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Tomato bushy stunt virus Co-Opts the RNA-Binding Function of a Host Metabolic Enzyme for Viral Genomic RNA Synthesis

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SUMMARY

Tomato bushy stunt virus **(TBSV), a plus-stranded [(+)] RNA plant virus, incorporates the host metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into the viral replicase complex. Here, we show that, during TBSV replication in yeast, the yeast GAPDH Tdh2p moves from the cytosol to the peroxisomal membrane surface, the site of viral RNA synthesis. In yeast cells lacking Tdh2p, decreasing the levels of its functionally redundant homolog Tdh3p inhibited TBSV replication and resulted in equivalent levels of (+) and minus-stranded [(−)] viral RNA, in contrast to the hallmark excess of (+)RNA. Tdh2p specifically bound an AU pentamer sequence in the (−)RNA, suggesting that GAPDH promotes asymmetric RNA synthesis by selectively retaining the (−)RNA template in the replicase complex. Downregulation of *GAPDH* in a natural plant host decreased TBSV genomic RNA accumulation. Thus, TBSV co-opts the RNA-binding function of a metabolic protein, helping convert the host cell into a viral factory.**

INTRODUCTION

Plus-stranded [(+)] RNA viruses depend on a long list of host factors to successfully replicate in infected cells. The host proteins might contribute to translation of viral replication proteins or be diverted to assist various steps in replication, including intracellular localization of viral proteins and RNA, and the assembly of the viral replicase complex. One of the major groups of host factors that likely affect (+)RNA virus replication is the abundant cellular RNA-binding proteins that play various roles in cells. The co-opted proteins include ribosomal proteins, translation factors, and RNA-modifying enzymes (Ahlquist et al., 2003; Noueiry and Ahlquist, 2003; Panavas et al., 2005b). The majority of the identified host proteins, such as poly(C)- and poly(A)-binding proteins, nucleolin, and the heterogeneous nuclear ribonucleoprotein A1, interact with (+)RNA of poliovirus, hepatitis C virus, or mouse hepatitis virus (Herold and Andino, 2001; Moradpour et al., 2007; Randall et al., 2007; Walter et al., 2002). Well-documented exceptions are TIAR and TIA-1, which are host-derived RNA-binding proteins involved in translation and RNA splicing, which bind to the negative-stranded [(−)] RNA replication intermediate of West Nile virus (Li et al., 2002). Based on genome-wide screens with *Brome mosaic virus* (BMV), *Tomato bushy stunt virus* (TBSV), and *Drosophila* virus C (Cherry et al., 2005; Kushner et al., 2003; Panavas et al., 2005b; Serviene et al., 2005), many host proteins are predicted to bind to viral RNAs and likely provide auxiliary functions to the viral replication proteins.

Another group of host factors interacts with the viral replicase, likely modulating its function. The viral replicase is a multisubunit complex, which consists of the viral RNA, the viral coded RNA-dependent RNA polymerase (RdRp), one or more viral auxiliary replication proteins, host proteins, and membranes (Ahlquist et al., 2003; Nagy and Pogany, 2006; Shi and Lai, 2005). Some host factors, such as cyclophilin B in the case of HCV, might modulate the RNA-binding activity of the viral replication proteins (Watashi et al., 2005). However, the functions of many host components of the viral replicase complex are currently unknown.

Tombusviruses, including TBSV, are small (+)RNA viruses infecting a wide range of host plants. TBSV has recently emerged as a model virus to study virus replication and recombination, based on the development of in vitro approaches and yeast (*Saccharomyces cerevisiae*) as a model host (Nagy and Pogany, 2006; Panavas and Nagy, 2003; Panaviene et al., 2004; White and Nagy, 2004). Robust replication of a TBSV replicon (rep) RNA in yeast facilitated identification of over 100 host genes affecting either TBSV replication or recombination (Panavas et al., 2005b; Serviene et al., 2005). A recent proteomics analysis of the highly purified tombusvirus replicase complex revealed the presence of the two viral replication proteins (i.e., p33 and $p92^{pol}$) and four to ten host proteins in the replicase complex, including protein chaperones (HSP70) and metabolic enzymes (Tdh2p, Tdh3p, Pdc1p), whose functions are currently under investigation.

Tdh2p and Tdh3p are yeast homologs of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in animals and plants, highly conserved, very abundant proteins that are ubiquitous in nature (Sirover, 1999). Tdh2p and Tdh3p are 96% identical and functionally redundant. GAPDH activity is a key component of cytosolic energy production. However, GAPDH displays many additional activities that are unrelated to its glycolytic function. These cellular activities include roles in apoptosis, endocytosis, nuclear tRNA transport, vesicular secretary transport, nuclear membrane fusion, modulation of the cytoskeleton, DNA replication and repair, maintenance of telomere structure, and

transcriptional control of histone gene expression (Sirover, 1999, 2005). One of its functions is to bind to various RNAs, such as AU-rich sequences at the 3′ terminus of mRNAs, which can lead to stabilization of the RNA in the cell (Bonafe et al., 2005).

In this work, we dissected the function of Tdh2p and Tdh3p in replication of TBSV. We found that downregulation of Tdh2/3p inhibited TBSV replication in yeast and resulted in symmetric (+) and (−)RNA synthesis, instead of the hallmark asymmetric RNA synthesis. Here, we demonstrate how one of the functions of a metabolic host protein is co-opted for (+)RNA virus replication.

RESULTS

Partial Relocalization of Tdh2p to Sites of TBSV Replication

A previous proteomics approach revealed that Tdh2/3p proteins are present in the highly purified tombusvirus replicase complex (Serva and Nagy, 2006). To determine if the interaction between Tdh2/3p and the viral replicase is cytoplasmic (where Tdh2/3p are normally localized in yeast cells in the absence of viral replication) (Sirover, 1999) or if it occurs at the sites of TBSV replication, we coexpressed YFP-tagged Tdh2p and CFP-tagged p33 replication protein together with $p92^{pol}$ and the TBSV DI-72 repRNA in yeast. Under inducing conditions, the transformed yeast cells supported robust TBSV replication (data not shown). Confocal microscopy of yeast cells 16 hr after the induction of Tdh2p-YFP expression and TBSV replication revealed partial relocalization of Tdh2p-YFP from the cytosol (Figure 1A) to punctate structures (Figure 1B). The redistributed Tdh2p-YFP colocalized with CFP-p33, suggesting that Tdh2p-YFP is located in the same subcellular compartment as CFP-p33, which is present in the viral replicase (Panaviene et al., 2004; Serva and Nagy, 2006). As expected based on earlier studies (McCartney et al., 2005; Panavas et al., 2005a; Rubino and Russo, 1998), this subcellular compartment is the peroxisomal membrane, as shown by the colocalization of GFP-p33 with RFP-tagged Pex3p in yeast coexpressing $p92^{pol}$ and the TBSV DI-72 repRNA (Figure 1C).

