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Novel Mechanism of Regulation of Tomato Bushy Stunt Virus Replication by Cellular WW-Domain Proteins

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ABSTRACT

Replication of (+)RNA viruses depends on several co-opted host proteins but is also under the control of cell-intrinsic restriction factors (CIRFs). By using tombusviruses, small model viruses of plants, we dissect the mechanism of inhibition of viral replication by cellular WW-domain-containing proteins, which act as CIRFs. By using fusion proteins between the WW domain and the p33 replication protein, we show that the WW domain inhibits the ability of p33 to bind to the viral RNA and to other p33 and p92 replication proteins leading to inhibition of viral replication in yeast and in a cell extract. Overexpression of WW-domain protein in yeast also leads to reduction of several co-opted host factors in the viral replicase complex (VRC). These host proteins, such as eEF1A, Cdc34 E2 ubiquitin-conjugating enzyme, and ESCRT proteins (Bro1p and Vps4p), are known to be involved in VRC assembly. Simultaneous coexpression of proviral cellular factors with WW-domain protein partly neutralizes the inhibitory effect of the WW-domain protein. We propose that cellular WW-domain proteins act as CIRFs and also as regulators of tombusvirus replication by inhibiting the assembly of new membrane-bound VRCs at the late stage of infection. We suggest that tombusviruses could sense the status of the infected cells via the availability of cellular susceptibility factors versus WW-domain proteins for binding to p33 replication protein that ultimately controls the formation of new VRCs. This regulatory mechanism might explain how tombusviruses could adjust the efficiency of RNA replication to the limiting resources of the host cells during infections.

IMPORTANCE

Replication of positive-stranded RNA viruses, which are major pathogens of plants, animals, and humans, is inhibited by several cell-intrinsic restriction factors (CIRFs) in infected cells. We define here the inhibitory roles of the cellular Rsp5 ubiquitin ligase and its WW domain in plant-infecting tombusvirus replication in yeast cells and in vitro using purified components. The WW domain of Rsp5 binds to the viral RNA-binding sites of p33 and p92 replication proteins and blocks the ability of these viral proteins to use the viral RNA for replication. The WW domain also interferes with the interaction (oligomerization) of p33 and p92 that is needed for the assembly of the viral replicase. Moreover, WW domain also inhibits the subversion of several cellular proteins into the viral replicase, which otherwise play proviral roles in replication. Altogether, Rsp5 is a CIRF against a tombusvirus, and it possibly has a regulatory function during viral replication in infected cells.

Plus-stranded (+)RNA viruses, which are widespread and emerging pathogens, replicate in the cytosol of infected cells by assembling membrane-bound viral replicase complexes (VRCs). The VRCs consist of the viral RNA and viral proteins, as well as co-opted host-coded proteins (1–8). Rapid progress has recently been made in understanding the functions of the viral replication proteins, including the viral RNA-dependent RNA polymerase (RdRp) and auxiliary replication proteins, and yet the functions of many subverted host proteins in VRC assembly are less well characterized (9, 10). The growing list of subverted host proteins contributing to VRC assembly includes translation factors, protein chaperones, RNA-modifying enzymes, and cellular proteins involved in lipid biosynthesis (11–15). The cellular ESCRT proteins, reticulons, and amphiphysins could be involved in membrane deformation occurring during VRC assembly (4, 16, 17). Altogether, it seems that the VRC assembly is a rather complex process driven by many factors; thus, it is likely regulated by viral and host factors for optimal replication in infected cells.

In addition to the subverted cellular replication proteins helping viral replication as susceptibility factors, many host proteins have been identified, which act as cell-intrinsic restriction factors (CIRFs) (11–13, 18–22). These factors might be components of the innate immune responses and used by the host for antiviral defense (23–26) or utilized by viruses as regulatory factors to keep the replication process under control (27).

Tomato bushy stunt virus (TBSV) is a small (+)RNA virus of plants. TBSV is used to study virus-host interactions using yeast (Saccharomyces cerevisiae) as a model host (5, 28–31). The auxiliary p33 replication protein, which is an RNA chaperone, recruits the TBSV (+)RNA to the site of replication, which occurs at the cytosolic surface of peroxisomal membranes (27, 32–36). The in-
teraction between the RdRp protein p92n\(^\text{pol}\) and the p33 replication protein is required for assembling the functional VRCs (30, 34, 37–39).

A dozen systematic genome-wide screens and global proteomics approaches in yeast or in vitro have led to the identification of ∼500 host proteins/genes involved in TBSV replication. The host proteins interacted with the viral replication proteins and viral RNA or affected TBSV replication and recombination when deleted/downregulated or overexpressed in host cells (11, 13, 40–48). Cataloging of the host factors affecting TBSV replication is one of the most complete among pathogens at a single cell level, thus facilitating mechanistic studies.

Several co-opted host factors are known to be involved in the assembly of the membrane-bound VRCs of tombusviruses. These proteins include the host heat shock protein 70 (Hsp70), the eukaryotic elongation factor 1A (eEF1A), Vps23p ESCRT (endosomal sorting complexes required for transport) protein, Bro1p ESCRT-associated protein, and Vps4p AAA + ATPase (39, 46, 47, 49–59). Cdc34p E2 ubiquitin-conjugating enzyme binds to p33, and it functions as a permanent member of the viral VRC, affecting the activity of the VRC (47).

Pex19p peroxisomal transport protein binds to p33 and promotes the recruitment of p33 to the peroxisomes (32, 36, 60). Interestingly, the Pex19p-p33 interaction is not essential for TBSV replication and, in the absence of Pex19p, p33 is recruited to the ER via another unidentified host protein/pathway (32, 60).

Several subverted cellular proteins are involved in viral RNA synthesis. The list includes eEF1A, the eukaryotic elongation factor 1B (eEF1B), the DDX3-, DDX5-, and eIF4AIII-like DEAD box helicases, and Tdh2p (GAPDH [glyceraldehyde-3-phosphate dehydrogenase]), all of which facilitate RNA replication (51, 53, 59, 61–63).

To test the possible regulatory functions of recruited cellular proteins, we chose the WW-domain-containing cellular proteins (64). The yeast Nedd4-type Rsp5p E3 ubiquitin ligase carrying WW domain was identified in several genome-wide screens previously (13, 42, 48). In addition to Rsp5p, several cellular WW-domain proteins, including Wwm1p, Prp40p, and plant AtDrh1, AtFCA, and AtPrp40c, bind to the tombusvirus replication proteins and inhibit their functions (64, 65). Accordingly, binding of Rsp5p and other WW-domain proteins to the p92n\(^\text{pol}\) replication protein leads to the degradation of p92n\(^\text{pol}\) (64, 65). The WW domain is a simple and highly conserved protein domain involved in protein–protein interactions (66, 67). The sequences of WW domains are highly variable (except from the conserved residues), which likely affect substrate specificity (66). The canonical WW domain contains two signature tryptophan residues and a conserved proline residue, which are part of a globular fold with three beta-sheets. WW-domain proteins, which are represented by multiple proteins in various organisms, including humans, bind to ligands usually carrying proline-rich sequences (66).

