

The GEF1 Proton-Chloride Exchanger Affects Tombusvirus Replication via Regulation of Copper Metabolism in Yeast

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Replication of plus-strand RNA viruses [(+)RNA viruses] is performed by viral replicases, whose function is affected by many cellular factors in infected cells. In this paper, we demonstrate a surprising role for Gef1p proton-chloride exchanger in replication of *Tomato bushy stunt virus* (TBSV) model (+)RNA virus. A genetic approach revealed that Gef1p, which is the only proton-chloride exchanger in *Saccharomyces cerevisiae*, is required for TBSV replication in the yeast model host. We also show that the *in vitro* activity of the purified tombusvirus replicase from *gef1Δ* yeast was low and that the *in vitro* assembly of the viral replicase in a cell extract was inhibited by the cytosolic fraction obtained from *gef1Δ* yeast. Altogether, our data reveal that Gef1p modulates TBSV replication via regulating Cu^{2+} metabolism in the cell. This conclusion is supported by several lines of evidence, including the direct inhibitory effect of Cu^{2+} ions on the *in vitro* assembly of the viral replicase, on the activity of the viral RNA-dependent RNA polymerase, and an inhibitory effect of deletion of *CCC2* copper pump on TBSV replication in yeast, while altered iron metabolism did not reduce TBSV replication. In addition, applying a chloride channel blocker impeded TBSV replication in *Nicotiana benthamiana* protoplasts or in whole plants. Overall, blocking Gef1p function seems to inhibit TBSV replication through altering Cu^{2+} ion metabolism in the cytosol, which then inhibits the normal functions of the viral replicase.

Recent genome-wide screens have revealed that replication of RNA viruses inside the infected cells depends on hundreds of factors, most of which have not been characterized (1–16). In addition, RNA viruses extensively rewire the cells by changing metabolic, secretory, and trafficking pathways, reorganize host membranes, and alter quality control mechanisms (17, 18).

Accordingly, several RNA viruses interfere with cellular pH-dependent metabolism and alter ion metabolism during the infection cycle (19). To achieve these changes in the host cells, some viruses carry genes that code for ion channel proteins, such as viroporins (20). To facilitate the endosomal uncoating of the viral particles, the M2 protein of influenza virus A increases membrane permeability of H^+ ions (21), and it also equilibrates pH between the lumen of the Golgi apparatus and the cytoplasm (22). A plus-strand RNA virus [(+)RNA virus], hepatitis C virus (HCV) carries a gene that encodes protein p7, which possesses ion channel activity (23) and which might function in virus assembly and release (24). The enterovirus 71 2B protein was shown to induce chloride-selective current and modify membrane permeability in *Xenopus* oocytes (25).

Albeit many other viruses do not carry genes that encode ion channel proteins, it is likely that different steps of viral life cycles might depend on cytoplasmic or organellar pH homeostasis and ion metabolism regulated by host ion channels and active ion transporters. For example, vacuolar ATPase, which is involved in maintaining pH homeostasis of the cell and regulating acidification of endosomes, is required for the replication of Sindbis virus and for the proper folding of Sindbis virus proteins (26). Although influenza virus A carries a gene that encodes its own proton channel protein M2, it still needs the proper function of the host V-ATPase to maintain the low pH in the late endosomes, which triggers fusion between viral and endosomal membranes and the release of viral ribonucleoprotein complexes into the cytosol (27). Thus, the host V-ATPase provides a promising target for antiviral research.

Voltage-gated chloride channels, the ClC family of proteins, have similar functions from *Saccharomyces cerevisiae* to plants and

humans (28). ClC proteins, which are localized either in the cell membrane or in the intracellular membranes, function in the maintenance of cytosolic and organellar pH and in the regulation of membrane potential via generating pH gradient through subcellular membranes. In humans, the ClC family consists of 9 separate genes expressed on the plasma membrane or in intracellular membranes of different organelles (29, 30). The *Arabidopsis thaliana* genome codes for 7 different ClC proteins designated AtClC-a to AtClC-g (At for *Arabidopsis thaliana*), and four of them might be active proton-anion exchangers (31). In contrast, yeast (*Saccharomyces cerevisiae*) has only one ClC protein, called Gef1p (32), that functions as a proton-chloride exchanger (33). The closest human homologue of *GEF1* is the *CLCN2* gene that codes for voltage-gated chloride channel ClC-2. The defective function of ClC proteins may lead to different pathological conditions called channelopathies. For example, the defective gene product of *CLCN2* has a neuropathological effect, and it plays a role in juvenile epilepsy (34). Human ClC-3 protein could also complement the slow growth phenotype of *gef1Δ* mutant yeast, suggesting functional similarities (34). Furthermore, mammalian ClC-4 and ClC-5, which are known chloride-proton antiporters, possess the same key residues as the yeast Gef1p protein. The closest plant homologue of Gef1p is AtClC-d, which is most abundantly expressed in the post-Golgi network, thus showing the same subcellular localization as Gef1p in yeast (35). AtClC-d also colocalizes with a V-type ATPase (36), suggesting a possible role of AtClC-d in organellar acidification. Gef1p protein also plays a major role in metal homeostasis. Its normal function is required for the balance

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of copper and iron transport system in yeast (37, 38). However, not much is known about the functions of plant ClC proteins in metal metabolism. Nonetheless, it was shown that out of the seven known *Arabidopsis* ClC proteins, AtClC-c and AtClC-d were able to rescue the Mn^{2+} -hypersensitive growth defect of *gef1Δ* mutant yeast (39).

Host factors affecting viral replication are intensively studied using *Tomato bushy stunt* tombusvirus (TBSV), which is among the most advanced model RNA viruses (10, 18, 40). The two replication proteins encoded by TBSV genes are the p33 replication cofactor, which is an RNA chaperone, and the p92^{Pol} RNA-dependent RNA polymerase (RdRp) (41, 42). p33 and p92^{Pol} membrane proteins are localized on the cytosolic surface of the peroxisomes or occasionally the endoplasmic reticulum (ER), where the replicase complexes form and the viral RNA replication takes place (43–46).

In this paper, we tested whether Gef1p proton-chloride exchanger is required for the replication of TBSV in yeast and *in planta*. The host proton-chloride exchanger was tested, because a previous work using a compound (guanabenz) known to affect ClC activity in cells (47) inhibited TBSV replication in yeast (Z. Sasvari and P. D. Nagy, unpublished data). Indeed, we found that TBSV replication is strongly inhibited in *gef1Δ* yeast lacking the only known ClC protein in yeast. In addition, pharmacological blocking of the function of ClC proteins in plant cells or in whole plants led to reduced levels of tombusvirus accumulation. Further detailed *in vivo* as well as *in vitro* experiments revealed that the altered intracellular copper ion metabolism in the cell hampered TBSV replication. Thus, this work supports the idea that intracellular environment and ion metabolism have major influence on the replication of a (+)RNA virus.

