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ROLE OF LIPIDS IN TOMBUSVIRUS REPLICATION

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ROLE OF LIPIDS IN TOMBUSVIRUS REPLICATION

DISSERTATION

A dissertation submitted in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

in the College of Agriculture at the University of Kentucky

By

Monika Sharma

Lexington, Kentucky

Director: Dr. Peter Nagy, Professor

Department of Plant Pathology

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ABSTRACT OF DISSERTATION

ROLE OF LIPIDS IN TOMBUSVIRUS REPLICATION

Positive-strand RNA virus group are the most abundant among viruses affecting plants and animals. To successfully achieve replication, these viruses usurp or co-opt host proteins. To facilitate the discovery of host factors involved in *Tomato bushy stunt virus* (TBSV), yeast has been developed as a surrogate model host. Genome-wide approaches covering 95% of yeast genes, has revealed approximately hundred factors that could affect virus replication. Among the identified host factors, there are fourteen yeast genes, which affect/regulate lipid metabolism of the host.

One of the identified host gene is ERG25, which is an important factor for sterol biosynthesis pathway, affecting viral replication. Sterols present in eukaryotes affect the lipid composition of membranes, where tombusviruses, similar to other plus-strand viruses of tobacco, replicate. Since potent inhibitors of sterol synthesis are known, I have tested their effects on tombusvirus replication. We demonstrated that these sterol-synthesis inhibitors reduced virus replication in tobacco protoplasts. Virus replication is resumed to the wild type level by providing phytosterols in tobacco protoplasts confirming the role of sterols in RNA virus replication in tobacco.

We have also identified INO2, a transcription factor for many phospholipid biosynthetic genes, reduces virus replication in its deletion background. When we provided this gene product in the mutant background, viral replication was back to normal, confirming the role of Ino2p in tombusvirus replication. Further biochemical assays showed that the viral inhibition is because of alteration in the formation of the viral replicase complex. Using confocal microscopy, we showed that the viral replication protein, termed p33, is forming large and few punctate structures rather than the small and many by overexpressing Ino2p in the wild type yeast cells. Over-expression of Opi1, an inhibitor of Ino2p led to greatly reduced viral replication, further supporting the roles of the phospholipid pathway in tombusvirus replication.

One of the phospholipid, which is regulated by this pathway, is cardiolipin an important component of the mitochondrial as well as peroxisomal membranes. We further characterized how cardiolipin is playing an important role for tombusvirus replication by using different biochemical approaches.

Key Words: Plant virus, RNA replication, lipid metabolism, phospholipids, cardiolipin

Monika Sharma

22nd November, 2011

ROLE OF LIPIDS IN TOMBUSVIRUS REPLICATION

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Chapter 1 Introduction

Viruses represent a major threat faced to plant, human and veterinary health. The heightened awareness and importance of the epidemiological potential of viruses, both in natural and man-caused outbreaks, has stimulated the search for both prophylactic and curative treatments. More than 800 million people do not have adequate food; 1.3 billion live on less than $1 a day and at least 10% of global food production is lost to plant diseases (Strange and Scott, 2005). Plant pathologists cannot ignore the juxtaposition of these figures for food shortage and the damage to food production caused by plant pathogens. Apart from cultural practice and resistant varieties scientists have nothing left in their arsenal to fight against plant viruses. Medical professionals have vaccines to prevent viral diseases. They even have few antiviral agents to use in therapy for viral insults; but so far neither vaccines nor antivirals have been reported to be effective against plant viruses. Classical targets for antiviral drugs are viral specific enzymes and metabolic pathways. But there is an increasing necessity to find novel targets for antivirals because many viruses evolve to escape the antivirals targeted against them. There are increasing number of evidences that viruses multiply by co-opting several cellular factors [reviewed by (Nagy, 2008)]. These usurped host factors can be excellent novel targets for antivirals (Schwegmann and Brombacher, 2008). Therefore, one of the research areas is to characterize host–virus interactions in order to invent new antiviral strategies and develop highly effective and specific antiviral agents. For this to happen, pioneering research is needed to characterize the intimate interaction of viruses with their hosts at the molecular level. Accordingly, the central theme of my research is to decipher the mechanisms how viruses co-opt and utilize the host factors, in particularly cellular

membranes and lipids for their own multiplication. I expect that this research about host and virus interaction will improve the approaches towards antiviral therapy. My research also opens up a vista in which virus infections can be understood in the context of the biology of their hosts.

INTRODUCTION TO THE RESEARCH SYSTEM

Tombusviruses, belong to RNA virus group (Russo et al., 1994) that are the most abundant among viruses affecting humans, animals and plants. RNA viruses encompass over one-third of all virus genera and include numerous pathogens, such as the severe acute respiratory syndrome coronavirus SARS, hepatitis C virus (HCV), Dengue virus, and many of the viruses on the U.S. Health and Human Services Department Select List of potential bioterrorism agents (Ahlquist, 2006). To facilitate the identification of host factors facilitating viral replication, our lab has developed yeast as a model host (Panavas and Nagy, 2003). Using genome-wide approaches covering 95% of yeast genes, we have reported approximately hundred host factors that could affect virus replication in yeast (Jiang et al., 2006; Panavas et al., 2005b). We confirmed that several of these host genes also facilitate tombusvirus replication in their host plants, validating our approach (Huang and Nagy, 2011; Sharma et al., 2010; Wang and Nagy, 2008; Wang et al., 2009). Altogether, tombusviruses have emerged as a one of the best characterized RNA viruses and a superior system to advance our understanding of virus – host interactions.

Among the identified host factors, there are fourteen yeast genes, which affect/regulate lipid metabolism of the host (Jiang et al., 2006; Panavas et al., 2005b). I used *in-vitro* assays, biochemical and genetics tools, confocal microscopy, plant

protoplast systems and whole plants, and I tried to find out the role of the critical host lipid metabolism genes affecting virus replication, including dissecting the mechanism(s) by which these factors affect virus replication.

OBJECTIVES AND HYPOTHESES

Viruses modify the host's normal physiological and metabolic pathways for their own benefit so that they can multiply at enormous rates. For e.g. many viruses in order to facilitate their own protein production, turn off the host protein machinery so that all the cellular resources are diverted for their multiplication (Kaariainen and Ranki, 1984). Like wise, when viruses multiply, they require continuous supply of lipids, which are the crucial components of cell membranes (Lazarow, 2011; Lorizate and Krausslich, 2011). The cell membranes are site of multiplication for many important RNA viruses (Laliberte and Sanfacon, 2010; Miller and Krijnse-Locker, 2008a). We know little about how viruses force the cells to make so much of lipids and thus membranes. Gaining insight into the role of lipids/membranes in the virus replication was objective of my projects. Sterols and phospholipids are the two major components of cellular membranes (Osman et al., 2011). With this knowledge, I started with simple hypothesis:

"Knock down of genes involved in lipid biosynthesis in host should inhibit virus replication."

Objectives:

1. To characterize the lipid metabolic factors involved in TBSV replication in yeast and plants.

2. To find out the mechanism by which a particular lipid affects the TBSV replication.

POSITIVE STRAND RNA VIRUS REPLICATION

Replication of positive stranded RNA viruses is a complex process that needs an interaction between viral replication proteins, viral genome and host factors (Ahlquist et al., 2003). Plant viruses start their multiplication cycle after entering the plant cells. Their entry is facilitated by mechanical damage to the cell wall, or by insects, fungi and nematodes. Immediately after entry the genomic RNA disassembles of the coat proteins. Co-translational disassembly is the most accepted model for the same. Briefly, ribosome during translation helps to disassemble the coat proteins from the 5′ end of the RNA molecule. Early proteins translated are most critical for replication of the viral genome. Then the RNA is recruited to the site of replication, generally specialized structures derived from organellar membranes. Plus strand RNA is used to synthesize minus strand or complementary strand of RNA, which in turn acts as template for production of multiple copies of plus strand RNA. The newly synthesized plus RNAs have different fates. Some go back to translate new viral proteins. Some are again recruited for another round of replication. The movement proteins might grab another group of RNAs for transportation to neighboring cells and distant tissues. Remaining lot is assembled into new virion particles after encapsidation by the coat proteins. To achieve high levels of daughter RNAs, viruses use cellular proteins at each of above-mentioned steps.

TBSV REPLICATION COMPONENTS

Tombusviruses belong to one of the most studied positive strand RNA viruses of plants. TBSV the type species of *Tombusviridae* family within *Tombusvirus* genus has become a model virus to study replication. TBSV was one of the earliest model for which the structure was deduced (Harrison, 1969).

TBSV genome codes for five ORFs. ORF2 is translated as a read-through of ORF1 (encoding p33 a replication auxillary protein) resulting into p92 that has RdRp domains [reviewed by (White and Nagy, 2004)]. Only these two proteins are sufficient to support RNA replication in plants and yeast (a model host). P33 is emerging as master-regulator of TBSV replication owing to its multi-functionality. For e.g. (i) Selects viral RNA template via binding through Arginine-Proline-Arginine rich (RPR) domain (Panaviene et al., 2003), (ii) Contains transmembrane domain for membrane localization (Panavas et al., 2005a), (iii) Dimerizes/oligomerizes with other p33/p92 molecules, which is necessary for RNA binding and RNA replication (Rajendran and Nagy, 2004, 2006) (iv) targets replication complex to peroxisomal membranes by interacting with peroxisomal transporter pex19p via peroxisomal targeting signals on its NH_2-terminal half (Pathak et al., 2008). (v) P33 also interact to many host-factors to either recruit them for replication (Li et al., 2008; Li et al., 2009b; Mendu et al., 2010; Serva and Nagy, 2006) or to get modified (Barajas and Nagy, 2010). (vi) Recently it has been shown that p33 possesses RNA chaperone activity to facilitate replication via improving accessibility of the p92 to the template RNA (Stork et al., 2011).

Overall, the emerging picture is: TBSV utilizes various host factors, host membranes, viral RNA and its versatile p33 apart from p92 to accomplish high level of multiplication rate that enables it to accumulate up to million progenies in a single day.

TBSV REPLICATION IN YEAST AND *IN VITRO*

Panavas and co-workers developed yeast as a surrogate host to rapidly to facilitate the studies of host factors utilized by TBSV for its replication (Panavas and Nagy 2003). Easy access to the commercial gene knock-out and knock down libraries combined with the high levels of accumulation of TBSV replicon RNA in yeast lead to discoveries of host factors involved in TBSV replication. To launch RNA replication in yeast, two replication indispensable viral proteins p33 and p92 were expressed via separate plasmids under constitutive *ADH1* promoters. RNA replicon was expressed under GAL1 promoter, which is galactose inducible and is suppressed under glucose. Auxotrophic markers were utilized for selection of the transformed yeast strains. Replication was checked by visualizing the replicon RNAs on agarose gels stained with ethidium bromide followed by Northern blotting.

To check the efficiency of replicase complex assembly, in vitro reconstitution assays were used based on Panaviene and co-workers methods (Panaviene et al., 2005). As the membrane fractions contained the assembled replicase of TBSV, it was isolated and normalized for the presence of similar quantities of p33 and p92 followed by an *in vitro* RNA dependent RNA polymerase (RdRp) assay.

CELLULAR FACTORS FOR RNA VIRUS REPLICATION

Different Subcellular membranous sites for replication

All positive strand RNA viruses replicate their genome on cytoplasmic surface of different subcellular organellar membranes. Among many studied RNA viruses, *Brome mosaic virus* (BMV) replicates on the membranes of endoplasmic reticulum associated complexes in plants as well as yeast *Saccharomyces cerevisiae* (Lee et al., 2001). Similarly *Tobacco etch virus*, *Cow pea mosaic virus* and polio virus induce proliferation and rearrangement of ER membranes where replicase complex are formed (Carette et al., 2000; Gosert et al., 2000; Rust et al., 2001; Schaad et al., 1997). Replication of *Flock house virus* is associated with the outer mitochondrial membranes (Miller et al., 2001). *Turnip yellow mosaic virus* and *Alfalfa mosaic virus* replicates on chloroplast and vacuolar membranes respectively (Prod'homme et al., 2001; Van Der Heijden et al., 2001). For tobamovirus RNA replication subcellular localization of viral and host proteins has been observed to be associated with the tonoplast membranes (Hagiwara et al., 2003).

TBSV is well known to replicate in the spherules formed on the cytoplasmic surface of peroxisomes (Burgyan et al., 1996; Weber-Lotfi et al., 2002). Surprisingly, in our lab, genome wide screening showed TBSV replicating perfectly in ΔPEX3 and ΔPEX19 detletion strains, which is known to be extremely crucial for peroxisome biogenesis. (Jonczyk et al., 2007). The authors discovered that TBSV could also replicate on endoplasmic reticulum membranes in ΔPEX3 and ΔPEX19 deletion yeast strains where peroxisome biosynthesis is completely abolished (Jonczyk et al., 2007).

Importance of host membranes in for virus replication

Many characterized RNA viruses are known to assemble a replicase complex on membranes. Importance of formation of such a replication complex has multiple purposes. First, a membrane provides a stable platform to localize and concentrate different components essential for replicase to assemble. It also provides a separate compartment for sequestering the genomic RNAs and replication factors from processes like translation and competing with RNA templates (den Boon et al., 2001). Virus induced membrane vesicle also protects dsRNA replication intermediates induced host defense responses (Ahlquist et al., 2003)

Importance of host membranes for TBSV replication.

An electron microscopic image of cells replicating tombusviruses shows extensive remodeling of membranes (Barajas et al., 2009). Genome wide screen of *S. cerevisiae* identified around fourteen host factors (INO2, ADA2, UME6, SIN3, SWI3, SNF6, CHO2, SPT3, ERG4, MCT1, POX1, TGL2, ERG25 and FAS2) involved in host lipid metabolism, suggested the role of different lipids for tombusvirus replication and recombination. (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2006; Serviene et al., 2005). Lipids play an important role in membranes fluidity, permeability and rigidity, which might be important to maintain the appropriate replicase complex for efficient viral replication.

Importance of lipids

Lipid synthesis and lipid composition are important for positive RNA virus replication. Bromo Mosaic Virus 1a protein leads to both membrane proliferation and membrane lipid synthesis, which leads to total increase in lipids by 25%to 33% in yeast. (Lee and Ahlquist, 2003b). Ole1p is the Δ9 fatty acid desaturase; an integral ER membrane protein that converts saturated fatty acids into unsaturated fatty acids (Lee et al., 2001). Mutation in OLE1 led to reduced amount of unsaturated fatty acids and BMV replication gets severely compromised prior to minus strand synthesis. Supplementation of media with unsaturated fatty acids can rescue the defects (Lee et al., 2001).

Flock house virus (FHV) in Drosophila S2 cells showed transcriptional up regulation of the several genes involved in lipid metabolism (Castorena et al., 2010). Furthermore, genes involved in glycerophospholipid metabolism have been found to be important for FHV replication by using gene-silencing approach (Castorena et al., 2010). Down regulation of Cct1 or Cct2 which encodes the genes involved in phosphatidylcholine biosynthesis shows decrease in flock house virus replication (Castorena et al., 2010). Deletion of an important enzyme 3-hydroxy-methyglutaryl-CoA reductase involved in cholesterol biosynthesis pathway reduces the replication of West Nile virus (WNV) replication (Mackenzie et al., 2007)

Norwalk virus (NV) infection is also affected by the genes involved in similar pathway. HMG-CoA reductase inhibitor statins increases the NV proteins and RNA accumulation, whereas acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors reduces replication of Norovirus replication (Chang, 2009).

Subcellular membranes in response to virus infection

Positive RNA viruses induce changes in membranes and invaginate these membranes to a small membrane bound spherule like structures, which contains all the components essential for virus replication (Mackenzie et al., 2007). WNV requires a complex membranous structure. WNV modulates host cell cholesterol homeostasis by up regulating its synthesis and redirecting the already existing as well as newly made cholesterol to membranes where virus replication takes place (Mackenzie et al., 2007).

Chapter 2 Inhibition of sterol biosynthesis inhibits tombusvirus replication in yeast and plants

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INTRODUCTION

Plus-stranded (+)RNA viruses usurp various intracellular/organellar membranes for their replication. These cellular membranes are thought to facilitate building of "viral factories", promote high concentration of membrane-bound viral proteins and provide protection against cellular nucleases and proteases (Ahlquist et al., 2003). The membrane lipids and proteins may serve as scaffolds for targeting the viral replication proteins or for the assembly of the viral replicase complex. The subcellular membrane may also provide critical lipid or protein cofactors to activate/modulate the function of the viral replicase. Indeed, formation of spherules, consisting of lipid membranes bended inward and viral replication proteins as well as recruited host proteins, has been demonstrated for several (+)RNA viruses (McCartney et al., 2005). These viral-induced spherules serve as sites of viral replication. Importantly, (+)RNA viruses also induce membrane proliferation that requires new lipid biosynthesis. Therefore, it is not surprising that several genome-wide screens for identification of host factors affecting (+)RNA virus replication unraveled lipid biosynthesis/metabolism genes (Cherry et al., 2005; Kushner et al., 2003; Panavas et al., 2005b; Serviene et al., 2006). Yet, in spite of the intensive efforts, understanding the

roles of various lipids and lipid biosynthesis enzymes and pathways in (+)RNA virus replication is limited.

TBSV is among the most advanced model systems regarding identification of host factors affecting (+)RNA virus replication (Nagy, 2008). Among the five proteins encoded by the TBSV genome, only p33 replication co-factor and the $p92^{pol}$ RNA-dependent RNA polymerase (RdRp) are essential for TBSV RNA replication (White and Nagy, 2004). p33 and $p92^{pol}$ are integral membrane proteins and they are present on the cytosolic surface of the peroxisomes, the site of replicase complex formation and viral RNA replication [(Mccartney et al., 2005) and (Pathak et al., 2008)]. Electron microscopic images of cells actively replicating tombusviruses have revealed extensive remodeling of membranes and indicated active lipid biosynthesis (Mccartney et al., 2005).

Additional support for the critical roles of various lipids in TBSV replication is coming from a list of 14 host genes involved in lipid biosynthesis/metabolism, which affected tombusvirus replication and recombination based on systematic genome-wide screens in yeast, a model host. These screens covered 95% of the host genes (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2006; Serviene et al., 2005). The 14 identified host genes involved in lipid biosynthesis/metabolism included 8 genes affecting phospholipid biosynthesis, 4 genes affecting fatty acid biosynthesis/metabolism and 2 genes affecting ergosterol synthesis. These finding suggest that these lipids are likely involved, directly or indirectly, in TBSV replication in yeast.

In order to further understand the roles of cellular membranes, lipids and host factors in viral (+)RNA replication, we analyzed the importance of sterol biosynthesis in

tombusvirus replication. Sterols are ubiquitous and essential membrane components in all eukaryotes, affecting many membrane functions. Sterols regulate membrane rigidity, fluidity and permeability by interacting with other lipids and proteins within the membranes (Bloch, 1992; Bloch, 1983). They are also important for the organization of detergent-resistant microdomains, called lipid rafts (Roche et al., 2008). The sterol biosynthesis differs in several steps in animals, fungi and plants, but the removal of two methyl groups at C4 position is critical and rate limiting. The C4 demethylation steps are performed by *SMO1* (sterol4alpha-methyl-oxidase) and *SMO2* in plants and by the orthologous *ERG25* gene in yeast (Darnet and Rahier, 2004). Accordingly, *erg25* mutant yeast accumulates 4,4-dimethylzymosterol, an intermediate in the sterol biosynthesis pathway (Bard et al., 1996). However, sterol molecules become functional structural components of membranes only after the removal of the two methyl groups at C4. Therefore, *ERG25* is an essential gene for yeast growth.

Our previous genome-wide screens for factors affecting tombusvirus replication have identified two sterol synthesis genes, *ERG25* and *ERG4*, which participate in different steps in the sterol biosynthesis pathway (Daum et al., 1998). In this work, we further characterized the importance of *ERG25* in TBSV replication in yeast. Down regulation or pharmacological inhibition of *ERG25* in yeast led to 4-to-5-fold decreased TBSV RNA accumulation. The in vitro activity of the tombusvirus replicase was reduced when isolated from the above yeast cells. We also found that the stability of $p92^{pol}$ viral replication protein decreased by 3-fold in yeast treated with a chemical inhibitor of *ERG25*. Inhibition of sterol biosynthesis in plant protoplasts or in plant leaves with a chemical inhibitor or silencing of *SMO1/SMO2* genes also resulted in reduction in TBSV

RNA accumulation, supporting the roles of sterols in tombusvirus replication in plants as well.

MATERIALS AND METHODS

Yeast strains and expression plasmids.

Saccharomyces cerevisiae strain BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and single-gene deletion strain *Δpex19* as well as ERG25/THC (BY4741; URA3::CMV-tTA) strain with the regulatable TET promoter from the Hughes collection, were obtained from Open Biosystems (Huntville, AL). The following yeast expression plasmids have been generated before: pGAD-His92 (Panaviene et al., 2004), pGBK-His33/DI-72 (Jiang et al., 2006), pGBK-His33/CUP1(Jaag et al., 2007) pGAD-His92CUP1 (Li et al., 2008), and pYC/DI72 (Panavas and Nagy, 2003).

To generate pCM189-tetDI72, DI-72 sequence was PCR-amplified using the following primers: #1803 (GGCGAGATCTGGAAATTCTCCAGGATTTCTC) and #3176 (CGGTCAAGCTTTACCAGGTAATATACCACAACGTGTGT) and pYC/DI72 as a template. The obtained PCR product was purified and digested with *Hind*III and *Bgl*II, followed by ligation into *Hind*III and *BamH*I digested vector pCM189.

TBSV repRNA replication studies in yeast.

The ERG25/THC strain transformed with pGAD-His92 and pGBKHis33/DI-72 plasmids was pre-grown in a synthetic complete dropout medium lacking leucine and histidine (SC-LH$^-$ medium) containing 2% glucose and then cultured for 24 h at 29°C until OD_{600} of ~0.8 to 1.0 in SC-LH$^-$ medium containing 2% galactose. For maximum level of *ERG25* gene expression, yeast was grown in the absence of doxycycline, whereas

to reduce the expression level of the *ERG25*, yeast was grown in the same medium in the presence of 10 mg/liter doxycycline (Jiang et al., 2006).

To study the effect of 6-amino-2-n-pentylthiobenzothiazole (APB) on TBSV repRNA accumulation, we used different APB concentrations to treat both BY4741 and ERG25/THC strains. Briefly, the above strains were transformed with pGAD-His92 and pGBK-His33/DI-72 plasmids and pre-grown overnight at 29°C with or without APB in SC-LH$^-$ medium containing 2% glucose. The replication of TBSV repRNA was induced by transferring the culture to SC-LH$^-$ media containing 2% galactose (Panavas and Nagy, 2003) followed by growing at 29°C. After 24 h, cells were used to obtain total RNA extracts.

To do the time course study with APB, ERG25/THC strain carrying pGAD-His92 and pGBK-His33/DI-72 plasmids was grown with 30 μM APB added at different time points. Briefly, yeast was pre-grown overnight at 29°C in 6 different batches (treatments #1-6) in SC-LH$^-$ medium containing 2% glucose. TBSV repRNA was expressed by transferring the culture to SC-LH$^-$ media containing 2% galactose at 29°C. The APB inhibitor was removed and the pellet was washed with the growth media at the beginning of repRNA induction or after 12-hour treatment (treatments #4 and #5, respectively), followed by culturing as described above.

To study the effect of Lovastatin on TBSV DI-72 repRNA accumulation in yeast, we prepared a stock solution of Lovastatin (Sigma) according to (Lorenz and Parks, 1990). In our studies, we used 20 μg/ml (final concentration) of the inhibitor or 0.1% of the solvent in mock treated samples. BY4741 yeast was co-transformed with plasmids pGBK-His33 and pGAD-His92, and pYC/DI72 and pre-grown overnight at 29°C with 20

μg/ml of Lovastatin or 0.1% of solvent in synthetic complete dropout medium lacking uracil, leucine and histidine (SC-ULH- medium) containing 2% glucose. TBSV repRNA replication was started by transferring the yeast culture to SC-ULH- media containing 2% galactose along with inhibitor. After 24 h culturing at 29°C, cells were collected and processed for RNA isolation to analyze repRNA accumulation as described below.

In vitro replicase assay.

To obtain the membrane enriched (ME) fraction, containing the assembled replicase complex with the repRNA template, from ERG25/THC strain, yeast transformed with pGAD-His92 and pGBK-His33/DI-72 plasmids was grown for 24 h at 29°C in SC-LH$^-$ medium containing 2% galactose with or without doxycycline (10 mg/L) until reaching ~0.8 to 1.0 OD_{600}. To collect the ME fraction from the APB treated yeast, BY4741 strain was transformed with plasmids pGBK-His33/CUP1 and pGAD-His92/CUP1 expressing 6xHis-tagged CNV p33 and 6xHis-tagged p92, respectively, from the inducible *CUP1* promoter, as well as pCM189tetDI72 expressing DI-72 repRNA from the doxycycline-repressible *TET* promoter. Briefly, yeast cells BY4741 were grown overnight with APB or without APB in SC-ULH$^-$ medium containing 2% glucose at 29°C. Then, repRNA replication was induced with 50 μM copper sulfate for 24 h at 23 °C with shaking at 250 rpm. After 24 h growth, yeast samples were collected at ~0.8 OD_{600} by centrifugation at 1,100 x g for 5 min. The procedure used to obtain the ME fractions from the above strains was described earlier (Jaag et al., 2007; Panaviene et al., 2005; Panaviene et al., 2004). Briefly, the yeast pellet was washed with 20 mM Tris–HCl, pH 8.0 and resuspended in 1 ml of 20 mM Tris–HCl, pH 8.0, followed by centrifugation at 21,000 x g for 1 min. Yeast cells were broken by glass beads in a

Genogrinder (Glen Mills Inc., Clifton NJ) for 2 min at 1,500 rpm. After mixing with 600 μl chilled extraction buffer (200 mM sorbitol, 50 mM Tris–HCl [pH 7.5], 15 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mix; Sigma), the samples were centrifuged at 100 x g for 5 min at 4°C. The supernatant was moved to a new microcentrifuge tube and centrifuged at 21,000 x 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, 0.2 μl RNase inhibitor, 1 mM ATP, CTP, GTP, 0.3 μl radioactive ^{32}P-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) and 100 μl phenol-chloroform (1:1). After isopropanol precipitation of the RNA products, the RNA samples were electrophoresed under denaturing conditions (5% PAGE containing 8 M urea) and analyzed by phosphoimaging using a Typhoon (GE) instrument as described (Jaag et al., 2007; Panaviene et al., 2005; Panaviene et al., 2004).

Northern blot and Western blot analyses.

Total RNA isolation from yeast and Northern blot analyses of the accumulation of TBSV repRNA were performed as described previously (Panavas and Nagy, 2003; Panaviene et al., 2004). Western blotting for measuring p33/p92 levels was performed using anti-His antibody whereas the secondary antibody was alkaline phosphatase conjugated anti-mouse immunoglobulin G (Sigma), as described previously (Serva and Nagy, 2006).

