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# An inhibitory function of WW domain-containing host proteins in RNA virus replication

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ABSTRACT

To identify new genes affecting *Tomato bushy stunt virus* (TBSV) replication in yeast model host, we are studying protein families, whose members have been identified during previous high throughput screening. In this paper, we have characterized the WW domain-containing protein family from yeast and plants. We find that, in addition to Rsp5 E3 ubiquitin ligase, yeast Wwm1 and Prp40 and three *Arabidopsis* WW domain-containing proteins are strong inhibitors of TBSV replication. The tombusvirus replicase complex isolated from yeast with down-regulated Wwm1 protein level was more active. Accumulation of viral $p92^{pol}$ was reduced when Wwm1 was over-expressed, suggesting that the stability of $p92^{pol}$ might be reduced, as observed with Rsp5. Moreover, replication of two insect RNA viruses is also inhibited by Wwm1 and Rsp5, suggesting that WW domain-containing proteins might have broad regulatory effects on RNA viruses. Thus, artificial antiviral proteins with WW domains could be useful antiviral strategy.



## Introduction

Plus-stranded (+)RNA viruses must interact with the host cells, which are critical for the outcome of viral infections. On one side, (+)RNA viruses have to utilize the host translation machinery to produce viral proteins, and they also have to use the resources of the host cells, co-opt host proteins and subvert host membranes (Bartenschlager et al., 2010; den Boon and Ahlquist, 2010; Li and Nagy, 2011; Miller and Krijnse-Locker, 2008; Nagy, 2008; Nagy and Pogany, 2010, 2012; Nagy et al., 2011; Novoa et al., 2005). On the other side, interaction between virus and host cells is affected by antiviral responses, which may involve innate immunity as well as other antiviral factors. To map all the interactions between viruses and hosts, genome-wide approaches have been applied with several model host viruses, including tombusviruses, *West Nile virus*, *hepatitis C virus*, *Dengue virus*, *Drosophila C virus*, and *Brome mosaic virus* (Cherry et al., 2005; Hao et al., 2008; Jiang et al., 2006; Krishnan et al., 2008; Kushner et al., 2003; Panavas et al., 2005; Randall et al., 2007; Serviene et al., 2005; Sessions et al., 2009; Tai et al., 2009). Accordingly, a large number of host genes have been identified, which either facilitate or inhibit (+)RNA virus replication. However, the roles and functions of most of the identified host proteins involved in RNA virus replication are currently unknown.

*Tomato bushy stunt virus* (TBSV) is among the most advanced model systems used to identify host factors affecting (+)RNA virus replication. The one component genomic RNA of TBSV codes for only five proteins, including two replication proteins translated directly from the genomic RNA (White and Nagy, 2004). One of the replication proteins is the abundant p33 replication co-factor, functioning as an RNA chaperone (Stork et al., 2011); while the other is $p92^{pol}$ RNA-dependent RNA polymerase (RdRp) (Pogany et al., 2008). Due to the overlapping expression strategy, p33 is identical with the N-terminal portion of the larger $p92^{pol}$ protein. The replication proteins share a common RNA-binding motif (Arginine–Proline-rich motif, RPR), a p33:p33/p92 interaction domain and two transmembrane domains (Nagy and Pogany, 2006, 2008; Rajendran and Nagy, 2006; Shapka et al., 2005; Stork et al., 2005). These proteins also interact with a surprisingly large number of host proteins (Li et al., 2008, 2009; Mendu et al., 2010; Nagy and Pogany, 2010; Serva and Nagy, 2006). These proteins include heat shock protein 70 (HSP70) needed for the assembly of the viral replicase complex (VRC) (Pogany et al., 2008;

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Wang et al., 2009a, 2009b); elongation factor 1A (eEF1A) and eEF1Bγ, required for (−)strand synthesis (Li et al., 2010; Sasvari et al., 2011); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that stimulates (+)-strand synthesis (Huang and Nagy, 2011; Wang and Nagy, 2008); and Pex19p host protein that facilitates the transport of p33 and $p92^{pol}$ from the cytosol to the cytosolic surface of the peroxisomes, the site of viral RNA replication (Pathak et al., 2008). Thus, multiple, dynamic protein–protein, protein–RNA and protein–membrane interactions must be required for robust tombusvirus replication. These interactions are likely inhibited by some host proteins that could lead to inhibition and/or regulation of virus replication.

Previous genome-wide screens and global proteomics approaches have identified over 300 host genes/proteins affecting TBSV replication (Jiang et al., 2006; Li et al., 2008, 2009; Mendu et al., 2010; Nagy and Pogany, 2010; Panavas et al., 2005; Serva and Nagy, 2006; Serviene et al., 2005, 2006). The list includes inhibitors of tombusvirus replication, such as nucleolin inhibiting the recruitment of the tombusviral (+)RNA for replication or cyclophilins (Jiang et al., 2010; Mendu et al., 2010). Indeed, one of the identified host proteins is Rsp5p E3 ubiquitin ligase, which have previously been found to interact with p33 based on the yeast protein array (Li et al., 2008). Interestingly, Rsp5p inhibits TBSV replication when over-expressed in yeast cells whereas its down-regulation leads to increased TBSV accumulation (Barajas et al., 2009b). The inhibition is caused by Rsp5p-mediated selective degradation of $p92^{pol}$, and inhibition of the TBSV replicase activity in a cell-free replication assay, likely due to the ability of Rsp5p to bind to both p33 and $p92^{pol}$. The inhibitory function of Rsp5p is not caused by the HECT domain involved in protein ubiquitination, but its WW domain, which is involved in protein–protein interactions (Barajas et al., 2009b). The observations suggest that direct binding of the WW domains in Rsp5p to p33 and $p92^{pol}$ is likely involved in inhibition of TBSV replication. Since there are many WW

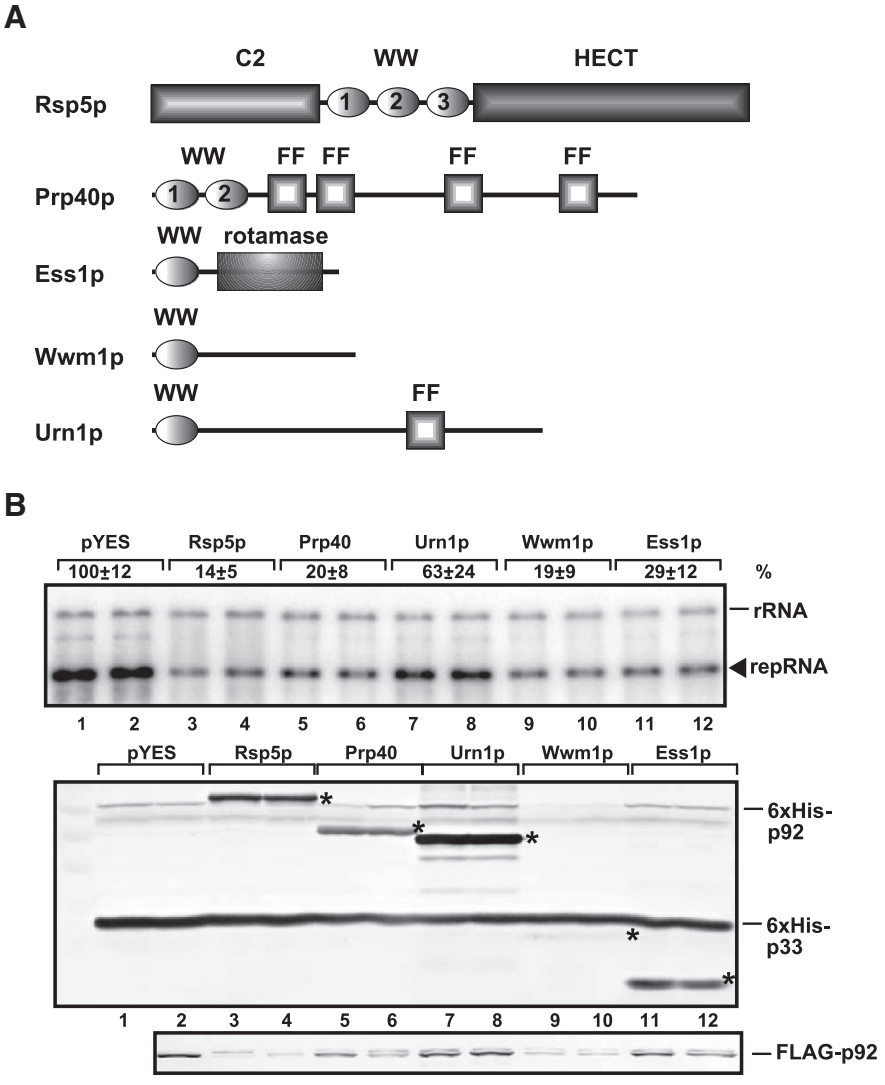


**Fig. 1.** The effect of over-expression of 5 WW domain-containing yeast proteins on TBSV replication. (A) Schematic representation of 5 WW domain-containing yeast proteins. The C2 domain of Rsp5p is involved in membrane-binding, the three WW domain repeats are needed for protein–protein interaction and substrate recognition, while the HECT domain has the E3 ubiquitin ligase function. Prp40p contains two, while Ess1p, Wwm1p and Urn1p carry one WW domain at their N-terminal regions. The FF domain (two phenylalanine-motifs) may be involved in protein–protein interactions, while the rotamase domain catalyzes *cis–trans* isomerization of prolines. (B) Top panel: Northern blot analysis was used to detect DI-72(+) repRNA accumulation in BY4741 yeast strain over-expressing one of the five WW domain-containing yeast proteins from the high copy number pYES plasmid as shown. The continuous expression of WW domain-containing yeast proteins 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. Each experiment was repeated 2–4 times. The average and standard deviation of the independent repeats are shown. 100% is the repRNA level in yeast expressing a short peptide from the empty pYES vector. Middle panel: Western blot analysis of the accumulation level of 6xHis-tagged p33, 6xHis-tagged p92 and 6xHis-tagged WW domain-containing yeast proteins using anti-6xHis antibody. The asterisk (*) indicates WW domain-containing yeast proteins. Bottom panel: Western blot analysis of the accumulation level of FLAG-tagged p92 using anti-FLAG antibody. This approach allows the detection of p92 without interference from Rsp5p, which migrates close to p92 in the SDS-PAGE gel (see middle panel).

domain-containing proteins in eukaryotes, we have decided to study if additional yeast and plant proteins with WW domain might also be able to inhibit TBSV replication in yeast.

