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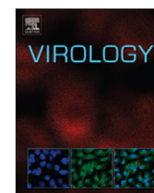

Characterization of dominant-negative and temperature-sensitive mutants of tombusvirus replication proteins affecting replicase assembly

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ABSTRACT

The assembly of the viral replicase complex (VRC) on subcellular membranes is a key step in the replication process of plus-stranded RNA viruses. In this work, we have identified lethal and temperature sensitive (ts) point mutations within the essential p33:p33/p92 interaction domain of p33 and p92 replication proteins of *Cucumber necrosis virus*, a tombusvirus. Mutations within the p33:p33/p92 interaction domain also affected viral RNA recombination in yeast model host. An *in vitro* approach based on yeast cell free extract demonstrated that several p33 and p92 mutants behaved as dominant-negative during VRC assembly, and they showed reduced binding to the viral (+)RNA and affected activation of the p92 RdRp protein, while they did not directly influence (−) or (+)-strand synthesis. Overall, the presented data provide direct evidence that the p33:p33/p92 interaction domains in p33 and p92 are needed for the early stage of virus replication and also influence viral recombination.

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Introduction

Positive-stranded (+)RNA viruses infecting eukaryotic hosts share many fundamental processes during their replication. The similarities include (i) the participation of (+)RNA in translation to produce essential viral replication proteins as well as in genome replication as a template; (ii) the use of (−)RNA as template for production of (+)-strand RNA progeny in an asymmetric manner; (iii) the need for virus-specific replicase complexes (VRC), which are associated with intracellular membranes; and (iv) the use of subverted host proteins to facilitate viral replication (Bartenschlager et al., 2010; den Boon and Ahlquist, 2010; Miller and Krijnse-Locker, 2008; Nagy and Pogany, 2011; Novoa et al., 2005; Salonen et al., 2005; Shi and Lai, 2005). Indeed, all (+)RNA viruses likely depend on or require host factors to replicate their genomic RNAs in infected cells (Bartenschlager et al., 2010; den Boon et al., 2010; Li and Nagy, 2011; Nagy and Pogany, 2010, 2011, 2012; Nagy et al., 2011; Shi and Lai, 2005). Due to the above similarities, our understanding of replication of (+)RNA viruses has advanced significantly in the last decade based on a small number of model viruses, including tombusviruses, such as *Tomato bushy stunt virus* (TBSV) and *Cucumber necrosis virus* (CNV).

Similar to other (+)RNA viruses, the genomic RNA of TBSV and other tombusviruses is replicated by the membrane-bound VRC (Nagy, 2008; Nagy and Pogany, 2010, 2011; White and Nagy, 2004). The tombusvirus VRC contains the viral-coded p33 and p92 replication proteins and host-coded proteins, including glyceraldehyde-3-phosphate dehydrogenase, the heat shock protein 70 chaperones (Hsp70), pyruvate decarboxylase (Serva and Nagy, 2006), Cdc34p E2 ubiquitin conjugating enzyme, Ded1 DEAD-box RNA helicase, eukaryotic translation elongation factor 1A (eEF1A), eukaryotic translation elongation factor 1Bgamma (eEF1Bγ), Pex19p shuttle protein and the Vps23p adapter ESCRT protein (Barajas et al., 2009; Barajas and Nagy, 2010; Huang and Nagy, 2011; Kovalev et al., 2012; Li et al., 2008, 2009, 2010; Nagy and Pogany, 2012; Pathak et al., 2008; Sasvari et al., 2011; Serva and Nagy, 2006; Wang and Nagy, 2008). The roles of several of these proteins in tombusvirus replication have been studied in some detail (Nagy and Pogany, 2010; Pogany et al., 2008; Wang and Nagy, 2008; Wang et al., 2009a, b). In addition to the above protein components, the tombusviral VRC also contains the viral plus-strand RNA, which likely serves as an assembly platform for the proteins (Panaviene et al., 2005; Pathak et al., 2012; Pogany et al., 2008). Due to the central role of the assembly of the VRC in virus replication, detailed studies have been conducted with several model RNA viruses to identify protein and RNA factors affecting VRC assembly and to characterize interactions

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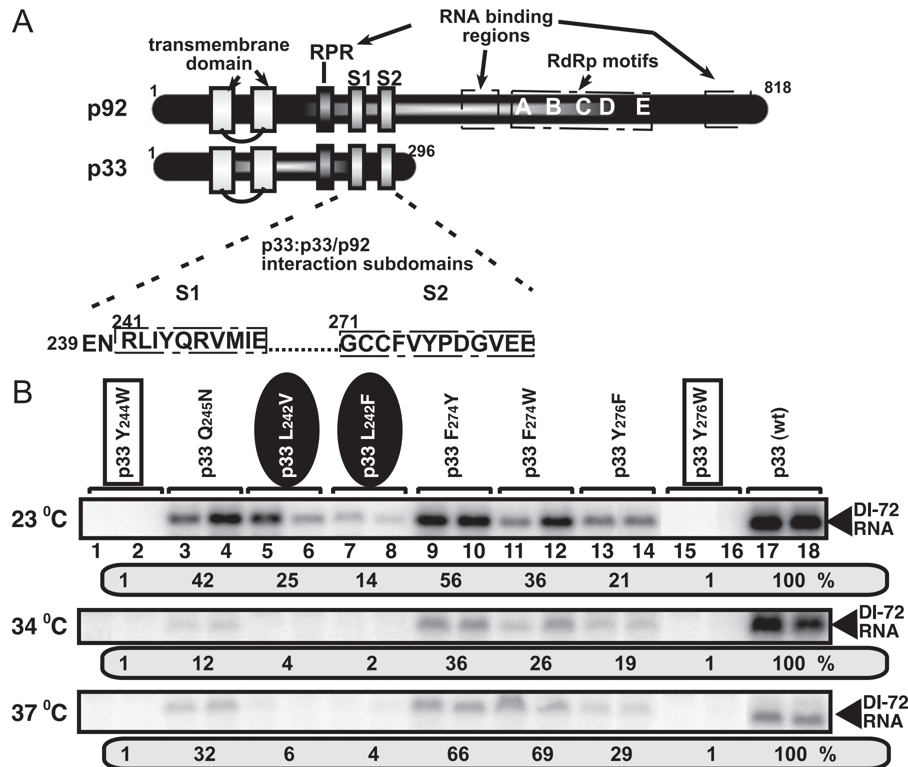

Fig. 1. Mutations in p33:p33/p92 interaction domain in p33 replication co-factor inhibits TBSV repRNA replication in yeast. (A) Cartoons of p33 (1–296 aa) and p92 (1–818 aa, a readthrough product of the p33 ORF, sharing the N-terminal 296aa with p33) replication proteins depicting various domains, such as S1 and S2 protein-protein interaction subdomains, an RNA binding (RPR) and a transmembrane domains. The sequences of S1 subdomain (241–251 aa) and S2 subdomain (271–283 aa) are shown. Quick-change mutagenesis approach was used to introduce specific mutations (see *Materials and methods*) into the S1/S2 subdomains. (B) Northern blot analysis of TBSV repRNA accumulation in BY4741 yeast cells co-expressing one of the mutated CNV His$_6$-tagged p33s, wt His$_6$-p92 and DI-72 repRNA. The names of the S1/S2 subdomain mutants indicate the mutations present in His$_6$-p33. Yeast was grown at three different temperatures to identify temperature-sensitive mutants (shown in black ovals). Nonfunctional p33 mutants are boxed, while the remaining mutants supported reduced repRNA accumulation. The control yeast expressed the wt His$_6$-tagged p33, and the replication level was taken as 100%. The 18S ribosomal RNA was used as a loading control (not shown). Quantification was done with Imagequant software. The experiments were repeated 2 times.

among the above factors (den Boon et al., 2010; Li and Nagy, 2011; Nagy and Pogany, 2012).

The assembly of the tombusviral VRC likely starts with specific interaction between the tombusviral (+)RNA and the p33 and p92^{pol} proteins, which results in selection of the viral RNA for replication and the recruitment of the viral RNA to the site of replication [*i.e.*, the cytoplasmic surface of the peroxisomal membrane (McCartney et al., 2005; Panavas et al., 2005; Pathak et al., 2008)]. The specific binding of p33 to the TBSV (+)RNA requires the p33 recognition element (p33RE), which includes a C•C mismatch within a large, conserved stem-loop structure, denoted RII(+)-SL (Monkewich et al., 2005; Pogany et al., 2005). p33 and the overlapping p92^{pol} contains an RNA-binding region (RPR domain, Fig. 1A) needed for binding to p33RE of TBSV (+)RNA (Pogany et al., 2005; Rajendran and Nagy, 2003). P33 and p92^{pol} also contain two small p33:p33/p92 interaction domains, denoted S1 and S2 (Fig. 1A), which are also required for the specific recognition of p33RE *in vitro* (Rajendran and Nagy, 2004, 2006). The requirement for p33:p33/p92 interaction domain for specific viral RNA binding indicates that p33 and p92^{pol} likely binds RNA as a dimer (or multimer). Because the N-terminal region of p92^{pol} has the same sequence as p33, it has been proposed that p92^{pol} might also be part of the above protein:RNA complex (Rajendran and Nagy, 2004, 2006). In addition to binding to both p92^{pol} and to the TBSV (+)RNA, p33 also binds to host factors, such as Hsp70 (Nagy and Pogany, 2010), and it has peroxisomal targeting sequences and membrane-spanning domains that facilitate the association of p33 with peroxisomal membrane surfaces, the sites of TBSV replication (McCartney et al., 2005; Panavas et al., 2005). In spite of the above advances, our current understanding of the assembly of TBSV VRC and the factors involved are incomplete.

To further characterize the multifunctional nature of the p33 replication protein, in the first part of the paper, we identify mutants within the S1 and S2 subdomains of the p33:p33/p92 interaction domain that render the replication protein nonfunctional, temperature sensitive (ts) or dominant-negative. Testing the accumulation of TBSV RNA in yeast, a model host led to the identification of critical amino acid residues within the p33:p33/p92 interaction domain as well as generation of ts mutants, which supported TBSV replication only at the permissive temperature. Using temperature shift experiments, we demonstrated that p33:p33/p92 interaction is required at an early step in tombusvirus replication. Then, in the second part of the paper, we dissect the mechanism of the inhibitory effect of p33/p92 mutations on tombusvirus replication. We find that mutations within the p33:p33/p92 interaction domain reduced the efficiency of the *in vitro* assembly of the functional tombusvirus VRC, affected the *in vitro* activation of the p92 RdRp protein, and reduced the ability of p33 to bind to the viral (+)RNA. Altogether, the presented data provide direct functional roles for the p33:p33/p92 interaction domains in p33 and p92 that are required for replication of tombusviruses.

Results

Identification of critical amino acid residues within p33:p33/p92 interaction domain of p33 replication protein affecting tombusvirus replication in yeast

Previous works have defined the S1 and S2 subdomains of the p33:p33/p92 interaction sequence (Fig. 1A), which are known to be essential for TBSV replication and the assembly of the tombusvirus

Table 1
The effect of mutations in the p33:p33/p92 interaction domain on p33 function.

