Identification of Essential Host Factors Affecting Tombusvirus RNA Replication Based on the Yeast Tet Promoters Hughes Collection†

Yi Jiang, Elena Serviène, Jozsef Gal, Tadas Panavas, and Peter D. Nagy*

Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546

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To identify essential host genes affecting replication of *Tomato bushy stunt virus* (TBSV), a small model plant virus, we screened 800 yeast genes present in the yeast Tet promoters Hughes Collection. In total, we have identified 30 new host genes whose down-regulation either increased or decreased the accumulation of a TBSV replicon RNA. The identified essential yeast genes are involved in RNA transcription/metabolism, protein metabolism/transport, or other cellular processes. Detailed analysis of the effects of some of the identified yeast genes revealed that they might affect RNA replication by altering (i) the amounts/functions of p33 and p92^{pol} viral replication proteins, (ii) the standard 10 to 20:1 ratio between p33 and p92^{pol} in the viral replicase, (iii) the activity of the tombusvirus replicase, and (iv) the ratio of plus- versus minus-stranded RNA replication products. Altogether, this and previous genetic screening of yeast (Panavas et al., *Proc. Natl. Acad. Sci. USA* 102:7326–7331, 2005) led to the identification of 126 host genes (out of ~5,600 genes that represent ~95% of all the known and predicted yeast genes) that affected the accumulation of tombusvirus RNA.

Replication of plus-stranded RNA viruses requires many components of the host cells, including host proteins and intracellular membranes, which serve as sites of virus replication in infected cells (1, 3, 38). Accordingly, the virus-specific replicase complex (RC) consists of virus- and host-coded proteins and the viral RNA, which assemble on intracellular membranes into functional complexes. In addition, the viral replication proteins and host factors likely play roles in template selection for replication and recruitment (intracellular transport/targeting) of the viral RNA into replication (2, 20). Host factors could also affect the stability/degradation of viral proteins and the viral RNA (4, 40, 41). Overall, viruses utilize/depend on many diverse resources of the host cells.

To identify the roles and/or effects of host genes on virus replication, systematic genome-wide screens were conducted in yeast, a model host, using the single-gene deletion library (YKO) with two distantly related plus-strand RNA viruses, namely *Brome mosaic virus* (BMV) and *Tomato bushy stunt virus* (TBSV) (12, 27). These studies led to the identification of ~100 host genes for each virus that either stimulated or inhibited virus replication. Interestingly, most of the identified genes had a virus-specific effect, whereas only a small number of genes affected the replication of both BMV and TBSV. These observations suggest that BMV and TBSV, belonging to different supergroups within plus-stranded RNA viruses, could use and/or be affected by mostly different host factors (12, 27). Altogether, the above systematic screens tested only ~80% of all the known genes, which are not essential for yeast growth, whereas the effect of essential yeast genes remained untested.

Tombusviruses, such as TBSV and *Cucumber necrosis virus*

(CNV), are single-component RNA viruses of ~4,800 nucleotides (nt). Among the five virus-coded proteins, only two, termed p33 and p92^{pol}, are essential for TBSV replication (44). p92^{pol} is the viral RNA-dependent RNA polymerase (RdRp), whereas p33 replication cofactor (which overlaps with the N-terminal pre-readthrough segment of p92^{pol}) is an RNA-binding protein (28, 32, 33). Earlier work defined that p33 is involved in template selection and recruitment of viral RNA into replication (18, 25, 32). These proteins interact with each other, the viral RNA, and the host proteins in cells (25, 34, 35, 39), which leads to the assembly of RC on peroxisomal membranes (22, 25). The CNV replication proteins can support the replication of TBSV defective interfering (DI) RNA, a small deletion derivative of the genomic RNA, as efficiently as TBSV replication proteins can (19, 24). A recent genome-wide screen of the YKO library for tombusvirus replication led to the identification of 96 host genes, whose separate deletions affected replication of a TBSV replicon RNA (repRNA), which is based on a DI RNA, in yeast (27). Based on the large number of host genes identified, the emerging picture is that the host likely plays a complex role in virus replication (20, 27).

In this paper, we extended the genome-wide screening to essential yeast genes to identify those affecting tombusvirus replication. Among the 800 essential host genes present in the yeast Tet promoters Hughes Collection (yTHC) (out of ~1,100 predicted essential yeast genes) (17), we found that 30 genes (when down-regulated) affected the accumulation of tombusvirus RNA. These essential yeast genes, which either increased or decreased the accumulation of tombusvirus RNA, are involved in protein metabolism/transport, RNA transcription/metabolism, or other cellular processes. Detailed analysis of the effect of a selected group of yeast genes revealed that they could affect the amount of replication proteins or the initial level of RNA templates as well as the activity of the tombusvirus replicase. Overall, this and previous genome-wide screening of ~95% of all yeast genes defined that ~2% of yeast genes affected the accumulation of TBSV RNA. In addition, docu-

* Corresponding author. Mailing address: Department of Plant Pathology, University of Kentucky, Plant Science Building, Lexington, KY 40546. Phone: (869) 257-7445. Fax: (859) 323-1961. E-mail: pdnagy2@uky.edu.

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mented interaction between the identified host proteins will facilitate future research aimed at dissecting their roles in tombusvirus replication.

MATERIALS AND METHODS

Yeast strains and expression plasmids. The Tet promoter-based Hughes Collection (yTHC) of yeast strains were obtained from Open Biosystems. yTHC was provided in the haploid strain R1158 background (*URA3::CMV-tTA MATa his3-1 leu2-0 met15-0*). This strain was created by a one-step integration of the tTA transactivator, under the control of the cytomegalovirus (CMV) promoter, at the *URA3* locus. The kan^R-tetO7-TATA cassette was then integrated into the yeast genome, replacing the endogenous promoter for each gene (17).

The expression plasmids pGAD-His92 (containing CNV p92^{pol} gene and *LEU2* marker) (30) and pGBK-His33/DI-AU-FP (coexpressing p33 from the *ADH1* promoter and DI-AU-FP RNA from the *GAL1* promoter) have been previously described (40). Construction of dual expression plasmid pGBK-His33/DI-72 (coexpressing p33 from the *ADH1* promoter and DI-72 RNA from the *GAL1* promoter) was done by inserting DI-72 sequence including the ribozyme at the 3' end (26) together with the *GAL1* promoter sequence between the *ADH1* promoter and the F1 origin in pGBK-His33 plasmid (30). These sequences were amplified by PCR using primers #1546 (CCGCGAATTCACGG ATTAGAACCGCGGAGCGGT) and #1069 (CCGGTCGAGCTCTACCA GGTAATATACCACAACGTGTGT) on pYC/DI-72 as a template. The 5' end of the *ADH1* promoter from pGBK-His33 was amplified with primers #1543 (CCGCGAATTCGGTGGCGGCTCTTCGCTATTACGCCA) and #1544 (G GAAGTCCATATTGTACACCGGAACA). The two PCR products were ligated through the *EcoRI* site, and the full-length 5'-P-ADH-P-Gal1-DI72 DNA was reamplified with primers #1069 and #1544. To obtain His3-F1ori DNA, the *His3* gene together with F1 origin from pGBK-His33 was amplified with PCR using primers #1081 (CGGCGTCCGGATAATTCGTTTAAAGAGCTT GGT) and #1545 (CCGGTCGAGCTCATCGCCCTTCCCAACAGTTGC GCA) to introduce a *SacI* site. The obtained PCR products of 5'-P-ADH-P-Gal1-DI72 and His3-F1ori were digested with Bsp1407I/*SacI* and BspEI/*SacI*, respectively, and cloned simultaneously into pGBK-His33 (30) digested with BspEI/Bsp1407I.