To test if p33 replication cofactor is sufficient for the relocalization of Tdh2p-YFP in yeast, we coexpressed Tdh2p-YFP and CFP-p33 in the absence of $p92^{pol}$ and the TBSV DI-72 repRNA. Under these conditions, Tdh2p-YFP and CFP-p33 did not colocalize in yeast (Figure 1D). While Tdh2p-YFP was mostly cytosolic, CFP-p33 formed punctate structures, which are located in the peroxisomal membrane (Jonczyk et al., 2007; Panavas et al., 2005a). These observations suggest that Tdh2p is only relocalized to the sites of tombusvirus replication in the presence of the tombusvirus replicase complex, whereas its subcellular localization is not altered in the presence of p33 replication protein.

Downregulation of TDH3 Expression Inhibits TBSV Replicon RNA Accumulation in the Absence of TDH2

Yeast strains with single deletion of the redundant *TDH2* (*tdh2Δ*) or *TDH3* (*tdh3Δ*) genes supported TBSV repRNA replication almost as efficiently as the WT BY4741 strain (Panavas et al., 2005b), suggesting that Tdh2/3p might not have significant function in the tombusvirus replicase or they might complement each other during TBSV replication. Double deletion of *TDH2* and *TDH3* is lethal (Sirover, 1999), therefore we had to use an alternative strategy to test more rigorously the roles of Tdh2/3p in TBSV replication. To this end, we altered Tdh3p levels in yeast by placing *TDH3* under a titratable *TET* expression system in the *tdh2Δ* background (*tdh2Δ TET::TDH3*, Figure 2). In this system, the expression of *TDH3* can be downregulated by addition of various amounts of doxycycline to the yeast growth medium, as is widely used for the construction of the yeast essential gene library (yTHC) (Mnaimneh et al., 2004). When we launched TBSV replication in the presence of increasing amounts of doxycycline in yeast (*tdh2Δ TET::TDH3*), then both the level of Tdh3p and accumulation of TBSV repRNA decreased significantly. This contrasted with p33 and $p92^{pol}$ viral replication proteins whose levels were unaltered (Figure 2A). The greater than 4-fold reduction in TBSV repRNA accumulation in the presence of reduced levels of Tdh3p strongly supports the model that Tdh2/3p play significant roles in TBSV replication.

To identify the possible step affected by Tdh2/3p in TBSV replication, we isolated the active membrane-bound tombusvirus replicase complex containing the copurified viral RNA template from yeast (*tdh2Δ TET::TDH3*) expressing decreasing amounts of Tdh3p. In vitro replicase assays using the copurified

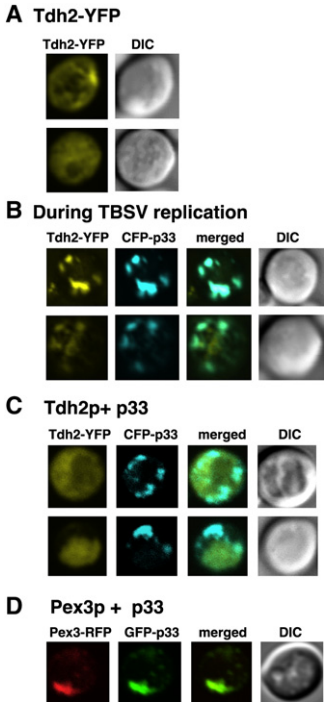

Figure 1. Tdh2p Relocalizes from the Cytosol to the Site of TBSV Replication

(A) Cytosolic localization of Tdh2p-YFP in the absence of TBSV replication.

(B) Relocalization of Tdh2p-YFP to punctate structures, also containing the viral CFP-p33, in yeast supporting TBSV replication. TBSV repRNA replication was launched from plasmids pGAD92, pYC-DI72, and pHisGBKCFP-p33 after addition of galactose to the growth medium.

(C) Cytosolic localization of Tdh2p-YFP in the presence of the viral CFP-p33. Note that $p92^{pol}$ and the repRNA are not expressed in these yeast cells.

(D) Peroxisomal localization of the viral CFP-p33 replication protein. The Pex3-RFP peroxisomal marker protein was expressed from its native promoter from the chromosome (Huh et al., 2003). The images were taken at 16 hr after induction of repRNA replication.

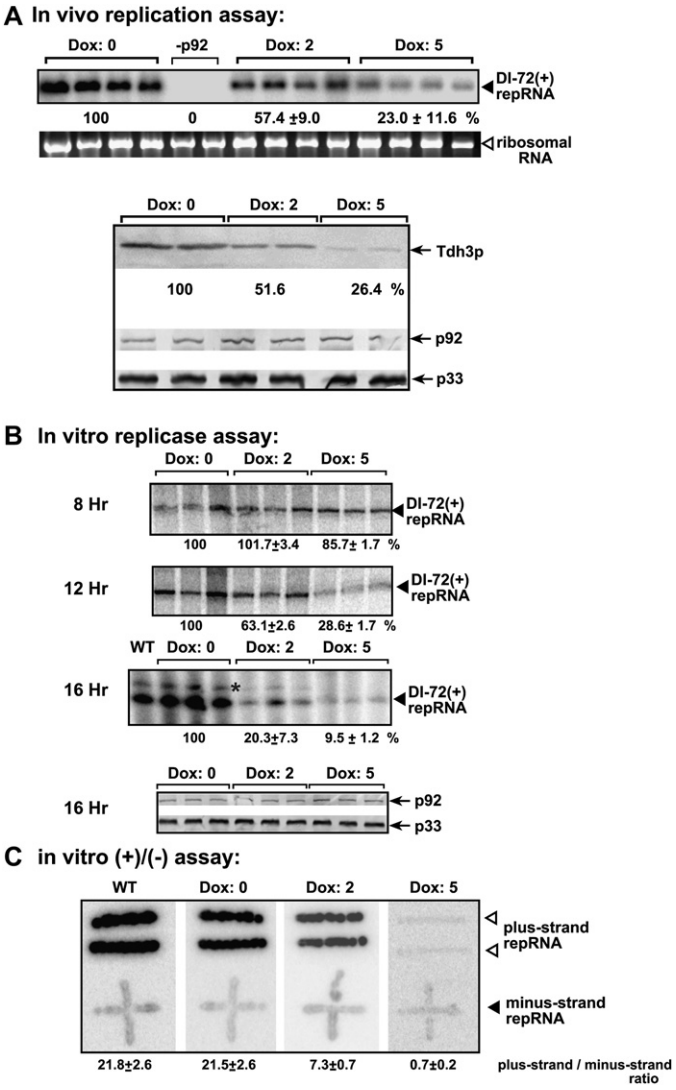

Figure 2. Reduced TBSV repRNA Replication after Downregulation of Tdh3p Expression in Yeast

(A) Yeast cells (*tdh2Δ TET::TDH3*) was treated with either 2 or 5 μg/ml doxycycline for 6 hr to downregulate Tdh3p level prior to launching TBSV repRNA replication. (Top panel) Northern blot analysis of total RNA extracts obtained from yeast 16 hr after induction of repRNA replication. Middle panel shows the ribosomal RNAs as loading controls for the northern blots. (Bottom panel) Western blot analysis with anti-6xHis antibody to detect the levels of Tdh3p, p33, and $p92^{pol}$ in the yeast extracts. Note that Tdh3p, p33, and $p92^{pol}$ contain a 6xHis-tag.

