navarro et al., 2004) and an N-terminal domain with unknown but essential function in replication (Panavas et al., 2005a). In contrast, the corresponding domains in p92 modulate the function of p92 protein, but they are not essential (Panavas et al., 2005a). In contrast, the p33:p33/p92 interaction domain, which is important for multimerization of p33 molecules and binding between p33 and p92 (Rajendran and Nagy, in press), is essential for tombusvirus replication (Panavas et al., 2005a). Both p33 and p92 are part of the active tombusvirus replicase, which is most active when it contains a 10–20-fold larger amount of p33 than p92 (Rajendran and Nagy, in press). Based on biochemical and cellular studies, the emerging picture is that, in spite of the overlapping sequences, p33 and p92 perform noncomplementary functions during tombusvirus replication.

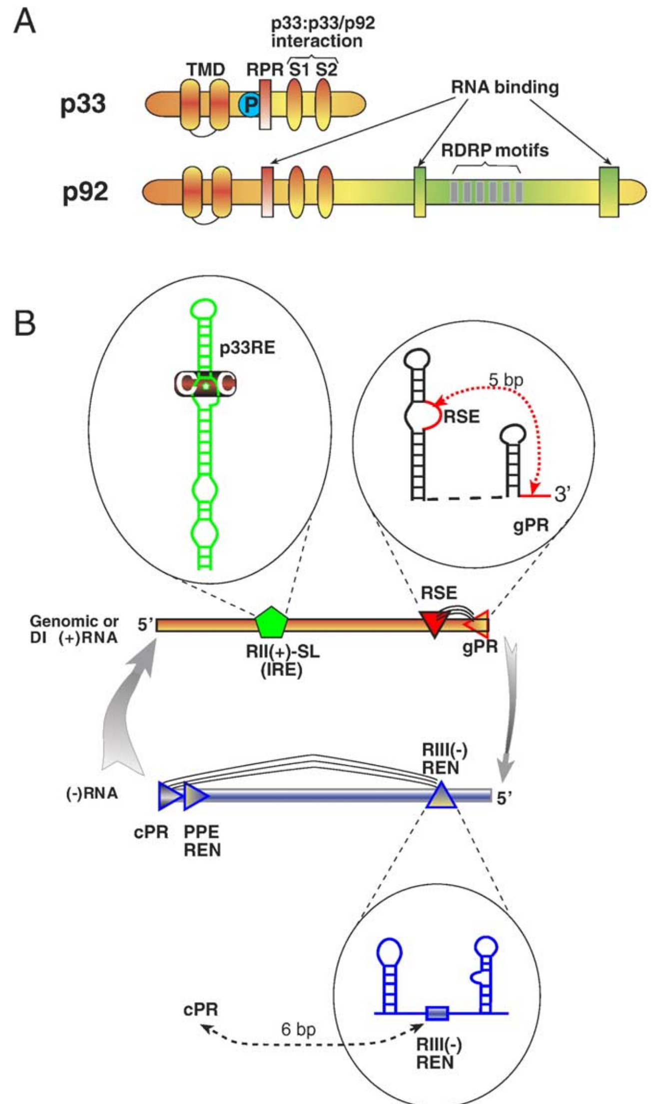

Fig. 1. Schematic representation of the known domains in the tombusvirus replication proteins (A) and *cis*-acting elements in the viral RNA (B). The asymmetrical minus-strand and plus-strand syntheses, respectively, are shown with arrows, whereas thin lines indicate long range RNA–RNA interactions between RSE and gPR as well as between cPR and RIII(−) REN (see text for details). TMD, trans-membrane domains; P, phosphorylation sites; RPR, arginine-proline-rich RNA-binding domain; S1 and S2 are subdomains of the p33:p33/p92 interaction domain; p33RE, p33 recognition element; IRE, internal replication element; RSE, replication silencer element; gPR, genomic promoter; cPR, complementary promoter; PPE, promoter proximal enhancer; REN, replication enhancer.

In addition to their simplicity, tombusviruses are also useful model systems due to the presence of efficient replicon RNAs, termed defective interfering (DI) RNAs (Finnen and Rochon, 1993; Hillman et al., 1987; Russo et al., 1994). DI RNAs are generated by RNA recombination during replication of the genomic (g)RNA, which gives rise to efficient replicon RNAs (White and Morris, 1994a, 1994b). The prototypical DI RNA contains four noncontiguous regions of the gRNA, with each region carrying *cis*-acting replication sequences. Overall, DI RNAs are useful model templates to study the interaction between the viral RNA and protein factors (White and Nagy, 2004).