Mutants	Replication		Recombination
	In vivo	*In vitro*	*In vivo*
S1 subdomain			
$R_{241}A$	Lethal	< 1%	
$L_{242}V$	ts		
$L_{242}F$	ts		
$LI_{242-243}CT$	Lethal	< 5%	
$LI_{242-243}SS$	Lethal		
$I_{243}V$	ts	Reduced	Reduced
$Y_{244}A$	lethal		
$Y_{244}F$	ts	Reduced	
$Y_{244}S$	Lethal	< 1%	
$Y_{244}W$	Lethal		
$Q_{245}A$	Lethal	< 1%	
$Q_{245}F$	Lethal	< 1%	
$Q_{245}N$	Reduced		
$R_{246}A$	Lethal	< 1%	
$R_{246}K$	Reduced	Reduced	
$M_{248}F$	ts	Reduced	Reduced
$M_{248}I$	Reduced		
$I_{249}A$	Reduced	Increased	
$E_{250}A$	Lethal	< 1%	
$KDC_{254-256}ASA$	Lethal		
$RY_{258-259}AA$	Lethal		
S2 subdomain			
$C_{272}T$	ts	< 15%	wt-like
$C_{273}F$	Lethal	< 5%	
$F_{274}Y$	Reduced		
$F_{274}W$	Reduced		
$F_{274}A$	ts	< 5%	
$Y_{276}F$	Reduced		
$Y_{276}W$	Lethal		
$Y_{276}A$	ts	< 5%	
$P_{277}F$	ts	< 1%	Reduced
$D_{278}A$	Reduced	wt-like	Reduced
$D_{278}F$	Reduced		
$PD_{277-278}SS$	Reduced		
$G_{279}A$	Reduced	wt-like	
$V_{280}A$	Reduced		
$E_{281}A$	Reduced	wt-like	

VRC *in vivo* (Rajendran and Nagy, 2004, 2006). To test in detail the critical amino acid residues within the S1 and S2 subdomains for tombusvirus replication, we introduced 34 site-specific mutations into the p33 ORF in plasmid pGBK-His33, which expresses the p33 replication protein from the constitutive *ADH1* promoter in yeast (Panaviene et al., 2004). To launch TBSV replication in yeast model host, we expressed the wt or mutated p33 together with $p92^{pol}$ RdRp protein as well as the TBSV replicon (rep)RNA, termed DI-72, as described earlier (Panavas and Nagy, 2003; Panaviene et al., 2004). Northern blot analysis of total RNA preparations obtained from yeast was used to estimate the accumulation level of TBSV repRNA in yeast expressing either mutated or wt p33 (Fig. 1B).

To this end, we tested 19 S1 and 15 S2 subdomain mutants of p33 using different temperatures (23, 29 and 34 or 37 °C) to identify possible temperature-sensitive (ts) mutants in this work (Table 1). The list of p33 mutants included conservative and alanine mutants carrying single or double point mutations. Interestingly, more than half of the S1 subdomain mutants were replication incompetent (10 out of 19, Table 1) and 5 showed ts behavior in replication, confirming that this sequence is critical for tombusvirus replication (Fig. 1B and Fig. 2) (Rajendran and Nagy, 2006). On the contrary, only 2 were replication incompetent and 4 ts mutants (out of 15, Table 1) within the S2 subdomain, suggesting that this sequence is, albeit playing a key role in replication, somewhat more tolerant to mutations than the S1 sequence in tombusvirus replication.

The third group of p33 mutants supported TBSV repRNA accumulation at all three temperatures tested (Fig. 2). Most of these mutants, however, showed reduced level of repRNA accumulation when compared to the wt p33 in yeast, indicating that changes of these amino acid residues were still detrimental for tombusvirus replication. Altogether, the above experiments illustrated that the p33:p33/p92 interaction domain is critical for TBSV repRNA replication in yeast, confirming previous results obtained with a small number of p33 and p92 mutants (Rajendran and Nagy, 2004, 2006).

Mutations within p33:p33/p92 interaction domain of p33 replication protein affect tombusvirus recombination in yeast

To test if viral RNA recombination is affected by mutations within the p33:p33/p92 interaction domain, we launched the replication of DI-AU-FP repRNA, which supports recombination at an increased rate when compared to DI-72 repRNA (Serviene et al., 2006, 2005; Shapka and Nagy, 2004), in the presence of co-expressed mutated p33 and wt $p92^{pol}$ replication proteins in yeast. We performed the experiments at 23 and 34 °C to identify ts mutants (Fig. 3). To this end, we identified 2 S1 and 2 S2 subdomain mutants (Fig. 3A and C) with decreased and 1 S1 mutant ($I_{249}A$, Fig. 3A) showing increased level of RNA recombination (Table 1). Mutants $I_{243}V$ and $M_{248}F$ (Fig. 3B) did not support recombination at detectable level at 34 °C after 48 h incubation, suggesting that these mutants behave as ts mutants not only for replication but for recombination as well. Altogether, these data indicate that the p33:p33/p92 interaction domain is also important in tombusviral RNA recombination.

Use of ts mutant of p33 to define the early requirement of p33:p33/p92 interaction for TBSV repRNA replication

Based on the ts nature of $Y_{276}A$ mutant of p33 (Table 1), we used temperature shift experiments to test the effect of nonpermissive temperature on TBSV repRNA replication. In the first set of experiments, we used the permissive temperature (20 °C) for 6, 8, 10, 12, and 14 h prior to shifting to the nonpermissive temperature (34 °C) that continued up to 28 h total incubation time (Fig. 4A). The data obtained suggested that tombusvirus replication is especially dependent on p33:p33/p92 interaction domain (*i.e.*, on p33:p33 or p33:p92 interactions) within the first 8 h, while it is less sensitive at latter time points.

In the second set of experiments, we used the nonpermissive 34 °C for 6, 8, 10, 12, and 14 h prior to downshift to the permissive 20 °C that continued up to 28 h total incubation time (Fig. 4B). This experiment showed that tombusvirus replication could "recover" if the nonpermissive treatment only lasted for 6 h (Fig. 4B, lanes 3–4). We observed only low level replication when the nonpermissive treatment was longer than 6 h (Fig. 4B, lanes 5–12). Based on these data, we suggest that p33:p33 or p33:p92 interactions might be important at the beginning of tombusvirus replication, possibly during the VRC assembly step. The relatively efficient replication when the nonpermissive treatment lasted only for 6 h could be explained that the nonfunctional p33 mutant expressed at 34 °C might rapidly become functional after the downshift. Altogether, the interpretation of obtained data is complicated by the nonsynchronized nature of tombusvirus replication due to the continuous production of p33 and p92 proteins that can potentially assemble into functional VRCs even at latter time points. Therefore, we needed to develop a more synchronized *in vitro* assay to dissect the dependency of various replication steps on p33:p33 and p33:p92 interactions (see below).

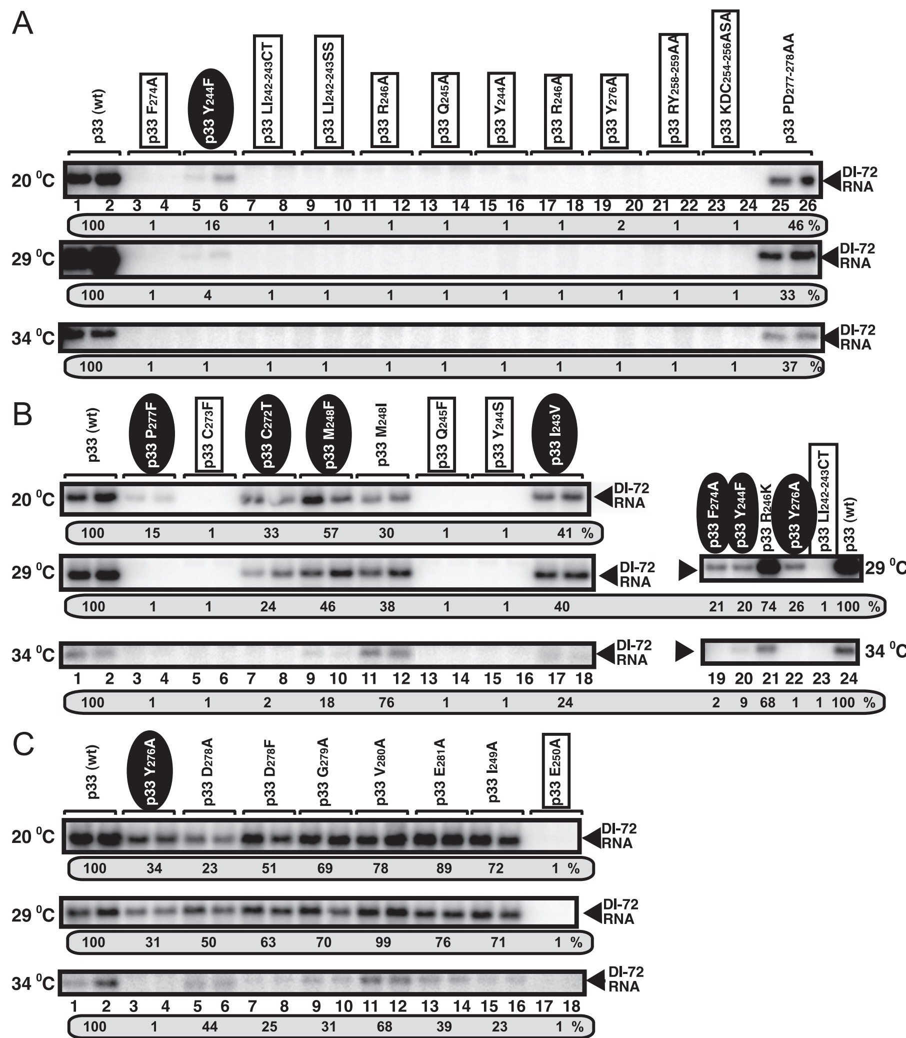

Fig. 2. Identification of ts mutants in p33:p33/p92 interaction domain in p33 replication co-factor in yeast. (A–C) Northern blot analysis of TBSV repRNA accumulation in BY4741 yeast cells co-expressing one of the mutated CNV His_6-tagged p33s, wt His_6-p92 and DI-72 repRNA. See further details in Fig. 1B.

