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The Nedd4-Type Rsp5p Ubiquitin Ligase Inhibits Tombusvirus Replication by Regulating Degradation of the p92 Replication Protein and Decreasing the Activity of the Tombusvirus Replicase▽

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Recent in vitro proteomics screens revealed that many host proteins could interact with the replication proteins of *Tomato bushy stunt virus* (TBSV), which is a small, plus-stranded RNA virus (Z. Li, D. Barajas, T. Panavas, D. A. Herbst, and P. D. Nagy, J. Virol. 82:6911–6926, 2008). To further our understanding of the roles of host factors in TBSV replication, we have tested the effect of Rsp5p, which is a member of the Nedd4 family of E3 ubiquitin ligases. The full-length Rsp5p, via its WW domain, is shown to interact with p33 and the central portion of $p92^{pol}$ replication proteins. We find that overexpression of Rsp5p inhibits TBSV replication in *Saccharomyces cerevisiae* yeast, while downregulation of Rsp5p leads to increased TBSV accumulation. The inhibition is caused by Rsp5p-guided degradation of $p92^{pol}$, while the negative effect on the p33 level is less pronounced. Interestingly, recombinant Rsp5p also inhibits TBSV RNA replication in a cell-free replication assay, likely due to its ability to bind to p33 and $p92^{pol}$. We show that the WW domain of Rsp5p, which is involved in protein interactions, is responsible for inhibition of TBSV replication, whereas the HECT domain, involved in protein ubiquitination, is not necessary for Rsp5p-mediated inhibition of viral replication. Overall, our data suggest that direct binding between Rsp5p and $p92^{pol}$ reduces the stability of $p92^{pol}$, with consequent inhibition of TBSV replicase activity.

Various interactions with their host cells are critical for plus-stranded (+)RNA viruses as they attempt to utilize the host translation machinery to produce viral proteins, gain access to the resources of the host cells, co-opt host proteins, and subvert host membranes (1, 17). Additional levels of interaction between virus and host reflect antiviral responses which may involve innate immunity, as well as other antiviral processes and factors. On-going research with several model viruses is striving to map all the interactions between viruses and hosts and characterize the functions of the co-opted host factors. In this regard, recent research has led to the identification of a large number of host proteins which affect the replication of various (+)RNA viruses and minus-stranded RNA viruses (4, 5, 9, 11, 22, 35, 39). The roles and functions of most of the host proteins identified as being involved in RNA virus replication, however, are currently unknown.

Tombusviruses, such as *Tomato bushy stunt virus* (TBSV), are among the most advanced model systems in relation to the identification of host factors affecting (+)RNA virus replication. The TBSV genome codes for only five proteins, two of which are the replication proteins translated directly from the genomic RNA (45). One of these replication proteins is the abundant p33 replication cofactor; the other is the RNA-dependent RNA polymerase (RdRp) $p92^{pol}$. Due to the overlapping expression strategy, p33 is identical with the N-terminal portion of the larger $p92^{pol}$ protein (Fig. 1A). Both replication proteins contain an RNA-binding motif (arginine-proline-rich motif), phosphorylation sites that affect RNA binding by the p33 protein, a p33-p33/p92 interaction domain, and two transmembrane domains (Fig. 1A) (18, 19, 32, 36, 37). Three short stretches of amino acids in p33 and $p92^{pol}$ are involved in binding to the Pex19p host protein that facilitates the transportation of p33 and $p92^{pol}$ from the cytosol to the cytosolic surface of the peroxisomes, the site of replicase complex formation and viral RNA replication (25). The essential nature of the above-named domains for obtaining functional replicase complexes suggests that multiple dynamic protein-protein, protein-RNA, and protein-membrane interactions must be required for robust tombusvirus replication.

In order to identify host genes involved in tombusvirus replication and recombination, systematic genome-wide screens that covered 95% of the host genes were performed in the model host *Saccharomyces cerevisiae* yeast (9, 22, 34, 35). These screens led to the identification of over 150 host genes, although the functions of these genes in TBSV replication are largely unknown. In addition, proteomics analysis of the highly purified tombusvirus replicase, as well as the use of yeast protein arrays containing ~4,100 purified proteins to identify host proteins interacting with p33 and/or $p92^{pol}$, led to the identification of ~60 pertinent yeast proteins (12, 33). Current efforts are focused on characterizing the functions of key host proteins in TBSV replication.

Most of the host factors identified facilitate tombusvirus replication, though some are inhibitory. The list of characterized host factors includes heat shock protein 70 (Hsp70), which is required for the assembly of the viral replicase in vitro, as

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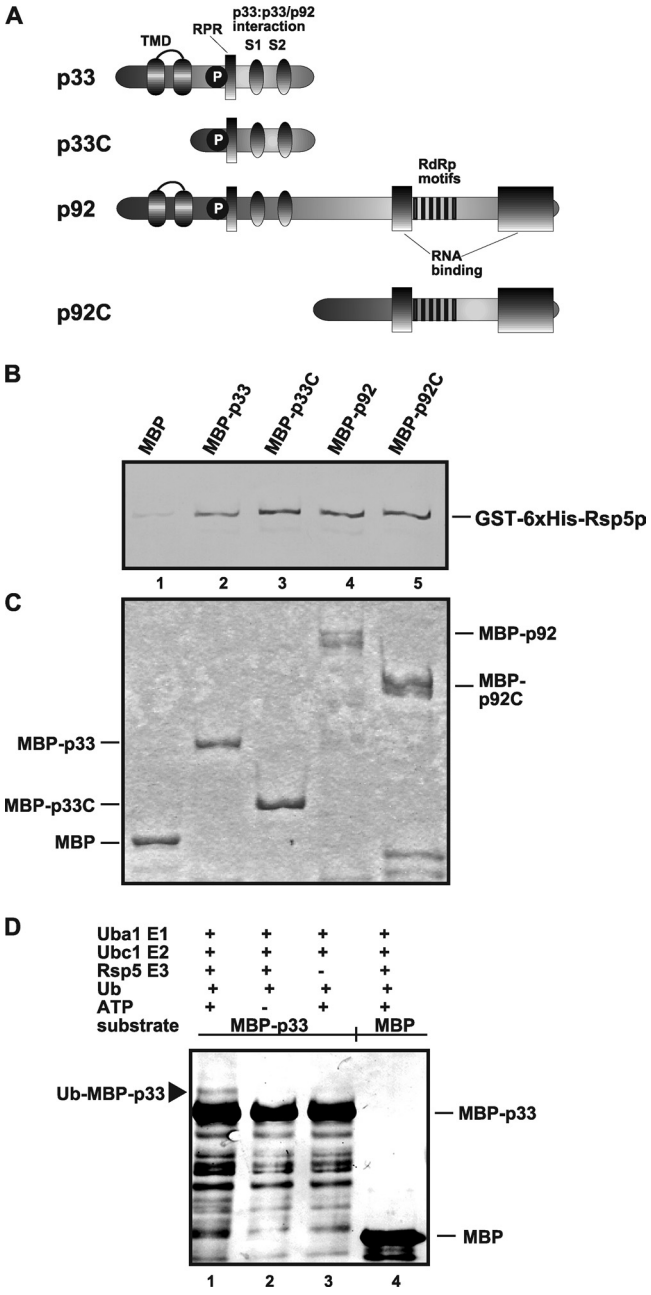


FIG. 1. Binding of Rsp5p to TBSV p33 and p92 proteins in vitro. (A) Schematic representation of viral proteins and their derivatives used in the binding assay. The various domains include the transmembrane domain (TMD), arginine-proline-rich RNA-binding domain (RPR), phosphorylated serine and threonine (P), and S1 and S2 subdomains involved in p33-p33/p92 interaction. The two RNA-binding regions in p92 are shown with boxes. (B) Affinity binding (pulldown) assay to detect interaction between GST–six-His–Rsp5p and the MBP-tagged viral proteins. The MBP-tagged viral proteins and MBP produced in *E. coli* were immobilized on amylose affinity columns. Then, GST–six-His–tagged Rsp5p expressed in *E. coli* was passed through the amylose affinity columns with immobilized MBP-tagged proteins. The affinity-bound proteins were specifically eluted with maltose from the columns. The eluted proteins were analyzed by Western blotting with anti-six-His antibody to detect the amount of GST–six-His–Rsp5p specifically bound to MBP-tagged viral proteins. (C) The amounts of MBP-tagged proteins eluted from the columns were analyzed by Coomassie blue staining of SDS-PAGE gels. (D) SDS-PAGE analysis of in vitro ubiquitination of replication protein p33 by purified recombinant Rsp5p. The components in the assays are indicated at the top. The ubiquitin-MBP-p33 product, detected by anti-six-His antibody, is marked by an arrowhead. Ub, ubiquitin; +, present; −, absent.

well as for membrane insertion and intracellular targeting of the viral replication proteins in vivo (29, 43). Another important host protein is GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which affects plus-strand synthesis (42). The functions of two other host factors that are also present in the replicase complex, namely, Cdc34p E2 ubiquitin-conjugating enzyme, which ubiquitinates p33 replication protein in vitro, and translation elongation factor 1A (eEF1A), which binds to a 3′ *cis*-acting regulatory element in the TBSV (+)RNA, are not yet characterized with respect to their roles in viral replication (12, 13). Downregulation of all four of the above-described host factors inhibited TBSV accumulation in the yeast model host and in plants (12, 13, 33, 42, 43), suggesting that they are significant players in TBSV replication.

In order to further the understanding of host factor roles in viral RNA replication, this paper addresses the effect of Rsp5p E3 ubiquitin ligase on TBSV accumulation. Rsp5p was selected since we have previously found an interaction between p33 and Rsp5p, based on the yeast protein array (12). Also, p33 is mono- and biubiquitinated in yeast cells (12), and Rsp5p is known to ubiquitinate select host proteins (3). These features of Rsp5p suggest its relevance to TBSV replication. Indeed, we found that Rsp5p inhibits TBSV replication when overexpressed in yeast cells, whereas its downregulation leads to increased TBSV accumulation. The inhibition is primarily caused by Rsp5p-mediated selective degradation of $p92^{pol}$. Its negative effect on the level of p33 is substantially less. However, the inhibitory function of Rsp5p is more complex, since the purified recombinant Rsp5p also inhibited RNA replication in a cell-free TBSV replication assay, likely due to the ability of Rsp5p to bind to both p33 and $p92^{pol}$. Surprisingly, the inhibitory function of Rsp5p is not caused by the HECT domain, which is involved in protein ubiquitination, but by its WW domain, which is involved in protein interactions. The observations suggest that direct binding between Rsp5p and p33 and, more importantly, $p92^{pol}$ is likely involved in the inhibition of TBSV replication.