In the present study, we dissected the detailed function of the WW-domain proteins in viral replication. We show that the expression of the WW-domain protein interfered with complex formation between the p33 replication protein and several cellular proteins that act as susceptibility factors during TBSV replication. In addition, the WW domain inhibited the binding of p33 to the viral RNA and p33-p33 self-interaction in yeast. We also show that the WW domain can efficiently block tombusvirus replication in yeast or in a cell-free replication assay. We propose models on the CIRF activity and regulatory role of the WW-domain proteins in controlling TBSV replication via inhibition of VRC assembly at the late stage of replication.

**MATERIALS AND METHODS**

**Yeast strains and plasmids.** The *Saccharomyces cerevisiae* strain BY4741 (MAT\(a\) his3\(\Delta 1\) leu2\(\Delta 0\) met15\(\Delta 0\) ura3\(\Delta 0\)) was obtained from Open Biosystems. Yeast strains expressing C-terminal hemagglutinin (HA)-tagged proteins were made by homologous recombination using BY4741 as a parental strain. PCRs for 3\(\times\)HA tagging of SSA1, TEF1, and TDH2 were performed with the primer pairs 5075/2947, 5076/5077, and 5078/5079, respectively, using plasmid pYM-24 (Euroscarf) as the template. PCRs for 6\(\times\)HA tagging of CDC34, PEX19, and VPS4 were performed with the primer pairs 5080/5081, 5180/5181, and 3258/3259, respectively, using plasmids pYM-14 (Euroscarf) as the template for CDC34 and PEX19 and pYM-16 for VPS4. The obtained PCR products were transformed into BY4741 strain. Recombinant yeasts were selected on YPD plates supplemented with hygromycin or with G418-Geneticin. The BRO1-6\(\times\)HA strain has been described before (4).

To create the yeast strain GAL1-CDC34, containing an extra copy of CDC34, whose expression driven by the GAL1 promoter integrated at retrotransposon sites, CDC34 sequence was PCR amplified with the primer pair 1846/1847. The PCR product was digested with BglII and Xhol and ligated into BamHI/Xhol-digested pESC-HIS (Agilent Technologies). The cassette comprising GAL1::CDC34-CYCt was amplified with the primer pair 3674/3654. The resulting product was digested with BgII and ligated to BgHII-digested pA6-hphNT1 (Euroscarf). The ligation was used as the template for PCR with the primers 3633 and 3634. The resulting PCR product was transformed into yeast strain BY4741. Recombinant yeast was selected on yeast extract–peptone–dextrose plates supplemented with hygromycin.

Plasmids pGBK-Hisp33-CUP1/DI72-GAL1, pGAD-His92-CUP1, pGAD-Flag92-CUP1, and pGBK-His33-CUP1 have been described before (47, 64, 68). To create plasmid pGAD-CFP-p92-CUP1, the 6\(\times\)His-CFP-p92 cassette was amplified by PCR with the primers 807 and 952 using plasmid pGAD-CFP-p92 as the template (34). The PCR product was digested with Ncol and Xhol and ligated to pGAD-His92-CUP1 previously digested with Ncol and Xhol. To create pGBK-CFP-p33-CUP1, the 6\(\times\)His-CFP-p33 cassette was PCR amplified with the primers 807 and 992B from plasmid pGBK-His-CFP-p33 (34). The product was digested with Ncol and PstI and ligated into pGBK-His33-CUP1 previously digested with Ncol and PstI.

To make plasmid pGAD-WW-p92-CUP1, the RSP5 WW region was amplified from plasmid pYES-Rsp5 (64) with the primers 3045 and 3492. The resulting product was digested with NcoI and PstI and ligated into pGBK-His33-CUP1 previously digested with NcoI and PstI.

The Yeast strains and plasmids.

**Plasmids and yeast strains.** The yeast strains used for in vitro assays were obtained from Open Biosystems or constructed as described above.

**Experimental protocols.** Plasmids were constructed using standard techniques. Plasmid DNA was purified using a Miniprep Kit (Qiagen). Yeast transformations were performed as described (47). Western blot analysis was performed as described (47).

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pMAL-p33, expressing the maltose-binding protein (MBP) fused with TBSV p33, was made as follows. The TBSV p33 ORF was PCR amplified with the primers 788 and 2460. The PCR product was digested with BamHI and XbaI and ligated into EcoRI/XbaI-digested pMALc2x (New England Biolabs). To make pMAL-p92, TBSV p92 was PCR amplified with the primers 4688 and 2744. The PCR product was digested with EcoRI and XhoI and ligated into EcoRI/XhoI-digested pMALc2x. To make pMAL-WW-p33, the WW region of TBSV p33 was PCR amplified with the primers 2805 and 4608 and digested with BglII. TBSV p92 was PCR amplified with the primers 4619 and 826 and digested with BglII. The two PCR products were ligated together and reamplified with PCR using the primers 2805 and 826. The product was digested with EcoRI and XhoI and ligated into EcoRI/XhoI-digested pMALc2x.

pMAL constructs expressing truncated versions of TBSV p33 fused to MBP have been described before (69). pMAL-p33ΔΔRPR was made as follows. TBSV p33 (amino acids [aa] 168 to 296) was PCR amplified with the primers 48 and 1134 and digested with XbaI. TBSV p33 (aa 221 to 296) was PCR amplified with the primers 3564 and 10 and digested with NheI. The two PCR products were ligated and reamplified with PCR using primers 48 and 10. The obtained PCR product was digested with BamHI and Xbal and ligated into pMALc2x digested with BamHI and Xbal. pMAL–p33ΔΔRPR was made using a similar strategy. TBSV p33 (aa 1 to 209) was PCR amplified with the primers 788 and 1134, digested with XbaI, and ligated with NheI-digested TBSV p33 (aa 221 to 296) product. The ligation products were used for PCR with the primers 788 and 10. The generated PCR product was digested with EcoRI and XhoI and ligated into EcoRI/XhoI-digested pMALc2x. The plasmid pGEX-His-Rsp5, for expression of GST (glutathione S-transferase)-His8-Rsp5p in Escherichia coli, has been described before (64).

The plasmid pESC-URA (Agilent Technologies) was modified to replace the GAL10 promoter with the ADH1 promoter. To do so, the GAL10 promoter was amplified by PCR with the primers 1147 and 5003. The ADH1 promoter was amplified with the primers 953 and 5003, using pGAD-His92 as the template (30). The resulting PCR products were digested with BspI and ligated, together and reamplified with PCR using the primers 5002 and 5003. The PCR product was digested with BamHI and EcoRI and ligated into BamHI/EcoRI-digested pESC-URA.