Development of yeast as a host for studies on tombusvirus replication

Due to the complex nature of virus–host interactions, genome-wide screens for host genes affecting viral RNA replication would be useful for identifying host factors (Kushner et al., 2003). For genome-wide studies, yeast (*Saccharomyces cerevisiae*) is the preferable host, because of the small genome-size (~5800 genes) and reduced level of redundancy in host genes. In addition, yeast is the best-known model eukaryotic cell with the largest percentage of characterized versus uncharacterized genes in the genome that can be quite useful to study some aspects of virus–cell interactions. In addition to viruses that are naturally present in yeast (Esteban and Fujimura, 2003; Esteban et al., 2005; Wickner, 1996a, 1996b), yeast can also be developed as an artificial host to study replication of plant [*Brome mosaic virus* (BMV), *Tomato bushy stunt virus* (TBSV), and other tombusviruses] and animal viruses (nodaviruses) (Ishikawa et al., 1997; Panavas and Nagy, 2003b; Pantaleo et al., 2003; Price et al., 2002, 2005). For replication studies in yeast, the essential replicase proteins and the viral RNA (termed replicon RNA) are all expressed from plasmids to launch viral RNA replication. Studies with BMV based on yeast mutant screens have been reviewed recently (Ahlquist et al., 2003; Noueiry and Ahlquist, 2003), and they are not covered in detail here.

The advantage of the recently developed TBSV-based replicon system in yeast is that the TBSV replicon RNA does not carry any selection factor and thus, its sequence is identical with the replicon used in the natural plant hosts (Panavas and Nagy, 2003b). Yet, the replicon RNA is stably maintained as a cytoplasmic replicon in yeast for extended periods in the absence of transcription from plasmid DNA (Panavas and Nagy, 2003b). In addition, translation of the replication proteins is driven from separate plasmids, allowing separate studies on replication proteins and the template RNA and on replication and translation that simplifies interpretation of data. Overall, the replication of the replicon RNA in yeast closely mimics the authentic replication taking place in plants or plant protoplasts, as demonstrated by the following observations: (i) comparable rates of RNA accumulation in time course experiments (maximum level of RNA accumulation is reached after 24–48 h) (Panavas and Nagy, 2003b), (ii) high levels of replicon RNA accumulation that can exceed ribosomal RNA levels in both yeast and plant hosts, (iii) the asymmetric nature of RNA accumulation (20–30-fold more plus-strands over minus-stranded intermediates) (Panaviene et al., 2004), (iv) formation of RNA recombinants during replication (Serviene et al., 2005; White and Morris, 1994a, 1995), and (v) the comparable in vitro activity of purified replicase complexes (Panaviene et al., 2004, 2005). Altogether, yeast has proven to be a useful and powerful tool to study the fundamental aspects of tombusvirus replication.

Identification of host factors affecting tombusvirus replication based on genome-wide screens

The systematic genome-wide screen of host genes has identified 96 nonessential (Panavas et al., 2005b) and 30 essential yeast genes (Jiang et al., unpublished), out of ~5500 host genes tested, that affected tombusvirus replication. Interestingly, deletion or down-regulation of 16 of the identified genes increased the level of replicon RNA accumulation, suggesting that these factors normally inhibit tombusvirus replication. On the contrary, the remaining 110 host factors identified, which inhibited replication when absent or present in reduced amount, likely facilitate tombusvirus replication by providing useful functions directly or indirectly (see below). The identified host factors are known to be involved in various cellular processes, such as metabolism/modifications of RNAs, lipids, and proteins; in protein intracellular transport/targeting; or in general metabolism (Panavas et al., 2005b) (Jiang et al., unpublished). Interestingly, the set of host genes affecting TBSV replication is vastly different from those affecting BMV replication, suggesting that viruses developed different ways to utilize the immense resources of cells. Inspite of the differences in the host genes involved, we predict that many of the genes might have analogous functions in TBSV and BMV replication. For example, molecular chaperones, albeit different members of the chaperone family, have been found to affect BMV (Tomita et al., 2003) and TBSV replication (Serva and Nagy, submitted for publication).

The genome-wide screens confirmed that TBSV depends on the intracellular components of the infected hosts for robust replication. The finding that many host factors with vastly different functions can affect TBSV accumulation indicates: (i) dependence of TBSV RNA or replication proteins on diverse cellular processes/proteins to perform various functions, (ii) competition between the virus and the host for limited cellular resources/factors, and (iii) the presence of targeted and general antiviral factors in host cells. The emerging picture is that the interaction between the host cell and TBSV during replication is rather complex, likely including numerous replication-associated factors with direct or indirect effects. Below, we illustrate the complexity of TBSV–host interactions.