MATERIALS AND METHODS

Yeast strains and expression plasmids. *Saccharomyces cerevisiae* strain InvSc1 was obtained from Invitrogen. Strain BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and the *pdr5*Δ, *vps27*Δ, *vps4*Δ, and *atg8*Δ single-gene deletion strains, as well as the R1158 (BY4741 URA3::CMV-tTA) and RSP5/THC strains with the regulatable TET promoter from the Hughes collection (yeast Tet promoters Hughes Collection [yTHC] library), were obtained from Open Biosystems. To create plasmid pGBK-His33-CUP1/DI72-GAL1, coexpressing six-His-tagged *Cucumber necrosis virus*-(CNV) p33 from the *CUP1* promoter and DI-72 plus-stranded replicon RNA [(+)repRNA] from the *GAL1* promoter, the *CUP1* promoter sequence was amplified by PCR from plasmid pSAL1 using primers 1598 and 1600 (8) (Table 1). The resulting PCR product was digested with Bsp1407I and NcoI, while plasmid pGBK-His33/DI72 (9) was digested with Bsp1407I and NcoI to remove the *ADH1* promoter. Then, the Bsp1407I/NcoI-digested PCR product was ligated to the above-described plasmid. Plasmid pGAD-His92-CUP1 expressing six-His-tagged CNV $p92^{pol}$ from the *CUP1* promoter (12) and plasmids coding for full-length or truncated TBSV p33 or p92 fused to the maltose binding protein (MBP) have been described previously (30).

To express Rsp5p in *Escherichia coli*, first the full-length *RSP5* gene was amplified by PCR using primers 2453 and 2454 and a yeast genomic DNA

TABLE 1. Sequences of primers used in this study

Primer	Sequence
0424	CGACGGATCCGATACCATCAAGAGGATGCTGTG
0952	CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA
1598	GCGTGTACAGGATCCCATTACCGACAT
1600	GCGCCATGGAATTCGTTACAGTTTGT
1849	GGCGCTCGAGTCATTCTTGACCAAACCCTATGG
2144	GTAATACGACTCACTATAGGGAAGCTATACCAAGCATACAATC
2145	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAACCTAAGAGTCAC
2203	CGTGCGGCCGATGGGTCATCATCATCATCATCACGAATTCCCTTCATCCATATCCGTCAAG
2204	CGTGCTCGAGCCTGCAGGTCAGCTAGCTTCTTGACCAAACCCTATGG
2260	GGCGAATTCATGCCTTCATCCATATCCGTCAAG
2388	CGTGCGGCCGAAGCTTACCATGCCTTCATCCATATCCGTCAAG
2450	GGCAAGCTTACCATGGGTCGGGATTACAAGGAC
2453	GGCAGATCTCATCATCATCATCATCATGGATCCTCTAGAAAGCTTACCATGCCTTCATC
2454	GCCGAATTCCTCGAGGCTAGCCCCGGGAAGCTTTCATTCTTGACCAAACCCTATG
2628	TAATACGACTCACTATAGGAAGCAATGGCGAGTGACT
2796	GCCGAATTCAAGCTTACCATGCCTTCATCCATATCCGTCAAG
2799	GCCGAATTCAAGCTTACCATGGCTAGCCAGCCCGCTCTTAGAATATTG
2800	CGGCTCGAGTCAGCTAGCCCTGAAATAAATAACCTTACGTC
2801	GCCGAATTCAAGCTTACCATGGCTAGCCAATTAGGTCCTTTGCCTTC
2802	CGGCTCGAGTCAGCTAGCGACCGGTTGTTGCTGAATGG
2803	GCCGAATTCAAGCTTACCATGGCTAGCAATATACCTCCTGTTAATGGTG
2804	CGGCTCGAGTCAGCTAGCACCACCTCCCACTTGAACTG
2805	GCCGAATTCAAGCTTACCATGGCTAGCCACCGTTCCACCAATTCCAC
2806	CGGCTCGAGTCAGCTAGCGGTTGTTGCCGTATTAGTAG

preparation. Second, the PCR product was digested with BglII and EcoRI and cloned into pGEX-2T (Novagen) treated with BamHI and EcoRI. The resulting plasmid, pGEX-His-Rsp5, was used to express the glutathione *S*-transferase (GST)–six-His-tagged Rsp5 protein in *E. coli*. To express derivatives of Rsp5p, pGEX-His-Rsp5 was digested with HindIII to excise the *RSP5* coding region and then religated to generate pGEX-His. A set of plasmids expressing the truncated *RSP5* derivatives was produced by using PCR with the primers listed in Table 2. The PCR products were digested with HindIII and XhoI and cloned into HindIII/XhoI-digested pGEX-His.

To express MBP-Rsp5 in *E. coli*, we generated plasmid pMAL-Rsp5 by PCR, amplifying the RSP5 coding sequence with primers 2203 and 2204. The PCR product was digested with EcoRI and XhoI and cloned into pMALc2x (New England Biolabs) digested with EcoRI and SalI.

To express Ubc1 (an E2 ubiquitin-conjugating enzyme) and Rsp5 (an E3 ubiquitin ligase) as GST fusion proteins in *E. coli*, the full-length sequences of yeast *UBC1* and *RSP5* were amplified by PCR using primer pair 2229 (GGCGGGATCCATGTCTAGGGCTAAGAGAATTATG)/2230 (GGCCCTCGAGTCACTTCAACAATTCCTCGATGAT) or 1848 (GGCGAGATCTATGCCTTCATCCATATCCG)/1849(GGCGCTCGAGTCATTCTTGACCAAACCCTATGG). The PCR products were digested by BamHI/XhoI or BglII/XhoI and ligated into similarly treated pGEX-2T.

To generate pYES-RSP5 and its derivatives, PCRs were performed as indicated in Table 2. The products were digested with EcoRI and XhoI and cloned into EcoRI/XhoI-digested pYES2/NT/C (Invitrogen). The resulting plasmids could express the six-His-tagged portions of Rsp5 using the *GAL1* galactose-inducible promoter. To produce pYES-Rsp5 (with no tag), we PCR amplified the coding region of Rsp5 with primers 2388/2204, followed by digesting the PCR products with HindIII/XhoI and cloning them into pYES2/NT/C digested with HindIII/XhoI. Note that the resulting plasmid expresses an untagged Rsp5 protein. pYC-Rsp5 is a low-copy-number plasmid that expresses untagged Rsp5 protein from a GAL1 promoter. To create pYC-Rsp5, the Rsp5 open reading frame was amplified with PCR using primers 2260/2204, digested with EcoRI/XhoI, and cloned into pYC2/CT/C (Invitrogen) digested with EcoRI/XhoI. The sequence coding for Rsp5-C777S was amplified by PCR using plasmid pKM103 (6) and primers 2203 and 2204. The PCR product was digested with EcoRI/XhoI and cloned to the similarly treated pYES2/NT/C. The constructs have been confirmed by sequencing.

TABLE 2. Primers used for construction of RSP5 derivatives for expression in *E. coli* and yeast

Primer pair	RSP5 derivative (amino acid position)
2796/1849	Full-length Rsp5 (1–809)
2796/2806	C2 (1–206)
2796/2800	C2/WW (1–443)
2799/1849	HECT (445–809)
2805/1849	WW/HECT (208–809)
2805/2800	WW1-3 (208–443)
2805/2802	WW1-2 (208–383)
2803/2800	WW2-3 (308–443)
2796/2804	C2/WW1 (1–306)
2801/1849	WW3/HECT (385–809)

Analysis of protein-protein interactions in vitro. The MBP-tagged p33 and p92 and derivatives were purified from *E. coli* as described previously (30). Briefly, expression of the MBP-tagged recombinant proteins was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in Epicurion BL21-codon-plus (DE3)-RIL cells (Stratagene). Cells were broken by sonication, and the cleared lysate was passed through amylose columns to bind the MBP-tagged viral proteins. The columns were washed three times with cold column buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 25 mM NaCl, 10 mM β-mercaptoethanol) (30). For the pulldown assay, we passed the cleared *E. coli* lysates containing GST–six-His–Rsp5 (full-length) or the truncated GST–six-His-Rsp5 derivatives through the amylose columns carrying the prebound MBP-tagged viral proteins. The columns were washed five times with cold column buffer, and the bound protein complexes were eluted with column buffer supplemented with 10 mM maltose. The presence of GST–six-His–Rsp5 proteins in the eluates was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using an anti-six-His antibody followed by an alkaline phosphatase-conjugated anti-mouse antibody (24). The amounts of MBP-tagged viral proteins in the eluates were analyzed by SDS-PAGE and Coomassie blue staining.

Analysis of TBSV repRNA replication in yeast. *S. cerevisiae* strain RSP5/THC was transformed with plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1. Transformed yeasts were grown in yeast synthetic dropout (SD) minimal medium without Ura/Leu/His (ULH^-) supplemented with 2% glucose at 29°C for approximately 16 h. Doxycycline was added to the cultures at the final concentrations (mg/liter) indicated below, and incubation continued for 7 to 8 h at 29°C to downregulate Rsp5 expression (34). The cultures were subsequently centrifuged, washed in ULH^- medium supplemented with 2% galactose, and then adjusted to an optical density at 600 nm of 0.5 in ULH^- 2% galactose supplemented with doxycycline and 50 μM $CuSO_4$ to induce viral replication and incubated for 24 h at 29°C (8, 34).

For Rsp5p overexpression, strain InvSc1 was cotransformed with plasmids pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1, and pYES-Rsp5 (full-

length) or pYES-Rsp5 derivatives. The transformed yeasts were grown in ULH$^-$ SD minimal medium supplemented with 2% galactose for 16 h at 29°C. Then, the cultures were supplemented with 50 μM $CuSO_4$ to induce virus replication and incubated for an additional 24 h at 29°C.

Total RNA was extracted from yeast and subjected to electrophoresis and Northern blot analysis as described previously (21). DI-72 (+)repRNA and 18S rRNA were detected by using ^{32}P-labeled riboprobes as described previously (22). The levels of mRNAs for p92^{pol} and p33 were detected with an RNA probe complementary to a 5′ portion of the p33 gene. This probe was synthesized by T7 transcription from a PCR product made with primers 0424 and 2628, using pGBK-His33-CUP1/DI72-GAL1 as template.