To create pESC-His-WW-GAL1/ADH1, a cassette comprising the GAL1 promoter and the His8-tagged WW region of TBSV p33 was obtained by PCR using pYES-Rsp5 (64) with the primers 1147 and 2800. The ADH1 promoter was amplified with the primers 953 and 5003 as described above. The two PCR products were digested with BspI and ligated, together and reamplified with PCR using the primers 2800 and 5003. The generated PCR product was digested with XhoI and NotI and ligated into XhoI/NotI-digested pESC-URA. The His8-tagged YFP, SSA1, TEF1, and TDH2 were PCR amplified with the primers 5035/5036, 4960/4961, 4958/4959, and 5090/5005, respectively. The obtained PCR products were digested with NotI and SacI and ligated into NotI/Sacl-digested pESC-GAL1/ADH1 or into pESC-His-WW-GAL1/ADH1.

For the YTH-based studies, we created plasmids pGBK-Hisp33-CUP1/D172-GAL1 and pGBK/Rsp5-H11001/CUP1/D172-GAL1, plus pGAD-His92-CUP1 and pYES-Rsp5-WW1-3 (64). Transformed yeasts were pregrown in liquid medium supplemented with 2% glucose for 16 h at 29°C, washed in 2% galactose medium, and used to inoculate 2% galactose cultures (the starting optical density at 600 nm was 0.3). These cultures were incubated for 8 h at 29°C and then supplemented with 50 μM CuSO4, followed by incubation for an additional 24 h at 29°C.

The accumulation of p33 and p92 viral replication proteins was analyzed by Western blotting. Total proteins were extracted from the aliquots of cultures used to analyze repRNA by using NaOH and SDS-PAGE loading buffer, as described previously (30). Proteins were detected by using anti-His antibody, followed by alkaline phosphatase-conjugated anti-mouse antibody and NBT-BCIP detection (30). p33 and p92 mRNAs were detected by Northern blotting as described previously (64).

In vivo protein–protein interaction assays. Yeast two-hybrid assays were performed as described previously (70).

Confocal microscopy. Yeast strain BY4741 was cotransformed with plasmids pESC-HisYFP-p33-GAL1/DI-72-GAL10 and pGAD-pestx13-CFP or plasmids pESC-WW-YFP-p33-GAL1/DI-72-GAL10 plus pGAD-FLAGp92-CUP1; the transformed yeasts were then grown as described above, and the accumulation of DI-72 (+)repRNA was analyzed by Northern blotting (64).

Analysis of TBSV replication and p33 and p92 expression in yeast. Yeast strain BY4741 was transformed with plasmids pGBK-Hisp33-CUP1/D172-GAL1 and pGAD-His92-CUP1, and pESC-GAL1/ADH1 plasmids coexpressing His8-tagged WW and other host proteins. Transformed yeasts were pregrown in liquid medium supplemented with 2% glucose for 16 h at 29°C, washed in 2% galactose medium, and used to inoculate 2% galactose cultures (the starting optical density at 600 nm was 0.3). These cultures were incubated for 8 h at 29°C and then supplemented with 50 μM CuSO4, followed by incubation for an additional 24 h at 29°C.

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Copurification of selected host factors with the tombusvirus replicase from yeast. Yeast strains expressing HA-tagged host proteins from their chromosomal locations were transformed with plasmids pGBK-Hisp33-CUP1/D172-GAL1 or pGBK-FLAGp33-CUP1/D172-GAL1, plus pGAD-His92-CUP1 and pYES-Rsp5-WW1-3 (64). Transformed yeasts were pregrown for 16 h at 29°C in SC minimal medium containing 2% glucose and 100 μM Bathocuproine disulfonate (BCS; Acros Organics) to chelate the copper ions in the media. Cultured yeasts were transferred to SC medium supplemented with 2% galactose and 100 μM BCS, followed by incubation for 8 h at 29°C. The yeasts were then transferred to SC medium supplemented with 2% galactose and 5 μM CuSO4, followed by incubation for an additional 24 h at 29°C. The cultures were centrifuged, washed with phosphate-buffered saline (PBS), and incubated in PBS plus 1% formaldehyde for 1 h on ice to cross-link proteins. Formaldehyde was quenched by addition of glycine (0.1 M final concentration), and the yeast was recovered by centrifugation. The viral replicase complex was purified as described previously (47) based on FLAG-tagged p33 replication protein using anti-FLAG M2 agarose. Purified p33 was analyzed by Western blotting with anti-FLAG antibody, followed by anti-mouse antibody conjugated to alkaline phosphatase. Co-
purified HA-tagged host proteins were analyzed with anti-HA antibody, followed by alkaline phosphatase-conjugated anti-rabbit antibody and detection with NBT-BCIP as described previously (4, 30).

**TBSV replication in yeast cell extracts.** Cell-free extracts (CFE) from yeast strain BY4741 were prepared as described previously (56). The MBP-tagged proteins were purified from *E. coli* as described previously (69). The in vitro CFE assays were carried out with 0.1 µg of each purified protein, 0.5 µg of in vitro-transcribed DI-72 (+) repRNA, and 2 µl of CFE in a 20-µl final volume. The mixtures were incubated at 25°C for 3 h, and the amount of newly synthesized 32P-labeled repRNA was analyzed in denaturing polyacrylamide/urea gels as described previously (56).

In another set of experiments, affinity-purified GST or GST-tagged WW-domain proteins were preincubated with MBP-p33 for 10 min at room temperature; then, all other components (not including MBP-p33) were added. The mixtures were incubated at 25°C for 3 h, and the amount of newly synthesized 32P-labeled repRNA was analyzed in denaturing polyacrylamide/urea gels as described previously (56). Each experiment was repeated three times.

**Analysis of membrane association of p33 replication protein.** Membrane fractionation was performed according to the same procedure for membrane-enriched fractions described elsewhere (64). Briefly, BY4741 yeast transformed with plasmids pESC-hiaFP-p33-GAL1/DJ-72/GAL10 or pESC-WW-p33-GAL1/DJ-72/GAL10 plus pGAD-Hisp92 was grown in 2% glucose minimal medium for 16 h at 29°C, transferred to 2% galactose medium, and then grown for 24 h at 29°C. Yeasts were collected by centrifugation, resuspended in buffer E, and broken with glass beads. Unbroken cells were removed by low-speed centrifugation (100 x g for 5 min). Membrane fractions were pelleted by centrifugation at 15,000 x g for 20 min. Both the membrane fractions and the supernatant were used to analyze His6-YFP-p33 and WW-YFP-p33 accumulation by SDS-PAGE and Western blotting with anti-His antibody.

