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Exploiting alternative subcellular location for replication: Tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes

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Abstract

Plus-strand RNA virus replication takes place on distinct membranous surfaces in infected cells via the assembly of viral replicase complexes involving multiple viral and host proteins. One group of tombusviruses, such as *Tomato bushy stunt virus* (TBSV), replicate on the surfaces of peroxisomal membranes in plant and yeast cells. Surprisingly, previous genome-wide screen performed in yeast demonstrated that a TBSV replicon RNA replicated as efficiently in yeast defective in peroxisome biogenesis as in the wt yeast (Panavas et al., Proc Natl Acad Sci U S A, 2005). To further test how the lack of peroxisomes could affect tombusvirus replication, we used yeast cells missing either *PEX3* or *PEX19* genes, which are absolutely essential for peroxisome biogenesis. Confocal microscopy-based approach revealed that the wild-type tombusvirus p33 replication protein accumulated in the endoplasmic reticulum (ER) in *pex3Δ* or *pex19Δ* yeast, suggesting that tombusvirus replication could take place on the surface of ER membrane. The activities of the isolated tombusvirus replicase preparations from wt, *pex3Δ* or *pex19Δ* yeasts were comparable, demonstrating that the assembly of the replicase was as efficient in the ER as in the authentic subcellular environments. The generation/accumulation of tombusvirus recombinants was similar in wt, *pex3Δ* and *pex19Δ* yeasts, suggesting that the rate of mistakes occurring during tombusvirus replication is comparable in the presence or absence of peroxisomes. Overall, this work demonstrates that a tombusvirus, relying on the wt replication proteins, can efficiently replicate on an alternative intracellular membrane. This suggests that RNA viruses might have remarkable flexibility for using various host membranes for their replication.

Keywords: Peroxisome; Endoplasmic reticulum; Site of replication; RNA-dependent RNA polymerase; *Cucumber necrosis virus*; *Tomato bushy stunt virus*; Yeast

Introduction

Replication of positive-strand RNA viruses takes place on the cytoplasmic faces of distinct membranous surfaces inside the infected cells. Many viruses use the endoplasmic reticulum (ER), while other viruses utilize mitochondrial, peroxisomal, chloroplast or vacuolar membranes (Mackenzie, 2005; Novoa et al., 2005). Therefore, one or more replication proteins of viruses contain intracellular targeting sequences to facilitate the recruitment of viral RNA and various viral and host factors to the sites of replication (Ahlquist et al., 2003; den Boon et al., 2001; dos Reis Figueira et al., 2002). Albeit the actual mechanism of replicase assembly for positive-stranded RNA viruses is incompletely understood, the emerging picture is that viral replication proteins, the viral RNA, various host proteins and membrane surfaces play essential/critical roles in replicase assembly (Ahlquist et al., 2003; Buck, 1996; Mackenzie, 2005; Nagy and Pogany, 2006; Novoa et al., 2005).

The assembly of the viral replicase, which includes the viral coded RNA-dependent RNA polymerase (RdRp), viral-coded auxiliary proteins and host factors, requires the recruitment of individual components of the replicase complex to the sites of replication in infected cells (Ahlquist, 2002; Buck, 1996; Nagy and Pogany, 2006). In case of several plant viruses, an auxiliary viral protein, such as 1a of *Brome mosaic virus* (BMV) and 140K protein of *Turnip yellow mosaic virus* (TYMV), can localize to the site of replication when expressed alone

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(Jakubiec et al., 2004; Restrepo-Hartwig and Ahlquist, 1999). The auxiliary proteins are also involved in targeting the viral RdRp to the site of replication via protein:protein interaction (Chen and Ahlquist, 2000; Jakubiec et al., 2004) that takes place either between the helicase domain and the unique N-terminal region of the RdRp protein [BMV (Kao et al., 1992; O'Reilly et al., 1997)] or between the proteinase domain and the polymerase domains [TYMV (Jakubiec et al., 2004)]. Similarly, the 6-kDa protein of potyviruses and the NTB-protein of nepoviruses are transmembrane proteins that serve to anchor the viral replicase to membranes (Han and Sanfacon, 2003; Schaad et al., 1997). In addition, the BMV 1a protein is also implicated in targeting the viral RNA to the sites of replication (Chen et al., 2001). The N-terminally overlapping p27 and p88 replication proteins of *Red clover necrotic mosaic virus* (RCNMV) have been shown to localize to the endoplasmic reticulum (ER), cause membrane restructuring and membrane proliferation (Turner et al., 2004). In contrast with RCNMV, other members of the Tombusviridae family are known to replicate on the surfaces of either peroxisomes or mitochondria (Burgyan et al., 1996; Weber-Lotfi et al., 2002). It is currently unclear what is the advantage for various viruses to utilize different membrane surfaces for their replication.

One of the least understood factors in the replication process is the role of the membrane surfaces. It has been suggested that the role of the membranes could be to facilitate the formation of characteristic virus-induced structures, such as spherules and vesicle-like structures in virus-infected cells, which contain the replicase complex and represent the site of viral RNA replication (Ahlquist, 2002; Mackenzie, 2005; Novoa et al., 2005). These membranous structures might also serve to increase the local concentration of replication factors and viral RNA templates by sequestering them to the site of replication in the cells. In addition, the membranous structures could "hide" viral replication, including putative double-stranded RNA replication intermediates and the viral RNA-dependent RNA polymerase (RdRp), away from the host antiviral surveillance system and general antiviral factors, such as ribonucleases and proteases.

Tombusviruses are small positive-stranded RNA viruses of plants, which are used intensively to dissect viral and host factors involved in replication (Miller and White, 2006; Nagy and Pogany, 2006). Replication of TBSV replicon (rep)RNA in yeast (a model host) and the TBSV genomic RNA in plants requires only two viral replication proteins, termed p33 and $p92^{pol}$ (Nagy and Pogany, 2006; Scholthof et al., 1995; White and Nagy, 2004). p33 replication co-factor is involved in selection of the viral RNA template for replication by recognizing an internal *cis*-acting element (p33RE), in template recruitment to the site of replication (Monkewich et al., 2005; Pogany et al., 2005) and in assembly of the functional viral replicase (Panaviene et al., 2005, 2004) on the peroxisomal membrane in both plant and yeast hosts. p33 is also known to bind to $p92^{pol}$ and to host proteins (Ssa1/2p, heat shock protein 70) (Serva and Nagy, 2006). Protein $p92^{pol}$ is the functional RdRp that is part of the replicase complex (Rajendran and Nagy, 2004, 2006; Serva and Nagy, 2006). Due to the expression strategy of tombusviruses, $p92^{pol}$ has an N-terminal overlapping sequence with p33 (Scholthof et al., 1995). Both proteins have peroxisomal targeting sequences within their overlapping region, which facilitate their targeting to the peroxisomal membrane in cells (McCartney et al., 2005; Panavas et al., 2005a; Rubino and Russo, 1998).