To test the level of ERG25 mRNA expression, ERG25/THC yeast strain was grown for 12 hrs in YPD media at 29°C with shaking at 250 rpm. Doxycycline was added and samples were collected at 0, 5, 11, 24 hrs time points. After the cells were pelleted, total RNA was extracted by using a modified hot-phenol method (see above). For the Northern blot analysis, the total RNA samples were diluted 100 times (except for the detection of *ERG25* mRNA, for which undiluted samples were used) before electrophoresis, followed by transfer of RNA to membranes. The ^{32}P-labeled RNA probes were prepared by in vitro transcription with T7 RNA polymerase from appropriate PCR products. The PCR product used to transcribe the labeled 18S rRNA probe was amplified from the yeast genome with primers #1251 (GGTGGAGTGATTTGTCTGCTT) and #1252 (TAATACGACTCACTATAGGTTTGTCCAAATTCTCCGCTCT). The template for the probe to detect *ERG25* mRNA was obtained by PCR with primers #2793 (GCCGGATCCATGTCTGCCGTTTTCAACAAC) and #2794 (GTAATACGAGTCACTATAGGGAGATAGAAGAACGGATTTCAAAC) using yeast genomic DNA as template.

Measuring p33/p92 stability in yeast treated with APB.

Yeast strain BY4741 was transformed with either pGBK-His33/CUP1 or pGAD-His92/CUP1 expressing 6xHis-tagged CNV p33 and 6xHis-tagged p92, respectively, from the inducible *CUP1* promoter. Yeast transformants were cultured overnight in SC H^- media (or SC L^- media for p92 expression) containing 2% glucose with or without 30 μM APB at 29 °C. To induce the expression of p33 or p92, 50 μM CuSO4 was added to the yeast cultures for 30 min at 29 °C, followed by the addition of cycloheximide to a

final concentration of 100 μg/ml to inhibit protein synthesis. Equal amounts of yeast cells were collected at given time points as indicated in figure legends after cycloheximide treatment (Li et al., 2009b), and cell lysates were prepared by NaOH method as described previously (Panavas and Nagy, 2003). The total protein samples were analyzed by SDS/PAGE and Western blotting with anti-His antibody using ECL (Amersham) as described (Jaag et al., 2007)

In vitro translation assay.

The in vitro translation reactions were based on a wheat germ extract containing of 1 mM amino acid mixture without methionine (Promega) and ^{35}S methionine (10 mCi/ml). We added 0.3 picomoles of p33 or p92 mRNAs to program the assay [modified from (Li et al., 2009b)], while APB was added at 30 μM final concentration. After 1 h incubation at room temperature, samples were mixed with SDS-PAGE loading dye and incubated at 100 °C for 2 min and electrophoresed in an SDS-PAGE gel followed by phosphoimaging as described (Li et al., 2009b).

Plant and protoplast experiments.

Preparation of *Nicotiana benthamiana* protoplasts, electroporation with TBSV and CNV RNA as well as viral RNA analysis were performed as described previously (Panaviene et al., 2003). APB was dissolved in dimethyl sulfoxide (DMSO) and was added at a concentration of 60 μM (or as indicated in the figure legend) before or after electroporation to the *N. benthamiana* protoplasts. Campesterol (4 mg/ml) and stigmasterol (4 mg/ml) (from Steraloids Inc, Newport, RI) were prepared using a 1:1 mixture of DMSO and 95% ethanol and then added to protoplast preparations at 40 μg/ml just before electroporation.

APB (1,500 μM) or DMSO (1.5% as a control) was used for infiltration into *N. benthamiana* leaves, followed by inoculation of the same leaves with inoculum containing TBSV, CNV or TMV. Total RNA extraction from the inoculated leaves was done at 4 dpi (Cheng et al., 2007).

VIGS constructs.

pTRV1 and pTRV2 plasmids to launch *Tobacco rattle virus* (TRV) infection through agroinfiltration were kindly provided by Dinesh-Kumar (Dinesh-Kumar et al., 2003). To amplify partial *SMO1/2* cDNA fragments from *N. benthamiana,* primers were designed according to consensus regions of aligned *SMO1/2* sequences (Darnet and Rahier, 2004). To generate plasmid pTRV2-SMO1, a 387 bp fragment of *NbSMO1* was amplified by RT-PCR with primers #2901 (GGCGGAATTCACAAGTTTGCCCCTGCCGTC) and #2902 (GGCGCTCGAGAACATAGTGATGGTAGTCATGGTAATC) (Darnet and Rahier, 2004) using total RNA extract obtained from uninfected *N. benthamiana* leaves. The obtained RT-PCR product was treated with *EcoR*I *and Xho1* and then was cloned at the *EcoR*I *and Xho1* sites of pTRV2 vector. Construct pTRV2-SMO2 carrying a 450 bp of *NbSMO2* sequence was generated as described for pTRV2-SMO1 except using primers #2903 (GGCGGAATTCATGGCTTCCATGATCGAATCTGCTTGG) and #2904 (GGCGCTCGAGGACAAGAAAAAGAATTTCAGCAGGGTGAGC) (Darnet and Rahier, 2004).

Virus-induced gene silencing (VIGS).

The VIGS assay to silence *SMO1/2* in *N. benthamiana* was performed as described previously (Wang et al., 2009). Eleven days after agroinfiltration of pTRV2-

SMO1 and pTRV2-SMO2, the accumulation levels for *NbSMO1/2* mRNAs in *N. benthamiana* were determined by semi-quantitative RT-PCR with primers #3273 (CCTAATCTTCTCTTGTGTCCCTC) and #2902 and primer pair of #3274 (CAATTGACTTGTCTTGGTGGGTTT) and #3275 (CAAGGACTCTAAGTGAGACCC), respectively (Darnet and Rahier, 2004). The accumulation level of the control tubulin mRNA was measured by semi-quantitative RT-PCR using primers #2859 (TAATACGACTCACTATAGGAACCAAATCATTCATGTTGCTCTC) and #2860 (TAGTGTATGTGATATCCCACCAA). The obtained RT-PCR products were sequenced to confirm their identities. The VIGS silenced leaves were sap inoculated with TBSV or TMV as described (Wang et al., 2009). Total RNA extracts from the infected leaves were prepared at 3 dpi (day post-inoculation) and from systemically infected leaves at 5 dpi to analyze TBSV and TRV accumulation.

RESULTS

Down regulation of ERG25 mRNA level in yeast reduces TBSV RNA accumulation and inhibits the replicase activity in vitro.

To confirm the role of *ERG25* in TBSV replication, we used ERG25/THC (*TET::ERG25*) strain from the yTHC collection (Open Biosystems). The expression of the essential *ERG25* gene is under the control of a doxycycline titratable promoter in the yeast genome in the ERG25/THC strain. Therefore, the expression of the *ERG25* gene can be down regulated/turned off by the addition of doxycycline to the yeast growth medium (Mnaimneh et al., 2004). This approach allowed us to test tombusvirus RNA

replication in the presence of high level of Erg25p (when yeast was grown without added doxycycline after the induction of tombusvirus repRNA replication) or various reduced level of Erg25p (when yeast was grown in the presence of doxycycline) (Mnaimneh et al., 2004).

To test the replication of the TBSV replicon (rep)RNA, which is an efficiently replicating surrogate RNA template derived from the TBSV genomic (g)RNA (Panavas and Nagy, 2003; White and Morris, 1994), we expressed the p33 and $p92^{pol}$ replication proteins and DI-72 repRNA from plasmids in ERG25/THC yeast strain. These experiments revealed efficient replication of DI-72 repRNA in ERG25/THC strain grown under standard growth conditions without added doxycycline (see Materials and Methods) (Fig. 2.1A, lanes 1 and 3). Addition of doxycycline to the growth media led to rapid decrease in *ERG25* mRNA levels at the beginning of repRNA replication (5 hour time point, Fig. 2.1B, lane 5). The level of *ERG25* mRNA was undetectable with Northern blotting 11 hours after the addition of doxycycline (Fig. 2.1B, lane 6) as well as at the 24-hour time point (Fig. 2.1B, lane 7), when the samples for TBSV repRNA analysis were collected (Fig. 2.1A). The accumulation level of DI-72 repRNA was ~3-fold lower in ERG25/THC yeast lacking detectable level of *ERG25* mRNA (Fig. 2.1A, lane 4), suggesting that high expression level of *ERG25* promotes TBSV repRNA replication in yeast.

To test if Erg25p affected the tombusvirus replicase activity, we generated enriched tombusvirus replicase preparations containing the co-purified repRNAs form ERG25/THC yeast grown in the absence or presence of doxycycline. Using comparable amounts of p33 replication protein during the assay (Fig. 2.1C, lower panel), we found

that the tombusvirus replicase preparation obtained from yeast with the undetectable level of *ERG25* mRNA was ~5-fold less active than the comparable replicase preparation obtained from yeast grown in the absence of doxycycline (Fig. 2.1C, lanes 5-8 versus 1-4). These experiments demonstrated that expression of Erg25p in yeast is important for the activity of the tombusvirus replicase.

Inhibition of Erg25p by APB chemical inhibitor in yeast reduces TBSV RNA accumulation.

To further test the role of *ERG25* in TBSV replication, we took advantage of a potent chemical inhibitor, namely 6-amino-2-n-pentylthiobenzothiazole (APB). APB has been shown to bind to Erg25p and inhibits its catalytic function in a competitive manner (Darnet and Rahier, 2003; Kuchta et al., 1995). Treatment of yeast with APB inhibited the biosynthesis of ergosterol and led to the accumulation of the methylated sterol precursors, such as ergosta-5,7-dienol and squalene, but had no significant effect on the composition and the rate of biosynthesis of fatty acids (Kuchta et al., 1997). We found that applying increasing amounts of APB to the growth media affected both yeast growth and TBSV accumulation (Fig. 2.2A). Normalization of TBSV repRNA accumulation based on yeast rRNA level indicated that APB, when applied between 20-40 mM concentration, had inhibited TBSV repRNA accumulation 4-to-5-times more than it inhibited yeast growth (Fig. 2.2A). This finding was confirmed in both ERG25/THC (TET::ERG25) (Fig. 2.2A) and BY4741 (wt) yeast strains (Fig. 2.2B).

TBSV replicates on the peroxisomal membrane surface in yeast and in plant cells [(Mccartney et al., 2005) and (Pathak et al., 2008)]. However, TBSV replication can shift

to the ER membrane in the absence of peroxisome, which results in as efficient replication as that occurring on the peroxisomal membrane surface (Jonczyk et al., 2007; Pathak et al., 2008). To test if the APB-driven inhibition of sterol biosynthesis could also reduce TBSV replication occurring on the ER membrane, we used *pex19D* yeast strain, which lacks peroxisomes (Jonczyk et al., 2007; Pathak et al., 2008). The accumulation of the TBSV repRNA decreased to ~20% in 30 mM APB-treated *pex19D* yeast strain, a level of inhibition comparable to that in the wt BY4741 strain (Fig. 2.2B, lanes 10-12 versus 4-6). Therefore, TBSV replication seems to be equally dependent on sterol biosynthesis when takes place on the peroxisomal membrane surface as on the ER membrane.

Since down-regulation of *ERG25* mRNA level (Fig. 2.1) or the use of APB inhibitor of Erg25p is known to lead to the accumulation of methylated sterol precursors, it is possible that these compounds directly inhibit TBSV repRNA replication in stead of the lack of sterols in the above yeast cells resulting in reduction of viral replication. To test this possibility, we also applied lovastatin, which is a potent inhibitor of hydroxymethylglutaryl-CoA (HMG-CoA) reductase (coded by the *HMG1* and *HMG2* genes in yeast), a rate-limiting enzyme in the mevalonate pathway that regulates cholesterol synthesis. Lovastatin acts as a competitive inhibitor of HMG-CoA reductase, effectively lowering sterol levels in yeast (Daum et al., 1998; Lorenz and Parks, 1990). Application of lovastatin reduced TBSV repRNA accumulation in yeast by four-fold (Fig. 2.2C, lanes 5-8) without affecting p33 level. The strong inhibitory effect of lovastatin on TBSV replication is not compatible with the model that the accumulation of

methylated sterol precursors in yeast with down-regulated *ERG25* mRNA level is responsible for direct inhibition of TBSV repRNA replication.

Inhibition of sterol biosynthesis in yeast at an early time point of TBSV replication is the most detrimental to TBSV RNA accumulation.

Previous EM images indicated that tombusvirus replication likely utilize the preexisting peroxisomal membrane surfaces in the infected cells at the beginning of infection, followed by induction of new membranes and possibly utilizing other than peroxisomal membranes at late time points of infection (Navarro et al., 2006). To test when TBSV is the most sensitive to inhibition of sterol biosynthesis, we devised a scheme for time-restricted inhibition of sterol biosynthesis via treatment with APB, followed by removing the inhibitor at given time points (see Fig. 2.3A for the experimental scheme).

Surprisingly, inhibition of sterol biosynthesis only during the period of TBSV replication was as effective as inhibition of sterol biosynthesis prior to and during TBSV replication (treatment #2 versus #3 in Fig. 2.3B-C, lanes 5-8 versus 9-12). Also, treatment of yeast cells with APB inhibitor only prior to the beginning of TBSV replication had limited inhibitory effect on TBSV accumulation (treatment #4, Fig. 2.3B-C, lanes 13-16). These data suggest that the pre-existing sterol level might not be as critical as the newly synthesized sterols during TBSV replication.

To further define when sterol biosynthesis is the most critical during TBSV replication, we used shorter treatments with APB. These experiments revealed that sterol biosynthesis is the most critical during the beginning of TBSV replication (between 1-12 hours, treatment #5, Fig. 2.3B-C, lanes 17-20) and less effective at the late time point

(between 12-24 hours, treatment #6, Fig. 2.3B-C, lanes 21-24). These data indicate that TBSV replication requires new sterol biosynthesis the most at an early stage of infection.

Inhibition of sterol biosynthesis by APB inhibits the replicase activity in vitro and reduces the half-life for p92pol in yeast.

In the experiments shown in Fig. 2.1., we have expressed the p33 and $p92^{pol}$ replication proteins from the constitutive *ADH* promoter, which could potentially allow some limited assembly of the replicase complex prior to down-regulation of *ERG25* level. Therefore, we have retested the replicase activity when the p33 and $p92^{pol}$ replication proteins were expressed from the inducible *CUP1* promoter to allow the replicase assembly to take place only when ergosterol biosynthesis was inhibited (Fig. 2.4). Testing the activity of tombusvirus replicase preparations obtained from APB-treated ERG25/THC yeast grown in the absence of doxycycline revealed that 40 mM concentration of APB inhibited the replicase activity by ~90% at a late time point (24 hour) when we adjusted the preparations to contain comparable amounts of p33 replication protein (Fig. 2.4A, top panel, lanes 4-6 versus 1-3). Interestingly, the amount of $p92^{pol}$ was lower in the replicase samples obtained from the APB-treated than from the untreated yeast (Fig. 2.4A, middle panel, lanes 4-6 versus 1-3). These experiments demonstrated that APB treatment of yeast could inhibit the in vitro activity of the tombusvirus replicase and decrease the level of $p92^{pol}$.

To test the stability of p33 and $p92^{pol}$ replication proteins when sterol biosynthesis is inhibited, we treated yeast cells first with APB and, then with cyclohexamide (to inhibit new protein synthesis), followed by measuring protein levels (Fig. 2.4B-C). We found that the half-life of p33 did not change significantly (Fig. 2.4C), while that of

$p92^{pol}$ was reduced by ~3-fold in APB-treated yeast in comparison with the DMSO-treated control yeast (based on reduction of the half-life of p92 from ~100 min to ~30 min, Fig. 2.4B). Interestingly, APB treatment did not affect translation of p33 or $p92^{pol}$ in vitro (Fig. 2.4D), suggesting that degradation of $p92^{pol}$ might be accelerated in APB-treated yeast, leading to reduced stability selectively for $p92^{pol}$ but not for p33.

Inhibition of sterol biosynthesis in plant protoplasts reduces TBSV RNA accumulation.

The above experiments demonstrated that sterol level is important for TBSV repRNA accumulation in yeast cells and in vitro. To test if sterol biosynthesis is also important for TBSV RNA accumulation in plant cells, we inhibited sterol biosynthesis by APB treatment based on the conserved function of Erg25p protein in yeast and Smo1/2 proteins in plants (Darnet and Rahier, 2003, 2004). We found that 40 mM APB concentration applied either before (Fig. 2.5A, lanes 2 and 5) or after electroporation (not shown) of TBSV genomic (g)RNA into *N. benthamiana* protoplasts inhibited TBSV gRNA as well as subgenomic (sg)RNA1 and sgRNA2 accumulation by ~3-fold. Time-course experiments showed that treatment with APB that started at the 0 time point (Fig. 2.5B, lanes 6-7) was more effective than treatments starting from 3 or 6 hour-post-electroporation time points. These experiments indicated that sterol biosynthesis is also important in plant cells for TBSV RNA accumulation, especially at the early time point.

Stigmasterol complements TBSV accumulation in plant protoplasts treated with APB.

To test if the negative effect on TBSV RNA accumulation by the APB-mediated inhibition of sterol biosynthesis could be complemented by addition of phytosterols to the growth media of plant protoplasts, we used stigmasterol (the major phytosterol in plant cell membranes) and campesterol in various concentrations prior to or after

electroporation of the TBSV gRNA. Interestingly, we found that 20 mM stigmasterol (Fig. 2.6A, lane 9) or 10 mM stigmasterol (not shown) applied in combination with 60 mM APB before electroporation of TBSV gRNA into *N. benthamiana* protoplasts increased TBSV gRNA accumulation from 27% (APB treatment, Fig. 2.6A, lanes 5-6) to 91% (20 mM stigmasterol treatment, lane 9) and 75% (10 mM stigmasterol treatment, not shown). The complementation was less pronounced with 40 mM campesterol (Fig. 2.6B, lane 10), which resulted in 52% TBSV gRNA accumulation from 25% (APB treatment, Fig. 2.6B, lanes 5-6) in *N. benthamiana* protoplasts. Overall, these experiments demonstrated that phytosterols, especially stigmasterol, could complement the inhibitory effect of APB treatment on TBSV gRNA replication in plant protoplasts.

Inhibition of sterol biosynthesis by APB reduces TBSV RNA accumulation in N. benthamiana plants.

To demonstrate that inhibition of sterol biosynthesis is important for TBSV RNA accumulation in plants, we infiltrated leaves of *N. benthamiana* with 60-to-1,500 mM of APB prior to inoculation with infectious TBSV virion preparations. Isolation of total RNA from the inoculated leaves 4 days after inoculation, followed by Northern blotting, revealed that APB treatment, when applied in 1,500 mM concentration, inhibited TBSV RNA accumulation by ~90% (Fig. 2.7A, lanes 11-17). Similar treatment with APB of *N. benthamiana* prior to inoculation with infectious CNV (a close relative of TBSV) virion preparations reduced CNV gRNA accumulation below detection limit at 4 dpi (Fig. 2.7C, lanes 10-21). Similar to TBSV, the CNV infected and APB treated plants showed a delay in symptom development in systemically-infected leaves when compared to the DMSO-treated and CNV or TBSV-inoculated plants (Fig. 2.7B, D). In contrast, APB treated

leaves supported TMV (an unrelated plus-strand RNA virus of the alphavirus supergroup) RNA accumulation almost as efficiently as the DMSO-treated leaves (Fig. 2.7E). As expected, there was no delay in symptom appearance in APB- or DMSO-treated plants infected with TMV (Fig. 2.7F). The APB treatment had no obvious effect on the leaves or the whole plants (panel F in Fig. 2.7). These experiments demonstrated that APB-treatment could lead to significant reduction of TBSV and CNV RNA accumulation in the treated leaves and delay in symptom development, whereas similar treatments had no significant effect on TMV RNA accumulation and did not delay symptom development in TMV-infected plants. Thus, the requirement for sterol biosynthesis seems to be different for tombusviruses and TMV.

Inhibition of sterol biosynthesis by silencing of SMO1 and SMO2 genes in N. benthamiana plants reduces TBSV RNA accumulation.

The two orthologs of yeast *ERG25* gene in plants are the *SMO1* and *SMO2* genes, which are 4alpha-methyl oxidases involved in phytosterol biosynthesis (Darnet and Rahier, 2004). The *SMO1* and *SMO2* genes are involved in different steps of phytosterol biosynthesis and they only show ~50% sequence identity, allowing for separate silencing of these genes (Darnet and Rahier, 2004). Accordingly, we silenced individually or in combination the expression of *SMO1* and *SMO2* genes, by using a VIGS strategy in *N. benthamiana*. Indeed, RT-PCR analysis has shown decreased levels of *SMO1* and *SMO2* mRNAs 11 days after infiltration of *Agrobacterium* carrying the VIGS constructs (Fig. 2.8G, lanes 4-6; and lanes 10-12). Inoculation of the *SMO1* and *SMO2* silenced leaves with TBSV led to ~3-fold reduced TBSV gRNA accumulation in the inoculated leaves

when compared with the nonsilenced leaves (plants infiltrated with *Agrobacterium* carrying the pTRV empty vector) (Fig. 2.8A, lanes 8-14 versus 1-7; and C, lanes 8-14 versus lanes1-7). Silencing of both *SMO1* and *SMO2* mRNAs led to even further reduction in TBSV RNA accumulation in the inoculated leaves that reached only ~10% of the TBSV RNA in the nonsilenced plants (Fig. 2.8E, lanes 8-14 versus 1-7). The development of the TBSV symptoms in *SMO1*, *SMO2* and *SMO1*/*SMO2* silenced plants was significantly delayed when compared with the pTRV-treated plants (Fig. 2.8B, D and F). However, silencing of the *SMO1*, *SMO2* and *SMO1*/*SMO2* mRNAs did not lead to complete protection of the plants from TBSV infections, as shown by the appearance of systemic symptoms (Fig. 2.8B, D and F) and the accumulation of TBSV gRNA in systemically-infected upper leaves (not shown).

On the contrary to the above results, the *SMO1* and *SMO2* silenced leaves accumulated TMV RNAs almost as efficiently in the inoculated leaves as in the nonsilenced leaves (~60-74%; Fig. 2.8I). Also, symptom development was comparable in the silenced versus nonsilenced *N. benthamiana* plants inoculated with TMV (Fig. 2.8J, L). The systemically-infected leaves supported TMV RNA accumulation in the silenced plants as efficiently as in the nonsilenced plants (Fig. 2.8K). Co-silencing of both *SMO1* and *SMO2* genes in *N. benthamiana* plants also had no detectable effect on TMV accumulation (Fig. 2.8M, N and O). These results show that inhibition of sterol biosynthesis by silencing of *SMO1* and *SMO2* genes reduces specifically tombusvirus replication, but the effect on TMV accumulation is weaker in the silenced plants. In addition, the above results make it unlikely that the *SMO1* and *SMO2* silenced plants inhibit tombusvirus replication due to nonspecific effects (such as "sick plant phenotype-

based general inhibition of virus accumulation"), since these plants are compatible for supporting TMV replication. Moreover, it seems that various plant viruses show different levels of dependence on sterol biosynthesis for their replication.

DISCUSSION

Since the replicase complexes of (+)RNA viruses are membrane bound, the lipid composition of membranes influencing membrane fluidity, rigidity and permeability is expected to affect the activity of the viral replicase. Accordingly, we demonstrate that down regulation of Erg25p, a critical enzyme in the sterol biosynthesis pathway (Bard et al., 1996), or inhibition of the activity of Erg25p by APB (Darnet and Rahier, 2003) reduced TBSV repRNA replication in yeast by 3-to-5-fold. Moreover, inhibition of sterol biosynthesis by Lovastatin also resulted in 4-fold reduction in TBSV repRNA replication in yeast. In addition, a previous genome-wide screen for the identification of host factors for TBSV revealed reduced TBSV repRNA accumulation in *erg4D* yeast (Panavas et al., 2005b), suggesting that the sterol biosynthesis pathway is required for TBSV replication.

TBSV replication is dependent on newly synthesized sterols.

The reduced level of sterols might have direct inhibitory effect on the tombusvirus replicase activity, since the isolated replicase complex with the co-purified repRNA showed ~5-fold reduced activity in vitro when obtained from yeast with down regulated Erg25p or treated with APB inhibitor. Interestingly, similar level of inhibition of TBSV RNA accumulation by APB treatment was observed in yeast lacking peroxisomal membranes, in which TBSV replication occurs on the ER membrane (Fig. 2.2B). These

data suggest that TBSV replication is greatly affected by sterols in yeast regardless of the subcellular location of the replicase complexes.

Pre-treatment of yeast cells with APB had only minor inhibitory effect on TBSV repRNA replication (Fig. 2.3B-C, treatment #4), while APB treatment after the induction of TBSV replication had larger inhibitory effect, especially when applied in the first 12 hours (Fig. 2.3B-C, treatment #5). These data suggest that TBSV replication mostly depends on the newly synthesized sterols in yeast, while inhibition of sterol biosynthesis prior to TBSV replication to reduce the level of pre-existing sterols in the cellular membranes had only minor inhibitory effect. Similarly, we found that APB-treatment was the most effective in *N. benthamiana* protoplasts when applied from the beginning of TBSV RNA replication (Fig. 2.5). Based on these data, it is possible that sterols regulate TBSV replication by not only affecting the structure and features of the subcellular membranes supporting TBSV replication, but also playing additional functions during TBSV replication.

Although the functions of sterols during TBSV replication are not yet known, it seems that sterols are needed for the stability of $p92^{pol}$ in yeast (Fig. 2.4). It is possible that the bulky $p92^{pol}$ replicase protein might be exposed more to cytosolic proteases in sterol-poor micro-environment or the structure of $p92^{pol}$ is different under sterol-depleted condition, leading to premature degradation of $p92^{pol}$. It is also possible that subcellular localization of $p92^{pol}$ could be different if less than normal level of sterols was available in cells.

Inhibition of sterol biosynthesis has similar effect on TBSV replication in plants and yeast.

Although the sterols synthesized in yeast (ergosterol) and in plants (phytosterols, among sterols stigmasterol is the most abundant) are different, they might play comparable roles in TBSV replication. Accordingly, down regulation of *ERG25* expression in yeast or silencing the orthologous *SMO1/SMO2* genes in *N. benthamiana* or APB treatment of yeast and plant cells had comparable inhibitory effects on TBSV RNA accumulation. Also, the negative effect of the APB treatment on TBSV RNA accumulation could be complemented in plant protoplasts by exogenous stigmasterol, strongly suggesting that sterols are the active compounds that affect TBSV RNA replication. It is intriguing that TBSV replication can take advantage of different sterols in yeast and in plants, suggesting high flexibility for TBSV in different subcellular environments.

Interestingly, sterols seem to be needed for tombusvirus replication, but less critical for TMV replication based on silencing *SMO1/SMO2* genes in *N. benthamiana* or the APB treatment of plant leaves. This different effect could be due to: (i) different subcellular compartments (tonoplast/vacuole and peroxisome, respectively) where TMV and TBSV replicate; and (ii) different features of the replication proteins or their abilities to bind to sterols. For example, the TMV replication proteins are likely peripheral membrane proteins (Hagiwara et al., 2003; Komoda et al., 2007), while TBSV replicaton proteins are integral membrane proteins (McCartney et al., 2005; Panavas et al., 2005a).