Characterization of loss-of-function mutants of tombusvirus p33 and p92 replication proteins based on a cell-free replication assay

To further characterize the functions of p33 and p92 proteins in tombusvirus replication, we tested p33 mutants carrying debilitating mutations within the p33:p33/p92 interaction region *in vitro* (Table 1). The assay included a cell-free extract (CFE) prepared from wt yeast (BY4741), which can support a single full cycle of TBSV RNA replication, producing (−)RNA and abundant (+)RNA progeny based on added (+)repRNA template and affinity-purified recombinant MBP-tagged p33 and p92 from *E. coli* (Pogany and Nagy, 2008; Pogany et al., 2008). We found that p33 mutant C_{273}F (CF) and Q_{245}F (QF) reduced the level of TBSV repRNA replication to 4% and 2%, respectively, close to the level obtained with our negative control MBP (containing MBP-p92, but missing MBP-p33 from the assay) (Fig. 5B, lanes 1–3). Additional mutations in p33, such as I_{243}V (IV) and C_{272}T (CT) reduced TBSV repRNA replication to 46 and 14%, respectively, when compared to wild type (wt) levels (Fig. 5B, lanes 4, 5 *versus* 6, 7).

Altogether, we found that the 8 replication incompetent p33 mutants (based on the replication level in yeast) tested in the CFE-based replication assay, all showed less than 5% activity when compared with the wt p33 (Table 1). Among the 7 ts p33 mutants tested, we found more variations (between 1 and 50% of wt level activity at 23 °C) in *in vitro* activities, while the 3 p33 mutants supporting reduced level of TBSV repRNA accumulation in yeast, showed wt or close to wt level of activities *in vitro* (Table 1). It is important to note that while there is only a single cycle replication in the CFE-based replication assay, the yeast-based assay support a number of sequential replication cycles, where the progeny (+)RNA from an early cycle becomes a new template in a latter cycle. Thus, the differences in repRNA accumulation in yeast among the various p33 mutants are expected to be larger than in the CFE-based assay, where the amount of the added template RNA is the same in each assay. Nevertheless, the activity of the p33 mutants in the *in vitro* CFE-based experiments correlates well with TBSV repRNA replication levels observed in wt yeast cells (Table 1).

A similar approach based on CFE and p92 mutated within the S1 and S2 subdomains in combination with wt p33 and TBSV (+)repRNA revealed that R_{241}A (RA) and $LI_{242-243}$CT (LT) mutations within the p33:p33/p92 interaction region (Fig. 1A) caused p92 to become nonfunctional (Fig. 5B, lanes 8 and 10), indicating that this region is also critical for the p92 activity *in vitro*. Interestingly, the same C_{273}F (CF) mutation, which rendered p33 barely functional (4% activity, Fig. 5B, lane 1), only reduced the activity of p92 by five-fold (Fig. 1B, lane 9). SDS-PAGE analysis (Fig. 5B) showed that the different levels of activities were not due to variations in the amounts of purified recombinant

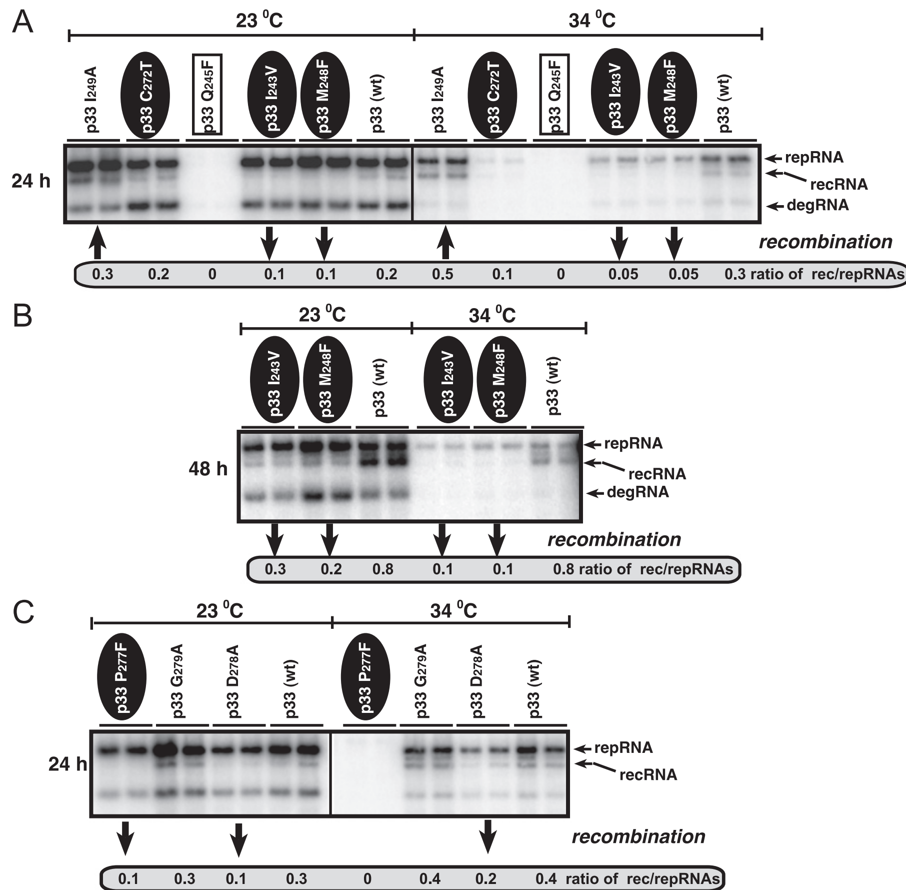

Fig. 3. Identification of mutants in p33:p33/p92 interaction domain in p33 replication co-factor that affects TBSV repRNA recombination in yeast. (A–C) Northern blot analysis of accumulation of TBSV repRNA and recombinant (rec)RNAs in BY4741 yeast cells co-expressing one of the mutated CNV His_6-tagged p33s, wt His_6-p92 and DI-AU-FP repRNA, which is highly recombinogenic. Yeast was grown at two different temperatures to identify temperature-sensitive mutants (shown in black ovals). Nonfunctional p33 mutants are boxed, while the mutants that supported increased or reduced recRNA levels are indicated with arrows. The control yeast samples expressed the wt His_6-tagged p33. Note that we calculated the ratio of recRNA/repRNA in these studies. Quantification was done with Imagequant software. The experiments were repeated 2 times.

proteins. Thus, both the S1 and S2 sub-domains of p33:p33/p92 interaction region are critical for the replication functions of p33 and p92 replication proteins and there is some difference between p33 and p92, based on the different characteristic of CF mutant when present in either p33 or p92.

Identification of dominant-negative mutants of tombusvirus p33 and p92 replication proteins based on a cell-free replication assay

Since the inactivity of the above p33 and p92 mutants could be due to inefficient formation of VRCs when the p33/p92 dimerization/oligomerization is inhibited by the particular mutation (Rajendran and Nagy, 2006), we wanted to complement the mutant p33 replication protein with wt p33 in the CFE-based replication assay. Therefore, to further characterize the deficiency in the nonfunctional p33 mutants, we used mixtures of wt and mutated replication proteins to program the CFE-based replication assay as shown in Fig. 6A. These experiments, however, showed that the deficient mutants of p33 interfered with the normal replication functions of wt p33. For example, adding more p33–Q_{245}F mutant to the CFE assay containing constant amount of wt p33 resulted in 93–97% inhibition of TBSV repRNA replication in the CFE assay (compare lanes 17–18 with 1, Fig. 6B). Mutants I_{243}V and C_{272}T also inhibited TBSV repRNA replication by ~60–70% (Fig. 6B), while addition of purified MBP had no inhibitory effect. In contrast, addition of more wt p33 or MBP did not change repRNA replication (Fig. 6B, lanes 1–3 and 22–24), suggesting that the CFE is capable of supporting high level of repRNA accumulation in the presence of high amount of recombinant proteins. Thus, the inhibitory role of p33–Q_{245}F mutant is unlikely due to nonspecific effect, such as the presence of too much p33 protein in the CFE assay. Based on these observations, we conclude that p33–Q_{245}F mutant has a strong dominant-negative function by interfering with TBSV repRNA replication.

To learn what stage of replication the dominant-negative function of p33–Q_{245}F mutant manifests, we performed a time-course experiment using the CFE-based replication assay. The tombusvirus replicase complexes assemble in 20–60 min in the CFE (after mixing all of the required components), followed by RNA synthesis up to 180 min (Pogany and Nagy, 2008; Pogany et al., 2008). Addition of p33–Q_{245}F mutant at the beginning of the replication assay (0 time point, Fig. 6C, lane 8) had the largest inhibitory effect by reducing TBSV repRNA level to 5% of the control (when purified recombinant MBP was added to the assay, Fig. 6C, lane 2). Moreover, addition of p33–Q_{245}F mutant at the 10 and 20 min time point was still effective in inhibition of TBSV repRNA accumulation *in vitro* (Fig. 6C, lanes 9–10), while its inhibitory effect started to weaken when added at later time points (~60% inhibition when added at 40 and 60 min time points, lanes 11–12, Fig. 6C). We observed a similar pattern for inhibition of TBSV repRNA accumulation by another dominant-negative mutant of p33 (ΔRPR that misses critical residues required for RNA binding, Fig. 1A), which showed strong inhibitory effect when added within the first 20 min of the CFE assay

(Fig. 6C, lanes 14–16). The addition of a purified MBP preparation had no significant inhibitory effect at any time points (lanes 2–6, Fig. 6C). Based on these findings, we conclude that the dominant-negative p33 mutants (Q_{245}F and ΔRPR) can interfere most efficiently with TBSV repRNA replication during the early stages of replication, likely the replicase assembly step. The remaining inhibitory effect of these dominant-negative mutants at latter time points could be due to the inhibition of still on-going replicase complex assembly, which is not synchronized in this assay and likely continues during the entire time of the 3 h-long assay (Pogany and Nagy, 2008; Pogany et al., 2008). Alternatively, the dominant-negative p33 mutants might directly inhibit the RNA synthesis step (see below).

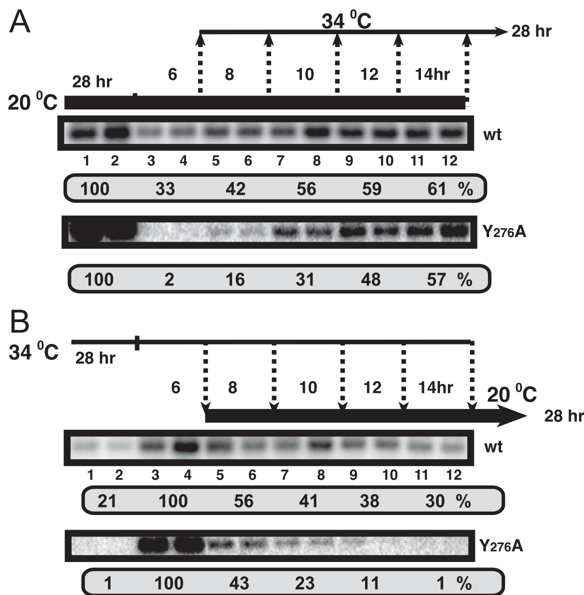

Fig. 4. Temperature-shift experiments with ts p33 mutants indicate that the function of the p33:p33/p92 interaction domain in p33 replication co-factor is required in the early cycle of TBSV replication in yeast. (A and B) Northern blot analysis of TBSV repRNA accumulation in BY4741 yeast cells co-expressing wt His_6-tagged p33 or p33-Y_{276}A, wt His_6-p92 and DI-72 repRNA. The time point for the temperature shift is indicated. Note that all the yeasts were grown up to 28 h before sampling. The control yeast expressed the wt His_6-tagged p33 and was grown at the given temperature (no temperature shift), and the replication level of repRNA was taken as 100%. See further details in Fig. 1B.