MATERIALS AND METHODS

Yeast strains and expression plasmids. *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the *ccc2Δ* single-gene deletion strain were obtained from Open Biosystems (Huntsville, AL). The *GEF1* gene in BY4741 strain was replaced with *URA3* by homologous recombination. For this, the *URA3* gene was amplified from pYES NTC2 plasmid using primers 4290 (GAATGTTGATCTTGACATACTATAAAATTATTTGACACTATTAGCTTTTCAATTCAATTCA) and 4291 (ATTAGTGGAACCTTTTGATTATGAAATGGATAACTGACGCATTTACTATAATACAG) by PCR. The correct insertion of *URA3* in strain BY4741 resulting in *gef1Δ* yeast was confirmed by PCR using primers 4541 (AAAGCACCGCCTGTTCTC) and 4695 (TAATACGACTCACTATAGGGATTACCCAGCCTGCTTTTCTGTAA).

Plasmids expressing p33 or p92 TBSV proteins and DI-72 replicon RNA (repRNA) have been prepared before: pHISGBK-CUP1-FLAG-His-p33/GAL1-DI-72 (*HIS3* selection), pGAD-CUP1-p92 (*LEU2* selection) (48), pGAD-His92 (pGAD-ADH1-p92; *LEU2* selection), and pESC-His-p33-DI-72 (pESC-GAL1-p33/GAL1-DI-72; *HIS3* selection) (43, 49). Yeast transformation was done by the standard lithium acetate method (50).

Inhibition of Gef1p activity in *Nicotiana benthamiana* protoplasts and whole plants by SITS. We applied SITS (4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene), an anion-channel inhibitor (51, 52), which was purchased from Sigma and dissolved in water to a final stock concentration of 200 mM. To isolate protoplasts from *N. benthamiana* callus culture, 1 g cellulysin and 0.2 g macerace (Calbiochem) were used as described previously (53, 54). Freshly prepared protoplasts were treated with SITS (final concentration of 4 mM) and then electroporated with TBSV or turnip crinkle virus (TCV) genomic RNA (gRNA) made by T7 transcription (53). Protoplasts were incubated in the dark for 40 h at room

temperature, and then total RNA was extracted and Northern hybridization was carried out using TBSV (+)RNA or TCV (+)RNA-specific probes (53).

Two leaves of *N. benthamiana* were infiltrated with SITS (final concentration of 1 mM or 7 mM), and 2 h later, the sap of SITS-treated leaves was inoculated with TBSV virion preparation as described previously (55). Total RNA was extracted 3 days postinoculation (dpi) from the inoculated leaves and 6 dpi from the systemically infected (upper) leaves. Northern blot analysis and quantification with Typhoon 9400 and ImageQuant (GE Healthcare) were done as described earlier (55).

Affinity purification of TBSV replicase from yeast strains. *S. cerevisiae* BY4741 and *gef1Δ* strains were transformed with pGBK-CUP1-FLAG-His-p33/GAL1-DI-72 together with pGAD-CUP1-p92 and cultured for 72 h at 23°C in galactose-containing media supplemented with 50 μM $CuSO_4$ and lacking the relevant amino acids. The volume of the cultures was gradually increased from 2 ml to a final volume of 100 ml. The membrane-bound tombusvirus replicase was solubilized and FLAG affinity purified via FLAG-tagged p33 viral replication protein using anti-FLAG M2-agarose affinity resin as described previously (56). The purified tombusvirus replicase preparation was programmed with 0.5 μg/μl DI-72 minus-strand RNA [(−)RNA] or with a shorter version of repRNA, (−)RI/III RNA (57). The *in vitro* replicase assay was carried out at 25°C for 3 h in the presence of ³²P-labeled UTP as described in reference 49, except that no dithiothreitol (DTT) was added. Then, the RNA was purified by phenol-chloroform followed by isopropanol-ammonium acetate precipitation, followed by 5% denaturing PAGE as described previously (49).

TCV RdRp assay in the presence of Cu^{2+} and Mg^{2+} ions. The TCV RdRp assay was based on affinity-purified recombinant TCV p88C (an N-terminally truncated version of p88) as described previously (58), except that DTT was omitted. The assay mixture contained 16 pmol repRNA (86-nucleotide [nt]-long 3' noncoding region of TBSV genomic RNA) (59) and 2 pmol of affinity-purified maltose-binding protein (MBP)-tagged p88C RdRp protein expressed in *Escherichia coli*. The RNA template was generated with T7 polymerase reaction using a PCR product produced with primers 1662 (TAATACGACTCACTATAGGACACGGT TGATCTACCCCTTC) and 1190 (GGGCTGCATTCTGCAATG) on a plasmid harboring cDNA of DI-72 repRNA (pHISGBK-CUP1-FLAG-His-p33/GAL1-DI-72). Different concentrations (0.01, 0.1, and 1 mM) of $CuSO_4$ were added to the assay buffer along with 10 mM $MgSO_4$. The RdRp reaction was carried out for 3 h at 25°C, followed by denaturing PAGE as described previously (58).

***In vitro* TBSV replication assay based on CFE.** Yeast cell-free extracts (CFEs) from BY4741 and *gef1Δ* strains were prepared, and the replication assay was carried out as described previously (60), except that no DTT was added. Membrane and soluble fractions of these CFEs were separated by centrifugation at 35,000 × g and then mixed them in various combinations as described in the legend to Fig. 5. The TBSV replication assay was performed for 3 h at 25°C, followed by phenol-chloroform extraction and denaturing PAGE as described earlier (60, 61).

Use of BPS to confer iron deficiency in yeast. Yeast BY4741 and *gef1Δ* strains were transformed with pHISGBK-CUP1-FLAG-HIS-p33/GAL1-DI-72 and pGAD-CUP1-p92. Transformed cells were grown at 23°C in media containing galactose and 50 μM copper sulfate and lacking the appropriate amino acids in the presence or absence of an iron chelator, bathophenanthroline sulfonate (BPS) (Molecular Probes) at a final concentration of 7.5 μM (51). The yeast strains were harvested after 40 h, and TBSV RNA accumulation was measured by Northern blotting (62).

The failure to grow under iron depletion is a well-documented phenotype of the *gef1Δ* yeast strain. Therefore, we confirmed the application of a biochemically active BPS by the method of Braun et al. (51). Briefly, wild-type (WT) and *gef1Δ* yeast strains were grown overnight in minimal selective galactose-containing media in 2-ml precultures at 23°C. The precultures were then used to inoculate fresh 2-ml media containing 0 or 7.5 μM BPS that resulted in cultures of 0.2 final optical density at 600 nm

(OD₆₀₀). Yeast cultures were grown for 48 h at 23°C, and OD₆₀₀ was measured at the following time points: 12, 24, 40, and 48 h.