Similar to tombusviruses, replication of other viruses, such as Dengue virus, Norwalk virus and hepatitis C virus (HCV), also depends on sterols (Chang, 2009;

Kapadia et al., 2007; Rothwell et al., 2009; Sagan et al., 2006). It has been shown that the HCV replicase complex is associated with cholesterol-rich lipid rafts (Aizaki et al., 2004). Infection with West Nile virus has been demonstrated to lead to redistribution of cholesterol to the sites of virus replication, possibly from the plasma membrane, and result in reduced antiviral responses (Mackenzie et al., 2007). Cholesterol is also important for animal virus entry to cells, infections and the exit of virus particles from cells (Lee et al., 2008; Marquardt et al., 1993; Phalen and Kielian, 1991; Simon et al., 2009). These findings invite further studies on dissecting the functional and mechanistic roles of sterols during virus infections. This could then lead to development of novel, broad range antiviral strategies in animals and plants.

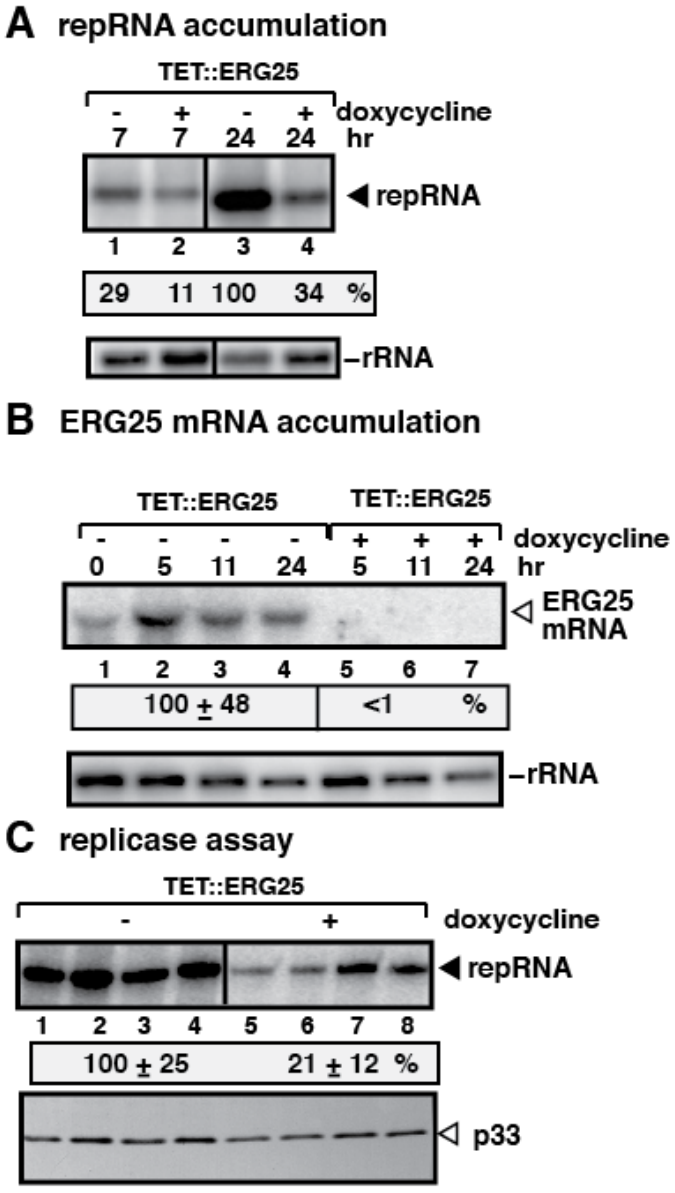
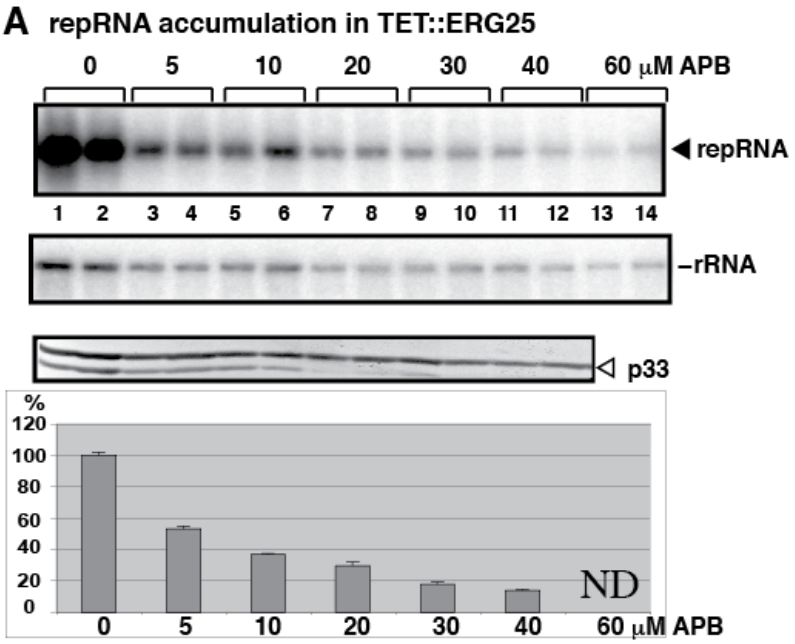
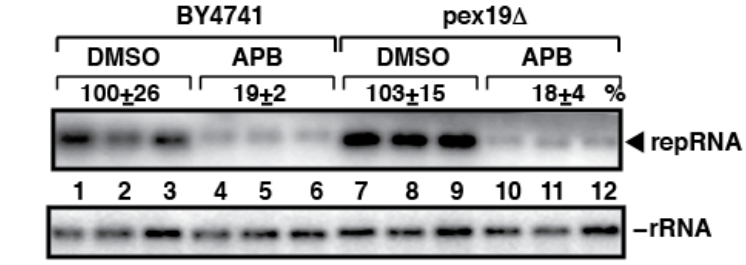

A repRNA accumulation
TET::ERG25
- + - + doxycycline
7 7 24 24 hr
repRNA
1 2 3 4
29 11 100 34 %
rRNA
B ERG25 mRNA accumulation
TET::ERG25
TET::ERG25
- - - - + + + doxycycline
0 5 11 24 5 11 24 hr
ERG25 mRNA
1 2 3 4 5 6 7
100 ± 48
<1 %
rRNA
C replicase assay
TET::ERG25
- + doxycycline
repRNA
1 2 3 4 5 6 7 8
100 ± 25
21 ± 12 %
p33

Figure 2.1 Down regulation of *ERG25* expression reduces TBSV repRNA accumulation in yeast

(A) Northern blot analysis with a 3' end specific probe was used to detect the accumulation level of the TBSV repRNA. *ERG25* expression was down regulated by doxycycline 12 hours prior to expressing the TBSV-derived DI-72 repRNA in ERG25/THC yeast, which contains a doxycycline regulatable promoter replacing the native *ERG25* promoter. To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the *ADH1* promoter and DI-72(+) repRNA from the galactose-inducible *GAL1* promoter. After pre-growing in the presence of doxycycline, yeast cells were cultured for additional 24 hours at 29ºC on 2% galactose SC minimal media containing doxycycline (as indicated by a "+" sign). The yeast was collected for total RNA extraction at the indicated time points. The accumulation levels of repRNA were calculated using Imagequant software. rRNA was used as a loading control (panel at the bottom). (B) Northern blot analysis to estimate the level of *ERG25* mRNA in yeast grown in the absence/presence of doxycycline. ND means non-detectable signal (less than 1%). (C) Decreased replicase activity in the presence of low Erg25p level. A replicase activity assay with membrane-enriched preparations obtained from yeast expressing high or low levels of Erg25p, based on the addition of 10 mg/L doxycycline to the growth media 12 hours prior to launching TBSV repRNA replication. The yeast samples were taken 24 hours after the induction of TBSV replication. The membrane-enriched fraction contains the endogenous repRNA template that is used during the in vitro replicase assay in the presence of ^{32}P-UTP and the other unlabeled rNTPs. Note that the in vitro activities of the tombusviral replicase were normalized based on p33 levels.


A repRNA accumulation in TET::ERG25
0 5 10 20 30 40 60 μM APB
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
–rRNA
p33
%
120
100
80
60
40
20
0
ND
0 5 10 20 30 40 60 μM APB


B repRNA accumulation on the ER
BY4741
pex19Δ
DMSO
APB
DMSO
APB
100±26
19±2
103±15
18±4 %
repRNA
1 2 3 4 5 6 7 8 9 10 11 12
–rRNA

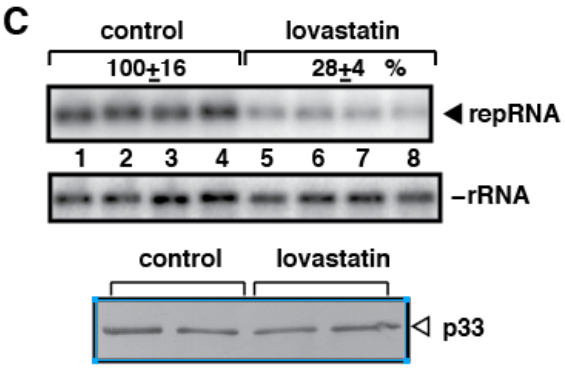

C
control
lovastatin
100±16
28±4 %
repRNA
1 2 3 4 5 6 7 8
–rRNA
control
lovastatin
p33

Figure 2.2 Inhibition of TBSV repRNA accumulation in yeast treated with APB.

(A) Yeast was treated with APB (0-to-60 mM, as shown), and TBSV repRNA replication was launched as described in Fig. 2.1 legend. Samples for viral RNA analysis were taken at 24 hours after the induction of TBSV replication. Northern blotting (top panel) shows the level of TBSV repRNA accumulation in individual samples using a 3' end specific probe. The middle panel shows a Northern blot indicating the level of 18S rRNA. Each experiment was repeated three times. Yeast treated with DMSO (the lanes marked by "0") was chosen as 100%. The bottom panel shows a Western blot for 6xHis-tagged p33 level in the samples. The graph shows the accumulation level of DI-72(+) repRNA in percentage, which was normalized based on 18S rRNA. Note that the APB treatment at the highest concentration inhibited yeast growth, so we did not quantify TBSV repRNA accumulation for this treatment. (B) Northern blot showing the accumulation level of DI-72 repRNA in yeast lacking peroxisome (*pex19D*) and wt yeast (BY4741) treated with 30 mM APB or DMSO as shown. Note that the total RNA samples were loaded based on adjusted 18S rRNA level. See further details in panel A. (C) Inhibitory effect of lovastatin treatment on TBSV repRNA accumulation. Lovastatin was used at 20 mg/ml concentration. See further details in panel A.

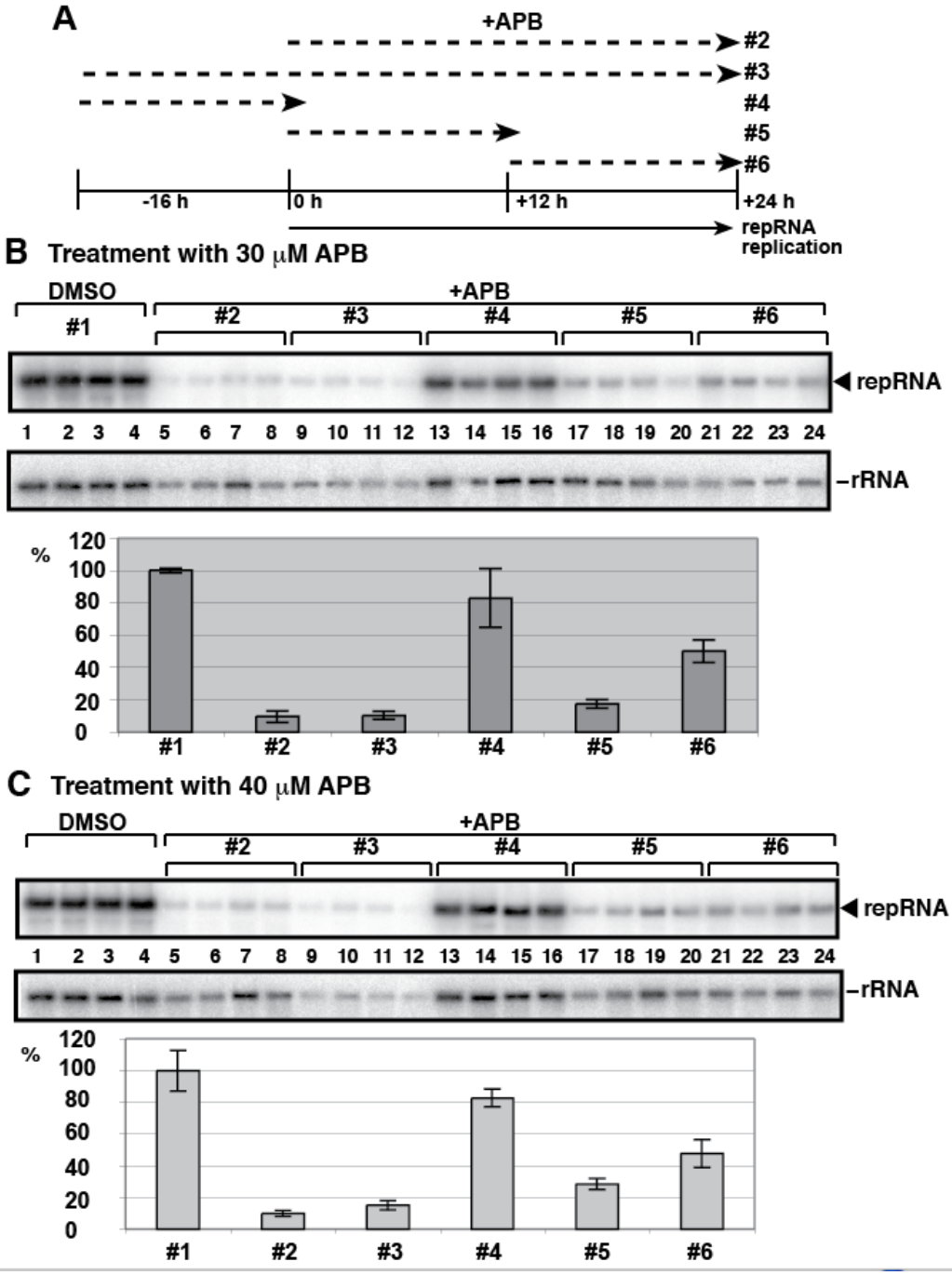
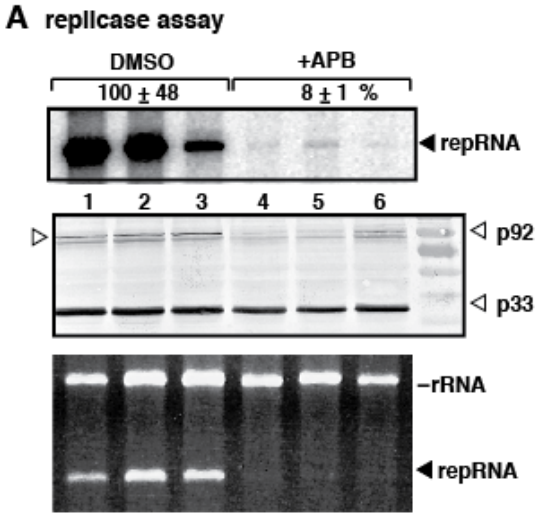
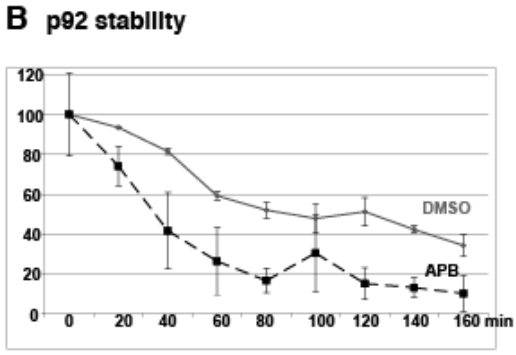
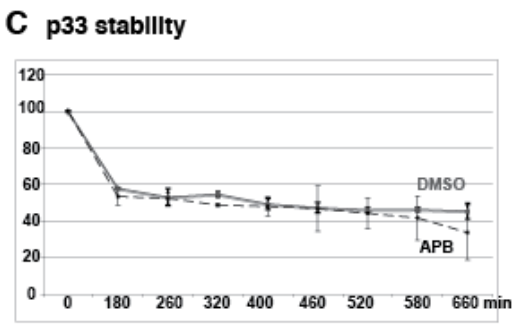

A
+APB
#2
#3
#4
#5
#6
-16 h
0 h
+12 h
+24 h
repRNA replication
B Treatment with 30 μM APB
DMSO
+APB
#1
#2
#3
#4
#5
#6
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
–rRNA
%
120
100
80
60
40
20
0
#1
#2
#3
#4
#5
#6
C Treatment with 40 μM APB
DMSO
+APB
#2
#3
#4
#5
#6
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
–rRNA
%
120
100
80
60
40
20
0
#1
#2
#3
#4
#5
#6

Figure 2.3 APB inhibits TBSV repRNA accumulation most effectively when applied at the beginning of virus replication in yeast.

(A) The scheme of APB treatment relative to initiation of repRNA replication. APB was added/removed to/from the growth media as shown with dotted lines. repRNA replication took place for 24 hours at 29 ºC before RNA analysis. (B) Yeast was treated with 30 mM APB or (C) with 40 mM APB. Northern blotting (top panel) shows the level of TBSV repRNA accumulation. DMSO-treated yeast (-APB, #1) was chosen as 100%. The graph shows the accumulation level of DI-72(+) repRNA in percentage, which was normalized based on 18S rRNA. See other details in Fig. 2.1.


A replicase assay
DMSO
+APB
100 ± 48
8 ± 1 %
repRNA
1 2 3 4 5 6
p92
p33
–rRNA
repRNA


B p92 stability
120
100
80
60
40
20
0
DMSO
APB
0 20 40 60 80 100 120 140 160 min


C p33 stability
120
100
80
60
40
20
0
DMSO
APB
0 180 260 320 400 460 520 580 660 min

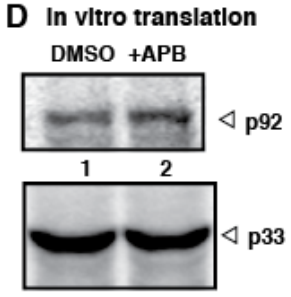

D In vitro translation
DMSO +APB
p92
1 2
p33

Figure 2.4 APB treatment of yeast inhibits the in vitro activity of the tombusvirus replicase and shortens the half-life of $p92^{pol}$ replication protein.

(A) To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the *CUP1* promoter and DI-72(+) repRNA from the *TET* promoter in BY4741 yeast strain. Yeast cells treated with either 30 mM APB or DMSO were cultured for 24 hours at 23ºC on 2% glucose SC minimal media containing 50 mM copper sulfate. Top panel: The replicase activity in the membrane-enriched preparations obtained from yeast was measured in the presence of ^{32}P-UTP and the other unlabeled rNTPs. Note that the in vitro activities of the tombusviral replicase were not normalized to p33 levels in these experiments. Middle panel: Western blotting analysis to show p33/p92 levels in the above replicase preparations from APB- or DMSO treated yeast. Note that the band migrating slightly faster than p92 is caused by heat/SDS-resistant p33 homodimers. Bottom panel: An ethidium-bromide stained gel showing the accumulation level of TBSV repRNA and rRNA in APB- or DMSO treated yeast based on total RNA extracts. (B) Shortened half-life of $p92^{pol}$ due to APB treatment of yeast. Yeast was pre-grown at 29 ºC for 12 hours in SC L^- with 2% glucose and 30 mM APB or DMSO (0.03%), followed by addition of 50 mM copper sulfate for 30 min to induce the expression of $p92^{pol}$. After removal of copper sulfate, 30 mM APB and cyclohexamide (100 mg/ml) were added and samples were collected at the shown time points. The amount of $p92^{pol}$ was estimated via Western blotting based on anti-His antibody and ECL-Plus. The images were analyzed by a phosphorimager and quantitated via Imagequant. The experiments were repeated three times. (C) Estimation of half-life of p33 after APB

treatment of yeast. See details in panel B. (D) Lack of inhibition of in vitro translation of TBSV RNA by APB. The wheat germ translation assay was programmed with 0.3 mg artificial uncapped p92 or p33 mRNA carrying a poly(A) tail in the presence of 30 mM APB or DMSO as a control. The radiolabeled p92 and p33 products were analyzed on SDS-PAGE. The experiment was repeated three times.

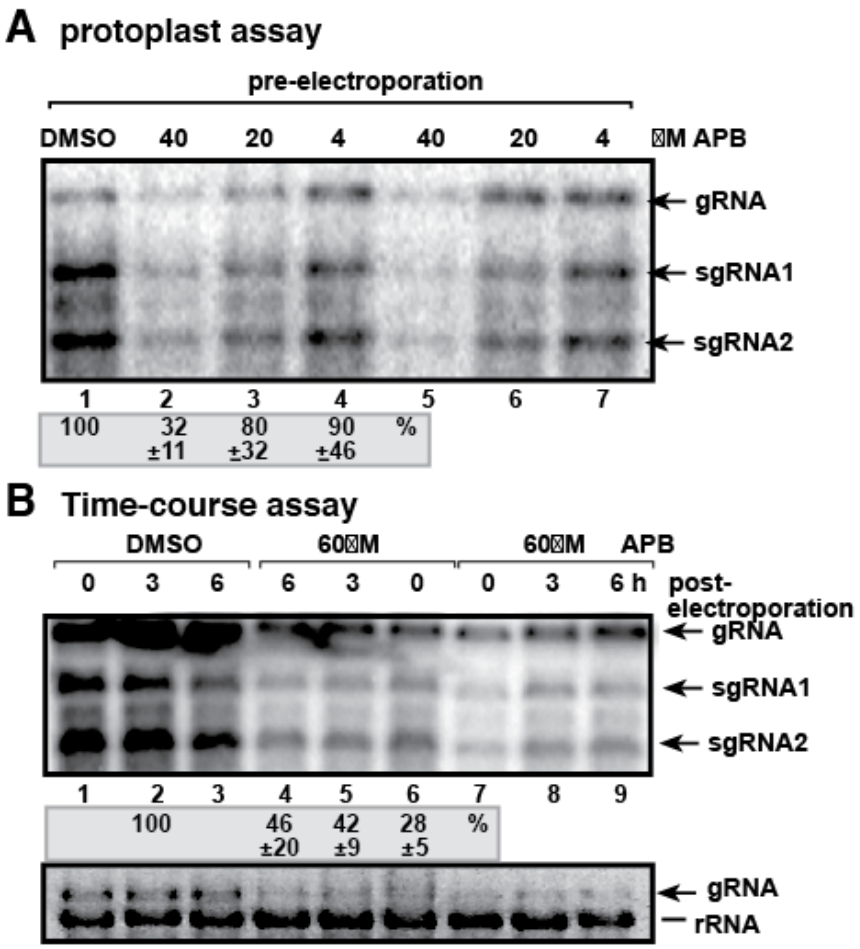

A protoplast assay
pre-electroporation
DMSO 40 20 4 40 20 4 M APB
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7
100 32 ±11 80 ±32 90 ±46 %
B Time-course assay
DMSO 60M 60M APB
0 3 6 6 3 0 0 3 6 h post-electroporation
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7 8 9
100 46 ±20 42 ±9 28 ±5 %
gRNA
rRNA

Figure 2.5 Inhibition of TBSV gRNA accumulation in *N. benthamiana* protoplasts by treatment with APB.

(A) Northern blot analysis was used to detect the accumulation levels of TBSV gRNA and subgenomic (sg)RNAs based on a 3' end specific probe. *N. benthamiana* protoplasts were treated with the shown concentrations of APB before electroporation. The samples were harvested 40 hours after electroporation. The average values of sgRNA2 accumulation for the same treatments are shown under the image. (B) Time course assay to test the effectiveness of APB treatment. *N. benthamiana* protoplasts were treated with APB after electroporation (0, 3 or 6 hours later). DMSO (0.06% solution), the solvent for APB, was used as a control. Northern blot analysis was done as in panel A. The ethidium-bromide stained gel at the bottom shows the ribosomal (r)RNA levels as loading controls. Note that the gRNA can reach rRNA levels in *N. benthamiana* protoplasts. The survival of the plant cells (after electroporation and treatment) was checked by measuring rRNA levels in total RNA extracts.

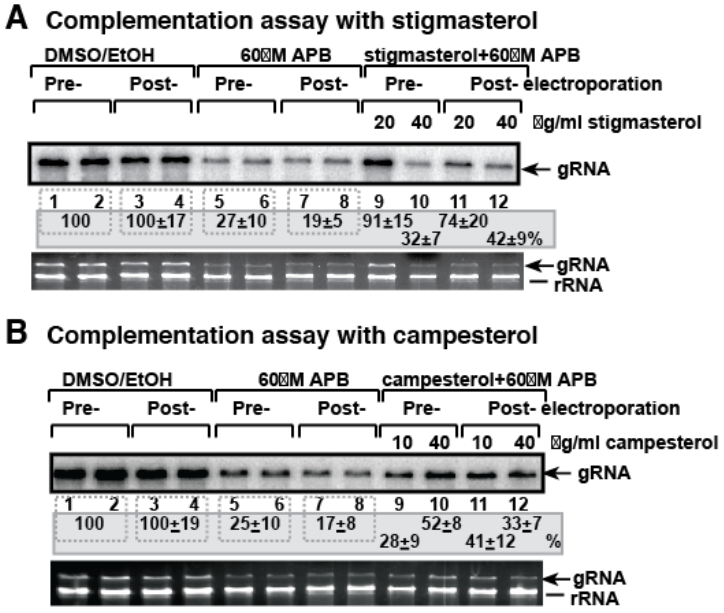

A Complementation assay with stigmasterol
DMSO/EtOH
60□M APB
stigmasterol+60□M APB
Pre-
Post-
Pre-
Post-
Pre-
Post- electroporation
20 40 20 40 □g/ml stigmasterol
gRNA
1 2 3 4 5 6 7 8 9 10 11 12
100 100±17 27±10 19±5 91±15 74±20
32±7 42±9%
gRNA
rRNA
B Complementation assay with campesterol
DMSO/EtOH
60□M APB
campesterol+60□M APB
Pre-
Post-
Pre-
Post-
Pre-
Post- electroporation
10 40 10 40 □g/ml campesterol
gRNA
1 2 3 4 5 6 7 8 9 10 11 12
100 100±19 25±10 17±8 52±8 33±7
28±9 41±12 %
gRNA
rRNA

Figure 2.6 Complementation of the inhibitory effect of the APB treatment of *N. benthamiana* protoplasts on TBSV gRNA accumulation by phytosterols.

(A) The stimulatory effect of stigmasterol on TBSV RNA accumulation. Northern blot analysis showing the accumulation levels of TBSV gRNA in *N. benthamiana* protoplasts treated with DMSO + ethanol (Lanes 1-4), APB alone (lanes 5-8), or with APB + stigmasterol (lanes 9-12) before or after electroporation. The samples were harvested 40 hours after electroporation. Bottom panel: An ethidium-bromide stained gel shows the ribosomal (r)RNA levels as loading controls. (B) The stimulatory effect of campesterol on TBSV RNA accumulation. Northern blot analysis showing the accumulation levels of TBSV gRNA in *N. benthamiana* protoplasts treated with DMSO + ethanol (lanes 1-4), APB alone (lanes 5-8), or with APB + campesterol (lanes 9-12) before or after electroporation. See further details in panel A. Each experiment was repeated.