Similar CFE-based replication assay using wt p33 and wt p92 and increasing amounts of p92 mutants (Fig. 7A) revealed that p92–$LI_{242–243}$CT mutant showed a strong dominant negative effect by reducing TBSV repRNA replication by ~90% (Fig. 7B, lanes 1–3 *versus* 4–6). Thus, similar to p33, the p33:p33/p92 interaction domain in p92 is likely critical for the assembly of the tombusvirus VRC.

Inhibition of the tombusvirus replicase by short peptides in a cell-free replication assay

Several mutations within the p33:p33/p92 interaction subdomains had dominant-negative effects, thus suggesting that interaction between the TBSV p33 and $p92^{pol}$ molecules is likely critical for replicase activity *in vitro*. To further test if interruption of the interaction between the tombusvirus p33 and $p92^{pol}$ molecules interferes with the function of the VRC, we measured the inhibitory effect of peptides on the *in vitro* activity of the tombuvirus replicase based on the CFE assay. Interestingly, the S1/S2 peptide representing the p33:p33/p92 interaction domain inhibited the *in vitro* activity of the tombusvirus replicase by ~3-fold (Fig. 8B, lanes 3–4), while the RPR peptide representing the RNA binding domain in p33/p92 also inhibited the activity by more than ~2-fold (lanes 5–6). On the contrary, the activity of the unrelated Flock house virus (FHV) replicase was not inhibited

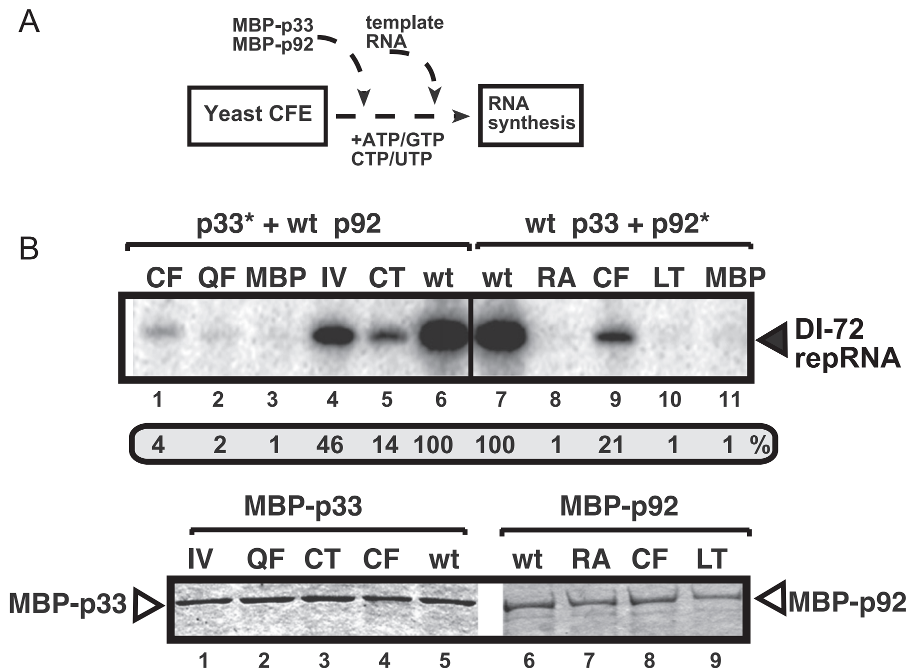

Fig. 5. Mutations within the p33:p33/p92 interaction domain in p33 and $p92^{pol}$ replication proteins inhibits RNA synthesis based on cell-free TBSV replication assay. (A) A scheme of the CFE-based TBSV replication assay with added purified recombinant p33 and $p92^{pol}$ replication proteins of TBSV and *in vitro* transcribed TBSV DI-72 (+)repRNA. The whole cell extract (CFE) was prepared from yeast. The *in vitro* assays contained ATP/CTP/GTP and ^{32}P-UTP. (B) A representative denaturing PAGE gel of newly synthesized ^{32}P-labeled repRNA in an *in vitro* CFE-based replication assay using purified recombinant wt or mutated p33 and p92 proteins. The mutated protein is indicated with an asterisk. The new full-length repRNA product is shown with arrowhead. Experiments were repeated three times and quantified using Imagequant. (C) Bottom image: Coomassie blue staining of SDS-PAGE shows similar amounts of affinity-purified recombinant MBP-p33 proteins (left panel) or MBP-p92 (right panel) used in the above CFE replication assays.

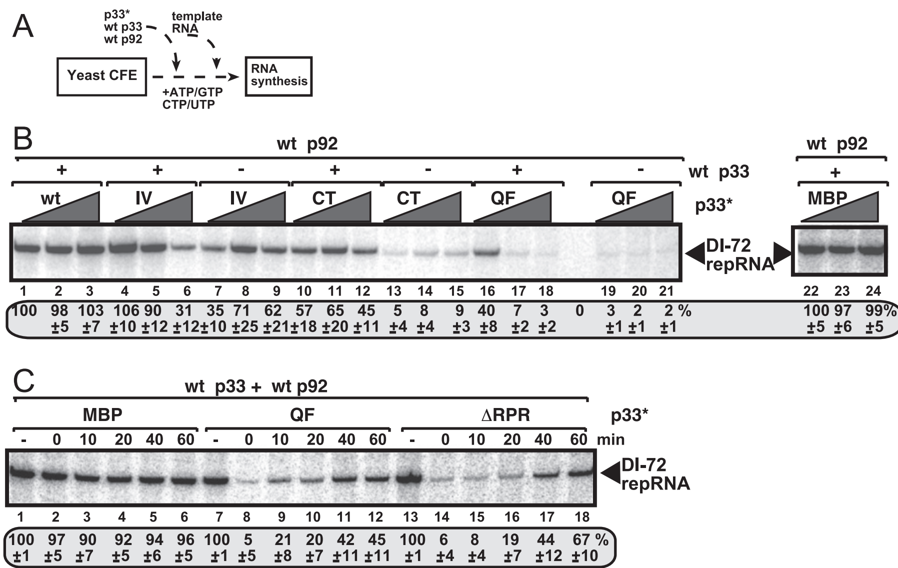

Fig. 6. Identification of dominant-negative p33 mutants *in vitro*. (A) A scheme of the yeast CFE-based assay. (B) Increasing amounts of recombinant p33 mutants (2, 3 and 4 pmol) were added to the CFE-based replication assay simultaneously with wild type p33, p92, repRNA, and ribonucleotides. The new repRNA product is depicted with arrowhead. Note that purified recombinant MBP and MBP-p33 were used as controls. (C) The maximum dominant-negative effect of the p33 mutants is exerted during VRC assembly. The *in vitro* CFE-based replication assay was performed using p33-Q_{245}F and p33-ΔRPR mutants as shown. We provided the recombinant proteins (3 pmol of p33, 1 pmol of p92 and 3 pmol of mutants or MBP) at the 0, 10, 20, 40 or 60 min time points followed by continuation of the *in vitro* replication assay up to 2 h. The denaturing PAGE analysis of the ^{32}P-labeled repRNA products obtained is shown. RepRNA accumulation in yeast CFE lacking the mutant p33 has been chosen as 100%.

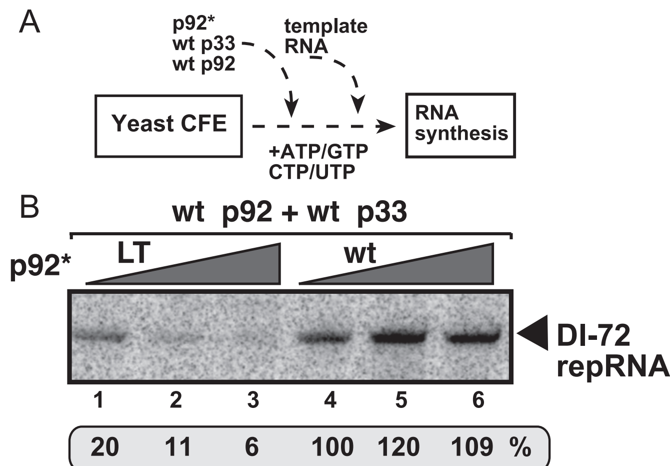

Fig. 7. Identification of a dominant-negative p92 mutant *in vitro*. (A) A scheme of the yeast CFE-based assay. (B) Increasing amounts of recombinant p92-$LI_{242-243}$CT mutant (1, 2, and 3 pmol) were added to the CFE-based replication assay simultaneously with wild type p33 (3 pmol), wt p92 (1 pmol), repRNA (2.5 pmol), and ribonucleotides. The new repRNA product is depicted with arrowhead. Note that purified recombinant wt MBP-p92 was used as a control. See further details in Fig. 6.

by these peptides in the CFE-based assay (Fig. 8C), confirming that the inhibitory effects of S1/S2 and RPR peptides on the tombusvirus replicase are specific. Based on these data, we propose that interaction between p33 and $p92^{pol}$ molecules are required for the tombusvirus replicase activity, suggesting that p33 and $p92^{pol}$ molecules functions as multimers *in vitro*.

Mutations within the p33:p33/p92 interaction region inhibit the in vitro assembly of the tombusvirus replicase

To obtain more direct evidence that mutations within the p33:p33/p92 interaction region interfere with the assembly of the functional tombusvirus replicase complex, we performed an *in vitro* replicase assembly assay based on the yeast CFE and purified recombinant p33 and p92 proteins (Fig. 9A). For this assay, we used only rATP and rGTP in the CFE assay, which facilitate the assembly process (Pogany and Nagy, 2008; Pogany et al., 2008), followed by solubilization and affinity-purification of the assembled tombusvirus replicase (Pathak et al., 2012). Then, the activity of the purified replicase preparations is compared by using the TBSV-derived RI/III(−) as a template (Fig. 9B). Since functional p33, p92 and (+)repRNA is required for the *in vitro* assembly and activation of the tombusvirus replicase (Pathak et al., 2012; Pogany et al., 2008), this test is suitable to measure how functional the various p33 and p92 mutants are in this process. Accordingly, we found that p33-Q_{245}F, p92-R_{241}A and p92-$LI_{242-243}$CT were nonfunctional, while other mutants, such as p33-C_{272}T, p33-C_{273}F and p92-C_{273}F, were poorly functional in the *in vitro* replicase assembly (Fig. 9B). As expected, p33-I_{243}V supported reduced, but significant activity in this test (52% of wt p33, Fig. 9B, lane 1). Altogether, these data confirmed that the p33:p33/p92 interaction region in p33 and p92 is critical during the tombusvirus replicase assembly process.