Expression of p33 alone or in combination with $p92^{pol}$ leads to major membrane rearrangements and membrane proliferation in cells. First, inward vesiculation of the peroxisomal boundary membrane leads to characteristic vesicle-like structures (McCartney et al., 2005). Then, the number of the peroxisomes decreases when compared to wt cells, while their sizes increase due to aggregation of several individual peroxisomes (McCartney et al., 2005). Eventually, larger structures, called peroxisomal multivesicular bodies (pMVBs), containing several globular or elongated vesicles of 40 to 170 nm in diameter, are formed also engulfing portion of cytosol. The integrity of the peroxisomal membrane was compromised by p33 expression, leading to degradation of peroxisomal matrix proteins. All other organelles, including ER and mitochondria, remained unaltered in TBSV-infected cells (McCartney et al., 2005).

Recent genome-wide screens that analyzed the effect of ~95% of yeast genes on tombusvirus replication revealed that single deletion of the known major peroxisome biogenesis proteins, termed peroxins (pex), affected TBSV replication less than 2-fold in yeast (Jiang et al., 2006; Panavas et al., 2005b). This is surprising because replication of TBSV and the closely related *Cucumber necrosis virus* (CNV) occurs on the peroxisomal membrane and causes membrane proliferation (McCartney et al., 2005; Panavas et al., 2005a). Therefore, in this paper, we analyzed in detail the effect of the two essential pex proteins, namely Pex3p and Pex19p, which are necessary for maintenance of peroxisomes in yeast and in plants (Hoepfner et al., 2005; Kragt et al., 2005). We found using confocal microscopy that similar to a peroxisomal marker (Pex13p) p33 was "mislocalized" to the ER in yeast lacking either *PEX3* or *PEX19* genes. However, the replication of the TBSV replicon was almost as efficient in *pex3Δ* or *pex19Δ* strains than in the wt yeast, suggesting the formation of active replicase complexes in the ER membrane. This was confirmed by showing comparable viral replicase activity in vitro with purified viral replicase complexes obtained from *pex3Δ*, *pex19Δ* and wt yeasts, respectively. In addition, we have shown that recombinant viral RNA accumulation was comparable in *pex3Δ*, *pex19Δ* and wt yeasts, suggesting that recombination might occur with similar frequency regardless of the subcellular location of viral replication. Altogether, this work demonstrates that TBSV, relying on the wt replication proteins, can efficiently replicate on an alternative subcellular membrane in the absence of the primary membrane.

Results

Replication protein p33 is re-localized to the ER membrane in the absence of peroxisomes in yeast

Previous genome-wide screen in yeast revealed that single deletions of *PEX3* or *PEX19* peroxisomal biogenesis genes, which lead to the complete elimination of peroxisomes

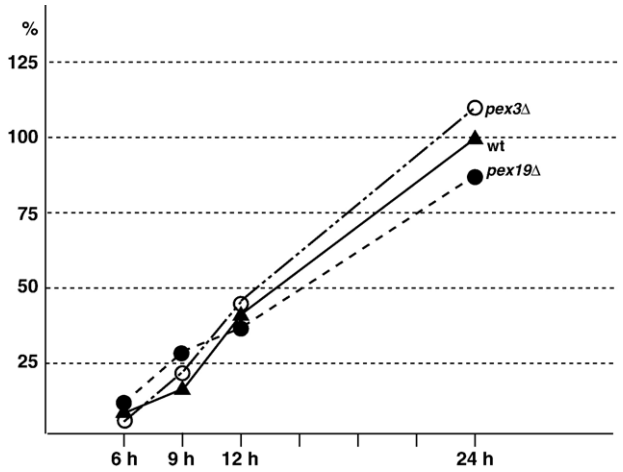


Fig. 1. Relative accumulation of TBSV repRNA in wt, *pex3Δ* and *pex19Δ* yeasts. TBSV repRNA replication was launched by addition of 2% galactose for 6 h to the medium, followed by growing yeast in 2% glucose to suppress transcription of the repRNA. The essential p33 and p92 replication proteins were produced from plasmids carrying the constitutive *ADH1* promoter. Total RNA was extracted at time points shown (0 time point is the addition of galactose to the media), followed by Northern blotting using a (+)repRNA-specific probe. Quantification was performed using phospho-imaging. The accumulation level of repRNA in wt yeast (BY4741) at the 24-h time point was taken as 100%. Four or more independent samples for each time point were analyzed.