In vitro ubiquitination of p33 by recombinant Rsp5p. GST-Uba1 (E1) was expressed in yeast from plasmid pEG(KT/KG)-UBA1 obtained from the GST–six-His open reading frame library (a generous gift from Brenda Andrews). The purification of GST-Uba1 from yeast was performed as described previously (12). Briefly, the expression of GST-Uba1 was induced by incubation with galactose overnight at 29°C, and yeast cells were harvested and resuspended in a lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol [DTT], 10% glycerol and 1% [vol/vol] yeast protease inhibitor cocktail). The cells were homogenized in liquid nitrogen and centrifuged at 21,000 × *g* for 20 min at 4°C, and then the supernatant was incubated with 0.2 ml glutathione Sepharose beads (Novagen) for 3 h at 4°C on a rotating table. The glutathione Sepharose beads were then placed into a chromatography column and washed extensively with the lysis buffer. The GST-Uba1 was eluted using 50 mM Tris-HCl, pH 7.5, 10 mM reduced glutathione, 1 mM DTT, and 10% glycerol. GST-Ubc1 and GST-Rsp5 were obtained from *E. coli* by use of affinity purification based on glutathione Sepharose beads as described previously (12). The purified recombinant proteins were dialyzed in 40 mM Tris-HCl (pH 7.5), 10% glycerol, 50 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM DTT overnight at 4°C (two changes of 0.5 liters of buffer), aliquoted, and stored in a −80°C freezer. The purified six-His-tagged human ubiquitin was obtained from Boston Biochem. In vitro ubiquitination was carried out in 20 μl ubiquitination buffer (40 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 10 mM creatine phosphate [Roche], and 1 U creatine kinase [Roche]) containing 50 nM GST-Uba1, 500 nM GST-Ubc1, 100 nM GST-Rsp5, 100 μM six-His–ubiquitin, and 0.5 μg purified recombinant MBP-p33 or MBP as substrates (12). The ubiquitination assays were performed at 30°C for 60 min and then terminated by boiling for 5 min with SDS sample buffer containing 0.1 M DTT, followed by Western blot analysis with anti-MBP antibody (12). Ubiquitination of p33 was analyzed as described previously (12).

In vitro tombusvirus replicase assay using yeast membrane-enriched fraction. The in vitro replicase assays containing replicase/RNA complexes preassembled in vivo in yeast were performed as described previously (23, 24). For downregulation of Rsp5p, yeast strains RSP5/THC and R1158 were transformed with plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1. For overexpression of Rsp5p, yeast strain InvSc1 was transformed with plasmids pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1, and pYES2/NT/C or pYES-Rsp5. The transformed yeast strains were grown as described under "Analysis of TBSV repRNA replication in yeast." Then, yeast cultures were collected by centrifugation, resuspended in 1.5 volumes of extraction buffer E (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), and broken with glass beads in a Talboys homogenizer. The yeast extracts obtained were centrifuged at low speed (100 × *g*) to remove cell debris and then at high speed (21,000 × *g*) to pellet the membrane-enriched fraction containing the viral replicase complex and the copurified RNA template. The membrane-enriched fractions were washed with extraction buffer plus 1.2 M NaCl, centrifuged, and finally resuspended in extraction buffer E. The replicase activity with the copurified RNA template was assayed as described previously (23, 24).

Analysis of the accumulation of p33, p92, and Rsp5p derivatives in yeast. Yeast strains RSP5/THC and InvSc1 were transformed with plasmids and grown as described above for replication studies. Proteins were extracted and analyzed by SDS-PAGE and Western blotting as described previously (21, 24), using anti-six-His and anti-FLAG antibodies and alkaline phosphatase-conjugated anti-mouse antibody.

To analyze p92^{pol} stability, the yeast strain BY4741 and the *vps27*Δ, *vps4*Δ, and *atg8*Δ strains (Open Biosystems) were transformed with pGBK-His33-CUP1/DI72-GAL1, pGAD-FLAG-92-CUP1, and pYES2/NT/C, pYES-Rsp5, or pYES-Rsp5-(WW1-3). To create pGAD-FLAG-92-CUP1, the FLAG-tagged p92 was amplified from plasmid pGAD-92HF (33) by PCR using primers 2450 and 0952. The product was digested with NcoI and XhoI and cloned into NcoI/XhoI-digested pGAD-His92-CUP1. Transformed yeasts were pregrown in ULH$^-$ SD minimal medium supplemented with 2% glucose for 20 h at 29°C and then changed to ULH$^-$ supplemented with 2% galactose and incubated for 24 h at 29°C to overexpress Rsp5p. Then, the yeast cultures were supplemented with 50 μM $CuSO_4$ to induce FLAG-tagged p92^{pol} expression and incubated for 4 and 24 h before samples were collected. For proteosome inhibition, the *pdr5*Δ yeast strain was transformed with pGBK-His33-CUP1/DI72-GAL1, pGAD-FLAG-92-CUP1, and pYES2/NT/C, pYES-Rsp5, or pYES-Rsp5-(WW1-3) and grown as stated above, with the only difference being that the cultures were treated with 75 μM MG132 (from a 500× stock in dimethyl sulfoxide) for 2 h at 29°C to inhibit the proteosome (14) before p92^{pol} expression was launched by the addition of 50 μM $CuSO_4$. Samples were collected 5 h after the addition of $CuSO_4$ to analyze protein accumulation.

In vitro translation of p92^{pol} in a wheat germ extract. To obtain the mRNA for p92^{pol} expression, the CNV p92 gene was amplified by PCR with primers 2144 and 2145 from plasmid pGAD-His92 (24). The PCR product carrying the T7 promoter sequence was used for a T7 polymerase-based transcription to generate uncapped p92^{pol} mRNA that was used for in vitro translation. The translation assay using a wheat germ extract (Promega) was performed following the manufacturer's recommendations. One hundred nanograms of affinity-purified MBP or MBP-Rsp5 proteins were added to the in vitro translation reaction mixtures to test for inhibitory effect on p92 translation.

Replicase assay in cell extracts. MBP-tagged Rsp5 was purified from *E. coli* (30). The preparation of yeast cell extracts capable of supporting TBSV RNA replication in vitro was described previously (29). The replicase assays were performed in mixtures with a 20-μl total volume and included 2 μl yeast cell extract (cell-free extract [CFE]), 500 ng DI-72 (+)repRNA, 50 ng purified MBP-p33, 50 ng purified MBP-p92, and 100 ng purified MBP or MBP-Rsp5 proteins, added to the reaction mixtures as indicated in Fig. 9 below. The mixtures were incubated at 25°C for 3 h to support TBSV RNA replication in vitro, and the amount of newly synthesized radiolabeled repRNA was analyzed in polyacrylamide-urea gels as described previously (28).

RESULTS

Yeast Rsp5p E3 ubiquitin ligase binds to p33 and p92^{pol} replication proteins in vitro and ubiquitinates p33. To confirm the interaction between Rsp5p and p33, as well as p92^{pol}, we used a pulldown assay with purified proteins expressed in *E. coli*. The recombinant p33 and p92^{pol} proteins carrying the MBP tag at their N terminus were separately immobilized on affinity columns, followed by the application of *E. coli* lysate containing Rsp5p double-tagged with GST and six-His (Fig. 1B). The bound protein complexes were eluted with maltose and analyzed by Western blotting using anti-His antibody to detect GST–six-His–Rsp5p (Fig. 1B) and by SDS-PAGE (Fig. 1C). Unlike the MBP negative control, the MBP-tagged p33 and p92^{pol} bound to Rsp5p efficiently in vitro (Fig. 1B, lanes 1 versus 2 and 4). The N-terminally truncated p33C also bound to Rsp5p (Fig. 1B, lane 3), suggesting that Rsp5p recognizes the portion of p33 that is predicted to face the cytosol when inserted into the membrane. We also found that the unique portion of p92^{pol}, termed p92C (Fig. 1A), bound to Rsp5p efficiently (Fig. 1B, lane 5).

To demonstrate whether the binding between p33 and Rsp5p leads to ubiquitination of p33, we performed in vitro experiments with purified components. Since ubiquitination of protein substrates by Rsp5p is known to require E1 and E2 enzymes as well (2), we used purified yeast Uba1p (E1) and Ubc1p (E2) together with Rsp5p and MBP-p33 in the in vitro ubiquitination assay (Fig. 1D). We observed the expected ~8-kDa increase in the migration of a fraction of MBP-p33 molecules that is due to the ligation of ubiquitin to MBP-p33 when all the required compounds were present during the reaction (Fig. 1D, lane 1). In contrast, the omission of either ATP or Rsp5p from the reaction mixture eliminated the formation of ubiquitin-MBP-p33 (Fig. 1D, lanes 2 and 3), suggesting that