The p33:p33/p92 interaction domain in p92 is critical for activation of RdRp activity in vitro

In addition to the requirement for assembly of the membrane-bound tombusvirus replicase complex, the RdRp subunit (*i.e.*, p92) has to be activated prior to complementary RNA synthesis (Panaviene et al., 2004; Pathak et al., 2012; Pogany et al., 2008). We have previously developed an *in vitro* assay that can perform the activation of the tombusvirus RdRp in the presence of soluble yeast factors (Pogany and Nagy, 2012). The activation is based on an N-terminally truncated p92 RdRp protein, called p92-Δ167 (Fig. 10A), and does not require the membrane fraction of the yeast CFE (Pogany and Nagy, 2012). The activated p92-Δ167 is capable of producing a 3′terminal extension product with the TBSV (+)repRNA template (Fig. 10B) (Pogany and Nagy, 2012).

Testing p92-Δ167 mutants carrying mutations in the S1 and S2 subdomains in the *in vitro* RdRp activation assay revealed that R_{241}A and $LI_{242-243}$CT mutations fully interfered with the activation of the *in vitro* RdRp activity (Fig. 10B, lanes 6 and 8), while

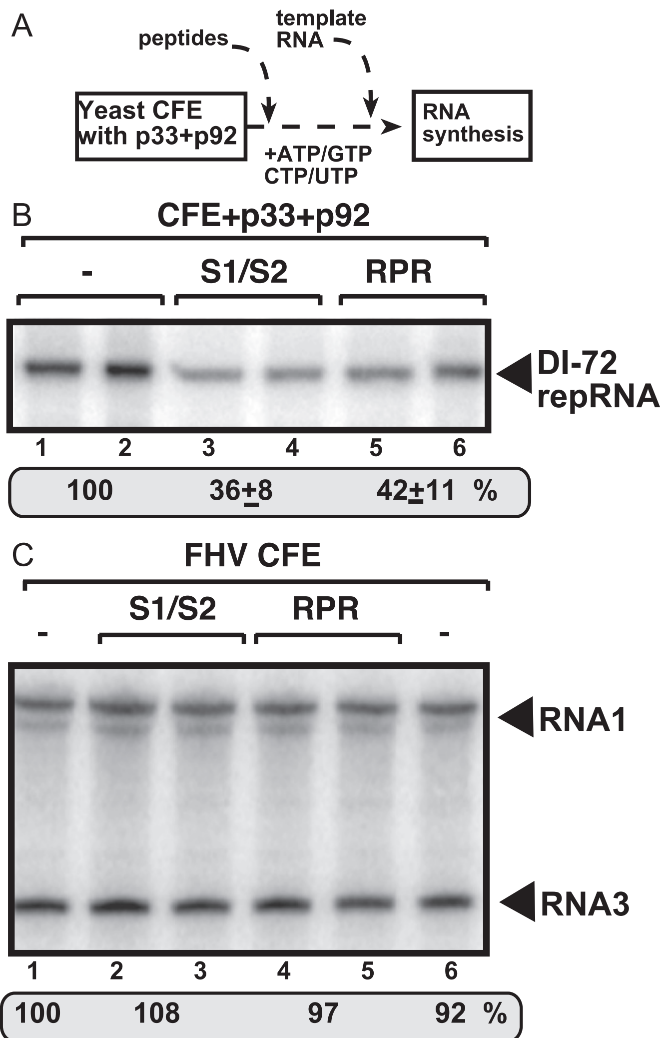

Fig. 8. Inhibition of tombusvirus replication by the S1/S2 peptide in the CFE-based assay. (A) A scheme of the CFE-based replication assay. The yeast used for preparation of CFE co-expressed the CNV p33 and p92 in the absence of the repRNA, which prevents full assembly and activation of the replicase in the cells. The *in vitro* assays contained the peptides, DI-72 (+)RNA, ATP/CTP/GTP and ^{32}P-UTP. (B) Denaturing PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with the tombusvirus replicase based on CFE. The assay contained 0.5 μg/μl (final concentration) of either S1/S2 (lanes 3–4) or RPR (lanes 5–6) peptides. (E) Lack of inhibition of the *in vitro* activity of the FHV replicase by the TBSV peptides in a CFE-based assay. Denaturing PAGE analysis of the ^{32}P-labeled RNA products shows the activity of the FHV replicase in the CFE assay containing 0.5 μg/μl (final concentration) of S1/S2 (lanes 2–3) or 0.5 μg/μl RPR (lanes 4–5) peptides. The positions of RNA1 and the subgenomic RNA3 are marked. Note that the yeast used for CFE preparation expressed the FHV RNA1.

C_{273}F mutation reduced the RdRp activity (lane 7). These data strongly support the model that the S1 and S2 subdomains of p92 also participate in the activation of the p92 RdRp function.

Dominant-negative p33 mutants cannot inhibit the tombusvirus replicase after the assembly step

To study the effect of the dominant-negative mutants on the RNA synthesis steps only (without them affecting the VRC assembly step), we performed *in vitro* tombusvirus replicase assembly by using only two (*i.e.*, rATP, rGTP) of the four ribonucleotides in the CFE assay (Fig. 11A), which facilitate the assembly process, but lead to an arrest of the replication process just after repRNA recruitment and replicase complex assembly (Pogany and Nagy, 2008; Pogany et al., 2008). These CFE-based replication assays consisted of wt p33 and wt p92 proteins (Fig. 11B and C, lanes 1–4) or p33 mutants (Fig. 11B, lanes 5–10) and p92 mutants (Fig. 11C, lanes 5–10). After removal of the supernatant (including all the viral and cellular proteins and repRNA template not bound to the membrane), we added the wt or mutated p33 and p92 to the CFE in addition to the four rNTPs to promote repRNA replication during the second step of the assay (Fig. 11A). We found that the addition of wt p33 at the second step improved the activity of the tombusvirus replicase only marginally from 39% to 47% in case of p33-I_{243}V (lane 5 *versus* 8 in Fig. 11B) and 10–17% in combination with p33-C_{272}T (lane 7 *versus* 10 in Fig. 11B), but did not complement the inactive p33-Q_{245}F mutant (lanes 6 *versus* 9, Fig. 11B). Based on these data, we suggest that the wt p33 cannot complement or only poorly complement the p33 mutants after the assembly of the tombusvirus replicase *in vitro*.

We also tested the reverse scenario for dominant-negative effect of the p33 mutants. Here, the wt p33 and wt p92 were used during the first step to assemble the tombusvirus replicase, followed by addition of the mutated p33 during the second step. Interestingly, the strong dominant-negative p33-Q_{245}F mutant or the other p33 mutants could not affect the activity of the tombusvirus replicase pre-assembled with wt p33 and wt p92 (compare lanes 1 to 2–4 in Fig. 11B).

We also performed similar experiments with p92 mutants (Fig. 11C). These experiments revealed that the tombusvirus replicase pre-assembled during the first step was not affected by addition of wt or mutated p92 during the second step (compare lanes 5–7 *versus* 8–10 in Fig. 11C). Also, similar to p33, the low activity of replication complexes assembled by mutated p92 mutants could not be rescued by wt p92 when provided after the assembly process.

In addition to the fraction of p33/p92 and repRNA, which were not bound to the membrane, the supernatant has also been removed at the end of the first step to prevent additional replicase assembly during the second step (Fig. 9A). Therefore, it is possible that the added replication proteins during the second step of the assay (Fig. 9A) could not associate with the membrane due to the absence of soluble host factors. This is because for the proper membrane insertion and function, tombusvirus replication proteins depend on host factors (*i.e.*, Hsp70 chaperone and eEF1A) (Li et al., 2009, 2010; Sasvari et al., 2011; Wang et al., 2009a, b), which are provided in the supernatant of the CFE. Therefore, we also performed experiments in which we provided the CFE supernatant fraction together with wt or mutated p33 to the tombusvirus replicase during the second step (Fig. 11D). The mutant p33 proteins, when added to the pre-assembled replicase with wt p33/wt p92 (Fig. 11E lanes 1–3), could not inhibit repRNA replication in case of p33-Q_{245}F and p33-C_{272}T, and only slightly in case of the dominant-negative p33-ΔRPR mutant. Also, the minimal level of repRNA replication supported by the p33 mutants was not complemented using the CFE supernatant in combination with wt p33 (Fig. 11E, lanes 4–6). Altogether, these results suggest that the tombusvirus replicase complexes pre-assembled using mutant replication proteins are permanently deranged beyond the capability of wt proteins to rescue functional replicases. Alternatively, the pre-assembled tombusvirus replicases consist of membrane-bound structures too closed to allow the entry of wt MBP-p33 (75 kDa) or MBP-p92 (134 kDa).

Dominant-negative p33 and p92 mutants do not inhibit the RNA synthesis by the purified tombusvirus replicase or carmovirus RdRp in vitro

To test further if the dominant-negative mutants could inhibit RNA synthesis step, we used purified tombusvirus replicase preparation obtained from yeast (Panaviene et al., 2004, 2005).

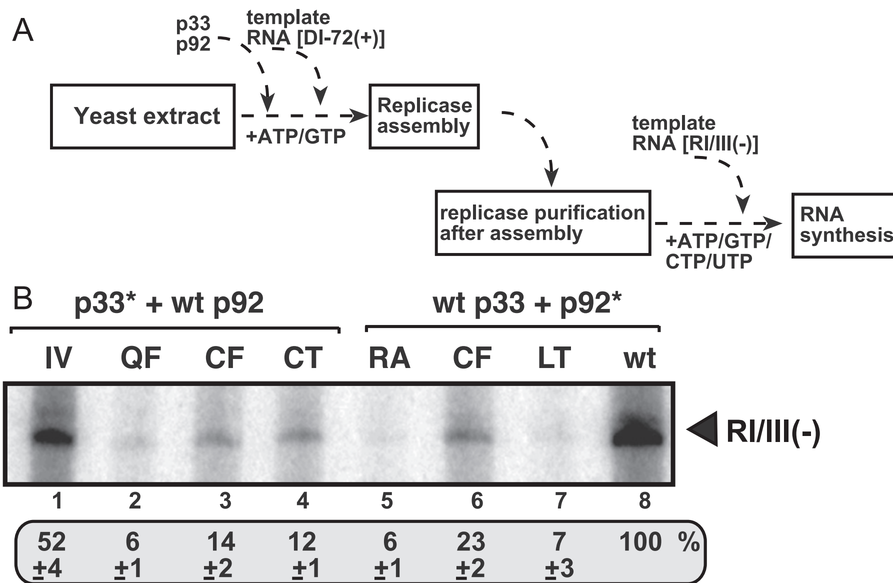

Fig. 9. Mutations within the p33:p33/p92 interaction domain in p33 and p92[pol] replication proteins inhibits the *in vitro* assembly of the CNV replicase complex. (A) A scheme of the CFE-based replicase assembly assay with added purified recombinant p33 and p92[pol] replication proteins and *in vitro* transcribed (+)repRNA. The whole cell extract (CFE) was prepared from yeast. After the *in vitro* assembly, which was performed in the presence of ATP and GTP, the membrane-bound viral replication complexes were purified by affinity chromatography and then the replicase activity was tested using RI/RIII(-) template. The *in vitro* assays contained ATP/CTP/GTP and ^{32}P-UTP. Note that the DI-72(+) repRNA serves only as an assembly factor in this assay since this repRNA is lost during solubilization of the membrane and affinity purification of the replicase (not shown). (B) Denaturing PAGE analysis of the replicase assay with RI/RIII(-) template. The full-length RNA product is depicted with an arrowhead. All the samples contained comparable amounts of wt or mutated p33 replication protein or wt or mutated p92 replication protein. The mutated protein is indicated with an asterisk.