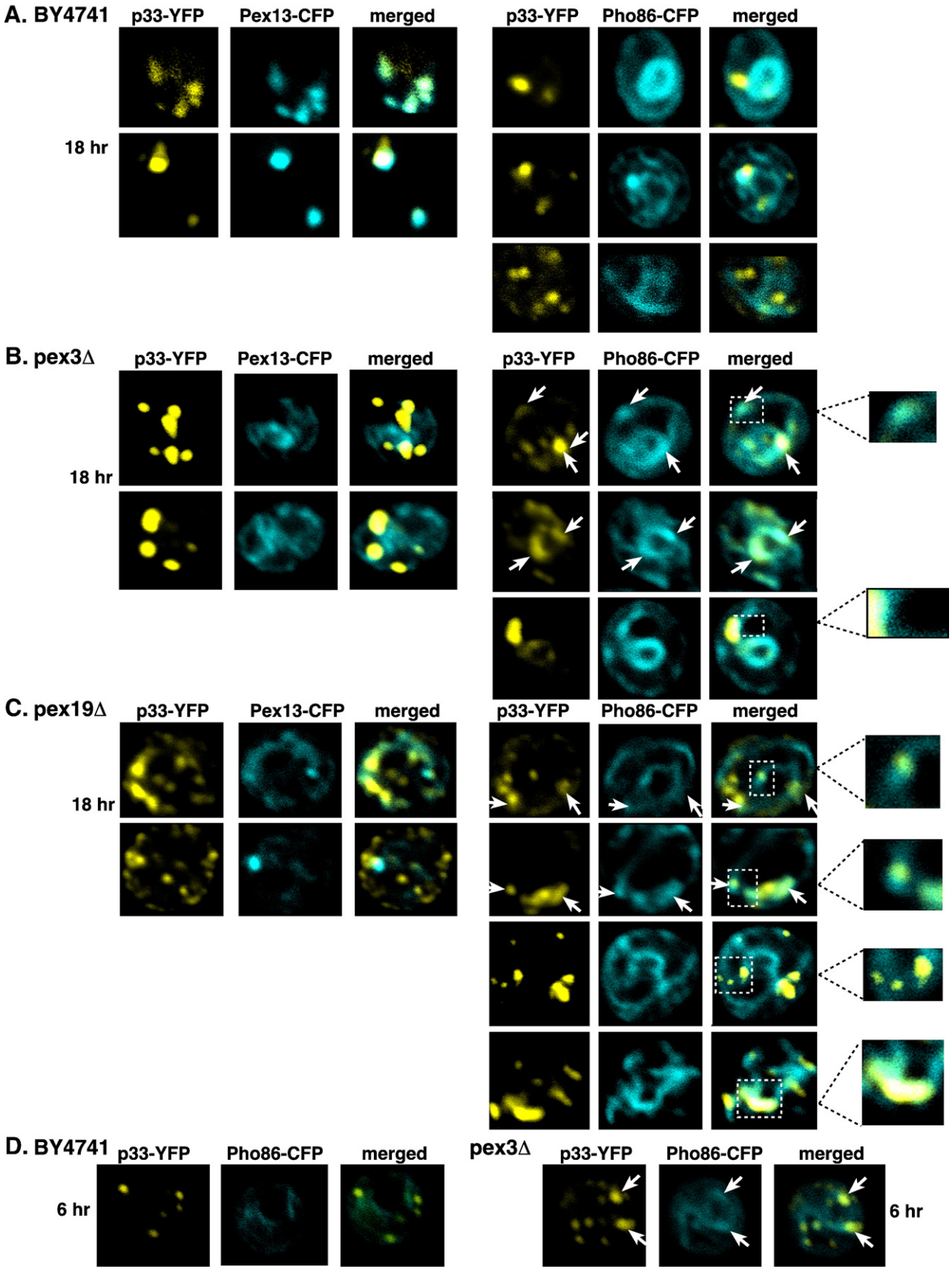
(including residual peroxisomal membrane structures, called "remnants" or "ghosts") (Hoepfner et al., 2005; Kragt et al., 2005), affected TBSV replication by less than 2-fold when compared to the wt yeast (Panavas et al., 2005b). To test if the kinetics of TBSV RNA replication was different in the presence versus in the absence of peroxisomes, we extracted total RNA 6, 9, 12 and 24 h after launching TBSV replication in wt, *pex3Δ* and *pex19Δ* yeasts, respectively. These yeasts co-expressed TBSV DI-72 replicon (rep)RNA from the galactose-inducible *GAL1* promoter, whereas p33 and $p92^{pol}$ replication proteins were co-expressed from the constitutive *ADH1* promoter (Panavas and Nagy, 2003; Panaviene et al., 2004). Northern blot analysis with a TBSV RNA-specific probe revealed that the accumulation of repRNA was comparable in wt, *pex3Δ* and *pex19Δ* yeasts at the various time points (Fig. 1), suggesting that TBSV RNA replication occurred with comparable kinetics in the presence versus absence of peroxisomes. We reasoned that the viral p33 replication protein, which is known to induce membrane proliferation in host cells, was either capable of inducing peroxisomal membrane synthesis even in the absence of *PEX3* and *PEX19* genes, or p33 and TBSV replication might be re-localized to a different intracellular membrane compartment(s) in *pex3Δ* or *pex19Δ* yeast cells.

The above models were tested using confocal microscopy with fluorescent protein fused with viral and yeast marker proteins co-expressed in wt, *pex3Δ* and *pex19Δ* yeast. To this end, we used either the combination of p33-YFP (yellow fluorescent protein)–Pex13p-CFP (cian fluorescent protein) or Pho86-CFP, which represent integral membrane proteins localized to either the peroxisomal membrane or the ER (Huh et al., 2003). The same cells also co-expressed the $p92^{pol}$ and DI-72 repRNA, which led to efficient replication of TBSV repRNA (not shown). The efficient accumulation of TBSV repRNA suggests that the p33-YFP is functional and the p33-containing punctate structures are the sites of TBSV replication in yeast, as shown earlier (Panavas et al., 2005a).

p33-YFP and Pex13p-CFP co-localized in wt yeast cells, whereas p33-YFP and Pho86p-CFP did not co-localize (although some level of partial co-localization is visible in some areas in the cells) (Fig. 2A). These observations are in agreement with previous data that showed p33-YFP is mainly localized to the peroxisomes and not to the ER. The punctate structures are usually bigger than regular peroxisomes due to membrane proliferation caused by p33 (McCartney et al., 2005; Panavas et al., 2005a). The limited partial co-localization of p33-YFP and Pho86p-CFP is likely due to the formation of large aggregated membranous structures induced by p33, which, in addition to the peroxisome, could also include mitochondrial and ER membranes as observed by Pantaleo et al. (2004) earlier.

We also examined the localization of p33 in *pex3Δ* and *pex19Δ* yeasts, which are known to lack peroxisomes and peroxisomal remnants (Hoepfner et al., 2005; Kragt et al., 2005). It was shown previously that peroxins are less stable and mislocalized in *pex3Δ* and *pex19Δ* yeasts, while the regular peroxisomal matrix proteins are mostly found in the cytoplasm (Hettema et al., 2000). Confocal microscopy of *pex3Δ* yeast co-expressing p33-YFP and Pex13p-CFP, $p92^{pol}$ and repRNA revealed that p33-YFP and Pex13p-CFP did not co-localize. While p33-YFP still formed large punctate structures, Pex13p-CFP was present in long elongated and/or round structures, which are characteristic of ER in yeast (Fig. 2B, left panel). Because p33 mutants were shown to accumulate in the ER (Panavas et al., 2005a) and the wt p33 is also proposed to have the ability to enter ER (McCartney et al., 2005), we tested if p33 is mislocalized to the ER in *pex3Δ* yeast by utilizing Pho86p-CFP ER marker protein, which is known to accumulate in both the perinuclear and cortical ER (Huh et al., 2003). Indeed, co-expressing p33-YFP and Pho86p-CFP, $p92^{pol}$ and repRNA in *pex3Δ* yeast revealed that p33-YFP was localized to the ER (both cortical, which is close to the cell membrane, and perinuclear). However, unlike Pho86p-CFP, p33-YFP formed punctate structures in *pex3Δ* yeast, suggesting that p33 is not distributed evenly in the ER, but rather it was sequestered to particular locations within the ER (Fig. 2B, right panel), possibly within the peroxisomal ER (pER) (McCartney et al., 2005). The area of the ER, where p33 accumulated, showed

Fig. 2. Subcellular localization of p33 replication protein in wt, *pex3Δ* and *pex19Δ* yeasts. Confocal microscopy images were taken at either 6 or 18 h after induction of p33 expression from a plasmid carrying *GAL1* promoter. The p33 replication protein was tagged with YFP, whereas the cellular marker proteins, Pex13p (peroxisomal marker protein) and Pho86p (an ER marker protein), were tagged with CFP as described (Panavas et al., 2005a). Panel A represent images from the wt yeast (BY4741), panel B from *pex3Δ* and panel C from *pex19Δ* yeasts. Arrows point at the area of the ER membrane, where membrane swelling is the most pronounced. (D) The confocal microscopy images were taken 6 h after the induction of p33 expression in wt (on the left) and *pex3Δ* yeasts.