**In vitro protein-protein interaction assays.** Pulldown assays were performed as previously described (64). Briefly, the MBP-tagged TBSV p33, expressed in *E. coli*, was bound to amylose columns. Lysates of *E. coli* expressing recombinant GST-His6-p33 were then passed through the columns. After washing, MBP-tagged proteins were eluted with maltose. The amount of GST-His6-p33 bound to MBP-tagged p33 was analyzed by SDS-PAGE and Western blotting with anti-His antibody as described previously (64).

**In vitro RNA-protein binding assays.** Electrophoresis mobility shift assays (EMSA) were performed as described previously (69). Briefly, reaction mixtures contained 5 ng of 32P-labeled DI-72 (+) RNA plus 50, 200, or 500 ng of purified MBP-tagged proteins. After incubation for 15 min at 25°C, samples were placed on ice and run on non-denaturing 5% polyacrylamide gels.

In another set of EMSA, we used GST (10 pmol) or GST-tagged Prp40p, Rsp5p, and WW-domain proteins (two values for each, 5 and 10 pmol), which were preincubated for 10 min at room temperature with MBP-p33C (5 pmol), and then all other components were added (including -0.1 pmol of 32P-labeled (+) -DI-72). After incubation for 15 min at 25°C, the samples were placed on ice and run on non-denaturing 5% polyacrylamide gels. Each experiment was repeated twice.

**In vitro replicase assay.** Yeast strains R1158 (wild type [wt] for yTHC library series) (41) and ΔWW (www1/Tet:RSP5/Tet:PRP40/65) (65) were cotransformed with plasmids pGBK-CUP1-Flag-p33/GAL1/DI72 and pGAD-Cup-Flag-p92, and colonies were selected using SC-LH− plates. After growing in 50 ml of SC-LH− medium supplemented with 2% glucose and 1 mg/ml doxycycline for 24 h at 29°C, yeast cells were pelleted, washed with SC-LH− medium supplemented with 2% galactose, and re-suspended in 50 ml of SC-LH− medium supplemented with 2% galactose, 1 mg/ml doxycycline, and 50 µM CuSO4. Yeast cells were grown for 16 or 64 h at 29°C and then pelleted. About 200 mg of pellet was used to isolate tombusvirus replicase (based on Flag-p33 and Flag-p92) with anti-Flag M2-agarose as described previously (49). Replicate preparations isolated from different yeast strains were balanced and their activities were measured *in vitro* using (-)DI1/3 template in the standard RdRp assay (30). The presence of host factors was detected by using the following primary antibodies: anti-eEF1A, anti-eEF1B, anti-CDC34 and by using alkaline phosphatase-conjugated anti-rabbit antibody (Sigma) and NBT-BCIP detection (30).

**Kinetic measurements with surface plasmon resonance.** Kinetic measurements were done using a BLITZ instrument (FortBio). Briefly, the GST-tagged yeast proteins (0.2 µM) were loaded onto the GST-chip-based biosensor for 5 min with shaking (1,000 rpm). Binding to chip was measured in MBP-elution buffer (30 mM HEPES-KOH [pH 7.4], 2.5 mM NaCl, 1 mM EDTA) at room temperature as follows. MBP-tagged proteins were diluted with MBP elution buffer to 0.1 to 12 µM, and 4 µl of protein was interacted with GST-tagged proteins bound to the biosensor. Kinetic data were obtained as recommended: 30 s for baseline (buffer), 2 min for association (MBP-tagged protein), 2 min for dissociation (buffer). Association rate constant (ka), dissociation rate constant (kd), and interaction affinity constant (Kd) values were calculated using the BLITZ software. The negative control was purified MBP binding to the immobilized GST-WW protein or GST-Cdc34 on the GST-chip-based biosensor.

**RESULTS**

**Inhibition of the RNA-binding and protein-interaction functions of tombusvirus replication proteins by the WW-domain protein.** Previous studies revealed that the yeast Rsp5p E3 ubiquitin ligase acts as a CIRF through binding via its WW-domain to tombusvirus p33 and p92pol replication proteins and inhibits TBSV replication in yeast and *in vitro*, while several WW proteins inhibit TBSV infection in plants (64, 65). To unravel the mechanism of WW-domain protein-driven inhibition on TBSV replication, we first defined the binding site for the WW-domain of Rsp5p in the p33 replication protein using a set of truncated proteins in a pulldown assay (Fig. 1A). These experiments defined that the arginine-rich domain (RPR) motif in p33 involved in viral RNA binding is the preferred binding site for Rsp5p WW-domain protein (Fig. 1B and C).

To test whether Rsp5p WW-domain protein affects the ability of p33 to bind to the viral RNA, we first expressed and purified the WW domain of Rsp5p, the full-length Rsp5p, and Prp40, another yeast protein with WW domain, which has moderate CIRF activity against tombusviruses (65). Using affinity-purified proteins and 32P-labeled viral RNA, we performed an electrophoretic mobility shift assay (EMSA). These experiments revealed that both WW-domain and full-length Rsp5p completely inhibited the RNA-binding function of p33 *in vitro* (Fig. 2A, lanes 7 to 10 versus lanes 1 and 2). Prp40 was less efficient inhibitor of p33 binding to RNA.

In case of the second approach, we made a fusion protein containing the WW domain of Rsp5p, and the C-terminal portion of p33, termed p33C, separated by a linker sequence (Fig. 2C). The fusion protein strategy is used to guarantee that each p33 sequence can efficiently interact with the WW domain. Another advantage of the fusion strategy is that the WW domain is known to fold efficiently in the absence of cofactors (67, 71, 72). Using affinity-purified proteins and 32P-labeled viral RNA in EMSA, we demonstrated that the WW domain completely inhibited the RNA-binding function of p33 *in vitro* (Fig. 2C, lanes 10 to 12 versus lanes 6 to 8).

Using a similar strategy with the fusion protein, we also showed that the WW domain inhibited the self-interaction between p33 molecules in a yeast two-hybrid assay (Fig. 2D). Similar to the observation with the above fusion strategy, copurification of His6-
tagged p33 with the FLAG-p33 from membranes was inhibited by the separate coexpression of the WW-domain protein in yeast (Fig. 2E, lane 3 versus lane 2). Altogether, these data suggest that the interaction of the WW domain with the C-terminal region of p33 inhibits both the RNA-binding and p33-p33 interaction functions of the viral replication protein. Because p92pol shares the same sequence with p33 in its N-terminal region, it is likely that the previously shown interaction between Rsp5p and p92pol (64, 65) also leads to the inhibition of the ability of p92pol to interact with p33 and the viral RNA.