Yeast transformation and cultivation. The parental strain (BY4741; Open Biosystems) and the strains in the yTHC collection were cotransformed with different combinations of plasmids using the LiAc-single-stranded DNA-polyethylene glycol method (11), and transformants were selected by complementation of auxotrophic markers.

For separate analysis of DI-AU-FP RNA and DI-72 RNA accumulation, each transformed yTHC strain was inoculated into synthetic complete dropout medium lacking leucine and histidine (SC-LH⁻ medium) containing 2% galactose and supplemented with Geneticin G418 (200 mg/liter) and cultured for 24 to 48 h at 29°C until an optical density at 600 nm of ~0.8 to 1.0. For maximum level of essential gene expression, yeast was grown in the absence of doxycycline, whereas to reduce the expression levels of the essential genes, yeast was grown in the same medium in the presence of 10 mg/liter doxycycline (17). Our preliminary experiments showed that the use of 10 mg/liter doxycycline was as good as 25 or 50 mg/liter doxycycline and better than 3.3 mg/liter to affect TBSV replication (not shown). Therefore, we used 10 mg/liter doxycycline throughout the experiments to turn the particular gene off.

RNA analysis. Total RNA isolation and Northern blot analysis were performed as described previously (26, 30). Briefly, for extraction of total RNA, yeast cells were broken by shaking for 1 to 2 min at room temperature with equal volumes of RNA extraction buffer (50 mM NaOAc, pH 5.2, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and water-saturated phenol and then incubated for 4 min at 65°C, followed by ethanol precipitation. The obtained RNA samples were separated on a 1.5% agarose gel and transferred to Hybond-XL membrane (Amersham) before hybridization with DI-72 RNA-specific probe. For detection of plus-strand repRNA, we prepared ³²P-labeled RIII/IV(-) probe with T7 transcription from PCR product obtained with primers #1165 (AGCGAGTAA GACAGACTCTTCA) and #22 (GTAATACGACTACTATAGGGCTGCAT TTCTGCAATGTTCC) on DI-72 templates.

Protein analysis. For protein analysis, yeast strains were cultivated as described above for RNA analysis. A total of 50 ml yeast culture was harvested, the pelleted cells were resuspended in 150 µl cold 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), and 250 µl of glass beads was added to each sample. The cells were broken with Genogrinder for 2 min at 1,500 rpm. Each sample was further mixed with 600 µl prechilled extraction buffer, and unbroken cells were

removed by centrifugation at 100 × *g* for 5 min. The supernatant was mixed with 1/2 volume of 3× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer followed by SDS-PAGE and Western blot analysis as described previously (26, 30). The primary antibody was anti-His₆ (Amersham), and the secondary antibody was alkaline-phosphatase-conjugated anti-mouse immunoglobulin G (Sigma).

CNV replicase assays. The "membrane-enriched" CNV replicase preparations, which are suitable to test the replicase activity on the endogenous templates present within the CNV replicase preparation, were obtained as previously described (30). Briefly, frozen yeast cells were homogenized with Genogrinder for 2 min at 1,500 rpm in 150 µl cold 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) plus 250 µl of glass beads. Each sample was further mixed with 600 µl prechilled extraction buffer, and unbroken cells were removed by centrifugation at 100 × *g* for 5 min at 4°C (26). The supernatant was centrifuged for 10 min at 21,000 × *g* at 4°C, and then the pellet was resuspended and used in a standard CNV replicase assay (29, 30). Because no template was added to the *in vitro* reaction, the replicase preparation could only use the endogenous template present within the enriched membrane fraction. The replicase products were phenol-chloroform extracted, precipitated with isopropanol-ammonium acetate, and analyzed under denaturing conditions (i.e., 5% PAGE containing 8 M urea) (31).

To test the ratio of plus- versus minus-strand synthesis on the endogenous templates by the CNV replicase, we obtained the membrane-enriched fraction (see above), followed by standard replicase assay in the presence ³²P-labeled UTP and the other three unlabeled rNTPs as described previously (30). Equal amounts of unlabeled *in vitro* transcripts of plus-strand and minus-strand DI-72 RNAs (prepared by T7 RNA transcription *in vitro*) were denatured separately by heating for 5 min at 85°C in Tris-EDTA (TE) buffer and formamide (in a 1:1 ratio). Then the DI-72 plus-strand and minus-strand RNAs were separately blotted onto a Hybond XL membrane (Amersham) and cross-linked with UV light (GS Gene Linker; Bio-Rad). Hybridization with the heat-denatured ³²P-labeled replicase products (see above) was done using ULTRAhyb solution (Ambion) at 68°C according to the supplier's instructions.

RESULTS

Screening of the yTHC collection for essential host genes affecting tombusvirus replication. The yTHC collection contains 800 out of ~1,100 essential yeast genes (17). In the yTHC collection, the expression of a given essential yeast gene is under the control of a Tet-titratable promoter in the genome (Open Biosystems). The expression of the essential gene can be turned off by the addition of doxycycline to the yeast growth medium (17). This approach allowed us to test tombusvirus RNA replication in the presence of the host protein (when yeast was grown without added doxycycline after the induction of tombusvirus repRNA replication) (Fig. 1A) or in its absence (when yeast was grown in the presence of doxycycline to turn off the expression of the particular gene) (Fig. 1B) (17).

To identify essential host genes affecting tombusvirus replication, we transformed each of the yeast strains present in the yTHC collection with two plasmids (pGAD-His92 and pGBK-His33/DI-AU-FP, Fig. 1A and B). These plasmids expressed the p33 and p92^{pol} replication proteins of CNV, which support TBSV RNA replication as efficiently as TBSV replication proteins (24, 26), from the constitutive *ADH1* promoter and DI-AU-FP RNA replicon (Fig. 1A and B) (42) from the galactose/glucose-inducible/repressible *GAL1* promoter (26). Expression of p33, p92^{pol}, and the DI-AU-FP repRNA in the parental yeast strain grown under standard growth conditions (see Materials and Methods) led to efficient replication of the 807-nt-long repRNA (~20,000 copies per yeast cells). The addition of 10 mg/liter doxycycline to the growth medium did not alter the accumulation of the repRNA (Fig. 2, row A1) in the case of the parental yeast strain (which carries all yeast genes that are expressed from their natural promoters), suggesting that the

FIG. 1. Schematic representation of launching replication of TBSV DI RNA replicon (repRNA) in yeast strains based on the yTHC collection. Tombusvirus p33 and p92^{pol} replication proteins are expressed constitutively from the *ADH1* promoter, whereas DI-AU-FP repRNA (or the wild-type DI-72 RNA; not shown) is expressed from the regulatable *GAL1* promoter. Replication of repRNA takes place in the cytoplasm (on peroxisomal membrane surfaces). The expression of a particular host gene occurs in the absence of doxycycline (–Dox) (as shown in panel A), whereas the expression of the particular host gene is switched off in the presence of doxycycline (+Dox) (as shown in panel B). Replication of DI-AU-FP repRNA with four noncontiguous regions (RI to RIV, also present in DI-72 RNA [not shown]) derived from TBSV genomic RNA and the artificial AU-FP region is shown via a minus-stranded intermediate RNA (42).

presence of doxycycline did not affect replication of the DI-AU-FP repRNA in the parental yeast strain.