A. BY4741
p33-YFP
Pex13-CFP
merged
p33-YFP
Pho86-CFP
merged
18 hr
B. pex3Δ
p33-YFP
Pex13-CFP
merged
p33-YFP
Pho86-CFP
merged
18 hr
C. pex19Δ
p33-YFP
Pex13-CFP
merged
p33-YFP
Pho86-CFP
merged
18 hr
D. BY4741
p33-YFP
Pho86-CFP
merged
pex3Δ
p33-YFP
Pho86-CFP
merged
6 hr
6 hr

swelling (enlargement), which could be indicative of membrane proliferation. We also performed co-localization experiments at an early time point (6 h after induction) in *pex3Δ* yeast co-expressing p33-YFP and Pho86p-CFP, $p92^{pol}$ and repRNA (Fig. 2D, right panel). The obtained data revealed that p33-YFP accumulated within limited areas of the ER even at the early time point, albeit the swelling of the ER was less pronounced than at the late (18 h after induction) time point (compare Figs. 2B and D, right panels).

Because yeast also lacks peroxisomes or peroxisomal remnants in the absence of Pex19p (Hoepfner et al., 2005), we examined the re-localization of p33-YFP in *pex19Δ* yeast. We observed ER location for p33-YFP, formation of punctate structures and swelling of ER in *pex19Δ* yeast, co-expressing p33-YFP and Pho86p-CFP, $p92^{pol}$ and repRNA (Fig. 2C, right panel). The sizes of the punctate structures were usually smaller, while their number was higher in *pex19Δ* yeast (Fig. 2C) than in *pex3Δ* yeast (Fig. 2B). Overall, the above data support the model that p33-YFP accumulates in punctate structures in ER in the absence of peroxisomes. Also, the distribution of p33-YFP is significantly different from the mislocalized Pex13p-CFP, which is also present in the ER.

The activity of the tombusvirus replicase complex obtained from pex3Δ and pex19Δ yeast is comparable to that obtained from wt yeast

To test if the viral replicase complex has similar activity when formed in the ER membrane versus on peroxisomal membranes, we obtained two different replicase preparations from wt, *pex3Δ* and *pex19Δ* yeasts co-expressing p33, $p92^{pol}$ and repRNA 12 and 24 h after induction of TBSV replication. The first preparation included a membrane-enriched fraction (ME), which contains the active tombusvirus replicase associated with the endogenous (co-purified) RNA template (Panaviene et al., 2004). The in vitro replicase assay with the ME fraction revealed that the replicase obtained from *pex3Δ* and *pex19Δ* yeasts were as active as that obtained from wt yeast 12 and 24 h after induction of TBSV replication (Fig. 3A). Western blot analysis of the ME fraction showed that p33 and $p92^{pol}$ replication proteins were as abundant in *pex3Δ* and *pex19Δ* yeasts as in the wt yeast at both time points examined (Fig. 3A).

In addition to the above total replicase activity of the ME fraction, which includes both plus- and minus-strand products synthesized in vitro, we also estimated the relative plus versus minus-strand synthesis by the ME fraction on the endogenous templates. This is done by performing standard in vitro replicase assay with the ME fractions in the presence of ^{32}P-labeled UTP and the other unlabeled ribonucleotides, followed by using the labeled in vitro replicase products as probes for RNA blotting (Panaviene et al., 2004). In these experiments, the nylon membranes have abundant, but equal amounts of plus- and minus-stranded DI-72 RNA transcripts (obtained by T7 RNA polymerase-based transcription) blotted separately (Panaviene et al., 2004; Stork et al., 2005). After washing the membranes to remove unhybridized products, phospho-imaging of the membranes can be used to estimate the relative amounts of plus- versus minus-stranded TBSV RNA products (i.e., the level of asymmetry in replication). These experiments revealed that the ME fractions obtained from *pex3Δ* and *pex19Δ* yeasts showed comparable levels of asymmetry in plus- and minus-strand synthesis (2.5:1 ratio) with that obtained from wt yeast at the 12-h time points (Fig. 3B, top), whereas the ME fractions from *pex3Δ* and *pex19Δ* yeasts produced ~40% more asymmetrical products than that of wt ME fraction 24 h after induction of TBSV replication (Fig. 3B, bottom).

The second replicase preparation was the purified replicase complex obtained after solubilization and affinity purification of the His_{6x}-tagged $p33/p92^{pol}$-containing replicase complex (Panaviene et al., 2005, 2004). The purified replicase complex contains host proteins (Ssa1/2p, Tdh2/3p, Pdc1p and an unidentified host protein) in addition to the viral replicase proteins (Serva and Nagy, 2006), but the endogenous viral RNA is mostly lost during purification (Panaviene et al., 2005, 2004). The purified replicase preparation can be programmed with exogenously added RNAs to measure its activity as described earlier (Panaviene et al., 2004).

The purified replicase preparations obtained from *pex3Δ* and *pex19Δ* yeasts were as active on the exogenous RNA template [RI/III(−) that contains RI carrying the promoter for plus-strand initiation and the RIII(−) replication enhancer (Panaviene et al., 2004)] as that obtained from wt yeast 24 h after induction of TBSV replication (Fig. 4A). The purified replicase preparations obtained from wt, *pex3Δ* and *pex19Δ* yeasts contained comparable amounts of p33 as well as $p92^{pol}$ replication proteins based on Western blot analysis (Fig. 4B).

Altogether, these in vitro replicase experiments with ME fractions (Fig. 3) and the purified replicase (Fig. 4) revealed that the tombusvirus replicase complex obtained from *pex3Δ* and *pex19Δ* yeasts have similar activities to that prepared from wt yeast. Therefore, the assembly of the wild-type tombusvirus replicase must be as efficient and its activity as high when present in the ER membrane as on peroxisomal membranes. Also, the amounts of both replicase proteins, thus the stability of the replicase proteins, are comparable in the ER and peroxisomal membranes.

Comparable level of accumulation of tombusvirus recombinants in wt, pex3Δ and pex19Δ yeasts

Features of the replicase complex can also affect RNA recombination, which is a major force in RNA virus evolution (Lai, 1992; Nagy and Simon, 1997; Worobey and Holmes, 1999). Thus, we wanted to compare if the altered subcellular localization of the tombusvirus replicase might affect the accumulation of viral recombinant RNAs (recRNA). To this end, we used a recombinogenic TBSV replicon, termed DI-AU-FP (Shapka and Nagy, 2004). This RNA is similar to DI-72 repRNA (Fig. 5A), but it carries a highly recombinogenic AU-rich sequence between RI and RII as shown schematically in Fig. 5A. In wt yeast, DI-AU-FP rapidly generates recRNAs with partial duplications (Fig. 5A) (Serviene et al., 2006, 2005), which can be estimated by Northern blotting. We have tested the

relative levels of recRNAs in *pex3Δ* and *pex19Δ* yeasts 24 h after induction of DI-AU-FP replication (Fig. 5B). Northern blot analysis of total RNA extracts showed that the recRNAs were as abundant in *pex3Δ* yeasts as in the wt yeast (Fig. 5B), whereas recRNAs accumulated to 32% higher levels in *pex19Δ* than in the wt strain. Similar results were obtained at the 12-h time point (not shown). The lengths of the recRNAs accumulating in *pex3Δ* and *pex19Δ* yeasts were similar to those seen in the wt yeast, suggesting that mechanism of recombinant generation is likely similar in *pex3Δ*, *pex19Δ* and the wt yeasts. Thus, the two different subcellular locations for virus replication have only moderate effect on viral RNA recombination.

