Cyclophilin A binds to the viral RNA and replication proteins resulting in inhibition of tombusviral replicase assembly

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Abstract:

Replication of plus-stranded RNA viruses is greatly affected by numerous host-coded proteins acting as restriction factors. Cyclophilins, which are a large family of cellular prolyl isomerases, have previously been found to inhibit *Tomato bushy stunt* tombusvirus (TBSV) replication in a yeast model based on genome-wide screens and global proteomics approaches. In this paper, we further characterized single-domain cyclophilins, including the mammalian Cyclophilin A and plant Roc1 and Roc2, which are orthologs of the yeast Cpr1p cyclophilin, a known inhibitor of TBSV replication in yeast. We found that recombinant CypA, Roc1 and Roc2 strongly inhibited TBSV replication in a cell-free replication assay. Additional *in vitro* studies revealed that CypA, Roc1 and Roc2 cyclophilins bound to the viral replication proteins and CypA and Roc1 to the viral RNA, too. These interactions led to inhibition of viral RNA recruitment, the assembly of the viral replicase complex and viral RNA synthesis. A catalytically inactive mutant of CypA was also able to inhibit TBSV replication *in vitro* due to binding to the replication proteins and the viral RNA. Over-expression of CypA and its mutant in yeast or plant leaves led to inhibition of tombusvirus replication, confirming that CypA is a restriction factor for TBSV. Overall, the current work revealed a regulatory role for the cytosolic single-domain Cpr1-like cyclophilins in RNA virus replication.
INTRODUCTION

Genome-wide screens have led to the identification of many host factors affecting positive-strand (+)RNA virus infections, including Tomato bushy stunt virus (TBSV), West Nile virus, Brome mosaic virus (BMV), Hepatitis C virus (HCV), Dengue virus and Droshophila virus C in yeast or animal cells (1-9). Among the identified host factors, there are numerous inhibitory host proteins serving as restriction factors for (+)RNA viruses. However, the functions of the majority of these host proteins during (+)RNA virus replication have not been fully revealed. The restriction factors likely interfere with the viral replication process, which takes place in membrane-bound viral replicase complexes (VRCs) in the cytoplasm of infected cells. The restriction factors could potentially target the viral replication proteins, the viral RNA or host-coded proteins usurped by (+)RNA viruses to aid the replication process (10-19).

Recently, a major effort to dissect host restriction factors has been done with TBSV, which is a small (+)RNA virus used as a model virus to study virus replication, recombination, and virus-host interactions based on yeast (Saccharomyces cerevisiae) model host (20-25). Genome-wide screens of yeast genes and global proteomics approaches have led to the identification of over 500 host genes/proteins that affected TBSV replication, recombination or interacted with the viral replication proteins or viral RNA (1, 3, 24-32). Interestingly, ~25% of the identified factors seem to inhibit TBSV replication or recombination, suggesting that many host proteins might work as intrinsic restriction factors.

The tombusvirus VRC consists of the two viral replication proteins (i.e., p33 and p92 pol) and ~10 host proteins (18, 30, 31, 33). The auxiliary p33 replication protein is an RNA
chaperone involved in recruitment of the TBSV (+)RNA to the site of replication, which is the
cytosolic surface of peroxisomal membranes (34-37). The RdRp protein p92\textsuperscript{pol} binds to the
essential p33 replication protein for assembling the functional VRC (22, 36, 38, 39). The
recruited host proteins include the heat shock protein 70 (Hsp70), eukaryotic elongation factor
1A (eEF1A) and the ESCRT (endosomal sorting complexes required for transport) family of host
proteins, which are involved in the assembly of VRC (10, 19, 33, 40-43). Other subverted host
proteins in the VRC include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eEF1A,
eEF1B\textgreek{Y} and Ded1 DEAD-box helicase, all of which have been shown to affect viral RNA
synthesis (19, 40, 41, 43-48).

Among the cellular restriction factors characterized is Nucleolin (Nsr1p in yeast), an
RNA binding protein, that interferes with the recruitment of the viral RNA into replication (49).
Additional restriction factors are the WW-domain proteins, such as Rsp5p, a Nedd4 family of E3
ubiquitin ligase, which regulate the degradation of p92\textsuperscript{pol} in yeast cells and inhibit the activity of
VRC \textit{in vitro} (50, 51). Pkc1p kinase phosphorylates p33 close to the RNA binding site and
inhibits binding of p33 to the viral RNA, leading to suppression of TBSV replication (28).

An intriguing group of cellular proteins that inhibits TBSV replication in yeast is
cyclophilins, such as the CypA-like single-domain Cpr1p and Cyp40-like multiple-domain
Cpr7p cyclophilins and Ess1p parvulin, which decrease TBSV RNA accumulation in yeast (29,
52). Cyclophilins are a ubiquitous, highly conserved protein family with prolyl isomerase
(PPIase) activity. Cyclophilins and the structurally unrelated FKB proteins (FK506-binding
proteins) and parvulins include 13 and 29 prolyl isomerases in yeast and in plants, respectively
(53, 54). PPIases catalyze \textit{cis-trans} isomerization of the peptidyl-prolyl bonds (i.e., rotation of X-
proline bonds from \textit{trans} to \textit{cis} conformation) that alter the structure, function or localization of
the client proteins (53-55). The isomerization of the peptidyl-prolyl bonds is often needed for protein refolding after trafficking through cellular membranes (55). Cyclophilins are also involved in the assembly of multidomain proteins, muscle differentiation, detoxification of reactive oxygen species, and immune response. Cyclophilins have been implicated in cancer, atherosclerosis, diabetes and neurodegenerative diseases (54, 56, 57). Cyclophilin expression is induced by both biotic and abiotic stresses including salt stress, heat and cold shock, wounding, viral and fungal infections (53, 58).

We have previously shown that the cytosolic Cyp40-like Cpr7p is a strong inhibitor of TBSV replication in yeast and in vitro (52). Cpr7p, through its TPR (tetra-tricopeptide repeats) domain and not the cyclophilin domain, binds to the RNA-binding domain of p33 replication protein and inhibits the p33/p92-driven recruitment of the TBSV RNA for replication and decreases the efficiency of VRC assembly (52). We demonstrated that the TPR domain of Cpr7p cyclophilin has antiviral activity, while its cyclophilin domain is not needed for viral restriction during RNA virus infections.

The best-characterized PPIase in eukaryotes is the cytosolic single-domain cyclophilin A (CypA in mammals) and the homologous Cpr1p in yeast. Unlike the Cyp40-like cyclophilins, CypA and Cpr1p lack the TPR domain. Therefore, in this paper, we have studied the mechanism of inhibition of TBSV replication by these single-domain cyclophilins. We find that CypA and the orthologous Arabidopsis Roc1 and Roc2 cyclophilins inhibit TBSV replication in a cell free assay. In addition, we find that CypA binds to the RNA-binding domain of p33 and, surprisingly, to the viral RNA. The binding of CypA to these viral components results in inhibition of viral (+)RNA recruitment in vitro and block VRC assembly. Over-expression of CypA or its PPIase inactive mutant led to reduced TBSV RNA accumulation in yeast and plant leaves, suggesting
that binding of CypA to the viral components and blocking their functions is the mechanism of inhibition by CypA.

**Materials and Methods**

**Yeast strains and expression plasmids.** *Saccharomyces cerevisiae* strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from Open Biosystems (Huntsville, AL, USA). Recombinant human GST-tagged CypA and H126Q CypA mutant proteins were produced in *E. coli* from pGEX-CypA and pGEX-H126Q CypA plasmids (a generous gift of Dr. Philippe Gallay) (59).