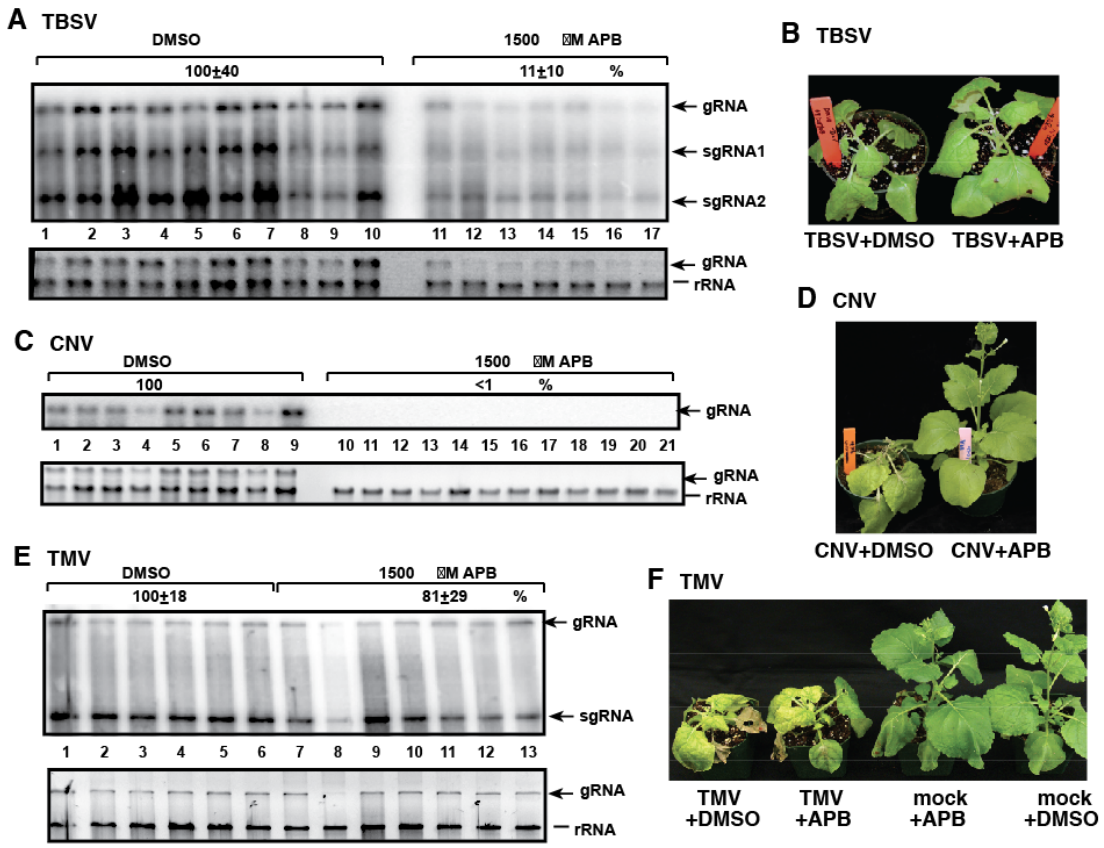

A TBSV
DMSO
1500 μM APB
100±40
11±10 %
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
gRNA
rRNA
B TBSV
TBSV+DMSO TBSV+APB
C CNV
DMSO
1500 μM APB
100
<1 %
gRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
gRNA
rRNA
D CNV
CNV+DMSO CNV+APB
E TMV
DMSO
1500 μM APB
100±18
81±29 %
gRNA
sgRNA
1 2 3 4 5 6 7 8 9 10 11 12 13
gRNA
rRNA
F TMV
TMV +DMSO
TMV +APB
mock +APB
mock +DMSO

Figure 2.7 Inhibition of TBSV and CNV gRNA accumulation in *N. benthamiana* plants treated with APB.

(A) Leaves were first infiltrated with DMSO (1.5%) or APB (1,500 mM), followed by inoculation of the same leaves with TBSV virion preparation. Samples for viral RNA analysis were taken from the infiltrated leaves at 4 dpi. Northern blotting (top panel) shows the level of TBSV gRNA and sgRNAs accumulation in individual samples using a 3' end specific probe. The bottom panel: an ethidium bromide stained gel indicating the levels of rRNA and TBSV gRNA. Each experiment was repeated three times. DMSO sample was chosen as 100%. (B) The delay in symptom development due to TBSV infections in the APB treated plant (shown on the right) at 6 dpi that indicates the potent antiviral activity of APB. Comparable DMSO treatment of plant leaves prior to inoculation with TBSV did not protect the plants from infection. (C) APB treatment inhibits CNV RNA accumulation in *N. benthamiana* plants. Treatment with DMSO or APB, inoculation of leaves with CNV, sample preparation and Northern blotting were done as described in panel A for TBSV. (D) The delay in CNV-induced symptom development in the APB treated plant at 10 dpi indicates the potent anti-CNV activity of APB. See further details in panel B. (E) Moderate inhibitory effect of APB treatment on TMV RNA accumulation in *N. benthamiana* plants. Treatment with DMSO or APB, inoculation of leaves with TMV, sample preparation and Northern blotting with a TMV-specific probe were done as described in panel A. (D) The lack of delay in TMV-induced symptom development in the APB treated plant at 10 dpi indicates the weak anti-TMV

activity of APB. Plants mock inoculated and infiltrated with APB (1,500 mM) or DMSO (1.5%) are shown on the right. See further details in panel B.

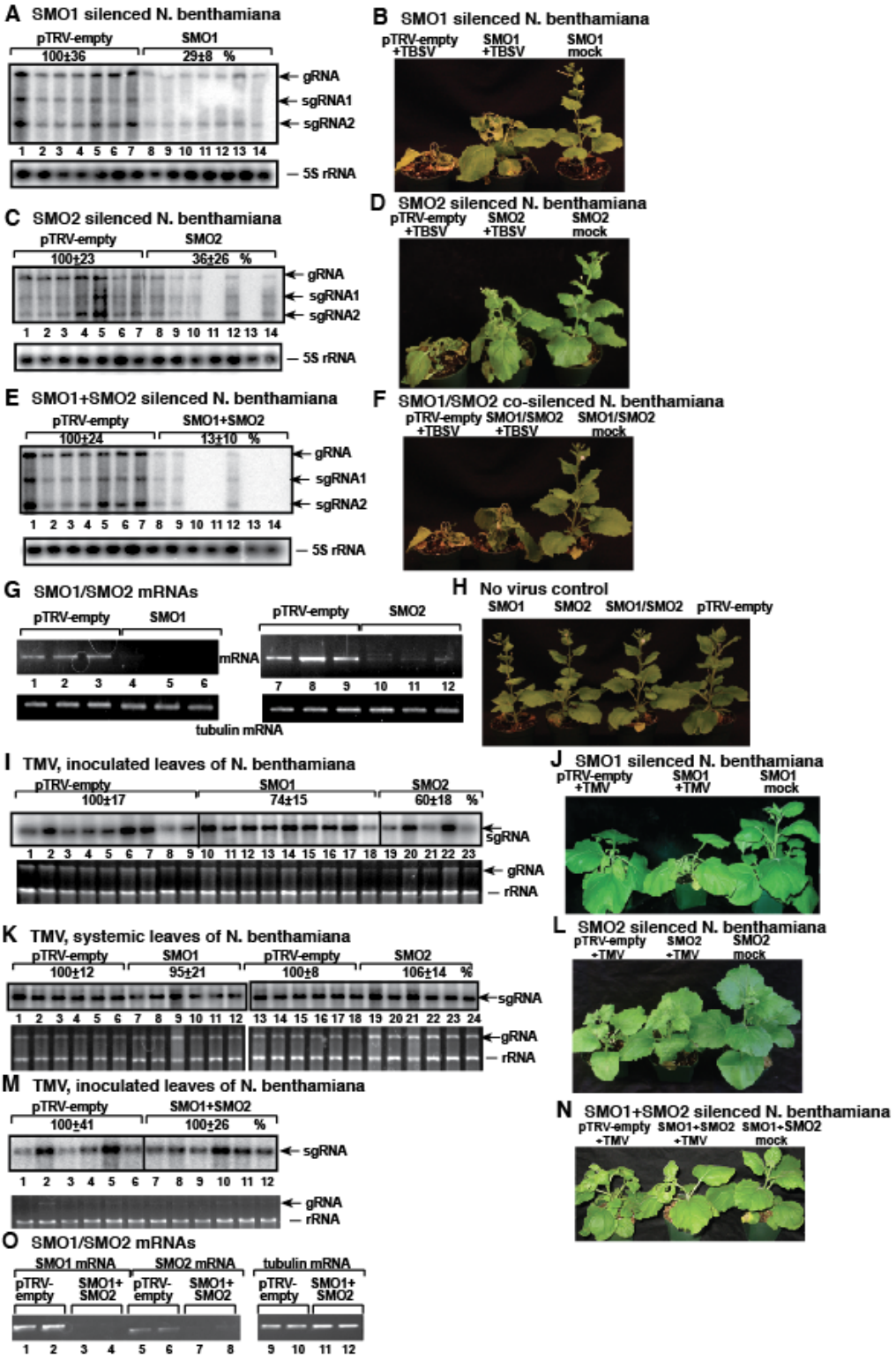

A SMO1 silenced N. benthamiana
pTRV-empty
SMO1
100±36
29±8 %
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
5S rRNA
B SMO1 silenced N. benthamiana
pTRV-empty +TBSV
SMO1 +TBSV
SMO1 mock
C SMO2 silenced N. benthamiana
pTRV-empty
SMO2
100±23
36±26 %
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
5S rRNA
D SMO2 silenced N. benthamiana
pTRV-empty +TBSV
SMO2 +TBSV
SMO2 mock
E SMO1+SMO2 silenced N. benthamiana
pTRV-empty
SMO1+SMO2
100±24
13±10 %
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
5S rRNA
F SMO1/SMO2 co-silenced N. benthamiana
pTRV-empty +TBSV
SMO1/SMO2 +TBSV
SMO1/SMO2 mock
G SMO1/SMO2 mRNAs
pTRV-empty
SMO1
pTRV-empty
SMO2
mRNA
1 2 3 4 5 6 7 8 9 10 11 12
tubulin mRNA
H No virus control
SMO1
SMO2
SMO1/SMO2
pTRV-empty
I TMV, inoculated leaves of N. benthamiana
pTRV-empty
SMO1
SMO2
100±17
74±15
60±18 %
sgRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
gRNA
rRNA
J SMO1 silenced N. benthamiana
pTRV-empty +TMV
SMO1 +TMV
SMO1 mock
K TMV, systemic leaves of N. benthamiana
pTRV-empty
SMO1
pTRV-empty
SMO2
100±12
95±21
100±8
106±14 %
sgRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
gRNA
rRNA
L SMO2 silenced N. benthamiana
pTRV-empty +TMV
SMO2 +TMV
SMO2 mock
M TMV, inoculated leaves of N. benthamiana
pTRV-empty
SMO1+SMO2
100±41
100±26 %
sgRNA
1 2 3 4 5 6 7 8 9 10 11 12
gRNA
rRNA
N SMO1+SMO2 silenced N. benthamiana
pTRV-empty +TMV
SMO1+SMO2 +TMV
SMO1+SMO2 mock
O SMO1/SMO2 mRNAs
SMO1 mRNA
SMO2 mRNA
tubulin mRNA
pTRV-empty
SMO1+SMO2
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2.8 The role of *SMO1/SMO2* sterol biosynthesis genes in TBSV RNA replication in whole plants.

(A) Accumulation of TBSV gRNA in the inoculated leaves of *SMO1* knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis. VIGS was performed via agroinfiltration of TRV vectors carrying *SMO1* sequence or the TRV empty vector (as a control). Inoculation with TBSV gRNA was done 11 days after agroinfiltration. (B) Delay in TBSV-induced symptom development in the *SMO1* knockdown plant (shown in the middle) at 10 dpi when compared to the control plant infiltrated with pTRV empty vector (shown on the left) that indicates the requirement of *SMO1* for TBSV infection. (C) Accumulation of TBSV gRNA in the inoculated leaves of *SMO2* knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis. See further details in Panel A. (D) Delay in TBSV-induced symptom development in the *SMO2* knockdown plant. See further details in Panel B. (E) Accumulation of TBSV gRNA in the inoculated leaves of *SMO1/SMO2* knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis. See further details in Panel A. (F) Delay in TBSV-induced symptom development in the *SMO1/SMO2* knockdown plant. See further details in Panel B. (G) Semi-quantitative RT-PCR analysis of the accumulation of *SMO1* or *SMO2* mRNAs in the knockdown *N. benthamiana* plants and in the control plants, which were agroinfiltrated with the TRV empty vector 11 days after agroinfiltration. RT-PCR analysis of the tubulin mRNA from the same samples served as a control. (H) Minor phenotypic effect, such as moderately increased leaf-size, of *SMO1, SMO2,* or *SMO1/SMO2* knockdown on *N. benthamiana*

plants when compared to the control plants, which were agroinfiltrated with the pTRV empty vector. (I) Accumulation of TMV sgRNA in the inoculated leaves of *SMO1* or *SMO2* knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis (top image). The accumulation level of TMV gRNA and the rRNA (as a loading control) are shown in an ethidium-bromide stained gel (bottom image). See further details in Panel A. (J) Lack of delay in TMV-induced symptom development in the *SMO1* knockdown plant. See further details in Panel B. (K) Accumulation of TMV sgRNA in the systemically infected leaves of *SMO1* or *SMO2* knockdown *N. benthamiana* plants 5 days post-inoculation, based on Northern blot analysis (top image). The accumulation level of TMV gRNA and the rRNA (as a loading control) are shown in an ethidium-bromide stained gel (bottom image). See further details in Panel A. (L) Lack of delay in TMV-induced symptom development in the *SMO2* knockdown plant. See further details in Panel B. (M) Accumulation of TMV sgRNA in the inoculated leaves of *SMO1/SMO2* knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis. See further details in Panel I. (N) Lack of delay in TMV-induced symptom development in the *SMO1/SMO2* knockdown plant. See further details in Panel J. (O) Semi-quantitative RT-PCR analysis of the accumulation of *SMO1* or *SMO2* mRNAs in the knockdown *N. benthamiana* plants and in the control plants, which were agroinfiltrated with the TRV empty vector 11 days after agroinfiltration. RT-PCR analysis of the tubulin mRNA from the same samples served as a control.

Chapter 3 Inhibition of phospholipid biosynthesis decreases the activity of the tombusvirus replicase and alters the subcellular localization of replication proteins.

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INTRODUCTION

The host cell's organellar membranes are efficiently subverted by plus-stranded (+)RNA viruses for their replication (Miller and Krijnse-Locker, 2008b). High concentrations of membrane-bound viral proteins and co-opted host proteins lead to the formation of "viral replication organelles" that provide protection against cellular nucleases and proteases (Ahlquist, 2003). In addition, the membrane lipids and proteins could also serve as scaffolds for the assembly of the viral replicase complex or they can facilitate the targeting of the viral replication proteins to a particular microdomain in the membrane. Moreover, the subcellular membranes may provide critical lipid or protein cofactors to regulate the function of the viral replicase. Indeed, dynamic remodeling/deforming membranes to give rise to unique structures, called spherules (i.e., invaginations of lipid membranes), is a characteristic feature for many (+)RNA viruses These viral-induced spherules serve as sites of viral RNA replication. Importantly, (+)RNA viruses also induce membrane proliferation that requires new lipid biosynthesis. Indeed, several genome-wide screens identified lipid biosynthesis/metabolism genes

affecting (+)RNA virus replication (Cherry et al., 2005; Krishnan et al., 2008; Kushner et al., 2003; Panavas et al., 2005b; Serviene et al., 2006).

The best characterized examples of virus-induced modification of cellular lipid metabolism include the recruitment of host enzymes such as PI4PKIIIß, which is involved in phosphatidylinositol-4-phosphate (PI4P) synthesis, to modify the lipid composition of membranes during poliovirus replication (Belov and Ehrenfeld, 2007; Belov et al., 2007; Hsu et al., 2010; Sasvari and Nagy, 2010). Hepatitis C virus (HCV) modulates phospholipid biosynthesis by recruiting PI4PKIIIα, which is also involved in PI4P synthesis, to facilitate the formation of the "membranous-web" (cellular vesicles), which serves as the site of HCV RNA replication (Berger et al., 2009). Dengue virus co-opts FASN, a major rate-limiting enzyme in fatty acid biosynthesis, by retargeting it to the ER membrane, the site of dengue virus replication (Heaton et al., 2010). Another example is Drosophila C virus (picorna-like virus), whose replication was blocked by depletion of the HLH106 regulator of fatty acid metabolism and fatty acid synthase (Cherry et al., 2006). Mutation of Ole1p, which affects the amount of unsaturated fatty acids, reduced the activity of the BMV replicase, and possibly altered the binding of the BMV 1a replication protein to the membrane due to a reduced ratio of unsaturated fatty acids in *S. cerevisiae* (Lee and Ahlquist, 2003a). Infection with West Nile virus (WNV) was shown to result in redistribution of cholesterol from the plasma membrane to the sites of virus replication (Mackenzie et al., 2007). Yet other change in lipid metabolism is induced by Dengue virus, which promotes autophagy and beta-oxidation of lipids released from lipid droplets to generate extra ATP needed for virus replication (Heaton and Randall, 2010).

TBSV, a tombusvirus, is among the most advanced model RNA viruses regarding characterization of host factors (Nagy, 2008). Among the five proteins encoded by the TBSV genome, only p33 replication co-factor, which is an RNA chaperone, and the $p92^{pol}$ RNA-dependent RNA polymerase (RdRp) are essential for TBSV RNA replication (Stork et al., 2011; White and Nagy, 2004). p33 and $p92^{pol}$ are integral membrane proteins with a topography facing the cytosolic surface of the peroxisomes or occasionally ER, the sites of replicase complex formation and viral RNA replication (Panavas et al.,2005a)

Electron microscopic images of cells replicating tombusvirus demonstrated extensive remodellng (Barajas et al., 2009). Previously, our lab identified 14 host genes involved in lipid biosynthesis metabolism affecting tombusvirus replication and recombination, suggesting that tombusviruses depend on active lipid biosynthesis (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2006; Serviene et al., 2005). The identified lipid biosynthesis/metabolism genes included 8 genes affecting phospholipid biosynthesis, 4 genes affecting fatty acid biosynthesis/metabolism and 2 genes affecting sterol synthesis (Nagy, 2008). These finding suggest that lipids are likely involved, directly or indirectly, in TBSV replication in yeast. Accordingly, previous studies showed that sterols are critical for TBSV replication (Sharma et al., 2010).

In order to demonstrate the roles of phospholipids in TBSV RNA replication, we selected *INO2* for additional in-depth studies from the pool of identified genes in the genome-wide screens with TBSV (Jiang et al., 2006; Nagy, 2008; Panavas et al., 2005b). Ino2 is a basic helix-loop-helix transcription activator for phospholipid synthesis genes (Block-Alper et al., 2002). The phospholipid biosynthesis in *S. cerevisiae* is based on

biochemical pathways conserved in higher eukaryotes [(Carman and Han 2009) and (Nohturfitt and Zhang 2009)]. The expression of phospholipid biosynthesis genes is controlled at the mRNA transcription and stability steps. The expression of many genes involved in phospholipid biosynthesis is controlled by a cis-acting DNA sequence (UAS_{INO}); the transcription activator Ino2, which forms a heterodimer with Ino4; and a repressor, named Opi1 (Fig. 3.1A) [(Carman and Han 2009) and (Nohturfitt and Zhang 2009)]. When low amount of phosphatidic acid (PA, a precursor of phospholipids) is present in the ER membrane, then Opi1 is released from the ER membrane and after its translocation into the nucleus, Opi1 binds to Ino2 and represses the mRNA transcription of the phospholipid biosynthesis genes (Fig. 3.1A) [(Carman and Han 2009) and (Nohturfitt and Zhang 2009)].

Our approach to test the effect of phospholipid biosynthesis regulators on TBSV RNA replication is justified by the data from the genome-wide screens that *INO2* and additional transcription regulators of the phospholipid biosynthesis genes [called SAGA complex and HAT (histone acetyltransferase) complex] have been identified, while single structural genes for phospholipid biosynthesis were not (Jiang et al., 2006; Nagy, 2008; Panavas et al., 2005b). This is expected because two parallel pathways, the *de novo* and the Kennedy, exist to produce phospholipids in yeast (Nohturfft and Zhang, 2009). Thus, single deletion of structural genes for phospholipid biosynthesis could be partially complemented under the conditions we performed the genome-wide screens (Jiang et al., 2006; Panavas et al., 2005b). However, deletion of *INO2* affects both pathways [(Carman and Han 2009) and (Nohturfitt and Zhang 2009)]. Accordingly, we demonstrate in this paper that co-deletion of *INO2-INO4* leads to a reduced level of TBSV repRNA

accumulation in yeast. The tombusvirus replicase complexes isolated from *ino2*Δ*ino4*Δ yeast show poor activity, suggesting that phospholipids are important for the assembly/activity of the tombusvirus replicase. In addition, we demonstrate altered cellular localization of the tombusvirus replication protein in *ino2*Δ*ino4*Δ yeast. Moreover, to expand our findings to other RNA viruses, we also show that the replication of FHV is inhibited in *ino2*Δ*ino4*Δ yeast. Thus, the emerging picture from the current work is that phospholipid biosynthesis is required for efficient replication of some (+)RNA viruses.

MATERIALS AND METHODS

Yeast strains and expression plasmids.

Saccharomyces cerevisiae strain BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and single-gene deletion strain *ino2Δ* and *ino4Δ* strains were obtained from Open Biosystems (Huntville, AL). The following yeast expression plasmids have been generated before: pHisGBK-His33 (Panaviene et al., 2004); pGAD-His92 (Panaviene et al., 2004); pYC-DI72sat (Panavas and Nagy, 2003); pHisGBK-CUP1::His33-ADH::DI72 (Mendu et al., 2010); pESC-His-p33-DI-72 (Jonczyk et al., 2007); pGBK-His33/DI-72 (Jiang et al., 2006); pYES-HisGFP-33 (Panavas et al., 2005a); pex13-CFP (Panavas et al., 2005a); pho86-CFP (Panavas et al., 2005a); pGAD-His92-Cup1 and pHisGBKHis33Cup1/GAL-DI (provided by K. Pathak). pESC-His-Cup-FHV-RNA1-$TRSV_{Rz}$ was provided by J. Pogany.

To create double deletion strain *ino2Δino4Δ*, we used homologous recombination in *ino2Δ* strain by replacing *INO4* ORF with hphNT1 (Hygromycin resistance) gene. The

hphNT1 ORF was PCR amplified from pFA6a-HPH (Euroscarf) using the following primers: #3693 (CGAAGGAGTTAAGAGGGCGGCTTGAACTAAAAAGAGAAAAGCA-cgtacgctgcaggtcga) and #3694 (AGAATTTCTTCGCTTATATTAC-TTACTTTACCCTACTCCTTGatcgatgaattcgagctc). The obtained PCR product was used to transform *ino2Δ* strain. The new strain *ino2Δino4Δ* was confirmed with primers #2501 (ATCCACGCCCTCCTACATC) and #3695 (GGGTACCTCCAAATCTGCGAAGGTA).

To express Ino2 in yeast, the full-length ORF of *INO2* was cloned into pYES/NT/C (Invitrogen). First, *INO2* ORF was amplified by PCR from yeast genomic DNA by using primers #2311 (CAGCGGATCCATGCAACAAGCAACTGGGAACGAATTACT) and #2312 (GACCCTCGAGTCAGGAATCATCCAGTATGT) that were appended with *BamH*I and *Xho*I recognition sequences, respectively, to facilitate directional cloning. To express Ino2, we PCR amplified the *INO2* ORF and digested with *Hind*III and *Xho*I sites and cloned into pYC2/CT low copy vector digested with the same pair of enzymes.

To obtain pYES-His92, which contains *GAL1*-p92 and *URA3* auxotrophic marker, p92 ORF was amplified using primers #788 (GAGGGATCCGAGACCATCAAGAGAATG) and #952 (CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA) appended with *BamH*I and *Xho*I restriction enzyme recognition sites to facilitate directional cloning and then ligated and cloned into pYES vector digested with the same pair of enzymes. Similarly, pYES-His33, which contains *GAL1*-p33 and *URA3* auxotrophic marker, the p33 ORF was

amplified using primers #788 and #1403 (gccgCTCGAGCTATTTCACACCAAGGGACTCA) appended with *BamH*I and *Xho*I restriction enzyme recognition sites to facilitate directional cloning and then ligated and cloned into pYES vector digested with the same pair of enzymes. Construct BG1805-Opi1-zz carries *OPI1* ORF behind the *GAL1* promoter and fused to a tandem affinity tag that includes a His-tag and the zz domain of protein A at the C terminus (Li et al., 2008).

TBSV replication assay in yeast.

Yeast strains by4741, ino2Δ, ino4Δ and ino2Δino4Δ were transformed with phisgbkhis33 and pgadhis92 (Panaviene et al., 2004) as well as with pyc-di72 (Panavas and Nagy, 2003). replication assay was performed by measuring the accumulation of di-72(+) reprna relative to the 18s rrna (Panavas and Nagy, 2003). to precisely regulate the amount and timing of expression of replication proteins p33 and p92 and to measure their subsequent effect on rna accumulation levels, ino2Δino4Δ and by4741 yeast strains were transformed with the following combination of plasmids: (a) phisgbkhis33 and pgadhis92 and pyc-di72; (b) pyes-92 and pesc-his33/gal-di; and (c) pgad-92cup1, phisgbkhis33cup1-gal-di. yeast cells were pre-grown at 29°c with shaking for 15 hrs and then transferred to media containing 2% galactose supplemented with 50μm cu++. standard rna extraction and northern blot was performed as mentioned in previous publications (Panavas and Nagy, 2003; Panaviene et al., 2004).

In vitro replicase assay using membrane-enriched (ME) fraction of yeast.

The procedure used to obtain functional ME fractions was the same developed by us earlier (Panaviene et al., 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 and incubated at 23 °C with shaking at 250 rpm. After 24 h growth, yeast samples were collected by centrifugation at 3000g 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 21,000g 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 100g for 5 min at 4 °C. The supernatant was moved to a new microcentrifuge tube, followed by centrifugation at 21,000g 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 MgCl2, 10 mM DTT, 100 mM potassium glutamate, 0.2μl Rnase inhibitor, 1 mM ATP, CTP, GTP, 0.1 μl radioactive P32-UTP (3000 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) and 100 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).

RNA and protein stability assays:

Yeast strains BY4741 and *ino2*Δ*ino4*Δ were transformed with pYC2-DI72. The transformed yeast strains were grown at 29°C in SC-U (synthetic complete without

uracil) with 2% galactose. After 20 h, the cultures were re-suspended in SC-U supplemented with 2% glucose and collected after indicated time-points. Northern blotting was performed to measure repRNA levels at various time points.