RESULTS

Deletion of Gef1p proton-chloride exchanger in yeast inhibits TBSV RNA accumulation. To explore the importance of Gef1p in TBSV replication, *gef1Δ* and its isogenic wild-type BY4741 yeast strains were transformed with plasmids expressing p33 and p92^{pol} viral proteins together with DI-72 repRNA (49, 62). Northern blot analysis revealed that the lack of Gef1p hindered TBSV replication by ~75% compared to the WT yeast (Fig. 1A, lanes 1 and 2 versus lanes 3 and 4). Though the accumulation of repRNA is impaired, the expression level of p33/p92^{pol} is unaltered in the *gef1Δ* strain (Fig. 1B). Since Gef1p affects copper homeostasis in yeast (37) and we used CuSO₄ to induce the expression of p33 and p92^{pol} viral proteins in the experiments described above (Fig. 1A and B), we repeated these experiments using 25-fold-less CuSO₄ (Fig. 1C) to reduce the copper-induced stress in yeast. In addition, we also initiated TBSV replication in yeast using galactose-inducible (*GAL1* for p33 expression) and constitutive (*ADH1* for p92 expression) promoters (Fig. 1D). In all these experiments, regardless of the promoters used or the amount of CuSO₄ applied, we observed ~4- to 12-fold-lower levels of TBSV repRNA accumulation in the *gef1Δ* strain. Thus, the above genetic approaches indicate a positive role for Gef1p in TBSV replication in yeast.

Pharmacological inhibition of ClC chloride channel reduces TBSV genomic RNA accumulation in the *Nicotiana benthamiana* host. To test whether blocking of chloride channels has an effect on TBSV genomic RNA (gRNA) accumulation in *planta*, a ClC inhibitor was applied to plant protoplasts and to whole plants. We used SITS (4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene), a known chemical to block the function of ClC-1 chloride channel, a functional homologue of yeast Gef1p protein, in *Nicotiana tabacum* (51, 52). *N. benthamiana* protoplasts were treated with SITS, and then protoplasts were transfected with TBSV gRNA or with the related *Turnip crinkle virus* (TCV) gRNA made by *in vitro* T7 transcription. Northern blot analysis showed that, in the presence of SITS, TBSV gRNA replication was reduced by ~50% (Fig. 2A, lanes 1 and 2 versus lanes 3 and 4) and TCV gRNA replication by ~60% (Fig. 2B, lanes 1 and 2 versus lanes 3 and 4).

Infiltration of leaves of whole plants with SITS also triggered significant drop in TBSV RNA accumulation and delayed symptom development in systemic leaves (Fig. 3A). The results of Northern blot analysis showed that TBSV gRNA accumulation at 3 dpi was reduced to ~15% in the inoculated leaves by treatment with SITS (Fig. 3B, lanes 9 to 16 versus lanes 1 to 8). Analysis of viral RNA accumulation in the systemically infected leaves at 6 dpi revealed that the TBSV gRNA accumulation was dropped to ~30% after SITS treatment (Fig. 3C, lanes 5 to 8 versus lanes 1 to 4). These data suggest that inhibition of chloride channels reduces TBSV accumulation in plant cells and in whole plants.

Purified TBSV replicase from *gef1Δ* yeast has impaired activity *in vitro*. Since p33/p92 levels were similar in WT and *gef1Δ* yeast (Fig. 1B), the reduced accumulation of TBSV repRNA in *gef1Δ* yeast might be due to the presence of less-efficient replicase complexes. To test this possibility, we affinity purified TBSV replicase from *gef1Δ* and WT yeasts. The solubilized and FLAG-affinity-purified tombusvirus replicase can efficiently utilize externally added (–)repRNA to make cRNA products (49, 63). Using either full-length DI-72 (–)repRNA or RI/RIII (–)RNA as the tem-

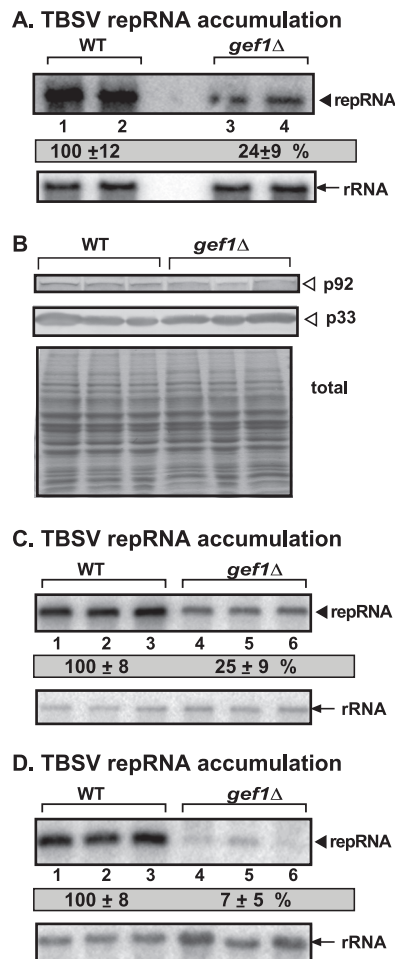
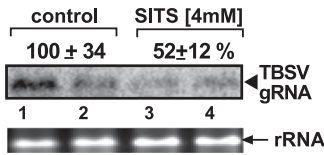


FIG 1 Deletion of the *GEF1* ClC gene inhibits TBSV repRNA accumulation in yeast. (A) Northern blot analysis of TBSV repRNA using a 3'-end-specific probe shows the reduced accumulation of repRNA in *gef1Δ* yeast. The viral proteins His₆-p33 (six-His-tagged p33) and His₆-p92 were expressed from plasmids from the copper-inducible *CUP1* promoter, while DI-72 (+)repRNA was expressed from the galactose-inducible *GAL1* promoter. We started TBSV replication by growing yeast cells in media containing 2% galactose and 50 μM CuSO₄ at 23°C for 48 h. Northern blotting with a 18S rRNA-specific probe was used as a loading control. The mean values of the two lanes are shown below the two lanes in the blot on a gray shaded background. (B) Western blot analysis of the levels of His₆-p33 and His₆-p92 with anti-His antibody in WT and *gef1Δ* yeasts (see panel A). The bottom panel shows a Coomassie brilliant blue-stained SDS-PAGE gel as a loading control. (C) Same as panel A, except the yeast cells were grown in media containing 2% galactose and 2 μM CuSO₄ at 23°C for 40 h. (D) Northern blot analysis of TBSV repRNA in *gef1Δ* yeast. Viral His₆-p92 was expressed from a plasmid using the constitutive *ADH1* promoter, while His₆-p33 and DI-72 (+)repRNA were expressed from the galactose-inducible *GAL1* and *GAL10* promoters. We started TBSV replication by growing yeast cells in media containing 2% galactose at 23°C for 40 h.

plates (57), the *in vitro* activity of the tombusvirus replicase obtained from the *gef1Δ* yeast strain decreased by ~70% compared to the replicase from WT yeast (Fig. 4A, lanes 3 and 4 versus lanes 1 and 2, and Fig. 4B, lanes 3 and 4 versus lanes 1 and 2). These two tombusvirus replicase preparations contained comparable amounts of p33/p92^{pol} (Fig. 4C), excluding the possibility that the observed differences in activity in these preparations are due to differences in replication protein levels. On the basis of these data, we suggest that either the assembly or activity of the tombusvirus replicase is hindered in *gef1Δ* yeast.