Identification of 30 essential host genes affecting tombusvirus replication. We performed high-throughput screening of the 800 strains present in the yTHC collection. Total RNA was extracted from samples (at least six samples per yeast strain, three each from yeast grown with or without doxycycline), electrophoresed in 1.5% agarose gel, stained with ethidium bromide, transferred to nylon membrane, and analyzed by Northern blotting specific for the 3' end of DI-AU-FP (data for 15 selected strains are shown in Fig. 2, rows A1 and B1, and Fig. 3, row 1). We found that 9 of the yTHC strains were not transformable with the two plasmids and 35 strains grew too slowly to obtain enough viral and host RNAs for subsequent analysis (the list of genes omitted is shown in Table S1 in the supplemental material). Out of the remaining yeast strains, we found that 19 strains showed a 2- to 5-fold-decreased level and 11 strains showed between a 50 and 450% increased level of repRNA accumulation in the presence of doxycycline (Table 1). We scored only those strains that showed at least 50% or more change in repRNA accumulation (27). It is important to note that we compared the accumulation levels of repRNA for each yeast strain grown in the absence versus in the presence of doxycycline, instead of comparing with the repRNA accumulation in the parental yeast strain. This is because the expression level of the particular host protein from the TET promoter is expected to differ from its expression from the original promoter, which could be either higher or lower than that from the TET promoter.

To confirm the above findings, we retested the above 30 yTHC yeast strains with DI-72 repRNA, which is capable of ~5-fold more robust replication (~100,000 RNA copies per cell) than DI-AU-FP repRNA used above (41). DI-72 repRNA is the prototypical DI RNA carrying four noncontiguous regions (RI to RIV) from the TBSV genomic RNA, whereas DI-AU-FP repRNA contains an artificial AU-rich sequence between RI and RII that could promote recombina-

tion in yeast and in plants (Fig. 1) (40–42). Northern blot analysis of total RNA extracts obtained from the selected yeast strains coexpressing DI-72 repRNA, p33, and p92^{pol} revealed that DI-72 repRNA replication was also affected by the above identified 30 yeast genes (data for 15 selected strains are shown in Fig. 2, rows A2 and B2, and Fig. 3, row 2). Based on these screens, we conclude that the 30 identified host factors affected the accumulation of two different TBSV repRNAs.

The 30 essential host genes identified in the above screen code for proteins with different molecular functions in various cellular processes (Yeast Genome Database, SGD; <http://www.yeastgenome.org>). These include RNA binding/processing (*UTP9*, *UTP15*, *NAB2*, *PRP39*, *RNA14*, *MEX67*, *NOP4*, *RPL15A*, and *RRP9*), RNA helicase/unwinding (*DED1*, *PRP5*, and *SEN1*), RNase (*RRP42*), or RNA polymerase/RNA transcription (*RPO21*, *MED6*, *RPB11*, *TFA2*, and *ARP9*) (Table 1). Others are involved in protein modification (*SLN1*, *MOB1*, and *EPL1*), protein synthesis (*RPL17A*), protein transport (*COP1*), or lipid biosynthesis (*ERG25*). *LPD1* codes for a pyruvate dehydrogenase, *RSC8* is involved in chromatin remodeling, and *NOG1* and *NOG2* code for putative GTPases. We also identified two genes with currently unknown functions (*GRC3* and *YDR327W*) (Table 1). Altogether, the identified host factors could have either direct or indirect effects on tombusvirus replication (see below).

Effect of the identified host factors on transcription of viral RNA and on amounts of p33 and p92^{pol} replication proteins. To determine if the identified host factors affected the amount of viral RNA transcripts (the initial templates) generated from the yeast expression plasmid (pGBK-His33/DI-72), we performed Northern blot analysis on nine selected strains, which decreased (Fig. 2A and B), and five strains that increased repRNA levels in the presence of doxycycline (Fig. 3). The selected yeast strains expressed only DI-72 RNA and p33, but not p92^{pol}, in these experiments to facilitate detection of the DI-72 RNA transcripts in the absence of replication. Comparison of the accumulation of DI-72 transcripts in nine of the

FIG. 2. Representative group of yTHC yeast strains supporting a decreased level of TBSV repRNA accumulation in the presence of doxycycline. Panels A and B include different sets of host genes as shown. Rows A1 and B1 show Northern blot analysis of total RNA extracts from the shown yeast strains was performed with a radiolabeled RNA complementary to RIII/IV. Four independent samples are shown for each strain; two samples were grown without (–Dox) and two with (+Dox) doxycycline to illustrate the reproducibility of repRNA accumulation. An arrow points at the DI-AU-FP replicon RNA. Rows A2 and B2 show Northern blot analysis of DI-72 repRNA accumulation. The probe was complementary to RIII/IV. Rows A3 and B3 show ethidium bromide-stained agarose gel to demonstrate DI-72 repRNA accumulation in selected yeast strains. Rows A4 and B4 show ethidium bromide-stained agarose gel with rRNA as a control for yeast growth. Rows A5 and B5 show Northern blot analysis of DI-72 RNA transcripts in the absence of replication from total RNA extracts obtained from selected yTHC yeast strains coexpressing p33, but lacking p92^{pol}. Rows A6 and B6 show Western analysis of p33 replication protein in total protein samples using anti-His-tagged antibody. Rows A7 and B7 show Western analysis of p92^{pol} and rows A8 and B8 show Western analysis of p33 replication proteins in membrane-enriched fractions as described in Materials and Methods. Note that the left sample is shown for “no doxycycline” and the right sample for “plus doxycycline” in each experiment.

yTHC strains that decreased replication of DI-72 RNA (Fig. 2, rows A5 and B5) in the presence of doxycycline revealed that the amount of DI-72 RNA transcripts decreased in six strains (*DED1*, *COP1*, *SLN1*, *RPO21*, *NAB2*, and *MED6*), increased in two strains (*UTP15* and *UTP9*), and did not change in one strain (*MOB1*), whereas the transcripts increased slightly in the parental strain in the presence of doxycycline. Altogether, we did not find good correlation between altered DI-72 transcript levels and the alteration in replication of the repRNA.

Testing six of the yTHC strains that showed increased replication of DI-72 RNA (Fig. 3, row A5) in the presence of doxycycline, however, revealed a correlation between the elevated levels of DI-72 transcripts and increased levels of

repRNA accumulation in five out of six strains (*NOG1*, *NOP4*, *ARP9*, *GRC3*, and *RPL15A*) in the presence of doxycycline. Therefore, it is possible that the initial amount of repRNA template could affect subsequent accumulation of repRNA in some yeast strains.

Since the amount of p33 replication cofactor could affect replication and p33 is the most abundant protein in the tombusvirus RC (13, 21), we tested the level of p33 present in total protein extracts obtained from 15 selected yTHC strains in comparison with the parental yeast using Western blotting (Fig. 2, rows A6 and B6, and Fig. 3, row 6) (30). We also tested the membrane-enriched fraction (29, 30), which contains the active tombusvirus replicase, for the levels of p33 (Fig. 2, rows

FIG. 3. Identification of yTHC yeast strains supporting increased level of repRNA accumulation in the presence of doxycycline. See further details in the legend to Fig. 2.

A8 and B8, and Fig. 3, row 8) and the less abundant p92^{pol} (Fig. 2, rows A7 and B7, and Fig. 3, row 7), as described in Materials and Methods.