(B) In vitro replicase assay using the membrane-enriched fraction carrying the copurified repRNA. (Top three panels) In vitro synthesis of the repRNA products by the tombusvirus replicase preparations obtained from yeast, which were treated with doxycycline (see [A]). Yeast cells were harvested 8, 12, or 16 hr after induction of TBSV replication, as shown. Asterisk points at a recombinant RNA species characterized earlier (Cheng et al., 2006). (Bottom panel) Western blot analysis of p33 and $p92^{pol}$ levels in the replicase preparations.

(C) The ratio of (+) versus (−)RNAs synthesized by the membrane-enriched replicase preparations obtained 16 hr after induction of TBSV replication. Standard replicase assay with ^{32}P-UTP produced the RNA probe used for RNA analysis. The blots contained equal amounts of unlabeled (+) and (−)RNA of TBSV DI-72 generated by T7pol transcription. Based on the hybridized probes, we calculated the ratio of (+) versus (−)RNAs produced by the viral replicase in vitro.

All values represent the mean of three independent experiments. Error bars indicate standard deviation of the mean.

TBSV repRNA revealed 2- to 4-fold and 5- to 10-fold decreased activity with downregulated Tdh3p at the 12 and 16 hr time points, respectively (Figure 2B, samples Dox:2 and Dox:5), when compared with the control preparation obtained from the same yeast strain grown in the absence of doxycycline (i.e., maximum level of *TDH3* expression) (Figure 2B, samples Dox:0). The in vitro replicase data suggest that the viral RNA synthesis declines rapidly over time in the presence of a limiting amount of Tdh3p. Thus, most of the TBSV repRNA accumulating in doxycycline-treated yeast (Figure 2A, Dox:5) has likely been synthesized early in the replication process. The levels of p33 and $p92^{pol}$ tombusviral replication proteins were comparable in these replicase preparations (Figure 2B, panels in the middle), suggesting that Tdh3p likely has a direct function within the viral replicase complex.

Downregulation of TDH3 Results in a Loss of Asymmetric TBSV Replication

One of the hallmark features of (+)RNA viruses is the asymmetric nature of viral RNA synthesis leading to the production of 10- to 100-fold more (+)RNA progeny than the level of the (−)RNA replication intermediate (Buck, 1996). To determine if *TDH2/3* affect asymmetric replication of TBSV, we used the ^{32}P-labeled TBSV repRNA products from the above in vitro replicase assays as probes to determine the extent of (+) versus (−)RNA synthesis by the isolated viral replicase. The RNA blot analysis revealed that the ratio of (+) versus (−)RNA products from the WT replicase preparation changed from ~22:1 to 0.7:1 in the case when the replicase was obtained from yeast (*tdh2Δ TET::TDH3*) grown in the presence of doxycycline (Figure 2C, samples WT and Dox:5, respectively). Based on the total replicase activity (Figure 2B) and the ratio of (+) versus (−)RNA products, we calculated that the decreased ratio of RNA strands was mainly due to decreased level of (+)RNA products by the replicases derived from yeast with reduced expression of *TDH3*. Altogether, these data support the model that Tdh2/3p are involved in the regulation of asymmetric RNA replication by facilitating (+)RNA synthesis.

Tdh2p Binds to an AU Pentamer Sequence Present in (−)repRNA

Since Tdh2p did not colocalize with the p33 replication protein when expressed in the absence of $p92^{pol}$/repRNA (Figure 1C), we reasoned that Tdh2p, which is a known RNA-binding protein (Bonafe et al., 2005; Nagy et al., 2000; Nagy and Rigby, 1995), might interact with the viral RNA within the replicase complex. To test interaction between the repRNA and Tdh2p, we first performed in vitro gel mobility shift experiments with purified components. This assay demonstrated that Tdh2p bound weakly to (+)repRNA, whereas Tdh2p binding to (−)repRNA was efficient, resulting in a complete band shift (Figure 3B). Further systematic mapping of the Tdh2p-binding sequence

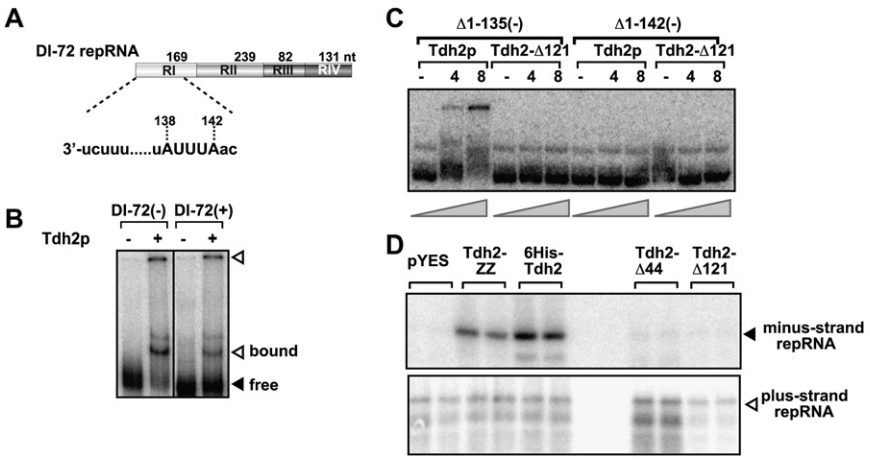

Figure 3. Binding of Tdh2p to an AU-Pentamer Sequence in the TBSV (−)repRNA

(A) Schematic presentation of the 3′ proximal AU-pentamer sequence in (−)repRNA.

(B) Gel mobility shift analysis of the purified recombinant Tdh2p binding to the ^{32}P-labeled DI-72(−) and DI-72(+) RNA transcripts obtained by T7 transcription.

(C) The purified recombinant Tdh2p binds specifically to the 3′ proximal AU-pentamer in vitro. Probe Δ1-135(−) contains the AU-pentamer, whereas probe Δ1-142(−) lacks the AU-pentamer [in addition to containing part of RI(−) and RII(−)]. Tdh-Δ121 is an N-terminally truncated Tdh2p, which lacks the two known RNA-binding regions. Note that the recombinant proteins were used in either 4 μg or 8 μg amounts during the binding assay.