To study the stability of p33 in yeast, BY4741 and *ino2*Δ*ino4*Δ were transformed with pYES-33 expressing 6xHis-tagged CNV p33 from the inducible *GAL1* promoter. Yeast transformants were cultured overnight in SC U^- medium containing 2% glucose at 29°C. Yeast cultures were transferred to U- 2% Galactose medium for 6 hrs 29°C. To study stability of protein A of FHV, BY4741 and *ino2*Δ*ino4*Δ strains were transformed with pGAD/CUP/PtnA/C-HA/FLAG construct. After pre-growing the cells in L^- media containing 2% glucose, protein A expression was induced with 50μM Cu^{++} for 12 hrs. Then, cycloheximide was added to a final concentration of 100 μg/ml to inhibit protein synthesis. Equal amounts of yeast cells were collected at given time points after cycloheximide treatment and cell lysates were prepared by the NaOH method as described previously (Sharma et al., 2010). The total protein samples were analyzed by SDS-PAGE and Western blotting with anti-His and anti-FLAG antibody as described previously (Panaviene et al., 2005; Panaviene et al., 2004).

Complementation assay and over-expression of Ino2p and Opi1p.

pYC-INO2 or pYC empty plasmids were transformed into BY4741 and ino2Δ strains containing pGAD-His92, pGBK-His33/DI-72 (to co-express p33 protein from the constitutive ADH1 promoter and DI-72 repRNA under inducible GAL1 promoter). pYC-INO2, pYC-empty, pYES-INO2 or pYES-empty plasmids were transformed into BY4741 strain containing pGAD-His92-Cup, pHisGBK-Cup1::His33-ADH1::-DI72 (Mendu et al., 2010), which expresses p33 protein from CUP1 promoter and DI-72

repRNA under constitutive ADH1 promoter. Cells were pre-grown at 29°C for 15 hrs in 2 % galactose media followed by supplementation with 50μM Cu^{++} and further incubation at 29°C for 24 hrs. For time course analysis, media was changed as described in figure legend. To over-express Opi1 above mentioned strategy was employed using BG1805-Opi1-zz.

Replication Protein Analysis.

Yeast strains were grown as described above for RNA analysis. A total of 2ml of yeast culture was harvested, the pelleted cells were resuspended in 200 μl of 0.1M NaOH and incubated at 23°C for 10 min. The supernatant was aspirated following a short centrifugation, and the pellet was resuspended in 100μl, 1X SDS-polyacrylamide gel electrophoresis (PAGE) buffer containing 5% β-mercaptoethanol and boiled for 5 min. The supernatant was used for SDS/PAGE and Western blot analysis as described (Panavas et al., 2005a). The primary antibodies were anti-6xHis (Invitrogen) for tombusvirus and anti-FLAG (Sigma) for FHV, and the secondary antibodies were alkaline-phosphatase-conjugated anti-mouse immunoglobulin-G (Sigma).

Membrane fractionation.

To check membrane association of p33 in BY4741 and *ino2*Δ*ino4*Δ yeast, cells were broken using Fast Prep[24] MP Bio. After removing debris by centrifugation at 1,000g for 5 min, membranes were collected at 40,000g for 1 hr. Supernatant and membrane fractions were analyzed for their p33 content by western blotting. In another set of fractionation experiments, we performed alkaline treatment to remove the proteins that bound peripherally to the membranes (Whitley et al., 1996). Briefly, after removal of cell

debris, supernatant (250μl) was incubated on ice with Na_2CO_3 (250 μl) 200mM pH 11.5, for 30 min and then loaded on the 300 μl cushion (200mM sucrose in100mM Na_2CO_3 pH 11.5) followed by centrifugation for 30 min at 40,000g. Separated soluble and membrane fractions were analyzed for p33 content by western blotting. Different cellular organellar protein markers (Sec61, Ssa1 and Pgk1) were also visualized by using their respective antibodies.

Yeast spheroplasting and membrane flotation.

ino2Δino4Δ yeast transformed with pESC-HisY-p33-DI-72 (for inducible expression of YFP-p33) was pre-grown in 2% glucose H^- media and then transferred to 2% galactose media and grown to OD_{600} of 0.8 to 1.0. Spheroplasting was performed using Zymolyase digestion as described (Daum et al., 1982; Wang et al., 2009). Briefly, cells were harvested at 3000g, then washed with water and re-suspended in 0.1M $TrisSO_4$ pH 9.4 containing 10mM DTT. This was followed by incubation at 30°C. Cells were collected by centrifugation and washed with 1.2M sorbitol. Pellet was resuspended in 1.2M sorbitol containing 20mM potassium phosphate (pH 7.4) and Zymolyase (5mg per 1 g of wet yeast cells), then incubated at 30°C with very gentle shaking for 50-90 min. Spheroplasting efficiency was checked under a microscope. Yeast spheroplasts were harvested at 500g and washed twice with 1.2M sorbitol.

Using 15 strokes in Dounce homogenizer, 100 milligram of yeast cells was broken in 600 μl of yeast lysis buffer (200 mM sorbitol, 50 mM Tris-HCl [pH 7.5], 15 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mi [Sigma]), followed by centrifugation for 5 min at 100*g* to pellet unbroken cells.

Supernatant was centrifuged at 16,000g for 15 minutes and the pellet containing membranes were collected for the flotation experiments.

For sucrose flotation gradient analysis, samples were adjusted to 52% (wt/wt) sucrose in the lysis buffer, and 400 µl was loaded to the bottoms of ultraclear polycarbonate ultracentrifuge tubes (Beckman), overlaid with 900 µl of 45% sucrose in lysis buffer, topped with 100 µl of 10% sucrose in lysis buffer, and subsequently centrifuged at 40,000 rpm at 4°C for 16 h by using an TLS55 Ti rotor in a Beckman Optima-Max-XP ultracentrifuge. Ten fractions (140 µl each) were manually collected from the top to the bottom, followed by protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western-blotting procedures as described previously using antibodies against Sec61p (an integral ER membrane protein) and anti-Pgk1p (cytosolic protein) (Wang et al., 2009).

Confocal Laser Microscopy.

Yeast cells were transformed with yfp- or gfp-tagged p33 (pesc-yfp-33 or pyes-hisgfp-33) *(Panavas et al., 2005a)*. To visualize peroxisome and er, pex13-cfp and pho86-cfp, respectively, were used as markers *(Panavas et al., 2005a)*. Transformed yeast cells were pre-grown overnight at 29°c and then transferred to media containing 2% galactose and samples were collected for microscopy after 6 hrs *(Panavas et al., 2005a)*. to test the effect of ino2 overexpression on p33 localization pattern, above yeast transformants were re-transformed with pyc-ino2 or pyc (as a control). Samples were collected for confocal microscopy 6 and 24 hrs post induction.

FHV replication assay.

BY4741 and ΔINO2/ΔINO4 strains were transformed with pESC-His-Cup-FHV-RNA1-TRSV$_{Rz}$. After pregrowing at 29°C, media (SC-H$^-$ with 2% galactose) was supplemented with 50μM Cu^{++} and harvested after 48 hrs for RNA analysis. To study the effects of Ino2 overexpression, above yeast strains were transformed with pYC-INO2 or pYC-empty plasmids along with pESC-His-Cup-FHV-RNA1-TRSV$_{Rz}$. Cells were grown in media SC-UH$^-$ 2% galactose media at 29°C and then supplemented with 50μM Cu^{++} and harvested after 48 hrs for RNA analysis.

RESULTS

Deletion of Ino2 and Ino4 transcription activators in yeast reduces TBSV RNA accumulation and inhibits the viral replicase activity in vitro.

To confirm the role of phospholipid biosynthesis in TBSV replication, we tested replication of the TBSV replicon (rep)RNA, which is an efficiently replicating surrogate RNA template derived from the TBSV genomic (g)RNA (Panavas and Nagy, 2003; White and Morris, 1994), in *ino2*Δ yeast, co-expressing the p33 and p92^{pol} replication proteins and DI-72 repRNA from plasmids. Northern blot analysis revealed ~60% less efficient replication of DI-72 repRNA in *ino2*Δ yeast in comparison with the parental yeast (Fig. 3.1B, lanes 1-4 versus 5-8), confirming the data from the previous genome-wide screen (Panavas et al., 2005b). The expression level of p33 also decreased by ~50% in *ino2*Δ yeast (Fig. 3.1B), which could be one of the reasons for the reduced accumulation level of the TBSV repRNA in *ino2*Δ yeast. To test if the activity of the

tombusvirus replicase complex was similar in *ino2*Δ and wt yeast strains, we isolated the membrane-bound tombusvirus replicase from these yeast strains and tested the in vitro template activity on the co-purified endogenous template RNA. These experiments revealed that the tombusvirus replicase showed only 22% activity when obtained from *ino2*Δ yeast, although the amount of p33/p92^{pol} was adjusted to comparable levels in the membrane fractions (Fig. 3.1C). The replicase-based in vitro data suggest that the reduced phospholipid synthesis in *ino2*Δ yeast inhibits the relative activity of the tombusvirus replicase. Thus, phospholipids are likely important for tombusvirus replication.

Additional control experiments showed that plasmid-born *INO2* could complement the negative effect of *INO2* deletion on TBSV RNA accumulation (Fig. 3.1D, lanes 1-4). Moreover, deletion of *INO4* also reduced TBSV replication significantly (Fig. 3.1E). This is not surprising since Ino4 is a transcription activator that forms a heterodimer with the Ino2 to control the transcription of phospholipid biosynthesis genes [(Carman and Han 2009) and (Nohturftt and Zhang 2009)].

Since inhibition of TBSV replication was consistently stronger in *ino2*Δ yeast than in *ino4*Δ yeast, we decided to test TBSV replication in a double-deletion yeast strain (*ino2*Δ*ino4*Δ). TBSV repRNA replication was even more debilitated in the double-deletion yeast strain (down to ~25% level Fig. 3.2A). In addition, we observed consistently reduced levels of p33/p92^{pol} in the double-deletion yeast (Fig. 3.2A), although both replication proteins were expressed from the constitutive *ADH1* promoter. To exclude the possibility that the reduced p33/p92^{pol} levels were due to inhibition of *ADH1* promoter-driven transcription, we also tested the accumulation of p33/p92^{pol} when

expressed from *GAL1* galactose inducible/glucose suppressible promoter (Fig. 3.2B) or from the copper-inducible *CUP1* promoter (Fig. 3.2C). Interestingly, repRNA replication as well as p33/p92^{pol} levels were also reduced in these yeasts, suggesting that viral RNA replication and reduction in replication protein levels is not promoter-specific (Fig. 3.2A-C). Testing the tombusvirus replicase activity in the membrane-enriched fraction obtained from *ino2*Δ*ino4*Δ yeast revealed that the normalized template activity (after adjustment of p33 to comparable levels in each sample) of the tombusvirus replicase on the endogenous template was low (Fig. 3.3, lanes 1-2 versus 3-4). Altogether, these data suggest that repRNA accumulation, the normalized activity of the tombusvirus replicase and the level of p33/p92^{pol} decreased markedly in *ino2*Δ*ino4*Δ yeast.

Reduced stability of p33 replication protein in ino2*Δ*ino4*Δ *yeast.

To test the stability of p33 replication protein when phospholipid biosynthesis is inhibited, we first expressed p33 from *GAL1* promoter for 6 hours, followed by adding cycloheximide (to inhibit new protein synthesis), followed by measuring protein levels (Fig. 3.4A). We found that the half-life of p33 decreased ~ 2-fold to 3.5 hours in *ino2*Δ*ino4*Δ yeast from more than 6 hours in wt yeast (Fig. 3.4A). Interestingly, the stability of the repRNA did not change in *ino2*Δ*ino4*Δ yeast (Fig. 3.4B), suggesting that the primary effect of reduced phospholipid synthesis is on the replication proteins and not on the stability of repRNA.

Over-expression of Ino2 increases TBSV RNA accumulation in yeast.

Over-expression of Ino2 has been shown to increase the phospholipid synthesis in yeast [(Carman and Han 2009) and (Nohturftt and Zhang 2009)]. We found that over-expression of Ino2 from either a low (Fig. 3.5, lanes 1-4) or high (lanes 9-12) copy

number plasmids led to 2-3-fold increase in TBSV RNA accumulation. The level of p33/p92pol replication proteins did not change in the over-expression strains, suggesting that the increased RNA accumulation was likely due to increased TBSV RNA replication.

To test when TBSV is the most dependent on phospholipid biosynthesis, we devised a scheme for controlled over-expression of Ino2, and thus regulated increase in phospholipid biosynthesis, followed by measuring TBSV RNA accumulation 24 hours after inducing repRNA replication (see Fig. 3.6A for the experimental scheme). The control experiments included the same expression plasmid and inducer (galactose), but without the *INO2* open reading frame (Fig. 3.6C), which leads to the over-expression of a small peptide. We found that Ino2 over-expression increased TBSV repRNA accumulation to the largest extent when it was over-expressed all the time (3x increase, from 69% to 225%, compare treatment #3 in Fig. 3.6B-C, lanes 7-9). We also observed ~2-fold increase in TBSV RNA accumulation when Ino2 was over-expressed for 12 h either before the induction of TBSV repRNA accumulation (from 88% to 151%, compare treatment #2, Fig. 3.6B-C, lanes 4-6) or the first half of TBSV replication period (treatment #4, lanes 10-12). Similarly, over-expression of Ino2 for 24 h during the entire TBSV replication period led to ~2x increase in TBSV RNA accumulation (compare treatment #6, Fig. 3.6B-C, lanes 16-18). In contrast, over-expression of Ino2 for 12 h during the second half of TBSV replication period (compare treatment #5, Fig. 3.6B-C, lanes 13-15) did not alter TBSV RNA accumulation. Altogether, these data are consistent with the model that TBSV replication is the most dependent on phospholipid biosynthesis at the early stage of the replication cycle and less dependent at the latter stage. However,

it is important to note that since we over-expressed Ino2 transcription activator, the actual effect on phospholipid levels in yeast cells could take place couple of hours after the induction of Ino2.

Over-expression of Opi1, a repressor of phospholipid synthesis, decreases TBSV RNA accumulation in yeast.

To further show that TBSV replication depends on active phospholipid biosynthesis, we over-expressed Opi1, which is a repressor of Ino2-Ino4 complex [(Carman and Han 2009) and (Nohturfitt and Zhang 2009)]. TBSV repRNA accumulation decreased by 2.5-fold in yeast over-expressing Opi1 (Fig. 3.7, lanes 1-4). The amount of p33 and $p92^{pol}$ replication proteins also decreased in this yeast (Fig. 3.7). Thus, the overall effect of over-expression of Opi1 was very similar to the situation seen with *ino2*Δ*ino4*Δ yeast (Fig. 3.2), suggesting that the phospholipid biosynthesis pathway is important in tombusvirus replication.

Phospholipid synthesis is required for proper subcellular localization of tombusvirus replication proteins.

To monitor the subcellular localization of the tombusvirus replication protein in *ino2*Δ*ino4*Δ yeast, first we used GFP-tagged p33 and confocal microscopy. Interestingly, the large majority of yeast cells showed unusual diffused pattern for GFP-p33 in *ino2*Δ*ino4*Δ yeast (Fig. 3.8A), which is in contrast with the punctate structures formed in wt BY4741 yeast (not shown and Fig. 3.8C, E, J-K) (Jonczyk et al., 2007; Panavas et al., 2005a). A smaller fraction of yeast cells showed GFP-p33 as part of punctate structures, as expected from cells replicating the TBSV repRNA (Fig. 3.8A).

To define the subcellular location of p33 in *ino2*Δ*ino4*Δ yeast, we co-expressed YFP-p33 with CFP-Pex13, which is a peroxisomal marker protein (Jonczyk et al., 2007; Panavas et al., 2005a). Surprisingly, both YFP-p33 and CFP-Pex13 showed the unusual diffused pattern in the majority of yeast cells (Fig. 3.8B), which is dramatically different from the usual punctate structures and co-localization as seen in the wt BY4741 strain (Fig. 3.8C). In a small fraction of cells (~25%), in which YFP-p33 and CFP-Pex13 showed punctate structures (Fig. 3.8B, bottom panel), their co-localization was detectable, suggesting that in a few cells tombusvirus p33 might be able to get transported to proper subcellular location, while it is mislocalized in the majority of *ino2*Δ*ino4*Δ yeast cells.

To further test the subcellular location of p33 in *ino2*Δ*ino4*Δ yeast, we co-expressed YFP-p33 with CFP-Pho86, which is an ER marker protein (Jonczyk et al., 2007; Panavas et al., 2005a). We did not observe co-localization of p33 and the ER marker protein in *ino2*Δ*ino4*Δ yeast (Fig. 3.8D), while we detected some co-localization of YFP-p33 with CFP-Pho86 in wt BY4741 yeast (Fig. 3.8E). Most of the punctate structures formed by YFP-p33 were located in the proximity of ER, but not co-localized with the ER marker, as shown previously (Jonczyk et al., 2007; Panavas et al., 2005a). This pattern is typical for tombusviruses, which is interpreted as peroxisomal location.

We also used cell-fractionation experiments to test if YFP-p33 is still membrane-bound in *ino2*Δ*ino4*Δ yeast. The obtained data from independent sets of experiments revealed that YFP-p33 sedimented with the membrane-containing fraction, not the supernatant containing the soluble proteins (Fig. 3.8F). Treating the membrane-fraction with alkaline to remove peripheral membrane-bound proteins did not remove p33, indicating that p33

is an integral membrane-protein in *ino2*Δ*ino4*Δ yeast (Fig. 3.8G). Membrane flotation experiments confirmed that p33 was the most abundant in the top fractions 2 and 3, which contains most of the membrane-bound proteins, such as Sec61p (Fig. 3.8H, lanes 2-3). Altogether, these data suggest that most YFP-p33 is likely membrane-associated in *ino2*Δ*ino4*Δ yeast.

Over-expression of Ino2 in BY4741 strain resulted in YFP-p33 pattern with frequent formation of large punctate structures both at 6 h and 24 h time points (Fig. 3.8J-K), which were co-localized with Pex13 peroxisomal marker at the 6h time point (Fig. 3.8I). In comparison, YFP-p33 is distributed to a large number, but smaller punctate structures in the wt yeast (Fig. 3.8J-K). Since Ino2 over-expression simulates TBSV repRNA accumulation, it is likely that the large punctate structures in Ino2 over-expressing cells are increasingly active in viral RNA synthesis.

***Phospholipid synthesis is needed for replication of* FHV.**

To test if the reduced phospholipid synthesis in *ino2*Δ*ino4*Δ yeast could also affect the replication of another RNA virus, we chose FHV (Castorena et al., 2010; Kopek et al., 2010; Odegard et al., 2010; Pogany et al., 2010; Price et al., 2002), which is an insect virus distantly related to TBSV, with both viruses belonging to the Flavivirus-supergroup among (+)RNA viruses. The accumulation of FHV RNA1 and the subgenomic RNA3 (produced from RNA1 during replication) was reduced dramatically in *ino2*Δ*ino4*Δ yeast (Fig. 3.9A). Interestingly, an N-terminally truncated protein A (PrtA) replication protein accumulated in *ino2*Δ*ino4*Δ yeast (Fig. 3.9B), suggesting that the FHV replication protein is unstable in presence of reduced amounts of phospholipids.

Time-course experiments showed that the full-length FHV PrtA was barely detectable even at the beginning of the protein stability experiments (Fig 9B).

Over-expression of Ino2 in wt BY4741 yeast resulted in ~3-fold increase in FHV RNA3 accumulation (Fig. 3.9C, lanes 1-4 versus 5-8). Thus, similar to TBSV, FHV replication is also dependent on phospholipid synthesis in yeast.

DISCUSSION

Phospholipids are major components of cellular membranes, affecting the size, shape and rigidity of cells and intracellular organelles (Nohturfft and Zhang, 2009). Replication of various RNA viruses, which hijack subcellular membranes to induce the formation of viral replication organelles (den Boon et al., 2001; Miller and Krijnse-Locker, 2008a; Novoa et al., 2005) likely depends on phospholipids. Indeed, all the genome-wide screens performed with (+)RNA viruses have led to the identification of a number of host genes affecting lipid biosynthesis or metabolism (Cherry et al., 2005; Krishnan et al., 2008; Kushner et al., 2003; Li et al., 2009a). Similarly, genome-wide screens with TBSV identified at least 14 host genes affecting phospholipid, sterol and fatty acid biosynthesis/metabolism (Jiang et al., 2006; Panavas et al., 2005b). The abundance of the identified host lipid synthesis genes suggests that lipids are important for TBSV replication. Indeed, we have shown previously that sterols affect TBSV replication in yeast, in plants and in vitro (Sharma et al., 2010).

The role of phospholipids in TBSV replication is supported by several pieces of evidence presented in this paper. First, single deletions of *INO2* and *INO4* or the double-

deletion of *INO2-INO4* inhibited TBSV replication in yeast (Figs. 1-2). *INO2* and *INO4* are activators of phospholipid biosynthesis genes, and, thus, their deletions are known to reduce phospholipid levels and prevent membrane proliferation (Block-Alper et al., 2002; Schuck et al., 2009). The *ino2*Δ*ino4*Δ yeast is viable due to the base-level phospholipid biosynthesis that occurs in the absence of Ino2/Ino4 transcription activators. We found that *ino2*Δ*ino4*Δ yeast still can support low level of TBSV replication (Figs. 1-2), likely due to the presence of some phospholipids in the cellular membranes. Second, over-expression of Opi1, which is a repressor of phospholipid biosynthesis by binding to Ino2 (Wagner et al., 1999; Wagner et al., 2001), also inhibited TBSV repRNA accumulation in yeast (Fig. 3.7). Third, over-expression of Ino2 increased TBSV RNA accumulation (Figs. 5-6).

The follow-up experiments revealed that the reduced phospholipid levels affected many steps/processes during TBSV replication. For example, the in vitro activity of the tombusvirus replicase in the membrane-enriched fraction from *ino2*Δ*ino4*Δ yeast was poor when compared with a similar preparation from wt yeast (Fig. 3.3). Since we adjusted the preparations to have comparable amounts of tombusvirus replication proteins, the differences in the template activity of these replicase preparations are likely due to either poor assembly of the replicase complex or the low activity of the replicase in *ino2*Δ*ino4*Δ yeast. Thus, phospholipids affect the activity of the replicase to make viral RNA products.

Another characteristic of TBSV replication in *ino2*Δ*ino4*Δ yeast is the reduced stability of the tombusvirus replication protein (Fig. 3.4A). This reduced stability could be due to incorrect localization of the replication proteins in *ino2*Δ*ino4*Δ yeast. Instead of

the usual punctate structures formed by p33 on the peroxisomal or ER membranes, p33 shows diffused distribution in *ino2*Δ*ino4*Δ yeast, albeit most of the p33 proteins seem to be still associated with membranes based on cell-fractionation and treatment of membranes with alkaline (Fig. 3.8). Based on these data, we propose that the tombusvirus replication proteins are not targeted to the proper subcellular locations in *ino2*Δ*ino4*Δ yeast. This interferes with the assembly of the viral replicase, resulting in reduced replicase activity and possibly faster turnover of the replication protein in *ino2*Δ*ino4*Δ yeast. On the contrary, the intracellular targeting of p33 and the assembly of the replicase might be facilitated by over-expression of Ino2, which resulted in enlarged punctate structures in yeast (Fig. 3.8H-I).

FHV replication also occured at a reduced level in *ino2*Δ*ino4*Δ yeast (Fig. 3.9). This confirms previous findings that phospholipids are important for FHV replication (Castorena et al., 2010). A new finding is the occurrence of a truncated prtA replication protein in *ino2*Δ*ino4*Δ yeast, suggesting that phospholipids could be important to protect prtA from cleavage by cellular proteinases. We did not observe similar abundant truncated products of p33 or $p92^{pol}$ in *ino2*Δ*ino4*Δ yeast, but this could be due to faster degradation of p33 or $p92^{pol}$ that rapidly removes the putative truncated protein products. Overall, the replication proteins of both viruses seem to require phospholipids for enhanced stability.

Although the data shown here support strongly the roles of phospholipids in TBSV and FHV RNA replication, we cannot yet pinpoint the critical phospholipids, since *INO2/INO4* transcription activators affect the production of many phospholipids in yeast (Carman and Han, 2009). A more detailed work on FHV demonstrated that genes

involved in the production of phosphatidylcholine are critical for FHV replication (Castorena et al., 2010). Additional experiments will be needed to identify the critical phospholipids for TBSV replication.

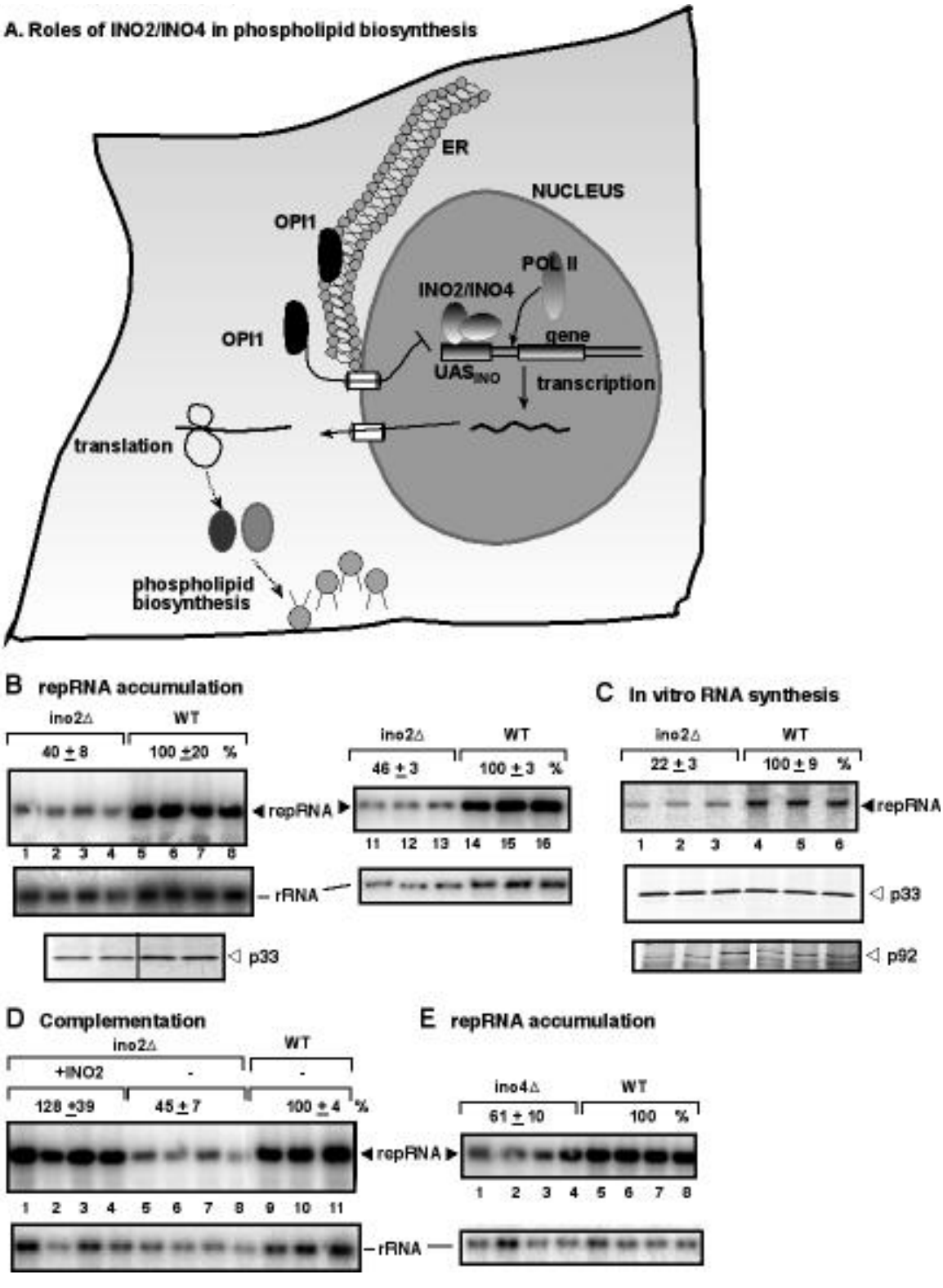

A. Roles of INO2/INO4 in phospholipid biosynthesis
ER
NUCLEUS
OPI1
OPI1
POL II
INO2/INO4
gene
UAS$_{INO}$
transcription
translation
phospholipid biosynthesis
B repRNA accumulation
ino2Δ
WT
40 ± 8
100 ± 20 %
repRNA
1 2 3 4 5 6 7 8
rRNA
p33
ino2Δ
WT
46 ± 3
100 ± 3 %
11 12 13 14 15 16
C In vitro RNA synthesis
ino2Δ
WT
22 ± 3
100 ± 9 %
repRNA
1 2 3 4 5 6
p33
p92
D Complementation
ino2Δ
WT
+INO2
-
-
128 ± 39
45 ± 7
100 ± 4 %
repRNA
1 2 3 4 5 6 7 8 9 10 11
rRNA
E repRNA accumulation
ino4Δ
WT
61 ± 10
100 %
repRNA
1 2 3 4 5 6 7 8
rRNA

Figure 3.1 Deletion of *INO2* and *INO4* inhibits TBSV repRNA accumulation in yeast.