These experiments revealed close correlation between level

of p33 in total protein extracts and the level of p33 in the membrane-enriched fraction. This is not surprising since most p33 was localized to peroxisomal membranes in yeast (25). We also found that three strains (*DED1*, *SLN1*, and *RPO21*) ac-

TABLE 1. Name and functions of the essential host genes affecting repRNA accumulation

Gene name	% repRNA accumulation ^a	Molecular function ^b
Decreased accumulation of repRNA		
<i>COP1</i>	29	Protein transporter, endoplasmic reticulum to Golgi, and retrograde transport
<i>DED1</i>	43	DEAD-box RNA helicase, translation
<i>EPL1</i>	46	Histone acetyltransferase activity
<i>ERG25</i>	43	Ergosterol biosynthesis
<i>LPD1</i>	50	Dihydrolipoyl dehydrogenase activity/pyruvate dehydrogenase
<i>MED6</i>	35	RNA polymerase II transcription mediator activity
<i>MEX67</i>	17	Poly(A) RNA binding protein
<i>MOB1</i>	18	Protein amino acid phosphorylation
<i>NAB2</i>	35	Nuclear polyadenylated RNA-binding protein, related to human hnRNPs
<i>PRP39</i>	36	RNA binding, nuclear mRNA splicing
<i>RNA14</i>	48	RNA binding/mRNA cleavage
<i>RPB11</i>	34	RNA polymerase
<i>RPO21</i>	27	RNA polymerase
<i>RRP42</i>	46	3'→5'-Exoribonuclease activity
<i>RSC8</i>	45	Chromatin remodeling
<i>SLN1</i>	41	Protein histidine kinase activity
<i>TFA2</i>	34	General RNA polymerase II transcription factor activity
<i>UTP9</i>	28	snoRNA binding, interacts with UTP15
<i>UTP15</i>	43	snoRNA binding, interacts with UTP9
Increased accumulation of repRNA		
<i>ARP9</i>	450	Actin-related protein involved in transcriptional regulation
<i>GRC3</i>	200	Unknown, possibly involved in rRNA processing
<i>NOG1</i>	211	Putative GTPase
<i>NOG2</i>	175	Putative GTPase
<i>NOP4</i>	184	RNA binding, rRNA processing
<i>PRP5</i>	164	RNA helicase in the DEAD-box family
<i>RPL15A</i>	290	Binds to 5.8S rRNA
<i>RPL17A</i>	200	Structural constituent of ribosome
<i>RRP9</i>	306	RNA binding/processing
<i>SEN1</i>	200	Putative helicase required for processing of tRNAs, rRNAs, and snoRNAs
<i>YDR327W</i>	159	Unknown

^a Percentage of repRNA accumulation in the particular yeast strain in the presence of doxycycline compared to that in the yeast strain grown in the absence of doxycycline (representing 100% for each strain).

^b Based on the *Saccharomyces* Genome Database, SGD, at <http://www.yeastgenome.org>.

TABLE 2. Effect of down-regulation of selected host genes on level of initial viral RNA, p33, and p92 replication proteins and on activity of the tombusvirus replicase^a

Gene	% DI-72 repRNA accumulation ^b	% Initial viral transcript level ^c	% Replicase protein ^d		% Replicase activity in vitro ^e	Plus/minus-strand ratio ^f
			p33	p92		
Parental	118	98.1	101	92	100.0	13.7/11.7
<i>ARP9</i>	450	343.0	212	325	517.0	2.6/11.0
<i>COP1</i>	29	43.5	95	>1	35.6	10.6/2.5
<i>DED1</i>	43	40.9	51	8	13.6	11.2/1.1
<i>GRC3</i>	200	300.0	102	50	NT ^g	NT
<i>MED6</i>	35	42.1	102	113	NT	NT
<i>MOB1</i>	18	93.0	120	37	5.8	6.7/2.0
<i>NAB2</i>	35	44.3	85	>1	19.4	10.6/4.4
<i>NOG1</i>	211	259.0	102	57	214.0	11.7/17.0
<i>NOP4</i>	184	307.0	88	59	NT	NT
<i>PRP5</i>	164	94.6	120	128	104.0	16.5/20.0
<i>RPL15A</i>	290	342.0	83	27	NT	NT
<i>RPO21</i>	27	25.5	69	43	NT	NT
<i>SLN1</i>	41	12.5	59	78	25.0	8.7/1.8
<i>UTP9</i>	28	232.0	109	76	15.6	8.3/2.7
<i>UTP15</i>	43	154.0	113	61	16.3	10.2/3.5

^a The above values reflect results from yeast grown in the presence of doxycycline in comparison with yeast grown in the absence of doxycycline (which represents 100% for each strain). Each experiment was repeated at least three times.

^b Percentage of DI-72 repRNA accumulation in the particular yeast strain in the presence of doxycycline compared to the yeast strain grown in the absence of doxycycline (which represents 100% for each strain) (Table 1).

^c Percentage of repRNA transcripts in the absence of p92 (Fig. 2 and 3, panels A5 and B5).

^d Accumulation levels of p33 and p92 replication proteins (Fig. 2 and 3, panels A7 and -8 and B7 and -8).

^e In vitro activity of CNV replicase preparations in the enriched membrane fractions (Fig. 4 and 5A).

^f Ratio of plus- versus minus-strand synthesis based on in vitro activity of CNV replicase preparations on endogenous RNA templates (Fig. 4 and 5B).

^g NT, not tested.

cumulated p33 (both in total and membrane-enriched fractions; Fig. 2, rows A6 and B6 and A8 and B8) at a reduced level in the presence of doxycycline among the nine selected strains that showed reduced levels of DI-72 RNA accumulation. The remaining six strains (*COP1*, *MOB1*, *UTP9*, *UTP15*, *NAB2*, and *MED6*) showed comparable p33 levels when grown in the absence or presence of doxycycline (Table 2). The level of p92^{pol} was reduced in five strains (*DED1*, *COP1*, *MOB1*, *NAB2*, and *RPO21*; Fig. 2, rows A7 and B7), while it did not change in one strain (*SLN1*). An additional two strains (*UTP9* and *UTP15*) contained small amount of p92^{pol} independent of doxycycline, whereas the *MED6* strain showed a somewhat higher p92^{pol} level in the presence of doxycycline (Fig. 2, rows A7 and B7). Therefore, it is possible that the reduced amount of either p33 or p92^{pol} or both inhibits the replication of DI-72 repRNA in several, but not all, of the nine selected strains that supported reduced DI-72 RNA accumulation in the presence of doxycycline.

The picture of the possible role of alteration of p33/p92^{pol} levels on tombusvirus replication, however, is more complex for those strains that showed an increased level of DI-72 RNA accumulation in the presence of doxycycline (Fig. 3, row 8). For example, among the six selected strains, the level of p33 increased in the *ARP9* strain and did not change in the *NOG1*, *NOP4*, *GRC3*, *PRP5*, and *RPL15A* strains in the presence of

doxycycline (Fig. 3, row 8). In the presence of doxycycline, the level of p92^{pol} decreased in *NOG1*, *NOP4*, *GRC3*, and *RPL15A* strains and increased in the *ARP9* strain, but it did not change in the *PRP5* strain (Fig. 3, row 7; Table 2). Altogether, the changes in p33 and p92^{pol} levels do not show good correlation with replication levels (Table 2). Therefore, these data do not support the idea that all the host genes identified in this work would affect replication of the repRNA by affecting p33 and p92^{pol} levels in host cells. However, it is possible that several genes, such as *DED1*, *COP1*, *SLN1*, *MOB1*, *NAB2*, *RPO21*, and *ARP9*, might affect repRNA accumulation via changing the accumulation levels of one or both of the p33 or p92^{pol} replication proteins (Table 2).

The tombusvirus RC contains 10- to 20-fold more p33 than p92^{pol} when purified from the parental yeast (30, 35). Changing the above ratio between p33 and p92^{pol} might affect the efficiency of replication (34, 35). Indeed, several of the identified yeast strains (*COP1*, *MOB1*, *NOG1*, *NOP4*, *GRC3*, and *RPL15A*; Fig. 2, rows A7 and -8 and Fig. 3, rows 7 and 8) produced p92^{pol} at reduced level, whereas the levels of p33 remained comparable in the absence versus in the presence of doxycycline (Table 2). For example, down-regulation of *COP1* and *MOB1* reduced only the level of p92^{pol}, but not that of p33 in the presence of doxycycline (Fig. 2, rows A7 and -8). Therefore, altering the ratio of p33 to p92^{pol} by the host factor might contribute to altered rate of tombusvirus replication in selected yeast strains.