The GST or MBP-tagged recombinant *A. thaliana* Roc1 and Roc2 cyclophilin proteins were produced in *E. coli* from plasmids pGEX-Roc1 or pGEX-Roc2 or pMAL-Roc1 or pMAL-Roc2 (pMalc-2X-based). To make these constructs, either pPR-N-Roc1 or pPR-N-Roc2 plasmids (29) were used as a template in PCR reactions with primers #3576 (CGAAGATCTATGGCGTTCCCTAAGGTATAC) and #3492 (CCGCTCGAGCTAAGAGAGCTGACCACAATC) for Roc1 and #3577 (CGCGGATCCATGGCGAATCCTAAAGTCTTC) and #3578 (CCGCTCGAGTTATGAACTTGGGTTCTTGAG) for Roc2. The obtained PCR products were digested with BglII and XhoI in case of Roc1 or BamHI and XhoI for Roc2, and were inserted between BamHI and XhoI sites in pGEX-His-Re (50) or BamHI and SalI in pMalc-2X.

Yeast over-expression plasmids pYC-His-CypA or pYC-His-Flag-CypA and pYC-His-H126Q (CypAmut) or pYC-His-Flag-H126Q were prepared as follows: plasmids pGEX-CypA or pGEX-H126Q were used as a template in PCR reaction with primers #5031...
(CCAGGGATCCATGGTCAACCCCACCGTGTTC) and #5032 (CCAGCTCGAGTTATTCGAGTTGTCCACAGTCAGCAATGG). The obtained PCR products were digested with BamHI and XhoI and were inserted between BamHI and XhoI sites in pYC-His or pYC-HisFlag. Plasmids pPR-N-CypA and pPR-N-H126Q (CypAmut) were prepared as follows: the above PCR products were digested with BamHI and XhoI and were ligated into pPR-N-Re (50) between BamHI and SalI sites.

The plant over-expression plasmids were constructed as described (60). Briefly, pGD-Roc1, pGD-Roc2, pGD-CypA and pGD-H126Q (CypA/mut) were obtained by digesting the PCR products of Roc1, Roc2, CypA and CypA- H126Q with BamHI and XhoI and were inserted between BamHI and SalI sites into pGD plasmid. Plasmids pGD-p19 and pGD-CNv were described earlier (60).

To study the effect of cyclophilin over-expression on tombusviral RNA replication, we transformed yeast parental strain (BY4741) or double mutant strain ΔCpr1/ts-Ess1 (29) with three plasmids: pYC-His-H126Q, pGBK-Cup-Flag-p33-Gal-DI72 (a gift from Dr. Barajas) and pGAD-Cup-Flag-p92 (50). Transformed yeast were selected on SC-ULH− plates, then pregrown for 12 hours in ULH− media containing 2% glucose at 29°C. After centrifugation at 2,000 rpm for 3 min and washing the pellet with selective media containing 2% galactose, yeast were grown for 24 hours in SC-ULH− media containing 2% galactose at 34°C (in case of ΔCpr1/ts-Ess1 yeast strain) or 23°C (in case of BY4741 strain). Then, yeast were grown for 8 hours in SC-ULH− media containing 2% galactose and 50 μmol CuSO4 at 23°C. Total RNA extraction from yeast cells and Northern blotting and Western blotting were done as previously described (22, 23).
Analysis of protein–protein interactions using the split-ubiquitin assay. The split-ubiquitin assay was based on the Dualmembrane kit3 (Dualsystems). Analysis of p33 interactions with cyclophilins was done as described (46). Briefly, the obtained prey constructs pPR-N-CypA, pPR-N-CypA/mut, pPR-N-Roc1 or pPR-N-Roc2 (29) or pPR-N-RE as a negative control or pPR-N-CPR1 (29) as a positive control, and the bait construct pGAD-BT2-N-His33 (50) were co-transformed into yeast strain NMY51/ADH-His92-Kan. Colonies were selected on TLUHAna (Trp−/Leu−/Ura−/His−/Ade−) synthetic minimal medium plates to test for p33:cyclophilin interactions.

Protein purification from E. coli. Expression and purification of the recombinant MBP-tagged TBSV p33, p33 derivatives and p92 replication proteins and MBP-tagged host proteins (Mal-Roc1 and Mal-Roc2) from E. coli were carried out as described earlier (61). Purification of GST-tagged CypA, H126Q, Roc1 and Roc2 was carried out using glutathione resin as described (52). The eluted recombinant proteins were aliquoted for storage at -80°C. The concentrations of the purified recombinant proteins were measured by Bio-Rad protein assay (62). Protein fractions used for the replication assays were at least 95% pure, as determined by SDS-PAGE.

Cyclophilin proteins purification from yeast. Yeast strain BY4741, transformed with plasmid pYC-His-Flag-CypA or yeast strain expressing GST-CPR1 (a generous gift from Dr. Brenda Andrews) (63) were streaked on SC-U− plates, and then were grown for 24 hours in 50 ml SC-U− media containing 2% galactose at 29°C. After dilution to 250 ml in SC-U− media containing 2% galactose, yeasts were grown for 18 hours at 29°C. 400 µg of pelleted yeast were
taken for affinity-purification using anti-FLAG M2 agarose in the case of HisFlag–CypA or GST-binding resin in the case of GST-CPR1 as described (22, 46, 52).

**In vitro pull-down assay.** MBP-binding columns were used to bind the MBP-p33, MBP-p33 derivatives or MBP-p92 (61). The sonicated extracts from *E. coli* containing the MBP-tagged viral proteins were added to the columns and incubated for 20 min at 4°C with mixing. After binding, the affinity columns were washed three times with cold column buffer prior to loading of purified GST (negative control), GST-tagged CypA, or GST-H126Q (100 µg or 200 µg) followed by incubation for 30 min at 4°C with mixing. The columns were washed three times with cold column buffer, followed by elution of the bound protein complexes with MBP-elution buffer (column buffer containing 0.18% maltose). The presence of GST-tagged proteins in the eluate was analyzed by SDS-PAGE, followed by Coomassie blue staining or Western blotting with an anti-GST antibody. A fraction of MBP-p33 and MBP-p92 proteins isolated with GST-tagged proteins were stored at -80°C and further used in CFE-based TBSV replication assays.

**RNA probes used for RNA:protein interactions.** In vitro T7 transcription reaction was used as described (61) to generate $^{32}$P-labeled or unlabeled full-length DI-72 (+) and (-)RNAs. Purification of transcripts for the CFE-based TBSV replication assays was done as described earlier (61). A UV spectrophotometer (Beckman) was used for the quantification of RNA transcripts.
Gel mobility-shift assay (EMSA). EMSA experiments were performed as described previously (35) with minor modifications. Briefly, the binding assay was done in the presence of 20 mM HEPES (pH 7.4), 50 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, 1 mM EDTA 5% glycerol, 6 U of RNasin and 0.1 µg tRNA in a 10 µl reaction volume. 0.1 pmol of $^{32}$P-labeled RNA probes was used together with 0.4 µg or 0.8 µg of recombinant proteins. The effect of CypA on binding of p33C to the $^{32}$P-labeled RNA (~0.1 pmol) was tested using 0.02 µg to 0.6 µg of recombinant GST-CypA, or GST-H126Q and 0.02 µg of p33C. Mixtures were incubated at room temperature for 15 min and loaded on 5% nondenaturing polyacrylamide gel as described previously (35).