A *N. benthamiana* protoplast



B *N. benthamiana* protoplast

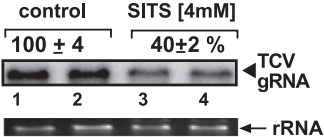
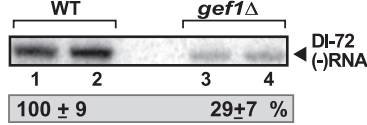


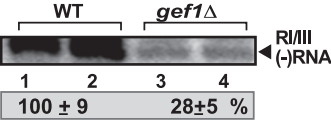
FIG 2 Anion channel blocker SITS inhibits TBSV RNA accumulation in *N. benthamiana* protoplasts. (A) Northern blot analysis of TBSV genomic RNA (gRNA) accumulation in SITS (4 mM)-treated or untreated (control) *N. benthamiana* protoplasts. The rRNA level is shown as the loading control. (B) Same as panel B with the exception that related TCV gRNA was electroporated into *N. benthamiana* protoplasts.

Decreased activity of TBSV replicase assembled in cell extract from *gef1Δ* yeast is determined by cytosolic factors. CIC proteins likely contribute to the ionic strength of the luminal side of the membranous compartments (28), and blocking CIC functions could influence different features of various membranes, such as membrane potential. Since (+)RNA viruses, like TBSV, replicate on intracellular membranes (18, 64, 65), we wanted to explore whether the condition of the intracellular membranes or that of the cytosol impedes tombusviral replication in *gef1Δ* yeast. To address these questions, we took advantage of an *in vitro* tombusvirus replication assay based on cell-free yeast extracts (CFEs) (60). The CFEs can be programmed with TBSV

A. replicase assay



B. replicase assay



C

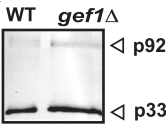
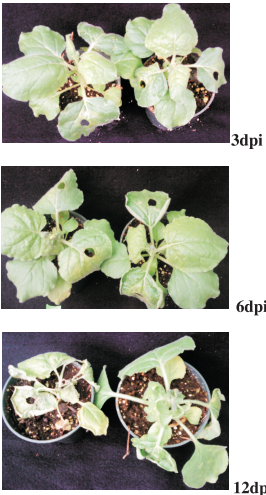


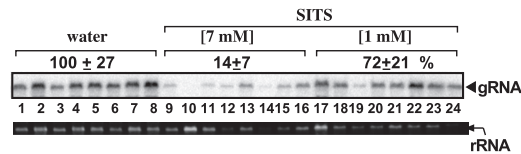
FIG 4 Reduced activity of the affinity-purified TBSV replicase derived from *gef1Δ* yeast. (A) TBSV replicase was FLAG affinity purified via His₆-Flag-tagged p33 viral replication protein from WT and *gef1Δ* yeast strains expressing p92^{pol}. His₆-Flag-tagged p33 expressed from the *CUP1* promoter and DI-72 repRNA from the *GAL1* promoter for 24 h before purification. Then DI-72 (-)RNA was used to program the purified replicase to produce ³²P-labeled cRNA product. *In vitro* replication was conducted for 4 h. The RNA products were analyzed by denaturing PAGE. (B) Same as panel A, except a short template, called RI/III (-)RNA (which contains the cPR promoter for plus-strand synthesis and the RIII replication enhancer region). (C) Western blot analysis to estimate the amount of His₆-Flag-tagged p33 and the copurified His₆-p92^{pol} in the FLAG-affinity purified TBSV replicase using anti-His antibody.

(+)repRNA together with recombinant tombusvirus p33 and p92^{pol} purified from *E. coli*, leading to *in vitro* assembly of active replicase complex, which supports a single full cycle of TBSV replication (60). We found that CFE prepared from *gef1Δ* yeast sup-

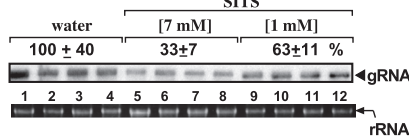
A water +TBSV SITS [7mM] +TBSV



B. inoculated leaves, 3dpi



C. systemic leaves, 6dpi



D water SITS [7mM]



FIG 3 SITS anion channel blocker also inhibits TBSV RNA accumulation in whole *N. benthamiana* plants. (A) Symptom development in *N. benthamiana* plants infected with TBSV. Leaves of *N. benthamiana* plants were infiltrated with 7 mM SITS or water, and then the same leaves were inoculated with the TBSV virion preparation. Pictures were taken at the indicated time points after TBSV inoculation. Note that the application of SITS at 7 mM concentration caused mild curliness of the edge of the infiltrated leaves after 4 or 5 days (middle panel [6dpi]). (B) Northern blot analysis of TBSV gRNA accumulation in inoculated leaves treated with SITS at the indicated concentration at 3 days postinoculation (dpi). The bottom panel shows an ethidium bromide-stained agarose gel showing rRNA levels. (C) Same as panel B with the exception that the samples were taken from systemically infected leaves at 6 dpi. (D) SITS treatment of *N. benthamiana* leaves causes mild curliness of the edge of the infiltrated leaves. The picture was taken 4 days after SITS treatment (applied at 7 mM concentration).