The WW domain is a highly conserved protein domain involved in protein–protein interaction (Hesselberth et al., 2006; Macias et al., 2000). The WW domain contains two signature tryptophan residues (at positions 13 and 36) and a conserved proline residue (at position 39). The domain adopts a globular fold with three beta-sheets that binds to ligands, which usually carry proline-rich sequences. It is present in multiple proteins in various organisms, including humans, in which dysfunction of these proteins contributes to various diseases (Hesselberth et al., 2006). Yeast has 10 WW-domain containing proteins with highly variable functions (Hesselberth et al., 2006). We have chosen 5 of these proteins, since they carry the signature motifs, while the other five proteins lack one of the tryptophane residues. The proteins studied here include Rsp5p, Prp40p mRNA splicing factor, Ess1p prolyl-isomarese, Wwm1p is a protein with unknown function, which is involved in peroxide-induced apoptotic response in yeast (Madeo et al., 2004; Szallies et al., 2002), and Urn1p, whose function has not been determined (Bonet et al., 2008; Hesselberth et al., 2006; Ingham et al., 2004; Otte et al., 2003). We find that over-expression of Prp40 and Wwm1p causes robust inhibition of replication, while Ess1p showed a lesser inhibitory effect on TBSV replication in yeast. These proteins also interact with the TBSV replication proteins. Down-regulation of the expression levels of the above proteins shows that Wwm1p is as strong inhibitor as Rsp5p in TBSV replication. In addition, we have identified three *Arabidopsis* WW domain-containing proteins, which are also strong inhibitors of tombusvirus replication in plants. Moreover, replication of two insect RNA viruses is also inhibited by Wwm1p and Rsp5p, suggesting that WW domain-containing proteins might have broad inhibitory or regulatory effects on various RNA viruses.

## Results

### *Over-expression of three WW domain-containing proteins in yeast reduces TBSV RNA accumulation*

Since the WW domains present in Rsp5p acted as strong inhibitors of TBSV replication in yeast (Barajas et al., 2009b), we wanted to test

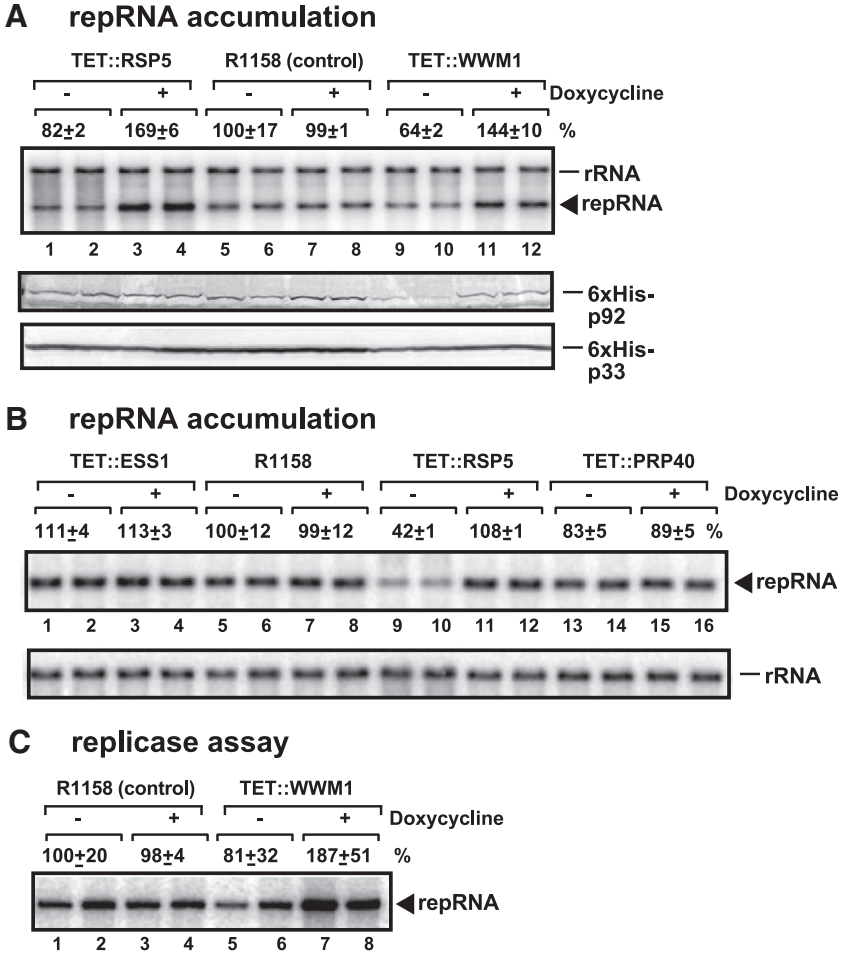


**Fig. 2.** The effect of down-regulation of WW domain-containing yeast proteins on TBSV repRNA accumulation in yeast. (A) Testing TBSV repRNA accumulation in TET-WWM1 and RSP5/THC yeast strains, which contain a doxycycline regulatable promoter (TET) replacing the native *WWM1* or *RSP5* promoters. Therefore, addition of doxycycline to the culture media can down-regulate Wwm1p or Rsp5p expression at the mRNA level. To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the copper-inducible *CUP1* promoter and DI-72(+) repRNA from the galactose-inducible *GAL1* promoter. First, Wwm1p or Rsp5p level was reduced by adding doxycycline 7 h prior to expressing the viral components. Then, yeast cells were cultured for 24 h at 29 °C on 2% galactose SC minimal media containing doxycycline (10 mg/l), plus 50 μM $CuSO_4$. Top panel: Northern blot analysis was used to detect DI-72(+) repRNA accumulation in these yeast strains. 100% is the repRNA level in the parental R1158 yeast cultured in the absence of doxycycline. The accumulation level of DI-72(+) repRNA was normalized based on 18S rRNA. Middle and bottom panels: Western blot analysis of the accumulation level of 6xHis-tagged p33 and 6xHis-tagged p92 proteins using anti-6xHis antibody. (B) Testing TBSV repRNA accumulation in TET-ESS1, TET-RSP5 and TET-PRP40 yeast strains. See details in panel A. (C) In vitro replicase assay based on the co-purified replicase and the endogenous repRNA in membrane-enriched fractions prepared from TET-WWM1 or control (R1158) yeast strains. The denaturing PAGE shows the level of repRNA synthesis by the tombusvirus replicase in vitro. Note that comparable amounts of membrane-bound replicase (based on the level of p33) were used in this assay (not shown). Each experiment was repeated 2–4 times. The average and standard deviation of the independent repeats are shown.

if additional yeast proteins carrying WW domains could inhibit viral replication. Over-expression of Prp40 carrying 2 WW domains and Wwm1p containing a single WW domain (Fig. 1A) proteins resulted in ~5-fold reduction (Fig. 1B, lanes 5–6 and 9–10, respectively), while that of Ess1p with a single WW domain led to ~3-fold reduction (lanes 11–12) in TBSV replication in yeast. These data indicated that several WW domain-containing proteins are strong inhibitors. In contrast, over-expression of Urn1p with a single WW domain decreased TBSV RNA accumulation only by ~35%, which does not seem to be significant since over-expression of proteins in general can lead to 20–30% reduction in TBSV RNA accumulation likely due to non-specific effect as shown previously (Li et al., 2008, 2009). Thus, only a select group of WW domain-containing proteins act as inhibitors of TBSV replication.

To test if Prp40, Wwm1p and Ess1p could affect $p92^{pol}$ levels, similar to Rsp5p (Barajas et al., 2009b), we performed Western blot analysis. Interestingly, over-expression of Wwm1p also led to as low $p92^{pol}$ level as that of Rsp5p (Fig. 1B, lanes 9–10 versus 3–4), while over-expression of Prp40p decreased $p92^{pol}$ level only moderately (lanes 5–6). In contrast, over-expression of Ess1p and Urn1p did not significantly decrease $p92^{pol}$ levels (Fig. 1B). These data suggest that Wwm1p and Prp40p might act similarly to Rsp5p resulting in decreased stability of $p92^{pol}$ during TBSV replication.

*The effect of down-regulation of WW domain-containing proteins on TBSV RNA accumulation in yeast*

To further test the significance of the WW containing proteins in TBSV replication, we down-regulated the expression of Rsp5p, Prp40, Wwm1p and Ess1p from a doxycycline-regulatable (*TET*) promoter. Interestingly, down-regulation of Wwm1 level resulted in 2.5-fold increase in replication (compare +doxycycline and −doxycycline samples, lanes 11–12 versus 9–10, Fig. 2A). This is comparable to that observed when Rsp5p was down-regulated (Fig. 2A, lanes 3–4 versus 1–2). In some experiments, Rsp5p behaved similarly to Wwm1p when expressed from the *TET* promoter by showing reduced TBSV RNA accumulation in the absence of doxycycline (Fig. 2B, lanes 9–10). This could be due to higher expression level of Rsp5p and Wwm1p from the TET promoter than from the native promoter (see below).

In contrast, down-regulation of Ess1p and Prp40p levels did not significantly change replication of TBSV repRNA in yeast (Fig. 2B). Altogether, these experiments suggest that Rsp5p and Wwm1p are significant in regulating TBSV replication, while Ess1p and Prp40 seem less important. Alternatively, the latter genes might have redundant function in TBSV replication in yeast.