Purification of tombusvirus replicase from yeast and in vitro replicase assay. Purification of the tombusvirus replicase was done as published previously (22, 46). The in vitro RdRp activity assay was performed as published previously (22, 46) by using full length DI-72 (~)RNA template obtained by T7 transcription in vitro. The effect of cyclophilin proteins was measured in the replicase assay with or without pre-incubation of the purified RdRp with GST-CypA, GST-H$_{126}$Q or GST for 15 min at room temperature. RNase I digestion to remove single-stranded $^{32}$P-labeled RNA was performed at 37°C for 30 min in a 1× RNase I buffer containing 0.1 µl of RNase I (Promega).

TBSV in vitro replication assay in cell-free yeast extract. Cell-free extract (CFE) from BY4741 strain able to support in vitro TBSV replication was prepared as published earlier (43). Recombinant cyclophilin proteins were added in different amounts (as indicated in the Figure legends) to the CFE-based replication assay as described (43).
Protein co-purification with the tombusviral replicase. Yeast strain BY4741 (200 mg of pelleted yeast) co-transformed with plasmids pYC-His-CypA (or pYC-His-H126Q), pGBK-Cup-Flag-p33-Gal-DI72 and pGAD-Cup-Flag-p92 were used for FLAG-affinity-purification of the membrane-bound tombusvirus replicase (Flag-p33 and Flag-p92) using anti-FLAG M2 agarose as published previously (33). Flag-p33 was detected with anti-Flag antibody (1/10,000 dilution) and AP-conjugated anti-mouse antibody (1/10,000). His$_6$-CypA or His$_6$-H126Q proteins were detected with anti-His$_6$ antibody from mouse (Amersham; 1/10,000 dilution) and AP-conjugated anti-mouse (1/10,000) followed by NBT-BCIP detection (33).

Northwestern assay. Northwestern was done as described (61) with modifications. Briefly, equal amounts (~1 µg) of recombinant proteins were run in an 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were renatured at room temperature in a renaturation buffer [10 mM Tris-Hcl [pH 7.5], 1 mM EDTA, 50 mM NaCl, 0.1% Triton X-100, and 1× Denhardt’s reagent, 0.1 mg/ml ssDNA and 2 µg/ml yeast RNA (USB)] with three buffer changes for 30 min each. The membranes were probed with $^{32}$P-labeled DI-72 (-)RNA for 2 h, washed three times with the renaturation buffer, air dried, and analyzed with a phosphorimager.

Over-expression of cyclophilins in N. benthamiana. Agrobacterium tumefaciens (strain C58C1) carrying separately pGD-Roc1, pGD-Roc2, pGD-CypA, pGD-H126Q (CypA/mut), pGD-p19 or pGD-CNV were prepared as published earlier (60). Young N. benthamiana leaves were infiltrated with agrobacterium cultures carrying either empty pGD-35S plasmid as a negative control or one of the plasmids pGD-Roc1, pGD-Roc2, pGD-CypA or pGD-H126Q (CypA/mut). The leaves were also agro-infiltrated with pGD-p19 for suppressing gene silencing.
On the next day, the same plants leaves were infiltrated with agrobacterium cultures carrying pGD-CNV to initiate CNV infection. Agrobacterium strains were used at the following OD$_{600}$: 0.25 for pGD-p19 and 0.75 for pGD-cyclophillins (or pGD-35S) and 0.2 for pGD-CNV. Samples for RNA extraction were taken from the agro-infiltrated leaves 72 hours after CNV agroinfiltration. Isolation of RNA and Northern blot analysis was performed using previously published method (60).

**RESULTS**

**Strong inhibition of TBSV replication by CypA and Arabidopsis Roc1 and Roc2 single-domain cyclophilins in a cell-free replication assay.** Although both Cpr1p and Cpr7p are members of the cyclophilin family (54), the mechanism of inhibition of TBSV replication by these two proteins must be different. This is because the inhibitory function of Cpr7p depends on its unique TPR domain, while its PPIase (cyclophilin) domain has a lesser inhibitory effect (52). In fact, the sole TPR domain is more potent inhibitor of TBSV replication than the full-length protein. In contrast, Cpr1p has only a single cyclophilin domain, but lacks the TPR domain (54).

To dissect the mechanism of inhibition of TBSV replication by a cytosolic single-domain cyclophilin, we have tested the yeast Cpr1p and the homologous human CypA protein. Cpr1p has been shown to inhibit TBSV replication in yeast. In addition, Cpr1p interacts with the p33 and p92 replication proteins (29). First, we used the purified recombinant Cpr1p and the human CypA protein to measure inhibition of TBSV replication in a cell-free replication assay. This assay is based on a cell-free extract (CFE) prepared from yeast that can be programmed with purified recombinant p33 and p92 replication proteins and the viral (+)repRNA to support a
single cycle full replication \textit{in vitro} (Fig. 1A) (43). Although, the recombinant yeast Cpr1p obtained from \textit{E. coli} had no inhibitory effect (52), the recombinant CypA showed remarkably strong inhibition of TBSV replication \textit{in vitro} (causing up to 10-fold reduction, Fig. 1B, lanes 7-8) on TBSV replication in the CFE-based assay. Interestingly, the purified recombinant \textit{Arabidopsis} Roc1 (AtCyp18-3) and Roc2 (AtCyp19-3) proteins (Fig. 1C), which are cytosolic cyclophilins and orthologs of Cpr1p (53), also showed up to 3-to-5-fold inhibitory effects on TBSV replication in the CFE assay (Fig. 1B, lanes 1-4 versus 5-6).

To test if minus- or plus-strand synthesis is inhibited by the above cyclophilins (Fig. 1D), we measured the levels of double-stranded (ds)RNA, which correlates with (-)-strand synthesis, and single-stranded (ss)RNA [representing the newly made (+)-strands] (38, 43) in the CFE assay. The recombinant CypA inhibited both dsRNA and ssRNA production to similar extent (Fig. 1E, lane 8 versus 5-6), suggesting that RNA synthesis in general was inhibited by CypA through possible interference with the assembly of the VRC \textit{in vitro}. Interestingly, the inhibition of TBSV RNA synthesis by CypA was as potent as that observed with the Cyp40-like multidomain Cpr7p protein (Fig. 1E, compare lanes 7-8 with 11-12).

Similar picture can be drawn for the single-domain \textit{Arabidopsis} Roc1 and Roc2 cyclophilins, both of which inhibited dsRNA and ssRNA production by ~3-fold (Fig. 1E, lanes 2 and 4 versus 5). Tagging of Roc1 and Roc2 with GST or MBP did not make major difference in their inhibitory potential (Fig. 1E, compare lanes 1-4 versus 15-18). Thus, we conclude that the closely-related single-domain CypA and Roc1 and Roc2 cyclophilins inhibit RNA synthesis by the tombusvirus VRC \textit{in vitro}. 
The PPIase function of CypA is not required for inhibition of TBSV replication in a cell-free replication assay. To test if the PPIase function of CypA is required for its inhibitory function, we used a purified recombinant CypA with a detrimental mutation (mutant H$_{126}$Q) (Fig. 1C-D) (59). Mutant H$_{126}$Q was as effective as the wt CypA in inhibition of TBSV replication in the CFE assay (Fig. 1B, lanes 9-10 versus 7-8). Mutant H$_{126}$Q was also able to reduce the production of dsRNA and ssRNA by up to 5-fold in vitro (Fig. 1E, lanes 9-10 versus 7-8). Based on these data, we suggest that mutant H$_{126}$Q is as potent inhibitor as the wt CypA, indicating that the PPIase activity is not needed for the inhibitory effect of CypA on TBSV replication in vitro.