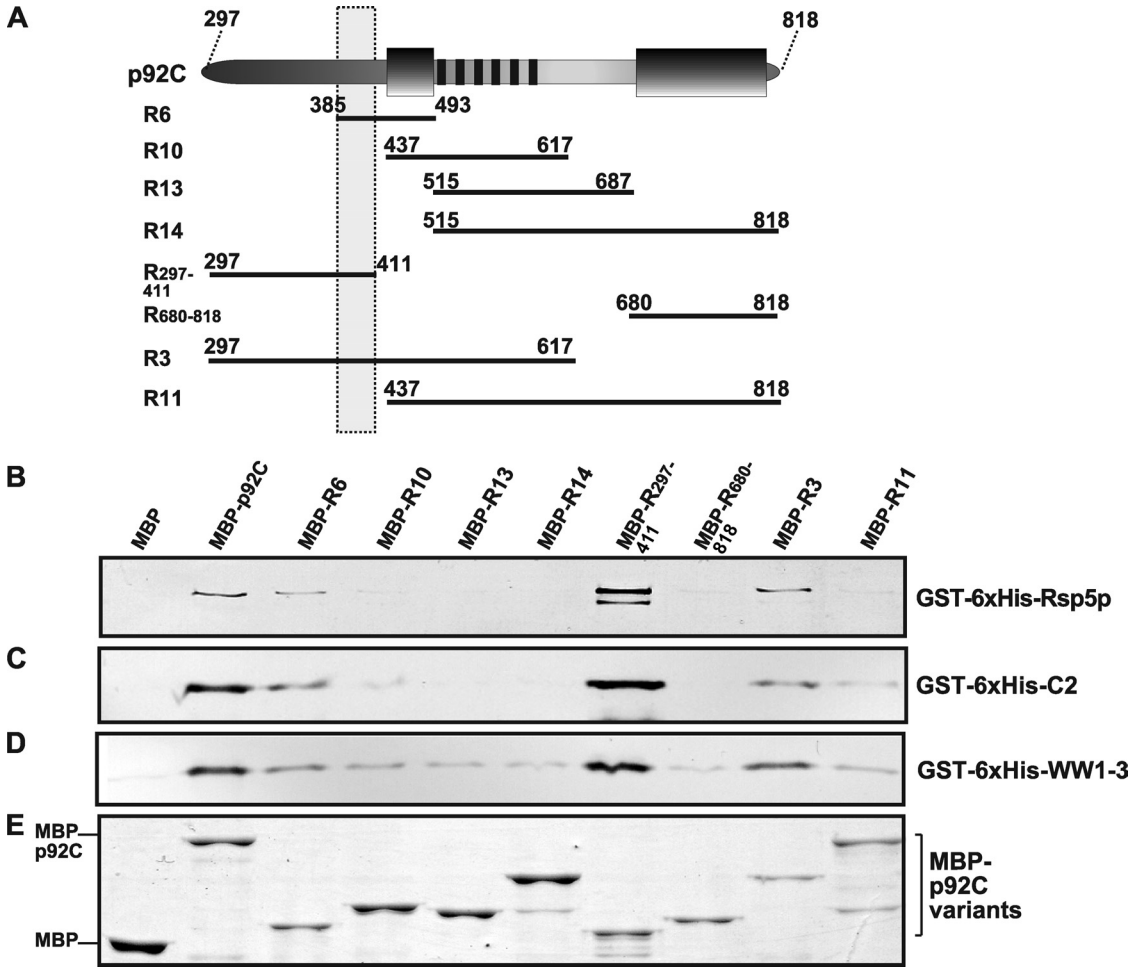


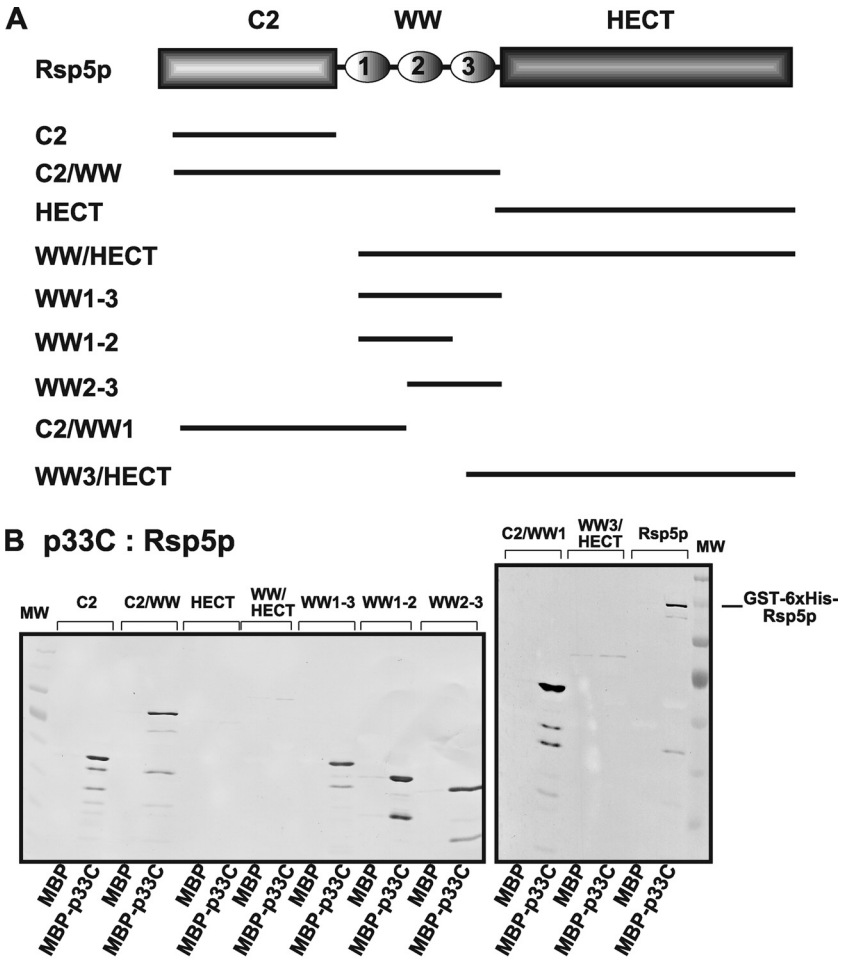
FIG. 2. Determination of the region in p92 binding to Rsp5p. (A) Schematic representation of the $p92^{pol}$ readthrough portion (p92C) and its truncated derivatives used in this assay. The amino acid coordinates are shown for each construct. The vertical box with the dotted outline shows the region required for Rsp5p binding; the p92C schema otherwise is as shown in Fig. 1A. (B) Western blot analysis of the full-length GST–six-His–Rsp5p bound to MBP-p92C and derivatives using anti-six-His antibody. See the Fig. 1B legend for further details. (C and D) Western blot analysis of the C2 domain (C) or the WW1-3 domain (D) of Rsp5p bound to MBP-p92C and derivatives using anti-six-His antibody. See the Fig. 2B legend for further details. (E) SDS-PAGE analysis of the amount of MBP-tagged p92C and derivatives eluted from the columns by Coomassie blue staining of gels.

Rsp5p is indeed required for ubiquitination of p33. The control assay with purified MBP as a substrate failed to detect a ubiquitinated product, excluding the possibility that the MBP portion of MBP-p33 becomes ubiquitinated in the reaction described above (Fig. 1D, compare lanes 1 and 4). Altogether, the results of these in vitro experiments confirmed that Rsp5p could ubiquitinate p33 under the conditions used.

To identify what portion of $p92^{pol}$ binds to Rsp5p, we performed additional pulldown experiments with a set of truncated MBP-p92 derivatives as shown in Fig. 2A. The C-terminally truncated mutants R3, $R_{297–411}$, and R6 of MBP-p92 bound to Rsp5p (Fig. 2B and E), the C2 domain (Fig. 2C), or the WW domain (Fig. 2D), whereas the other mutants did not bind to Rsp5p (Fig. 2B) or bound less efficiently to the C2 domain (Fig. 2C) or the WW domain (Fig. 2D). The results of these experiments demonstrated that Rsp5p binds to the central portion of $p92^{pol}$ (highlighted in Fig. 2A), a region that is proximal to the readthrough codon but does not overlap with the canonical RdRp motifs (Fig. 2B). Since R11 and R10 did not bind to Rsp5p, we conclude that Rsp5p does not bind to a previously defined RNA-binding region of $p92^{pol}$ (31, 32) which is located in a region shared by R6 and R11. Altogether, the results of these in vitro pulldown experiments demonstrated specific and efficient binding between $p33/p92^{pol}$ and Rsp5p.

The WW domain of Rsp5p is involved in binding to p33 and $p92^{pol}$ replication proteins in vitro. Rsp5p is a large protein with three functional domains. The N-terminal C2 domain binds to phospholipids, and this domain is implicated in membrane association. The central portion contains three repeats of the WW domain known to participate in protein interactions with a number of client proteins. The C-terminal HECT domain is responsible for the E3 ubiquitin ligase activity (38, 41).

To further define what domain(s) of Rsp5p is needed for interaction with p33 and/or $p92^{pol}$, we constructed nine trun-


A
C2
WW
HECT
Rsp5p
1
2
3
C2
C2/WW
HECT
WW/HECT
WW1-3
WW1-2
WW2-3
C2/WW1
WW3/HECT
B p33C : Rsp5p
MW
C2
C2/WW
HECT
WW/ HECT
WW1-3
WW1-2
WW2-3
C2/WW1
WW3/ HECT
Rsp5p
MW
GST-6xHis-Rsp5p
MBP
MBP-p33C
C Rsp5p
C2
C2/WW
HECT
WW/ HECT
WW1-3
WW1-2
WW2-3
C2/WW1
WW3/ HECT
MW
MBP
MBP-p92C
D E. coli extract
MW
Rsp5p
C2
C2/WW
HECT
WW/HECT
WW1-3
WW1-2
WW2-3
C2/WW1
WW3/HECT

cated versions of the recombinant GST–six-His–Rsp5p as shown schematically in Fig. 3A. The binding of the Rsp5p derivatives was tested in a pulldown assay with immobilized MBP-p33C (Fig. 3B) or MBP-p92C (Fig. 3C). The results of these binding experiments revealed that truncated Rsp5p protein constructs containing either the C2 or the WW domains (Fig. 3D) bound to both MBP-p33C (Fig. 3B) and MBP-p92C (Fig. 3C). Interestingly, the presence of the HECT domain in Rsp5p and its derivatives, such as WW/HECT and WW3/HECT, slightly inhibited the binding of the WW domain to MBP-p33C (Fig. 3B) or MBP-p92C (Fig. 3C) compared to the binding of constructs carrying the WW domain only (WW1-3, WW2-3, and WW2-3) or the C2 and WW domains (C2 and C2/WW). Altogether, the results of these experiments have established that the N-terminal half of Rsp5p is responsible for binding to p33 or $p92^{pol}$.

Downregulation of Rsp5p leads to increased TBSV repRNA accumulation in yeast. During our previous genome-wide screens covering 95% of yeast genes (9, 22), we did not test the essential *RSP5* gene, since it was not present in the early version of the yTHC library containing 800 of the 1,100 essential genes. Therefore, to test the effect of downregulation of *RSP5* gene expression on TBSV replication, we recently obtained a more complete yTHC library containing the *RSP5* strain from Open Biosystems. In the yTHC collection, the expression of a given essential yeast gene is under the control of a doxycycline-titratable promoter in the yeast genome. Therefore, the expression of the *RSP5* gene in the RSP5/THC strain can be downregulated/turned off by the addition of doxycycline to the yeast growth medium (15). This approach allowed us to test tombusvirus RNA replication in the presence of high levels of Rsp5p (when yeast was grown without added doxycycline after the induction of tombusvirus repRNA replication) or various reduced levels of Rsp5p (when yeast was grown in the presence of various concentrations of doxycycline) (15).

