SUPPLEMENT:

Supplementary materials and methods

Yeast strains and expression plasmids. *Saccharomyces cerevisiae* strain BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) was used during this work.

Recombinant protein purification from *E. coli*. Expression and purification of the recombinant MBP-tagged TBSV p33 and p92 replication proteins from *E. coli* were carried out as described earlier (1, 2). Briefly, the expression plasmids were transformed into *E. coli* strain BL21(DE3) CodonPlus. Isopropyl β-D-thiogalactopyranoside (IPTG) was used for inducing protein expression for 8 h at 16°C. Then, the cells were collected by centrifugation at 5,000 rpm for 5 min, followed by re-suspension of the pellet and sonication in a MBP column buffer (30 mM HEPES-KOH pH 7.4, 25 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol). The broken cells were centrifuged at 14,000 rpm for 5 min to remove cell debris, and then the supernatant was incubated with amylose resin (NEB) for 15 min at 4°C with mixing. The resin was washed 2 times with the column buffer and the resin-bound proteins were eluted with column buffer containing 0.18% (W/V) maltose. The eluted proteins were aliquoted and stored at -80°C. Proteins used for the replication assays were at least 95% pure, as determined by SDS-PAGE (not shown).

DNAzyme experiments. To test if the addition of Triton X-100 stimulates RNA annealing between (+) and (-)-strands, we mixed 1 μg of P^{32}-labeled DI-72 (-) repRNA transcript with unlabeled 250 μg of DI-72 (-)repRNA transcript and 500 μg of DI-72 (+)repRNA transcript. This ratio of (-)versus (+)repRNA reflects the approximate 1:2 ratio of (-)RNA to

(+)RNA inside the replicase complex based on our observations (Fig. 1B). Then, we added DNAzyme 10-23 (250 pmol final concentration) in 1x RdRp buffer (final concentration) in the presence of 0, 0.01 %, 0.1 % or 0.5 % of Triton X-100 (final concentrations). The RNA/DNAzyme mixture was incubated for 2 hours at room temperature. The reaction was stopped by addition of 50 mM EDTA and 1% SDS, followed by denaturing PAGE analysis.

Time-course experiments. In order to increase the sensitivity of the measuring ssRNA versus dsRNA amounts in the CFE-based replication assay, the soluble fraction was removed at the end of the assay by centrifugation at 14,000 rpm for 10 min. Membrane fraction (pellet) was re-suspended in buffer A. Half of the sample was left untreated, while the other half of the sample was hybridized with cold R1(-) transcripts in the presence of 0.1% Triton X-100. After addition of 5x volume of 1% SDS and 50 mM EDTA, we performed phenol/chloroform extraction and RNA precipitation, and then the samples were dissolved in 1x RNA loading dye.

Reverse pulse-chase experiments. The CFE-based TBSV replication assay was performed for 2 hours at 25°C in the presence of unlabeled rNTPs (10 mM each of ATP, CTP, GTP, and UTP). Then, the samples were centrifuged for 10 min at 14,000 rpm at room temperature, followed by re-suspension of the pellet (membranous fraction) in buffer A. Then, we added 0.4 µl actinomycin D (5 mg/ml), 2 µl of 150 mM creatine phosphate, 0.2 µl of 10 mg/ml creatine kinase, 0.2 µl of RNase inhibitor, 0.2 µl of 1 M DTT, 2 µl of rNTP mixture (10 mM ATP, CTP, and GTP, 0.25 mM UTP and 0.1 µl of [^{32}P]UTP) to continue the replication assay. The assay was incubated for 10 to 50 min at 25°C. After that 5x volume of 1% SDS and 50 mM EDTA was added, followed by phenol/chloroform extraction and RNA precipitation.