CypA inhibits VRC assembly in vitro. Since the recombinant CypA was a potent inhibitor of TBSV replication in vitro, we used a two-step replication assay based on yeast CFE (43) to determine what steps of TBSV replication could be inhibited by CypA. In this assay, the first step includes VRC assembly on the endogenous membranes present in the CFE. The assay also contains the viral (+)repRNA, the recombinant p33 and p92 replication proteins obtained from E. coli and rATP/rGTP (Fig. 2A, step 1). Under these conditions, the viral replication proteins recruit the (+)repRNA to the membrane and the VRC becomes partially RNase and protease insensitive. The pre-assembled VRC, however, cannot initiate minus-strand synthesis yet, due to the absence of rCTP/rUTP (43). Then, during the second step, centrifugation and washing the membranes will remove all the proteins and molecules not bound to the membrane. Addition of rATP/rCTP/rGTP/$^{32}$P-labeled rUTP to the membrane fraction of the CFE then initiates asymmetrical (-) and (+)-strand RNA synthesis in vitro.

We found that addition of purified recombinant CypA during the first step (Fig. 2A) inhibited TBSV replication up to ~90% (Fig. 2B, lanes 8-9 versus 5-7), similar to the strong
inhibitory effect of CypA during standard CFE replication assay (Fig. 1A-B). This suggests that
CypA strongly inhibits VRC assembly *in vitro*. Interestingly, the PPIase inactive mutant H126Q
was again as potent an inhibitor as the wt CypA (Fig. 2B, lanes 10-11), confirming that the
PPIase function is not required to block TBSV replication. Importantly, CypA was as strong an
inhibitor as Cpr7p (lanes 12-13), demonstrating that a single-domain cyclophilin can be as
effective inhibitor of TBSV replication as the TPR-domain containing Cpr7p. Roc1 and Roc2
also inhibited TBSV replication when added during the 1st step of the CFE assay (Fig. 2B, lanes
1-4 versus 5-7), further demonstrating that these *Arabidopsis* cyclophilins have similar activity to
CypA to inhibit TBSV replication *in vitro*.

In contrast, addition of CypA, its mutant or Roc1 and Roc2 exclusively during the second
step of the CFE assay inhibited TBSV RNA replication only by 10-to-20% (Fig. 2C). These data
are similar to that obtained with Cpr7p (Fig. 2C, lane 7) (52). We suggest that the lack of
inhibition of viral RNA synthesis by cyclophilins after the VRC assembly step (i.e., during the
second step of the CFE assay, Fig. 2A) is due to the inaccessibility of the membrane-bound pre-
assembled replicase by proteins (possibly due to physical hindrance and/or the membranous
structure of the VRC).

To further test the effect of CypA on VRC assembly, we performed a second CFE assay,
in which the tombusvirus p33 and p92 replication proteins were expressed in yeast prior to CFE
preparation. This approach allows the membrane insertion of p33 and p92 and the formation of
pre-VRCs in intact yeast cells in the absence of the viral RNA (38). Then, CFE is prepared and
programmed with (+)repRNA in the presence or absence of purified recombinant CypA. We
found that CypA or H126Q were able to efficiently inhibit TBSV ssRNA and dsRNA production
in this assay (Fig. 3A, lanes 5 and 7). This suggests that CypA inhibits TBSV replication not
through inhibiting the membrane association of the viral replication proteins or the pre-assembly of the protein components of the VRCs (both of which occurred in live yeast cells before CFE preparation in this assay), but one of the subsequent steps, such as (+)RNA binding, activation of polymerase function of p92 or RNA synthesis.

We also tested if the pre-assembled and active VRCs from yeast could be inhibited by addition of CypA in the CFE assay (Fig. 3B). These membrane-bound VRCs already contain the endogenous viral RNA (38). The added CypA or H126Q were unable to inhibit TBSV replication in this assay (Fig. 3B, lanes 3 and 4). This confirmed that the pre-assembled and active VRCs are not accessible to CypA.

CypA is associated with the viral replicase and binds to the RNA-binding region of the viral replication proteins. The inhibitory effect of a cellular protein on TBSV replication could be due to the effect of the given host protein on cellular factors co-opted for viral replication, on viral factors or due to indirect effects on host metabolism. To identify the target of CypA, first, we tested if CypA is present in the tombusviral VRCs. We isolated the membrane fraction from yeast containing the tombusviral VRCs, followed by affinity purification of the p33 replication protein, the most abundant component of the VRC (33). We found that CypA was co-purified with the tombusvirus replicase from yeast (Fig. 4A-B, lane 1 versus 2). The H126Q mutant was also co-purified (Fig. 4A, lane 3), suggesting that the PPIase activity is not needed for its recruitment.

To test if CypA interacts with p33 replication protein, we performed a MYTH (split-ubiquitin) membrane-based yeast two-hybrid assay. As expected based on prior Cpr1p studies (29), CypA interacted with p33 (Fig. 5A). The interaction of Roc1 and Roc2 with p33 was also
detected in the same assay (Fig. 5A). Interestingly, the interaction between H126Q and p33 was noticeably weaker than the binding of p33 to the wt CypA (Fig. 5A). This was also confirmed in a pull down experiment that indicated ~3 times less efficient binding between H126Q and either p33 or p92 than wt CypA (Fig. 5B, lanes 3 and 6 versus 2 and 5; see also 5C, lanes 5 and 8 versus 4 and 7). Based on these data, we propose that the sequence or the activity of the PPIase domain in CypA is critical for efficient binding to p33 and the over-lapping p92 replication proteins.

To study if the interaction between CypA and p33/p92 is relevant for the inhibitory effect on TBSV replication, we affinity-purified p33/p92:CypA complex, followed by addition of the complex as a sole source of p33 and p92 to the CFE-based TBSV replication assay (Fig. 5D). Interestingly, the p33/p92:CypA complex supported TBSV replication poorly (at 8% level), suggesting that binding of CypA to the replication proteins blocks their replication function. In contrast, H126Q mutant was less effective in this function than the wt CypA (Fig. 5D, lanes 3-4 versus 5). This is likely due to the reduced binding of H126Q mutant to p33/p92 complex in vitro.

To map the CypA binding site in the overlapping TBSV replication proteins, we have used pull-down experiments with immobilized MBP-p33 and its truncation derivatives (Fig. 6A) and E. coli lysate containing GST-tagged CypA. These experiments revealed that CypA binds to the arginine-proline-rich (RPR) motif in p33 (Fig. 6A-B). Indeed, deletion of the RPR motif inhibited p33 binding to CypA (p33ΔRPR, Fig. 6B, lane 2). The RPR-motif represents the RNA-binding site in p33 and p92 replication proteins required for specific viral (+)RNA recruitment and replicase assembly (35, 61, 64).
CypA binds to the viral RNA and inhibits the recruitment of the viral RNA for replication. The observation that H126Q mutant inhibits TBSV replication as efficiently as the wt CypA in the CFE assay (Fig. 1), yet H126Q mutant, unlike the wt CypA, binds inefficiently to the viral replication proteins (Fig. 5, 6B) suggests that there must be additional target for CypA and H126Q that affects the efficiency of TBSV inhibition. Therefore, we tested the binding activity of CypA to the viral RNA using purified recombinant protein (Fig. 7A). The EMSA assay revealed that both CypA and H126Q mutant bound to the viral (+)RNA efficiently, and even more to (-)RNA (Fig. 7A-C). Similarly, we also observed that the Arabidopsis Roc1 can bind to the viral RNA (Fig. 7B, lane 5), albeit less efficiently than CypA. Viral (-)RNA binding by the Arabidopsis Roc2 was undetectable (Fig. 7B, lane 4). In contrast, Cpr7p Cyp40-like cyclophilin did not bind to the viral RNA under the same conditions (Fig. 7B, lanes 11-12). We confirmed the viral RNA binding ability of the E. coli-expressed CypA and H126Q mutant (Fig. 7D, lanes 2-3) and the yeast-expressed CypA (Fig. 7D, lane 1) using Northwestern assay.