To test the replication of the TBSV repRNA, which is an efficiently replicating surrogate RNA template derived from the TBSV genomic RNA (21, 44), we expressed the p33 and $p92^{pol}$ replication proteins and DI-72 repRNA from plasmids in the RSP5/THC yeast strain. We found efficient replication of DI-72 repRNA in the RSP5/THC strain grown under standard growth conditions without added doxycycline (see Materials and Methods) (Fig. 4A, lane 1). The addition of increasing concentrations of doxycycline to the growth medium for RSP5/THC yeast led to decreases in *RSP5* mRNA levels at the beginning of repRNA replication (Fig. 4B), as well as after 24 h, when the samples for TBSV repRNA analysis were collected (Fig. 4C). Indeed, the level of *RSP5* mRNA at the highest doxycycline concentration was undetectable with Northern blotting at both time points (Fig. 4B and C, lane 4).

The level of accumulation of DI-72 repRNA was 2.5-fold higher in RSP5/THC yeast lacking a detectable level of *RSP5*

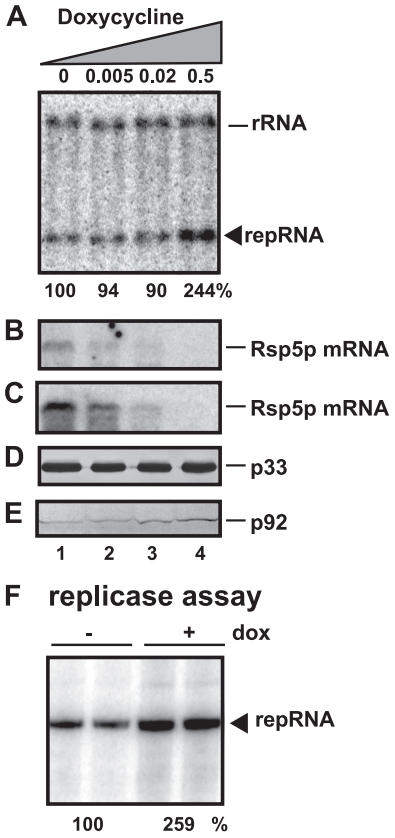


FIG. 4. Downregulation of *RSP5* expression increases TBSV repRNA accumulation in yeast. (A) For these experiments, RSP5/THC yeast was used, which contains a doxycycline-regulatable promoter replacing the native *RSP5* promoter. Therefore, the addition of doxycycline to the culture medium can downregulate Rsp5p expression at the mRNA level. To launch TBSV repRNA replication, we expressed six-His–p33 and six-His–p92 from the copper-inducible *CUP1* promoter and DI-72 (+)repRNA from the galactose-inducible *GAL1* promoter. First, the Rsp5p level was reduced by adding doxycycline (using the final concentrations indicated [mg/liter]) 7 h prior to expressing the viral components. Then, yeast cells were cultured for 24 h at 29°C on 2% galactose SC minimal medium containing the indicated amounts of doxycycline (mg/liter) plus 50 μM $CuSO_4$. Northern blot analysis was used to detect DI-72 (+)repRNA accumulation in RSP5/THC yeast. The level of accumulation of DI-72 (+)repRNA, with the level with no doxycycline set as 100%, was normalized based on the level of 18S rRNA. (B and C) Northern blot analysis of *RSP5* mRNA levels before launching TBSV repRNA replication (B) or at the end of the experiment (C) using the same samples for which results are shown in panel A. (D and E) Western blot analysis of the levels of accumulation of six-His-tagged p33 (D) and six-His-tagged p92 (E) proteins using anti-six-His antibody. (F) In vitro replicase assay based on the copurified replicase and the endogenous repRNA in membrane-enriched fractions prepared from RSP5/THC yeast. The denaturing PAGE assay shows the level of repRNA synthesis by the replicase in vitro. Note that comparable amounts of membrane-bound replicase (based on the level of p33) were used in this assay. dox, doxycycline (10 mg/liter); +, present; −, absent.

FIG. 3. Analysis of Rsp5p domains involved in binding to p33 or p92. (A) Schematic representation of Rsp5p domains and the various Rsp5p derivatives used in this assay. (B and C) Western blot analysis of the full-length GST–six-His–Rsp5p and derivatives bound to MBP-p33C (B) or to MBP-p92C (C) using anti-six-His antibody. See the Fig. 1B legend for further details. (D) The amounts of GST–six-His–Rsp5p and derivatives loaded to the columns were comparable, as determined by Western blot analysis of *E. coli* lysates. MW, molecular weight.

mRNA (Fig. 4A, lane 4), suggesting that a higher level of expression of Rsp5p inhibits TBSV repRNA replication in yeast. Testing the expression levels of p33 and p92^{pol} revealed that reduced Rsp5p levels did not affect the accumulation of p33 (Fig. 4D), whereas the level of p92^{pol} increased in RSP5/THC yeast lacking a detectable level of *RSP5* mRNA (Fig. 4E, lane 4). The negative correlation between Rsp5p and p92^{pol} levels indicates that Rsp5p might be involved in the regulation of p92^{pol} accumulation in yeast cells.

To test whether Rsp5p directly affected the tombusvirus replicase activity, we obtained enriched tombusvirus replicase preparations carrying copurified repRNAs from RSP5/THC yeast grown in the absence or presence of doxycycline. The in vitro replicase assay containing comparable amounts of p33 replication protein revealed that the tombusvirus replicase preparation obtained from yeast with the undetectable level of *RSP5* mRNA was ~2.5-fold more active than a comparable replicase preparation obtained from yeast grown in the absence of doxycycline (Fig. 4F). These experiments demonstrated that the level of Rsp5p affects the activity of the tombusvirus replicase.

To test the effect of Rsp5p under normal conditions (not an overexpression situation), we compared the activities of the viral replicase preparations derived from the parental yeast (R1158) expressing the wild-type (wt) Rsp5p from its natural promoter and RSP5/THC yeast grown in the absence or presence of doxycycline. The results of the in vitro replicase assay containing comparable amounts of p33 replication protein revealed that the replicase preparation obtained from the parental yeast was as active as the replicase preparation obtained from RSP5/THC yeast grown in the absence of doxycycline (Fig. 5A). However, the replicase activity increased by approximately fourfold when Rsp5 was downregulated (Fig. 5A, lanes 7 and 8). These data suggest that Rsp5 has some inhibitory effect on the tombusvirus replicase under normal conditions (i.e., wt expression level). We propose that the inhibitory effect of Rsp5p could be important to (i) keep the optimal ratio of p33/p92 for preserving the fidelity of the replicase complex; (ii) facilitate the assembly of the replicase; or (iii) prevent premature activation of replicase in the cytosol, before binding to the membrane.

In addition, we have tested the ubiquitination level of p33 in the parental yeast expressing the wt Rsp5p from its natural promoter and in RSP5/THC yeast grown in the absence or presence of doxycycline. The ubiquitination levels of p33 were comparable in the parental yeast and in RSP5/THC yeast grown in the absence of doxycycline (Fig. 5B, lanes 2 and 4). However, downregulation of Rsp5p led to decreased ubiquitination of p33 (Fig. 5B, lane 3), suggesting that Rsp5p could be involved in p33 ubiquitination in yeast.

Overexpression of Rsp5p decreases the accumulation of TBSV repRNA in yeast. To test the effect of Rsp5p overexpression on TBSV repRNA accumulation in yeast, we expressed Rsp5p from the galactose-inducible *GAL1* promoter 16 h earlier than we launched TBSV repRNA replication via the expression of p33/p92^{pol} proteins from the copper-inducible *CUP1* promoter (Fig. 6A). The high level of Rsp5p expression from a high-copy-number plasmid (pYES-RSP5) (Fig. 6B, lanes 7 to 10) led to efficient inhibition of TBSV repRNA accumulation (down to 4% of the level in the pYC control) (Fig. 6A, lanes 1 and 2). On the other hand, a low level of overexpression of Rsp5p from a low-copy-number plasmid (pYC-RSP5, expressing untagged Rsp5p) (Fig. 6B, lanes 3 and 4) did not alter the level of accumulation of TBSV repRNA. Interestingly, the accumulation level of p92^{pol} decreased dramatically, whereas the level of p33 was reduced to ~50% in yeast overexpressing Rsp5p at high levels (Fig. 6B, lanes 7 to 10). These data indicated that overexpression of Rsp5p can inhibit the accumulation of TBSV repRNA and that abundant Rsp5p is mostly inhibitory to the accumulation of p92^{pol} in yeast.

To study whether the activity of the tombusvirus replicase is affected in the presence of overexpressed Rsp5p, we isolated the enriched replicase fractions carrying the endogenous repRNA template from yeast cells. The in vitro replicase assay with replicase preparations that were adjusted for comparable levels of p33 showed only 22% activity in the Rsp5p overexpression yeast compared with the level of activity in the control yeast (expressing a short peptide from plasmid pYES) (Fig. 6C). These experiments confirmed that Rsp5p overexpression significantly reduces the activity of the tombusvirus replicase.

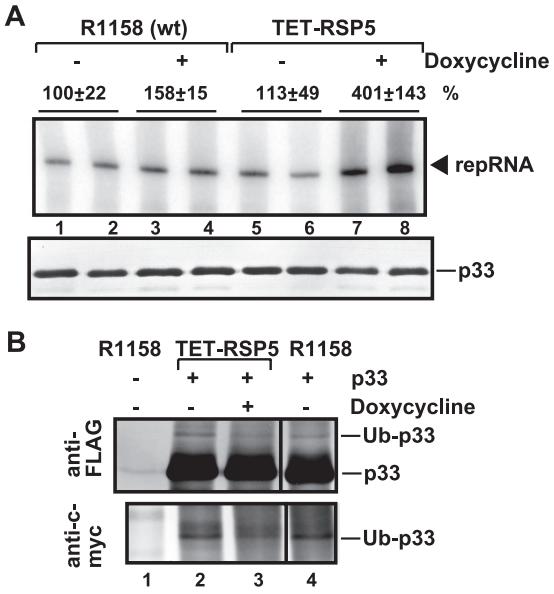


FIG. 5. Comparison of the effects of *RSP5* expression in wt (R1158) and RSP5/THC (TET-RSP5) yeast on the replicase activity and p33 ubiquitination. (A) In vitro replicase assay based on the copurified replicase and the endogenous repRNA in membrane-enriched fractions prepared from R1158 (wt) and RSP5/THC yeast grown with or without doxycycline. Top panel: the denaturing PAGE assay shows the levels of repRNA synthesis by the replicase in vitro, quantified as mean percentages and standard deviations by setting the activity in wt yeast without doxycycline as 100%. Note that comparable amounts of membrane-bound replicase (based on the level of p33) were used in this assay, as shown in the bottom panel. The level of six-His-tagged p33 in the replicase preparations was detected by Western blot analysis. The panel shows experiments performed in duplicate. (B) Ubiquitination of the six-His- and FLAG-tagged p33 protein was tested via Western blotting using anti-FLAG antibody (top panel) and anti-c-myc antibody (bottom panel). Note that monoubiquitination causes an ~8-kDa shift in p33 protein migration. The membrane-bound p33 was purified via FLAG affinity chromatography after solubilization of yeast coexpressing c-myc-tagged ubiquitin from a plasmid. Ub, ubiquitin; +, present; −, absent.