(D) Copurification of the minus-stranded repRNA with Tdh2p from yeast cells. Yeast (BY4741) expressed the zz-tagged Tdh2p, the 6His-tagged Tdh2p, or the N-terminally truncated Tdh2-Δ44 or Tdh2-Δ121 (tagged with 6His). pYES sample is the control yeast, carrying the empty expression plasmid. The Tdh2p variants were purified from yeast cells via affinity chromatography, followed by RNA extraction and analysis with northern blotting using probes specific for TBSV (−) or (+)RNA detection. Note that the signal with the (+)RNA probed (bottom image) is nonspecific, likely due to high levels of (+)RNA, since it also appeared in pYES control. The shorter than full-length (+)repRNA is likely a degradation product.

localized RI(−) carrying an AU pentamer sequence (Figure 3C and Figure S1 available online). Deletion of the AU pentamer in (−)repRNA [construct Δ1–142(−)] or deletion of the RNA-binding region in Tdh2p (the N-terminal 121 aa, construct Tdh-Δ121, Figure 3C) (Nagy et al., 2000; Nagy and Rigby, 1995) eliminated the RNA-Tdh2p interaction in vitro.

To confirm the Tdh2p:viral RNA interaction in vivo, we coexpressed the zz-tagged or His-tagged Tdh2p and the repRNA together with p33 and p92^{pol}, followed by affinity-based purification of Tdh2p (Figure 3D). Interestingly, the purified Tdh2p preparation contained the (−)repRNA, whereas the control Tdh2-Δ121 lacking both RNA-binding regions (Nagy et al., 2000; Nagy and Rigby, 1995) and Tdh2-Δ44 lacking one of the two RNA-binding regions did not bring down (−)repRNA (Figure 3D). Also, (−)repRNA was copurified with Tdh2p from yeast when we used a nickel-affinity approach (construct 6His-Tdh2, Figure 3D), confirming specific interaction between Tdh2p and (−)repRNA. The above Tdh2p preparations also contained a small amount of (+)repRNA, which bound nonspecifically (Figure 3D, bottom panel). Collectively, the above in vitro and in vivo data support the model that Tdh2p binds specifically to an AU pentamer sequence within the (−)repRNA.

Deletion of the AU Pentamer Sequence from the repRNA Results in Reduced Level of (+)RNA Synthesis

Because Tdh2p binds with high efficiency to the AU pentamer present in (−)repRNA, deletion of the AU pentamer is predicted to inhibit its interaction with Tdh2/3p, which then should result in less asymmetric RNA synthesis. This was tested by coexpressing Δpenta repRNA (Figure 4A) lacking the AU pentamer sequence with p33 and p92^{pol} replication proteins in WT yeast. In vitro replicase assay with isolated replicase preparations revealed that Δpenta repRNA resulted in a loss of asymmetric RNA synthesis [~1:1 ratio of (+) versus (−)RNA synthesis] (Figure 4B). In contrast, another repRNA, termed double-penta (Figure 4A), carrying two AU pentamer sequences in proximal locations supported highly asymmetric RNA synthesis [~35:1 ratio of (+) versus (−)RNA synthesis] (Figure 4B). These results are in agreement with the model that interaction between the AU pentamer sequence in the repRNA and Tdh2/3p is a critical factor regulating the (+):(−) ratio during RNA synthesis.

To determine if replication of the full-length TBSV genomic (g)RNA also depends on the 5′ proximal AU-pentamer sequence, we introduced the Δpenta and double-penta mutations to gRNA (the corresponding position in gRNA as shown for repRNA in Figure 4A). Viral RNA accumulation was estimated after electroporation of *Nicotiana benthamiana* (a host of TBSV) protoplasts with in vitro transcripts of WT and mutated TBSV gRNA, followed by northern blot analysis of total RNA extracts with TBSV-specific probes. The (+)gRNA of Δpenta construct accumulated ~36% of the WT gRNA levels at the 8 hr time point, whereas (−)RNA accumulation was comparable with WT (Figure 4C). Interestingly, both (+) and (−)RNAs of the double-penta construct accumulated as efficiently as WT gRNA, suggesting that the mutations per se did not affect TBSV gRNA replication, but the AU-rich nature of the sequence was an important factor. Overall, these data confirmed that the replication of TBSV gRNA in plant cells is regulated by a 5′ proximal AU pentamer, similar to that observed for replication of repRNA in yeast.

Dominant-Negative Mutants of Tdh2p Destabilize the Viral Replicase Complex In Vitro

Plasmid-based expression of WT Tdh2p in *tdh2Δ* yeast resulted in a moderately more active replicase preparation (54% increased activity) than that obtained from *tdh2Δ* yeast not expressing Tdh2p (compare Tdh-His with pYES control, Figure 5A). Interestingly, expression in *tdh2Δ* yeast of C-terminally truncated Tdh2p carrying the RNA-binding regions only (constructs Tdh-N121 and Tdh-N201) gave replicase preparations with vastly decreased activities (down to 3%–5% of that of the control) (Figure 5A). The reduced activity of the replicase preparations derived from *tdh2Δ* yeast expressing either the Tdh-N121 or Tdh-N201 truncated proteins could be due to loss of the viral RNA template during isolation, or to partial

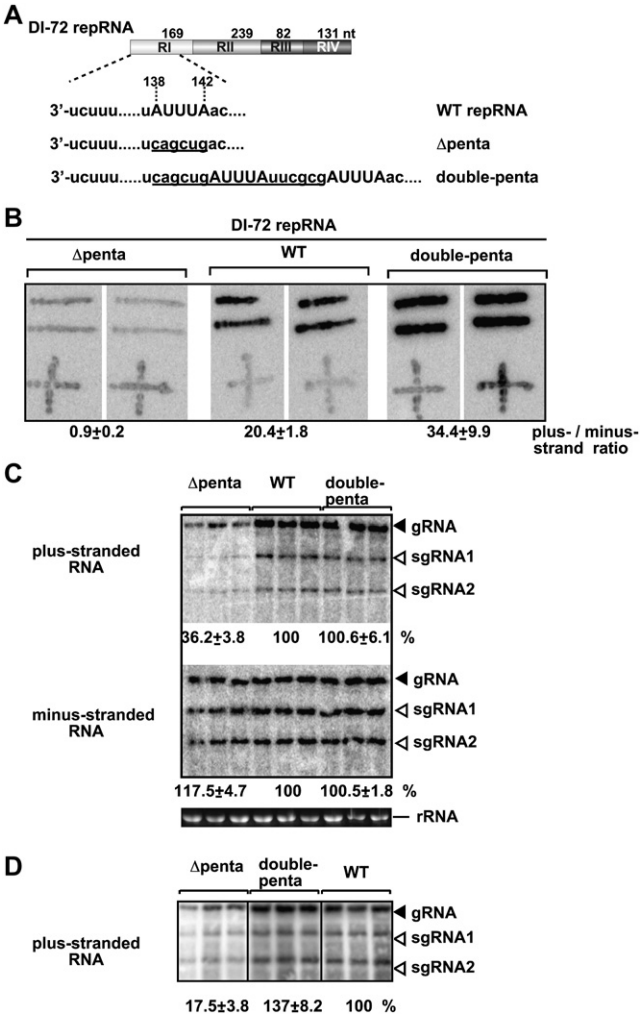

Figure 4. Mutagenesis of the AU-Pentamer Reduces (+)RNA Synthesis

(A) Schematic representation of the AU-pentamer and the mutations generated in the repRNA. Double-penta RNA contains the same mutations at the original position as Δpenta RNA, but it carries two proximal AU-pentamers. Note that the sequences represent (−)RNA sequences, since Tdh2/3p binds more strongly to (−) than to (+)RNA (Figure 3).