Viral RNA binding by CypA suggests that CypA might affect various functions of the viral RNA in cells. Therefore, we tested if CypA could affect the selective binding of p33 to the p33RE (p33 recognition element), which is required for replication, using an EMSA assay. As expected, p33 efficiently binds and shifts the (+)RNA carrying the p33RE (Fig. 8A, lanes 6 and 8) (35). However, addition of increasing amounts of recombinant CypA inhibited the formation of p33:RNA complex in vitro (Fig. 8, lanes 2-3). Interestingly, H126Q mutant also efficiently inhibited the formation of p33:RNA complex in vitro (Fig. 8A, lanes 11-12). These data are consistent with the model that CypA can inhibit the selective recognition of the viral (+)RNA by p33 replication protein, thus likely interfering with the recruitment of the viral (+)RNA for replication.
To test this model directly, we performed *in vitro* viral (+)RNA recruitment assay based on yeast CFE with endogenous membranes (43) (Fig. 8B). We found the purified recombinant CypA and H$_{126}$Q mutant strongly inhibited the recruitment of (+)repRNA by p33 and p92 replication proteins to the cellular membranes (Fig. 8B, lanes 5-6 and 7-8 versus 3-4). These data strongly support that CypA can inhibit (+)repRNA recruitment, which is critical for TBSV replication (35, 39, 65, 66). Moreover, the PPIase activity is not needed for this inhibitory function.

CypA inhibits *de novo* initiation by the tombusvirus replicase *in vitro*. By binding to the viral RNA and to the replication proteins, CypA might also interfere with RNA synthesis. To test this, we performed an *in vitro* assay with affinity-purified tombusvirus replicase on added (−)-strand template in the presence or absence of recombinant CypA (Fig. 9A). The detergent-solubilized and affinity-purified tombusvirus replicase from yeast lacks endogenous RNA, which is removed during purification, and can only synthesize complementary RNA products on added TBSV templates (22, 39, 67). The purified replicase, unlike the membrane-bound replicase in the CFE-based assay, cannot perform a complete cycle of RNA synthesis (22, 39, 67). The purified tombusvirus replicase produces three types of products on the exogenous DI-72 RNA: a full-length *de novo* initiated product (FL), internal initiation products (not shown) and 3′ terminal extension product (3′TEX) (Fig. 9B, lane 1). Interestingly, the addition of CypA strongly inhibited the FL product (up to 5x reduction, Fig. 9B, lanes 3 and 6). The H$_{126}$Q mutant was also a strong inhibitor (Fig. 9B, lanes 4-5), suggesting that CypA inhibits *de novo* initiation, which is required for standard TBSV replication in infected cells.
CypA inhibits tombusvirus replication in yeast. To compare the activity of the yeast Cpr1p with the mammalian CypA, we over-expressed these proteins in wt yeast or in yeast lacking CPR1 and carrying a temperature-sensitive ESS1 parvulin (which complements CPR1 in TBSV replication in yeast) (29). Over-expression of CypA in wt yeast led to ~50% reduction in TBSV repRNA accumulation (Fig. 10A, lanes 1-4). The inhibitory effect of CypA over-expression was even more pronounced in cpr1Δ/tts-ess1 yeast, resulting in 25% TBSV replication only (Fig. 10B, lanes 1-4). Similar to Cpr1p (29), the over-expression of CypA did not affect the level of p33 replication protein in the two yeast strains tested, indicating that CypA is unlikely to destabilize the replication proteins. These data established that, similar to Cpr1p (29), CypA is a potent inhibitor of TBSV replication in yeast.

Interestingly, H126Q mutant was as potent an inhibitor of TBSV replication as the wt CypA, when over-expressed in both yeast strains (Fig. 10A-B, lanes 9-12). Thus, the PPIase activity of CypA is not required for its inhibitory effect on TBSV replication. This finding is in agreement with the CFE-based data.

Inhibition of tombusvirus accumulation in N. benthamiana plants expressing Roc1 and Roc2 cyclophilins. To test if the single-domain cyclophilins can inhibit tombusvirus accumulation in plants, we separately over-expressed Roc1, Roc2 and human CypA in leaves of N. benthamiana. Testing tombusvirus RNA accumulation revealed up to 12-fold reduction in leaves over-expressing either Roc1 or Roc2 cyclophilins (Fig. 11A, lanes 13-20 versus 9-12). Over-expression of Roc1 (Fig. 11B) or Roc2 (not shown) did not have phenotypic effects on the N. benthamiana plants. The plants over-expressing Roc1 were protected from the lethal necrosis caused by tombusvirus infection (Fig. 11B). Similarly, expression of CypA or H126Q mutant also led to close to 10-fold reduction in tombusviral RNA (Fig. 11A), and greatly reduced necrosis.
DISCUSSION

The emerging picture from recent genome-wide screens and global proteomics approaches with tombusviruses that many host proteins act as intrinsic restriction factors by inhibiting virus replication (28, 44, 49, 68). Among the host restriction factors are cyclophilins, which are a large family of peptidyl-prolyl cis-trans isomerases with protein chaperone-like function. Through isomerization of peptidyl-prolyl bonds, cyclophilins play important roles in protein folding, maturation and conformational changes in client proteins and affect protein trafficking (54, 55). All cyclophilins share a common 109 aa long cyclophilin-like domain (CLD) performing the PPIase activity, while additional unique sequences or domains in each member of the family are important for selection of protein substrates and for subcellular compartmentalization.

Previous works have identified 6 yeast PPIases, including four cyclophilins among the 8 S. cerevisiae cyclophilins, which bound to the tombusvirus p33 replication protein (29). Evidence was obtained that the cytosolic Cpr1p and Cpr7p, the mitochondrial Cpr3p as well as the parvulin Ess1p, inhibited TBSV replication in yeast (29, 52). However, the Cyp40-like Cpr7p inhibited TBSV replication via its unique TPR domain, which is absent in Cpr1p and its orthologous mammalian CypA and Arabidopsis Roc1 and Roc2 cyclophilins (52). This suggests the small Cpr1p and the homologous plant and human proteins with a single cyclophilin domain
have to function using different mechanisms than the TPR domain in Cpr7p in restricting TBSV replication. Since the *E. coli* expressed Cpr1p lacked inhibitory activity in the CFE-based TBSV replication assay (52), we decided to dissect the mechanism by using the orthologous mammalian CypA and the *Arabidopsis* Roc1 and Roc2 cyclophilins. Detailed mechanistic studies revealed that CypA inhibits TBSV replication by inhibiting the viral (+)RNA recruitment for replication and by blocking the VRC assembly. CypA also inhibits the RNA synthesis steps if it gets recruited to the VRC during assembly. However, CypA does not seem to be able to enter the already assembled VRC, suggesting that CypA cannot remodel the VRC once the membrane-bound VRC is formed. Altogether, these events lead to reduced RNA synthesis and potent inhibition of TBSV replication.