(A) Schematic representation of the regulation of expression of phospholipid biosynthesis genes by Ino2/Ino4 transcription activators and Opi1 repressor. (B) Top panel: Northern blot analysis with a 3' end specific probe was used to detect the accumulation level of the TBSV repRNA in *ino2*Δ or wt (BY4741) yeast. To launch TBSV repRNA replication, we expressed both 6xHis-p33 and 6xHis-p92 from the *ADH1* promoter as well as DI-72(+) repRNA from the galactose-inducible *GAL1* promoter from plasmids. Yeast cells were cultured for 24 hours at 23ºC in 2% galactose SC-ULH$^-$ media. The accumulation levels of repRNA were calculated using Imagequant software. Middle panel: Northern blot analysis to probe ribosomal rRNA, which was used as a loading control. Bottom panel: Western blot analysis of p33 accumulation using anti-His antibody. (C) Decreased tombusvirus replicase activity in *ino2*Δ yeast. An in vitro replicase activity assay was performed with membrane-enriched preparations obtained from *ino2*Δ or wt yeasts grown as in Panel B. The membrane-enriched fraction contains the tombusvirus replicase bound to the endogenous repRNA template that is used during the in vitro replicase assay in the presence of ^{32}P-UTP and the other unlabeled rNTPs. Note that the in vitro activities of the tombusviral replicase were normalized based on p33 (middle panel) levels. Bottom panel shows Western blot analysis of p92 present in the membrane-enriched replicase preparations. (D) Northern blot analysis of TBSV repRNA in *ino2*Δ yeast complemented with Ino2 expression from the low copy pYC-

INO2 plasmid (lanes 1-4), or wt yeast (with the empty pYC plasmid). (E) Northern blot analysis of TBSV repRNA in *ino4*Δ yeast. See further details in Panel B.

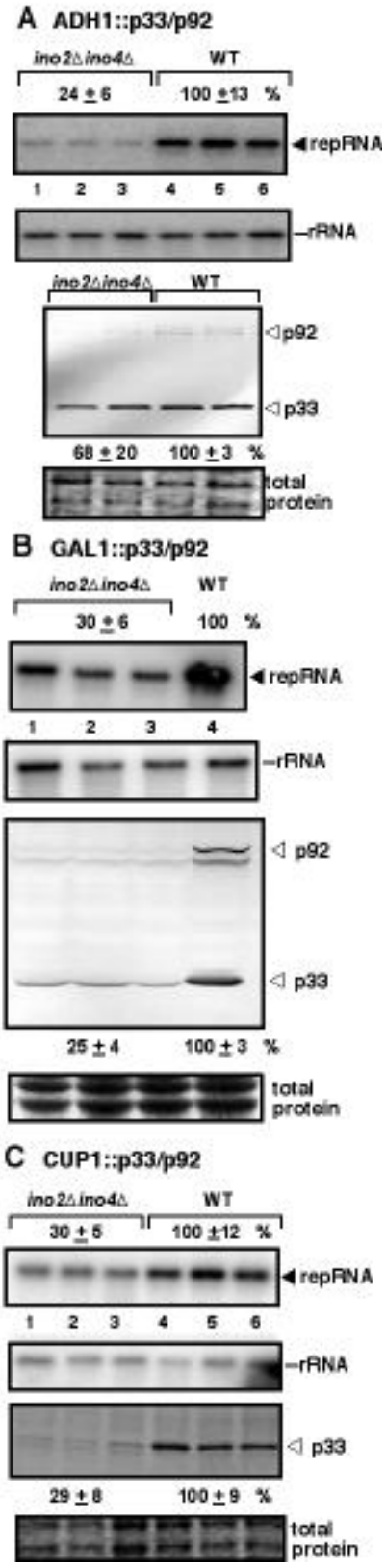

A ADH1::p33/p92
ino2Δino4Δ
WT
24 ± 6
100 ±13 %
repRNA
1 2 3 4 5 6
rRNA
ino2Δino4Δ
WT
p92
p33
68 ± 20
100 ± 3 %
total protein
B GAL1::p33/p92
ino2Δino4Δ
WT
30 ± 6
100 %
repRNA
1 2 3 4
rRNA
p92
p33
25 ± 4
100 ± 3 %
total protein
C CUP1::p33/p92
ino2Δino4Δ
WT
30 ± 5
100 ±12 %
repRNA
1 2 3 4 5 6
rRNA
p33
29 ± 8
100 ± 9 %
total protein

Figure 3.2 Reduced TBSV repRNA accumulation in ino2Δino4Δ yeast.

(A) Northern blot analysis of TBSV repRNA in *ino2*Δ*ino4*Δ or wt yeast. To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the *ADH1* promoter and DI-72(+) repRNA from the galactose-inducible *GAL1* promoter. Yeast cells were cultured for 24 hours at 23ºC in 2% galactose SC- ULH$^-$ media containing 2mg/L inositol. See further details in Fig. 3.1B. (B) To induce TBSV repRNA replication, we expressed 6xHis-p33, 6xHis-p92, and DI-72(+) repRNA from the *GAL1* promoter. See further details in Fig. 3.1B. (C) To induce TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the copper-inducible *CUP1* promoter and DI-72(+) repRNA from *GAL1* promoter. See further details in Fig. 3.1B.

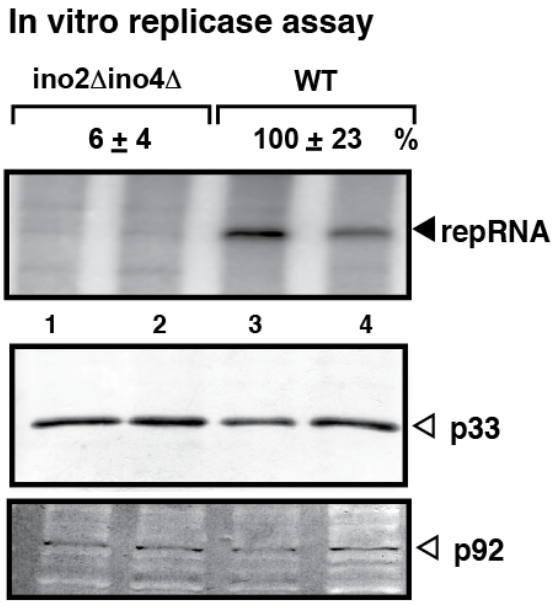

In vitro replicase assay
ino2Δino4Δ
WT
6 ± 4
100 ± 23 %
repRNA
1
2
3
4
p33
p92

Figure 3.3 Reduced tombusvirus replicase activity in *ino2*Δ*ino4*Δ yeast.

TBSV repRNA replication was induced by expressing 6xHis-p33 and 6xHis-p92 from the *ADH1* promoter and DI-72(+) repRNA from *GAL1* promoter in yeast for 24 hours at 23ºC in 2% galactose SC-ULH$^-$ media containing 2mg/L inositol. See further details in Fig. 3.1C.

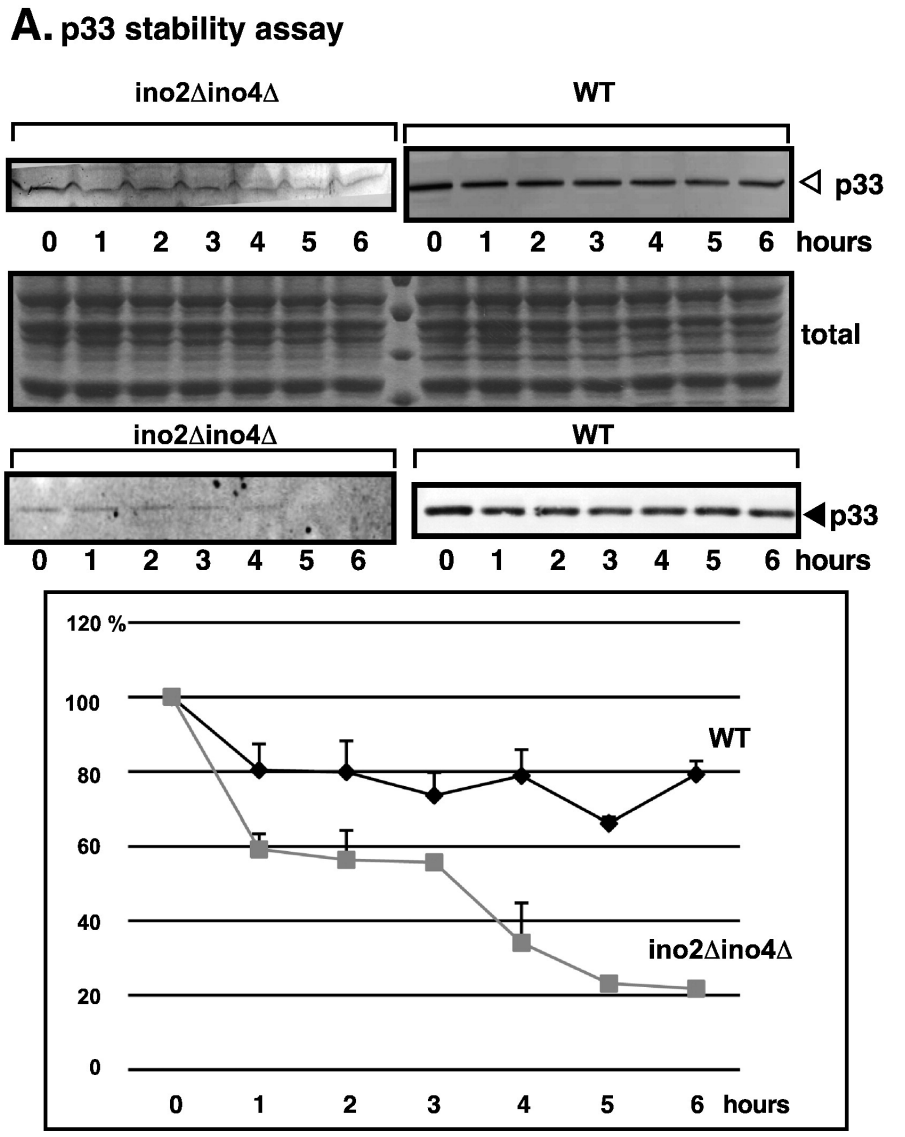

A. p33 stability assay
ino2Δino4Δ
WT
p33
0 1 2 3 4 5 6 hours
total
ino2Δino4Δ
WT
p33
0 1 2 3 4 5 6 hours
120 %
100
80
60
40
20
0
WT
ino2Δino4Δ
0 1 2 3 4 5 6 hours
B. RNA stability assay
ino2Δino4Δ
WT
ino2Δino4Δ
WT
0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 min
*
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
rRNA

Figure 3.4 Reduced half-life of p33 replication protein in *ino2*Δ*ino4*Δ yeast.

(A) Yeast was pre-grown at 29 ºC for 12 hours in SC-U^- with 2% glucose, followed by replacing the media with SC-U^- with 2% galactose for 6 hours to induce the expression of p33, followed by addition of cycloheximide (100 mg/ml). Samples were collected at the shown time points. The amount of p33 was estimated via Western blotting based on anti-His antibody and ECL-Plus. The images were analyzed by a phosphorimager and quantitated via Imagequant. The experiments were repeated three times (two repeats are shown). The error bars represent the upper half of standard error. (B) Yeast was pre-grown at 29 ºC for 12 hours in SC-U^- with 2% glucose, followed by replacing the media with SC-U^- with 2% galactose containing 78mg/L inositol for 12 hours at 23 ºC to induce the expression of DI-72(+) repRNA, followed by replacing the media with SC-U^- with 2% glucose. Samples were collected at the shown time points. The amount of DI-72(+) repRNA was estimated by Northern blotting. * marks the full-length transcripts (uncleaved) carrying nonviral sequences at the 3' end, while the repRNA (which was quantified) carries the authentic TBSV 3' end due to cleavage of the 3' extension by a ribozyme and is pointed at by a solid arrowhead.

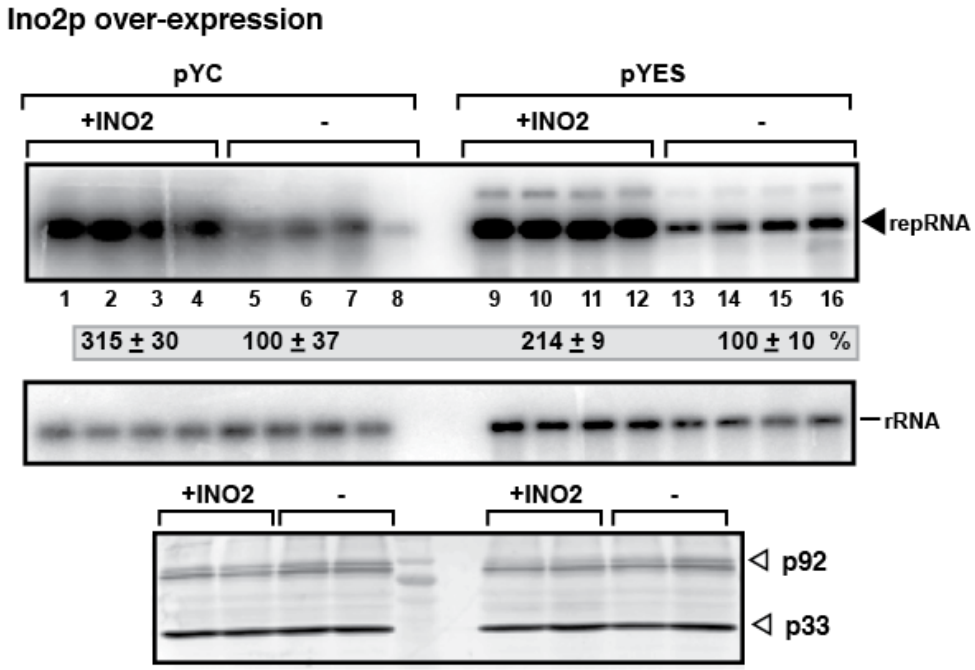

Ino2p over-expression
pYC
pYES
+INO2
-
+INO2
-
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
315 ± 30
100 ± 37
214 ± 9
100 ± 10 %
rRNA
+INO2
-
+INO2
-
p92
p33

Figure 3.5 Over-expression of Ino2 enhances TBSV repRNA replication in yeast.

Top panel: Northern blot analysis of TBSV repRNA in BY4741 yeast over-expressing Ino2 from the GAL1 promoter from the low copy pYC-INO2 plasmid (+INO2, lanes 1-4), or from the high copy pYES-INO2 (+INO2, lanes 9-12). The control yeast carried either the empty pYC or pYES plasmids as shown. The expression of Ino2 started 15 hours before launching TBSV repRNA replication, which started by expressing 6xHis-p33 and 6xHis-p92 from the CUP1 promoter and DI-72(+) repRNA from the GAL1 promoter, and continued to the end of the experiment (24 hours of TBSV replication at 29 ºC). We omitted inositol from the growth media. In addition, we used 100 μM BCS for 15 hours before inducing TBSV replication (to prevent leaky transcription from the CUP1 promoter). See further details in Fig. 1B. Bottom panel shows the Western blot analysis of p33 and p92 replication proteins using anti-His antibody.

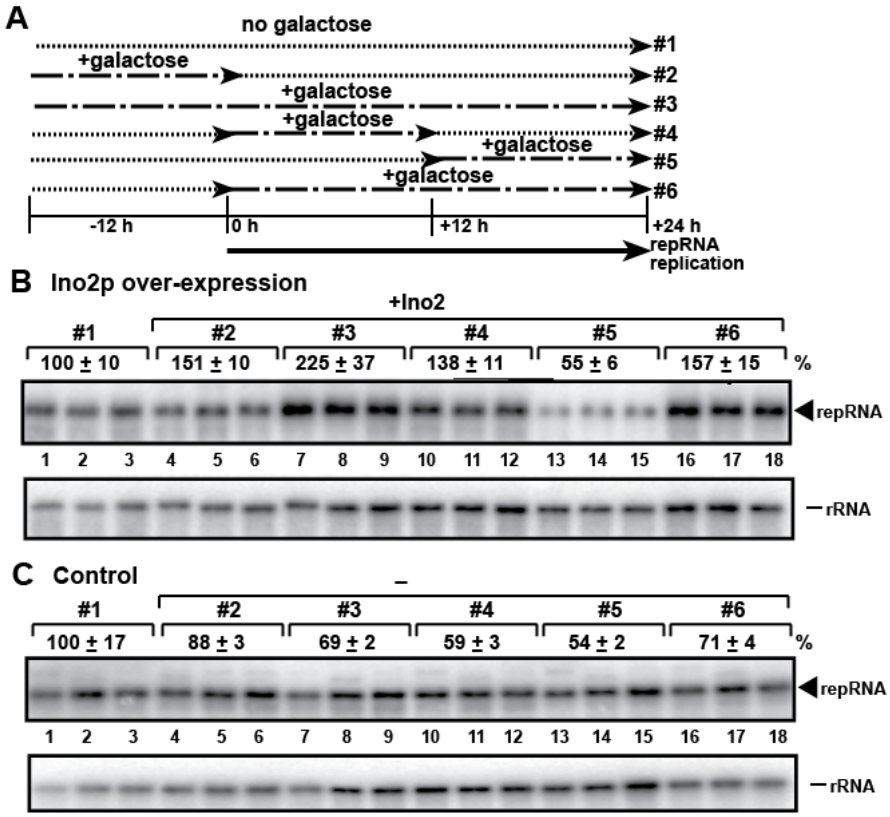

A
no galactose
#1
+galactose
#2
+galactose
#3
+galactose
#4
+galactose
#5
+galactose
#6
-12 h
0 h
+12 h
+24 h
repRNA
replication
B Ino2p over-expression
+Ino2
#1
#2
#3
#4
#5
#6
100 ± 10
151 ± 10
225 ± 37
138 ± 11
55 ± 6
157 ± 15
%
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
rRNA
C Control
–
#1
#2
#3
#4
#5
#6
100 ± 17
88 ± 3
69 ± 2
59 ± 3
54 ± 2
71 ± 4
%
repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
rRNA

Figure 3.6 Over-expression of Ino2 facilitates TBSV repRNA accumulation the most effectively when expressed continuously in yeast.

(A) The scheme of Ino2 over-expression from the *GAL1* promoter from the low copy pYC-INO2 plasmid relative to initiation of repRNA replication. repRNA replication took place for 24 hours at 29 ºC before RNA analysis. (B) Northern blot analysis of TBSV repRNA in yeast samples over-expressing Ino2 as shown schematically in panel A. We omitted inositol from the growth media, which always contained 2% raffinose plus 0 or 2% galactose as shown in panel A. In addition, we used 100 μM BCS for 12 hours before inducing TBSV replication (to prevent leaky transcription from the *CUP1* promoter). Note that treatment #1 (no Ino2 is expressed) is the same as in panel C and it is chosen as 100% to allow comparison between the two panels. (C) Northern blotting shows the level of TBSV repRNA accumulation when a small peptide was over-expressed from the *GAL1* promoter in pYC as shown schematically in panel A. The accumulation level of DI-72(+) repRNA (shown in percentage) was normalized based on 18S rRNA.

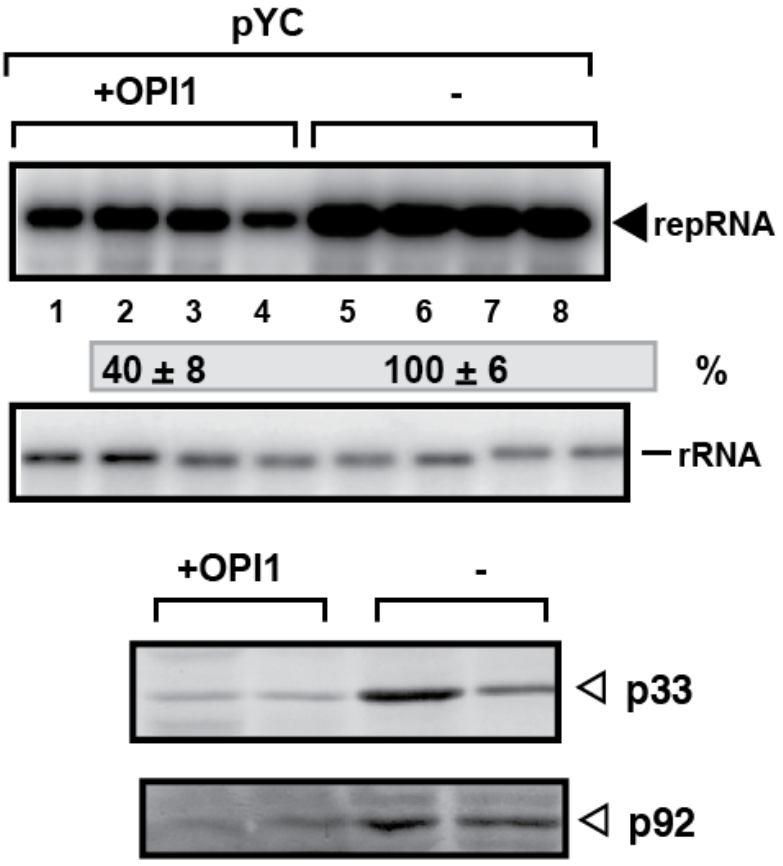

pYC
+OPI1
-
repRNA
1 2 3 4 5 6 7 8
40 ± 8
100 ± 6
%
rRNA
+OPI1
-
p33
p92

Figure 3.7 Over-expression of Opi1 repressor inhibits TBSV repRNA replication in yeast.

Top panel: Northern blot analysis of TBSV repRNA in BY4741 yeast over-expressing Opi1 from the *GAL1* promoter from the low copy pYC-OPI1 plasmid (+OPI1, lanes 1-4). The control yeast carried the empty pYC plasmid (lanes 5-8). The expression of Opi1 started 15 hours before launching TBSV repRNA replication, which started by expressing 6xHis-p33 and 6xHis-p92 from the *CUP1* promoter and DI-72(+) repRNA from the *GAL1* promoter, and continued to the end of the experiment (24 hours of TBSV replication at 29 ºC). We used 100 μM BCS in the media for 15 hours before inducing TBSV replication (to prevent leaky transcription from the *CUP1* promoter). See further details in Fig. 3.1B. Bottom panel shows the Western blot analysis of p33 and p92 replication proteins using anti-His antibody.

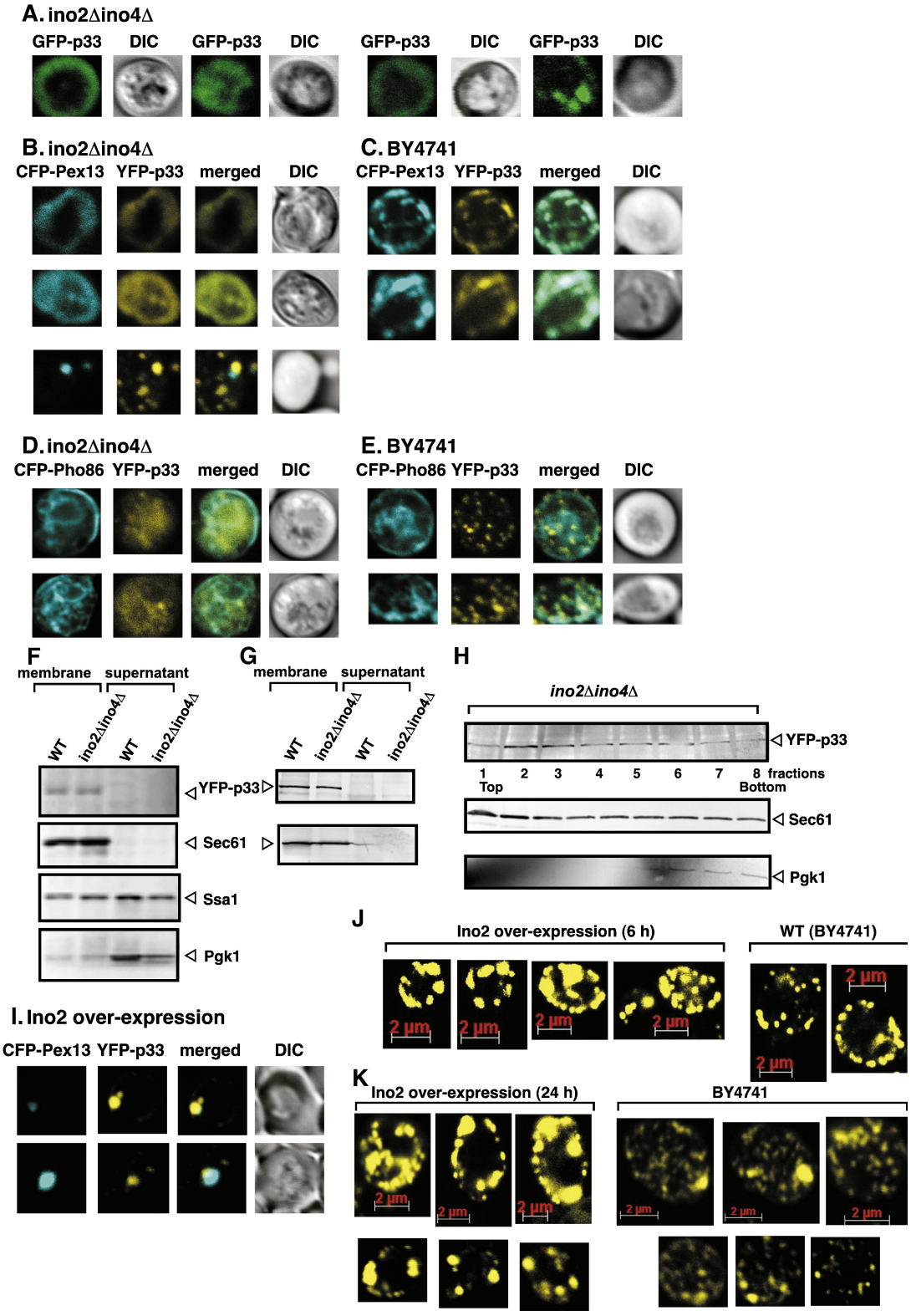

A. ino2Δino4Δ
GFP-p33
DIC
GFP-p33
DIC
GFP-p33
DIC
GFP-p33
DIC
B. ino2Δino4Δ
CFP-Pex13
YFP-p33
merged
DIC
C. BY4741
CFP-Pex13
YFP-p33
merged
DIC
D. ino2Δino4Δ
CFP-Pho86
YFP-p33
merged
DIC
E. BY4741
CFP-Pho86
YFP-p33
merged
DIC
F
membrane
supernatant
WT
ino2Δino4Δ
WT
ino2Δino4Δ
YFP-p33
Sec61
Ssa1
Pgk1
G
membrane
supernatant
WT
ino2Δino4Δ
WT
ino2Δino4Δ
H
ino2Δino4Δ
YFP-p33
1
2
3
4
5
6
7
8 fractions
Top
Bottom
Sec61
Pgk1
I. Ino2 over-expression
CFP-Pex13
YFP-p33
merged
DIC
J
Ino2 over-expression (6 h)
WT (BY4741)
2 μm
K
Ino2 over-expression (24 h)
BY4741
2 μm

Figure 3.8 Phospholipid synthesis is essential for the proper subcellular localization of tombusvirus p33 replication protein in yeast.