The WW domain inactivates the replication function of both p33 and p92 in the CFE-based replication assay. To test the inhibitory function of the WW domain (derived from Rsp5p and contains three WW motifs) under defined conditions in vitro, we used the purified recombinant WW-domain protein in a CFE-based TBSV replication assay (Fig. 3A). The TBSV repRNA can go through a single full cycle of replication (producing double-stranded RNA intermediate on added plus-stranded template and excess amount of new plus-stranded RNAs) in yeast CFE when purified recombinant p33 and p92pol proteins are provided (Fig. 3B, lane 1) (37, 56, 73). When WW-domain protein or Rsp5p were added to the CFE assay, then TBSV replication was ~10% of the control assay containing purified GST protein (Fig. 3B, lane 2 versus lane 1). Preincubation of the WW-domain protein and p33 replication protein did not increase further the inhibitory effect of the WW-domain protein (Fig. 3B, lane 6).

However, the above-described experiments did not define whether the WW-domain protein or Rsp5p inhibited the function of p33, p92pol, or both. Therefore, we used the fusion protein approach in the CFE-based replication assay (Fig. 3C). When
WW-p33 was provided with p92pol in the CFE assay, then TBSV replication was undetectable (Fig. 3C, lanes 3 and 4). The WW-p33 in combination with p33 supported a low level of TBSV RNA replication (Fig. 3C, lanes 5 and 6), suggesting that the replication function of p92pol is also inhibited by the fusion with the WW domain. Altogether, these data confirmed that the WW domain efficiently inhibits the replication functions of both p33 and p92pol in vitro.

To test whether the WW domain makes p33 molecules dominant negative (i.e., also inhibiting wt p33 or p92pol molecules that are part of the VRCs), we added the functional p33 and p92pol, together with WW-p33, to the CFE assay. Interestingly, we observed no inhibitory effect by the WW-p33 in the CFE assay, suggesting that WW-p33 had no dominant-negative effect on TBSV replication (Fig. 3D, lanes 3 and 4 versus lanes 1 and 2). This is in contrast with the dominant-negative effect of the p33 mutant lacking the RPR region responsible for RNA binding (mutant p33ΔRPR, lanes 5 and 6, Fig. 3D) (74). The lack of dominant-negative effect of WW domain in WW-p33 on the viral replicase activity could be important during regulation of tombusvirus replication in infected cells (see Discussion).

Inhibition of tombusvirus replication by the WW-domain protein in yeast. Using the fusion protein approach, we tested the effect of the WW domain on TBSV replication to define if the WW-domain protein inhibited the function of p33, p92pol or both in yeast cells (Fig. 4A). Expression of WW-p33 with His6-p92 or His6-p33 with WW-p92 completely blocked TBSV repRNA accumulation (Fig. 4B, lanes 7 to 12 versus lanes 1 to 6). Western blot analysis showed that WW-p33 accumulated in yeast (Fig. 4C, lanes 5 and 6), suggesting that the lack of TBSV replication in the
presence of WW-p33 was likely due to the inactivation of p33 functions by the WW domain. On the other hand, WW-p92 did not accumulate at a detectable level in yeast cells (Fig. 4C, lanes 7 and 8), suggesting that p92pol was degraded in the presence of the WW domain, as shown previously with separately expressed proteins (64, 65). Indeed, we were able to detect mRNA expression for WW-p92 (Fig. 4B, bottom panel). In contrast, either His6-p33 and His6-p92 or His6-CFP-p33 and His6-CFP-p92 supported TBSV RNA accumulation efficiently (Fig. 4B, lanes 1 to 6), suggesting that the N-terminal tags attached to p33/p92 pol, which do not interact with the p33 and p92 pol sequences, do not inhibit TBSV replication.

We also used a different fusion protein, in which the YFP sequence separated the WW domain from the p33 sequence (construct WW-YFP-p33, Fig. 4A). This protein, when coexpressed with p92pol, could not support TBSV repRNA accumulation in yeast (Fig. 4D, lanes 3 to 4), confirming that the WW domain interferes with the functions of p33 replication protein. Similar to wt p33 (34, 75) and the YFP-p33, the WW-YFP-p33 is associated with membranes (was inserted into the lipid bilayer) in yeast based on fractionation and washing with 1 M NaCl, which could remove peripheral membrane proteins (Fig. 4E). Also, confocal microscopic analysis showed the mostly peroxisomal localization of WW-YFP-p33 (Fig. 4F), similar to YFP-p33 (Fig. 4G) (32, 34, 60). Thus, the WW sequence is correctly folded within the WW-YFP-p33 fusion protein and the peroxisomal targeting sequence and membrane association of p33 have also remained functional. These data are in agreement with the prediction that the WW domain precisely inactivates p33 functions, such as the RNA-binding function, p33-p33 interaction, and p33-host protein interactions, when present in the fusion protein.