Comparison of the mechanism of inhibition of TBSV replication by the TPR-containing Cpr7p (52) and the Cpr1-like CypA revealed several similarities as well as differences in their activities. The similarities include the inhibition of early steps of TBSV replication, including RNA recruitment and VRC assembly, efficient binding of both cyclophilins to the RPR sequence of p33/p92 involved in RNA-binding, and the lack of requirement of PPIase activity for the inhibitory function. Also, neither protein can inhibit the function of the pre-assembled tombusvirus VRCs in vitro. The differences in the mechanism between CypA and Cpr7p are also substantial and include: the domains involved in inhibition is the TPR domain in case of Cpr7p and the cyclophilin domain in case of CypA; also CypA binds to the viral RNA, which seems to be important, while Cpr7p-driven inhibition is through binding to p33/p92 replication proteins; CypA becomes recruited to the VRC, while this is not yet shown for Cpr7p. Those mechanistic features of CypA, which are based on RNA binding, are actually similar to the cellular nucleolin,
which is also a restriction factor inhibiting the recruitment of the viral (+)RNA into replication through binding to the viral (+)RNA (49). Thus, it seems that different cellular restriction factors might target similar early steps during tombusvirus replication. These antiviral activities by these restriction factors might be possible since the tombusvirus (+)RNA and replication proteins could be more exposed at the early stage before VRC assembly, than during the latter stages when the membrane-bound VRCs are tightly protected (38, 43).

Based on the in vitro, yeast and plant data with wt and PPIase mutant CypA, we suggest that the RNA binding function of CypA could also be critical for its viral restriction function. This is supported by the following observations: (i) The de novo initiated FL product decreased in the presence of CypA (Fig. 9B); (ii) The H126Q mutant behaved similarly to the wt CypA in inhibiting tombusvirus replication in vitro, in yeast and plant, although the H126Q mutant binds to the viral replication proteins much less efficiently then the wt CypA; (iii) Also, both wt CypA and H126Q mutant inhibit the selective binding of p33 replication protein to the p33RE (Fig. 8). These observations could be explained by the ability of CypA to bind viral RNA that hinders the interaction of the viral RNA with the viral replication proteins. However, the possibility also remains that the affinity of CypA(H126Q) to TBSV replication proteins (Fig. 5) is sufficient to inhibit the RNA-binding activity of p33. Masking the RPR motif of p33 by CypA and even by CypA(H126Q) rather than their viral RNA binding function is still reasonable to explain their inhibitory effects on RNA replication. Although refolding of the replication proteins by the PPIase activity of CypA, resulting in inhibition of their functions, cannot be excluded, the observation that the PPIase inactive H126Q mutant is also an effective inhibitor of TBSV replication makes the importance of PPIase activity more difficult to demonstrate. We suggest that cyclophilins are effective inhibitors of TBSV replication most likely at the beginning of
natural infections, when the amounts of viral proteins and the viral (+)RNA is still low in cells—so the relative amount of cyclophilin is very high, resulting in blocking recruitment of the viral (+)RNA for replication and inhibition of the replicase function.

Although most of the mechanistic studies were performed with the mammalian CypA protein due to its high activity in vitro, we also obtained evidence that the Arabidopsis CypA-like Roc1 and Roc2 cyclophilins also have similar inhibitory function in the CFE assay (Fig. 1). In addition, Roc1 and Roc2 also bind to both the viral replication proteins and the viral RNA (Fig. 7B), suggesting that these Arabidopsis cyclophilins play comparable role to the CypA protein. Arabidopsis has 29 PPIases including 7 cytosolic single-domain (CypA-like) cyclophilins (53), so it is possible that additional plant members of this large family could have restriction function during tombusvirus replication in plants.

Works with many viruses indicate that cellular cyclophilins are either co-opted to facilitate viral infections or used by the host as intrinsic restriction factors (69). For example, similar to our findings with Cpr1p CypA-like proteins and Cyp40 homologs, cyclophilins have been shown to inhibit accumulation of several RNA viruses, including influenza A virus, West Nile virus, alfamodaviruses, HIV-1 (human immunodeficiency virus-1) virions (52, 70, 71). Interestingly, HIV targets CypA via the retroviral Vif protein, which inhibits the incorporation of CypA into the viral particles (72). Overall, cyclophilins are potent inhibitors of several RNA viruses and they might be part of the innate response of the host against some viruses. Since PPIases are conserved and ubiquitous proteins, their roles could be widespread against many viruses.

The picture is more complex, however, since several RNA viruses subvert cyclophilins to facilitate their replication. The list of these viruses includes flaviviruses and coronaviruses (70, 73). HCV usurps the cytosolic CypA to facilitate the assembly of the HCV VRC through
influencing the cleavage of NS5A-NS5B fusion protein and the folding of the NS5A and NS5B RdRp proteins (59, 74-76). CypA was shown to bind to the flavivirus NS5 replication protein and is likely a component of the flaviviral VRC (77).

Overall, cyclophilins seem to play important roles during RNA virus infections. The current work revealed a new role for CypA-like proteins as intrinsic inhibitors of tombusvirus replication. This function for CypA is likely conserved from yeast to plants.

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REFERENCES


Figure legends

Fig. 1. Cell-free TBSV replication assay supports an inhibitory role for CypA and two Arabidopsis single-domain cyclophilin orthologs. (A) Scheme of the CFE-based TBSV replication assay. (B) Denaturing PAGE analysis of the 32 P-labeled TBSV repRNA products obtained in the CFE-based assay programmed with in vitro transcribed TBSV DI-72 (+)repRNA and purified recombinant MBP-p33 and MBP-p92pol replication proteins of TBSV. Purified recombinant GST-tagged CypA, the GST-H126Q PPIase-deficient mutant, GST-Roc1, GST-Roc2 or GST (10 and 20 pmol) were added to CFE prepared from BY4741 yeast strain. Each
experiment was repeated three times. (C) SDS-PAGE analysis of the purified recombinant proteins used in the above CFE-based assay. (D) Schematic representation of domains present in yeast Cpr1p (17 KDa), human CypA (18 KDa) and the yeast Cpr7p Cyp40-like cyclophilins. (E) Recombinant CypA inhibits the production of viral dsRNA and ssRNA in a CFE-based TBSV replication assay. Nondenaturing PAGE analysis of single- and double-stranded RNA products produced in the cell-free TBSV replication assay. The assay contained the purified recombinant GST-CypA, the GST-H126Q mutant, GST-Roc1, GST-Roc2 or GST-Cpr7p (7.5 and 15 pmol). Note that the dsRNA product represents the annealed (-)RNA and the (+)RNA, while the ssRNA products represents the newly made (+)RNA products. Samples were not heat-treated (thus both ssRNA and dsRNA products are present). The % of dsRNA and ssRNA in the samples are shown. Each experiment was repeated three times.