To test for possible redundant effects among WW containing proteins, we generated various combinations of yeast strains with two or three of the selected proteins under expression control. For example, simultaneous down-regulation of both Rsp5p and Wwm1p resulted in 2.5-fold increase in TBSV repRNA accumulation (Fig. 3A, lanes 11–12 versus 9–10). Similarly, down-regulation of Rsp5p, Prp40p and deletion of *WWM1* also led to 2.5-fold increase in TBSV accumulation (Fig. 4A, lanes 11–12, versus 9–10). The largest (~3-fold) increase in TBSV accumulation was seen with a yeast strain with down-regulated Rsp5p, Prp40p, Ess1p and deletion of *WWM1* (Fig. 4A, lanes 15–16, versus 13–14). Additional combinations of two down-regulated genes resulted in modest increase in TBSV RNA accumulation (Fig. 3A).

In these experiments, we used the regulatable *TET* promoter for expression of *RSP5*, *ESS1* and *PRP40*, which are essential for yeast growth. Also, we used the TET promoter for *WWM1* in some experiments, because we found that yeast strain with deletion of *WWM1* was difficult to grow (not shown). Due to this expression strategy, it is possible that the expression of these genes is higher from the *TET* promoter (in the absence of doxycycline) than from the native promoter. Therefore, we measured the expression of these genes in comparison with expression from the original promoter. Surprisingly, the expression of Rsp5p, Prp40 and Ess1p was higher from the *TET* promoter in the absence of doxycycline than from the native promoter (Fig. 4B). Another observation is that the expression of Wwm1p increased when Rsp5p was placed under the *TET* promoter (Fig. 4B, lanes 3–4). Since Wwm1p is an interacting partner and substrate of Rsp5p (Hesselberth et al., 2006), it is possible that these two genes could affect each other roles in TBSV replication. Nevertheless, these experiments suggest that Rsp5p and Wwm1p are important regulators of TBSV replication,

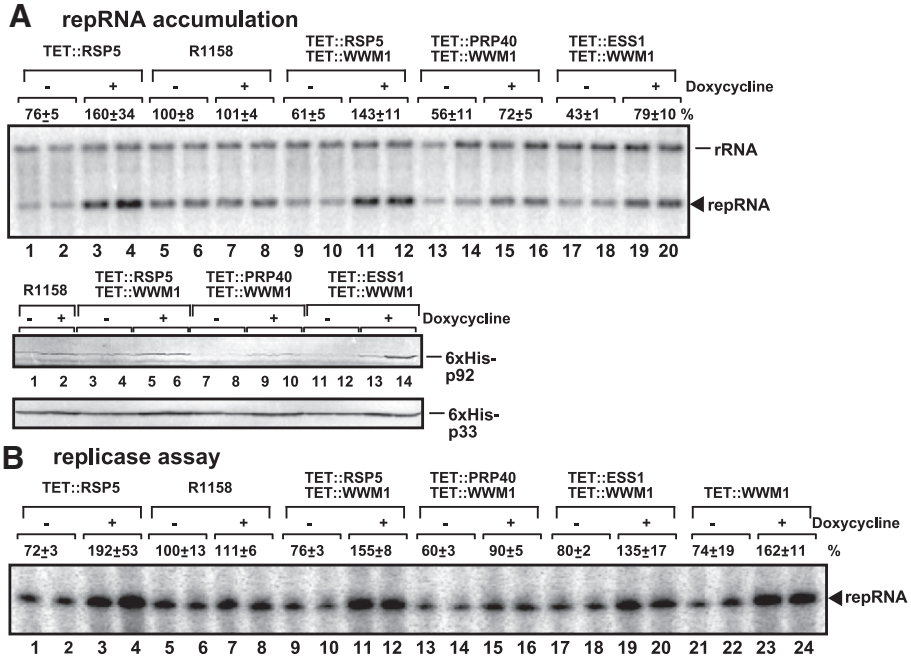


**Fig. 3.** The effect of simultaneous down-regulation of two WW domain-containing yeast proteins on TBSV repRNA accumulation in yeast. (A) Testing TBSV repRNA accumulation in the shown yeast strains by Northern blotting (top panel). Middle and bottom panels: Western blot analysis of the accumulation level of 6xHis-tagged p33 and 6xHis-tagged p92 proteins using anti-6xHis antibody. (B) In vitro replicase assay based on the isolated replicase and the co-purified endogenous repRNA in membrane-enriched fractions prepared from the shown yeast strains. See further details in Fig. 2.

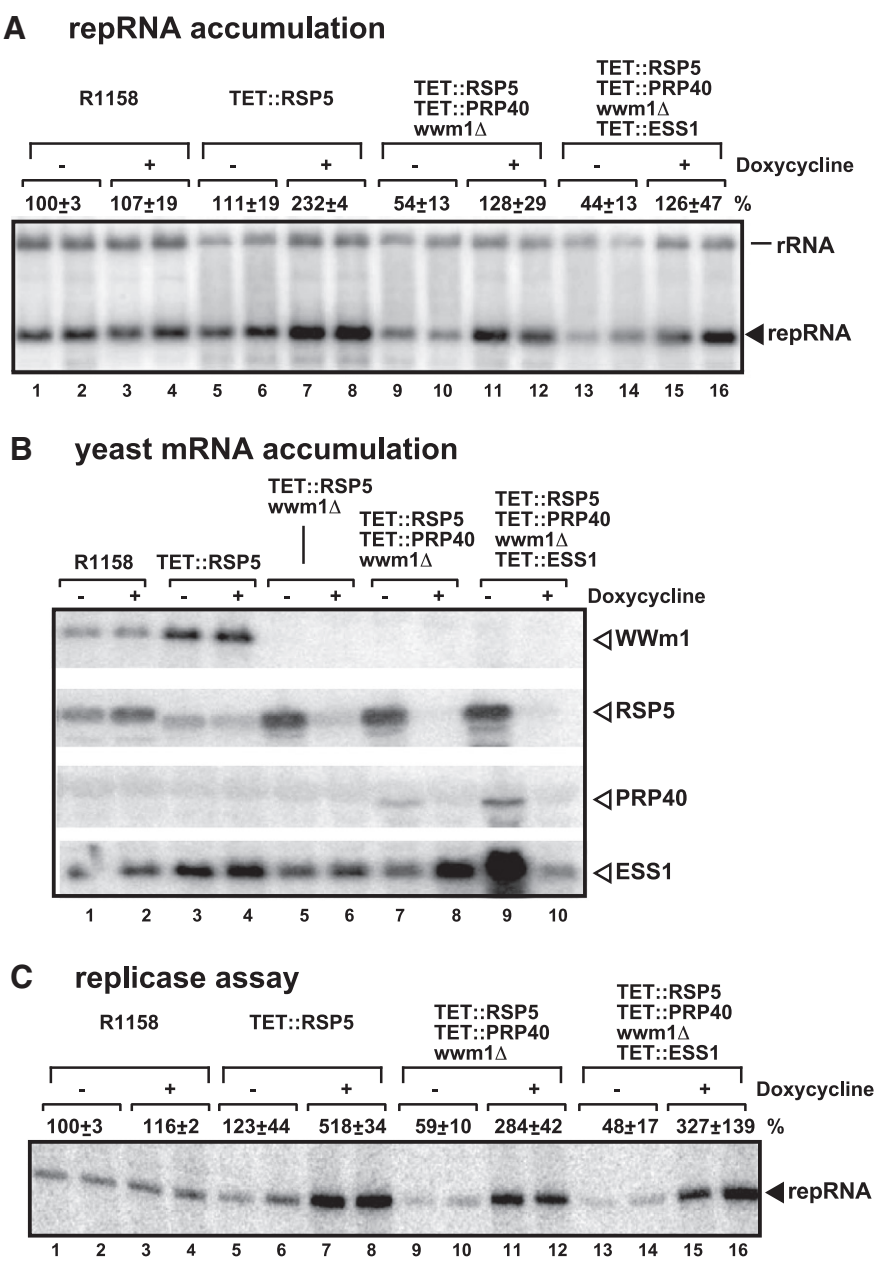


**Fig. 4.** The effect of simultaneous down-regulation (or deletion) of three and four WW domain-containing yeast proteins on TBSV repRNA accumulation in yeast. (A) Testing TBSV repRNA accumulation in the shown yeast strains by Northern blotting. See further details in Fig. 2. (B) Northern blot analysis of *WWM1*, *RSP5*, *PRP40* and *ESS1* mRNA levels at the end of the experiment (the same samples as in panel A). (C) In vitro replicase assay based on the co-purified replicase and the endogenous repRNA in membrane-enriched fractions prepared from the shown yeast strains. See further details in Fig. 2.

but redundant roles for the various WW domain proteins are not conclusive based on the above approaches. Since TBSV replication is robust in yeast, it is possible that simultaneous down-regulation of Rsp5p and Wwm1p could not lead to further increase in TBSV replication due to other limiting host factors under these conditions.

*The effect of down-regulation of WW domain-containing proteins on TBSV RNA accumulation in vitro*

We also tested the in vitro activity of the membrane-bound tombusvirus replicase isolated from various yeast strains with altered expression of the WW domain proteins (Figs. 3B and 4C). Down-regulation of Wwm1p level in yeast resulted in tombusvirus replicase preparations with ~2-fold increased in vitro activity on the co-purified RNA template (Fig. 2C, lanes 7–8 versus 5–6). The largest (~7-fold) increase in in vitro TBSV replication was seen with tombusvirus replicase preparation obtained from a yeast strain with down-regulated Rsp5p, Prp40p, Ess1p and deletion of *WWM1* (Fig. 4C, lanes 15–16, versus 13–14). But other combination of down-regulated WW domain proteins also led to increased TBSV replication in vitro (Figs. 3B and 4C). Altogether, the in vitro and yeast data indicate that Rsp5p and Wwm1p are important regulators of TBSV replication, while the other WW domain proteins had lesser effects.