This preparation contains the assembled and activated replicase, which is solubilized and purified that leads to the loss of the endogenous RNA template, and it can be programmed with added TBSV RNA (Panaviene et al., 2004, 2005). We found that the addition of the purified recombinant p33 and p92 mutants did not inhibit the plus-strand synthesis activity of the tombusvirus replicase programmed with RI/RIII(−) RNA template [derived from DI-72 (−)RNA (Panavas et al., 2002)] (Fig. 12A). Similarly, the addition of the purified recombinant p33 and p92 mutants did not inhibit the plus-strand synthesis activity of the purified TCV RdRp programmed with RI/RIII(−) RNA template, which is used by the TCV RdRp efficiently (Rajendran et al., 2002) (Fig. 12B). Based on these *in vitro* data, we conclude that p33 and p92 mutants cannot inhibit plus-strand synthesis.

Dominant-negative p33 mutants show reduced RNA-binding in vitro

Although the dominant-negative p33 mutants tested in this work have mutations within the p33:p33/p92 interaction region (Fig. 1A), it is possible that these mutations might have altered the RNA-binding capacity of the p33 protein as well. This was tested by using EMSA with two TBSV RNA probes. The wt RNA probe contained a short region [RII(+)-SL) involved in selective binding to p33, while the mutated RNA [RII(+)-SL:G/C] carried a debilitating G-to-C mutation, which interferes with binding to p33 (Monkewich et al., 2005; Pogany et al., 2005). We found that, unlike the wt p33 [we used the wt sequence representing the soluble portion of p33, called p33C (Pogany et al., 2005; Rajendran and Nagy, 2003)] (compare lanes 5–8 with 1–4, Fig. 13A) and p33-C_{272}T (Fig. 13B, lanes 7–9) that bound selectively to the wt RNA probe, the p33-Q_{245}F mutant bound to neither RNA probes (Fig. 13A, lanes 9–16). While p33-I_{243}V bound inefficiently to the wt RNA probe (lanes 21–24, Fig. 13A), but not to the mutated probe (lanes 17–20). These findings suggest that the p33:p33/p92 interaction region is involved in binding of p33 to the viral RNA.

Discussion

Replication of (+)-strand RNA viruses is performed by viral replicases assembled from viral and co-opted host-coded components on subverted subcellular membrane surfaces. These viruses only code for a small number of replication proteins, which then perform a surprisingly large number of functions in infected cells (Nagy et al., 2012; Nagy and Pogany, 2012). The tombusvirus p33 replication protein is such factor that provides an increasing number of functions during replication. Accordingly, p33 is regarded as a master regulator of tombusvirus replication that is involved in selective binding to the tombusvirus (+)RNA, and recruitment of the viral RNA into replication (Monkewich et al., 2005; Pathak et al., 2012; Pogany et al., 2005). p33 is also component of the viral replicase complex and affects VRC assembly and regulates viral RNA synthesis by acting as an RNA chaperone (Panaviene et al., 2004, 2005; Pogany and Nagy, 2008; Pogany et al., 2008; Stork et al., 2011). p33 also interacts with a large number of host proteins that is needed for co-opting host proteins and altering cellular pathways (Li et al., 2008; Mendu et al., 2010; Nagy, 2008; Nagy and Pogany, 2010, 2012).

To perform so many different functions, p33 contains multiple functional domains (Fig. 1A) (Nagy and Pogany, 2008; Stork et al., 2011). The C-terminal p33:p33/p92 interaction domain, consisting of the S1 and S2 subdomains, is critical for tombusvirus replication, subcellular localization of VRC and the selective binding to the viral (+)RNA (Panavas et al., 2005; Pogany et al., 2005; Rajendran and Nagy, 2006). We find that changing conserved amino acids within the S1 and S2 subdomains has frequently led to substantial effect on p33 functions, resulting in lethal (nonfunctional), ts and dominant-negative features during replication in yeast model host (Table 1). Interestingly, mutations within the S1 subdomain were more frequently detrimental to p33 functions then mutations within the S2 subdomain, albeit both subdomains are critical for tombusvirus replication (Table 1) (Rajendran and Nagy, 2004, 2006). We also show that several

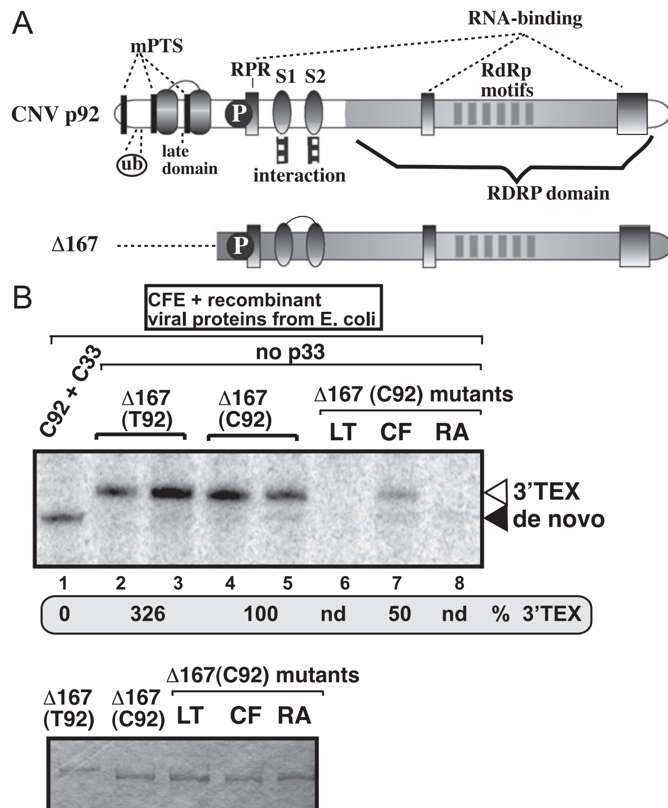

Fig. 10. The p33:p33/p92 interaction domain is required for the RdRp activity of the N-terminally truncated p92^{pol} replication protein in the cell-free yeast extract. (A) Schematic representation of the N-terminally truncated p92^{pol} replication protein, termed Δ167, and the location of known functional domains in the wt p92. The N-terminal segment in the CNV p92^{pol} contains the same sequence as in p33 due to the overlapping expression strategy of the CNV genome. The various domains in the shared sequences are termed as: ub, monoubiquitinated sequence; mPTS, peroxisomal membrane targeting sequences; TMD, trans-membrane domain; late domain, whose sequence is recognized by the host ESCRT factors; *P*, phosphorylation sites; RPR, arginine-proline-rich RNA-binding domain; S1 and S2 are subdomains of the p33:p33/p92 interaction domain. The C-terminal region of p92^{pol} carries the RdRp domain and two RNA-binding sequences. (B) Top image: denaturing PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with recombinant CNV proteins (the wt p33 and p92 are shown here as C33 and C92, while the N-terminally truncated p92^{pol} replication protein is Δ167). The results with the CNV p92-Δ167 mutants with mutations within the p33:p33/p92 interaction domain is shown in lanes 6–8 (1 pmol). The 3′-TEX (terminal extension) products produced by the CNV p92-Δ167 RdRp are pointed at with an open arrowhead, while the *de novo* products (supported in the presence of C33) were depicted with black arrowhead. Each experiment was repeated two or three times. Note that we used the previously characterized TBSV p92-Δ167 RdRp as a control. Bottom image: Coomassie blue staining of SDS-PAGE shows similar amounts of affinity-purified recombinant wt or mutated MBP-Δ167 RdRp proteins used in the above CFE replication assays.

mutations within the S1 and S2 subdomains affected tombusvirus repRNA recombination in yeast, supporting that the interaction domain is involved in regulation of template-switching events, the major pathway for RNA recombination in tombusviruses (Cheng and Nagy, 2003; Jaag et al., 2010; Nagy, 2011).

Many mutants within the S1 and S2 subdomains have been shown to affect p33:p33 and p33:p92 interactions (Rajendran and Nagy, 2004, 2006), suggesting that the detrimental effects of these mutations on tombusvirus replication are due to the reduced level of interaction between p33 and p33 as well as p33 and p92. The critical role for the p33:p33 and p33:p92 interactions are also supported by the inhibitory effect of the S1/S2 peptide on the activity of tombusvirus replicase (Fig. 8). But what is the functional role(s) of the p33:p33 and p33:p92 interactions during replication?

Detailed *in vitro* characterization of selected S1 and S2 subdomain mutants revealed that these subdomains are directly involved in critical functions, such as: (i) binding to the viral (+)RNA (Fig. 13); (ii) assembly of the functional VRC (Fig. 9); and (iii) activation of the p92 RdRp (Fig. 10), while they are not directly affecting (−) or (+)-strand synthesis, based on experiments using the CFE replication assay (Fig. 11) or data obtained with the purified tombusvirus replicase (Fig. 12). These findings are further supported by replication data from yeast based on ts p33 mutants, which unraveled an early function for the S1 and S2 subdomains in tombusvirus replication. The yeast data did not support a major role for the S1 and S2 subdomains in the late steps of tombusvirus replication, which include (−) or (+)-strand synthesis and the release of new (+)RNA progeny from the site of replication.

An unusual feature of tombusviruses is the overlapping sequence shared by p33 and N-terminal portion of the p92 protein, which includes the S1 and S2 subdomains (Fig. 1A) (White and Nagy, 2004). This overlaps makes it more challenging to dissect the actual functions of S1 and S2 subdomains when present in either p33 or p92 proteins. Mutations in S1 and S2 subdomains in both proteins, however, defined that these regions are critical for the functions of both p33 and p92 proteins (Fig. 5) [see also Ref. (Panavas et al., 2005; Rajendran and Nagy, 2006) for replication data from yeast]. This conclusion is further supported by the identification of dominant-negative mutants within the S1 subdomain of p33 and p92 (Figs. 6 and 7), indicating that improper interactions between the dominant-negative mutant and wt replication proteins could interrupt the normal replication functions of the wt replication proteins. Surprisingly, mutations within the p33:p33/p92 interaction domain also inhibited the *in vitro* activity of a truncated p92 RdRp, suggesting that activation of the viral RdRp likely requires interaction between two or more p92 RdRp proteins.