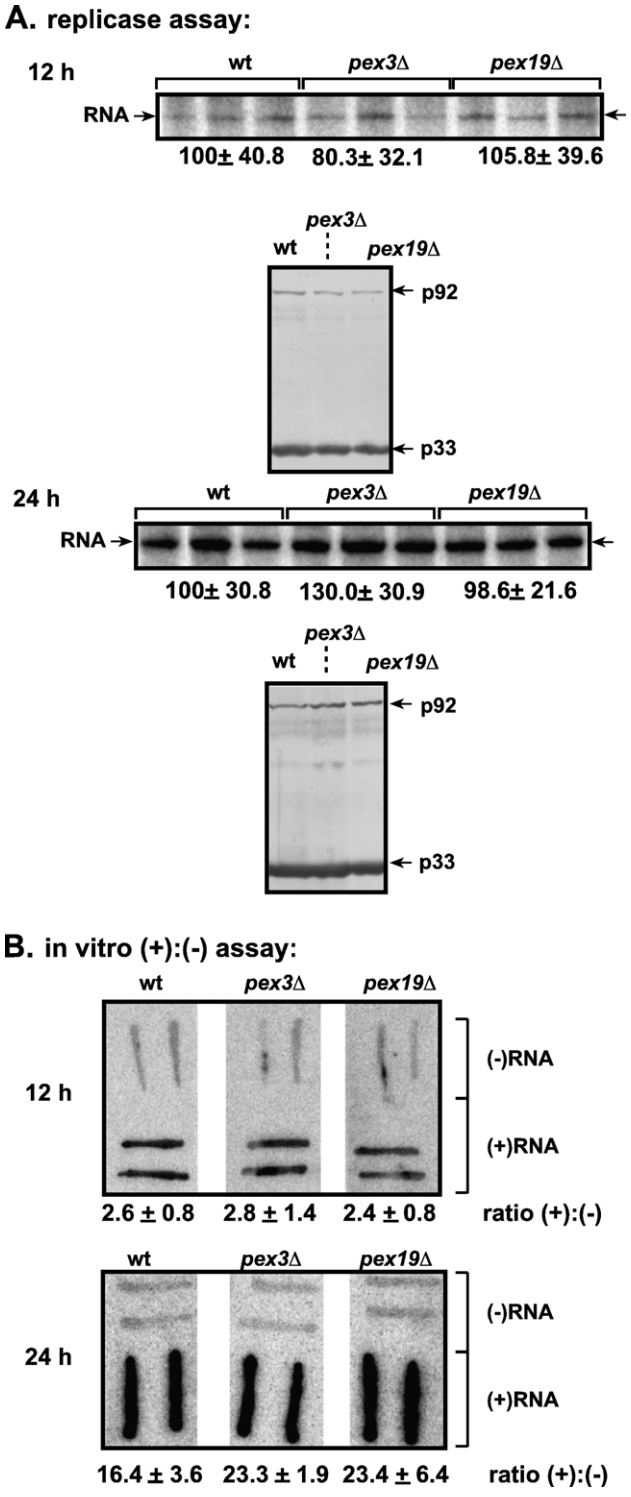


Fig. 3. Comparison of the tombusvirus replicase obtained from wt, *pex3Δ* and *pex19Δ* yeasts. The membrane-enriched (ME) fraction, which contains the replicase bound to the endogenous template RNA, was prepared and tested in vitro as described in the Materials and methods section. (A) The level of in vitro total RNA synthesis (combination of plus- and minus-strands) supported by the ME fraction obtained from wt, *pex3Δ* and *pex19Δ* yeasts at 12- and 24-h time points. Western blotting shows the accumulation level of p33 and p92 in the ME fractions at 12- and 24-h time points. (B) The extent of asymmetrical RNA synthesis supported by the ME fraction on the endogenous templates obtained from wt, *pex3Δ* and *pex19Δ* yeasts at 12- and 24-h time points. The ratio of plus- versus minus-strands was determined via RNA blotting using the in vitro synthesized replicase products, labeled with ^{32}P-UTP, as probes on membranes carrying the same amount of plus and minus-stranded viral RNA transcripts. (+)RNA and (−)RNA indicate the newly synthesized (labeled) (+)RNA and (−)RNA products. Note that, due to the asymmetrical synthesis, the (+)RNA was 2-to-23-fold more abundant product than the (−)RNA in the in vitro reaction.

Discussion

One of the current unsolved puzzles in plus-strand RNA virus replication is the role of various subcellular membranes/compartments. Different RNA viruses use different subcellular locations, frequently causing membrane deformations and proliferations. It is known that viruses in the same genus might utilize different membranes: for example, some tombusviruses, such as TBSV and CNV, replicate on the peroxisomal membranes (McCartney et al., 2005; Panavas et al., 2005a), whereas the closely related *Carnation Italian ringspot virus* (CIRV) usurps the mitochondrial membrane for replication (Pantaleo et al., 2004). What is the evolutionary advantage of one subcellular location over another, like replication in ER versus on the peroxisomal membrane? The answer to this question is not trivial because viral replication proteins with new targeting sequences could support virus replication efficiently. For example, Flock house virus replication could be re-targeted to the ER from mitochondrial membrane by replacing the mitochondrial targeting sequence with ER targeting sequences (Miller et al., 2003). Interestingly, the ER targeted FHV replicated to higher level than the wt FHV did. Also, introducing the peroxisomal targeting sequence from the *Cymbidium ringspot tombusvirus* (CyRSV) to the CIRV replication proteins resulted in re-targeting of CIRV replication to peroxisome (Burgyan et al., 1996; Rubino and Russo, 1998). Since the above studies were based on incorporation of new sequences to viral replication proteins, we currently do not know if the unmodified (wild-type) viral replication proteins could re-target replication to new intracellular locations in case the original location is not suitable for replication any longer.