*Binding of WW domain-containing proteins to p33 and p92$^{pol}$ replication proteins in vitro*

The five WW domain proteins tested here contain various numbers of WW domain repeats: the central portion of Rsp5p contains three repeats of the WW domain known to participate in protein interactions

with a number of client proteins (Sudol and Hunter, 2000; Wang et al., 1999), Prp40 has two WW domains at the N-terminus, while Wwm1p, Ess1p and Urn1p have only a single WW domain (Fig. 1A). To test if these host proteins can interact with p33/p92, we constructed *Escherichia coli* expression vectors to produce recombinant GST-6xHis-Rsp5p and the other WW domain-containing proteins (Figs. 5A–B). Binding of the recombinant proteins was tested in a pull down assay with immobilized MBP-p33C (the C-terminal domain) or MBP-p92C (the C-terminal unique domain, no sequence overlap with p33) (Rajendran and Nagy, 2003, 2004). These binding experiments revealed that all five WW domain-containing yeast proteins bound to both MBP-p33C and MBP-p92C in vitro (Fig. 5A).

*The number of WW domain repeats in Rsp5p affects TBSV RNA accumulation in yeast*

To test if each WW domain repeat in Rsp5p contributes to its inhibitory function, we expressed various truncated versions of Rsp5p in yeast (as shown in Fig. 6A), followed by measuring TBSV repRNA accumulation. Expression of a C-terminally truncated Rsp5p with all three WW domain repeats was almost as efficient inhibitor (by 65%, Fig. 6B, lanes 10–12) as the full-length Rsp5p (by 85%, lanes 13–15). Interestingly, the truncated Rsp5p with all three WW domain repeats also decreased the accumulation of $p92^{pol}$ as efficiently as the full-length Rsp5p. The truncated Rsp5p carrying one or two WW domain repeats was less efficient, showing reduction in TBSV RNA accumulation from 23 to 50% (Fig. 6B, lanes 4–9).

Since the above data suggest that either all three WW domain repeats are needed for maximum level of inhibition or the 3rd WW repeat is critical, we tested additional Rsp5p-derived constructs. Expression of the WW2–3 (2nd and 3rd WW repeats, Fig. 6A) or WW3 (3rd repeat only in WW3/HECT) resulted in ~50 and 10%, respectively, reduction in TBSV replication (Fig. 6D, lanes 7–12). Also, expression of the WW1–2 (1st and 2nd WW repeats, Fig. 6A) was as inhibitory as that of WW2–3 (Fig. 6D, lanes 13–15 versus 7–9) and both were less full inhibitory than WW1–3 (construct C2/WW1–3, Fig. 6A) carrying all three WW domain repeats (Fig. 6D versus Fig. 6B). These data support the idea that all three WW domain repeats are necessary for the full inhibitory effect of Rsp5p on TBSV replication. Moreover, Rsp5p sequences carrying two or three WW domain repeats decreased the accumulation level of $p92^{pol}$, indicating that regulation of $p92^{pol}$ stability is one of the functions of Rsp5p. However, the accumulation of $p92^{pol}$ did not perfectly correlate with TBSV repRNA accumulation (see WW2–3 with only ~50% inhibition of TBSV accumulation but as efficient reduction in accumulation of $p92^{pol}$ as the full-length Rsp5p, Fig. 6E, lanes 5–6 versus 3–4). These findings confirm the previous observation that Rsp5p also has a direct inhibitory effect on the viral replicase in addition to regulation of $p92^{pol}$ stability (Barajas et al., 2009b).

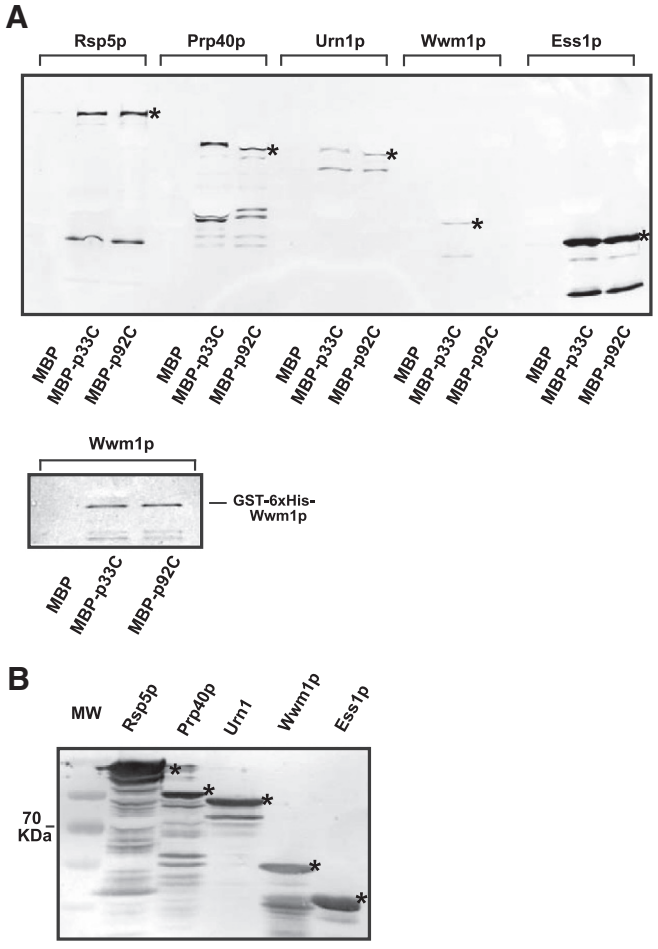


**Fig. 5.** Binding of WW domain-containing yeast proteins to TBSV p33 and p92 proteins in vitro. (A) Affinity binding (pull down) assay to detect interaction between GST-6xHis-WW domain-containing yeast proteins and the MBP-tagged viral proteins. The MBP-tagged viral proteins and MBP produced in *E. coli* were immobilized on amylose-affinity columns. Then, GST-6xHis-tagged WW domain-containing proteins 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-6xHis antibody to detect the amount of GST-6xHis-WW domain-containing yeast proteins specifically bound to MBP-tagged viral proteins. Asterisks mark the expected bands for the WW domain-containing yeast proteins. The second smaller panel is an additional experiment with longer exposure to visualize Wwm1p. (B) The total proteins were analyzed by Western blotting with anti-6xHis antibody to detect the amount of GST-6xHis-WW domain-containing yeast proteins expressed in *E. coli*. Asterisks mark the expected bands for the WW domain-containing yeast proteins.

*WW domain-containing proteins from Arabidopsis inhibit tombusvirus RNA accumulation in Nicotiana benthamiana*

To test if WW domain-containing proteins from *Arabidopsis* can inhibit TBSV repRNA accumulation, we expressed the WW domains of 4 *Arabidopsis* proteins in *N. benthamiana*. Interestingly, similar to the yeast proteins, three *Arabidopsis* WW domain-containing proteins [AtDRH1, AtFCA and AtPrp40c (At3g19840), Fig. 7A] inhibited *Cucumber necrosis virus* (CNV, a very close relative of TBSV) genomic RNA accumulation, while one of them (At2g41020) was not inhibitory.

To test if *Arabidopsis* WW domain-containing proteins can interact with p33 replication protein, we used MYTH (membrane yeast two-hybrid) split-ubiquitin assay. The MYTH assay revealed that the three members of the *Arabidopsis* WW domain-containing protein family, which inhibited CNV accumulation when over-expressed, also interacted with the p33 replication protein (Fig. 7B). We did find correlation between the strength of binding and the inhibitory effect, but this does not exclude that other features of the *Arabidopsis* WW domain-containing proteins are also needed for their inhibitory functions. Only *Arabidopsis* At3g19840 and yeast Prp40p show clear sequence similarity throughout the whole protein and are likely orthologues. Other *Arabidopsis* WW proteins show no remarkable similarity to yeast WW proteins besides the WW domain. Nevertheless, these data confirm that three *Arabidopsis* WW domain-containing proteins can also bind the p33 replication protein, suggesting that the yeast and plant WW domain-containing proteins have similar inhibitory/regulatory functions in tombusvirus replication.

*WW domain-containing proteins also inhibit alfanodavirus replication in yeast*

To test if replication of other RNA viruses could also be regulated by WW domain-containing proteins, we tested if over-expression of