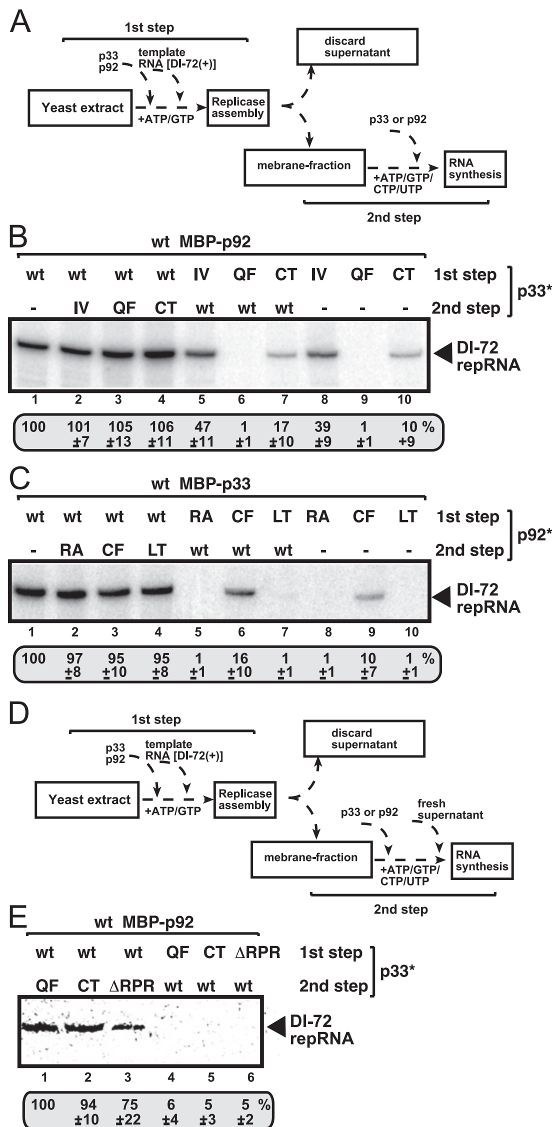

Fig. 11. Lack of complementation or dominant-negative effects of p33 and p92 mutants after the assembly of the tombusvirus VRC based on step-wise cell-free replication assay. (A) Scheme of the CFE-based tombusvirus VRC assembly and replication assays. Purified recombinant wt or mutated p33 (3 pmol, each) and p92^{pol} replication proteins of CNV (1 pmol) and *in vitro* transcribed TBSV DI-72 (+)repRNA were added in step 1 to the CFE. The assay was performed with or without the purified recombinant mutated p33 or p92 during step 1. Note that the assay was performed in the presence of ATP/GTP to facilitate CNV VRC assembly, but inhibit the RNA synthesis in step 1. After finishing step 1, the samples were centrifuged to collect the membrane fraction of the CFE. This was then followed by washing the membranes, and performing step 2 in the presence of ATP/CTP/GTP and ^{32}P-UTP to allow repRNA replication. Note that we also added the purified recombinant mutated p33 or p92 at the beginning of step 2 as shown. (B) Denaturing PAGE analysis of the ^{32}P-labeled repRNA products obtained in the CFE assays in the presence of the shown purified recombinant wt or mutated MBP-p33 and wt MBP-p92. Each experiment was repeated at least three times and the data were used to calculate standard deviation. (C) Denaturing PAGE analysis of the ^{32}P-labeled repRNA products obtained in the CFE assays in the presence of the shown purified recombinant wt or mutated MBP-p92 and wt MBP-p33. See further details in panel B. (D) Modified scheme of the CFE-based tombusvirus VRC assembly and replication assays. Note that these experiments are similar to those described in panel A, except we added fresh supernatant of the yeast CFE with purified recombinant wt or mutated p33 (3 pmol, each) and wt p92^{pol} (1 pmol) replication proteins of CNV at the beginning of step 2 as shown. (E) Denaturing PAGE analysis of the ^{32}P-labeled repRNA products obtained in the CFE assays (see panel D) in the presence of the shown purified recombinant wt or mutated MBP-p33 and wt MBP-p92. Note that the addition of the CFE supernatant (containing soluble host factors) obtained after centrifuging yeast extract at 35,000 g served to help membrane insertion of the added wt p33 (lanes 4–6) or the p33 mutants (lanes 1–3). See further details in panel B.

Overall, the presented data provide direct evidence that the p33:p33/p92 interaction domains in p33 and p92 replication proteins affect several functions that are needed for the early stage of virus replication and also influence viral recombination. It is possible that many p33 and p92 molecules form large complexes via the p33:p33/p92 interaction domains in infected cells that facilitate protein localization, VRC assembly, and the activation of the viral RdRp. The possible role of the S1 and S2 subdomains in binding and interaction with host factors is currently under investigation.

Materials and methods

Yeast, Plasmids, Reagents and Antibodies

Saccharomyces cerevisiae strain BY4741α (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used for this work. Plasmids expressing His_6-tagged p33 (pGBK-His33), p92 (pGAD-His92), p33 point mutants $Y_{244}A$, $R_{246}A$, $F_{274}A$, $Y_{276}A$ as well as pYC-DI-72 expressing transcript RNA were described earlier (Panavas and Nagy, 2003; Panaviene et al., 2004; Rajendran and Nagy, 2004, 2006). Also, pMAL-TCV-88c and pMAL-CNVΔRPR were described earlier (Rajendran and Nagy, 2003; Rajendran et al., 2002).

For obtaining pMAL-CNV-33, the CNV p33 ORF was amplified using primers #25 (GGAGTCTAGAGATACCATCAAGAGGATG)/ #992B (GAGCTGCAGCTATTTCACACCAAGGGA) appended with *Xba*I and *Pst*I restriction enzymes to facilitate directional cloning into pMAL-C2 vector (NEB) digested with the same pair of enzymes. This expression results in a recombinant protein with N-terminal Maltose Binding Protein (MBP) fusion. Same strategy was used to clone the various S1 and S2 mutants. To generate pMAL-CNV-92, the CNV p92 ORF was fused with MBP using the same strategy as above except for changing the reverse primer to #26 (GGAGCTGCAGTCATGCTACGGCGGAGTC). The same set of primers was used to clone the various p92 mutants.

Primers #633 (CGACGGATCCATGTACGCTACCCTACCCAGGGA)/ #992B carrying *BamH*I and *Pst*I sites were used for PCR to clone the C-terminal half of p33 ORF that resulted in pMAL-CNV-33C. The p33C mutants were fused with MBP in gel shift experiments.

To obtain pET-HisMBP-CNV33, we PCR-amplified the MBP-p33 ORF with primers #889 (GGAGCCATGGCTAAAATCGAAGAAGG-TAAAC)/ #1403 (GCCGCTCGAGCTATTTCACACCAAGGGACTCA) using pMAL-CNV-33 template. The PCR product was then cloned into *Nco*I/*Xho*I digested pET vector resulting in a dually His_6-MBP-tagged p33 or p33 mutants. Similar strategy was used to obtain pET-HisMBP-CNV92 and its His_6-MBP-tagged mutants in pET vector except from changing the reverse primer to #952 (CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA).

To generate pMAL-Δ167CNV-92, which expresses the CNV Δ167 RdRp lacking the N-terminal 167 aa, we used primers #4914 (CCAGGGATCCGCCCGAGCACATATGGAGGATG)/ #870 (CCCGTCTA-GATTTGTGTTTGCTTGGTCATGCTACGGCGGAGTC) appended with *BamH*I and *Xba*I for PCR. The obtained PCR product was cloned into pMAL digested with the same pair of enzymes. Same primers were used to clone the S1/S2 mutants of CNV Δ167 RdRp.

Site directed mutagenesis

Plasmids expressing mutant p33 and p92 were created using Quick Change Mutagenesis Kit (Stratagene) with pGBK-His33 or pGAD-His92 as template DNAs (Rajendran and Nagy, 2006). The PCR reactions included the *Pfu* Turbo DNA polymerase, different sets of primer pairs designed for each mutant and 40 ng of DNA as a template. PCR products were digested with *Dpn*I before

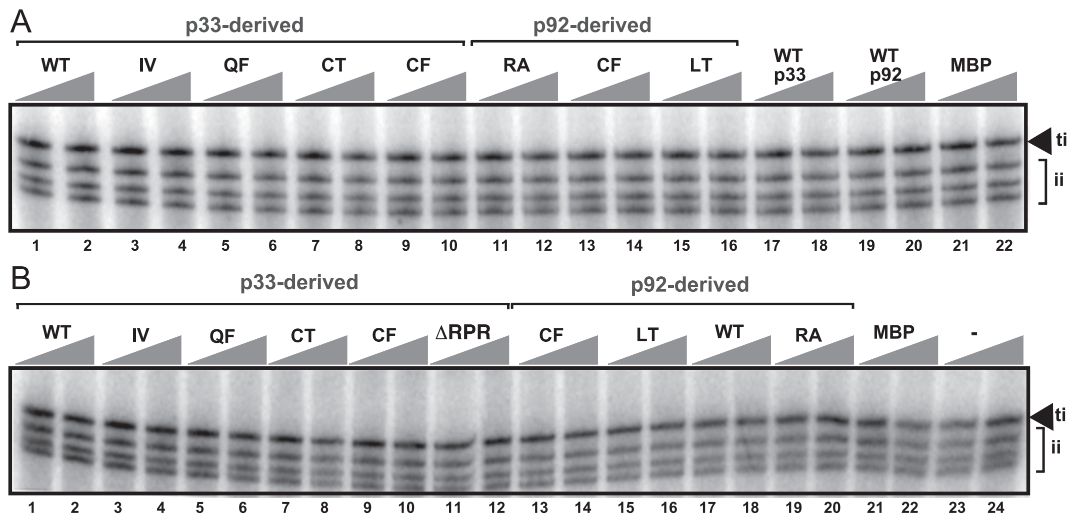

Fig. 12. Mutants within the p33:p33/p92 interaction domain in p33 or p92 does not have dominant-negative effect on the plus-strand synthesis activity of the affinity-purified tombusvirus replicase. (A) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified CNV replicase *in vitro* in the presence of 2 or 4 pmol of purified recombinant p33 and p92 mutants is shown. The level of complementary RNA synthesis producing "repRNA" (marked as "ti", the full-length product, made via *de novo* initiation from the 3′-terminal promoter) in each sample was compared to that of the replicase activity obtained with the recombinant MBP protein (lane 21–22). Note that this replicase preparation also synthesizes internal initiation products ("ii"). The *in vitro* assays contained the DI-72 (-)repRNA, the affinity-purified recombinant p33 or p92 mutants in addition to ATP/CTP/GTP and ^{32}P-UTP. Yeast co-expressing p33 and p92^{pol} replication proteins and DI-72 (+)repRNA was used to affinity-purify the RNA-free CNV replicase. Each experiment was repeated two times. (B) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified TCV RdRp *in vitro* in the presence of 2 or 4 pmol of purified recombinant p33 and p92 mutants is shown. The TCV RdRp protein (named p88C) was purified from *E. coli*.

transformation into *E. coli* (DH5α). The presence of the desired mutations was confirmed by sequencing.