Fig. 2. CypA inhibits the assembly of the TBSV VRC in a step-wise CFE-based TBSV replication assay. (A) Scheme of the CFE-based TBSV replicase assembly and replication assays. Purified recombinant MBP-p33 and MBP-p92 replication proteins of TBSV and in vitro transcribed TBSV DI-72 (+)repRNA were added to CFE prepared from BY4741 yeast strain in step 1. The assay either contained or lacked the purified recombinant GST-CypA or GST (7.5 and 15 pmol) during step 1. Note that the assay was performed in the presence of rATP/rGTP to facilitate TBSV VRC assembly, but not to support RNA synthesis in step 1. Centrifugation was used to collect the membrane fraction of the CFE after step 1 and after washing the membranes, step 2 was performed in the presence of rATP/CTP/rGTP and 32P-rUTP to support TBSV RNA replication. (B) Denaturing PAGE analysis of the 32P-labeled TBSV repRNA products obtained in the CFE-based assays when CypA and other cyclophilins
were added during the 1st step. See further details in Fig. 1. (C) Denaturing PAGE analysis of the $^{32}$P-labeled TBSV repRNA products obtained in the CFE-based assays when CypA and other cyclophilins were added at the 2nd step. Three repeats of each experiment were performed. Note that all the cyclophilins used in panel B-C are GST-tagged at the N-terminus.

**Fig. 3.** Recombinant CypA cannot inhibit the activity of the pre-assembled VRC in a CFE-based TBSV replication assay. (A) Nondenaturing PAGE analysis of single- and double-stranded RNA products produced in the cell-free TBSV replication assay. Note that the wt yeast expressed p33 and p92 replication proteins, allowing the pre-assembly of the VRC in living yeast cells prior to CFE preparation. The CFE was programmed with DI-72(+)repRNA only for the *in vitro* assay. The % of dsRNA and ssRNA in the samples are shown. Each experiment was repeated three times. (B) Denaturing PAGE analysis of repRNA products obtained in the cell-free TBSV replication assay. Note that the wt yeast expressed p33 and p92 replication proteins and the DI-72 repRNA, thus completely assembled VRCs are formed in yeast prior to CFE preparation. Therefore, here, we did not add viral components (p33/p92/repRNA) to the CFE prior to the *in vitro* assay. Note that all the cyclophilins used in panel A-B are GST-tagged at the N-terminus.

**Fig. 4.** Co-purification of CypA with the p33 replication protein from yeast. (A) The membrane-bound VRCs were collected by centrifugation, followed by solubilization and FLAG-affinity purification from yeast co-expressing His$_6$-CypA or His$_6$-H$_{126}$Q. Top two panels: Western blot analysis of co-purified His$_6$-CypA or His$_6$-H$_{126}$Q with the FLAG-tagged p33 from yeast extracts using a FLAG-affinity column with anti-His and anti-FLAG antibodies, respectively. Bottom panels: Western blot of His$_6$-CypA or His$_6$-H$_{126}$Q and the FLAG-tagged p33 in the total yeast.
extract using anti-His and anti-FLAG antibodies, respectively. (B) Negative control showing the lack of nonspecific binding of His<sub>6</sub>-CypA to the FLAG-affinity column. See details in panel A.

**Fig. 5.** Interaction between CypA and p33 replication protein. (A) Split ubiquitin MYTH assay was used to test binding between p33 and the shown full-length cyclophilin proteins. The bait p33 was co-expressed with the prey proteins in yeast. Cpr1 and the empty prey vector (NubG) were used as positive and negative controls, respectively. (B) Affinity binding (pull-down) assay to detect interaction between GST-CypA and the MBP-tagged viral replication proteins. The MBP-p33 and MBP-p92 produced in *E. coli* were immobilized on amylose-affinity columns. Then, GST-CypA expressed in *E. coli* was passed through the amylose-affinity columns with immobilized MBP-tagged proteins. The affinity-bound proteins were eluted with maltose from the columns. The eluted proteins were analyzed by SDS-PAGE and Coomassie-staining. Lane 7 shows a sample where MBP-p92 was better visualized than in lanes 4-6. Panel on the right shows the input purified recombinant proteins based on Coomassie-stained SDS-PAGE. (C) The eluted proteins were also analyzed by Western blotting with anti-GST antibody to confirm the binding of GST-CypA to MBP-p92. Lanes 1-3 show the input purified recombinant proteins. (D) Inhibition of TBSV replication by a preformed GST-CypA:MBP-p33:MBP-p92 complex. As shown schematically on the top, we incubated the GST-CypA or GST-H<sub>126</sub>Q PPIase-deficient mutant (100 and 200 µg) with the immobilized MBP-tagged proteins to form the protein complex on the column. Then, the bound protein complexes were eluted with maltose from the columns. The eluted proteins were used together with (+)repRNA to program the yeast CFE as shown. The image shows denaturing PAGE analysis of RNA products obtained in the cell-free TBSV replication assay.
Fig. 6. Mapping the binding site in TBSV p33 protein to CypA. (A) Schematic representation of viral p33 and its derivatives used in the binding assay (each MBP-tagged at the N-terminus). The various domains include: TMD, transmembrane domain; RPR, arginine-proline-rich RNA binding domain; P, phosphorylated serine and threonine; S1 and S2 subdomains involved in p33:p33/p92 interaction. The results of the in vitro binding experiments are summarized (“+” or “-”, based on two repeats). (B) Pull-down assay to detect interaction between GST-CypA and the MBP-tagged viral p33 protein derivatives. See further details in Fig. 5B.

Fig. 7. CypA binds to the TBSV RNA. (A) In vitro EMSA binding assay with purified CypA and viral RNA template. The assay contained the ^32^P-labeled DI-72 (-)repRNA (~0.1 pmol), plus 0.4 or 0.8 µg of purified recombinant CypA and H_{126}Q mutant, as shown. The free or CypA-bound RNA was separated on nondenaturing 5% acrylamide gels. (B) EMSA binding assay with the GST-tagged Roc1, Roc2, Cpr7p, and Cpr1p, which were expressed in E. coli. The assay contained the ^32^P-labeled DI-72 (-)repRNA (~0.1 pmol), plus 0.4 or 0.8 µg of purified recombinant cyclophilins, as shown. See further details in panel A. (C) Comparison of CypA or H_{126}Q binding to either (+) or (-)-stranded RNA based on EMSA. See further details in panel A. (D) Left image: Northwestern analysis of RNA-binding by the purified recombinant CypA. The GST-tagged CypA, GST-H_{126}Q mutant, and the p33C (the soluble C-terminal RNA-binding region only) replication protein were expressed in E. coli, while the His_{6}-Flag-tagged HF-CypA was expressed in yeast. The probe was the ^32^P-labeled template DI-72 (-)RNA (~50 pmol). Right image: SDS-PAGE analysis of the affinity-purified recombinant proteins. The gel was
stained with Coomassie blue. Each experiment was repeated at least two times. Note that all the cyclophilins used in panel A-D are GST-tagged at the N-terminus.