In another set of CFE-based replication assay (Fig. 5) was performed with 10 mM ATP, CTP, and GTP, 0.25 mM UTP. After the reaction mixture was incubated for 2 hours at 25°C, 0.1

µl of [^{32}P]UTP was added to the reaction mixture. After incubation 10 to 50 min at 25°C, 5x volume of 1% SDS and 50 mM EDTA was added with following phenol/chloroform extraction and RNA precipitatation. Half of each sample was loaded onto the gel directly, while another half was annealed with cold R1(-) in the presence of 0.1% Triton X-100. After addition of 5x volume of 1% SDS and 50 mM EDTA, we performed phenol/chloroform extraction and RNA precipitation, and then the samples were dissolved in 1x RNA loading dye.

Figure legends

Figure S1. Two different types of cell-free TBSV replication assays show the accumulation of RNase protected viral dsRNA. (A) Scheme of the first CFE-based TBSV replication assay. The CFE was prepared from BY4741 yeast expressing tombusvirus p33 and p92^{pol} replication proteins (thus representing the pre-assembled but not yet activated/functional membrane-bound viral replicase in yeast). The CFE was then programmed with *in vitro* transcribed TBSV DI-72 (+)repRNA in the presence of ATP, CTP, GTP and ^{32}P-UTP. After repRNA synthesis, ssRNA-specific ribonuclease RNase I and dsRNA-specific V1 nuclease were added to the assay in the presence or absence of Triton X-100 to destroy the unprotected RNAs. (B) PAGE analysis of the ^{32}P-labeled TBSV dsRNA products produced in the first CFE-based TBSV replication assay. The odd numbered lanes represent replicase products, which were not heat-treated (thus both ssRNA and dsRNA products are present), while the even numbered lanes show the heat-treated replicase products (only ssRNA is present). Note that, in the nondenatured samples, the dsRNA product represents the annealed (-)RNA and the (+)RNA, while the ssRNA products represents the newly made (+)RNA products. Each experiment was repeated three times. (C) Scheme of the second CFE-based TBSV replication assay. The CFE was prepared from BY4741 yeast

expressing tombusvirus p33 and $p92^{pol}$ replication proteins and the TBSV DI-72 (+)repRNA (thus representing the fully assembled membrane-bound viral replicase in yeast). The CFE-based assay was then initiated by adding ATP, CTP, GTP and ^{32}P-UTP. After repRNA synthesis, ssRNA-specific ribonuclease RNase I and dsRNA-specific V1 nuclease were added to the assay in the presence or absence of Triton X-100 to destroy the unprotected RNAs. (D) PAGE analysis of the ^{32}P-labeled TBSV dsRNA products produced in the second CFE-based TBSV replication assay. See additional details in panel B.

Figure S2. The effect of detergent-based disruption of membranes on RNase I-sensitivity of repRNA products in the CFE-based TBSV replication assay. (A) Scheme of the CFE-based TBSV replication assay. The CFE was prepared from BY4741 yeast expressing tombusvirus p33 and $p92^{pol}$ replication proteins and the TBSV DI-72 (+)repRNA. See further details in Fig. S1, panel C. (B) Nondenaturing PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays. The concentrations of detergents applied are shown. See further details in Fig. S1, panel B. (C) The presence of detergent does not affect p33 binding to the viral RNA. Gel mobility shift assay with affinity-purified MBP-p33C (the soluble C-terminus of p33) and a portion of the ^{32}P-labeled DI-72(+) RNA, representing the R2(+)-SL. The bound and unbound RNAs are marked. Note the full shift of the RNA in the presence of increasing amounts of Triton X-100. (D) Scheme of the DNAzyme-based cleavage of the ^{32}P-labeled (-)repRNA in the presence of (+)repRNA. Note that the DNAzyme must anneal to the free (-)repRNA to induce cleavage of the target (-)repRNA as shown. (E) Representative denaturing PAGE analysis of the DNAzyme-treated ^{32}P-labeled (-)repRNA. The DNAzyme plus 0 to 0.5% Triton X-100 were mixed with 1 μg of P^{32}-labeled DI-72 (-) repRNA transcript, 250 μg of unlabeled DI-72 (-

)repRNA transcript and 500 μg of unlabeled DI-72 (+)repRNA transcript that represents the 1:2 ratio of (-)versus (+)repRNA inside the replicase complex based on our observations (Fig. 1B). Note that the presence of Triton X-100 did not change the efficiency of DNAzyme cleavage of (-)repRNA *in vitro,* suggesting that the detergent did not promote the annealing between (-) and (+)repRNAs under the test conditions.