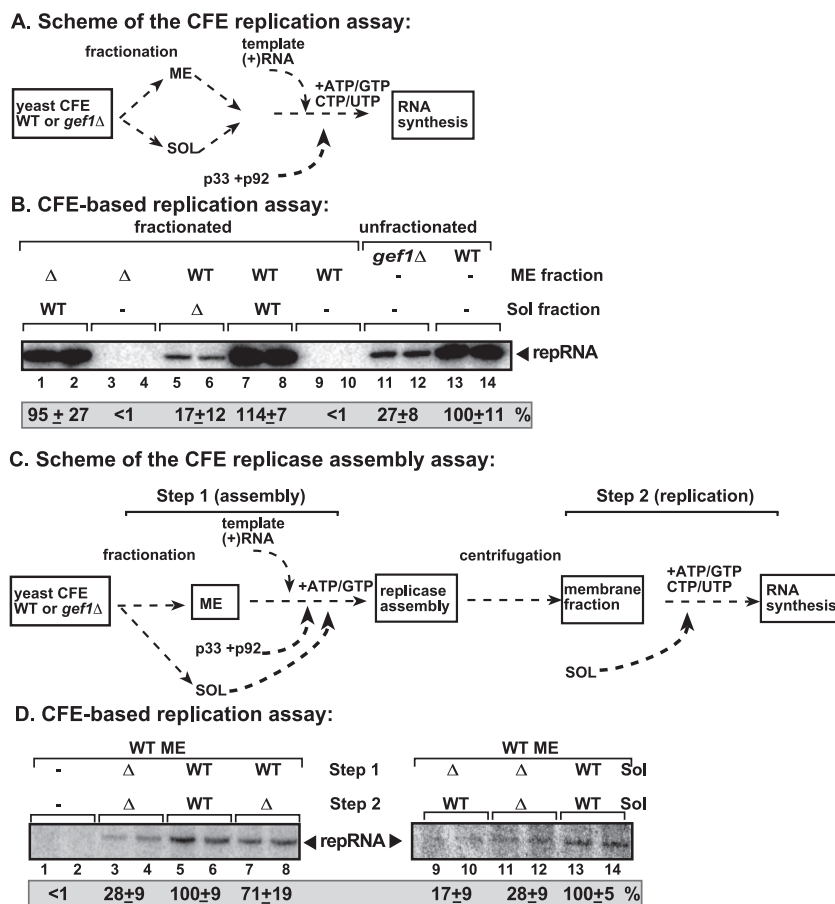


FIG 5 Supernatant fraction of the CFE derived from *gef1Δ* yeast inhibits TBSV repRNA replication *in vitro*. (A) Scheme of the experiments. The supernatant (soluble [SOL] fraction) and the membrane (ME) fraction of CFE were separated by high-speed centrifugation (35,000 × g), and then the various fractions were mixed as indicated. Purified recombinant MBP-p33, MBP-p92^{pol}, and T7 transcripts of TBSV DI-72 (+)repRNA were added to yeast CFE together with isotope-labeled and unlabeled nucleotides to initiate viral replication. (B) The ³²P-labeled repRNA products were analyzed by denaturing PAGE. Note that lanes 11 to 14 show repRNA replication in the original CFEs (i.e., the ME and SOL fractions were not separated). (C) Scheme of the tombusvirus replicase assembly assay. Fractionation was done as in panel A. Step 1 includes VRC assembly, while RNA synthesis by the assembled VRC takes place in step 2 in the presence of ³²P-labeled and unlabeled ribonucleotides. (D) The ³²P-labeled repRNA products were analyzed by denaturing PAGE.

ported TBSV repRNA replication by ~4-fold less efficiently than CFE from WT yeast (Fig. 5B, lanes 11 and 12 versus lanes 13 and 14). This finding suggests that the CFE from *gef1Δ* yeast has some inhibitory molecules to reduce TBSV replication *in vitro*.

To test whether the putative inhibitory factors are present either in the membrane or soluble (cytosolic) fractions of the *gef1Δ* CFE, we separated the membrane and cytosolic fractions of the CFEs with high-speed centrifugation prior to the replication assay (Fig. 5A). As expected, the membrane (ME) fractions (Fig. 5B, lanes 3 and 4 and lanes 9 and 10) or the soluble (SOL) fractions alone (not shown) did not support tombusvirus replication. When we mixed the ME fraction derived from *gef1Δ* yeast with the cytosolic fraction from WT CFE, then TBSV replication was close to 100% (Fig. 5B, lanes 1 and 2). In contrast, mixing the WT ME fraction with the SOL fraction from *gef1Δ* yeast led to ~5-fold reduction in *in vitro* TBSV replication (Fig. 5B, lanes 5 and 6). On the basis of these results, we propose that the soluble fraction of CFE prepared from *gef1Δ* yeast contains factors inhibiting TBSV replication, while the membrane fraction of the CFE from *gef1Δ* yeast seems compatible with TBSV repRNA replication *in vitro*.

To test whether the soluble factors in CFE from *gef1Δ* yeast

inhibit the assembly of the tombusvirus replicase complex (VRC) or its RNA synthesis activity, we used a two-step TBSV replication assay (60, 66). The first step includes the VRC assembly in the ME fraction of the WT CFE in the presence of ATP and GTP and the SOL fraction from either WT or *gef1Δ* yeasts (Fig. 5C). Then, centrifugation of the membrane fraction collects the membrane-bound assembled VRC but removes the SOL fraction and all the viral and host proteins not bound to the membrane. The second step is then performed with the membrane fraction containing the preassembled VRC in the presence of all four ribonucleoside triphosphates (rNTPs) and the SOL fraction from either WT or *gef1Δ* yeasts (Fig. 5C).

Interestingly, the SOL fraction of CFE from *gef1Δ* yeast inhibited *in vitro* TBSV replication the most when added during the first step (Fig. 5D, lanes 9 to 12). In contrast, the inhibition by the SOL fraction of CFE from *gef1Δ* yeast was only moderate (~30%) when added during the second step of the replication assay (Fig. 5D, lanes 7 and 8). On the basis of these data, we propose that the SOL fraction of CFE from *gef1Δ* yeast contains factors that greatly inhibit the VRC assembly step and also affect the RNA synthesis step to some extent.

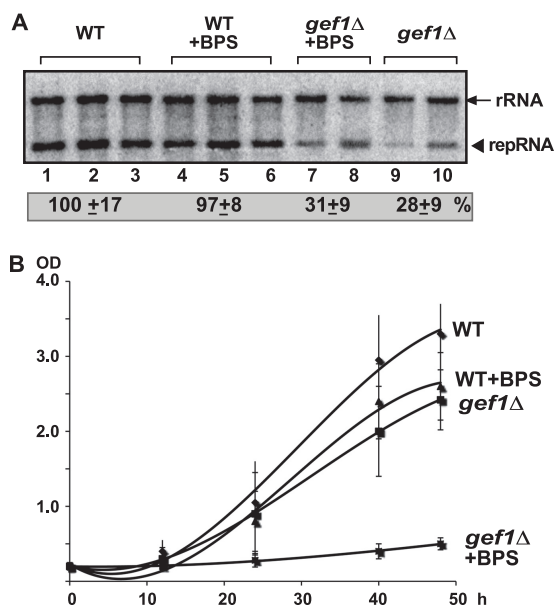


FIG 6 Iron starvation does not influence TBSV repRNA accumulation in *gef1Δ* or WT yeast strains. (A) Northern blot analysis shows the accumulation of TBSV repRNA in WT yeast without iron chelator (BPS) or with iron chelator (7.5 μ M). See further details in the legend to Fig. 1. (B) Positive control for the BPS treatment. The assay is based on the lack of yeast growth under iron depletion in *gef1Δ* yeast strain. The growth of the yeast cultures shown at 23°C was measured at OD₆₀₀ at the following time points: 12, 24, 40, and 48 h.

Altered iron metabolism in *gef1Δ* yeast does not influence TBSV replication. Gef1p is also involved in iron and copper metabolism (35, 37, 67). The fundamental role of Gef1p in iron homeostasis raised the question whether the impaired iron trafficking system in *gef1Δ* yeast has any effect on TBSV replication. To test this question, we took advantage of a specific iron chelator, called bathophenanthroline sulfonate (BPS) (51). The presence of BPS causes iron starvation and the cytosol of WT yeast cell and the Gef1p-containing compartments become more neutral compared to the acidic nature of the cytosol without BPS treatment.

However, TBSV replication is not affected by the presence of BPS in WT yeast (Fig. 6A, lanes 1 to 3 versus lanes 4 to 6, and Fig. 6B). Although deletion of *GEF1* gene also elevates cytosolic pH, the presence of BPS leads to further alkalization of the cytosol (51). Yet, TBSV repRNA accumulation was comparable in *gef1Δ* yeast in the absence of BPS (Fig. 6A, lanes 9 and 10) or presence of BPS (Fig. 6A, lanes 7 and 8). Since BPS did not further reduce viral RNA accumulation in *gef1Δ* yeast, we suggest that the alkalization of the cytosol or the altered iron homeostasis does not affect TBSV repRNA accumulation significantly in yeast.