(B) The ratio of (+) versus (−)RNAs synthesized by the membrane-enriched replicase preparations obtained from yeast expressing one of the shown repRNAs. See further details in Figure 2C.

(C) Accumulation of TBSV gRNA carrying Δpenta or double-penta mutations. *N. benthamiana* protoplasts were electroporated with 2 μg of WT or mutant TBSV gRNA, and samples were harvested 8 hr later for RNA analysis. The positions of the gRNA and sgRNAs on the northern blots are depicted with arrowheads. rRNA was used as a loading control.

(D) Accumulation of TBSV gRNA carrying Δpenta or double-penta mutations in *N. benthamiana* protoplasts at the 40 hr time point. See other details in (C).

All values represent the mean of three independent experiments. Error bars indicate standard deviation of the mean.

inactivation of the viral replicase complex. To distinguish between these models, we used RNA blotting to detect viral (+) and (−)repRNAs in the replicase preparations. These assays confirmed that both the (+) and the (−)repRNAs were undetectable in the replicase preparations obtained from yeast expressing Tdh-N121 or Tdh-N201, whereas both RNAs were easily detectable in the control WT or *tdh2Δ* replicase preparations (Figure 5B). All the purified replicase preparations from the above yeast strains supported RNA synthesis on added repRNA templates (Figure S2), demonstrating that the viral replicases were active when obtained from yeast expressing Tdh-N121 or Tdh-N201. Therefore, we conclude that the viral replicase complexes could dissociate from the RNA component during the isolation of the replicase from yeast expressing Tdh-N121 or Tdh-N201. These observations suggest that expression of Tdh-N121 or Tdh-N201 in yeast makes the viral replicase less stable and prone to dissociate from the RNA template.

To test if the less stable complex of replicase:viral RNA template formed in yeast expressing Tdh-N121 might affect repRNA replication, we measured the level of repRNA accumulation at 23°C and 34°C. Interestingly, the repRNA accumulation decreased ~2-fold in yeast expressing Tdh-N121 at 34°C when compared to yeast expressing Tdh2p (Figures 5C and 5D). This decrease in repRNA level is not due to the production of less p33 and $p92^{pol}$ replication proteins in yeast expressing Tdh-N121 (Figure 5C, bottom panel). Interestingly, the accumulation of repRNA in yeast expressing Tdh-N121 at 23°C was comparable to that observed in yeast expressing Tdh2p (Figure 5E), suggesting that the inhibition of repRNA accumulation by expression of Tdh-N121 is a temperature-sensitive phenomenon. This can be explained by the less stable nature of the replicase:viral RNA template complex formed in yeast expressing Tdh-N121 when compared to the more stable replicase complex containing the full-length Tdh2p.

Inhibition of TBSV Accumulation by Downregulation of GAPDH-C via Gene Silencing in *Nicotiana* Host

To demonstrate that interaction between Tdh2/3p and TBSV repRNA within the viral replicase complex is also relevant for the replication of the full-length TBSV RNA in a natural plant host, we used complementation and gene silencing approaches. First, we demonstrated that the cytoplasmic GAPDH-C protein of *Arabidopsis thaliana*, the ortholog of the yeast Tdh2/3p, complemented the inhibitory effect of downregulation of Tdh3p in yeast (*tdh2Δ TET::TDH3*) on the replication of TBSV repRNA (Figure S3). For the second approach, we used *Nicotiana benthamiana* as a host. We cloned two regions of the *NbGAPDH-C* separately into a *Tobacco rattle virus*-based gene silencing vector (Dinesh-Kumar et al., 2003). The obtained TRV-GAPDHC1 and TRV-GAPDHC2 vectors were launched in *N. benthamiana* via Agro-infiltration (Dinesh-Kumar et al., 2003). We confirmed using RNA blot analysis that *NbGAPDH-C* mRNA levels decreased to ~3%–15% of the mock-silenced (using TRV2 empty vector) plants 9 days after agro-infiltration (Figure 6A). New leaves of these *N. benthamiana* plants were inoculated with TBSV virion preparation, followed by total RNA isolation from both the TBSV-inoculated and uninoculated (systemically infected) leaves 6 days postinoculation. RNA analysis revealed 90% inhibition of TBSV RNA accumulation in the inoculated leaves (Figure 6A), whereas TBSV RNA was undetectable in the uninoculated leaves (data not shown). A similar level of inhibition was observed when the *NbGAPDH-C*-silenced plants were inoculated with *Cucumber necrosis virus* (CNV), a closely related tombusvirus (Figure 6A). Northern blot analysis of total RNA extracts obtained from CNV-20KSTOP (a less aggressive

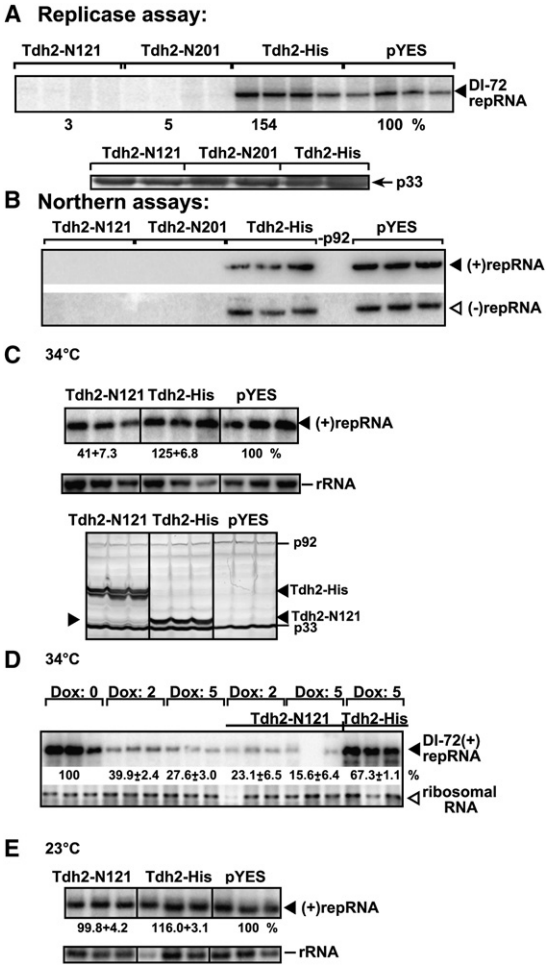

Figure 5. Reduced Stability of the Viral Replicase and Decreased Replication of TBSV repRNA in Yeast Expressing Truncated Variants of Tdh2p

(A) (Top panel) In vitro replicase assay using the membrane-enriched fraction carrying the copurified repRNA obtained from yeast, expressing C-terminally truncated Tdh2p, the full-length Tdh2p, or empty expression plasmid (pYES). (Bottom panel) Western blot analysis of the comparable p33 levels in the replicase preparations.