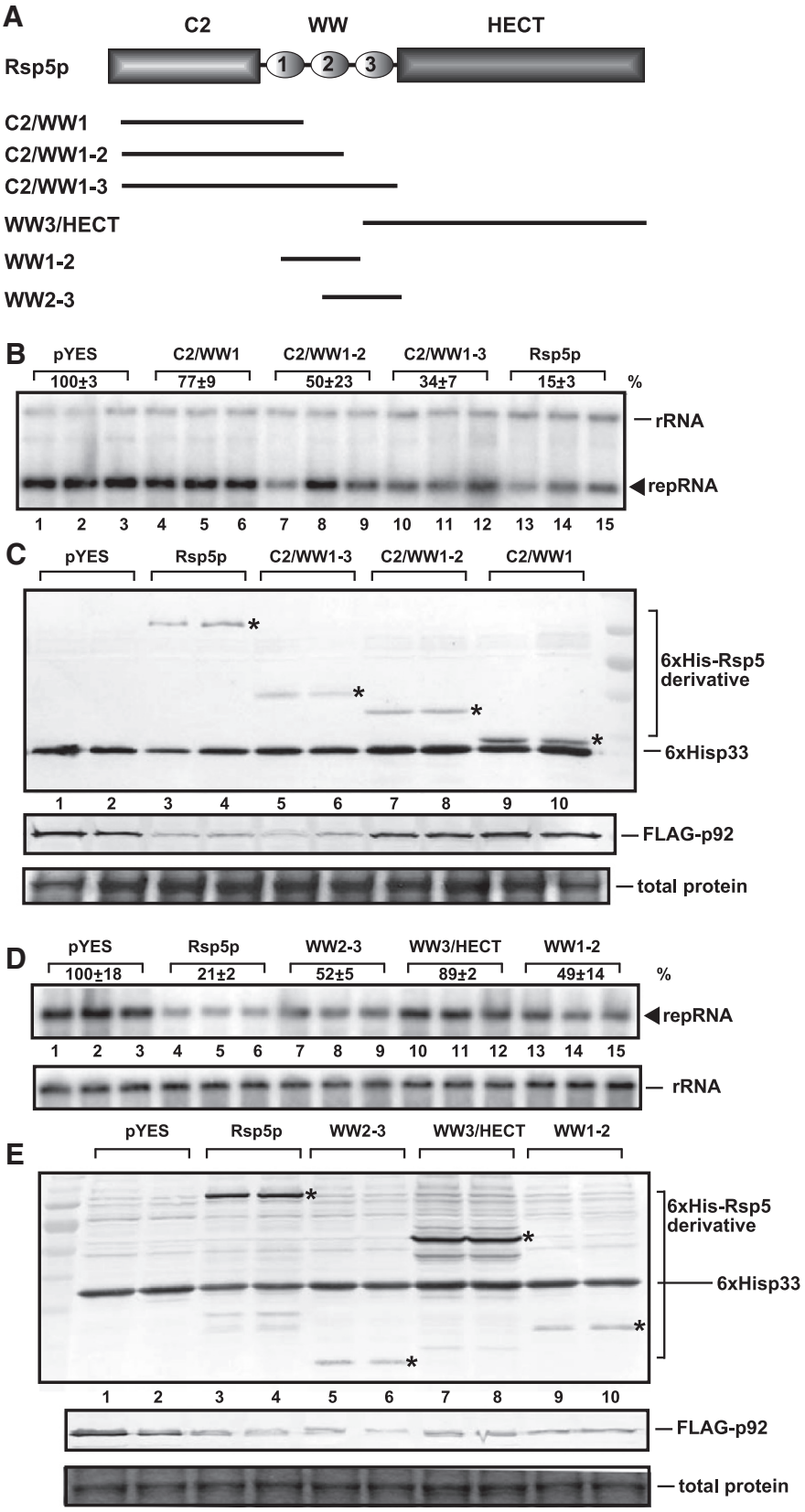


**Fig. 6.** The effect of over-expression of WW domain repeats from Rsp5p on TBSV replication. (A) Schematic representation of various domains of Rsp5p and the constructs used for expression in yeast. See further details in Fig. 1. (B) Northern blot analysis was used to detect DI-72(+) repRNA accumulation in BY4741 yeast strain over-expressing Rsp5p or one of its derivatives from *GAL1* promoter in the high copy number pYES plasmid as shown. See further details in Fig. 1. (C) Top panel: Western blot analysis of the accumulation level of 6xHis-tagged p33, 6xHis-tagged p92 and 6xHis-tagged Rsp5p or one of its derivatives using anti-6xHis antibody. The asterisk (*) indicates WW domain-containing yeast proteins. Middle panel: Western blot analysis of the accumulation level of FLAG-tagged p92 using anti-FLAG antibody. Bottom panel: Coomassie blue-stained SDS-PAGE gel used to show the amount of total proteins loaded. (D) The same as panel B, except different Rsp5p derivatives are expressed (as shown). (E) The same as panel C, except different Rsp5p derivatives are expressed (as shown).

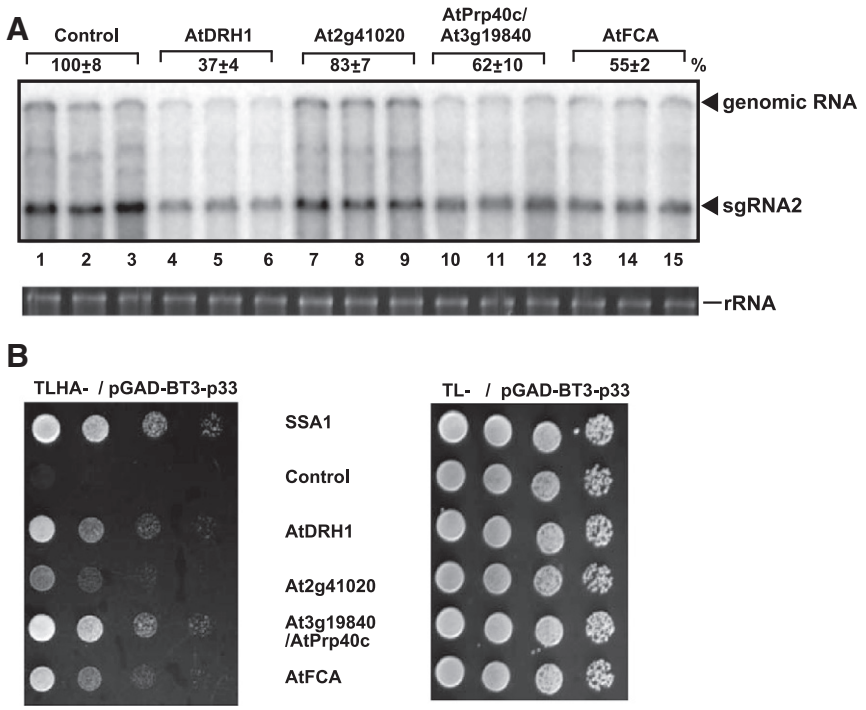


**Fig. 7.** Inhibition of tombusvirus RNA accumulation in plants by expression of WW domains of *Arabidopsis* proteins. (A) WW domains of *Arabidopsis* proteins were expressed from the 35S promoter by agro-infiltration of *N. benthamiana* leaves. One day later, the same leaves were agro-infiltrated with *Agrobacterium* carrying a plasmid expressing CNV (a tombusvirus closely related to TBSV) from the 35S promoter. The control samples were obtained from leaves expressing no proteins (lanes 1–3). Total RNA was extracted from leaves 68 h after agro-infiltration of CNV construct. The accumulation of CNV gRNA and subgenomic (sg)RNAs in *N. benthamiana* leaves was measured by Northern blotting (top panel). The ribosomal RNA (rRNA) was used as a loading control and shown in agarose gel stained with ethidium-bromide (bottom panel). (B) Confirmation of interaction between WW domains of *Arabidopsis* proteins and the p33 replication protein. In the split ubiquitin assay, the bait p33 was co-expressed with the prey proteins in yeast. SSA1 (HSP70 chaperone), and the empty prey vector (NubG) were used as positive and negative controls, respectively. Left panel shows yeast growth under selective media, while yeasts were grown on a nonselective media in the right panel.

these proteins could affect *Nodamura virus* (NoV) and the related *Flock house virus* (FHV), which are insect viruses, replication in yeast model host (Pogany et al., 2010). Interestingly, FHV RNA replicated ~5-fold less efficiently in yeast over-expressing Rsp5p, its three WW domain repeats or Wwm1p proteins (Fig. 8A, lanes 4–9 and 16–18) when compared with the control yeast cells (Fig. 8A, lanes 1–3). Similar to FHV, NoV RNA also replicated ~3-to-5-fold less efficiently in yeast over-expressing Rsp5p, the three WW domain repeats or Wwm1 proteins (Fig. 9A, lanes 1–3, 7–9 and 16–18). On the other hand, over-expression of Prp40p was moderately inhibitory to FHV or NoV replication, while Ess1p and Urn1p were not inhibitory (Figs. 8A and 9A). Interestingly, over-expression of Rsp5p and Wwm1 proteins also reduced the amount of NoV and FHV protein A replication protein levels (Figs. 8–9), suggesting that these WW domain-containing proteins might affect protein A stability during virus replication, similar to that for TBSV.

To test if WW domain-containing yeast proteins can interact with replication proteins, called protein A, of FHV and NoV, we used *E. coli* expression vectors to produce recombinant GST-6xHis-Rsp5p and the other WW domain-containing proteins. Binding of the recombinant proteins was tested in a pull down assay with immobilized FHV MBP-protein A or NoV MBP-protein A. These binding experiments revealed that all five WW domain-containing yeast proteins bound to both FHV and NoV protein A in vitro (Figs. 8B and 9B).

Overall, these experiments confirmed that replication of two insect RNA viruses is also affected by WW domain-containing yeast proteins and these proteins can interact with the protein A replication protein of FHV and NoV. Therefore, it is possible that replication of other RNA viruses could be regulated by WW domain-containing yeast proteins.

## Discussion

### *A regulatory role for WW domain-containing proteins in TBSV replication*

Based on our experience with TBSV, genome-wide screens and global proteomics approaches do not lead to the identification of all the host factors affecting TBSV replication or RNA recombination (Nagy, 2011; Nagy and Pogany, 2010). An additional approach to identify new genes affecting TBSV replication is the detailed characterization of protein families, whose selected members have been identified during the high throughput screens. Accordingly, in this paper we have characterized 5 members of the WW domain-containing protein family, based on the previous finding that the WW domains in Rsp5p E3 ubiquitin ligase were found to be critical for inhibition of TBSV replication in yeast (Barajas et al., 2009b). This approach led to the identification of Wwm1p, a protein of unknown function, as a strong inhibitor of TBSV replication. Moreover, a similar approach using *Arabidopsis* WW domain-containing protein family led to the identification of three novel plant proteins (AtDRH1, AtFCA and AtPrp40c, Fig. 7A) that strongly inhibited tombusvirus replication. In addition, we have found an additional yeast protein (i.e., Prp40p splicing factor) that has moderate inhibitory effect. Altogether, these data indicate that studying a whole family of host proteins could be a fruitful approach to identify new host factors.