Deletion of Ccc2p copper pump inhibits TBSV replication in yeast. Gef1p is essential for Cu²⁺ ion transfer to apoFet3p in post-Golgi vesicles, and this process is mediated by a copper pump called Ccc2p ATPase. Ccc2p translocates copper to the lumen of the late Golgi and prevacuole compartments, thus reducing cytosolic Cu²⁺ level. Gef1p and Ccc2p function in concert in copper homeostasis (28). This is accomplished by Gef1p by staying non-functional until the membrane potential reaches a certain level (due to anion accumulation at the cytosolic site of the membrane and cation accumulation inside the lumen) that renders the Gef1p functional. The functional Gef1p is then thought to allow Cl⁻ ions

A. TBSV repRNA accumulation

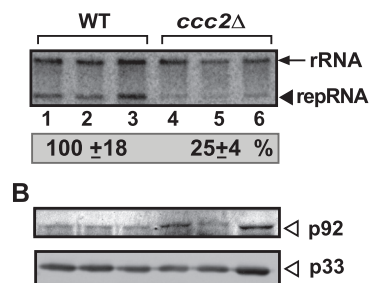


FIG 7 Deletion of CCC2 ATPase, an intracellular copper pump, hinders TBSV repRNA accumulation in yeast. (A) Northern blot analysis was used to detect the accumulation of repRNA in WT and *ccc2Δ* yeast strains. See further details in the legend to Fig. 1. (B) Western blot analysis of the expression level of His₆-p92^{pol} and His₆-p33 in WT and *ccc2Δ* yeast strains.

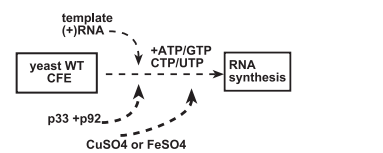
to enter the lumen, which, in turn, reduces the membrane potential and promotes further Cu²⁺ and H⁺ uptake to the organelle from the cytosol by keeping Vma1p and Ccc2p transporters active (35, 37, 67). In the absence of Gef1p, the intravesicular voltage will remain high without compensation and extra Cu²⁺ and H⁺ cannot enter the subcellular organelle. We predicted that the resulting altered Cu²⁺ ion metabolism in the cytosol might affect the viral replicase function in that location.

To test this possibility, we measured TBSV repRNA accumulation in *ccc2Δ* yeast. Interestingly, RNA accumulation was decreased by ~75% in *ccc2Δ* yeast (Fig. 7A, lanes 4 to 6 versus lanes 1 to 3), although the expression level of p33 did not change and p92^{pol} level was higher (Fig. 7B, lanes 4 to 6). These results strongly suggest that the increased Cu²⁺ level (in the free or bound form) in the cytosol might be the inhibitory factor for TBSV replication in *gef1Δ* and *ccc2Δ* yeasts.

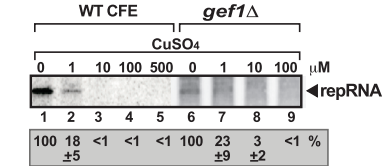
Cu²⁺ ion inhibits the assembly of the tombusvirus VRC *in vitro*. Since deletion of either *GEF1* or *CCC2* leads to decrease of Cu²⁺ ions in the secretory apparatus and in the vacuole and increase in the cytosol (37, 51), it is possible that the cytosolic Cu²⁺ ions in *gef1Δ* or *ccc2Δ* yeasts could inhibit the tombusvirus replicase activity. This was tested by adding increasing amounts of Cu²⁺ ions to the CFE programmed with TBSV replication proteins and repRNA (Fig. 8A). We found very strong inhibition of TBSV RNA production even in the presence of small amounts of Cu²⁺ ions (1 to 10 μ M [Fig. 8B, lanes 2 and 3 versus lane 1]). On the other hand, a much larger amount of FeSO₄ (100 μ M) was needed to block TBSV RNA replication based on the CFE assay (Fig. 8C). These results support the model that Cu²⁺ ions accumulating in the cytosol of *gef1Δ* or *ccc2Δ* yeasts are likely responsible for inhibition of TBSV RNA replication.

To test what step of the TBSV replication process is inhibited by Cu²⁺ ions, we performed the two-step CFE-based TBSV replication assay as shown schematically in Fig. 9A. Addition of Cu²⁺ ions during the VRC assembly step (step 1) fully inhibited TBSV RNA replication in CFE (Fig. 9B, lanes 1 to 4). In contrast, addition of 5 μ M Cu²⁺ ions after the VRC assembly step (step 2 [Fig. 9B]), which involves RNA synthesis by the preassembled TBSV VRC, had only moderate inhibitory effect on TBSV RNA replication in the CFE (Fig. 9B, lanes 7 and 8), suggesting that the RNA synthesis step is less sensitive to the presence of Cu²⁺ ions than the VRC assembly step. However, addition of 10 μ M Cu²⁺ ions after

A. Scheme of the CFE-based replication assay:



B. CFE-based replication assay:



C. CFE-based replication assay:

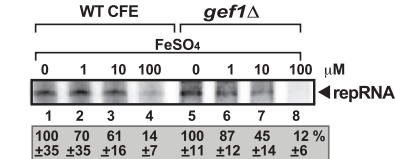
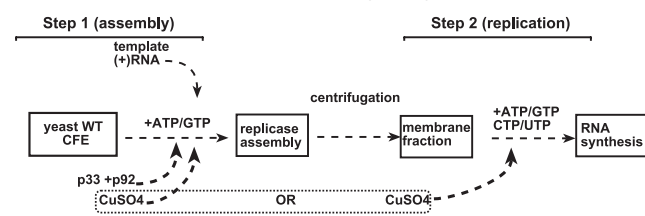


FIG 8 Inhibition of *in vitro* replication of TBSV RNA in CFE by CuSO₄. (A) Scheme of the experiments shown in panels B and C. Purified recombinant MBP-p33, MBP-p92^{pol}, and T7 transcripts of TBSV DI-72 (+)repRNA were added to yeast CFE together with ³²P-labeled and unlabeled ribonucleotides to initiate viral replication. The CFEs were prepared from untransformed WT or *gef1Δ* yeast strains. (B and C) The ³²P-labeled repRNA products were analyzed by denaturing PAGE. The amounts of CuSO₄ (B) or FeSO₄ (C) added at the beginning of the assay are indicated above the lanes. Each experiment was performed 2 or 3 times.

the VRC assembly step resulted in strong inhibition, indicating that TBSV RNA synthesis is sensitive to higher levels of Cu²⁺ ions.

The inhibitory effects of the soluble fraction of *gef1Δ* yeast (Fig. 5) and the addition of small amount of Cu²⁺ ions to the CFE from WT yeast (Fig. 8 and 9) on the *in vitro* TBSV replicase activity are remarkably similar. Both treatments strongly inhibit the assembly of the TBSV VRC and moderately reduce viral RNA synthesis *in*

A. Scheme of the CFE replicase assembly assay:



B. CFE-based replication assay:

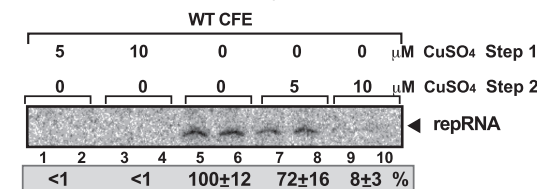


FIG 9 Inhibition of *in vitro* VRC assembly by CuSO₄. (A) Scheme of the *in vitro* CFE-based tomosvirus replicase assembly assay. Note that CuSO₄ was added either during step 1 or step 2 as indicated. See further details in the legend to Fig. 5C. (B) The ³²P-labeled repRNA products from the replication assay described in the legend to Fig. 5C were analyzed by denaturing PAGE.