(B) Northern blot analysis of the membrane-enriched replicase preparation to detect the copurified (+) and (−)repRNAs from yeast expressing C-terminally truncated Tdh2p, the full-length Tdh2p, or empty expression plasmid (pYES).

(C) Accumulation of TBSV repRNA in yeast expressing the shown Tdh2p variants using northern blots (top panel). The yeast cells were incubated at 34°C. rRNA was used as a loading control. The accumulation level of the Tdh2p variants was estimated using western blotting (bottom panel).

(D) Accumulation of TBSV repRNA in yeast (*tdh2Δ TET::TDH3*) expressing the shown Tdh2p variants using northern blot. Yeast cells were treated with either 2 or 5 μg/ml doxycycline for 6 hr to downregulate Tdh3p level prior to launching TBSV repRNA replication. See further details in the legend to Figure 2.

(E) Accumulation of TBSV repRNA in yeast expressing the shown Tdh2p variants using northern blots (top panel). The yeast cells were incubated at the standard 23°C.

All values represent the mean of three independent experiments. Error bars indicate standard deviation of the mean.

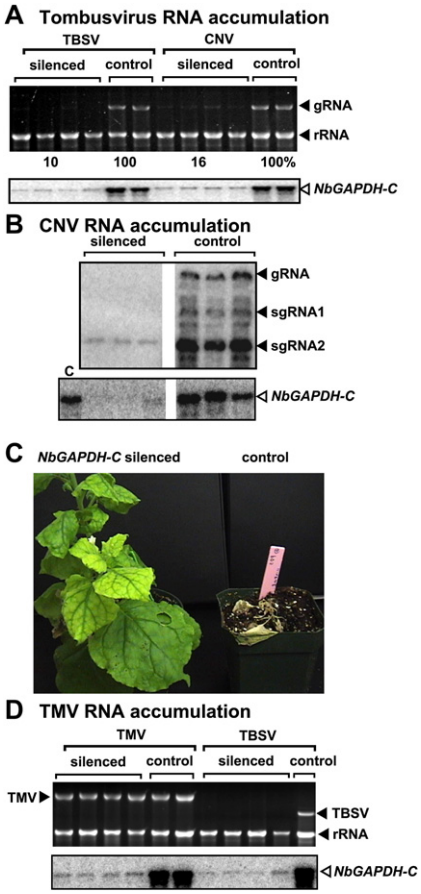

Figure 6. Knocking Down the Cytosolic GAPDH in *N. benthamiana* Reduces Tombusvirus RNA Accumulation

(A) A gene-silencing vector based on TRV carrying cDNA of the cytosolic GAPDH was Agro-infiltrated to the lower leaves of *N. benthamiana*. The control plants were Agro-infiltrated with the empty TRV vector. The accumulation level of the GAPDH-C mRNA was estimated by northern blotting from the upper leaves 9 days after Agro-infiltration. Leaves of *N. benthamiana* above the Agro-infiltrated leaves were inoculated with either TBSV or CNV 9 days after Agro-infiltration. The systemically infected upper leaves were tested 6 days after inoculation for accumulation of TBSV or CNV gRNA from total RNA samples (in ethidium-bromide-stained agarose gels) or via northern blotting using a CNV specific probe (B).

(C) Attenuated symptoms of TBSV-infected *N. benthamiana* plant 30 days after inoculation. Note that the control plant (unsilenced for GAPDH-C) rapidly died due to TBSV infection.

(D) Knocking down GAPDH-C in *N. benthamiana* did not affect TMV replication. Total RNA samples for the ethidium-bromide-stained agarose gel (top panel) and the northern blot (bottom panel) to detect NbGAPDH-C mRNA were obtained as described in (A) and (B).

mutant of CNV lacking functional suppressor of gene silencing [Rochon, 1991])-infected plants showed that the gRNA as well as the subgenomic RNAs accumulated to reduced levels in the *NbGAPDH-C*-silenced plants when compared to the control nonsilenced plants (Figure 6B). These results demonstrate that downregulation of *NbGAPDH-C* significantly reduces tombusvirus accumulation. The TRV2-infected plants did not inhibit TBSV accumulation, excluding that either Agro-infiltration or TRV infection could inhibit TBSV accumulation in *N. benthamiana*.

Development of viral symptoms was also inhibited in *NbGAPDH-C*-silenced plants (Figure 6C). Small areas in the inoculated leaves occasionally showed necrosis in the inoculated leaves, but the younger, uninoculated leaves were symptom

free. In contrast, the young leaves went through rapid necrosis in nonsilenced plants after CNV or TBSV infections, followed by death of the whole plants (Figure 6C). Thus, knocking down *NbGAPDH-C* level in *N. benthamiana* leads to resistance against tombusviruses.

Since downregulation of *NbGAPDH-C* level in *N. benthamiana* could affect the metabolism of the host (indeed, we did observe moderate stunting and yellowing of the silenced plants), it is possible that the inhibition of TBSV and CNV accumulation in *NbGAPDH-C*-silenced plants is due to indirect effects on plant physiology. To test this possibility, we infected *NbGAPDH-C*-silenced *N. benthamiana* plants with TMV, which is only distantly related to tombusviruses [a different supergroup of (+)RNA viruses] (Koonin and Dolja, 1993). We found using RNA blot analysis that TMV, unlike TBSV and CNV, accumulated as efficiently in *NbGAPDH-C*-silenced *N. benthamiana* plants as in the control *N. benthamiana* plants (Figure 6D). Therefore, we conclude that *NbGAPDH-C* is unlikely to be involved in TMV replication. Moreover, the physiology of the *NbGAPDH-C*-silenced *N. benthamiana* is suitable to support viral accumulation, thus inhibition of TBSV and CNV accumulation is likely due to a specific defect in tombusvirus replication as suggested based on the yeast model host.

DISCUSSION

Revealing the functions of host proteins within the amazing replicases of (+)RNA viruses of plants and animals has led to limited progress in the last 30 years. Although several host proteins have been identified to be part of the viral replicase complex, the activity of these host proteins have only been characterized for a small number of host proteins (Ahlquist et al., 2003; Lai, 1998; Shi and Lai, 2005). The replicase complex of tombusviruses contains four to ten host proteins, including HSP70 chaperone and Tdh2/3p glycolytic enzyme (Serva and Nagy, 2006). To dissect the function of host proteins in TBSV replication, in this work we characterized the role of the redundant Tdh2p and Tdh3p, two homologous GAPDH proteins. The in vitro RNA-binding studies (Figure 3) and in vivo copurification approach (Figure 3D) showed that Tdh2/3p is mostly associated with the viral RNA component of the replicase complex. We mapped that Tdh2/3p interacts with the AU pentamer sequence in (−)repRNA, albeit Tdh2/3p also can bind weakly to (+)repRNA. The subcellular localization experiments with Tdh2p-YFP also support a role for the viral RNA, since Tdh2p was redistributed to punctate structures (i.e., the peroxisomal membrane surface) containing p33 replication protein only when the viral repRNA in combination with $p92^{pol}$ to induce TBSV replication was also coexpressed in yeast cells (Figure 1B).