Three major groups of host factors affecting TBSV replication

The identified host factors affecting TBSV replication fall into three categories. First are those factors that include TBSV replication-associated host proteins, host membranes, the intracellular transport and trafficking system, the translation apparatus, various intracellular compartments, such as the ER, peroxisome and vesicles, and so on, which tombusviruses require and/or utilize to complete their replication. For example, Ssa molecular chaperone, which is present in the viral replicase complex, might be involved in replicase assembly (Serva and Nagy, submitted for

publication). Also, the number of host factors known to play a role in protein transport (Panavas et al., 2005b), might facilitate localization/targeting of viral and/or other host factors. The second group includes direct inhibitory factors, such as those comprising the host antiviral defense mechanism, which affect virus replication by destroying/modifying TBSV RNA and/or viral replication proteins in targeted or in general manners. For example, Ngl2p and related endoribonucleases and Xrn1p 5′–3′ exoribonuclease were found to affect degradation, and thus stability, of TBSV RNA (Serviene et al., 2005) (Cheng et al., submitted for publication). The third group of replication-modulating factors includes indirect or general factors that affect virus replication by competing for the same cellular resources, host proteins, and intracellular compartments, which TBSV also depends on for its replication in the cell. For example, transcription factors might affect the level of host factors available in the cell, thus indirectly affecting TBSV replication. All these direct and indirect groups of factors will influence the outcome of TBSV replication and they could also be targets for development of antiviral strategies. Therefore, studies aimed at identifying and dissecting all these replication-associated activities/factors are expected to increase the number and efficiency of our methods to interfere with successful viral replication/infection. One of the next major challenges will be to determine what roles the identified host factors play during TBSV replication.

Replication cycle of tombusviruses consists of multiple distinct steps

Recent advances in tombusvirus replication, including new findings on viral replication proteins and on *cis*-acting elements in TBSV RNA, as well as identification of host factors, have allowed for the division of tombusvirus replication to six separate steps (Fig. 2). These include: (1) selection of the viral RNA template for replication; (2) targeting of essential viral replication proteins and the viral RNA to the site of replication; (3) assembly of the functional viral replicase; (4) synthesis of the viral RNA progeny; (5) release of the viral progeny from replication; and (6) disassembly of the viral replicase. Our current knowledge on the role of viral and host factors in each of the above steps will be discussed below.

Selection of TBSV RNA template for replication

During translation, the plus-stranded genomic (g)RNA of tombusviruses, which lack a 5′ cap and 3′ poly(A) tail, is used as a mRNA to produce the p33 and p92 replication proteins (Fabian and White, 2004). Unlike other mRNAs, TBSV gRNA, however, has to be saved from degradation and then recruited into replication to produce viral RNA progeny. How is the gRNA selected for replication among the thousands of mRNAs associated with host ribosomes and other host RNAs present in the cell cytoplasm and heterologous viral RNAs in case of mixed viral infections? Are the template selection performed by viral or host proteins?

To answer these questions, a combination of in vivo and in vitro experiments was performed. First, in vitro RNA: protein interaction studies with purified components identified highly specific binding of the p33 replication protein to a conserved sequence within TBSV gRNA, termed the p33 recognition element (p33RE) (Fig. 1B) (Pogany et al., 2005). Interestingly, p33RE is part of a large, conserved internal replication element (IRE) that forms a stem-loop structure with an internal loop, termed RII(+)-SL (Monkewich et al., 2005). The critical part of the RII(+)-SL structure is a C•C mismatch (Fig. 1B), which is recognized by p33 likely by both base-specific and structure-specific features. The recognition of p33RE by p33 depends on both the RPR RNA binding and the p33:p33/p92 interaction domains of p33, suggesting that dimerization of p33 is important for specific recognition. Indeed, a peptide carrying only the RPR motif bound nonspecifically to RNA (Pogany et al., 2005).

The p33:TBSV RNA interaction via the p33RE is absolutely essential for TBSV RNA replication in yeast or virus replication in plant protoplasts or whole plants (Monkewich et al., 2005; Pogany et al., 2005). Moreover, a satellite (sat)RNA associated with the distantly related *Turnip crinkle virus* (TCV, which also belongs to Tombusviridae) (Simon et al., 2004) lacking RII(+)-SL with the C•C mismatch could not bind to p33 in vitro and it was not replicated by TBSV replicase in vivo (Pogany et al., 2005). This finding is in contrast with in vitro experiments with partially purified TBSV and CNV replicases, which could efficiently recognize the TCV satRNA and produce complementary RNA products in an in vitro assay (Nagy and Pogany, 2000; Panavas et al., 2002a). Therefore, the absence of TCV satRNA replication by TBSV replicase in vivo is likely due, at least in part, to the lack of selection of TCV satRNA by TBSV p33 for replication.