**Fig. 8.** CypA inhibits the binding of p33 replication protein to the TBSV RNA and (+)RNA recruitment. (A) *In vitro* EMSA binding assay with purified MBP-p33C [an N-terminally truncated version of p33, which shows selective binding to the viral (+)RNA] in the presence of CypA or H₁₂₆Q. The $^{32}$P-labeled RNA template was RII(+)-SL (~0.1 pmol), which is the p33RE [part of RII(+)], and binds selectively to p33. The assay contained 0.02 µg of purified recombinant MBP-p33C, plus 0.02, 0.06, 0.2 or 0.6 µg of purified recombinant GST-CypA or GST- H₁₂₆Q, as shown. The samples in lanes 1 and 13 contained 0.6 µg of purified recombinant GST-CypA or GST- H₁₂₆Q in the absence of p33C. Purified recombinant GST does not affect p33:RNA interaction (not shown). See further details in Fig. 7. (B) The viral (+)RNA recruitment assay was based on CFE. The $^{32}$P-labeled TBSV (+)repRNA template was added together with purified p33/p92 and CypA or H₁₂₆Q to CFE prepared from BY4741 yeast. The membrane-association of $^{32}$P-labeled DI-72 (+)repRNA template is measured using denaturing PAGE gels. Note that $^{32}$P-labeled TBSV (+)repRNA template can inefficiently associate with the membrane even in the absence of p33/p92 (lane 1), likely due to nonspecific binding to an RNA-binding host protein in the membrane. Note that all the cyclophilins used in panel A-B are GST-tagged at the N-terminus.

**Fig. 9.** CypA inhibits *de novo* RNA synthesis by the affinity-purified tombusvirus replicase. (A) Scheme of the replicase preparation and the *in vitro* replication assay. Note that the original viral template RNA in the replicase from yeast is removed during replicase solubilization/purification.
Therefore, an added (-)repRNA is tested for replicase activity. (B) Representative denaturing gel of $^{32}$P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 0.5 µg of purified recombinant GST-CypA or GST- H$_{126}$Q or GST. The in vitro assays were programmed with DI-72 (-)repRNA, and they also contained ATP/CTP/GTP and $^{32}$P-UTP. All the components were added at the same time (lanes 1-4) or the replicase is preincubated with GST-CypA, GST- H$_{126}$Q or GST for ~5 min prior to the assay (lanes 5-8). The level of complementary RNA synthesis producing “FL” (the full-length product, made via de novo initiation from the 3’-terminal promoter) is shown as % of FL product in the control sample. Note that this replicase preparation also synthesizes 3’-terminal extension products (“3’TEX”). Each experiment was repeated three times. Note that all the cyclophilins used in panel B are GST-tagged at the N-terminus.

**Fig. 10.** Over-expression of CypA or H$_{126}$Q inhibits TBSV repRNA accumulation in yeast. (A) The wt or (B) cpr1Δ/ts-ess1 yeast strains were used for these experiments. Top panel: Northern blot analysis of TBSV repRNA accumulation in yeast overproducing the His$_6$-tagged CypA or H$_{126}$Q. the repRNA levels were normalized based on rRNA loading. Middle panel: Northern blot analysis shows the level of ribosomal RNA loading. Bottom panels: Detection of Flag-tagged p33 by Western blotting using anti-Flag antibody. Detection of the overproduced His$_6$-tagged CypA or H$_{126}$Q by Western blotting using anti-His antibody in yeast. The total protein level in each sample was analyzed by SDS-PAGE and Coomassie-blue staining. Note that all the cyclophilins expressed in yeast (panel A-B) are His$_6$-tagged at the N-terminus.
Fig. 11. Over-expression of single-domain cyclophilins inhibits tombusvirus RNA accumulation in *Nicotiana benthamiana*. (A) Top panel: Northern blot analysis of Cucumber necrosis virus (CNV, a very close relative of TBSV) RNA accumulation in plants overproducing *Arabidopsis* Roc1, Roc2, human CypA or H126Q mutant. The repRNA levels were normalized based on rRNA loading. Bottom panel: Ethidium bromide-stained agarose gel shows the level of ribosomal RNA loading. Note that *N. benthamiana* leaves transiently expressed the cyclophilins from the 35S promoter introduced via Agro-infiltration. Samples were taken 3 days after agro-infiltration with CNV. (B) The phenotypes of plants over-expressing Roc1 and the symptoms induced by CNV infection. The picture was taken 10 days after agro-infiltration with CNV. Note that all the cyclophilins expressed in plants (panel A-B) are untagged.
Fig. 2

A. Scheme of the CFE replication assay:

1. assembly step
   - Yeast CFE
   - repRNA+p33+p92

   CypA (panel B)
   - ATP/GTP

2. RNA synthesis step
   - Replication assembly
   - RNase P/CTP/GTP/32P/UTP

   CypA (panel C)

B. CypA - 1st step with CFE:

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repRNA

42 33 90 19 100 93 84 18 9 16 10 51 17 % repRNA

+6 +3 +8 +4 ±7 ±11 ±3 ±2 ±4 ±3 ±7 ±2

C. CypA - 2nd step with CFE:

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repRNA

93 89 100 99 82 79 96 % repRNA

±5 ±8 ±9 ±11 ±12 ±7
Fig. 3

A. CFE with pre-expressed Hist-p33/Hist-p92

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100 88 96 61 17 57 20 % ss RNA
16 16 15 11 3 12 5 % ds RNA

B. CFE with pre-expressed Hist-p33/Hist-p92/DI-72 repRNA

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<td>4</td>
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100 98 97 95 % repRNA

±3 ±1 ±2 ±3 ±1 ±2
Fig. 5

A. SC-TLH AU

Cpr1
CypA
H126Q
Roc1
Roc2

B. pull-down

MBP-p33
MBP-p92
MBP-p92
MBP-p33
MBP-p92
MBP-p92
MBP-p92

C. pull-down / Western

total
MBP-p33
MBP-p92
GST-CypA
GST-CypA
GST-CypA

D. Scheme of the CFE replication assay:

1. binding step

2. CFE assay

<table>
<thead>
<tr>
<th>Immobilized MBP-p33 and MBP-p92</th>
<th>C FE protein complex</th>
<th>C FE protein complex</th>
<th>RNA synthesis</th>
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<tbody>
<tr>
<td>+ ATP/CTP</td>
<td>wash</td>
<td>+ GTP/32P-UTP</td>
<td>+ ATP/CTP</td>
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<table>
<thead>
<tr>
<th>GST-CypA</th>
<th>GST-H126Q</th>
<th>repRNA</th>
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<tbody>
<tr>
<td>39±8</td>
<td>8±3</td>
<td>84±20</td>
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<tr>
<td>56±16</td>
<td>100% repRNA</td>
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Fig. 8

A. MBP-p33C

B. RNA recruitment assay:

% bound

% repRNA
Fig. 9

A. Scheme of the in vitro replicase assay:

In vivo

Replicase assembly

template (-)RNA

+ATP/GTP
CTP/32P-UTP

replicase purification

RNA synthesis
in RfR assay

Recombinant
CypA

yeast
cosexpressing
tombusvirus replicase
repRNA+p33+p92

B. in vitro tombusvirus replicase assay:

<table>
<thead>
<tr>
<th></th>
<th>GST</th>
<th>CypA</th>
<th>H1-HQ</th>
<th>H1-HQ CypA</th>
<th>GST</th>
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<tr>
<td>tombusvirus replicase</td>
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<td>92</td>
<td>21</td>
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<td>±9</td>
<td>±6</td>
<td>±4</td>
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Fig. 10

A. over-expression

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<td>Flag-p33</td>
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<tr>
<td>His6-CypA</td>
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B. over-expression

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<tr>
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<tr>
<td>Flag-p92</td>
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<tr>
<td>His6-CypA</td>
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<tr>
<td>total</td>
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Fig. 11

A. over-expression in *N. benthamiana*

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<tr>
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<th>Roc2</th>
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<td>11±3</td>
<td>100±19</td>
<td>7±2</td>
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</tbody>
</table>

B

-  CNV
-  Roc1
-  CNV
-  Roc1