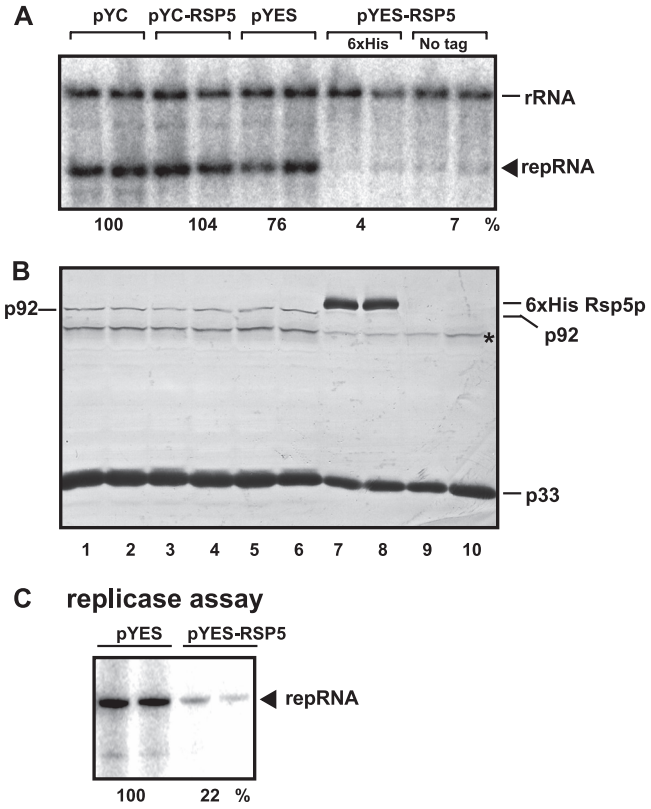


FIG. 6. Inhibition of TBSV repRNA accumulation by overexpression of Rsp5p in yeast. (A) Northern blot analysis was used to detect DI-72 (+)repRNA accumulation in the Sc1 yeast strain overexpressing Rsp5p or a small peptide from the low-copy-number plasmid pYC or from the high-copy-number plasmid pYES, as shown. The levels were quantified by setting the accumulation with plasmid pYC as 100%. The continuous expression of Rsp5p from the *GAL1* promoter started 16 h prior to launching repRNA replication at 29°C. Note that samples were obtained after 24 h of repRNA replication. See the Fig. 4 legend for further details. The panel shows experiments performed in duplicate. (B) Western blot analysis of the levels of accumulation of six-His-tagged p33, six-His-tagged p92, and six-His-tagged Rsp5p proteins using anti-six-His antibody. The asterisk indicates p33 homodimers. The panel shows experiments performed in duplicate. (C) In vitro replicase assay based on the copurified replicase and the endogenous repRNA in membrane-enriched fractions prepared from yeast overexpressing a peptide (pYES) or Rsp5p (pYES-RSP5). The denaturing PAGE assay shows the level of repRNA synthesis by the replicase in vitro. Note that comparable amounts of membrane-bound replicase (based on the level of p33) were used in this assay (not shown).

This effect is likely due to the reduced level of $p92^{pol}$ in yeast and/or direct inhibition of the replicase activity via binding of Rsp5p to the replication proteins (see below).

Overexpression of the WW domain is sufficient to decrease TBSV repRNA accumulation in yeast. To test what domain(s) of Rsp5p is important for inhibition of TBSV repRNA replication, we expressed various combinations of domains of Rsp5p in yeast replicating TBSV repRNA. Northern blot analysis of yeast total RNA revealed that Rsp5p derivatives carrying all three WW motifs, such as C2/WW, WW/HECT, and WW1-3, inhibited TBSV repRNA accumulation by four- to fivefold compared to the level of accumulation in the control expressing a small peptide from plasmid pYES (Fig. 7A). This level of inhibition is comparable to that caused by overexpression of the full-length Rsp5p. Importantly, the C2/WW, WW/HECT, and WW1-3 proteins also inhibited the accumulation of $p92^{pol}$ as much as the full-length Rsp5p (Fig. 7B, lanes 5, 7, and 8 versus 1 and 3).

The expression of the C2 and HECT domains did not significantly inhibit TBSV repRNA accumulation (Fig. 7A, lanes 5, 6, 9, and 10). Therefore, the results of these experiments conclusively demonstrated that the central domain with the three WW motifs in Rsp5p is critical for the biological activity of Rsp5p in TBSV replication, while the ubiquitin ligase HECT domain is not. This is further supported by the results for a ubiquitination-defective mutant Rsp5p (with a mutation of the cysteine at position 777 that is required for ubiquitin transfer) (6) (Fig. 7C) which had inhibitory effects on TBSV repRNA accumulation that were as strong as those of the wt Rsp5p. In addition, expression of the Rsp5p-C777S mutant decreased the $p92^{pol}$ level as much as expression of the wt Rsp5p did (Fig. 7D). Therefore, the ubiquitination function of Rsp5p does not seem to be important for inhibiting tombusvirus replication or reducing the level of $p92^{pol}$ in yeast.

Rsp5p facilitates the degradation of $p92^{pol}$ replication protein in yeast. To test how overexpression of Rsp5p can inhibit the accumulation of $p92^{pol}$, we used an in vitro translation assay with p92 mRNA (12, 28). The addition of purified recombinant Rsp5p to the in vitro translation assay did not affect the production of $p92^{pol}$ in vitro (Fig. 8A). Also, Rsp5p overexpression did not affect the mRNA level for $p92^{pol}$ compared with the level in yeast carrying plasmid pYES (Fig. 8B). This indicates that $p92^{pol}$ mRNA is likely available for translation in yeast overexpressing Rsp5p. Overall, the above-described data suggest that Rsp5p is unlikely to inhibit the translation of $p92^{pol}$. Alternatively, overexpression of Rsp5p might facilitate the degradation of $p92^{pol}$.

To test whether the degradation of $p92^{pol}$ is facilitated by Rsp5p, we overexpressed the full-length Rsp5p in yeast expressing $p92^{pol}$ while inhibiting the proteosome, which degrades many host proteins, by applying MG132 inhibitor (14). Measuring the levels of accumulation of $p92^{pol}$ in yeast overexpressing Rsp5p revealed approximately threefold-reduced levels of accumulation of $p92^{pol}$ in the absence or presence of MG132 (Fig. 8C, lanes 2 and 5) relative to the levels in the respective pYES controls (Fig. 8C, lanes 1 and 4). These data indicate that inhibition of the proteosome did not lead to increased levels of $p92^{pol}$ in yeast overexpressing Rsp5p. Thus, the Rsp5p-mediated degradation of $p92^{pol}$ might not be performed by the proteosome.

To further test the degradation of $p92^{pol}$, we also inhibited the endosome and autophagosome pathways (both occurring via the vacuole) by using *vps27*Δ, *vps4*Δ, and *atg8*Δ yeast strains. The protein degradation pathways that are mutated in these strains play roles in the degradation of membrane proteins (7, 20, 27). Interestingly, the accumulation level of $p92^{pol}$ increased by approximately twofold in *vps27*Δ and *vps4*Δ yeast strains (Fig. 8D and E, lanes 4 and 7). Moreover, the levels of $p92^{pol}$ were ~5- to 10-fold higher in *vps27*Δ and *vps4*Δ yeast strains than in the wt yeast strains, each overexpressing Rsp5 (Fig. 8D and E, lanes 5 and 8 versus 2). These data support the model that Rsp5p-mediated degradation of $p92^{pol}$ might be performed by the vacuole. This is further supported by the