This p33:TBSV RNA interaction likely influences the translation of p92 as well, based on utilization of temperature-sensitive RNA mutants defective within RII(+)-SL (Monkewich et al., 2005). The temperature-sensitive RNA mutants were also useful to demonstrate that p33:p33RE interaction is required at the beginning of infection of plant protoplasts, but not at latter stages (Monkewich et al., 2005). Altogether, the available in vitro and in vivo evidence firmly supports the role of a highly specific p33:p33RE interaction as the major factor in selection of tombusvirus RNA for replication. However, it is possible that host proteins are also important for affecting and/or regulating the selection of viral RNA for replication as demonstrated for BMV (Ahlquist et al., 2003; Diez et al., 2000). In addition, the actual mechanism of the escape of tombusvirus gRNA from translation and degradation is currently unknown, albeit p33 likely plays a central role in this process, too.

Overall, the results obtained with tombusviruses support the model that template selection for replication is the

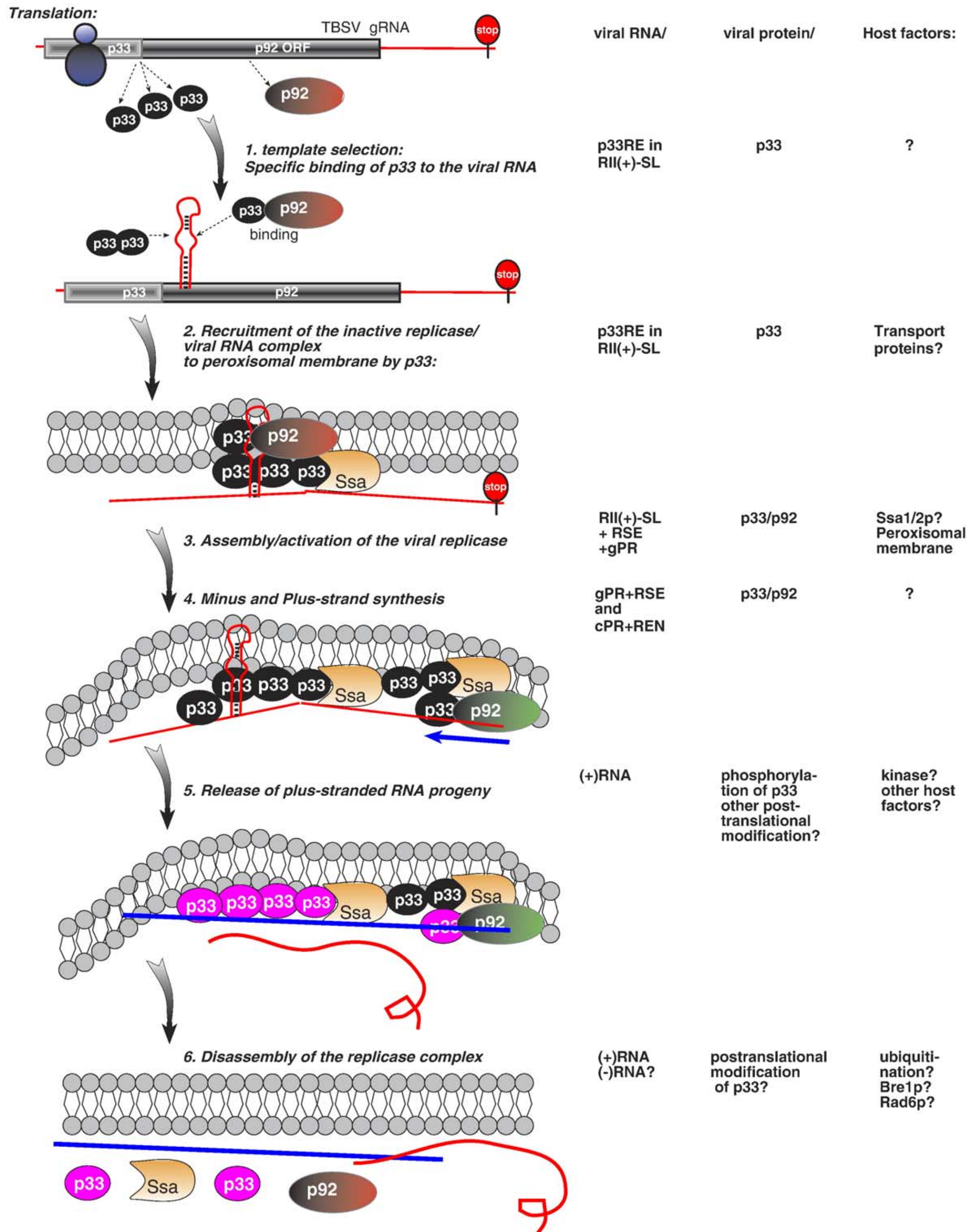

Fig. 2. A model of TBSV replication and the proposed roles of viral and host factors. The six separate steps proposed during TBSV replication are listed, including the viral and putative cellular factors involved in each step. The replicase complexes are shown schematically, but they likely contain more protein components. Stop sign at the 3′ end of the TBSV (+)RNA indicates that the RNA forms an inactive confirmation due to RSE–gPR interaction (Fig. 1B), which likely gets opened by an unknown factor in step 4. The red coloring for p92 suggests that p92 is inactive (replication incompetent) at the beginning, while it gets activated (shown in green color) during the replicase assembly process, possibly with the help of Ssa molecular chaperone. The active form of p33 (competent in RNA binding) and the inactive form (incompetent in RNA binding after phosphorylation), is shown with black and pink circles, respectively. Red line indicates plus-stranded, while blue line shows the minus-stranded viral RNAs. The RII(+)-SL harboring the p33RE is shown as a hairpin structure. Circles show the peroxisomal membranes. Question marks indicate the putative nature/function of the particular factor. See text for further details of each replication step.

major mechanism responsible for template specificity observed in virus replication. The model (Fig. 2) predicts that p33RE-containing RNAs will have the best chance to be replicated by the tombusvirus replicase, since they will be efficiently selected from the diverse RNA pool present in the host cell. The selection of templates for replication