The current work further addressed the role of subcellular location on virus replication and evolution. But unlike the previous cases (see above), in this work, we have not altered the viral replication proteins by artificially adding new targeting sequences. Instead, we have used mutated yeast host, which either contained peroxisomes (and peroxisomal membranes) or

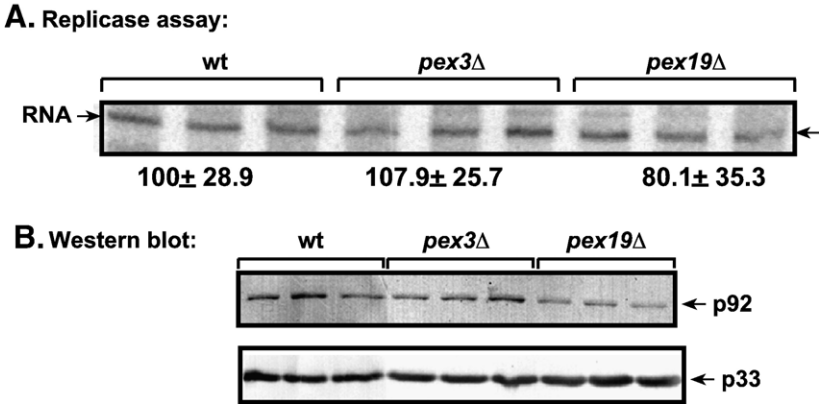


Fig. 4. In vitro activity of the purified tombusvirus replicase obtained from wt, *pex3Δ* and *pex19Δ* yeasts. The affinity purified tombusvirus replicase preparation, which lacks the endogenous template RNA, was prepared and tested in vitro as described in the Materials and methods section. (A) The purified replicase was programmed with RI/III(−) template. Note the comparable activities of the purified replicase preparations obtained from wt, *pex3Δ* and *pex19Δ* yeasts. (B) Western blotting shows comparable levels of p33 and p92 in the purified replicase obtained from wt, *pex3Δ* and *pex19Δ* yeasts.

lacked peroxisomes and peroxisomal remnants completely. Indeed, previous work demonstrated that peroxisome biogenesis is completely defective in *pex3Δ* or *pex19Δ* yeasts, which lack peroxisomes and peroxisomal remnants (Hoepfner et al., 2005; Kragt et al., 2005). Moreover, all peroxins and peroxisomal matrix proteins are mislocalized and sometime show decreased stability in *pex3Δ* and *pex19Δ* yeasts (Hettema et al., 2000). Accordingly, we found that Pex13p, an integral peroxisome membrane protein, is re-localized to the ER in *pex3Δ* and *pex19Δ* yeasts (Figs. 2B–C) (Hettema et al., 2000). The tombusviral p33 replication protein is also mislocalized to both perinuclear and the cortical ER in *pex3Δ* and *pex19Δ*

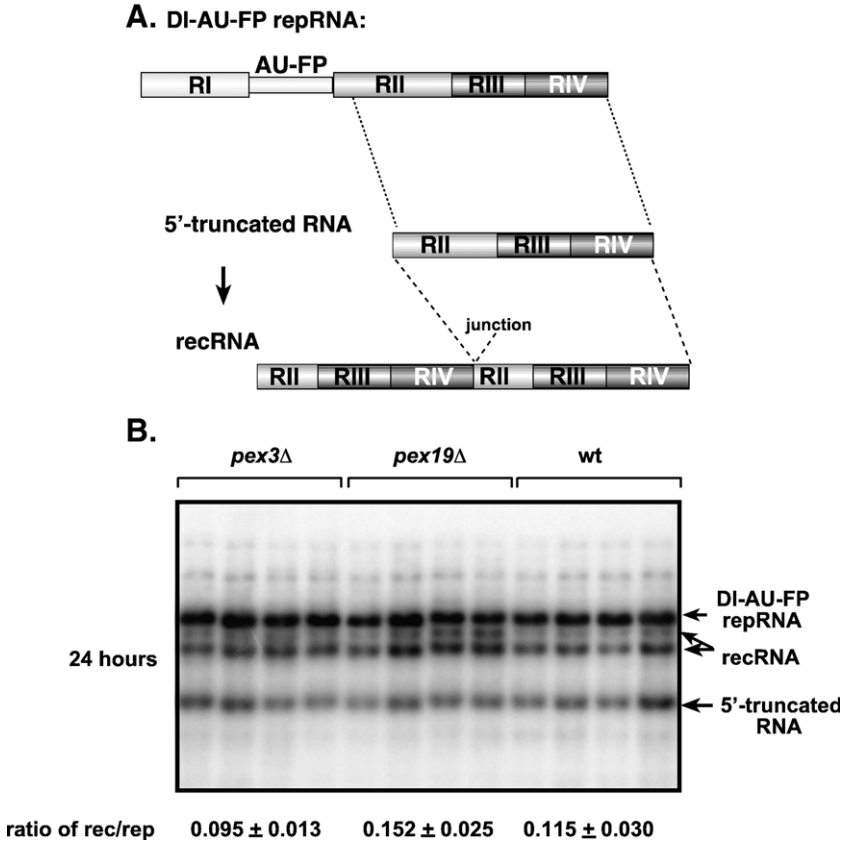


Fig. 5. TBSV recRNA accumulation in wt, *pex3Δ* and *pex19Δ* yeasts. (A) Schematic representation of the DI-AU-FP repRNA, the 5′ truncated RNA, which is recombination intermediate, and the recRNA. The recRNA is a dimer formed between two 5′ truncated repRNA (Cheng et al., 2006). (B) TBSV repRNA (DI-AU-FP) replication was launched by addition of 2% galactose (for 6 h) to the medium, followed by growing yeast in 2% glucose to suppress transcription of the repRNA. Total RNA was extracted at the 24-h time point, followed by Northern blotting using a (+)TBSV RNA-specific probe. Quantification was performed using phospho-imaging. The accumulation level of recRNA was compared to the level of repRNA in each sample.

yeasts. Unlike Pex13p, which distributed almost evenly within the ER membrane, p33 formed punctate structures in the ER (Figs. 2B–D), similar to the punctate structures on the peroxisomal membrane in wt cells (Panavas et al., 2005a). Similar observation was also noted in case of CyRSV (Navarro et al., 2006). The Russo group found that the GFP-tagged p33 of CyRSV mislocalized to the ER based on fluorescent microscopy in *pex19Δ* yeast (Navarro et al., 2004). We found that the punctate structures containing p33 became larger at the late stage of viral replication (18 h after the induction of TBSV repRNA replication) in *pex3Δ* and *pex19Δ* yeasts (Fig. 2D), suggesting active membrane proliferation as noted before for peroxisomal membranes (McCartney et al., 2005; Navarro et al., 2006; Panavas et al., 2005a). The punctate structures containing p33 consist of swelled ER membrane and they likely constitute the sites of TBSV replication as shown previously in case of the peroxisomal membrane (McCartney et al., 2005; Panavas et al., 2005a). A nonfunctional p33 mutant lacking the p33:p33/p92 interaction sequence was also found in the ER, but its distribution was more even, not forming or only forming few punctate structures (Panavas et al., 2005a). Thus, formation of the punctate structures might be a characteristic feature of the active tombusvirus replicase.

In vitro analysis of the tombusvirus replicase, either the ME fraction with the endogenous template or the purified replicase complex with added RNA template, showed comparable activities regardless of its subcellular locations (from peroxisome in wt and from the ER membrane in *pex3Δ* and *pex19Δ* yeasts) (Fig. 3). Also, replicase preparations obtained from *pex3Δ* and *pex19Δ* yeasts at the early and late time points, respectively, were similar to those from wt yeast. In addition to the total activity, the levels of asymmetry in plus- versus minus-strand syntheses by these replicase preparations were comparable (Fig. 3B). These data suggest that there is no delay in tombusvirus replicase assembly on the ER membranes when compared to the peroxisome membrane. Also, the recruitment of host factors to the ER membrane must be as efficient as to the peroxisome membrane.