Wwm1p seems to be a potent inhibitor of TBSV replication based on over-expression studies in yeast (Fig. 1). In addition, down-regulation of Wwm1p expression led to increased TBSV replication (Fig. 2), suggesting that Wwm1p has a unique (not redundant) function in regulation of TBSV replication. Also, the tombusvirus replicase complex

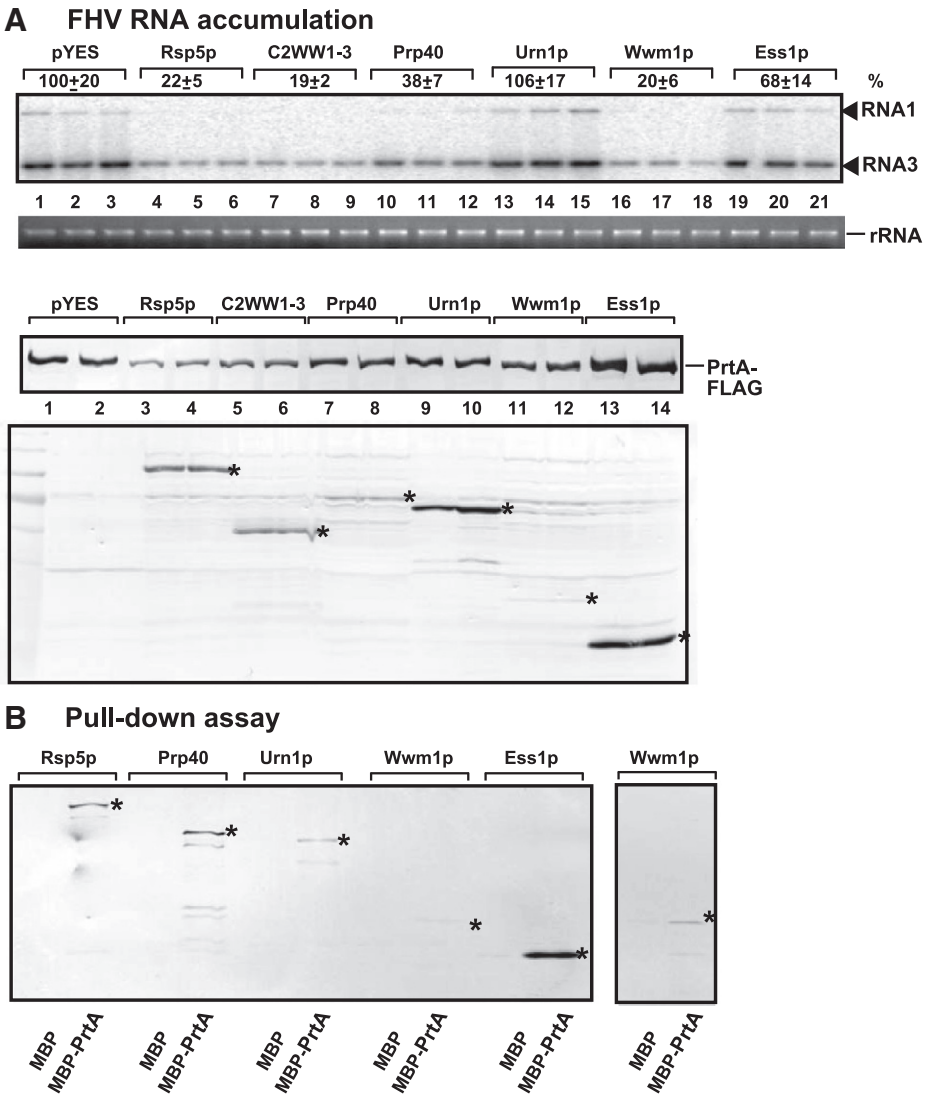


**Fig. 8.** The effect of over-expression of WW domain-containing yeast proteins on FHV RNA accumulation in yeast. (A) Top panel: Northern blot analysis was used to detect RNA1/RNA3 accumulation for FHV. To launch FHV RNA1 replication, we expressed FHV RNA1 from the copper-inducible *CUP1* promoter in BY4741 yeast strain. The continuous expression of WW domain-containing yeast proteins from the *GAL1* promoter started 16 h prior to launching FHV RNA replication at 29 °C. The yeast cells were cultured for 24 h at 29 °C in 3 ml SC-ULH$^-$ with 2% galactose media containing 50 mM $CuSO_4$. The accumulation level of FHV RNAs was normalized based on 18S rRNA. Each experiment was repeated. The average and standard deviation of the repeats are shown. Middle panel: Western blot analysis of the accumulation level of FLAG-tagged protein A using anti-FLAG antibody. Bottom panel: Western blot analysis of the accumulation level of 6xHis-tagged WW domain-containing yeast proteins using anti-6xHis antibody. The asterisk (*) indicates WW domain-containing yeast proteins. (B) Affinity binding (pull down) assay to detect interaction between GST-6xHis-WW domain-containing yeast proteins and the MBP-tagged FHV protein A replication protein. The bound proteins were analyzed by Western blotting with anti-6xHis antibody to detect the amount of GST-6xHis-WW domain-containing yeast proteins specifically bound to MBP-tagged viral protein. Asterisks mark the expected bands for the WW domain-containing yeast proteins. The second smaller panel is an additional experiment with longer exposure to visualize Wwm1p. See further details in Fig. 5A.

isolated from yeast with down-regulated Wwm1p protein level was ~2-fold more active than from wt yeast. Moreover, the accumulation level of $p92^{pol}$ was reduced when Wwm1p was over-expressed, suggesting that the stability of $p92^{pol}$ might be reduced— similar to that observed with Rsp5p (Barajas et al., 2009b). It is possible that Wwm1p plays a role in TBSV replication by forming a complex with Rsp5p. Indeed, Wwm1p was shown to interact with Rsp5p and Wwm1p serves as a substrate for Rsp5p (Hesselberth et al., 2006). This interaction between Wwm1p and Rsp5p could explain why single down-regulation of Wwm1p has similar effect on TBSV replication as dual down-regulation of Wwm1p/Rsp5p. However, the formation of Wwm1p/Rsp5p does not necessarily prevent that single (not in a complex) Wwm1p or Rsp5p proteins might inhibit TBSV replication as well.

Alternatively, Wwm1p and Rsp5p as well as other WW domain-containing proteins may have partially redundant function in yeast. This might not be easily documentable, possibly because TBSV replication cannot further increase from the level reached by TBSV RNA accumulation in a yeast with single down-regulated protein when compared with a yeast strain, in which three or four members of WW domain-containing proteins are down-regulated/deleted, due to restriction of viral replication by other limiting host factors. Indeed, we observed more substantial differences (~7-fold) in in vitro activities of the tombusvirus replicase when the replicase preparations were obtained from a yeast strain with down-regulated Rsp5p, Prp40p, Ess1p and deletion of *WWM1* (Fig. 4C). Altogether, this work demonstrates that several members of WW domain-containing protein family are inhibitors/regulators of TBSV replication.