is mechanistically somewhat similar to viral RNA encapsidation, which is also based on the requirement of a specialized viral protein (i.e., viral coat protein versus p33 replication cofactor) to sequester the viral RNA into virus-induced macromolecular structures that are mostly inaccessible for other processes.

Targeting of essential viral replication proteins and the viral RNA to the site of replication

Although the selection of the viral gRNA for replication likely takes place in the cytoplasm during and/or after translation, replication of tombusviruses occurs on the cytoplasmic surfaces of peroxisomal (for TBSV, *Cucumber necrosis virus* and *Cymbidium ringspot virus*) or mitochondrial (*Carnation Italian ringspot virus*, which will not be discussed further below) membranes (Burgyan et al., 1996; Navarro et al., 2004; Panavas et al., 2005a; Rubino and Russo, 1998). Therefore, the viral RNA and the replication proteins must be targeted to these compartments (Fig. 2, step 2). p33 likely plays a major role in this process, because it has a peroxisomal targeting sequence and it is localized to the peroxisomal membranes in yeast (Navarro et al., 2004; Panavas et al., 2005a). Interestingly, p33 lacking the S1 subdomain within the p33:p33/p92 interaction domain accumulates mainly in the ER, albeit a portion of the mutant p33 still makes it to the peroxisomes (Panavas et al., 2005a). These observations suggest that p33 likely forms multimolecular p33:p33 complexes in the cytoplasm and/or in the ER before its targeting to the peroxisomal membranes occurs (Panavas et al., 2005a). It is possible that targeting of multimolecular p33:p33 complexes is more efficient (requires proportionately less host resources) than the targeting of individual p33 molecules. Colocalization data also suggest that p92 is also part of the above p33:p33 multimolecular complexes, because p92 is also partitioned between the ER and peroxisome when it lacks the p33:p33/p92 interaction domain (Panavas et al., 2005a). Also, p92 can be targeted to the peroxisomal membranes even in the absence of the peroxisomal targeting domain when coexpressed with wt p33 (Panavas et al., 2005a), suggesting that the mutant p92 might be "piggy-backing" on p33 to the site of replication. Interestingly, this mutant p92 protein is still functional, albeit at a reduced level, in the absence of peroxisomal targeting or the RPR RNA-binding domains; however, it is nonfunctional if the p33:p33/p92 interaction domain is absent (Panavas et al., 2005a).