Figure S3. Testing the RNase I-sensitivity of repRNA products in the CFE-based TBSV replication assay. (A) Scheme of the CFE-based TBSV replication assay. The CFE was prepared from BY4741 yeast expressing tombusvirus p33 and $p92^{pol}$ replication proteins and the TBSV DI-72 (+)repRNA. See further details in Fig. S1, panel C. (B) PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays. The RNase I treatment was done either before or after phenol/chloroform extraction, as indicated. See further details in Fig. S1, panel B.

Figure S4. Additional evidence for the lack of free TBSV (-)repRNA among the TBSV RNAs produced in the CFE assay. (A) Scheme of the CFE-based TBSV replication assay. In experiment 1, at the end of the replication assay (60 min or 120 min), we added unlabeled R2(+) or R2(-) RNAs to the membrane-fraction of the CFE assay in the presence of 0.1% Triton X-100 prior to phenol/chloroform extraction and RNA analysis. In experiment 2, at the end of the replication assay, we added 0.1% Triton X-100 to the membrane-fraction of the CFE assay, performed phenol/chloroform extraction, heat denatured the RNAs, and then added R2(+) or R2(-) RNAs. Note that experiment 2 tests the ability of R2(+) or R2(-) RNAs to specifically anneal to the target ^{32}P-labeled repRNA in the assay. (B) Top: Scheme of the annealed unlabeled R2(+) or R2(-) RNAs to the ^{32}P-labeled repRNA products. Note that the annealed RNA duplex

changes the migration of the RNA in PAGE. Bottom: Representative PAGE analysis of ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. The positions of shifted repRNAs [due to the annealing to R2(+) or R2(-) RNAs], ss repRNAs and ds repRNAs are shown. Each experiment was repeated three times.

Figure S5. Time-course experiments to detect the appearance of TBSV ss and ds repRNA products generated in the CFE assay. (A) Scheme of the CFE-based TBSV replication assay. First, we performed a cold replication assay to produce unlabeled TBSV dsRNAs. Then, we removed the supernatant by centrifugation to prevent new VRC assembly. This is then followed by the labeling assay for 10-to-50 min. At the end of the time-course replication assay, we added unlabeled R1(-) RNA (in the presence of 0.1% Triton X-100) to the CFE to test the polarity of the ^{32}P-labeled ssRNA products. (B) Representative PAGE analysis of ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. The positions of shifted repRNAs [due to the annealing to R1(-) RNA (in the even numbered rows)], ss repRNAs and ds repRNAs are shown. Note that dsRNA became labeled earlier (10 min) than that of bulk of the (+)ssRNA product (between 30-50 min), suggesting that the dsRNA might serve as a template for new (+)RNA synthesis. These results also make it less likely that the dsRNA is a dead-end product of replication. Asterisk indicates the accumulation of a 5' truncated repRNA (missing R1 and 5' portion of R2) during in vitro replication, which is not shifted in the presence of R1(-) RNA probe. Each experiment was repeated three times.

References:

1. **Rajendran KS, Nagy PD.** 2003. Characterization of the RNA-binding domains in the replicase proteins of tomato bushy stunt virus. J Virol **77:**9244-9258.
2. **Rajendran KS, Pogany J, Nagy PD.** 2002. Comparison of turnip crinkle virus RNA-dependent RNA polymerase preparations expressed in Escherichia coli or derived from infected plants. J Virol **76:**1707-1717.