Another potential effect of subcellular locations could be on the precision of the virus replicase during RNA synthesis (Cheng and Nagy, 2003; Cheng et al., 2006). This was tested in a recombination assay in yeast, which showed comparable levels of recRNA accumulation in *pex3Δ* and in wt yeasts (Fig. 5), whereas recRNA accumulated to moderately higher levels (an increase by 32%) in *pex19Δ* yeast. Also the lengths of the recRNAs were comparable in wt, *pex3Δ* and *pex19Δ* yeasts, suggesting similar mechanism during recombinant formation (Cheng et al., 2006). It seems that the tombusvirus replicase assembled in the ER might have similar recombination features to the peroxisome-localized replicase. Thus, at least within the short period tested, RNA recombination does not seem to be involved in accelerating tombusvirus adaptation to "peroxisome free" infections.

If TBSV can replicate as efficiently in the ER as on the peroxisomal membrane, then why the peroxisomal membrane is the preferred place for TBSV replication? It is possible that membrane alterations/proliferations within the peroxisomal membrane surfaces might be less detrimental to the cell than similar changes in the ER, which could lead to ER stress and apoptosis (He, 2006; Zhang and Kaufman, 2006). Reduced negative effect on the physiology of the cell might be beneficial to tombusviruses by allowing more robust viral replication.

Based on our data, we propose that subcellular locations (either peroxisomal or ER) are not critical during TBSV replication. It is possible that TBSV might be able to utilize more than one types of subcellular membranes during infections, when the preferred membrane becomes limited. Also, the observed flexibility of tombusvirus replication in subcellular locations might increase the host range of tombusviruses or the types of cells infected by these viruses by allowing access to more than one types of subcellular membranes. It seems that TBSV replication is readily prepared for overcoming different kind of obstacles raised by various cell/host types. This could be one of the reasons for the broad range of hosts infected by tombusviruses.

Materials and methods

Yeast strains and expression plasmids

Saccharomyces cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and the haploid deletion series (BY4741 strain background) were from Open Biosystems (Huntville, AL).

To express C-terminal fusions of Pex13-CFP or Pho86-CFP, we inserted the Pex13 and Pho86 ORFs, respectively, and the CFP-Cerulean ORF [(Rizzo et al., 2004) a generous gift by David W. Piston] into pGADT7 plasmid (Panavas et al., 2005a). Plasmids pGAD-His92, pHisGBK-His33 and pYES/DI-72(+) have been described elsewhere (Panavas and Nagy, 2003; Panaviene et al., 2004). The URA-selectable plasmid p92HFU, carrying the p92 ORF plus 6xHis and FLAG tags at the N-terminus behind the *ADH1* promoter, was the generous gift of Dr. Serva.

Dual expression plasmid pESC-HisY-p33-DI-72 (based on pESC, Invitrogen) was used to express p33-YFP and DI-72 (+) RNA from *GAL1* and *GAL10* promoters, respectively. YFP-Venus [(Nagai et al., 2002), a generous gift by Atsushi Miyawaki], was fused to the N-terminus of p33 as described (Panavas et al., 2005a). The DNA for p33-YFP fusion was amplified by PCR using primers #1402 (5′-GCGGCAGATCT-TACCATGGGGGGTTCTCA) and #1403 (5′-GCCGCTC-GAGCTATTTCACACCAAGGGACTCA), cleaved with *Bgl*II and *Xho*I, then cloned into pESC cleaved with *Bam*HI and *Xho*I (Panavas and Nagy, unpublished). In addition, the cDNA for DI-72(+)Rz-sat was introduced into the above pESC plasmid between *Xba*I and *Sac*I sites (Panaviene et al., 2004).

Transformation and culturing of yeast

Yeast strains were co-transformed with selected combination of plasmids using the standard LiAc method (Gietz and Woods, 2002; Panaviene et al., 2004). After transformation, yeast cells were plated on selective SC medium (supplemented with

200 mg/liter geneticin (G418) in case of *pex3Δ* and *pex19Δ* YKO strains) and incubated at 29 °C for 3–4 days. Yeast colonies were grown as described earlier (Panaviene et al., 2004).

Replication assay in yeast

The CNV p33 and p92 replication proteins with N-terminal 6xHis-tags were expressed constitutively in *S. cerevisiae* strains from pGBK-His33 and pGAD-His92 plasmids carrying *ADH1* promoters (Panaviene et al., 2004). The expression of the full-length DI-72(+) repRNA was launched from pYC-DI-72(+)Rz from the galactose-inducible *GAL1* promoter (Panavas and Nagy, 2003).

The yeast strains (BY4741, *pex3Δ* and *pex19Δ*) co-expressing p33, p92 and DI-72 RNA were grown in SC-ULH$^-$ medium containing 2% galactose (supplemented with 200 mg/liter geneticin G418 in case of *pex3Δ* and *pex19Δ* strains) at 23 °C with shaking at 300 rpm. After 6 h incubation, one batch of culture was pelleted (6-h time point, Fig. 1). Other batches were washed with SC-ULH$^-$ medium containing 2% glucose and resuspended in SC-ULH$^-$ containing 2% glucose and further incubated at 23 °C with shaking at 300 rpm. Samples were pelleted at 3, 6 and 18 h (9-, 12- and 24-h time points, respectively, Fig. 1) after medium change. Yeast samples were pelleted at 3000 g for 5 min and the total RNA was isolated using the hot phenol procedure, 1.5% agarose gel electrophoresis, followed by transfer to nylon membranes as described (Serviene et al., 2006, 2005). Hybridization was done as described (Serviene et al., 2005) using probe RIII/IV(−), which selectively binds to the 3′ end region of the plus-stranded TBSV repRNA. The quantitative analysis was performed using phospho-imaging with a Typhoon (GE) instrument as described (Serviene et al., 2006, 2005).

Confocal laser scanning microscopy

Yeast strains were transformed with Pex13-CFP or Pho86-CFP, in combination with p92HFU and pESC-HisY-p33-DI-72. Confocal laser scanning micrographs were acquired on an Olympus FV1000 microscope (Olympus America Inc., Melville, New York). ECFP was excited using 440 nm laser light, attenuated to 4.5% of the maximum laser power, while EYFP was excited using 515 nm laser line (3.5% of the maximum laser power). The images were acquired using sequential line-by-line mode in order to reduce excitation and emission cross-talk. The primary objective used was a water-immersion PLAPO60XWLSM (Olympus). Image acquisition was conducted at a resolution of 512×512 pixels and a scan-rate of 10 μs/pixel. Image acquisition and exportation of TIFF files were controlled by using Olympus Fluoview software version 1.5. Figures of micrographs were assembled using Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA).