Expression of the WW-domain protein decreases the amount of copurified cellular proteins in the tombusvirus replicase. To test the possible regulatory role of the WW-domain proteins in TBSV replication, we expressed the WW domain of Rsp5p in yeast. We chose to express only the WW domain and not the full-length protein, since the WW domain is the functionally relevant portion of Rsp5p during TBSV replication (65), and the presence of the E3 ubiquitin ligase domain of Rsp5p might affect the functions of numerous client cellular proteins when overexpressed. The same yeast cells also coexpressed p33 and p92pol replication proteins and the DI-72 replicon (rep)RNA and selected HA-tagged cellular proteins, those which are known to function as susceptibility factors for TBSV, from chromosomal locations. After affinity purification of the membrane-bound tombusvirus VRCs (via purification of p33 and p92pol), we analyzed the amount of copurified host proteins.
FIG 4 WW domain of Rsp5p blocks TBSV RNA replication in yeast. (A) Schematic representation of the fusion proteins used for expression in yeast. The WW domain of Rsp5p containing three WW repeats and the p33 sequence were fused as shown. The functional CFP-p33 fusion protein was chosen as control. (B) For the top panel, Northern blot analysis to detect DI-72(H11001) repRNA accumulation in yeast coexpressing the shown combination of p33 and p92 fusion proteins was performed. The accumulation level of DI-72(+) repRNA was normalized based on 18S rRNA. For the bottom panel, Northern blot analysis of p33 and p92 mRNA levels in yeast was performed. (C) Western blot analysis of total protein extracts with anti-His antibody. (D) For the top panel, Northern blot analysis to detect DI-72(+) repRNA accumulation in yeast coexpressing the shown combination of p33 and p92 fusion proteins was performed. For the middle and bottom panels, Western blot analysis of total protein extracts with anti-His or anti-Flag antibodies was performed. See further details in panels B and C. (E) Membrane association of the various fusion proteins in yeast. Broken yeast cells were fractionated to obtain supernatant (S, soluble fraction) and membrane fraction (P, pellet). Note that the yeast membrane fraction was washed with 1 M NaCl to remove peripheral membrane proteins. Lanes 9 and 10 represent the total, not fractionated, proteins as standards. (F) The WW-domain-p33 fusion protein shows mostly peroxisomal localization in yeast. Confocal laser microscopy images show the subcellular localization of WW-YFP-p33 fusion protein expressed from GAL1 promoter in the BY4741 yeast strain. The peroxisomes were visualized with Pex13p-CFP marker. The merged images show the colocalization of WW-YFP-p33 and Pex13p-CFP marker. Differential interference contrast (DIC) images are shown on the right. Each row represents a separate yeast cell. (G) Peroxisomal localization of YFP-p33 fusion protein. Yeast was grown under similar conditions and images were taken as in panel F. Each experiment was repeated two to three times.
Interestingly, the amounts of six subverted VRC-associated host proteins decreased by 45 to 90% in the VRC preparations purified from yeast coexpressing the WW-domain protein (Fig. 5B to G), while copurification of one host factor, the Ssa1p Hsp70, was not affected (Fig. 5A). The largest decrease in copurification was observed with the cellular ESCRT proteins (i.e., Vps4p and Bro1p) and Cdc34p E2 ubiquitin-conjugating enzyme, all of which are known to affect the assembly of the tombusvirus VRCs (4, 47, 51). The extent of inhibition of the additional copurified host proteins was also significant, but somewhat less pronounced for (i) Tef1p (eEF1A) translation elongation factor known to affect many viral functions, including the stability of p33, the recruitment of the viral RNA, and the assembly of the VRCs and (-)RNA synthesis (46, 51, 53); (ii) Tdh2p (GAPDH) involved in (+)RNA synthesis (62, 63); and (iii) Pex19p cytosolic shuttle protein that targets p33 and p92pol to the peroxisomes (60). Altogether, the inhibition of recruitment of multiple host factors to the VRCs by WW-domain protein suggests a regulatory function, possibly via competition of the WW-domain protein with these cellular proteins for binding to p33 and p92pol. It is possible that some of the proviral host proteins compete, while the rest of them just being disturbed by the active binding of the ectopically expressed WW domain to p33.

Overexpression of selected cellular proteins decreases the inhibitory effect of the WW-domain protein on TBSV replication. If competition for binding to p33 replication proteins exists among the WW-domain proteins and the subverted stimulatory host factors, then overexpression of selected stimulatory host proteins is expected to neutralize the inhibitory effect of the WW-domain proteins on TBSV replication in yeast cells. To test this model, we individually overexpressed four stimulatory host proteins in yeast, also coexpressing the WW-domain protein. The increase of TBSV replication was ~4-fold in yeast overexpressing Cdc34p and the WW-domain proteins in comparison with the overexpression of the WW-domain protein only (Fig. 6A, lanes 7 and 8 versus lanes 3 and 4). Thus, overexpression
of Cdc34p completely neutralized the strong inhibitory effect of the overexpressed WW-domain protein. This neutralization effect by Cdc34p likely due to efficient recruitment of the overexpressed Cdc34p by the p33 replication protein, because Cdc34p does not interact with Rsp5p (76). Overexpression of Ssa1p or Tef1p slightly increased TBSV replication in wt yeast (Fig. 6B, lanes 5 and 6 and 9 and 10), while both proteins partly neutralized the inhibitory effect of the WW-domain protein (leading to a 2- to 2.5-fold increase) in TBSV replication in yeast cells overexpressing the WW-domain protein (Fig. 6B, lanes 7 and 8 and 11 and 12 versus lanes 3 and 4). The incomplete neutralization of the inhibitory effect by WW-domain protein could be due to the physical interaction between Ssa1p or Tef1p and Rsp5p (76) that might be responsible for the reduced overexpression level of both Ssa1p and Tef1p in yeast coexpressing the WW-domain of the Rsp5p protein (Fig. 6B, lanes 4 and 6 versus lanes 3 and 5). Overexpression of the fourth host factor, Tdh2p, had lesser neutralization effect on the inhibitory function of the overexpressed WW-domain protein (Fig. 6B, lanes 15 and 16). However, overexpression of Tdh2p did not increase TBSV repRNA replication in yeast (lanes 13 and 14, Fig. 6B), suggesting that Tdh2p is not a limiting factor in wt yeast under the given experimental conditions.

Overexpression of the above host factors in yeast also overexpressing the WW-domain protein was not effective enough to increase p33 or p92<sub>pol</sub> levels to that observed in wt yeast cells coexpressing YFP (Fig. 6B). These observations suggest that the overexpressed WW-domain protein is a strong competitor against the stimulatory host factors in binding to the viral replication protein. This could be a reason why these stimulatory host proteins only had partial neutralizing effects against the inhibitory effect of the overexpressed WW-domain protein. Altogether, these data suggest that selected stimulatory host factors have neutralizing effects on the inhibitory WW-domain protein during TBSV replication.

Expression of the TPR-domain protein or cyclophilin A did not inhibit the amount of copurified cellular proteins in the tombusvirus replicase. In addition to the WW-domain-containing host factors, other cellular factors with CIRF functions might also be involved in regulation of TBSV replication (22). To further test the possible regulatory functions of recruited cellular proteins, we chose two additional cellular CIRF factors that inhibit TBSV replication. These were the TPR-domain-containing cellular proteins (77) and cyclophilins (45). The TPR domain from Cyp40-like Cpr7p chaperone and the CypA (homolog of the yeast Cpr1p) cyclophilin have been shown to bind to the tombusvirus replication proteins (45, 65, 78).

Similar to the strategy described above with the WW-domain protein (Fig. 5), we overexpressed either the TPR domain of Cpr7p or CypA cyclophilin in yeast coexpressing p33 and p92<sub>pol</sub> replication proteins, the TBSV repRNA, and selected HA-tagged stimulatory cellular proteins from chromosomal locations (Fig. 7). After affinity purification of the membrane-bound tombusvirus VRCs, we analyzed the amount of copurified stimulatory host proteins. We found that the copurification of Cdc34p E2 ubiqui-
tin-conjugating enzyme, Ssa1p HSP70 chaperone, and Bro1p ESCRT protein was not affected by the overexpression of the TPR-domain protein or CypA cyclophilin (Fig. 7A to C, lanes 3 and 4 versus lane 2). Moreover, copurification of Pex19 shuttle protein with the tombusviral VRC was increased from yeast expressing the TPR domain and especially CypA protein (Fig. 7D, lanes 3 and 4). Since Cdc34p and Bro1p cellular proteins were among those most affected by overexpression of the WW-domain protein (Fig. 5) and yet their recruitment into the VRCs was not affected by the overexpression of the TPR-domain protein or CypA (Fig. 7), we suggest that the CypA and TPR-domain proteins and the WW-domain proteins have different regulatory roles during TBSV replication in yeast. This is surprising because, similar to the WW domain, the TPR domain of Cpr7p and CypA also bind to the RPR region in p33 responsible for viral RNA binding (77, 78).