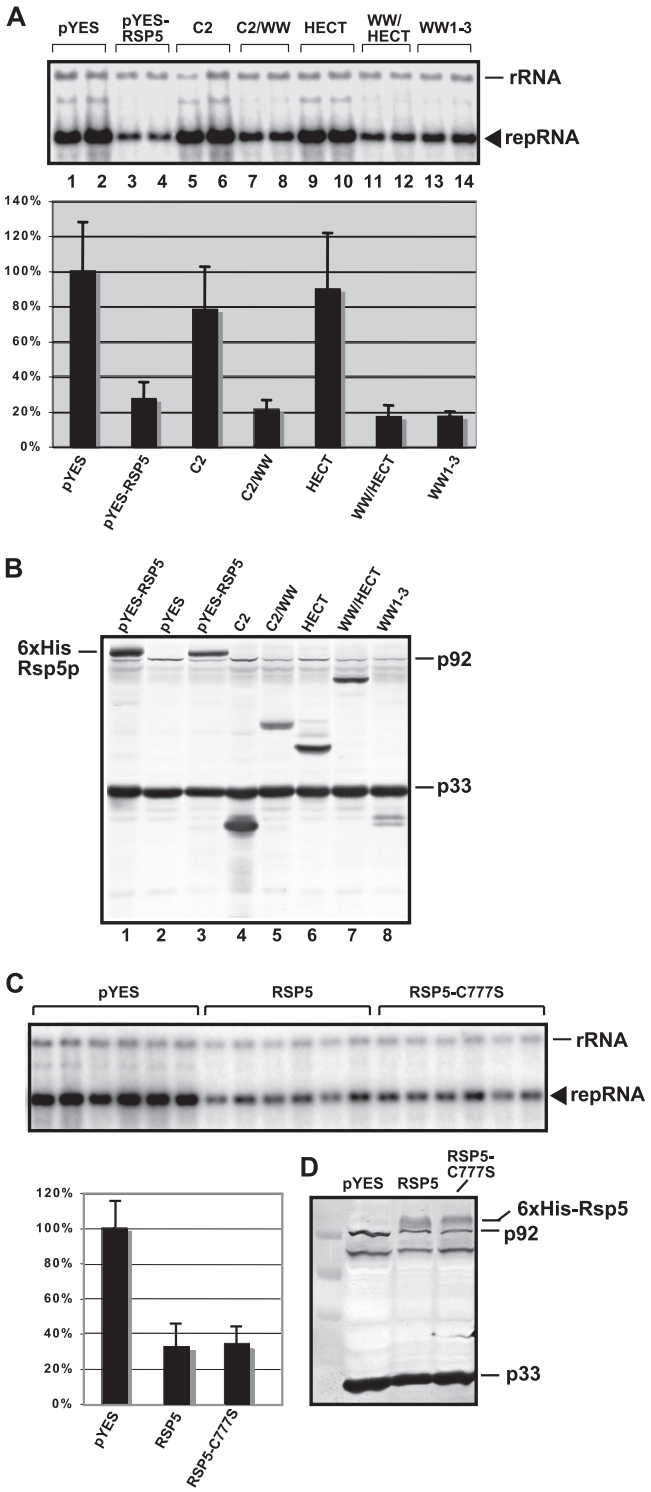


FIG. 7. Inhibitory effect of the WW domain in Rsp5 on TBSV repRNA accumulation in yeast. (A) Top panel: Northern blot analysis was used to detect DI-72 (+)repRNA accumulation in yeast strain Sc1 overexpressing various domains of Rsp5p from the high-copy-number plasmid pYES, as shown. See the Fig. 4 legend for further details. Bottom panel: percentage of TBSV repRNA accumulation in yeast overexpressing the indicated Rsp5p derivatives compared to the amount of peptide expressed from pYES. The graph represents averages and standard deviations obtained from eight samples for each Rsp5p derivative. The panel shows experiments performed in duplicate. (B) Western blot analysis of the levels of accumulation of six-His-tagged p33, six-His-tagged p92, and six-His-tagged Rsp5p derivatives using anti-six-His antibody. (C) Northern blot analysis was used to detect DI-72 (+)repRNA accumulation in yeast strain Sc1 overexpressing a ubiquitination-defective mutant of Rsp5p from the high-copy-number plasmid pYES, as shown. See the Fig. 4 legend for further details. The panel shows experiments performed in multiples. (D) Western blot analysis of the levels of accumulation of six-His-tagged p33, six-His-tagged p92, and six-His-tagged Rsp5p derivatives using anti-six-His antibody with the same samples for which results are shown in panel C. Error bars in the graph show standard deviations.

increased level of $p92^{pol}$ accumulation in *atg8*Δ yeast in comparison with the level in the wt yeast (Fig. 8D and E, lane 10 versus 1). Overexpression of Rsp5p had a similar inhibitory effect on the accumulation of $p92^{pol}$ in the *atg8*Δ yeast strain, but the level was still three times higher than in the wt yeast strain (Fig. 8D and E, lane 11 versus 2). Interestingly, overexpression of the WW domain of Rsp5p led to greater degrees of inhibition of $p92^{pol}$ accumulation (Fig. 8D and E, lanes 3, 6, 9, and 12), suggesting that the highly expressed WW domain is more effective than Rsp5p in facilitating the degradation of $p92^{pol}$ in yeast. This might be due to the stronger binding of the WW domain than of Rsp5p to $p92^{pol}$ (Fig. 2D and Fig. 3B and C), or some host proteins might compete with $p92^{pol}$ for Rsp5p, making Rsp5p less accessible than the WW domain protein. Overall, the data obtained support the model that the Rsp5p-mediated degradation of $p92^{pol}$ might occur via the endosome/vacuole pathway.

The recombinant Rsp5p inhibits the activity of the tombusvirus replicase in vitro. Recently, we have developed a cell-free TBSV replicase assay, based on a yeast extract and purified recombinant p33 and $p92^{pol}$ from *E. coli* (29). In this assay, the assembly of the active replicase takes place on the membrane surfaces present in the cell extract, and then the in vitro-assembled TBSV replicase is capable of supporting a complete cycle of replication, including minus- and plus-strand synthesis in a asymmetrical manner, similar to in vivo replication.

To test the effect of Rsp5p on the activity of the in vitro-assembled TBSV replicase, we affinity purified Rsp5p from *E. coli* and added it to the cell-free assay as shown in Fig. 9. We found that a large amount of Rsp5p was required to inhibit the activity of the TBSV replicase by 36% in vitro when all the components were mixed together simultaneously (Fig. 9, lane 2 versus lane 1). However, preincubation of Rsp5p and $p92^{pol}$ for 5 min in the cell extract (CFE), followed by the addition of the missing components, enhanced the inhibitory effect of Rsp5p (54% inhibition) (Fig. 9, lane 6). Similar preincubation of p33 and Rsp5p in the CFE had only a moderately inhibitory effect on the activity of the TBSV replicase (18%) (Fig. 9, lane 14). However, Rsp5p inhibited the function of p33 when preincubated with p33 in the replicase buffer, resulting in 60% inhibition of the activity of the TBSV replicase (Fig. 9, lane 18). Overall, the results of the in vitro experiments demonstrated that Rsp5p can directly inhibit the activity of the TBSV replicase, albeit the level of inhibition is significantly less than that observed in yeast overexpressing Rsp5p protein. The higher level of inhibition of TBSV repRNA replication by Rsp5p in yeast than in vitro is likely due to the inhibitory effect

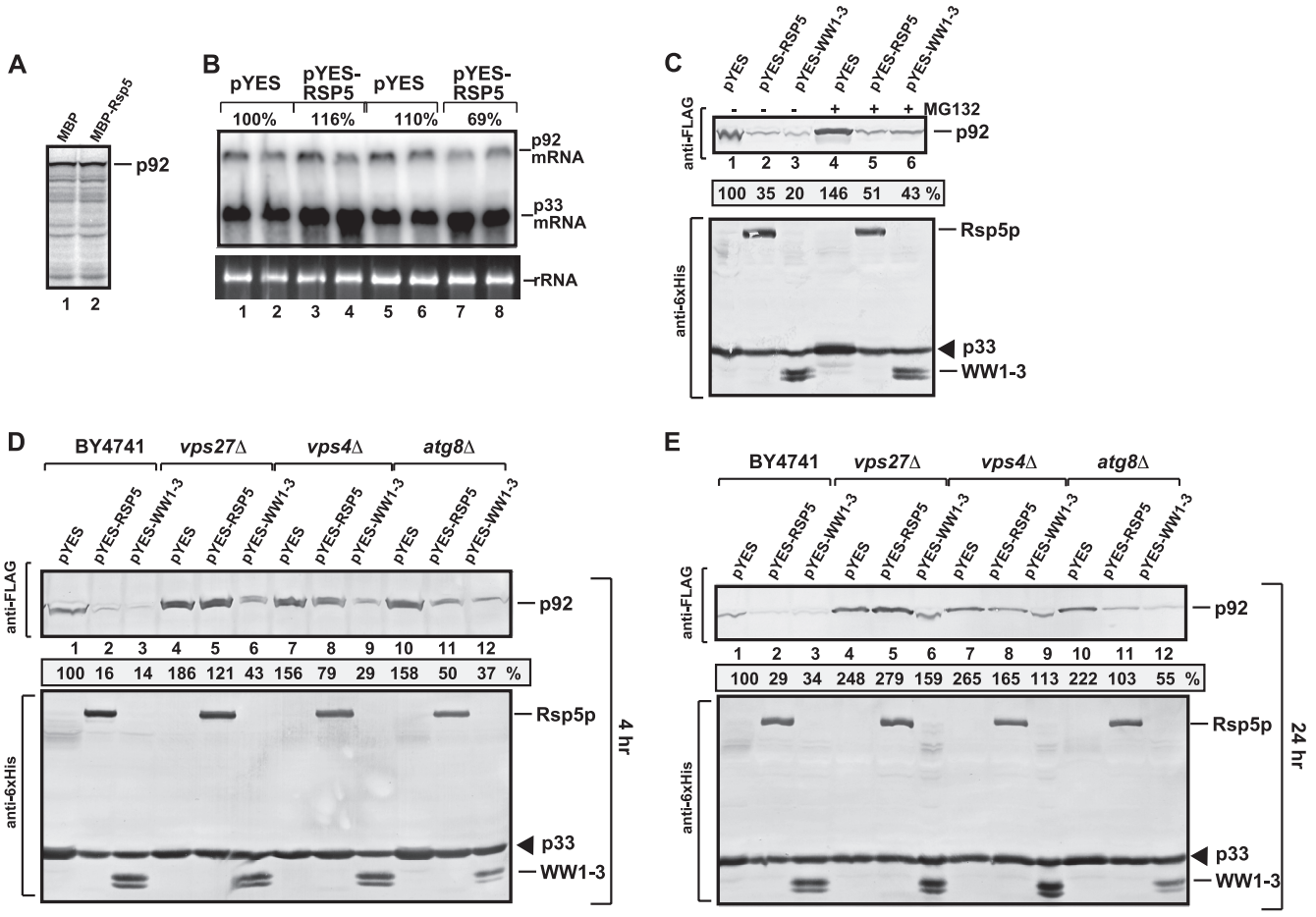


FIG. 8. Overexpression of Rsp5p affects degradation of p92 in yeast. (A) In vitro translation of p92 mRNA in wheat germ extract supplemented with 100 ng of purified MBP or MBP-Rsp5 protein. (B) Lack of effect of overexpression of Rsp5p on p92 mRNA level in yeast. The Northern blot analysis was done with riboprobes detecting both p33 and p92 mRNAs. The rRNA analyzed on an ethidium bromide-stained denaturing PAGE gel (bottom panel) was used as a loading control. The experiments were performed in duplicates. (C) Lack of effect of proteosome inhibition on p92 accumulation in yeast overexpressing Rsp5p. For these experiments, *S. cerevisiae* strain *pdr5*Δ expressing plasmid-borne FLAG-p92 from the *CUP1* promoter, six-His–p33 from the *CUP1* promoter, DI-72 (+)repRNA from the *GAL1* promoter, and six-His–Rsp5 or six-His–WW1-3 from the *GAL1* promoter was treated for 2 h with 75 μM MG132 proteasome inhibitor (+) or was not treated with MG132 (−). Subsequently, p92 expression was induced by the addition of 50 μM $CuSO_4$ for 5 h and the p92 accumulation level was analyzed by Western blotting using anti-FLAG antibody. p33, Rsp5p, and WW1-3 levels were analyzed by Western blotting using anti-six-His antibody. (D and E) Effect of inhibition of the endosomal and autophagosomal protein degradation pathways on the Rsp5p-mediated degradation of p92. Six-His–Rsp5p or six-His–WW1-3 was overexpressed in the parental strain BY4741 or the *vps27*Δ, *vps4*Δ, or *atg8*Δ yeast strain, while FLAG-p92 was launched by adding 50 μM $CuSO_4$ and incubating for 4 h (D) and 24 h (E). Western blot analyses with anti-FLAG antibody show the levels of accumulation of p92 in panels B to E, with the level with plasmid pYES set as 100%.

of Rsp5p on $p92^{pol}$ accumulation in yeast, which does not happen in vitro (not shown).