Replicase assay with the ME fraction

The procedure used to obtain functional ME fractions from BY4741, *pex3Δ* and *pex19Δ* strains was the same developed by Panaviene et al. (2004, 2005). Briefly, yeast was pre-grown in SC-ULH$^-$ medium containing 2% glucose for 24 h at 29 °C with shaking at 250 rpm. Then, yeast cells were transferred to SC-ULH$^-$ containing 2% galactose. The initial OD_{600} for each culture was 0.3 in case of the 12-h samples and 0.1 in case of the 24-h samples. Yeasts were incubated at 23 °C with shaking at 250 rpm. After 12 or 24 h growth, yeast samples were collected (OD_{600} was ~0.8) by centrifugation at 1,100×*g* for 5 min, followed by washing the pellet with 20 mM Tris–HCl, pH 8.0. The pelleted cells were resuspended in 1 ml of 20 mM Tris–HCl, pH 8.0, followed by centrifugation at 21,000×*g* for 1 min. Yeast cells were broken by glass beads in a Genogrinder (Glen Mills Inc., Clifton NJ) for 2 min at 1500 rpm. After mixing with 600 μl chilled extraction buffer (200 mM sorbitol, 50 mM Tris–HCl [pH 7.5], 15 mM MgCl2, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mix; Sigma), the samples were centrifuged at 100×*g* for 5 min at 4 °C. The supernatant was moved to a new microcentrifuge tube, followed by centrifugation at 21,000×*g* for 10 min at 4 °C. The pellet was resuspended in 0.7 ml extraction buffer, resulting in the ME fraction. The replicase assay with the ME fraction was performed in 100 μl volume containing RdRp buffer [40 mM Tris pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 100 mM potassium glutamate, 0.2μl Rnase inhibitor, 1 mM ATP, CTP, GTP, 0.3 μl radioactive P^{32}-UTP (800 mCi/mmol ICN) and 50 μl ME fraction. Samples were incubated at 25 °C for 2 h. The reaction was terminated by adding 70 μl SDS/EDTA (1% SDS, 50 mM EDTA pH 8.0) and100 μl phenol-chloroform (1:1). After standard isopropanol precipitation of the RNA products, the RNA samples were electrophoresed under denaturing conditions (5% PAGE containing 8 M urea) and analyzed by phospho-imaging using a Typhoon (GE) instrument as described (Panaviene et al., 2005, 2004).

Determination of plus-strand versus minus-strand activity of the replicase in vitro

After the above in vitro replicase assay with the ME fractions, RNA samples from 3 separate reactions were pooled together, mixed with equal volume of formamide and heated at 85 °C for 5 min. The obtained labeled RNA samples were used as probes in hybridization assays (Panaviene et al., 2004; Stork et al., 2005). We have prepared the membranes for hybridization by blotting 5 μl (300 ng/μl) of denatured DI-72(+) and (−)-strand RNAs produced by standard "cold" T7 polymerase reaction (Panaviene et al., 2004; Stork et al., 2005). The hybridization was performed in Ultra-Hyb solution (Ambion) at 68 °C. The RNA blots were analyzed by phospho-imaging using a Typhoon (GE) instrument as described (Panaviene et al., 2004; Stork et al., 2005).

Purification of replicase from yeast

First, we prepared the ME fraction from yeast cells as described above. Then, the ME fraction was resuspended in the replicase extraction buffer containing 1.2 M NaCl, followed by gentle rotation for 20 min at 4 °C and centrifugation at 21,000×*g*

for 15 min at 4 °C (Panaviene et al., 2005, 2004). The obtained pellet was resuspended in the solubilization buffer [extraction buffer plus 1% Triton X-100, 5% SB3-10 (caprylyl sulfobetaine) (Sigma) and 500 mM KCl] by gentle rotation for 1 h at 4 °C, followed by centrifugation at 21,000×*g* for 15 min at 4 °C (Panaviene et al., 2005, 2004). After centrifugation, the supernatant was applied to a column containing ProBond resin (Invitrogen) equilibrated with the solubilization buffer. The column was then rotated for 1 h, followed by washing twice with the solubilization buffer (without KCl), and then additional washing with the solubilization buffer containing 2 mM imidazole. The recombinant proteins were recovered from the column in the extraction buffer containing 300 mM imidazole and 0.1% Triton X-100 in a two-step elution. The obtained purified replicase preparation was then used in the standard replicase reaction (see above for the ME fraction) with 300 ng of external template RI/III(−) added (Panaviene et al., 2005, 2004). The RNA samples from the replicase assays were electrophoresed under denaturing conditions (5% PAGE containing 8 M urea) and analyzed by phospho-imaging using a Typhoon (GE) instrument as described (Panaviene et al., 2005, 2004).

Western blot

Various yeast samples, including the ME fractions or the affinity purified replicase samples, were mixed (in 1:1) with SDS–PAGE sample loading buffer, heated for 10 min at 85 °C, electrophoresed in SDS–8% PAGE gels and electro-transferred to a polyvinylidene difluoride membrane (Bio-Rad). Nonspecific binding sites on the membranes were blocked with 5% nonfat dry milk solution in Tris-buffered saline (TBS) buffer containing 0.1% Tween 20 (TTBS), and the membranes were washed three times with TTBS buffer and incubated with monoclonal anti-His antibodies (Amersham) for 1 h at room temperature (Panaviene et al., 2005, 2004). Following three 5-min washes with TTBS buffer, membranes were incubated for 1 h at room temperature with secondary alkaline phosphatase-conjugated antibody (Sigma). After three washes of the membranes with TTBS, His_{6x}-tagged p33 and p92 replication proteins were visualized by using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma) (Panaviene et al., 2004).

In vivo recombination assay

The CNV p33 and p92 replication proteins were expressed constitutively from pGBK-His33 and pGAD-His92 plasmids carrying *ADH1* promoters (Panaviene et al., 2004), whereas DI-AU-FP repRNA was launched from pYC/DI-AU-FP from the galactose-inducible *GAL1* promoter (Serviene et al., 2006). The yeast strains (BY4741, *pex3Δ* and *pex19Δ*) co-expressing p33, p92 and DI-AU-FP RNA, which contains an AU-rich recombination hot spot sequence that facilitates RNA recombination (Serviene et al., 2005; Shapka and Nagy, 2004), were grown in SC-ULH$^-$ medium containing 2% galactose at 23 °C with shaking at 300 rpm. After 6 h incubation, the cells were pelleted by centrifugation, washed with 2% glucose SC-ULH$^-$ medium and resuspended in SC-ULH$^-$ containing 2% glucose and further incubated at 23 °C with shaking at 300 rpm. Samples were pelleted at 6 and 18 h after medium change, respectively. The total RNA was isolated and Northern blotting was done as described above using probe RIII/IV(−), which selectively binds to the 3′ end region of the plus-stranded TBSV RNA (both the replicon and the recombinant RNAs).

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