(A) Confocal laser microscopy analysis of subcellular distribution of GFP-tagged p33 in *ino2*Δ*ino4*Δ yeast. DIC represents Differential interference contrast microscopic images. (B-C) YFP-tagged p33 co-expressed with CFP-tagged Pex13, a peroxisomal marker protein, in *ino2*Δ*ino4*Δ yeast and in wt BY4741. (D-E) Subcellular localization of YFP-p33 co-expressed with CFP-Pho86, an ER marker protein, in *ino2*Δ*ino4*Δ yeast and in wt BY4741, respectively. Throughout the experiments GFP- or YFP- tagged p33 was expressed from *GAL1* promoter and the marker proteins, CFP-Pex13 and CFP-Pho86 from the *ADH1* promoter. (F) Western blot analysis of p33 (6xHis-tagged) after fractionation from wt (BY4741) and *ino2*Δ*ino4*Δ yeast cells. Sec61 ER protein, Ssa1 both cytosolic and membranous protein, and Pgk1p cytosolic protein were used as controls and detected with specific antibodies. (G) Western blot analysis of p33 after fractionation from wt (BY4741) and *ino2*Δ*ino4*Δ yeast cells. Note that the membrane fraction was treated with alkaline to remove peripheral proteins from the membrane as described in Materials and Methods. (H) Western blot analysis of p33 from wt and *ino2*Δ*ino4*Δ yeast cells after membrane flotation. The top fractions contain the membrane-associated proteins, while the bottom fractions contain soluble or aggregated proteins. See further details in panel F. (I) Confocal laser microscopy analysis of subcellular localization of YFP-p33 co-expressed with CFP-Pex13 peroxisomal marker protein at 6 h time point in yeast over-expressing Ino2. (J) and (K) Localization of YFP-p33 at 24 h time point, in yeast over-expressing Ino2 or in wt (control) background as shown. The bottom row of images are shown at lower magnification of yeast cells to illustrate the presence of ~1-4

large punctate structures in yeast over-expressing Ino2, while smaller punctate structures form in the control BY4741 yeast transformed with the empty vector.

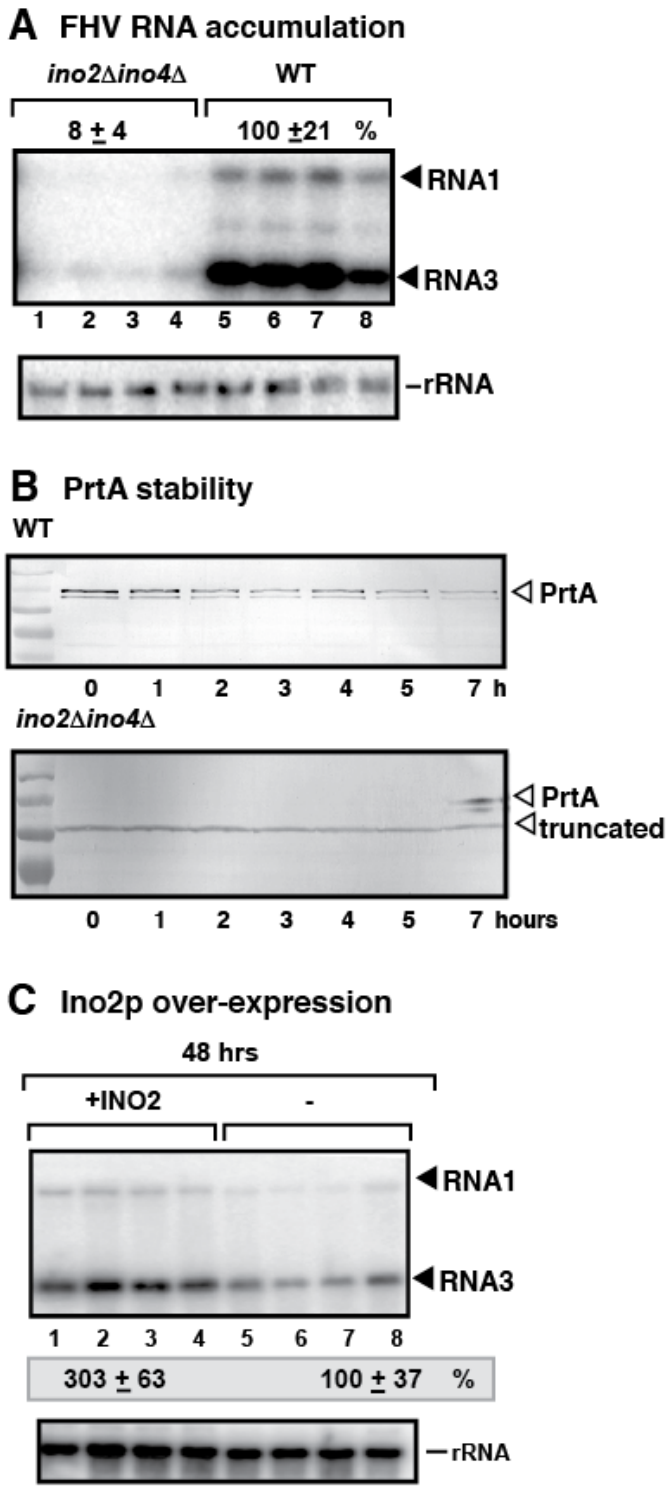

A FHV RNA accumulation
ino2Δino4Δ
WT
8 ± 4
100 ±21 %
RNA1
RNA3
1 2 3 4 5 6 7 8
rRNA
B PrtA stability
WT
PrtA
0 1 2 3 4 5 7 h
ino2Δino4Δ
PrtA
truncated
0 1 2 3 4 5 7 hours
C Ino2p over-expression
48 hrs
+INO2
-
RNA1
RNA3
1 2 3 4 5 6 7 8
303 ± 63
100 ± 37 %
rRNA

Figure 3.9 Deletion of *INO2* and *INO4* inhibits FHV RNA accumulation in yeast.

(A) Top panel: Northern blot analysis with a 3' end specific probe was used to detect the accumulation level of the FHV RNA1 and RNA3 in ino2Δino4Δ or wt (BY4741) yeast. To launch FHV RNA replication, we expressed FHV RNA1 from the copper-inducible CUP1 promoter from a plasmid. Yeast cells were cultured for 48 hours at 29ºC in 2% galactose SC-H^- media containing 2mg/L inositol. The accumulation levels of repRNA were calculated using Imagequant software. The accumulation level of FHV RNA3 (shown in percentage) was normalized based on 18S rRNA. Middle panel: Northern blot analysis to probe rRNA, which was used as a loading control. (B) Western blot analysis of protein A (prtA) accumulation using anti-FLAG antibody. Samples were collected at 0, 1, 2, 3, 4, 5 and 7 hours. Note that sample 7 (bottom panel) also contains a trace amount of full-length prtA, depicted by an arrowhead, as a size control to illustrate the difference in protein A products. (C) Over-expression of Ino2 facilitates FHV RNA accumulation in yeast. Ino2 was over-expressed from the GAL1 promoter from the low copy pYC-INO2 plasmid. FHV replication took place for 48 hours at 29 ºC before RNA analysis. The accumulation level of FHV RNA3 (shown in percentage) was normalized based on 18S rRNA. We omitted inositol from the growth media, which always contained 2% raffinose.

Chapter 4 Role of cardiolipin and its biosynthetic genes in tombusvirus replication

INTRODUCTION

One of the key features of RNA viruses is that they replicate on different host intracellular organeller membranes and assemble their replicase complexes on the cytoplasmic surface of these membranes (Stapleford et al., 2009). To facilitate the membrane associated replicase complex formation viruses contain replication proteins which encode the membrane targeting sequences; Several of them are well characterized (den Boon et al., 2001; Miller and Ahlquist, 2002; Miller et al., 2003; Schaad et al., 1997). However host factors that are necessary for membranes biogenesis and final components of membranes important for virus replicase assembly are not well studied. Identification of such host factors and its final products assisting virus to assemble replicase complex for its efficient replication can be an important target for antiviral (Stapleford et al., 2009).

Positive stranded RNA viruses often induce vesiculation, proliferation, and redistribution of specific intracellular membranes (Lee and Ahlquist, 2003b). A lipid synthesis inhibitor, cerulenin, is known to inhibit RNA replication of Semliki Forest virus, poliovirus, and cowpea mosaic virus, indicates a requirement for lipid and/or membrane synthesis (Lee and Ahlquist, 2003b). Lovastatin, an inhibitor of another lipid (cholesterol) also reduces the replication of WNV (Mackenzie et al., 2007). Furthermore, some of the in vitro studies demonstrated that complete in vitro replication of FHV requires glycerophospholipid (Wu et al., 1992). However, present knowledge of the

contributions of membranes and different lipids to the assembly and function of viral RNA replication complexes is not clear.

Identification of several factors in genome wide screens, suggest that lipids are likely involved, directly or indirectly, in TBSV replication in yeast. Accordingly, previous studies (Chapter 1 and 2) showed that *INO2/ INO4*, positive regulator of phospholipids and ergosterol are critical for TBSV replication (Sharma et al., 2010, 2011).

Ino2p/Ino4p transcription factors regulate the synthesis of hundreds of genes involved in phospholipid biosynthesis in yeast. Likewise several positive strand RNA viruses require phospholipids during their replication. Previous work from our lab established the importance of phospholipids in TBSV replication but could not pinpoint which phospholipids in particular are being utilized by tombusvirus. For e.g. FHV uses phosphotydyl choline and cardiolipin during replication (Stapleford et al., 2009). Polioviruses depend upon glycerotadyl choline (Vance et al., 1980). Recently it has been shown that PIP4 levels are critical for replication of poliovirus (Hsu et al., 2010) Similarly downregulation of phosphoinositol synthesizing genes in mammalian cells compromised the replication of Hepatitis C virus (Reiss et al., 2011). In this study, we wanted to know which phospholipids(s) are utilized by TBSV during its replication. During the genome wide studies conducted in our lab, specific genes were not found altering the replication of TBSV. It's not surprising because its known that different phopholipid synthesis pathways exist in the yeast cells and also that individual products can also be complemented for each other (Osman et al., 2010). In order to discover the individual phospholipids in TBSV RNA replication, we exploited the lipid arrays and

tested viral replication proteins for its binding to particular lipid. Cardiolipin positively bound to the TBSV replication proteins. Cardiolipin, is a dimeric phosphoglycerolipid mainly present in mitochondrial membranes. It plays important roles in the cellular energy metabolism mitochondrial dynamics and in the initiation of apoptotic pathways (Osman et al., 2010).

We further focused on cardiolipin and its biosynthetic pathways because peroxisomes are the second most abundant sink of cardiolipins in the yeast cells (Zinser et al., 1991). Moreover these are the only phospholipids in yeast cells with four different carbon chains rendering it highly anionic. Accordingly, we demonstrate in this paper that deletion of *crd1* and *gep4* leads to a drastically low level of TBSV repRNA accumulation in yeast. *crd1* and *gep4* are crucial factors for the synthesis of cardiolipins (Osman et al., 2010). We demonstrate that cellular localization of the tombusvirus replication protein changes in crd1Δ yeast. We also show that the replication of nodamuravirus is inhibited in crd1Δ yeast broadening our discovery to other positive strand RNA viruses. This work indicates that cardiolipin biosynthesis is required for efficient replication of some (+) strand RNA viruses.

MATERIAL AND METHODS

Yeast and bacterial strains:

Saccharomyces cerevisae strains s288c, *crd1*Δ, *gep4*Δ, and *pgc1*Δ were generous gifts from Dr. Thomas Langer of Max-Planck-Institute for Biology of Aging, Cologne, Germany. Top10 strain of *E.coli* was used for cloning purposes.

Plasmids:

To express Crd1, Gep4, Pgc1 in yeast, their ORFs were cloned into pYC vector containing 6X His residues on amide terminal end using *Bam*H1 and *Xho*I sites. Following primers appended with *Bam*H1 and *Xho*I recognition sequences respectively to facilitate directional cloning were used to amplify the desired ORFs: (i) Crd1: #4748 (AAAATGATTCAAATGGTGCCCATTTATTCATGCTCC) and #4749 (CTATTTTAAAAGTTTAAAAGCGTTTCTC) (ii) Gep4: #4752 (AAAATGAACATCAGTGGCACCTTAAATACGC) and #4753 (TCAAAATCCCAAAAAGTTGTATAAT) and (iii) Pgc1: #4750 (AAAATGGTTGAAATTGTGGGCCACAGAGCTTTTAAA) and #4751 (TCAAAGAAAATGAATGGTTCGAAGGAAC).

TBSV replication assay in yeast

Yeast strains s288c, *crd1*Δ, *gep4*Δ, and *pgc1*Δ were transformed with the plasmids pHisGBK-CUP-His33/GAL-DI72 (Jaag et al., 2010) and pGAD-CUP-Hisp92 (Sharma et al., 2011). Replication assay was performed by measuring the accumulation of DI-72(+) repRNA relative to the 18S rRNA (Panavas and Nagy, 2003a).

Protein Analysis

Yeast strains were grown as described above for RNA analysis. A total of 2ml of yeast culture was harvested, the pelleted cells were resuspended in 200 µl of 0.1M NaOH and incubated at 23°C for 10 min. NaOH was aspirated after a short centrifugation, and the samples were resuspended in 100µl, 1X SDS-polyacrylamide gel electrophoresis (PAGE) buffer containing 5% β-mercaptoethanol and boiled for 5 min. The supernatant was used for SDS/PAGE and Western blot analysis as described in by Panavas et al.,

(2005). The primary antibodies were anti-6x His (Invitrogen), and the secondary antibodies were alkaline-phosphatase-conjugated anti-mouse immunoglobulin-G (Sigma).

Confocal microscopy

Yeast cells were transformed with YFP tagged p33 (pESC-YFP-33). To visualize peroxisome, pex13-CFP was used. Cells were pregrown overnight at 29°C and then transferred to media containing 2% galactose at 32°C and samples were collected after 6 hrs and 24 hrs.

Nodamura virus replication assay

S288c and all deletion strains were transformed with **pESC-His-Cup-NOV-RNA1-TRSV** (provided by Dr. Judit Pogany, University of Kentucky). After pregrowing at 29°C, media was supplemented with 50μM Cu^{++} and samples were divided into two batches and transferred to 29°C and 32°C respectively and harvested after 48 hrs for RNA analysis.

Overexpression of host proteins

pYC-Crd1, pYC-Gep4, pYC-Pgc1, pYC-empty, plasmids were transformed into s288c strain containing pGAD-His92-Cup1, pHisGBK-CUP-His33/GAL-DI72, which expresses p33 protein from the regulatable CUP1 promoter and DI72 under inducible GAL promoter. Cells were pregrown at 29°C for 15 hrs in 2 glucose media followed by supplementation with 50μM Cu^{++} and further incubation at 29°C for 24 hrs. To over-

Complementation assay

pYC-Crd1, pYC-Gep4, pYC-Pgc1, pYC-empty plasmids were transformed into *crd1*Δ, *gep4*Δ, *pgc1*Δ and s288c strains containing pGAD-His92-Cup1, pHisGBK-CUP-His33/GAL-DI72 (expresses p33 protein from the inducible Cup1 promoter and DI72 under inducible GAL1 promoter). All the steps of yeast transformation, yeast cultivation and RNA analysis were same as described above.

RESULTS

TBSV replication proteins binds to cardiolipin

TBSV RNA is synthesized in close association of membranes and viral replication proteins, which include the p33 and p92. We tested whether these proteins had themselves any affinity for any kind of lipid. We assayed for binding of p33 and p92 by incubating MBP tagged purified recombinant MBP tagged p33 and p92 proteins with membrane strips spotted with different types of cellular lipids and MBP alone as a control. After washing and detecting with anti-MBP antibody we found that these replication proteins bound to cardiolipin and sulfatide (ceramide) lipids over all other phospholipids (Figure 4.1). Thus either p33 or p92 replication protein alone, independent of any other components of the viral replication complex, has an affinity for cardiolipin and sulfatide lipids that potentially can regulate both its binding to cellular membranes and its subsequent RNA synthesis activities.

Deletion of Gep4 and Crd1 cardiolipin biosynthetic genes in yeast reduces TBSV RNA accumulation whereas deletion of Pgc1 increases its accumulation.

To find out the relevance of cardiolipin in TBSV replication, we tested number of genes involved in the cardiolipin biosynthesis pathway. This pathway has two genes Crd1 and Gep4 involved in cardiolipin biosynthesis and Pgc1, which maintains the optimum amount of cardiolipin in cell by degrading excess phosphatidylglycerol (PG) (Simockova et al., 2008) (Fig. 4.2A). *gep4*Δ, *crd1*Δ, *pgc1*Δ along with wild type strain (s288c) were tested for their ability to accumulate TBSV RNA accumulation. Northern blot showed that in *gep4*Δ*, crd1*Δ yeast strains replication was 25% and 3% compared to parental strain respectively. (Fig. 4.2B, lanes 13-16, 5-8 versus 1-4, 9-12). *pgc1*Δ yeast strain showed increase in rep RNA accumulation compared to the parental strain (Fig. 4.2B, lanes 21-24 versus 17-20). Increase in rep RNA accumulation in *pgc1*Δ yeast strain is most likely because of increased amount of cardiolipin. The expression level of p33 was not affected in all of these deletion strains, while accumulation level of p92 was less in *crd1*Δ yeast strain (Fig. 4.2C, lanes 5-6 versus 3-4).

Overexpression of cardiolipin genes increases TBSV RNA accumulation in yeast

Overexpression of CRD1 has been shown to increase the cardiolipin synthase activity in yeast (Chen et al., 2010). We have found over expression of 6X His NH_2-tagged Gep4 and Crd1 from the low copy number plasmid led to around 1.5 fold (Fig. 4.3B, lanes 9-12 versus 5-8) and 2.5 fold (Fig. 4.3B, lanes1-4 versus 5-8) increase in TBSV replication respectively. Overexpressing Gep4 without any tag increases TBSV replication around 2 fold compared to an empty vector in wild type background (Fig. 4.3B, lanes 13-14 versus 5-8). The level of p33 and p92 replication proteins did not

change in these strains, suggesting that the increased RNA accumulation was likely due to increased activity of tombusvirus replicase. We have also found overexpression of Pgc1 decrease the TBSV replication to 27 % compared to wild type (Fig. 4.3B, lanes 18-21versus 15-17).

Providing cardiolipin back in the yeast cells via CRD1 and GEP4 can rescue viral replication.

Plasmid-born Crd1 and Gep4 could complement the negative effect of their deletion on TBSV RNA accumulation (Fig.4.4A (lanes 1–4) and B, (lanes 1–3) respectively. Where as control experiments showed decrease in RNA accumulation in *crd1* and *gep4* deletion yeast strain (Fig. 4.4A (lanes 5-8) and B, (lanes 5-7). We have found crd1 could increase RNA accumulation around 2 times compared to parental strain expressing empty plasmid (Fig.4.4A lanes 1–4 versus lanes 9-12).

Cardiolipin is required for proper subcellular localization of tombusvirus replication proteins

To find out the subcellular localization of p33 in *crd1*Δ yeast, we co-expressed YFP-p33 with CFP-Pex13, which is a peroxisomal marker protein YFP-p33 showed the diffused pattern in the majority of yeast cells while small amount of p33 proteins also found in punctate form especially after 6 hrs of induction of virus replication (Fig.4.5A) which is different from the usual number of punctate structures and co-localization as seen in the wt s288c strain (Fig.4.5B). These punctate structures after 24 hrs in *crd1*Δ were very less than wt s288c strain (Fig.4.5D) instead having unusual diffused pattern of p33 (Fig.4.5C) suggesting that these few punctate structures formed by p33 are able to support 10% of the replication we observe in experiments shown in Fig. 4.2. The

peroxisomal protein marker pex13p was very diffused in the *crd1*Δ cells (data not shown) probably because of deranged peroxisomal membranes.

Cardiolipin is needed for replication of Nodamura virus.

To test if the reduced cardiolipin synthesis in different deletion yeast strains could also affect the replication of another RNA virus, we chose *Nodamura virus* (NoV) (Price et al., 2005), which is virus distantly related to TBSV, can be efficiently replicated in yeast (Price et al., 1996), with both viruses belonging to the Flavivirus-supergroup among (+)RNA viruses. The accumulation of NoV RNA1 and the subgenomic RNA3 (produced from RNA1 during replication) was reduced in all the deletion yeast strains where cardiolipin is supposed to accumulate to a low level (Fig. 4.6). When I performed the experiments at 32°C the differences between wildtype and deletion strains became even more dramatic. (Fig. 4.6 Lanes 1-24). Interestingly, deletion of Pgc1 that produces a factor critical for removal of PG, increases NoV RNA accumulation (Fig 4.6 lanes 21-24).

DISCUSSION

In the previous chapter we found strong evidences of role of phospholipid biosynthesis genes INO2 and INO4 for TBSV and FHV. This chapter presents several evidences that suggest that cardiolipins are one of the particular phospholipids cruicial for TBSV and NoV replication in yeast. First, we found that cardiolipin bound to the TBSV replication proteins p33 and p93 (Fig. 4.1). Second, deletions of CRD1 or GEP4 or the double-deletion of the same inhibited TBSV replication in yeast (Figs. 2). *CRD1* and *GEP4* are critical for synthesis of cardiolipin and, thus, their deletions are known to reduce cardiolipin levels (Osman et al., 2010). Second, deletion of Pgc1, which is a factor

removing PG that is a precursor of CL increased TBSV repRNA accumulation in yeast (Fig. 4.2). Third, over-expression of CRD1 and GEP4 increased TBSV RNA accumulation whereas PGC1 reduced it as expected (Fig. 4.3). And lastly, all the phenotypes observed in deletion strains could be complemented when the missing proteins were provided via plasmid expression (Fig. 4.4).

The follow-up experiments revealed that the reduced cardiolipin levels affected the quality of punctate structures probably because of diffused localization of replication protein p33 (Fig. 4.5). Thus, phospholipids affect the activity of the replicase to make viral RNA products. Based on this data, I propose that the tombusvirus replication proteins are not targeted to the proper subcellular locations in cardiolipin deficient yeast.

NoV, replication is known to occur on the membranes of mitochondria (Garzon et al., 1990). Also highest concentration of cardiolipin is found in the outer mitochondrial membranes (second highest being peroxisomal membranes) (Zinser et al., 1991). Dramatic decrease in NoV RNA accumulation in cardiolipin deficient yeast strains further confirms the importance of cardiolipins in NoV replication.

Altogether, in this chapter I was able to pinpoint the exact phospholipid critical for tombusvirus and further extended its relevance to a related insect virus (can infect mammalian cells under artificial conditions). To decipher the molecular details of "How this lipid helps tombusvirus replication" needs further investigation. The features of cardiolpin that TBSV might be utilizing are its anionicity and high level of unsaturation apart from the presence of four aliphatic chains instead of two in the common lipids.

Gaining this kind of knowledge is proving very helpful for the virologists wanting to develop antivirals.

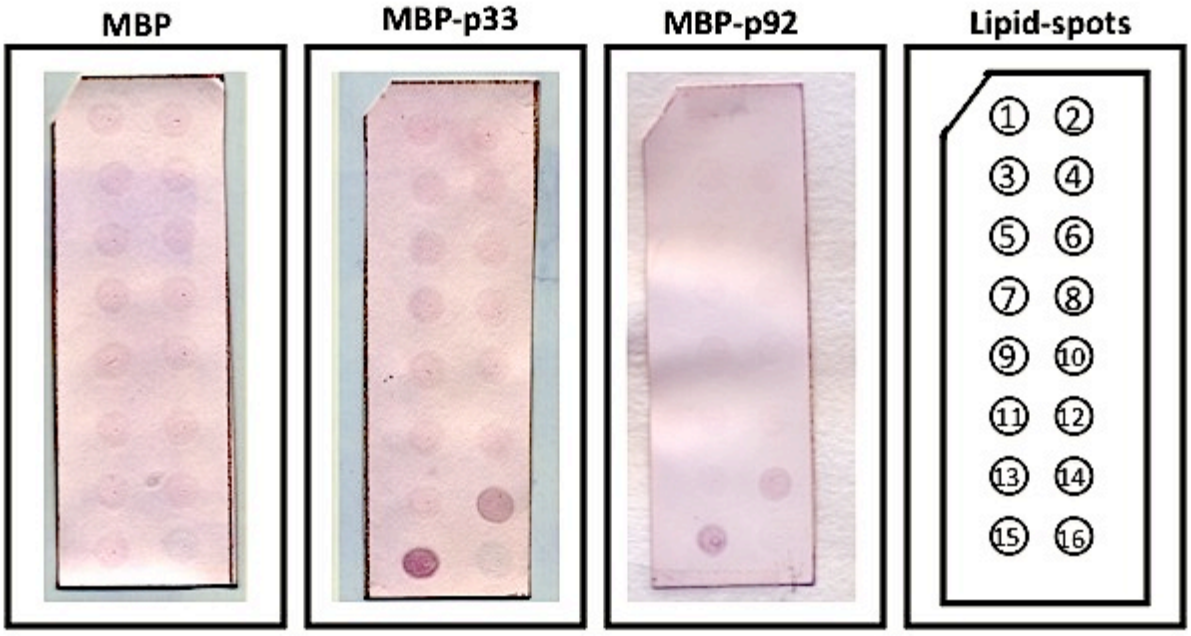
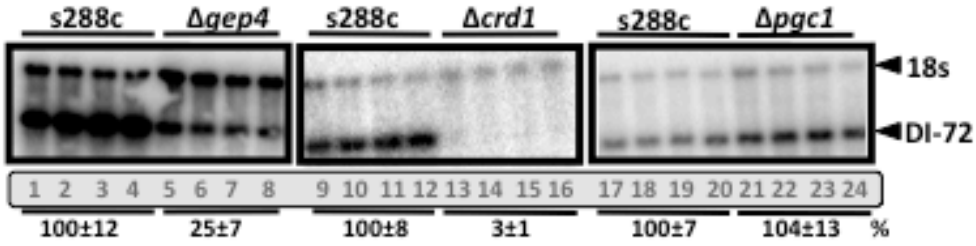

MBP
MBP-p33
MBP-p92
Lipid-spots
1 2
3 4
5 6
7 8
9 10
11 12
13 14
15 16

Figure 4.1 Cardiolipin binds to tombusviral replication proteins.