Reduced CNV replicase activity obtained from yeast strains that supported reduced DI-72 RNA accumulation in the presence of doxycycline. To test if some of the host factors identified above could affect replication of the repRNA via altering the activity of the CNV RC, we performed functional assays with the tombusvirus replicase obtained from selected yeast strains. First, we prepared membrane-enriched fraction (29, 30) from each selected yeast strain coexpressing p33, p92^{pol}, and DI-72 repRNA as described in Materials and Methods. Subsequently, the obtained membrane-enriched preparations containing the CNV RC were tested in the presence of four ribonucleotides, including ³²P-labeled UTP. Under these conditions, the CNV replicase completes RNA synthesis on the endogenous templates (i.e., on the viral RNA that was copurified with the RC, an in vitro reaction called "runoff synthesis"), which are part of the RC actively synthesizing viral RNA in the yeast cells at the time of extraction. The in vitro replicase runoff synthesis demonstrated that the CNV replicase synthesized 4- to 17-fold-decreased amounts of products in vitro when the replicase preparations were obtained from yeast strains with down-regulated expression of the *SLN1*, *MOB1*, *DED1*, *COP1*, *UTP15*, *UTP9*, and *NAB2* genes, respectively, versus the replicase obtained from the parental yeast (Fig. 4A). Reduction of in vitro CNV replicase activity and the reduced in vivo accumulation of repRNA with the above strains in the presence of doxycycline were in good correlation (Table 2), suggesting that down-regulation of these genes might affect repRNA levels via inhibition of CNV replicase activity (see Discussion).

Reduced ratio of plus- versus minus-strand synthesis by the CNV replicase obtained from selected strains. One of the hallmark features of plus-stranded RNA viruses is asymmetrical strand synthesis, which results in 10- to 100-fold more

FIG. 4. Down-regulation of expression of selected essential yeast genes inhibits the activity of CNV replicase in vitro. (A) (Top row) In vitro activity of CNV replicase present in membrane-enriched preparations. Each replicase preparation, obtained from the yeast shown coexpressing p33, p92^{pol}, and DI-72⁺ RNA, was tested with the copurified endogenous template. Strains were grown in the absence (–DOX) or presence (+DOX) of doxycycline, as indicated. ³²P-labeled RNA products from the above preparations were analyzed on denaturing 5% PAGE–8 M urea gels. For quantification, we measured the intensity of ³²P-labeled RNA products by using a PhosphorImager. Activity of the CNV replicase obtained from the parental yeast strain in the absence of doxycycline corresponds to 100%. Each experiment was performed three times. (Bottom row) A Western blot shows the accumulation level of p33 in the membrane-enriched preparations. (B) Effect of down-regulation of selected essential genes on asymmetrical RNA synthesis by the CNV replicase obtained from the shown yeast strains. Unlabeled T7 RNA polymerase transcripts of DI-72⁺ (marked as “+” or “||”) and DI-72[–] (marked as “=”) (400 ng each), respectively, were blotted on the membrane. The blotted RNAs were then hybridized with denatured ³²P-labeled RNA probes, which were generated by the CNV replicase in vitro in a runoff experiment on the endogenous templates present in the enriched membrane fractions obtained from selected yTHC strain grown in the absence (left row) or presence (right row) of doxycycline. The ratio between plus- and minus-stranded RNAs in the in vitro replicase assay was calculated based on PhosphorImager analyses from three separate experiments. For example, the value of 13.7 means that the CNV replicase produced 13.7-fold more plus-stranded RNA than minus-stranded RNA in vitro using the endogenous template.

abundant plus strands than the minus-stranded intermediates (1, 3). To test if down-regulation of the expression levels of the *SLN1*, *MOB1*, *DED1*, *COP1*, *UTP15*, *UTP9*, and *NAB2* genes could affect the ratio of plus- versus minus-strand RNA synthesis by the CNV replicase, we first performed replicase runoff experiments (see the above section) that produced ³²P-labeled RNA based on the copurified repRNAs present in the replicase fraction, followed by using the labeled RNAs as probes in RNA blotting (30). The target templates were the same amounts of denatured plus- and minus-stranded DI-72 repRNA fixed separately onto nylon membranes (Fig. 4B). After hybridization of the membranes with the ³²P-labeled runoff products obtained from the in vitro replicase reactions, we measured the ratio of plus- versus minus-strand-specific signals on the RNA blots using a PhosphorImager (19, 30, 43). These experiments demonstrated that the CNV replicase from *SLN1*, *MOB1*, *DED1*, *COP1*, *UTP15*, *UTP9*, and *NAB2* yeast

strains grown in the presence of doxycycline synthesized only ~1- to 4-fold more plus strands than minus strands, whereas the CNV replicase from the same strains grown in the absence of doxycycline produced ~7- to 11-fold more plus strands than minus strands (Fig. 4B). In comparison, the CNV replicase obtained from the parental strain produced ~12- to 13-fold more plus strands than minus strands under both conditions (Fig. 4B). The reduction in ratio of plus-stranded versus minus-stranded repRNA was the largest for *DED1* (11-fold) and intermediate for *SLN1*, *MOB1*, *COP1*, *UTP15*, *UTP9*, and *NAB2* (3- to 5-fold) (Fig. 4B). The altered ratio of plus- and minus-strand synthesis suggests that these host factors could affect the assembly and/or the functions of the replicase. Future experiments will be aimed at dissecting the role of these host factors in the asymmetrical RNA synthesis.

Down-regulation of *NOG1*, *ARP9*, and *PRP5* expression alters the activity of the CNV replicase. The in vitro replicase

FIG. 5. Down-regulation of *NOG1*, *ARP9*, and *PRP5* expression alters the in vitro activity of the CNV replicase. See further details in the legend to Fig. 4.

runoff synthesis performed with the membrane-enriched fraction showed two- and fivefold increases in the amount of in vitro RNA products when the replicase preparations were obtained from yeast strains with down-regulated expression of *NOG1* and *ARP9*, respectively, whereas the CNV replicase activity obtained from the *PRP5* strain was comparable to the replicase preparation obtained from the parental yeast in the absence or presence of doxycycline (Fig. 5A). The ratio of plus versus minus strand synthesized by the CNV replicase preparations obtained from *NOG1*, *ARP9*, and *PRP5* yeast strains grown in the absence of doxycycline synthesized was rather variable with the ratio of plus strands and minus strands ranging from ~3 to 16, whereas the CNV replicase from the same strains grown in the presence of doxycycline showed an increased ratio between plus strands and minus strands by ~20 to 400% (Fig. 5B). The increase in ratio of plus-stranded versus minus-stranded repRNA was the largest for the *ARP9* strain (fourfold), intermediate for the *NOG1* strain (45%), and less pronounced in the *PRP5* strain (20%) (Fig. 5B). In comparison, the CNV replicase obtained from the parental strain showed ~15% reduction in the ratio of plus strands versus minus strands when grown in the presence of doxycycline (Fig. 5B).

The above data with the CNV replicase preparations obtained with yeast strains supporting increased DI-72 RNA accumulation in the presence of doxycycline suggest that down-regulation of *ARP9* and, to a less extent, *NOG1* expression facilitated the assembly of more tombusvirus RC or increased the activity of those complexes, whereas down-regulation of

PRP5 had only a somewhat smaller effect (Table 2) (see Discussion). Moreover, the observations that the tombusvirus replicase was defective when obtained from the *ARP9* yeast strain in the absence of doxycycline and only partly improved in the presence of doxycycline (Fig. 5, lanes 5 and 6) suggest that expression of *ARP9* from the TET promoter has an inhibitory effect on replicase activity and on repRNA replication (Fig. 3, panels A1 and A2).

DISCUSSION

Two percent of all host genes affect tombusvirus replication.