The contrasting data on WW-domain protein versus cyclophilins suggest that WW-domain proteins have a unique role during TBSV replication. Nevertheless, these results indicate that WW domain selectively affects the recruitment of stimulatory cellular proteins into VRCs, while overexpression of the TPR-domain protein or CypA do not detectably influence these activities.

**Binding kinetics suggest a faster association of proviral cellular factors to p33 than Rsp5p.** In the infected cells, subverted proviral cellular factors and antiviral restriction factors, CIRFs, likely compete with one another for binding to the tombusvirus replication proteins. To test the binding kinetics of selected cellular proteins to the p33 replication protein, we used surface plasmon resonance measurements with purified recombinant proteins, which were separately immobilized on the chip, in analyzing binding constants to the soluble C-terminal portion of p33. Interestingly, five of the known co-opted cellular factors bound with ~2- to 7-fold-higher $k_a$ value to p33 than Rsp5p (Table 1). Among these host proteins Tdh2p and Tef1p are present in large amounts in cells, further increasing the chance that these host proteins bind to p33 first or earlier than Rsp5p. This suggests that p33 likely have a better chance to bind to the co-opted host factors at the early

**TABLE 1** Kinetics of interaction between p33 replication protein and cellular proteins

<table>
<thead>
<tr>
<th>Protein or control</th>
<th>$K_d$ (M)</th>
<th>Mean $k_a$ (1/Ms) ± SD</th>
<th>$k_d$ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rsp5</td>
<td>6.548e–7</td>
<td>2.203 ± 96</td>
<td>1.442e–3</td>
</tr>
<tr>
<td>WW</td>
<td>5.200e–7</td>
<td>7.035 ± 23</td>
<td>3.659e–3</td>
</tr>
<tr>
<td>Tdh2</td>
<td>3.806e–7</td>
<td>15.900 ± 457</td>
<td>6.052e–3</td>
</tr>
<tr>
<td>Vps4</td>
<td>1.907e–7</td>
<td>11,380 ± 234</td>
<td>2.170e–3</td>
</tr>
<tr>
<td>Tef1</td>
<td>3.633e–7</td>
<td>6,977 ± 72</td>
<td>2.535e–3</td>
</tr>
<tr>
<td>Bro1</td>
<td>6.529e–7</td>
<td>5,961 ± 151</td>
<td>3.892e–3</td>
</tr>
<tr>
<td>Cdc34</td>
<td>8.756e–7</td>
<td>4,549 ± 217</td>
<td>3.983e–3</td>
</tr>
<tr>
<td>Pex19</td>
<td>3.822e–6</td>
<td>1,446 ± 411</td>
<td>5.526e–3</td>
</tr>
<tr>
<td>Negative controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WW:MBP</td>
<td>3.746e–6</td>
<td>417</td>
<td>1.566e–3</td>
</tr>
<tr>
<td>Cdc34:MBP</td>
<td>7.959e–6</td>
<td>737</td>
<td>5.867e–3</td>
</tr>
</tbody>
</table>

Note: $K_d$, affinity of interaction constant; $k_a$, association rate constant; $k_d$, dissociation rate constant.
stage of infection when p33 concentration is low and the “free” proviral cellular factors are still abundant.

The tombusvirus replicase purified at late stage of infection is less precise when isolated from cells with depleted WW-domain proteins. To test whether Rsp5p might have a function in regulation of VRC assembly and whether this function is manifested at the late stage of infection, we purified tombusvirus replicase from yeast with depleted WW-domain proteins (TET::RSP5/TET::PRP40/wwm1Δ) at early and late time points. Then, the purified replicase preparations were tested with a (~)RNA template that is used to measure the precision of initiation events by the RdRp (79, 80). While the replicase preparation from yeast with depleted WW-domain proteins prepared at the early time point (16 h) was ~2-fold more active than the similar preparation from wt yeast, the ratio of internal initiation and 3’ terminal extension (3′-TEX) products versus full-length cRNA synthesis product (this product is required for viral replication) was comparable by these replicases (Fig. 8B, lane 3 versus lane 1). Thus, the replicase from yeast with depleted WW-domain proteins is more active, but comparable in precision with the wt replicase.

Interestingly, the replicase preparation from yeast with depleted WW-domain proteins prepared at the late time point (64 h) was ~4-fold more active than the similar preparation from wt yeast. However, the replicase with depleted WW-domain proteins was ~2-fold less precise than the wt replicase, based on the ratio of internal initiation and 3′-TEX versus full-length cRNA synthesis (Fig. 8B, lane 5 versus lane 4). These data suggest that the WW-domain proteins not only inhibit tombusvirus replicase assembly, but surprisingly, they also help making the replicase more precise at the late time points.

**DISCUSSION**

Rsp5p and WW-domain proteins act as CIRFs against tombusviruses. (+)RNA viruses replicate the viral RNA efficiently in infected cells via assembling membrane-bound VRCs consisting of viral and subverted host proteins (1–7, 36, 81, 82). However, some cellular factors could act as CIRFs restricting virus replication (22, 83, 84). Among the several CIRFs against tombusviruses, Rsp5p and the WW-domain proteins are strong inhibitors, which are present in both yeast and plant cells (64, 65). In the present study, we show data that support the model that the cellular WW-domain proteins could inhibit assembly of new tombusvirus VRCs. The presented data indicate that binding of the WW domain from Rsp5p to p33 and p92pol (that contains p33 sequence due to the overlapping expression strategy) blocks interaction of the replication proteins with (i) the viral RNA, (ii) the oligomerization with other p33 and p92pol molecules, and (iii) a set of stimulatory (susceptibility) host factors subverted for TBSV replication. All of these interactions are needed for the assembly of new VRCs and the activation of p92pol, which is initially inactive in the cytosol after translation (30, 37, 39, 56). Based on these features involving many of the VRC components, the WW-domain proteins seem to exhibit a complex mechanism as CIRFs.