Downregulation of Tdh3p in the absence of the redundant Tdh2p decreased TBSV repRNA replication four-fold in yeast cells, confirming an important function for Tdh2/3p in TBSV replication. Interestingly, we found that deletion/downregulation of Tdh2/3p mostly inhibited (+)RNA synthesis, whereas (−)RNA synthesis by the viral replicase was comparable. The defect in (+)RNA synthesis strengthen the model that the primary role of Tdh2/3p in TBSV replication is to promote asymmetric RNA synthesis, leading to the production of abundant (+)RNA progeny. In a way, TBSV became double-stranded RNA virus-like in the presence of limiting amount of Tdh2/3p by producing (+) and (−)RNAs in 1:1 ratio.

Further evidence supporting the central role of Tdh2/3p:viral RNA interaction in regulation of asymmetric RNA synthesis comes from RNA mutagenesis experiments. Altering the 5′ proximal AU-pentamer sequence, which showed the most efficient binding to the purified recombinant Tdh2p in vitro, resulted in decreased level of (+)repRNA accumulation [close to 1:1 ratio of (+) and (−)RNAs, Figure 4B]. Similar mutation of the 5′ proximal AU-pentamer in the TBSV gRNA also resulted in decreased accumulation level of (+)RNA progeny in plant cells, whereas (−)RNA accumulation was not affected at the time point examined (Figure 4C).

Based on the above findings, we propose that binding of Tdh2/3p to (−)repRNA likely plays a role in the retention of (−)repRNA replication intermediate in the replicase complex (Figure 7). Accordingly, expression of truncated Tdh2p carrying only the RNA-binding domain in yeast resulted in a viral replicase complex, which easily lost the viral RNA template during the replicase isolation process. Expression of truncated Tdh2p in yeast also decreased TBSV repRNA accumulation at 34°C (Figures 5C and 5D), which could be due to decreased stability of the replicase complex carrying the truncated Tdh2p. In contrast to (−)RNA, the fate of (+)RNA is different. This is because (+)RNA is released from the site of replication after RNA synthesis (Figure 7) (Panavas et al., 2005a). We also noticed that the Tdh2/3p-binding site is proximal, but not overlapping, with the known promoter and replication enhancer elements in (−)RNA (Panavas et al., 2002, 2003), suggesting that Tdh2/3p binding will not block replicase binding to the 3′ terminus of (−)RNA template as illustrated in Figure 7. In summary, our model predicts that the strong selective binding of (−)RNA to Tdh2/3p and the weak binding of (+)RNA progeny to Tdh2/3p could be the key regulatory step during viral RNA replication by allowing more efficient access of the (−)RNA replication intermediate to the viral RdRp than (+)RNA template, which then gets released into the cytosol. This could lead to asymmetric RNA synthesis, explaining this hallmark feature of (+)RNA viruses.

To demonstrate the relevance of the above yeast work in a natural plant host, we knocked down *NbGAPDH-C* levels in *N. benthamiana*, which led to 85%–90% reduction in TBSV and CNV gRNA accumulation. This level of reduction is comparable to that seen with the Δpenta gRNA of TBSV in *N. benthamiana* protoplasts at the 40 hr time point (Figure 4D), suggesting that the interaction between GAPDH and viral RNA is critical during virus replication and GAPDH is unlikely to affect the spread of the virus through the plant. Importantly, the silenced plants also showed resistance against tombusviruses, indicating that this approach might lead to a new antiviral approach. The same strategy did not interfere with TMV accumulation, which could be due to the lack of function for GAPDH in TMV replication. This is not surprising since TMV is more similar to BMV than to TBSV and we showed previously that TBSV is affected by a vastly different set of host factors as BMV (Panavas et al., 2005b). Further evidence for the role of the plant GAPDH in TBSV replication comes from complementation experiments in which expression of AtGAPDH, the ortholog of the yeast Tdh2/3p, increased replication of TBSV repRNA in yeast expressing reduced levels of Tdh3p (Figure S3). In conclusion, our work

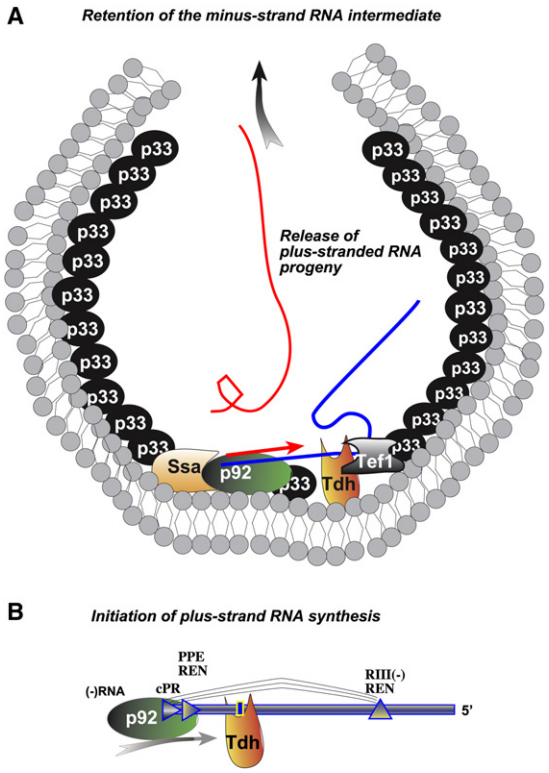

Figure 7. A Model for the Role of GAPDH in Tombusvirus Replication

(A) Tdh2/3p is part of the replicase complex (Serva and Nagy, 2006), which likely forms a membrane-containing spherule-like structure, similar to those shown for BMV (McCartney et al., 2005; Schwartz et al., 2002). By binding selectively to the 3′ proximal AU-pentamer sequence on TBSV (−)RNA, Tdh2/3p is proposed to facilitate the selective retention of the (−)RNA replication intermediate in the replication complex. On the contrary, the (+)RNA progeny is not bound by Tdh2/3p and it gets released from the site of replication into the cytosol (Panavas et al., 2005a). The (−)RNA then could be used repeatedly by the viral replicase as a template. Thus, this model predicts that Tdh2/3p-driven retention of (−)RNA is a major factor in (+)RNA synthesis and in regulation of asymmetric RNA replication, a hallmark of (+)RNA viruses. Tdh2/3p is likely retained within the replication complex by interacting with Tef1/2p translational elongation factor EF-1α (Gavin et al., 2006), which has been shown to be part of the complex, and to bind to p33 and to the viral RNA as well (Z. Li, R.Y.-L.W., and P.D.N., unpublished data).