In contrast to p33, the viral RNA is not colocalized with peroxisomal marker proteins when expressed without p33 (Panavas et al., 2005a). Coexpression of the TBSV replicon RNA together with p33, however, resulted in colocalization of the viral RNA with the peroxisomal marker (Panavas et al., 2005a). Interestingly, only a small number of peroxisomal sites contain viral RNA initially (in the absence of replication), suggesting that localization of the viral replicon RNA might be a limiting step (bottle neck) in the replication process despite the presence of abundant viral RNAs transcribed from launching plasmids. The relatively inefficient recruitment of the replicon RNA could be due to the requirement for *trans*-acting p33 molecules. However, during natural infections, the viral genomic RNA, unlike defective interfering RNAs, is likely recruited in "*cis*" by the newly produced p33, which could potentially bind to the genomic RNA present in the same location ("*cis*"-binding) (Oster et al., 1998; Panaviene et al., 2003). Altogether, recruitment in *cis* could be a more efficient process than recruitment in *trans* that could secure the "safe travel" of the limited amount of viral genomic RNA to the site of replication.

Overall, the existing evidence supports a master role for p33 in intracellular targeting of other p33 and p92 proteins as well as the TBSV RNA, likely in the form of multimolecular complexes, to the site of replication (Fig. 2). Formation of the multimolecular complex including p33, p92, the viral RNA, and possibly host factors in the cytoplasm could facilitate efficient transport and colocalization of all these essential components to the same replication sites to increase the probability of successful replicase assembly (see below). This structural pre-organization of replication factors could be especially important at the beginning of infections when limited amounts of viral factors are available, which should be targeted to the same compartments and at the same time to maximize the assembly of fully functional replicase complexes.

Assembly of the viral replication complex

The p92 RdRp protein is nonfunctional when expressed without the p33 cofactor in yeast (Panaviene et al., 2004, 2005), suggesting that p92 is getting "activated" in cells, likely during the assembly process (Fig. 2, step 3). Therefore, assembly of the viral replicase could be an important regulatory step in tombusvirus replication. Moreover, coexpression of the plus-stranded, but not the minus-stranded, viral replicon RNA has been shown to enhance replicase assembly/activity by ~40–100-fold in yeast (Panaviene et al., 2004, 2005). Thus, the plus-stranded viral RNA could serve as a platform to facilitate replicase assembly.

Systematic analysis of viral RNA elements affecting replicase assembly identified three major RNA elements: internal recognition element (IRE), which includes RII(+)-SL structure containing the p33RE, located internally within the p92 ORF; the 3′-terminal gPR promoter; and a 3′ proximal replication silencer element (RSE) (Fig. 1B). The RSE has been shown to interact with the 3′-terminal 5 nt within the gPR sequence in vitro and likely in vivo, too (Fabian et al., 2003; Pogany et al., 2003).

Separate mutagenesis of critical nucleotides within IRE, RSE, and gPR elements inhibited replicase assembly significantly, demonstrating that the sequences/structures of all these elements contribute to replicase assembly (Panaviene et al., 2005). Whereas p33 is known to bind efficiently to the p33RE (within the IRE), the actual roles of RSE and gPR in the replicase assembly have not yet been determined. It is possible that initiation of minus-strand synthesis from the gPR sequence might lead to the stabilization of the replicase

complex. On the other hand, RSE might serve as a switch to activate the gPR sequence, allowing minus-strand synthesis to take place in a highly regulated manner. The role of RSE can, however, be more complex than a switch, because the primary sequence of the 5 nt RSE seems to be important for the replicase assembly in yeast. Further studies will be needed to identify host or viral factors recognizing the RSE sequence.

In addition to the *cis*-acting RNA factors, *trans*-acting factors are likely needed for the assembly of the functional tombusvirus replicase. Accordingly, the p33 replication cofactor plays an essential role during the assembly process, because mutagenesis of the S1 subdomain, and to a lesser extent the S2 subdomain within the p33:p33/p92 interaction domain (Fig. 1A), inhibited replicase assembly/activity (Rajendran and Nagy, in press). The critical amino acid residues at the interface for p33:p33/p92 interaction include aromatic and positively charged amino acids (Rajendran and Nagy, in press). Kinetic measurements suggest moderate strength of binding (in the nanomolar range) that could support stable p33:p33 and p33:p92 binding, and thus replicase assembly (Rajendran and Nagy, in press). Interestingly, the most active purified replicase complexes contained over 10-fold more p33 than p92. The actual biochemical function of p33 in the replicase complex, however, is currently unknown. Unlike the case for p92, which contains the hallmark sequences of RdRp (O'Reilly and Kao, 1998), bioinformatics has not revealed any conserved enzymatic features for p33.