DISCUSSION

The p33 and $p92^{pol}$ replication proteins of TBSV likely interact with many host proteins that could be important for providing functions needed for the activity of the viral replicase, regulation of the virus replication process, reprogramming of the host cell to support virus replication, and/or as components of the host antiviral responses. The interaction between the yeast Rsp5p ubiquitin ligase and p33 is intriguing since p33 becomes ubiquitinated in yeast (12) and Rsp5p, as one of the major ubiquitin ligases in yeast (2), might be involved in the ubiquitination of p33. Indeed, purified Rsp5p was capable of ubiquitinating p33 in vitro (Fig. 1D). Also, downregulation of Rsp5p led to decreased levels of ubiquitinated p33 (Fig. 5B). However, the current work does not supply evidence that the ubiquitin ligase activity of Rsp5p is critical for TBSV replication. First, overexpression of Rsp5p inhibits TBSV RNA accumulation by fivefold or more (depending on the level of Rsp5p expression) (Fig. 6 and 7). However, overexpression of the wt Rsp5p, a truncated Rsp5p lacking the HECT E3 domain (Fig. 7A), or a ubiquitination-defective mutant Rsp5p (with a mutation of the cysteine at position 777 that is required for ubiquitin transfer) (Fig. 7C) had strong inhibitory effects on TBSV repRNA accumulation. Second, downregulation of Rsp5p in yeast leads to increased levels of TBSV repRNA accumulation and enhanced activity of the tombusvirus replicase in vitro (Fig. 4). Third, the inhibitory effect of

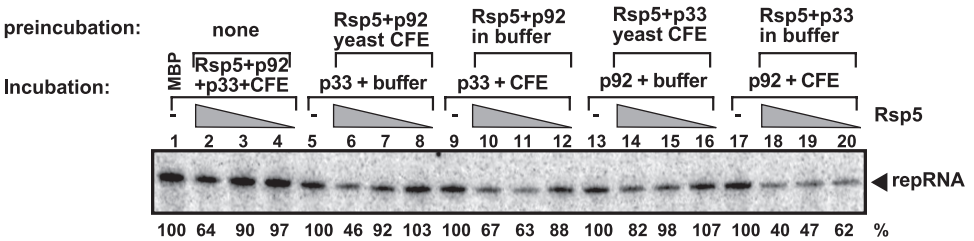


FIG. 9. Inhibitory effect of recombinant Rsp5p on TBSV replication in a yeast cell extract. Denaturing PAGE analysis shows the levels of repRNA accumulation in vitro, quantified by setting the level of accumulation with purified MBP as 100%. The yeast cell extracts (CFE) containing host factors and cellular membranes required for TBSV replication were mixed with in vitro-synthesized DI-72 (+)RNA and purified MBP-p33 and MBP-p92 proteins expressed in *E. coli*. The MBP protein (100 ng) purified from *E. coli* (indicated by −) or MBP-Rsp5 (100, 25, or 6 ng, as indicated by triangles) was added to the assay mixtures as indicated. Either all the recombinant proteins were added simultaneously to the CFE (lanes 1 to 4), or MBP-p92 or MBP-p33 was preincubated with MBP-Rsp5 or MBP in CFE (lanes 5 to 8 and 13 to 16, respectively) or preincubated in the replicase buffer (lanes 9 to 12 and 17 to 20, respectively) for 5 min at room temperature. Subsequently, the missing components (MBP-p33 or MBP-p92 and the CFE) not present during preincubation were added to the reaction mixtures. The reaction mixtures included [^{32}P]UTP to detect newly synthesized DI-72 repRNA. Note that the CFE is capable of supporting a full cycle of TBSV replication, leading to asymmetrical (+)RNA and minus-stranded RNA synthesis on the added (+)repRNA.

Rsp5p on TBSV replication depends on the presence of the WW domain, while the C-terminal ~40-kDa HECT domain of Rsp5p that is important for ubiquitin ligase activity is not necessary for this inhibitory activity (Fig. 7). Therefore, it is unlikely that Rsp5p could affect TBSV replication through its ubiquitin ligase activity, or this function might not be identified here due to the redundancy of ubiquitin ligase functions in yeast, which has more than 100 E2 and E3 enzymes (2). Indeed, Cdc34p E2 ubiquitin-conjugating enzyme, a host factor associated with the tombusvirus replicase, has also been shown to ubiquitinate p33 in vitro (12).

In addition to its direct role in biochemical catalysis of lysine ubiquitination, the HECT domain of Rsp5p is also known to be involved in interactions with E2-conjugating enzymes, such as yeast Ubc4, Ubc5, and other Ubc proteins (2). Yeast contains five HECT E3 ubiquitin ligases, but only Rsp5p has the WW domain. The other four are Ufd4p, Tom1p, Hul4p, and Hul5p, and they were not identified during the genome-wide screens to affect TBSV replication (9, 22). This further supports the model that the WW domain and not the HECT domain in Rsp5p is important for TBSV replication.

The C2 domain (amino acids 3 to 140) of Rsp5p, which is important for the ability of Rsp5p to bind to membrane phospholipids, inositol polyphosphates, and membrane-associated proteins, can bind to both p33 and p92^{pol} in vitro (Fig. 3). However, the interaction between the C2 domain and p33/p92^{pol} replication proteins does not seem to be important for the inhibitory effect of Rsp5p on TBSV replication (Fig. 7). So, the functional relevance of C2 domain-p33/p92^{pol} interaction is currently unknown.

The three-repeat WW domain (spanning amino acids 231 to 418) of Rsp5p, which is a protein-protein interaction module consisting of 38 to 40 amino acids per repeat with two highly conserved tryptophan residues, seems to be critical for the inhibitory effect of Rsp5p on TBSV replication (Fig. 7). When peptides containing all three WW repeats were expressed in yeast, we observed the inhibition of TBSV repRNA accumulation (Fig. 7). Also, the WW domain alone is sufficient to bind to both p33 and p92^{pol} (Fig. 3). Interestingly, the expression of the peptides with the WW domain or the full-length Rsp5p leads to reduced accumulation of the p92^{pol} replication protein in yeast, suggesting that Rsp5p could be involved in the degradation of the p92^{pol} replication protein in yeast. Accordingly, inhibition of the endosome-based membrane protein degradation pathway in yeast by using *vps27*Δ or *vps4*Δ yeast strains led to increased levels of p92^{pol} accumulation, even when Rsp5p was overexpressed (Fig. 8D and E). Inhibition of the autophagosome in *atg8*Δ yeast also led to increased accumulation of p92^{pol} (Fig. 8D and E), suggesting that the autophagosome might also be involved in the degradation of p92^{pol} in yeast. Altogether, the data obtained suggest that the Rsp5p-mediated degradation of p92^{pol} is likely based on the endosome/vacuole pathway (possibly with the involvement of the autophagosome as well). Overall, our observations support the idea that Rsp5p, via its WW domain, is a negative regulator of p92^{pol} accumulation and TBSV replication in yeast.

In addition to the proposed role of Rsp5p in the degradation of the p92^{pol} replication protein, we observed that the accumulation of the p33 replication protein is also decreased in yeast overexpressing Rsp5p at a very high level (Fig. 6). In addition, the purified recombinant Rsp5p was able to decrease the activity of the in vitro-assembled TBSV replicase in a yeast cell extract (Fig. 9). The inhibition was the greatest when Rsp5p and p92^{pol} were preincubated in the cell extract or when Rsp5p and p33 were preincubated in the RdRp buffer (Fig. 9). On the other hand, Rsp5p did not inhibit the activity of the in vitro-assembled TBSV replicase if it was preincubated with p33 in the cell extract (Fig. 9). Thus, the inhibitory function and the binding of Rsp5p to the p92^{pol} and p33 replication proteins might be facilitated by different conditions. Rsp5p binds to the unique portion of p92^{pol} that does not overlap with known RNA-binding or RdRp functions (Fig. 2). The importance of this binding, however, is still unknown. Overall, the above-described observations suggest that Rsp5p has a direct inhibitory effect on the TBSV replicase, but this activity of Rsp5p is probably a less significant factor than its proposed role in the degradation of the p92^{pol} replication protein, based on the much-higher efficiency of Rsp5p-driven inhibition of TBSV replication in yeast than in the cell extract (Fig. 6 and 9).

Since Rsp5p is involved in many cellular processes, including endocytosis, protein sorting at the trans-Golgi network, biosynthesis of fatty acids, mitochondrial inheritance, and main-

tenance of the actin cytoskeleton (10), it is possible that down-regulation or overexpression of Rsp5p affects TBSV replication via its pleiotropic effect on the host cell. However, the cellular functions of Rsp5p require the active E3 ubiquitin ligase function, whereas the overexpression of only the WW domain of Rsp5p was as effective in the inhibition of TBSV replication as the overexpression of the full-length Rsp5p (Fig. 7). Moreover, we showed direct binding of Rsp5p via its WW domain to p33 and $p92^{pol}$, as well as documenting an inhibitory effect of purified recombinant Rsp5p on the TBSV replicase assembled in a cell extract (Fig. 9). All these results suggest that Rsp5p likely regulates TBSV replication directly, albeit its pleiotrophic effect cannot be fully ruled out.

A growing list of observations suggests that HECT domain-type E3 ubiquitin ligases are involved in host-virus interactions. For example, the human E6-AP HECT E3 ubiquitin ligase interacts with the E6 protein of the human papillomavirus, leading to ubiquitination of tumor suppressor p53 that likely results in cell immortalization and cancer (26). The human Nedd4 HECT E3 ubiquitin ligase has been shown to ubiquitinate the Gag protein of human immunodeficiency virus, which has been proposed to facilitate the recruitment of endosomal proteins like ESCRT proteins for virus budding (16, 40).

This work confirms that, Rsp5p, a previously identified host protein which, based on a high-throughput protein array analysis, bound to replication protein p33 (12), is a negative regulator of TBSV replication in yeast. The inhibitory effect of Rsp5p is due to its WW protein interaction domain, suggesting that similar proteins with WW domains present in plants could play similar roles during TBSV replication in a native host. Future experiments will be needed to unravel whether Rsp5p plays a regulatory role in TBSV replication or whether it is a host protein having antiviral function.

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