Fig. S1

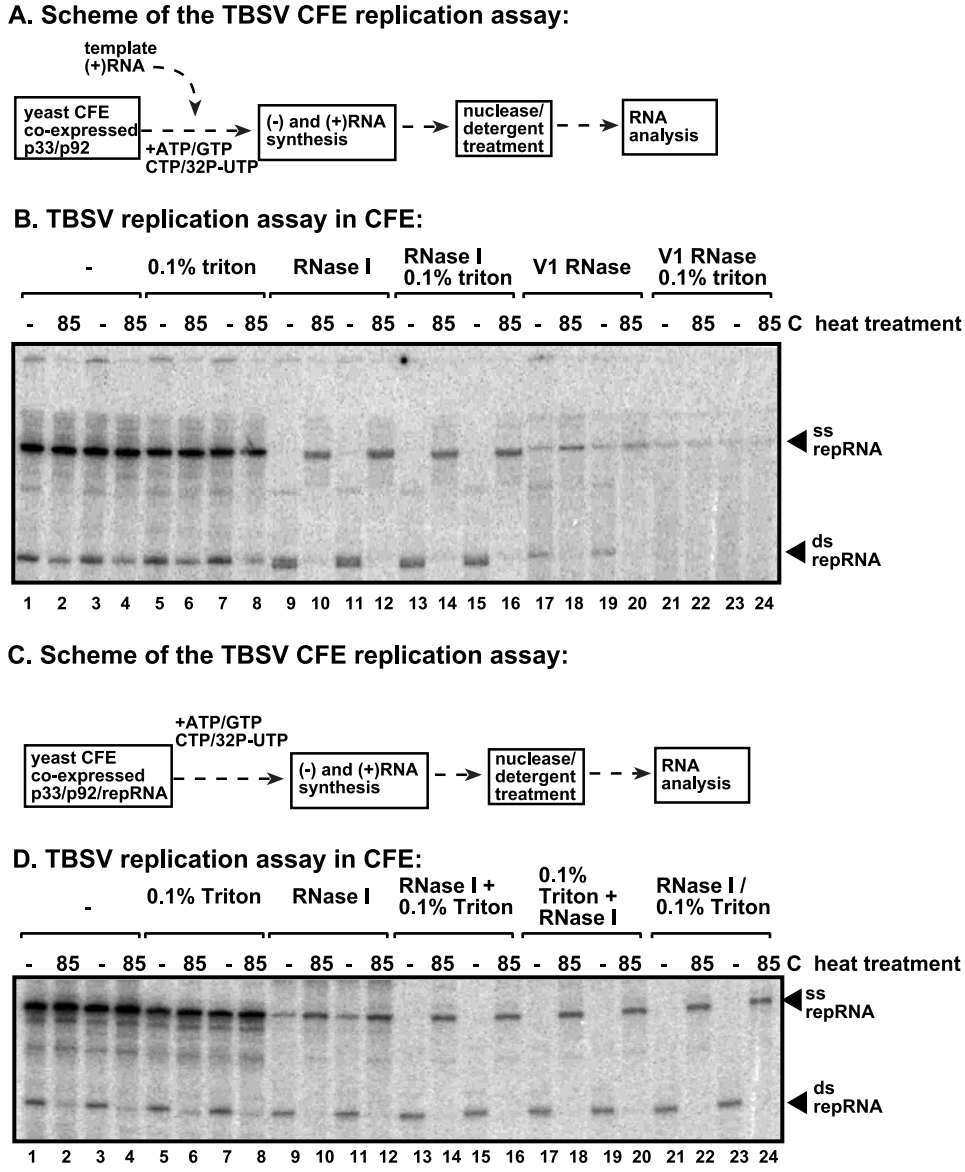

A. Scheme of the TBSV CFE replication assay:
template (+)RNA
yeast CFE co-expressed p33/p92
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
nuclease/ detergent treatment
RNA analysis
B. TBSV replication assay in CFE:
-
0.1% triton
RNase I
RNase I 0.1% triton
V1 RNase
V1 RNase 0.1% triton
- 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 C heat treatment
ss repRNA
ds repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
C. Scheme of the TBSV CFE replication assay:
+ATP/GTP CTP/32P-UTP
yeast CFE co-expressed p33/p92/repRNA
(-) and (+)RNA synthesis
nuclease/ detergent treatment
RNA analysis
D. TBSV replication assay in CFE:
-
0.1% Triton
RNase I
RNase I + 0.1% Triton
0.1% Triton + RNase I
RNase I / 0.1% Triton
- 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 C heat treatment
ss repRNA
ds repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Fig. S2