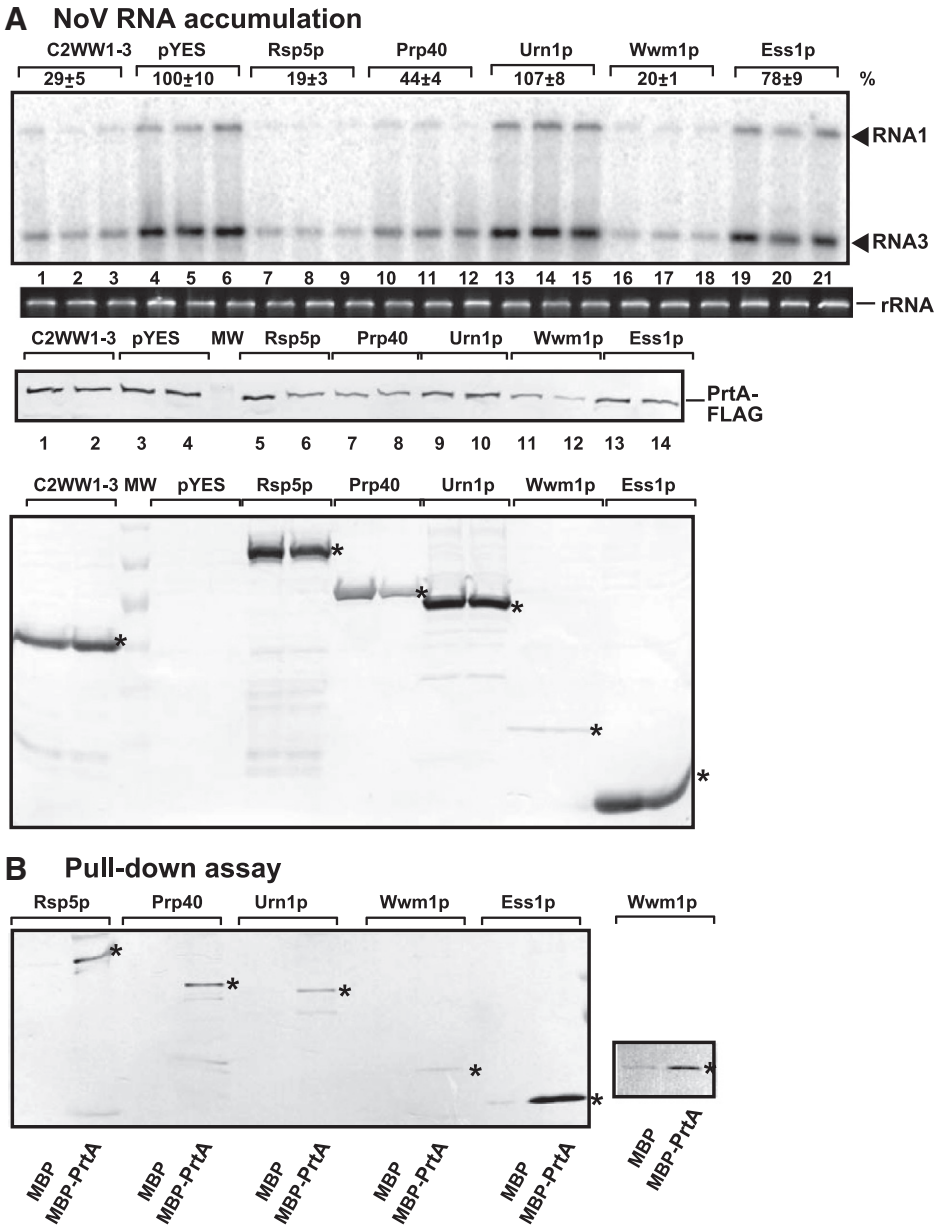


**Fig. 9.** The effect of over-expression of WW domain-containing yeast proteins on NoV RNA accumulation in yeast. (A) Top panel: Northern blot analysis was used to detect RNA1/RNA3 accumulation for NoV. To launch NoV RNA1 replication, we expressed NoV RNA1 from the copper-inducible *CUP1* promoter in the parental (BY4741) yeast strain. The yeast cultures were pre-grown in 3 ml SC-ULH$^-$ supplemented with 2% glucose for 20 h, then they were diluted to 0.25 $OD_{600}$. The yeast cells were cultured in 3 ml SC-ULH$^-$ with 2% glucose media for 20 h. Then, yeast was transferred to 3 ml SC-ULH$^-$ with 2% galactose media containing 50 mM $CuSO_4$ and cultured for 2 days at 29 °C. The accumulation level of NoV RNAs was normalized based on 18S rRNA. Each experiment was repeated. Middle panel: Western blot analysis of the accumulation level of FLAG-tagged protein A using anti-FLAG antibody. Bottom panel: Western blot analysis of the accumulation level of 6xHis-tagged WW domain-containing yeast proteins using anti-6xHis antibody. The asterisk (*) indicates WW domain-containing yeast proteins. (B) Affinity binding (pull down) assay to detect interaction between GST-6xHis-WW domain-containing yeast proteins and the MBP-tagged NoV protein A replication protein. The bound proteins were analyzed by Western blotting with anti-6xHis antibody to detect the amount of GST-6xHis-WW domain-containing yeast proteins specifically bound to MBP-tagged viral protein. Asterisks mark the expected bands for the WW domain-containing yeast proteins. The second smaller panel is an additional experiment with longer exposure to visualize Wwm1p. See further details in Fig. 5A.

*The role of WW domain repeats in inhibition of TBSV replication*

Sequences of WW domains are highly variable, except from the conserved residues (Hesselberth et al., 2006), which likely affect substrate specificity. However, we found that all 5 yeast and 4 *Arabidopsis* proteins carrying WW domains bound to p33/p92 (Figs. 5A and 7B). Yet, the inhibitory activity of these proteins on TBSV replication is highly variable. Moreover, Wwm1p with a single N-terminal WW domain is as potent inhibitor of TBSV replication as Rsp5p with three WW domain repeats. Thus, it seems that not all WW domains behave similarly to inhibit TBSV replication. It is important to note that protein localization and cellular expression level of a given WW domain-containing protein could also affect the effectiveness of the particular protein as an inhibitor, in addition to its direct effect of the particular WW domain sequence.

Interestingly, all three WW domain repeats of Rsp5p are needed for maximum level of inhibition (Fig. 6). This indicates that expression of artificial antiviral proteins with variable number of WW domains could be a useful antiviral strategy. Overall, the presented data firmly established that several WW domains are involved in inhibition of TBSV replication, while other WW domains are not important in this respect. Mutagenesis experiments will be needed

to determine the key features within WW domain sequences needed for inhibition of TBSV replication.

*Wide-spread roles of WW domain repeats in inhibition of (+)RNA virus replication?*

Interestingly, we found that not only TBSV, but also the distantly-related nodaviruses (insect RNA viruses), could be inhibited by over-expression of Rsp5p and Wwm1p (Figs. 8–9). Importantly, WW domain-containing proteins are frequent in animals and plants, making it likely that these proteins could affect the replication of similar viruses as well. Therefore, it seems that different (+)RNA viruses are regulated by selected WW domain proteins, opening up the possibility that this type of regulation is wide-spread among (+) RNA viruses.

## Materials and methods

*Yeast strains and expression plasmids*

Yeast (*Saccharomyces cerevisiae*) strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was obtained from Open Biosystems (Huntsville, AL, USA). Yeast strain NMY51 [*MAT***a** *his3Δ 200 trp1-901 leu2-3, 112 ade2 LYS2*::(*lexAop*)$_4$-*HIS3 ura3*::(*lexAop*)$_8$-*lacZ ade2*::(*lexAop*)$_8$-*ADE2 GAL4*] was obtained from Dualsystems. Yeast strains R1158 (BY4741 URA3::CMV-tTA) and the TET regulatable promoter strains (yTHC) TET-RSP5, TET-PRP40 and TET-ESS1 were obtained from Open Biosystems.

Strains TET-WWM1, TET-WWM1/TET-RSP5, TET-WWM1/TET-PRP40 and TET-WWM1/TET-ESS1 were generated by homologous recombination, inserting a TET-regulatable promoter upstream of the yeast *WWM1* gene. To do so the *WWM1* ORF was amplified by PCR from yeast genomic DNA using primers #3066 and #3067 (Table 1) and the product was digested with *BamH*I. A *TET* promoter-containing DNA fragment was excised from plasmid pCM189 (Gari et al., 1997) by *SpeI*/*BamH*I digestion. This DNA fragment was then ligated to the *BamH*I-digested *WWM1* PCR product and to *Spe*I-digested pFA6a-*hphNT1* plasmid (Janke et al., 2004) containing a hygromycin B resistance gene. The ligation product was used as template for PCR with primers #3362 and #4779. The resulting PCR product, containing the *hphNT1* gene, the *TET* promoter and the first 258 nucleotides from *WWM1* ORF, was transformed into yeast strains R5811 or the yTHC strains TET-RSP5, TET-PRP40 and TET-ESS1 and recombinants selected in YPD plates containing 300 mg/l hygromycin B. The correct insertion of the *TET* promoter upstream of *WWM1* in the resulting strains was confirmed by PCR.

To generate multiple TET/deletion yeast strains, first the *WWM1* gene was replaced by the *natNT2* gene, which confers resistance to nourseothicin. The *natNT2* gene was amplified from plasmid pFA6a-*natNT2* with primers #3362 and #3363. The resulting PCR product was transformed into the TET-PRP40 yTHC strain and the recombinants selected in YPD plates containing 100 mg/l nourseothicin. Second, the *TET* promoter was inserted upstream of the *RSP5* gene. A PCR was performed using TET-RSP5 yTHC strain genomic DNA as template and primers KanB and #3345. The resulting PCR product containing the *TET* promoter and the first 268 nucleotides of the RSP5 ORF was digested with BglII and ligated to BglII-digested pFA6a-*hphNT1* plasmid (Janke et al., 2004). The ligation product was used as template for PCR with primers #3344 and #3345, and the PCR product transformed into the TET-PRP40/wwm1Δ yeast strain. Recombinants were selected in YPD–hygromycin B plates to obtain TET-PRP40/wwm1Δ/TET-RSP5. Third, the TET promoter was inserted upstream of the *ESS1* gene. The TET promoter plus the first 269 nucleotides of the *ESS1* ORF were amplified from TET-ESS1 yTHC strain genomic DNA with primers KanB and #3717 and the resulting product was digested with BglII. The *ble* gene, which encodes resistance to phleomycin, was excised from plasmid pUG66 (Janke et al., 2004) by BglII and SacI digestion and inserted into BglII/SacI digested pAG-29 (Janke et al., 2004). The resulting plasmid was digested with BglII and ligated to the BglII-digested TET-ESS1 PCR product. The ligation product was used as template with primers #3723 and #3717 and the resulting PCR product transformed into yeast strain TET-PRP40/wwm1Δ/TET-RSP5. Recombinant yeasts containing the *ble*-TET-ESS1 cassette were selected in YPD plates with 20 mg/l phleomycin, resulting in the yeast strain TET-PRP40/wwm1Δ/TET-RSP5/TET-ESS1.

The plasmids pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1 and pGAD-FLAG-92-CUP1 used for TBSV replication in yeast have

**Table 1**
List of primer sequences.

| | |
|---|---|
| 3066 | 5′-GCCGGATCCATGGCTCAAAGTAAAAGTAATC-3′ |
| 3067 | 5′-CGGCTCGAGTTATCTAGAAAAGTCACTACCGTCAAATC-3′ |
| 3362 | 5′-ACTCACTACAAGAGAGTCGATAAAAAAAACCCAAAACATTTTAACGTACGCTGCAGGTCGA-3′ |
| 4779 | 5′-TTGGGCCCCTGCTTGCAC-3′ |
| 3363 | 5′-CGTATTCAGTATTATTTCAACAG-3′ |
| 3345 | 5′-GGACGTTAACCACGCCAAG-3′ |
| kanB | 5′-CTGCAGCGAGGAGCCGTAAT-3′ |
| 3344 | 5′-ATTTCAGTTCAGTCCTTCTTTAGAAGAGTGAGTGCGTAGCAAATCGATGAATTCGAGCTC-3′ |
| 3717 | 5′-GCGTCTTGCTTGGATATCG-3′ |
| 3723 | 5′-TACCTTTTTCCCTTCGATCTAAGTAGAAAAAGGACCTGAATGATCGATGAATTCGAGCTC-3′ |
| 2796 | 5′-GCCGAATTCAAGCTTACCATGCCTTCATCCATATCCGTCAAG-3′ |
| 2802 | 5′-CGGCTCGAGTCAGCTAGCGACCGGTTGTTGCTGAATGG-3′ |
| 3062 | 5′-GCCGGATCCATGTCTATTTGGAAGGAAGC-3′ |
| 3063 | 5′-CGGCTCGAGTCATCTAGAATAGTCCAATTCCACAGCC-3′ |
| 3064 | 5′-GCCGGATCCATGCGTGGAGAATGGCAG-3′ |
| 3065 | 5′-CGGCTCGAGCTATCTAGATTCCGGATTTATCGGCTCG-3′ |
| 3066 | 5′-GCCGGATCCATGGCTCAAAGTAAAAGTAATC-3′ |
| 3067 | 5′-CGGCTCGAGTTATCTAGAAAAGTCACTACCGTCAAATC-3′ |
| 3033 | 5′-CGCGGATCCATGCCATCTGACGTAGCATCG-3′ |
| 3034 | 5′-CCGCTCGAGCTAACCTACCCGCTTGATCAC-3′ |
| 3048 | 5′-GCCGGATCCATGGCTGCTACCGCTGCTG-3′ |
| 3049 | 5′-GCCCTCGAGGTCGACTCACTCAGAAAACCTGGACACAC-3′ |
| 3051 | 5′-GCCGGATCCATGTCAGCGGTTAGTTGGAATAG-3′ |
| 3052 | 5′-GCCCTCGAGGTCGACTCAACAATGAACACAATAACCCCA-3′ |
| 3053 | 5′-GCCGGATCCATGTCTCAACTAGTAGGAAATCG-3′ |
| 3054 | 5′-GCCCTCGAGGTCGACTCATGCGCTCAAACTTGTTAGATC-3′ |
| 3055 | 5′-GCCGGATCCATGGCTGGATCTGCAATTCCAAG-3′ |
| 3056 | 5′-GCCCTCGAGGTCGACTCAAAACGGCTGCTGTAATTGCTG-3′ |

been described before (Barajas et al., 2009b). The following yeast expression plasmids have been generated before: pHisGBK-His33 (Panaviene et al., 2004); pGAD-His92 (Panaviene et al., 2004); pYC-DI72sat (Panavas and Nagy, 2003). Plasmids pESC-Leu/Cup/5′-NR/NoV/proteinA/HA/FLAG and pESC-His/CUP/NoV/RNA1/fs/TRSVrz for NoV replication, pESC-His/Cup/FHV/RNA1/HA/FLAG/TRSVrz and pGAD/Cup/FHV/proteinA/C-term/HA/FLAG/YFP for FHV replication will be described elsewhere.