A. Scheme of the CFE replication assay:

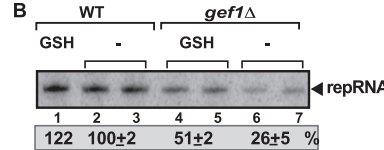
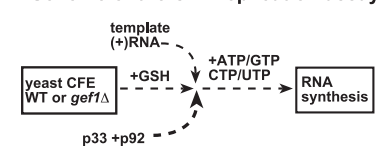


FIG 10 Glutathione stimulates the *in vitro* replication of TBSV RNA in CFEs. (A) Scheme of the experiments. Glutathione (GSH) (37 μM) and 5 min later, purified recombinant MBP-p33, MBP-p92^{pol}, and T7 transcripts of TBSV DI-72 (+)repRNA were added to yeast CFEs together with ³²P-labeled and unlabeled ribonucleotides to initiate viral replication. The CFEs were prepared from untransformed WT or *gef1Δ* yeast strains. (B) The ³²P-labeled repRNA products were analyzed by denaturing PAGE. The experiments were performed 2 times.

vitro. Therefore, it is possible that the soluble fraction of *gef1Δ* yeast contains Cu²⁺ ions (in free or bound form) that are inhibitory to the TBSV replicase. To test this idea, we added glutathione (GSH) to the CFE from *gef1Δ* yeast (Fig. 10A), which is known to bind efficiently to Cu²⁺ ions (68), thus protecting the viral replication proteins from exposure to free Cu²⁺ ions. We observed ~2-fold increase in TBSV replication in CFE prepared from *gef1Δ* yeast in the presence of GSH (Fig. 10B, lanes 4 and 5 versus lanes 6 and 7). These data suggest that Cu²⁺ ions in the cytosol of *gef1Δ* yeast could be responsible for decreased level of tomosvirus replication.

Cu²⁺ ion inhibits viral RNA-dependent RNA polymerase activity *in vitro*. The viral RdRps are known to require Mg²⁺ ions for their polymerase activities (69), and it seems plausible that Cu²⁺ ions, having similar ionic radius and charge, might compete with Mg²⁺ ions for binding to the active center of the viral RdRp. To test the direct effect of Cu²⁺ ion on the RNA synthesis activity of a viral RdRp, we also used a solubilized and affinity-purified tomosvirus replicase preparation, which is capable of cRNA synthesis on added minus-strand TBSV RNA templates (49, 63). We found that 10 μM or more Cu²⁺ ions strongly inhibited the cRNA synthesis by the purified tomosvirus replicase *in vitro* (Fig. 11A, lanes 4 to 6). These data are comparable to the inhibitory effect of 10 μM Cu²⁺ ions on TBSV replication in the CFE-based assay (Fig. 9B).

We also tested a purified recombinant version of p88^{pol} polymerase of TCV. The RNA specificity of TCV RdRp protein is similar to the affinity-purified TBSV replicase (49, 58, 70). We kept a comparable MgCl₂ level (10 mM concentration) in the TCV RdRp assay, but we added different amounts of CuSO₄ to test its effect (Fig. 11B). The application of 1/100th of CuSO₄ concentration (compared to that of MgCl₂) in the TCV RdRp assay almost completely blocked cRNA synthesis (Fig. 11B, lanes 5 and 6 versus lanes 1 to 4). This suggests that Cu²⁺ ions possibly not only replaced Mg²⁺ in a competitive manner in the active site of the viral RdRp, but the Cu²⁺ ions likely altered the structure of the RdRp, rendering it nonfunctional. Altogether, the above *in vitro* assays support the idea that an increased level of Cu²⁺ ions could greatly inhibit the activity of the viral RdRp.

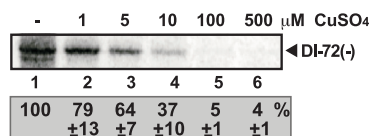
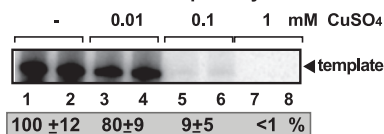
A. Purified TBSV replicase-based assay:**B. Purified TCV RdRp assay:**

FIG 11 Inhibition of *in vitro* activities of the purified tombusvirus replicase and the TCV RdRp by CuSO₄. (A) Denaturing PAGE shows the ³²P-labeled cRNA product from the tombusvirus replicase assay performed in the presence of 10 mM MgSO₄ plus an increasing amount of CuSO₄ as shown. The recombinant tombusvirus replicase expressed in yeast was solubilized and affinity purified, rendering the preparation template dependent. (B) Denaturing PAGE shows the ³²P-labeled cRNA product from the TCV p88C RdRp assay performed in the presence of 10 mM MgSO₄ plus an increasing amount of CuSO₄ as shown. The MBP-tagged p88C was purified from *E. coli*. The template was the 3' end (SL1/SL2/SL3) of the TBSV DI-72 (+)repRNA, which contains the promoter region and the replication silencer element. The RdRp activity in the absence of CuSO₄ was set at 100% (lanes 1 and 2). Each experiment was performed 2 or 3 times.

DISCUSSION

We demonstrate a surprising role for the Gef1p proton-chloride exchanger in replication of TBSV model (+)RNA virus. Gef1p is the only CIC protein in yeast, thus making it easier to delineate its significance in virus replication. We provide genetic evidence that functional Gef1p is required for TBSV replication (Fig. 1). Importantly, our findings are relevant for native host infections, since TBSV replication is also impeded in *N. benthamiana* protoplasts or in whole plants after applying SITS, a CIC blocker (Fig. 2 and 3).

Mechanistic studies revealed that the viral replicase is less functional when obtained from *gef1Δ* yeast than from the WT yeast (Fig. 4). In addition, the *in vitro* assembly of the tombusvirus replicase is greatly hindered in CFE prepared from *gef1Δ* yeast (Fig. 5). This is not due to the concentration of the viral replication proteins, since we used comparable amounts of recombinant proteins from *E. coli* in these assays. In addition, the low replicase activity is probably not due to putative membrane changes in *gef1Δ* yeast, since the ME fraction from *gef1Δ* yeast was as good at supporting *in vitro* TBSV replication as the WT ME fraction (Fig. 5). This is somewhat surprising, since altered intracellular Cu²⁺ ion metabolism in the cytosol caused by Gef1p deletion might damage intracellular membrane structures due to lipoperoxidation or destruction of membrane proteins (71). Any changes in lipid composition or lipid structure may cause severe defects in TBSV replication which, similar to other (+)RNA viruses, requires subcellular membranes for replication (18, 64, 65). On the basis of our *in vitro* results, we conclude that the membrane structure in *gef1Δ* yeast is suitable to fulfill the requirements for TBSV replication.