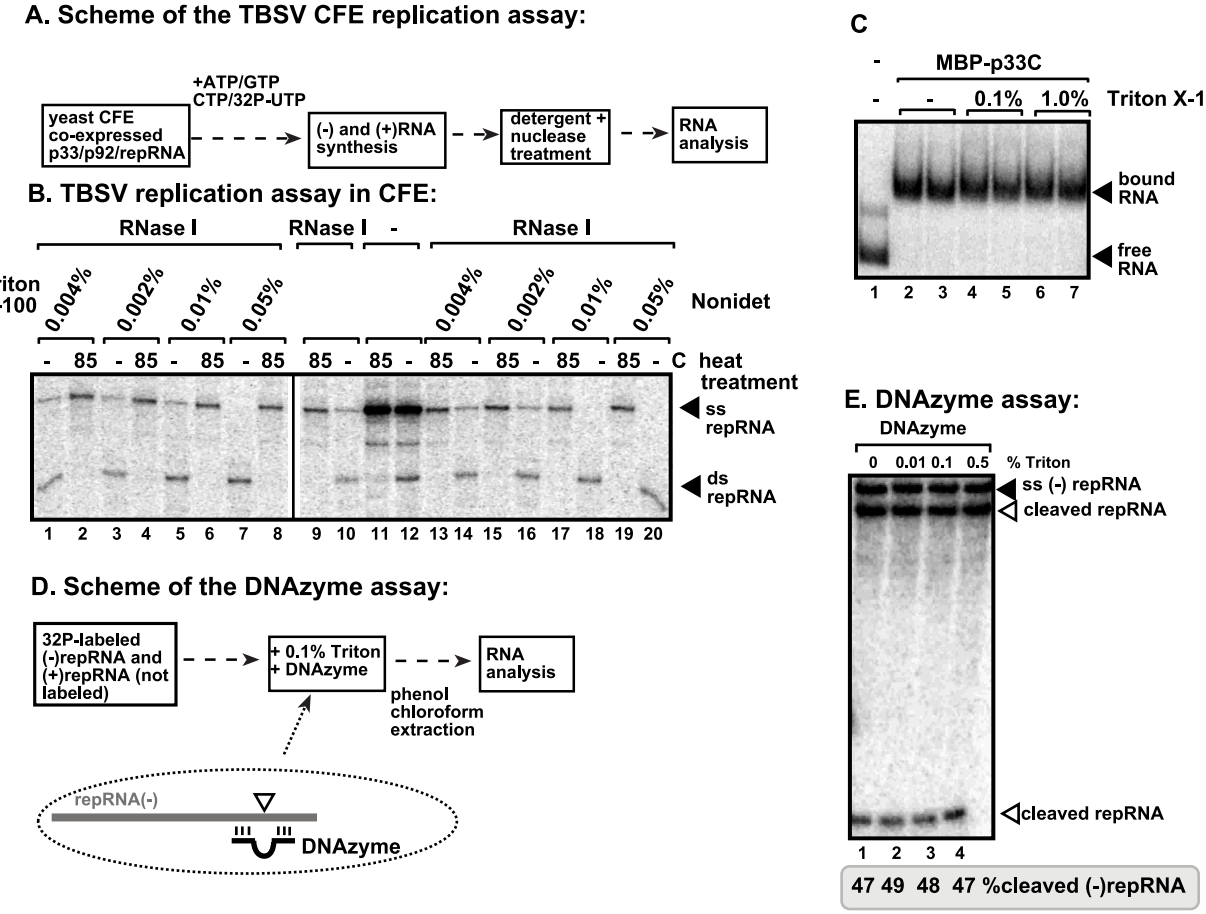

A. Scheme of the TBSV CFE replication assay:
yeast CFE co-expressed p33/p92/repRNA
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
detergent + nuclease treatment
RNA analysis
B. TBSV replication assay in CFE:
RNase I
RNase I
-
RNase I
Triton X-100
0.004%
0.002%
0.01%
0.05%
Nonidet
- 85 - 85 - 85 - 85 85 - 85 - 85 - 85 - 85 - 85 - C
heat treatment
ss repRNA
ds repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