For protein overexpression in yeast, the pYES2/NT/C based plasmids coding for the 6xHis-tagged Rsp5p or the 6xHis-Rsp5p fragments C2/WW1, C2/WW1-3(C2/WW), WW2-3, WW1-2 and WW3/HECT were made as described before (Barajas et al., 2009b). To create plasmid pYES-C2/WW1-2 a PCR was done with primers #2796 and #2802 using pYES-RSP5 (Barajas et al., 2009b) as template. The PCR product was digested with EcoRI and XhoI and inserted into *Eco*RI/*Xho*I-digested pYES2/NT/C.

The ORFs of *PRP40*, *URN1*, *WWM1* and *ESS1* genes were amplified from yeast genomic DNA with primers #3062/#3063, #3064/#3065, #3066/#3067 and #3033/#3034 respectively. The PCR products were digested with *BamH*I and *Xho*I and inserted into BamHI/XhoI-digested pYES2/NT/C for overexpression in yeast or into *BamH*I/*Xho*I-digested pGEX-His (Barajas et al., 2009b) for *E. coli* expression.

Plasmids pMAL-p33C and pMAL-p92C coding for truncated MBP-tagged TBSV p33 protein (amino acids 151–296) and MBP-tagged TBSV p92 protein (amino acids 297–818) have been described before (Rajendran and Nagy, 2003). Plasmids pMAL-FHV-PrtA and pMAL-NoV-PrtA coding for MBP-tagged FHV protein A and MBP-tagged NoV protein A, respectively (generous gift of Dr. Judit Pogany).

The fragments from *Arabidopsis thaliana* genes *At3g01540*/*DRH1*, *At2g41020*, *At3g19840*/*AtPrp40c*, and *At4g16280*/*FCA* containing WW domains, were amplified from *A. thaliana* genomic DNA using primers #3048/#3049, #3051/#3052, #3053/#3054, and #3055/#3056, respectively. All PCR products were digested with *BamH*I and *Xho*I. The digested products were inserted into the plasmid pGD-L (Barajas et al., 2009a) digested with BamHI and XhoI, for agroinfiltration into *N. benthamiana*. The same primers and restriction enzymes were used to clone the WW-containing gene fragments into pPR-N-RE plasmid (Li et al., 2008) for membrane yeast two hybrid analysis, except that the WW-containing fragments were amplified from *A. thaliana* cDNAs and the plasmid pPR-N-RE was digested with BamHI and SalI.

### *Replication analysis in yeast*

The TET-promoter yeast strains and R1158 were transformed with plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1. Transformed yeasts were grown in the presence or absence of doxycycline as described (Barajas et al., 2009b). Total RNA was extracted from yeast and repRNA accumulation analyzed by Northern blot as previously described (Barajas et al., 2009b; Panaviene et al., 2004).

For TBSV repRNA replication analysis in the presence of over-expressed WW proteins, strain BY4741 was transformed with pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1 (or alternatively with pGBK-His33-CUP1/DI72-GAL1 and pGAD-FLAG-92-CUP1) plus the pYES2/NT/C-based plasmids expressing 6xHis-tagged WW proteins or Rsp5p-derived fragments. The transformed yeasts were grown and repRNA accumulation was analyzed by Northern blot as previously described (Barajas et al., 2009b; Panaviene et al., 2004).

For the analysis of replication of the FHV system in the presence of over-expressed WW proteins, yeast strain BY4741 was transformed with plasmids pESC-His/Cup/FHV/RNA1/HA/FLAG/TRSVrz and pGAD/Cup/FHV/proteinA/C-term/HA/FLAG/YFP plus the pYES2/NT/C plasmids expressing the 6xHis-tagged WW proteins. Growing conditions were the same as used for TBSV repRNA replication analysis (Barajas et al., 2009b). For replication of the NoV system, yeast strain BY4741 was transformed with plasmids pESC-Leu/Cup/5′-NR/NoV/proteinA/HA/FLAG and pESC-His/CUP/NoV/RNA1/fs/TRSVrz plus the pYES2/NT/C plasmids expressing the 6xHis-tagged WW proteins. Transformed yeast colonies were grown in Uracyl$^-$/Leucine$^-$/Histidine$^-$ (ULH$^-$) SD minimal media supplemented with 2% glucose at 29 °C for 20 h. The cultures were centrifuged to remove the glucose media, then the yeast was diluted to $OD_{600}=0.25$ in ULH$^-$ media with 2% galactose plus 50 μmol $CuSO_4$ and grown for 2 days at 29 °C. Detections of FHV and NoV RNA1 and subgenomic RNA3 by Northern blots were done using specific $\alpha^{32}$P UTP-labeled RNA probes complementary to the 3′ terminus of each viral RNA.

### *Northern and Western blots for the analysis of viral and host proteins*

For the detection of host proteins mRNAs, $\alpha^{32}$P UTP-labeled RNA probes complementary to the 5′ regions of Rsp5, Prp40, Wwm1 and Ess1 were synthesized by T7 transcription. The same RNA preparations used to analyze TBSV repRNA accumulation were used to analyze mRNA accumulation by Northern blot as described before (Panaviene et al., 2004).

Accumulation of p92, p33, FHV protein A, NoV protein A and over-expressed host proteins was analyzed by Western blot as described (Panaviene et al., 2004), from the same cultures used to analyze repRNA accumulation. The 6xHis-tagged p33 and 6xHis-tagged p92 as well as the 6xHis-tagged WW proteins were detected using anti-6xHis antibody, followed by alkaline phosphatase-conjugated anti-mouse antibody. The FLAG-tagged p92 protein, the FHV protein A and the NoV protein A were detected using anti-FLAG antibody and alkaline phosphatase-conjugated anti-mouse antibody.

### *In vitro replicase assay using yeast membrane-enriched fractions*

The TET-promoter yeast strains and R1158 were transformed and grown in the same conditions as described above for TBSV repRNA replication on yeast. Yeasts were collected and processed as described (Barajas et al., 2009b) to obtain membrane-enriched fractions containing the in vivo-assembled replicase complexes. The relative amount of 6xHis-tagged p33 in each membrane fraction preparation was determined by Western blot with anti-6xHis antibody in order to use comparable amounts of replicase from each preparation in the subsequent replicase assay. The replicase assay was performed as described (Barajas et al., 2009b). The reactions (100 μl) included 25 μl of the normalized membrane fraction preparations, 50 mM Tris–Cl pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 0.2 RNase inhibitor, 10 mM ATP, 10 mM CTP, 10 mM GTP and 0.1 μl of $\alpha^{32}$P UTP (3000 Ci/mmol). Reaction mixtures were incubated 2 h at 25 °C, followed by phenol/chloroform extraction and isopropanol/ammonium acetate (10:1) precipitation. $\alpha^{32}$P UTP-labeled RNA products were resolved in 8% acrylamide/8 M urea gels.

### *Analysis of protein interactions in vitro*

Pull-down assays were performed as described (Barajas et al., 2009b). Briefly, the MBP-tagged p33C, p92C, FHV protein A or NoV protein A, separately, were bound to amylose columns. Then *E. coli* lysates containing the GST-6xHis-tagged WW proteins were passed through. Proteins were eluted from the columns with 10 mM maltose and the relative amounts of WW proteins bound to MBP-tagged viral proteins were analyzed by SDS-PAGE and Western blot using anti-6xHis antibody and alkaline phosphatase-conjugated anti-mouse antibody.

### *Analysis of TBSV replication in N. benthamiana*

Preparation of *Agrobacterium tumefaciens* cultures for agroinfiltration of *N. benthamiana* leaves was done as described (Wang and Nagy, 2008). Leaves were first agroinfiltrated with constructs expressing

WW-containing portions of *A. thaliana* proteins. One day later the same leaves were agroinfiltrated with construct pGD-35S-20Kstop to launch CNV-20Kstop infection (Barajas et al., 2009a). Three days later RNA was extracted from the infiltrated leaves and CNV-20Kstop accumulation was analyzed by Northern blot as described (Cheng et al., 2007).

*Analysis of protein interactions in vivo using the split-ubiquitin assay*

Yeast strain NMY51 was co-transformed with plasmid pGAD-BT3-N-His33 (Mendu et al., 2010) and the pPR-N-RE plasmids expressing WW-containing portions of *A. thaliana* proteins. Transformed yeast colonies were suspended in 100 μl of water and serially diluted (10 fold) in water. 5 μl of each dilution were spotted onto TL− plates, as loading control, or onto TLHA− plates to score protein interactions (Li et al., 2008).

## Acknowledgments

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