Among the major ongoing research areas with plus-strand RNA viruses are (i) cataloging all the host genes affecting virus replication and (ii) definition of the roles of the identified factors in various steps of the replication process (20). Utilizing the yeast-based efficient replication of a tombusvirus repRNA and the available yeast genomic libraries, the effect of most of the host genes on tombusvirus replication has been studied with ~4,800 nonessential single-gene deletions in the YKO library (27) and with 800 essential yeast genes in the yTHC library (this work). These genomic screens have led to the identification of 96 nonessential and 30 essential host genes, respectively, which affected the accumulation of the tombusvirus repRNA by 50% or more. Altogether, the two genetic screens combined have covered close to 95% of all the genes (estimated to be ~5,800) present in the yeast genome. Therefore, the identified 126 host genes that affected repRNA accumulation by more than 50% when compared to the parental strain represent ~2% of all the yeast genes tested. The identified host genes among the essential genes represent a higher ratio (3.75%) than among the nonessential genes (2%), suggesting that tombusviruses might have adapted to use and/or depend on essential genes to higher extent than nonessential genes. Altogether, this number of host genes is probably an underestimation, because genetic screens frequently overlook redundant genes (such as gene families) with similar functions whose deletion/down-regulation could be compensated for by other genes (39). Nevertheless, the identified host genes represent a diverse set of host genes shown to affect viral RNA replication, and thus they should be valuable for studies on the mechanism of tombusvirus replication.

Possible roles of the identified host factors in tombusvirus replication. Measuring the effect of down-regulation of selected essential host genes on (i) the level of initial viral RNA transcripts (Fig. 2 and 3), (ii) the amount of p33 and p92^{pol} produced (Fig. 2 and 3), (iii) the activity of the viral replicase to synthesize viral RNA products, and (iv) the ratio of the plus versus minus strands (Fig. 4 and 5) revealed that many of the identified genes, such as *DED1*, *SLN1*, *NAB2*, *RPO21*, and *ARP9*, might affect repRNA accumulation via changing the accumulation levels of both p33 and p92^{pol} replication proteins (Table 2). On the other hand, down-regulation of *COPI* and *MOBI* reduced only the p92^{pol} level but had less of an effect on the amount of p33 in the presence of doxycycline (Fig. 2, rows A7 and -8) (Table 2). Therefore, either reducing the amount of p92^{pol} or altering the ratio of p33 to p92^{pol} by *COPI* and *MOBI* might contribute to the altered rate of tombusvirus replication in these yeast strains. The altered p33/p92^{pol} levels or the changes in p33/p92^{pol} ratio could have direct effects on the

assembly of the tombusvirus replicase, as shown by us earlier (35). This is supported by the correlation between the reduced activity of the CNV replicase in vitro and the reduced in vivo accumulation of repRNA with the above strains in the presence of doxycycline. The above correlation is also valid for *ARP9* and, to a less extent, for *NOG1*, which enhanced CNV replicase activity and also increased DI-72 RNA accumulation in the presence of doxycycline. The correlation is weaker in case of *PRP5*, whose down-regulation had only small effect on CNV replicase activity yet led to 65% enhanced replication. It is possible that down-regulation of *PRP5* stimulates replication via enhancing the asymmetry of strand synthesis (Fig. 5), instead of affecting the total activity of the RC.

Roles of the identified host factors in tombusvirus replication might also be deduced from their known cellular functions. Therefore, below we will discuss briefly a group of identified host factors with known cellular functions in protein translation, modification, and stability. For example, *DED1* codes for an RNA helicase that is an essential translation factor (6, 14, 46). It is likely that the reduced level of Ded1p in the presence of doxycycline directly inhibits p33 and p92^{pol} translation, which in turn could lead to a reduced level of RC assembly, resulting in reduction in repRNA replication. Interestingly, Ded1p was also found to affect BMV 2a replicase production and BMV replication in yeast (23), suggesting that Ded1p might be an important, widely used regulator of viral protein synthesis. The core protein of hepatitis C virus has been shown to bind to DBX, a DEAD-box RNA helicase, which can complement ded1p mutation in yeast (15). The HCV core protein-DBX interaction might explain how HCV infections could inhibit host cell translation. In addition, Ded1p was shown to bind to the particles of the yeast L-A virus, a double-stranded RNA virus, and promote the L-A virus negative-strand RNA synthesis in vitro (5). It is important to point out that down-regulation of Ded1p likely affects translation of host genes too; therefore, further experiments will be needed to demonstrate if translation of the tombusvirus p33 and p92^{pol} is selectively inhibited in yeast with a reduced Ded1p level.

Reduced expression of *MOB1* selectively decreased the amount of p92^{pol}, but not that of p33, in the presence of doxycycline (Fig. 4, lanes 5 and 6). The Mob1/phocein family of proteins, which are activating subunits of Dbf2-related protein serine/threonine kinases, are essential in yeast, and they are also present in all eukaryotic cells (9). Therefore, it is possible that the reduced stability of p92^{pol} could be due to altered posttranslational modification of p92^{pol} when a reduced amount of Mob1p is present.

Down-regulation of *SLN1*, which codes for a membrane-bound histidine kinase, moderately decreased the level of p33 and had only a minor effect on p92^{pol} levels in the CNV replicase (Fig. 4, lanes 3 and 4), whereas replicase activity and repRNA accumulation were changed by 4- and 2.5-fold, respectively. It is possible that Sln1p might affect the activity/amount of p33, and thus replication of recRNA, directly. We cannot yet exclude, however, that the effect of Sln1p on repRNA replication is only indirect via affecting the activity of transcription factors, which could regulate the functions of many host proteins. Interestingly, however, Sln1p is known to

TABLE 3. Host factors affecting both replication and RNA recombination

Gene	Effect on replication ^a	Effect on recombination ^b
<i>COP1</i>	Decrease	Increase
<i>RPB11</i>	Decrease	Decrease
<i>ARP9</i>	Increase	Decrease
<i>RRP9</i>	Decrease	Decrease

^a Based on Table 1.

^b Based on the study by Serviene et al. (40).

interact with Cop1p, another host factor affecting repRNA accumulation (Table 1; also see below).

Comparison of host factors affecting tombusvirus replication versus RNA recombination based on genome-wide screens. One of the characteristic features of plus-stranded RNA viruses is high recombination frequency, which promotes rapid virus evolution, a major problem for the host antiviral defense and development of long-lasting antiviral strategies (36, 45). The most frequent RNA recombination is based on template switching by the viral replicase during virus replication (21, 45). Thus, an intriguing question is whether host genes that affect virus replication could also affect RNA recombination. To answer this question, we recently performed genome-wide screens with the YKO (41) and yTHC collections (40) to identify host genes affecting tombusvirus RNA recombination. Comparison of the identified essential host genes in the yTHC collection revealed that down-regulation of four of the identified genes affected both RNA recombination (40) and tombusvirus replication (this work) (Table 3). Among these host genes identified, *COP1* is the most interesting, because down-regulation of *COP1* decreased repRNA accumulation, but increased the accumulation of recombinant RNAs (recRNAs) (40). Because Cop1p is involved in intracellular protein transport (7), it is possible that it could affect the assembly of the viral RC and/or the intracellular targeting/localization of the viral RC. The altered RC then might be less efficient for replication, but more prone for recombination events. The possible role of Cop1p in tombusvirus replication has also been suggested by McCartney et al. (16), who found that inhibition of vesicle formation at peroxisomes (which requires Cop1p activity) by expression of a dominant-negative mutant of ADP-ribosylation factor 1 affected intracellular sorting of p33 in plant cells.

Another interesting gene is *ARP9*, which increased repRNA accumulation (Table 1), but decreased the ratio of recRNA versus repRNA (40) when down-regulated. Thus, a small amount of Arp9p might somehow increase the fidelity of virus replication, albeit the process could be indirect via an additional host factor.