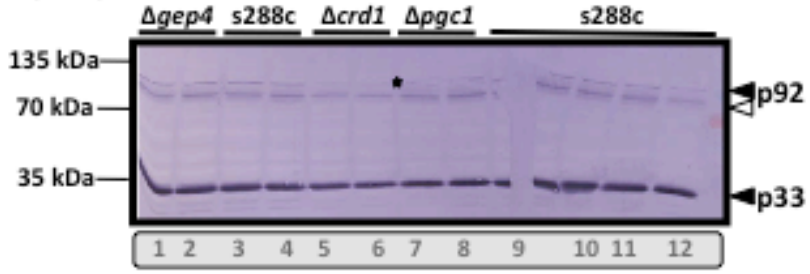
NH_2 – terminal MBP tagged p33 and p92 were expressed and purified using amylose column chromatography and normalized to the similar amounts as MBP that served as a negative control. Purified proteins were incubated with lipid strips pre-spotted (far right map, including cardiolipin at No. 15) with fifteen lipids followed by washing and detection using anti-MBP antibody. The other lipids spotted on the strip are as follows ①Triglyceride ②Phosphatidylinositol (P_i), ③Diacylglycerol (DAG), ④PtdIns(4)P, ⑤Phosphatidic acid (PA), ⑥PtdIns(4,5)P_2 , ⑦Phosphatidylserine (PE), ⑧PtdIns(3,4,5)P_3, ⑨Phosphatidylethanolamine (PE), ⑩Cholesterol, (11) Phosphatidylcholine (PC), (12) Sphingomyelin, (13) Phosphatidylglycerol (PG), (14) 3-sulfogalactosylceramide (Sulfatide), (15) Cardiolipin (CL) and (16) Blank

A. Cardiolipin biosynthesis pathway

B. TBSV repRNA accumulation



C. p33/p92 accumulation



D. Total protein

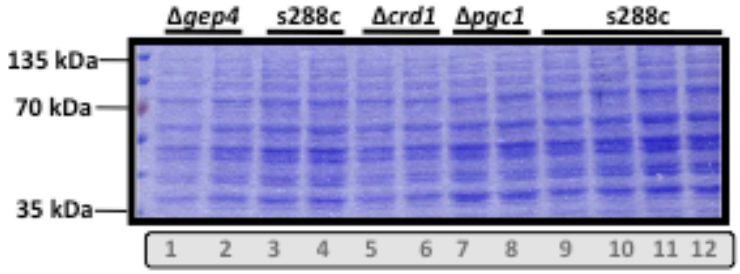
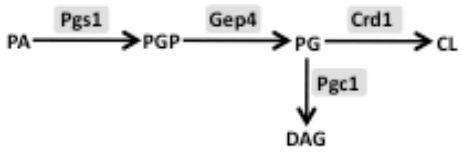


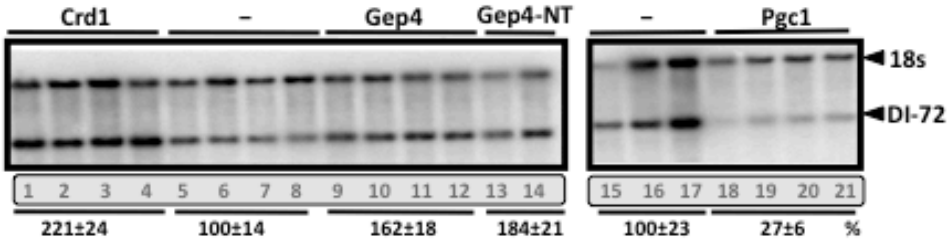
Figure 4.2 Effect of deletion of cardiolipin metabolic genes on TBSV RNA accumulation.

(A) Schematic representation of the cardiolipin biosynthesis pathway. (B) Northern blot analysis to detect TBSV repRNA in *gep4*Δ*, crd1*Δ, *pgc1*Δ and wt (s288c) yeast strains. To launch TBSV repRNA replication, we expressed both 6X His-p33 and 6XHis-p92 from the *CUP1* and DI-72(+) repRNA replication under the *GAL1* promoters respectively. Yeast cells were grown in induction media for 24 hrs at 32°C. RepRNA values were normalized as per their ribosomal RNA (18S) accumulation (C) Western blot analysis of p33 and p92 using anti-His antibody and (D) Coomassie blue stained gel showing total protein accumulation

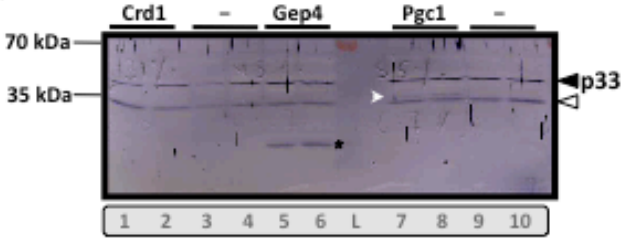
A. Cardiolipin biosynthesis pathway



B. TBSV repRNA accumulation



C. Western blot



D. Total protein

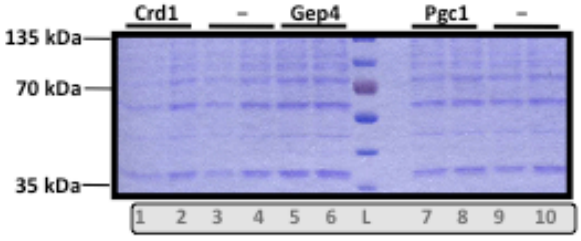
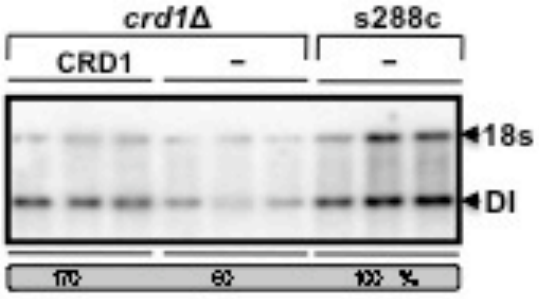


Figure 4.3 Over-expression of Cardiolipin biosynthesis factors TBSV in yeast.

(A) Scheme showing the metabolic factors in cardiolipin synthesis in yeast. (B) Northern blot analysis of TBSV repRNA in BY4741 yeast over-expressing Amide-terminally 6XHis tagged Crd1, Gep4, Gep4-NT (untagged) and Pgc1 from *GAL1* promoter from a low copy pYC-plasmid. The control yeast (–) carried the empty pYC plasmid. The expression of proteins started 15 h before launching TBSV repRNA replication, which started by expressing 6xHis-p33 and 6xHis-p92 from the *CUP1* promoter and DI-72(+) repRNA from the *GAL1* promoter, and continued to the end of the experiment (24 h of TBSV replication at 29 °C). We used 100 μM BCS in the media for 15 h before inducing TBSV replication (to prevent leaky transcription from the *CUP1* promoter). (C) Western blot analysis using anti-His antibody shows p33 (solid arrowhead) Gep4p (Star) and Pgc1p (white arrowhead). An empty arrowhead marks a putative host protein detected by the antibody (D) Coomassie staining showing total protein isolated from the yeast cells from panel (C).

Complementation assay

A



B

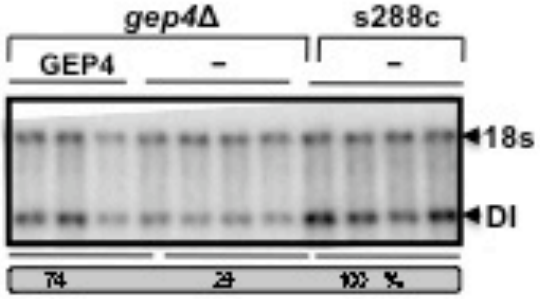


Figure 4.4 Viral replication complementation by cardiolipin biosynthetic genes.

(A) Northern blot analysis of TBSV repRNA in wt (s288c) and *crd1*Δ yeast strains expressing amide-terminally 6XHis tagged CRD1 or just the tag as a control (-) in a low copy centromeric plasmid. Growing conditions and other methods were similar as in Figure 4.3. (B) Similar experiments as in (A) were performed in *gep4*Δ and wt yeast strains.

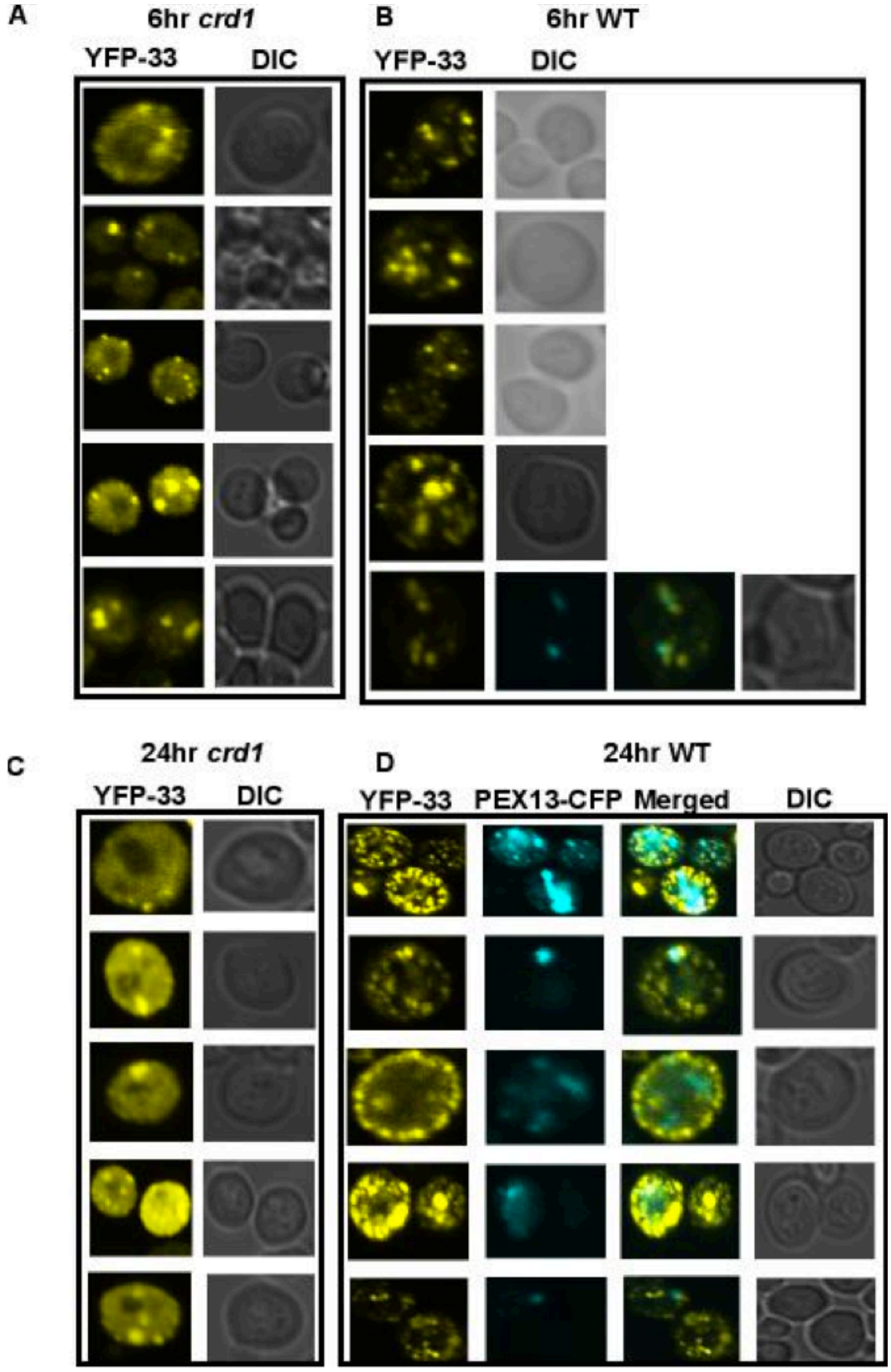

A
6hr crd1
YFP-33
DIC
B
6hr WT
YFP-33
DIC
C
24hr crd1
YFP-33
DIC
D
24hr WT
YFP-33
PEX13-CFP
Merged
DIC

Figure 4.5 Cardiolipin synthesis is essential for the proper subcellular localization of tombusvirus p33 replication protein in yeast.

YFP-tagged p33 co-expressed with CFP-tagged Pex13, a peroxisomal marker protein, in *crd1*Δ (A,C) yeast and in wt s288c (B,D). Throughout the experiments YFP-tagged p33 was expressed from *GAL1* promoter and the peroxisomal marker protein and Pex13-CFP from the *ADH1* promoter. Note that pex13-CFP was below detection limits in *crd1*Δ cells.

Nodamura virus RNA accumulation

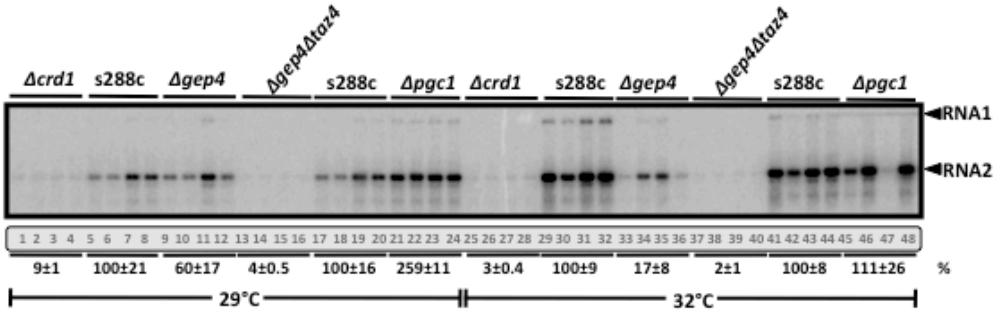


Figure 4.6 Effect of deletion of cardiolipin metabolic genes on *Nodamura virus* RNA accumulation.

Northern blot analysis to detect NoV RNA1 and RNA3 in *gep4*Δ, *gep4*Δ*taz4*Δ, *crd1*Δ, *pgc1*Δ and wt (s288c) yeast strains. A 3′ end specific probe was used to detect the accumulation level of the NoV RNA1 and RNA3 in deletion strains or wt (BY4741) yeast. To launch NMV RNA replication, we expressed NMV RNA1 under the copper-inducible *CUP1* promoter from a plasmid. After pre-growth, cells were cultured for 48 h at 29°C and 32°C in 2% galactose media containing 50μM Cu^{++}. The accumulation of RNA2 was calculated using Imagequant software.

Chapter 5 Summary and Discussion

Tombusvirus is a positive stranded RNA virus involves interaction of host factors as well as viral factors at different steps of its replication cycle. Viral RNA in early stages interacts to the cellular factors to produce viral replication proteins. These proteins along with host cellular factors and cellular membranes facilitate viral RNA to assemble replicase complex and helps in efficient replication. There are evidences that viral RNA components form different complexes with different proteins, membranes in host cell, so it is important to identify different host factors to dissect their role in viral replication.

Lot of efforts has been made in these areas in recent years. Many genetic and biochemical approaches has been performed and identified over 100 factors affecting tombusvirus replication. Identified host factors can be divided into two categories according to their effect on viral replication: One group has positive effect (enhancers) and other has negative effect (inhibitors) on virus replication. Among 'enhancers' around 14 factors have been identified which has role in different lipid biosynthesis pathway, which indicates the importance of lipids in viral replication. In the essential yeast library screen, we found a possible positive regulator ERG25, whose downregulation led to decreased TBSV rep RNA accumulation. ERG25 encodes for an intermediate enzyme C-4 methyle oxidase in the ergosterol biosynthesis pathway of yeast (Fig. 5.1). Ergosterol is an important component of cellular membranes involved in various functions like maintaining fluidity, flexibility, rigidity, and permeability. It also helps membranes to interact with different proteins and lipids. Sterols are conserved among different kingdoms like fungi mammals and plants minor differences in the several steps of its synthesis.

To see the effect of ERG25 on TBSV repRNA accumulation, I added doxycycline and observed rapid decrease in mRNA levels as early as 5 hrs and found an effect on rep RNA accumulation of virus. I also used one of the well-known inhibitor of ERG25 named APB (Fig. 5.1) that reduced replication in yeast by 3-to 5- folds. Similar inhibitory effects were observed using lovastatin, an inhibitor of another factor of the same pathway, suggesting that the sterol biosynthesis pathway is required for TBSV replication.

In order to find out this effect is specific to peroxisome or also effects endoplasmic reticulum, another replication site for TBSV, we used Δpex19 yeast strains where peroxisomes are absent and treated these cells with APB. This treatment decreased TBSV replication, which indicates that TBSV replication is hugely affected by sterols, regardless of the site of replication.

We also down regulated yeast orthologs of ERG25 in *N. benthamiana* SMO1 and SMO2 and observed comparable inhibition of viral RNA accumulation. This inhibition could be complemented in plant protoplasts by adding exogenous stigmasterol. These experiments strongly suggest that sterols are important component in tombusvirus replication.

The molecular mechanism by which the sterols affect TBSV replication is not known. The shortening of half-life of p92 molecules in the yeast knocked-down for sterol suggested the dependence of the bulky polymerase protein on perfect membrane structure thereby protecting it from the cellular proteases. Alternatively, deranged membranes could have changed the intra-cellular localization of p92. Its noteworthy, the stability of p33 (three times smaller molecule than p92) did not change in sterol-depleted cells. We

also performed *in vitro* wheat germ translation assay, which had no inhibitory effect on translation of p33 and p92 replication proteins.

We identified another lipid metabolism host gene Ino2 whose deletion halved the total rep-RNA accumulation. Ino2p is a positive transcriptional regulator of number of genes involved in phospholipids biosynthesis pathway (Fig. 5.1) Ino2p is a basic helix loop helix protein, forms heterodimer with another protein Ino4p for its activator activity and with Opi1p for its inhibitory function (Fig. 5.1). In order to find out if the inhibition in viral replication in absence of Ino2, was direct effect of a lack of ino2 or because of some other factors affected, I provided back Ino2p and observed complementation in replication. Double deletion of ino2 and ino4 reduced the TBSV replication in yeast up to five-folds. However we did not observed any effect on RNA stability in these yeast strains. Based on these observations we can conclude that this effect is not because of the effect of transcription factor on transcription of DI72 plasmid. I also tested stability of p33 in these strains and observed degradation is much faster.

Overexpresssion of Opi1p in BY4741 yeast strain, a negative regulator of phospholipid biosynthesis pathway also reduces virus replication, supports the importance of lipids in TBSV replication. Similarly over expression of Ino2 increases the DI accumulation around 3 times. I also tested the pattern of p33 localization in these cells and found bigger punctate structure compared to wild type. It might be possible that virus induced phospholipids may help p33 replication protein to concentrate efficiently and better replicase complex formation.

In the fourth chapter, I was able to pinpoint the lipid usage of tombusvirus and NoV to cardiolipin, a lipid enriched in mitochondrial and peoxisomal membranes (Fig.

5.1). The yeast strains lacking in the genes involved in cardiolipin biosynthesis pathway also were not able to support good level of tombusvirus replication. Furthermore, cardiolipin deficient yeast cells were unable to maintain the typical punctate structures by the TBSV replication auxiliary protein p33 (Fig. 5.1). These structures are the special structures where high level of TBSV replication occurs.

Overall, my research work shows that two different classes of lipids, cardiolipin a phospholipid and ergosterol are used for efficient replication of tombusvirus and other related viruses in yeast and in plants.

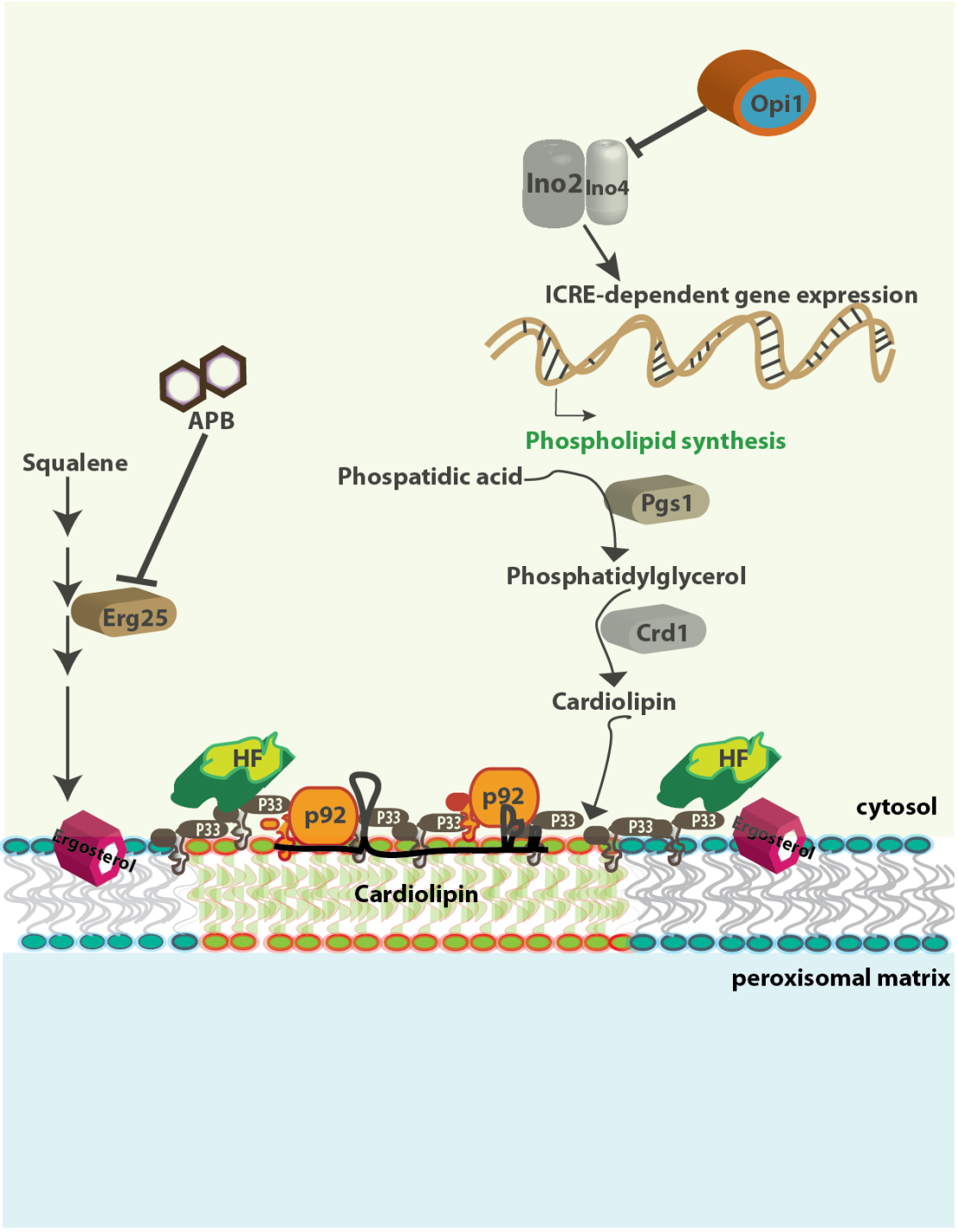

Opi1
Ino2
Ino4
ICRE-dependent gene expression
Phospholipid synthesis
APB
Squalene
Phospatidic acid
Pgs1
Phosphatidylglycerol
Erg25
Crd1
Cardiolipin
HF
HF
P33
p92
p92
cytosol
Ergosterol
Ergosterol
Cardiolipin
peroxisomal matrix

Figure 5.1 Tombusvirus utilizes host lipid factors and peroxisomal membranes to form replication complex.

Cartoon showing p33 and p92 targeted to peroxisomal membranes together with the viral RNA (black freeform) and other host factors (HF) to initiate the formation of replicase complex. Replication proteins bind to cardiolipin (middle half of the lipid bi-layer), which is generated via Pgs1, Crd1 metabolic pathway. It is under the ICRE gene expression system regulated by Ino2/Ino4 transcription factors. Opi1 binds to Ino2 and inhibits the Ino2/Ino4 dimer formation that turns off the transcription of the phophsolipid genes. Squalene is converted to ergosterol via multiple metabolic intermediates (sequential arrows) one of them being Erg25. APB, a potent inhibitor of Erg25 is known to dramatically decrease tombusvirus replication in yeast and plant hosts.

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PUBLICATIONS AND PRESENTATIONS

1) Sharma M, Sasvari Z and Nagy PD (2011). Inhibition of phospholipid biosynthesis decreases the activity of the tombusvirus replicase and alters the subcellular localization of replication proteins. Virology 415:141-52
2) Sharma M, Sasvari Z and Nagy PD (2011). Cardiolipin is critical for efficient assembly of tombusvirus replicase complex. (Manuscript in preparation).
3) Sharma M, Sasvari Z and Nagy PD (2010). Inhibition of sterol biosynthesis reduces tombusvirus replication in yeast and plants. J. Virol 84: 2270-81

4) Pathak KB, Jiang Z, Sharma M, Shapka N, Okanine V and Nagy PD (2011). Role of p33 in tombusvirus RNA recruitment and replicase assembly. (Manuscript in preparation).
5) Barajas D, Sharma M and Nagy PD (2011) Tombusvirus regulates the phospholipid biosynthesis by interacting to Scs2 and Opi1. (Submitted, PloS Pathogens).
6) Martinez N, Shah-Nawaz M, Sharma M, Pascal H, Jonczyk M, Baker J [authors' hierarchy may change (2011)]. Screening of yeast ORF overexpression library reveals the role of COF1 in tombusvirus replication. (Manuscript in preparation).
7) Sharma M, Sasvari Z and Nagy PD (2009). Role of sterols in tombusvirus replication. Oral presentation at American Society for Virology annual meeting in University of British Columbia, Vancouver, BC, Canada
8) Jonczyk M, Pathak KB, Sharma M and Nagy PD (2007). Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum. Virology 362:320-30.

AWARDS

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4) Student Travel Support Award by Graduate School, University of Kentucky, to attend ASV annual meeting at Vancouver, BC, Canada. July 2009.
5) Junior Research Fellowship from Department of Biotechnology, New Delhi, India.