In addition to the viral factors, host factors likely contribute to replicase assembly. For example, Ssa1/2p molecular chaperones, which are members of the heat shock 70 (Hsp70) protein family, were found to be part of the highly purified and active tombusvirus replicase complex (Fig. 2) (Serva and Nagy, submitted for publication). Double-deletion of *SSA1* and *SSA2* genes reduced the accumulation of TBSV replicon in yeast by 4-fold and resulted in less active replicase complexes (Serva and Nagy, submitted for publication). In contrast, the activity of the purified replicase from yeast overexpressing either Ssa1p or Ssa2p enhanced by 2-fold, in agreement with the proposed role of Ssa proteins in replicase assembly. It is possible that additional, yet undefined, host proteins might also contribute to the assembly of the tombusvirus replicase.

The unexpectedly complex assembly of tombusvirus replicase could be an important specificity factor (a secondary safe-guarding step behind the template selection step, see above) to prevent efficient replication of some defective tombusvirus RNAs or cellular and heterologous viral RNAs. This would ensure that efficient replication would occur only for those RNAs that contain all three critical *cis*-acting elements required for the assembly of the viral replicase. Therefore, defective (such as partially-degraded) tombusvirus RNAs missing one of the critical internal and/or 3′-terminal sequences would not be able to assemble functional replicase complex and thus could only be replicated in *trans*. This limitation could represent a "safeguard" mechanism against wasting limited viral/host components on amplification of defective viral RNA templates, which, when present in large amounts, could also trigger antiviral responses (Szittya et al., 2002).

One of the vastly understudied areas in virology is the effect of viruses on each other during mixed infections, which could be common in nature. For example, an interesting question is the possibility of assembly of chimeric replicase complexes when two related viruses infect the same host cells. How would these chimeric replicases function with various viral RNA templates? This question was tested in vitro between the closely related p33 and p92 replication proteins of TBSV and CNV and the more distantly related p28 and p88 of TCV. Data from surface plasmon resonance (SPR) measurements indicated that p33 and p92 of TBSV and CNV could bind to each other efficiently, suggesting that the assembly of chimeric replicases during TBSV and CNV infections is highly likely. Accordingly, the combined expression of CNV p33 and TBSV p92 was shown to function in DI RNA replication in plant protoplasts (Oster et al., 1998). In contrast, replication proteins p28 or p88 of TCV did not interact with p33 of TBSV in vitro, suggesting that the assembly of chimeric TBSV/TCV replicases is unlikely to occur (Rajendran and Nagy, in press). Therefore, interaction or the lack of interaction between replication proteins is another regulatory factor to control the assembly of functional replicase complexes. This might be important in virus evolution, because the assembly of chimeric replicases might result in RNA replication with low specificity and/or reduced fidelity, which in turn, could lead to error catastrophe (Domingo et al., 2005; Freistadt et al., 2004), thereby preventing successful virus replication.

Altogether, the assembly of functional replicase seems to be a highly regulated event during TBSV infections (Fig. 2, step 3). Both *cis*-acting RNA elements and *trans*-acting factors contribute to the fidelity of the process, which facilitates that the tombusvirus replicase contains the appropriate factors and it is assembled at the correct intracellular place and in the right time.

Factors affecting the synthesis of viral RNA progeny

The assembled tombusvirus replicase already contains the viral RNA template (see above), thus specific recognition of the template RNA might not be critical at this step. However, the tombusvirus replicase must efficiently recognize *cis*-acting elements in the viral RNA to be able to synthesize the progeny RNA in a regulated manner: first the full-length minus-stranded complementary RNA is produced, then this is followed by the synthesis of plus-stranded RNA progeny (Fig. 1B). Tombusviruses also produce two subgenomic RNAs from prematurely terminated minus-stranded intermediates (Lin and White, 2004). Production of all these RNAs requires initiation of RNA synthesis de novo (i.e., independent of primers) at specific sites (Kao et al., 2001; Nagy and Pogany, 2000). The *cis*-acting RNA elements required for initiation are called promoter (initiation) elements. These elements are well defined for tombusviruses based on in vivo (plant protoplasts) and in vitro approaches with partially purified tombusvirus replicases and viral RNA mutants (Fig. 1B) (Fabian et al., 2003; Havelda

and Burgyan, 1995; Nagy and Pogany, 2000; Panavas et al., 2003, 2002a, 2002b). More recent work using yeast and affinity-purified replicase preparations also confirmed previous findings (Panaviene et al., 2004). Because this topic is covered in a recent review (White and Nagy, 2004), we only briefly summarize the current models. The minus-strand synthesis is initiated de novo from the gPR promoter present at the 3′ terminus of the plus-strand (Fig. 1B). Then, the synthesized full-length minus-stranded RNA, which contains a promoter sequence, termed cPR, will result in production of full-length progeny (+)RNA. Initiation from gPR and cPR promoters, which differ in sequence, is regulated by other *cis*-acting elements, such as RSE and replication enhancers (REN). The RSE is present in the plus-strand RNA and it down-regulates initiation from gPR in vitro by masking the initiation site (Pogany et al., 2003). This is achieved by formation of a 5 bp long double-stranded structure between the 3′ end sequence in gPR and RSE (Fig. 1B). What opens up this base-paired structure is currently unknown. Nevertheless, the RSE–gPR interaction is essential for tombusvirus replication (see the chapter above on the replicase assembly).