RepRNA accumulation and recombination in yeast

The yeast-based replication and recombination experiments have been performed as described previously (Shapka and Nagy, 2004; Shapka et al., 2005). RNA accumulation was done with Northern blotting and specific probes (Shapka and Nagy, 2004; Shapka et al., 2005).

Protein purification from E. coli

The MBP-tagged CNV p33, CNV MBP-p92, or the p33/p92 mutants and TCV p88C were purified from *E. coli* as described previously (Rajendran and Nagy, 2003; Rajendran et al., 2002). Briefly, expression of the MBP tagged proteins was induced by isopropyl-d-thiogalactopyranoside (IPTG) in Epicurion BL21-codon-plus (DE3)-RIL cells (Stratagene). Cells were suspended in the column buffer (10 mM Tris–HCl [pH 7.4], 1 mM EDTA, 25 mM NaCl, 10 mM β-mercaptoethanol) and were broken by sonication. The cell lysate then was passed through the equilibrated amylose columns to bind MBP tagged proteins. After passing the cell lysate, the columns were washed three times with ice-cold column buffer and eluted with column buffer containing 10 mM maltose and stored at −80 °C until further use.

Cell free extract (CFE)-based in vitro replication assay

The CFE was prepared from untransformed yeast strain BY4741 as described (Pogany and Nagy, 2008; Pogany et al., 2008). Briefly, the CFE (1 μl) was pre-incubated on ice for 10 min in 10 μl cell-free replication buffer containing 0.2 M sorbitol, 50 mM HEPES-KOH, pH 7.4, 5 mM magnesium acetate, 150 mM potassium acetate, and 0.4 μl actinomycin D (5 mg/ml). Then, the reaction volume was adjusted to 20 μl using 1x cell-free replication buffer, also containing 2 μl of 150 mM creatine phosphate; 2 μl of 10 mM ATP, CTP, and GTP and 0.25 mM UTP; 0.3 μl of [^{32}P]UTP, 0.2 μl of 10-mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M dithiothreitol, and 0.5 μg RNA transcript. The reaction mixture also contained 4 pmol MBP-tagged CNV p33 and 1 pmol CNV MBP-p92 purified from *E. coli* cells (Rajendran and Nagy, 2003; Rajendran et al., 2002). The CFE-based replication assay was performed at 25 °C for 3 h. The reactions were terminated by adding 110 μl stop buffer (1% sodium dodecyl sulfate [SDS] and 0.05 M EDTA, pH 8.0), followed by phenol–chloroform extraction, isopropanol–ammonium actetate precipitation, and a washing step with 70% ethanol as described earlier. The RNA samples were electrophoresed under denaturing conditions (5% PAGE containing 8 M urea) and analyzed by phosphoimaging using a Typhoon (GE) instrument as described (Panaviene et al., 2005).

Purification of the recombinant tombusvirus replicase from yeast

Yeast cells (BY4741) transformed with pGBK-His33, pGAD-His92 and pYC-DI72 were pre-grown in SC-ULH$^-$ medium containing 2% glucose for 15 h at 29 °C with shaking at 250 rpm. The affinity-purification of the solubilized tombusvirus replicase was performed using ProBond resin (Invitrogen) as described (Panaviene et al., 2004, 2005). The obtained template-dependent CNV replicase was then used in a standard replicase assay using either DI-72(-) or RI/RIII(-) exogenous templates and [^{32}P]UTP (Panaviene et al., 2004, 2005).

In vitro assembly and purification of CNV replicase complex

The *in vitro* replicase assembly assay was conducted at 20 °C for 1 h as described (Kovalev et al., 2012). The reaction volume was increased to 200 μl, while the final concentration of DTT was reduced from 10 mM to 2.5 mM. In addition, only rATP and rGTP were used, while [^{32}P]UTP was omitted. The recombinant p33 was dually-tagged with both MBP and 6xHis. After incubation, the assay mixture was diluted with 800 μl chilled solubilization buffer and affinity-purification was done exactly as described (Panaviene et al., 2004, 2005).

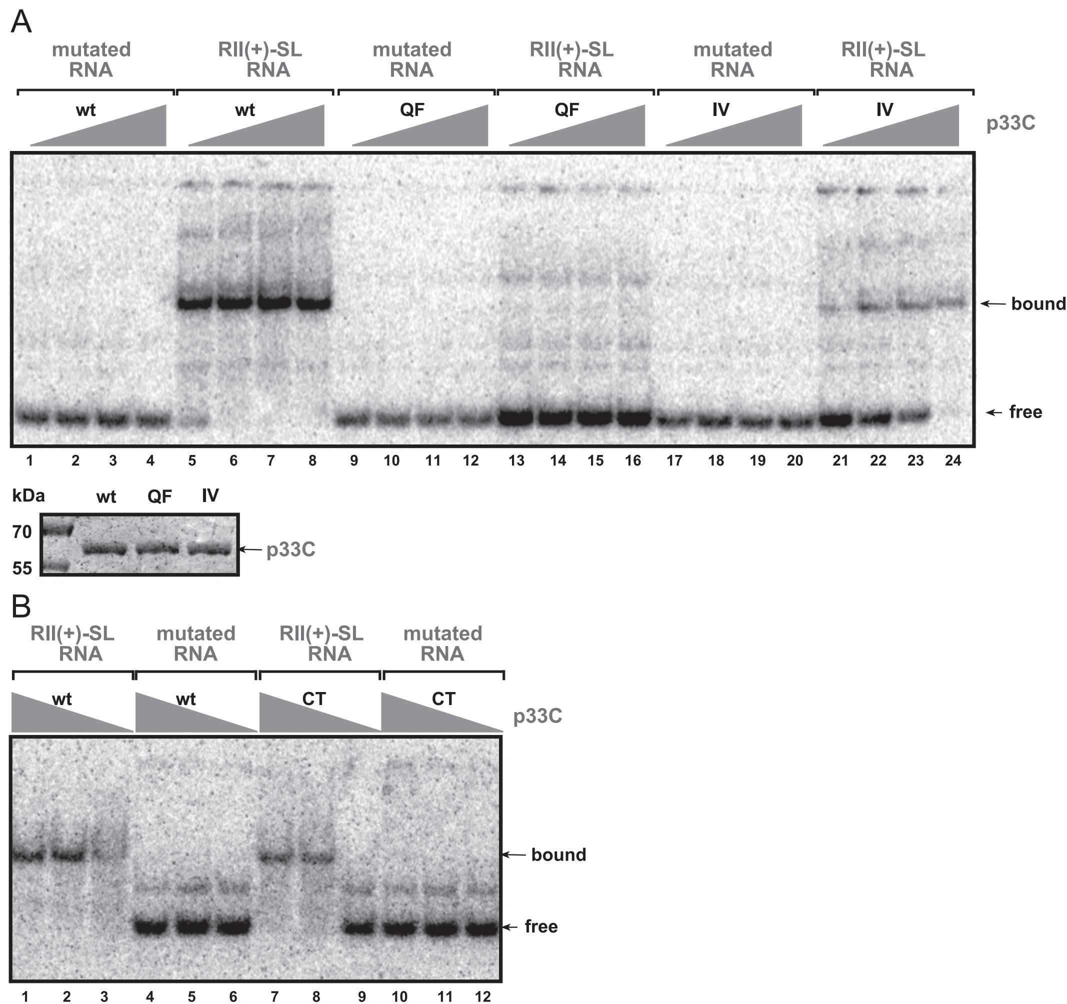

Fig. 13. Mutants within the p33:p33/p92 interaction domain show reduced RNA binding *in vitro*. (A) Top image: EMSA was performed with 2, 4, 8 or 16 pmol of purified MBP-tagged wt or mutant p33C (carrying 150–296 aa). The ^{32}P-labeled RNA probes included 10 nM of wt RII(+)SL RNA (a highly specific RNA binding site for p33C) or 10 nM of a mutated RNA [carrying C→G mutation within the C•C mismatch of RII(+)SL RNA]. Arrows depict the bound and unbound RNA probes. Note that p33-Q_{245}F mutant showed inefficient viral RNA binding. Bottom image: SDS-PAGE followed by Coomassie blue staining of the purified recombinant proteins used in panel A. (B) EMSA assay with purified recombinant p33-C_{272}T mutant, similar to that shown in panel A.

In vitro TCV RdRp assay

We used affinity-purified recombinant TCV p88C (Rajendran et al., 2002). Briefly, the RdRp reaction was performed in 100 μl volume containing RdRp buffer 40 mM Tris pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 0.2 μl RNase inhibitor, 1 mM ATP, CTP, GTP, 0.1 μl radioactive [^{32}P]UTP and 50 μl RdRp preparation. As an external template, 300 ng of DI-72(-) RNA or RI/III(-) RNA were added. Samples were incubated at 25 °C for 2 h. The reaction was terminated by adding 70 μl SDS/EDTA (1% SDS, 50 mM EDTA pH 8.0) and 100 μl phenol–chloroform (1:1). The analysis of RdRp products was by nondenaturing PAGE (Rajendran et al., 2002).

EMSA assay

The affinity-purified recombinant proteins (1 μg as the highest amount and twofold dilution series) were incubated with 1 ng of radioactively labeled probe (see above) in a binding buffer (50 mM Tris–HCl [pH 8.2], 10 mM $MgCl_2$, 10 mM dithiothreitol, 10% glycerol, 100 ng of yeast tRNA [Sigma], and 2 U of RNase inhibitor [Ambion]) at 25 °C for 15 min (Pogany et al., 2005; Rajendran and Nagy, 2003). After the binding reaction, the samples were analyzed by 4 or 5% nondenaturing PAGE in Tris-acetate-EDTA buffer at 200 V in a cold room. The gels were dried, exposed, and analyzed in a phosphorimager.

Replication assay based on CFE

The CFE was prepared from BY4741 yeast co-expressing CNV p33 and p92. The CFE-based replication assay was done according to (Pogany and Nagy, 2008; Pogany et al., 2008), except the pre-incubation step was omitted. Instead, the complete reaction was incubated on ice for 5 min before switching the incubation to the reaction temperature, usually room temperature. The reaction buffer contained 50 mM potassium acetate instead of the 150 mM. The S1/S2 peptide (RLIYQRVMIEIMDKDCVRYVDRDVIL-PLAIGCCFVYPDGVEES) and the RPR peptide (TKVIASTGRPRRR-PYAAKIAQ) were synthesized by Peptide2.0. These peptides were dissolved in the RdRp buffer (10 mg/ml final concentration). The peptides were added at the beginning of the replication assays to reach 0.5 μg/μl final concentration.

FHV replication assay

To obtain the FHV replicase preparation based on yeast CFE, BY4741 yeast strain was transformed with plasmid pESC-His-GAL1:FHV RNA1 (Kovalev et al., 2012). The preparation of CFE

was performed as described (Kovalev et al., 2012). The S1/S2 and RPR peptides were applied in the FHV replicase assay as described above.

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