The other two common host genes (*RPB11* and *RRP9*) reduced the accumulation of both repRNA and recRNAs in the presence of doxycycline (Table 3). The availability of less viral RNA templates due to reduced replication could also lead to reduced recombination (albeit recombination was affected to a higher extent than replication), suggesting that these factors might have an indirect effect on RNA recombination. Nevertheless, these studies should open new ways to study the involvement of host genes in virus replication/recombination and

TABLE 4. Interactions among the host proteins affecting tombusvirus replication or recombination^a

Protein	Interacting protein	Function (effect on TBSV replication or on recombination)
Ded1p	Xrn1p	5'→3' Exoribonuclease (replication, down; recombination, up)
Ded1p	Nsr1p	RNA binding/rRNA processing (replication, up)
Cop1p	Sln1p	Protein histidine kinase activity (replication, down)
Sln1p	Cop1p	Protein transporter (replication, down)
Mob1p	Mps1p	Protein threonine/tyrosine kinase (recombination, down)
Utp15p	Utp9	snoRNA binding (replication, down)
Utp9p	Utp15p	snoRNA binding (replication, down)
Utp9p	Gph1p	Glycogen phosphorylase (replication, down)
Lpd1p	Dci1p	Peroxisomal $\Delta(3,5)$ - $\Delta(2,4)$ -dienoyl-coenzyme A isomerase, involved in fatty acid metabolism (recombination, down)
Med6p	Rox3p	RNA polymerase II transcription mediator (replication, down)
Rsc8p	Sin3p	Histone deacetylase (replication, down)
Rsc8p	Arp9p	Actin-related protein involved in transcriptional regulation; subunit of the chromatin remodeling Snf/Swi complex (replication, up; recombination, down)
Nog1p	Nop4p	RNA bonding (replication, up)
Nop4p	Nog1p	Putative GTPase (replication, up)
Arp9p	Rsc8p	Chromatin remodeling (replication, down)

^a Based on references 26, 40, and 41.

they will be useful in understanding the mechanism of virus replication/recombination.

Interactions among host factors that might influence tombusvirus replication. Host proteins do not function alone in the host cells, but interact with many other proteins forming dynamic complexes or protein networks that perform various functions (8, 10, 37). Therefore, it is possible that viruses take advantage of some of these complexes (such as the ribosome for translation) during virus replication. In an opposite scenario, viruses might have to compete for selected host proteins with other host proteins in order to recruit the selected host proteins away from regular cellular processes (such as intracellular transport or those taking place in the nucleus). The advantage of genome-wide screens is that they could possibly identify host factors involved in either of the above scenarios. Therefore, we searched the SGD database for documented host protein interactions (Table 4).

The most intriguing interaction found is between Sln1p and Cop1p, both of which down-regulates repRNA accumulation in the presence of doxycycline (Table 4). It is possible that Sln1p kinase affects the intracellular transport activity of Cop1p, or vice versa, Cop1p might affect the intracellular location of Sln1p kinase, which then could affect the assembly of the functional tombusvirus RC.

An example for competition between host proteins and tombusvirus replication could involve Nog1p and Nop4p. Down-regulation of either of these proteins, which interact with each other (Table 4) with doxycycline, led to increased accumulation of repRNA. We speculate that, to perform a cellular function, Nog1p and Nop4p might associate with a common host factor, which is also needed for tombusvirus replication. When either Nog1p or Nop4p is present only in a reduced amount, then the tombusvirus replicase proteins or RNA might gain easier access to this putative common host factor.

Interestingly, we found host factors that affected replication and interacted with other host proteins that also influenced

tombusvirus recombination. For example, Lpd1p interacts with Dci1p, which is involved in fatty acid metabolism and localized to the peroxisome—where tombusvirus replication takes place (22, 25). Absence of Dci1p was found to reduce the accumulation of tombusvirus recombinants (41). Therefore, it is possible that interaction between host factors could influence not only tombusvirus replication, but RNA recombination as well.

Interactions among other host proteins might also help group host factors affecting tombusvirus replication only indirectly. For example, Med6p, Rsc8p, and Arp9p are known to interact with Rox3p, Sin3p, and Rsc8p, respectively, which could affect transcription in the host cells, possibly altering the amounts of other host factors necessary for virus replication and/or the amounts of viral proteins and viral RNA transcripts.

Conclusions. The identified host genes in the previous (27) and current (Table 1) works reveal a complex picture for the host in tombusvirus replication. For example, host proteins in rather diverse groups, such as (i) RNA-binding proteins, ribonucleases, helicases; (ii) intracellular transport proteins; (iii) kinases and phosphatases; (iv) protease, protein reductase, and endopeptidase; (v) transcription factors; (vi) DNA replication factors; and (vii) proteins with unknown functions, were implicated. In addition, some of the above proteins stimulated, while others inhibited, tombusvirus replication. Overall, the systematic genome-wide screens likely identified proteins playing direct or indirect functions in tombusvirus replication. Further detailed studies will establish the functional roles of many of the identified proteins in tombusvirus replication.

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REFERENCES

- Ahlquist, P. 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science* **296**:1270–1273.
- Ahlquist, P., A. O. Noueiry, W.-M. Lee, D. B. Kushner, and B. T. Dye. 2003. Host factors in positive-strand RNA virus genome replication. *J. Virol.* **77**:8181–8186.
- Buck, K. W. 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv. Virus Res.* **47**:159–251.
- Cheng, C.-P., E. Serviene, and P. D. Nagy. 2006. Suppression of viral RNA recombination by a host exoribonuclease. *J. Virol.* **80**:2631–2640.
- Chong, J. L., R. Y. Chuang, L. Tung, and T. H. Chang. 2004. Ded1p, a conserved DExD/H-box translation factor, can promote yeast L-A virus negative-strand RNA synthesis in vitro. *Nucleic Acids Res.* **32**:2031–2038.
- Cordin, O., N. K. Tanner, M. Doere, P. Linder, and J. Banroques. 2004. The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. *EMBO J.* **23**:2478–2487.
- Cosson, P., Y. Lefkir, C. Demolliere, and F. Letourneur. 1998. New COP1-binding motifs involved in ER retrieval. *EMBO J.* **17**:6863–6870.
- Cusick, M. E., N. Klitgord, M. Vidal, and D. E. Hill. 2005. Interactome: gateway into systems biology. *Hum. Mol. Genet.* **14**(Suppl. 2):R171–R181.
- Devroe, E., H. Erdjument-Bromage, P. Tempst, and P. A. Silver. 2004. Human Mob proteins regulate the NDR1 and NDR2 serine-threonine kinases. *J. Biol. Chem.* **279**:24444–24451.
- Dunker, A. K., M. S. Cortese, P. Romero, L. M. Iakouchava, and V. N. Uversky. 2005. Flexible nets. The roles of intrinsic disorder in protein interaction networks. *FEBS J.* **272**:5129–5148.
- Gietz, R. D., and R. A. Woods. 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350**:87–96.
- Kushner, D. B., B. D. Lindenbach, V. Z. Grdzishvili, A. O. Noueiry, S. M. Paul, and P. Ahlquist. 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. *Proc. Natl. Acad. Sci. USA* **100**:15764–15769.
- Lai, M. M. C. 1992. RNA recombination in animal and plant viruses. *Microbiol. Rev.* **56**:61–79.