The minus-stranded RNA contains two enhancer elements, a 3′ promoter proximal enhancer (PPE) (Panavas et al., 2003) and an internal enhancer [termed RIII(−) REN] (Ray and White, 1999, 2003), which stimulate plus-strand synthesis from the cPR promoter in vitro (Fig. 1B) (Panavas and Nagy, 2003a, 2005). The stimulation is due to recruitment (binding) of the replicase to REN and base pairing interaction between REN and cPR sequences, which likely make cPR more accessible to the replicase (Panavas and Nagy, 2005). Due to the presence of the REN elements, the minus-stranded RNA is a superior template for RNA synthesis in vitro (Panavas and Nagy, 2003a). These elements also promote RNA recombination, possibly via actively binding to the “jumping” replicase during template switching events (Cheng and Nagy, 2003; Panaviene and Nagy, 2003). Although the REN elements are important modulatory elements during replication, they are not essential. Yet, they are critical under competitive conditions when viral RNAs carrying the REN elements are also present in the same cells.

Altogether, the activities of the RSE and REN elements are likely responsible for regulation of asymmetrical RNA synthesis, leading to 20–30-fold more plus-stranded progeny than minus-stranded intermediates (White and Nagy, 2004).

Release of viral RNA from replication

After completion of the new plus-strand RNAs (both gRNA and subgenomic RNAs), these RNAs should be released from replication in order to play additional roles (Fig. 2, step 5). These roles for the plus-stranded RNA include a new cycle of translation, replication, viral RNA encapsidation (virion formation), cell-to-cell, and long distance movement in the plants. Accordingly, time point studies on the localization of plus-stranded TBSV RNA revealed that significant portion of the plus-stranded RNAs was associated with the sites of replication at an early time point (12 h), but not at a latter time point (48 h) in yeast (Panavas et al., 2005a). This is in contrast with the localization of minus-stranded RNA, which was found together with p33 replication protein at both early and late time points (Panavas et al., 2005a). These observations suggest that the release of RNA progeny from replication is not a spontaneous, but rather a highly regulated event.

Although the mechanism of viral RNA release from replication is currently not known, posttranslational modification, including phosphorylation, of p33 might play a role. For example, serine and threonine residues located in the vicinity of the RPR RNA binding domain in p33 could be phosphorylated in vitro (Shapka et al., in press) and phosphorylation was shown to reduce RNA binding by p33 (Stork et al., in press). If phosphorylation takes place reversibly, then the same replicase complex would be able to release the viral RNA progeny (Fig. 2, step 5), followed by new rounds of RNA synthesis and release. This process, however, should be able to release selectively the plus-stranded RNA progeny and not the minus-stranded RNA. This model does not exclude that host factors and/or other processes, such as replicase disassembly (see below) might also play a role in viral RNA release.

Disassembly of the viral replicase

At the end of replication, the replicase complex likely gets inactivated and disassembled (Fig. 2, step 6). As pointed out above, disassembly of the replicase might also promote the release of the plus-stranded viral RNA progeny from replication. Indeed, RNA synthesis declines at late time points in plant protoplasts and in plants. Phosphorylation of p33 and p92 could also play a role in disassembly, because the nonphosphorylatable alanine mutants supported replication better at late time points in plants than the phosphorylation competent wt virus (Shapka et al., in press). It is also possible that additional posttranslational modifications are involved. Indeed, genome wide screen in yeast identified yeast genes that could affect the ubiquitination pathway, such as *BRE1* and *RAD6* (Panavas et al., 2005b). This could alter the stability of p33 and/or the replicase complexes. In spite of these observations, we are only at the beginning in our understanding of replicase disassembly.

Conclusions

Development of new tools and assays, such as yeast as a model host and powerful in vitro assays with purified replicase complexes, together with reverse genetic approaches led to major advance in replication of tombusviruses. Genome-wide approaches have identified new players, and revealed the complex nature of virus–host interactions, which make possible the robust replication of tombusviruses. Future studies will be aimed at dissecting the roles of the identified factors in replication and this knowledge will likely stimulate the development of new antiviral methods and other approaches that could make tombusviruses useful in biotechnology.

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