Interestingly, overexpression of WW-domain protein inhibited the subversion of several host proteins and their recruitment into the VRCs (Fig. 5). Notably, the list of host factors affected by the WW-domain protein includes co-opted host proteins, such as eEF1A, Bro1p, Vps4p, and Cdc34p that play important roles in the assembly of the tombusvirus VRCs in vitro, in yeast and plant cells (4, 46, 47, 51, 53). We propose that overexpression of WW-domain proteins leads to strong competition between the WW-domain proteins and the co-opted susceptibility host factors in binding to the viral replication proteins even at the early stage of replication due to the high abundance of free WW-domain proteins in these cells (Fig. 9C). This should lead to inhibition of the VRC assembly and reduced level of replication. Accordingly, overexpression of Rsp5p and several other WW-domain proteins has resulted in strong inhibition of TBSV RNA replication in yeast and plants (64, 65). Simultaneous overexpression of both WW-domain protein and a co-opted host protein is also expected to lead to strong competition in binding to the viral replication proteins due to the high abundance of free WW-domain protein and the
given stimulatory host factor in these cells. Indeed, we observed that overexpression of selected co-opted host proteins increased TBSV replication even in the presence of abundant WW-domain protein (Fig. 6). On the contrary, deletion or downregulation of WW-domain proteins increased TBSV replication in yeast cells (64, 65) or in vitro (Fig. 3 and 8). Thus, our data support that Rsp5p and WW-domain proteins act as CIRFs under certain cellular conditions.

Rsp5p and WW-domain proteins might also function as regulatory factors for TBSV replication. The VRC assembly is likely highly regulated to prevent the extensive production of incomplete or truncated viral RNAs at the late stage of replication, when one or more cellular components become limiting or even depleted, making them unavailable for new rounds of VRC assembly and virus replication. Our multiple genome-wide screens with TBSV and yeast showed that missing a proviral host factor (due to depletion or gene deletion) does not necessarily prevent the assembly of the VRCs (11, 13, 40–42, 45, 48). Instead, it usually leads to incorrect VRC assembly that changes some of the activities of the viral replicase (such as increased rate of RNA recombination or reduced efficiency of replication). It could be advantageous for the virus to have an active mechanism(s) to regulate the assembly of new VRCs toward the late phase of infection when several subverted proviral host factors might be already depleted during the previous rounds of VRC assembly.

Based on these ideas, we predict that somehow an RNA virus could sense the state of the host cell and decide whether new VRCs should be assembled or not. Based on the features presented in the present study, Rsp5p and WW-domain proteins are likely highly suitable for regulatory functions during TBSV replication. For example, the regulatory role of the WW-domain proteins in TBSV replication is supported by several interesting observations. (i) Rsp5p binds to the p33 replication protein not as rapidly as several proviral cellular factors (based on $k_a$ values in Table 1), suggesting that the proviral factors might be favored by TBSV to act early during replication, while Rsp5p might function at a latter stage of infection when some of the proviral factors are mostly depleted. (ii) The binding of a single Rsp5p or WW-domain protein likely inhibits the function of a single p33 or p92pol replication protein but does not have dominant-negative effect on the VRCs, as
shown by the use of WW-p33 fusion protein in a CFE-based viral replication assay (Fig. 3). This could be important for tombusviruses, since the previously assembled VRCs should not be blocked or destroyed by the regulatory protein. Instead, only the formation of new VRCs should be inhibited at the late stage of infection. It is also possible that the WW-domain protein cannot access the previously assembled VRCs, because those are closed from cytosolic proteins due to the spherule structure (57) or, alternatively, the p33 and p92 proteins are already oligomerized (via p33-p33 self-interaction or p33-p92 interaction) or bound to proviral host factors. (iii) A regulatory protein is expected to block the interaction between the viral replication proteins and the viral RNA in order to facilitate a nonreplicative use of the viral RNA (e.g., for encapsidation or cell-to-cell movement), instead of keeping the viral RNA trapped in the translation/replication cycle. (iv) It could be useful if the regulatory protein would facilitate the degradation of excess amounts of viral replication proteins to prevent the functional interference of these replication proteins with other, nonreplicative functions. Indeed, p92\(^{\text{2ns}}\) is efficiently degraded when Rsp5p or Wwm1p WW-domain proteins are overexpressed in yeast (65). (v) It is predicted that VRCs assembled at the late stage of infection could be especially error-prone if one or more proviral cellular proteins are not recruited into VRCs due to their depletion in previous rounds of VRC assembly. Accordingly, we detected a higher error rate for incorrect initiation of RNA synthesis with the purified replicase when derived from yeasts with depleted WW-domain proteins at a late stage (Fig. 8). This aberrant feature of the replicase could be due to incorrect assembly of the replicase at the late stage, possibly due to depletion of one or more proviral factors. Interestingly, we have shown that the WW-domain proteins fulfill all of these features during TBSV replication.

Based on these observations, we suggest a new model for the interplay between proviral factors and WW-domain proteins, such as Rsp5p, in the regulation of tombusvirus VRC assembly. We propose that the tombusvirus replication proteins first interact with the host susceptibility factors, which are co-opted for virus replication at the beginning of infection when these susceptibility factors are abundant and/or accessible (Fig. 9A). These events lead to efficient assembly of VRCs and robust viral replication at the early stage of replication. As the amounts of newly produced p33 and p92\(^{\text{2ns}}\) replication proteins increase due to ongoing translation, the cell likely runs out of one or more available susceptibility factors at the late stage of replication. Depletion of the susceptibility factors allows the viral replication proteins to interact with the cellular WW-domain proteins (Fig. 9B). This will then lead to a blockage for the assembly of new VRCs and inhibition of the formation of new p33-viral RNA complexes and to the degradation of p92\(^{\text{2ns}}\) replication protein. Thus, viral replication, especially the formation of new VRCs, will be slowed down at the late stage, and the newly made viral (+)RNAs will be able to leave the translation/replication cycle and become committed to additional functions, such as encapsidation or cell-to-cell movement. Altogether, we propose that tombusviruses could sense the status of the infected cells via “measuring” the availability of cellular susceptibility factors versus cellular WW-domain proteins, which then determines whether new VRCs are assembled or the VRC assembly process is halted.

Why would an RNA virus select WW-domain proteins for such a regulatory function? We propose that the WW-domain proteins are very suitable for these functions, since they are present in the cytosol of all eukaryotic cells, and they also represent an ancient, very simple motif selected for protein-protein interactions (66, 72, 85). Indeed, we were able to identify several WW-domain proteins in both yeasts and plants (65), which could be used by tombusviruses for such regulatory roles. Interestingly, the unrelated nodaviruses (insect RNA viruses) could also be inhibited by overexpression of yeast Rsp5p and Wwm1p WW-domain proteins (65). The question remains if additional RNA viruses could also take advantage of WW-domain proteins or other cellular proteins for regulatory functions to optimize their replication in various cells and hosts.

Unlike the WW-domain proteins, other inhibitory cellular CIRF proteins, such as the TPR-domain containing Cyp40-like Cpr7p chaperone or CypA cyclophilin (45, 77), do not seem to affect the recruitment of stimulatory host factors into the VRCs (Fig. 7). Thus, these cellular restriction factors are not involved in regulation of tombusvirus replication in a manner similar to WW-domain proteins, or they function utilizing different regulatory mechanisms.

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REFERENCES