14. Linder, P. 2003. Yeast RNA helicases of the DEAD-box family involved in translation initiation. *Biol. Cell* **95**:157–167.
15. Mamiya, N., and H. J. Worman. 1999. Hepatitis C virus core protein binds to a DEAD box RNA helicase. *J. Biol. Chem.* **274**:15751–15756.
16. McCartney, A. W., J. S. Greenwood, M. R. Fabian, K. A. White, and R. T. Mullen. 2005. Localization of the tomato bushy stunt virus replication protein p33 reveals a peroxisome-to-endoplasmic reticulum sorting pathway. *Plant Cell* **17**:3513–3531.
17. Mnaimneh, S., A. P. Davierwala, J. Haynes, J. Moffat, W. T. Peng, W. Zhang, X. Yang, J. Pootoolal, G. Chua, A. Lopez, M. Trocheset, D. Morse, N. J. Krogan, S. L. Hiley, Z. Li, Q. Morris, J. Grigull, N. Mitsakakis, C. J. Roberts, J. F. Greenblatt, C. Boone, C. A. Kaiser, B. J. Andrews, and T. R. Hughes. 2004. Exploration of essential gene functions via titratable promoter alleles. *Cell* **118**:31–44.
18. Monkewich, S., H.-X. Lin, M. R. Fabian, W. Xu, H. Na, D. Ray, O. A. Chernysheva, P. D. Nagy, and K. A. White. 2005. The p92 polymerase coding region contains an internal RNA element required at an early step in tombusvirus genome replication. *J. Virol.* **79**:4848–4858.
19. Nagy, P. D., and J. Pogany. 2000. Partial purification and characterization of cucumber necrosis virus and tomato bushy stunt virus RNA-dependent RNA polymerases: similarities and differences in template usage between tombusvirus and carmovirus RNA-dependent RNA polymerases. *Virology* **276**:279–288.
20. Nagy, P. D., and J. Pogany. 2006. Yeast as a model host to dissect functions of viral and host factors in tombusvirus replication. *Virology* **344**:211–220.
21. Nagy, P. D., and A. E. Simon. 1997. New insights into the mechanisms of RNA recombination. *Virology* **235**:1–9.
22. Navarro, B., L. Rubino, and M. Russo. 2004. Expression of the *Cymbidium ringspot virus* 33-kilodalton protein in *Saccharomyces cerevisiae* and molecular dissection of the peroxisomal targeting signal. *J. Virol.* **78**:4744–4752.
23. Noueiry, A. O., J. Chen, and P. Ahlquist. 2000. A mutant allele of essential, general translation initiation factor DED1 selectively inhibits translation of a viral mRNA. *Proc. Natl. Acad. Sci. USA* **97**:12985–12990.
24. Oster, S. K., B. Wu, and K. A. White. 1998. Uncoupled expression of p33 and p92 permits amplification of tomato bushy stunt virus RNAs. *J. Virol.* **72**:5845–5851.
25. Panavas, T., C. M. Hawkins, Z. Panaviene, and P. D. Nagy. 2005. The role of the p33:p33/p92 interaction domain in RNA replication and intracellular localization of p33 and p92 proteins of cucumber necrosis tombusvirus. *Virology* **338**:81–95.
26. Panavas, T., and P. D. Nagy. 2003. Yeast as a model host to study replication and recombination of defective interfering RNA of tomato bushy stunt virus. *Virology* **314**:315–325.
27. Panavas, T., E. Serviene, J. Brasher, and P. D. Nagy. 2005. Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. *Proc. Natl. Acad. Sci. USA* **102**:7326–7331.
28. Panaviene, Z., J. M. Baker, and P. D. Nagy. 2003. The overlapping RNA-binding domains of p33 and p92 replicase proteins are essential for tombusvirus replication. *Virology* **308**:191–205.
29. Panaviene, Z., T. Panavas, and P. D. Nagy. 2005. Role of an internal and two 3'-terminal RNA elements in assembly of tombusvirus replicase. *J. Virol.* **79**:10608–10618.
30. Panaviene, Z., T. Panavas, S. Serva, and P. D. Nagy. 2004. Purification of the cucumber necrosis virus replicase from yeast cells: role of coexpressed viral RNA in stimulation of replicase activity. *J. Virol.* **78**:8254–8263.
31. Pogany, J., M. R. Fabian, K. A. White, and P. D. Nagy. 2003. A replication silencer element in a plus-strand RNA virus. *EMBO J.* **22**:5602–5611.
32. Pogany, J., K. A. White, and P. D. Nagy. 2005. Specific binding of tombusvirus replication protein p33 to an internal replication element in the viral RNA is essential for replication. *J. Virol.* **79**:4859–4869.
33. Rajendran, K. S., and P. D. Nagy. 2003. Characterization of the RNA-binding domains in the replicase proteins of tomato bushy stunt virus. *J. Virol.* **77**:9244–9258.
34. Rajendran, K. S., and P. D. Nagy. 2004. Interaction between the replicase proteins of tomato bushy stunt virus in vitro and in vivo. *Virology* **326**:250–261.
35. Rajendran, K. S., and P. D. Nagy. 2006. Kinetics and functional studies on interaction between the replicase proteins of tomato bushy stunt virus: requirement of p33:p92 interaction for replicase assembly. *Virology* **345**:270–279.
36. Roossinck, M. J. 2003. Plant RNA virus evolution. *Curr. Opin. Microbiol.* **6**:406–409.
37. Rual, J. F., K. Venkatesan, T. Hao, T. Hirozane-Kishikawa, A. Dricot, N. Li, G. F. Berriz, F. D. Gibbons, M. Dreze, N. Ayivi-Guedehoussou, N. Klitgord, C. Simon, M. Boxem, S. Milstein, J. Rosenberg, D. S. Goldberg, L. V. Zhang, S. L. Wong, G. Franklin, S. Li, J. S. Albala, J. Lim, C. Fraughton, E. Llamasas, S. Cevik, C. Bex, P. Lamesch, R. S. Sikorski, J. Vandenhaute, H. Y. Zoghbi, A. Smolyar, S. Bosak, R. Sequerra, L. Doucette-Stamm, M. E. Cusick, D. E. Hill, F. P. Roth, and M. Vidal. 2005. Towards a proteome-scale map of the human protein-protein interaction network. *Nature* **437**:1173–1178.
38. Salonen, A., T. Ahola, and L. Kaariainen. 2005. Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**:139–173.
39. Serva, S., and P. D. Nagy. 2006. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. *J. Virol.* **80**:2162–2169.
40. Serviene, E., Y. Jiang, C. P. Cheng, J. Baker, and P. D. Nagy. 2006. Screening of the yeast yTHC collection identifies essential host factors affecting tombusvirus RNA recombination. *J. Virol.* **80**:1231–1241.
41. Serviene, E., N. Shapka, C. P. Cheng, T. Panavas, B. Phuangrat, J. Baker, and P. D. Nagy. 2005. Genome-wide screen identifies host genes affecting viral RNA recombination. *Proc. Natl. Acad. Sci. USA* **102**:10545–10550.
42. Shapka, N., and P. D. Nagy. 2004. The AU-rich RNA recombination hot spot sequence of *Brome mosaic virus* is functional in tombusviruses: implications for the mechanism of RNA recombination. *J. Virol.* **78**:2288–2300.
43. Stork, J., Z. Panaviene, and P. D. Nagy. 2005. Inhibition of in vitro RNA binding and replicase activity by phosphorylation of the p33 replication protein of cucumber necrosis tombusvirus. *Virology* **343**:79–92.
44. White, K. A., and P. D. Nagy. 2004. Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination. *Prog. Nucleic Acid Res. Mol. Biol.* **78**:187–226.
45. Worobey, M., and E. C. Holmes. 1999. Evolutionary aspects of recombination in RNA viruses. *J. Gen. Virol.* **80**:2535–2543.
46. Yang, Q., and E. Jankowsky. 2005. ATP- and ADP-dependent modulation of RNA unwinding and strand annealing activities by the DEAD-box protein DED1. *Biochemistry* **44**:13591–13601.