C
- MBP-p33C
- - 0.1% 1.0% Triton X-100
bound RNA
free RNA
1 2 3 4 5 6 7
D. Scheme of the DNAzyme assay:
32P-labeled (-)repRNA and (+)repRNA (not labeled)
+ 0.1% Triton + DNAzyme
RNA analysis
phenol chloroform extraction
repRNA(-)
DNAzyme
E. DNAzyme assay:
DNAzyme
0 0.01 0.1 0.5 % Triton
ss (-) repRNA
cleaved repRNA
cleaved repRNA
1 2 3 4
47 49 48 47 %cleaved (-)repRNA

Fig. S3

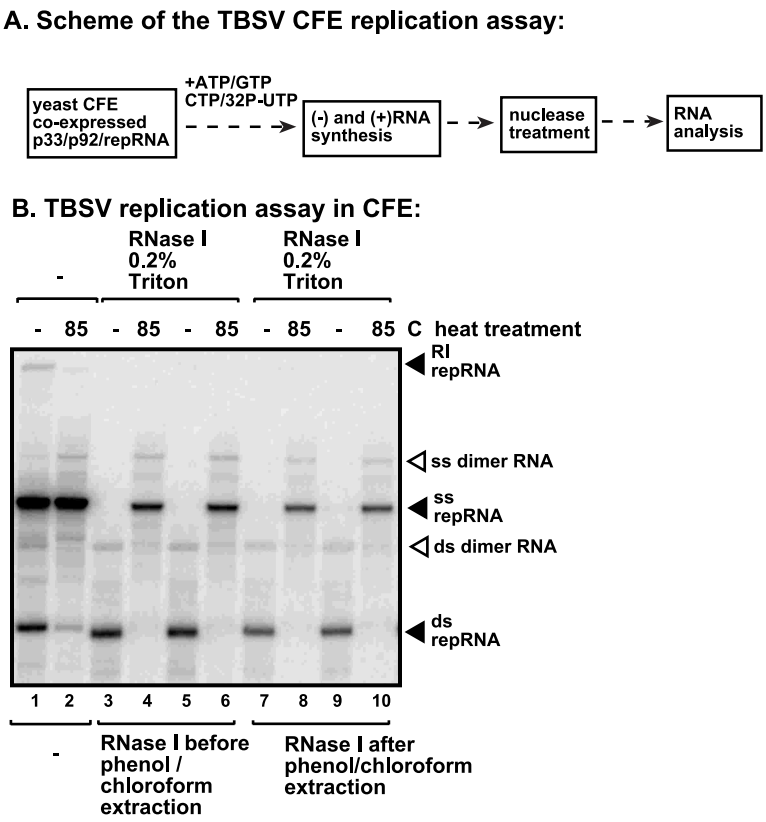
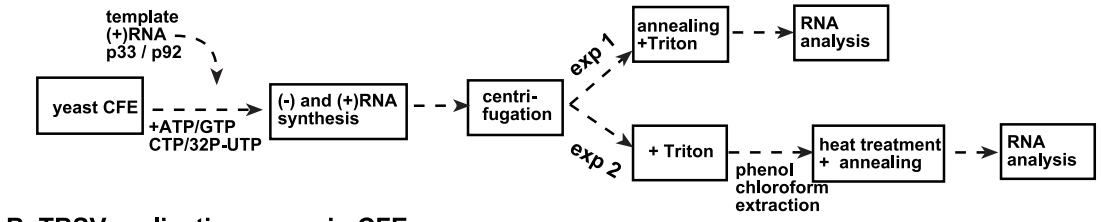