(B) A model for the initiation of (+)RNA synthesis. Shown are the known *cis*-acting elements in (−)RNA, including the cPR promoter sequence, the promoter proximal replication enhancer (PPE REN), and the RIII(−) REN, which have been shown to bind to the viral replicase and affect initiation of (+)RNA synthesis. Interestingly, the 3′ proximal AU-pentamer that binds to Tdh2/3p does not overlap with these elements. This arrangement of *cis*-acting elements likely facilitates efficient initiation of (+)RNA synthesis by the tombusvirus replicase.

provides strong evidence that a host factor identified and characterized in the yeast model host is also relevant for virus replication in the native plant infected with the full-length WT virus.

The novel functional role of GAPDH in viral RNA replication and RNA synthesis expands the battery of activities of this multifunctional enzyme, which is known to be involved in numerous cellular processes, including glycolysis, apoptosis, endocytosis, nuclear tRNA transport, vesicular secretary transport, nuclear membrane fusion, DNA replication and repair, maintenance of telomere structure, and transcriptional control of histone gene expression (Sirover, 1999, 2005). Probably the most important function of GAPDH for RNA viruses is to bind to AU-rich sequences (Nagy et al., 2000; Nagy and Rigby, 1995). For example, GAPDH has been shown to bind specifically to several cellular RNAs, including human lymphokine mRNA, which can lead to stabilization of the RNA in the cell (Bonafe et al., 2005; Nagy and Rigby, 1995; Singh and Green, 1993). Moreover, GAPDH has been shown to bind to AU-rich sequences present in various RNA viruses, including hepatitis A virus (HAV), hepatitis C virus, and human parainfluenza virus type 3 (De et al., 1996; Dollenmaier and Weitz, 2003; Randall et al., 2007). The functional role of binding of GAPDH to the above viruses is not clear. It has been proposed that binding of GAPDH to the internal entry site (IRES) element in HAV could suppress cap-independent translation of HAV RNA based on GAPDH overexpression experiments (Yi et al., 2000). GAPDH can also bind to a posttranscriptional regulatory element and may be involved in the posttranscriptional regulation of hepatitis B virus gene expression (Zang et al., 1998).

Selective retention of the viral (−)RNA by GAPDH in the replication complex could have major significance for viral replication. First, it could promote asymmetric RNA synthesis by allowing more efficient use of the (−)RNA at the expense of the (+)RNA (which would be released from the complex after its synthesis). Second, it could inhibit the recognition of (−)RNA, which might form partial dsRNA with (+)RNA by the dsRNA surveillance system of the host. Third, it could regulate the timing of viral RNA release from replication, which could be important for subgenomic RNA synthesis and RNA recombination. Altogether, we propose that GAPDH plays a major functional role in replication of tombusviruses. Moreover, GAPDH or host proteins similarly capable of binding viral RNA are likely present in replicases of other (+)RNA viruses of plants and animals.

EXPERIMENTAL PROCEDURES

Yeast Strains and Expression Plasmids

The single-gene deletion (*tdh2*Δ) yeast strain was purchased from Open Biosystems. The doxycycline-regulatable Tdh3p expression system was generated via homologous recombination as described in the Supplemental Data. Construction of Δpenta and double-penta repRNA is also described in the Supplemental Data.

RNA and Protein Analysis

Total RNA isolation and northern blot analysis were performed as described previously (Panaviene et al., 2003). The protein analysis with antibody against anti-His6 (GE Healthcare) was done as described previously (Panaviene et al., 2004).

Tombusvirus Replicase Assay

The TBSV replicon system in yeast is based on CNV p33 and p92^{pol} replication proteins, which support as efficient replication as the corresponding TBSV replication proteins. The "membrane-enriched" CNV replicase preparation, which is suitable to test the replicase activity on the copurified templates present within the CNV replicase preparation, was obtained as previously described (Panaviene et al., 2004; Stork et al., 2005). Because no template was added to the in vitro reaction, the replicase preparation could only use the copurified template present within the enriched membrane fraction.

To test the ratio of (+) versus (−)RNA synthesis on the copurified templates by the CNV replicase, we obtained the membrane-enriched fraction (see above), followed by standard replicase assay in the presence ^{32}P-labeled

UTP and the other three unlabeled rNTPs. This is followed by RNA hybridization to the (+) and (−)RNA probes blotted on membranes as described previously (Panaviene et al., 2004; Stork et al., 2005).

Gel Mobility Shift Assay

RNA templates were obtained using T7 RNA polymerase-based transcription on PCR templates in the presence of ^{32}P-labeled UTP and unlabeled ATP, CTP, and GTP as described (Pogany et al., 2005). The affinity-purified recombinant proteins (1 μg) were incubated with 1 ng of radioactively labeled RNA probe (see above) in a binding buffer (12 mM HEPES [pH 7.9], 15 mM KCl, 0.2 mM DTT, 10% glycerol, 200 ng yeast tRNA [Sigma] and 2 U of RNase inhibitor [Ambion]) at 25°C for 15 min (Rajendran and Nagy, 2003). The samples were analyzed by 5% nondenaturing polyacrylamide gel electrophoresis in TAE buffer performed at 200V in a cold room.

RNA Analysis of TBSV Accumulation in *N. benthamiana* Protoplasts

N. benthamiana protoplasts were prepared from callus cells by treatment with 0.5 g of cellulysin and 0.1 g of macerase (Calbiochem) as described (Panaviene and Nagy, 2003). Protoplasts were electroporated with 2 μg of in vitro transcribed gTBSV RNA as described (Panaviene and Nagy, 2003). Total RNA was extracted, and northern blot analysis was performed with gTBSV-specific probes as described (Panaviene and Nagy, 2003).

Silencing of the Cytosolic GAPDH-C in *N. benthamiana* Plants

The virus-induced gene silencing (VIGS) assay to knock down cytosolic GAPDH mRNA levels in *N. benthamiana* plants was done as described in the Supplemental Data.

Copurification of Tdh2p and the TBSV repRNA from Yeast

The copurification experiment was based on yeast cells expressing p33, p92^{pol}, DI-72 repRNA, and Tdh2p-zz (Open Biosystems) and affinity purification of Tdh2p-zz on IgG agarose as described in the Supplemental Data.

Subcellular Localization of Tdh2p and p33 Replication Protein by Confocal Laser Microscopy

To detect the subcellular localization of Tdh2p in yeast replicating TBSV repRNA, we transformed yeast with TDH2-YFP, in combination with pGAD-His92, pYC-DI72, and pHisGBKCFP-p33. The confocal microscopy was performed on an Olympus FV1000 (Olympus America Inc., Melville, NY) as described earlier (Jonczyk et al., 2007).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and three supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/3/3/178/DC1/.

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