A. Scheme of the TBSV CFE replication assay:
yeast CFE co-expressed p33/p92/repRNA
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
nuclease treatment
RNA analysis
B. TBSV replication assay in CFE:
RNase I 0.2% Triton
RNase I 0.2% Triton
- 85 - 85 - 85 - 85 - 85 C heat treatment
RI repRNA
ss dimer RNA
ss repRNA
ds dimer RNA
ds repRNA
1 2 3 4 5 6 7 8 9 10
-
RNase I before phenol / chloroform extraction
RNase I after phenol/chloroform extraction

Fig. S4

A. Scheme of the TBSV CFE replication assay:



B. TBSV replication assay in CFE:

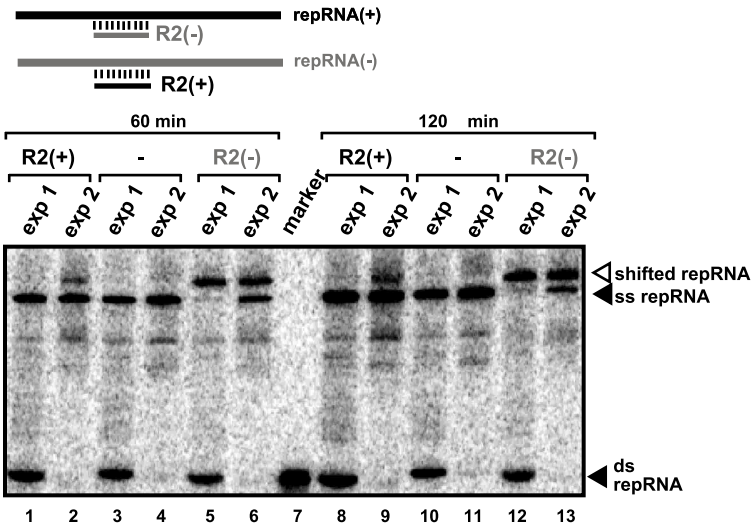
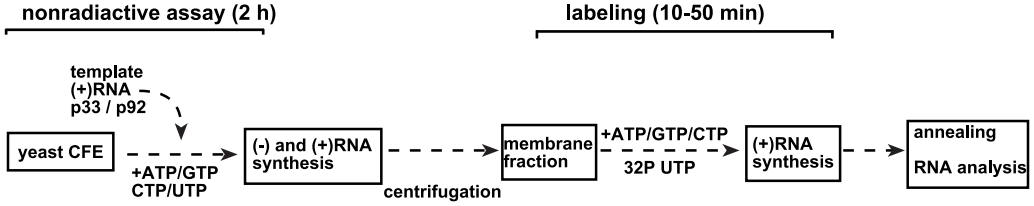
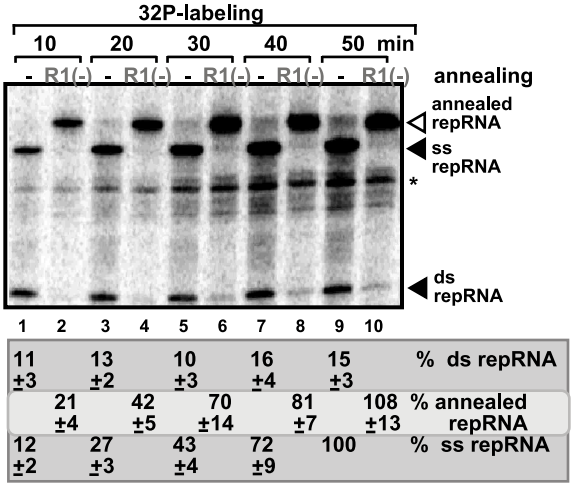


Fig. S5. Kovalev

A. Scheme of the CFE replication assay:



B. Chase experiment:



11 ±3		13 ±2		10 ±3		16 ±4		15 ±3		% ds repRNA
	21 ±4		42 ±5		70 ±14		81 ±7		108 ±13	% annealed repRNA
12 ±2		27 ±3		43 ±4		72 ±9		100		% ss repRNA