The presented data reveal that Gef1p likely modulates TBSV replication via regulating Cu²⁺ homeostasis in the cell. This possibility is supported by several pieces of data, including the following: (i) the direct inhibitory effect of Cu²⁺ ions on the *in vitro* replicase assembly (even in the presence of abundant Mg²⁺)

(Fig. 8 and 9); (ii) an inhibitory effect of the soluble fraction prepared from *gef1Δ* yeast on the VRC assembly *in vitro* (Fig. 5); (iii) the stimulatory effect of added glutathione, which binds to Cu²⁺ ions, on TBSV replication in CFE from *gef1Δ* yeast (Fig. 10); (iv) a similar inhibitory effect of deletion of CCC2 copper pump to deletion of *GEF1* on TBSV replication in yeast (Fig. 7); and (v) the lack of inhibition by the iron chelator BPS on TBSV replication in yeast, making the altered iron metabolism in *gef1Δ* yeast less likely responsible for the reduced TBSV replication. These data are important, since Gef1p plays major role in both iron and copper homeostasis (35, 67).

We also discovered that Ccc2p copper pump affects TBSV replication via regulating copper metabolism. Indeed, Gef1p works in concert with Ccc2p Cu²⁺ ATPase in metal homeostasis, via transporting copper ions through the *trans*-Golgi network (37). Mutations in the ATP7A gene that encodes the human homologue of Ccc2p Cu²⁺ ATPase cause Menkes syndrome. This disorder leads to copper accumulation in some tissues and deficiency in others due to defective export of copper from the cytosol (72). Similar to Gef1p, Ccc2p also localizes in the membranes of post-Golgi vesicles, and the duration of the active state of Ccc2p is most likely influenced by Gef1p (37). In the absence of Gef1p protein, copper import into the lumen of post-Golgi vesicles via Ccc2p is restricted, which may lead to accumulation of copper in the cytosol. Accordingly, in the liver cells of *Atp7b*^{-/-} mice, Cu²⁺ ions accumulated predominantly in the cytosol and nuclei (73). *Atp7b* is an ortholog of Ccc2p and can complement the defective phenotype of *ccc2Δ* yeast (74).

Experiments based on the ME and soluble fractions of CFE prepared from *gef1Δ* yeast suggested that the cytosolic fraction contained an inhibitory factor. This factor could be the elevated level of Cu²⁺ in the soluble fraction of CFE from *gef1Δ* yeast. Interestingly, the tombusvirus replicase purified from *gef1Δ* yeast showed poor activity *in vitro*, albeit the p33 and p92 content of the replicase looked similar to that obtained from the WT yeast. Therefore, it is possible that Cu²⁺ ions might inflict long-lasting damage in the tombusvirus replicase. Indeed, subsequent *in vitro* experiments with CFE revealed that the inhibitory effect is manifested mostly by blocking VRC assembly. In addition, Cu²⁺ ions also reduced the replicase activity, albeit to a lesser extent than inhibition of the VRC assembly step. Thus, it is possible that viral or host proteins present in the replicase complex could be subject to copper "poisoning."

Cu²⁺ ions are known to bind to proteins via their cysteine, histidine, or methionine residues in a pH-dependent manner, and high concentrations of copper ions can bind to proteins, nonspecifically leading to breakdown of protein structures (75). As Cu²⁺ and Mg²⁺ have similar ion radii, it is possible that the Cu²⁺ ion replaces the Mg²⁺ ion in the active center of p92 RdRp, rendering the enzyme inactive. Interestingly, our *in vitro* tombusvirus replicase and TCV RdRp experiments imply that Cu²⁺ does not bind to the RdRp in a competitive manner, since even 1/100th of Cu²⁺ concentration compared to Mg²⁺ concentration was sufficient to block the enzyme activity by 90% (Fig. 10). Therefore, it seems more likely that elevated amount of Cu²⁺ in cells causes excessive destructive changes in the structure of p92^{pol}. The possible deleterious effect of the increased copper ion level on other cytosolic host protein(s) that could affect recruitment of p92^{pol} to the viral replicase complex cannot be ruled out at this time.

Since Gef1p plays a fundamental role in iron homeostasis, we

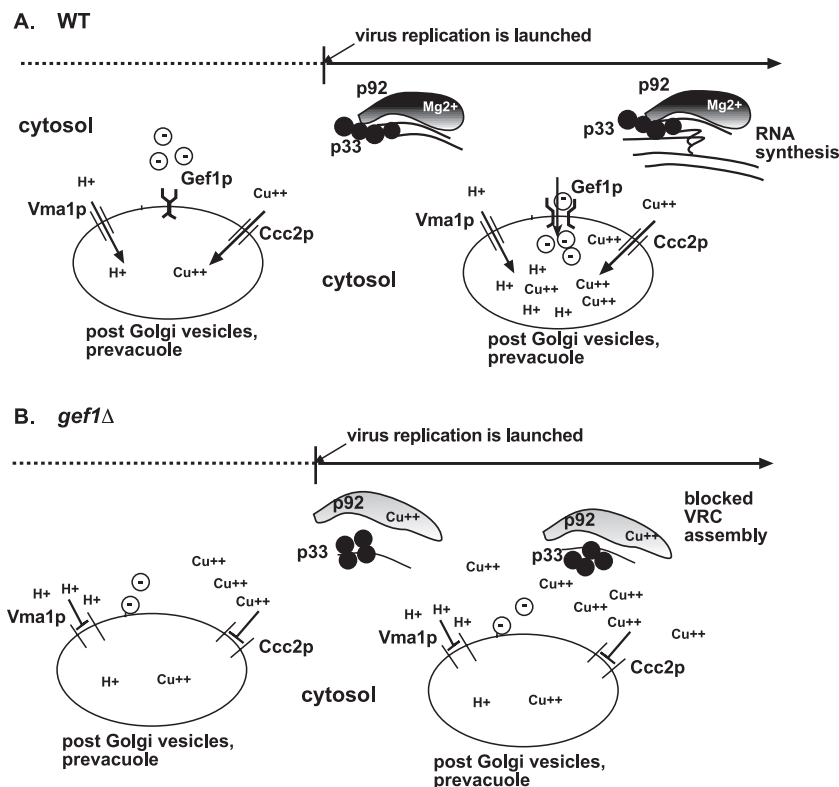


FIG 12 Model on the role of Gef1p in TBSV replication in yeast. (A) In WT yeast cells, the Gef1p proton-chloride exchanger is inactive, while H⁺ and Cu²⁺ ions are transported into the lumen of post-Golgi vesicles, prevesicles, and late endosomes. At a certain membrane potential, the Gef1p proton-chloride exchanger becomes activated and transports Cl⁻ ions to these compartments, which in turn allows further Cu²⁺ and H⁺ uptake into the lumen of these organelles. During this state, the Cu²⁺ concentration in the cytosol is kept at a low level that allows robust tombusvirus replicase (VRC) assembly and RNA replication. (B) Yeast with *gef1Δ* background or with blocked Gef1p function is not able to transfer Cl⁻ ions into the organelles, thus blocking the additional uptake of H⁺ and Cu²⁺ ions into the lumen of these organelles. Ultimately, this leads to increased Cu²⁺ accumulation in the cytosol, which in turn impairs the assembly of the TBSV VRC and inhibits viral RNA synthesis. Note that *gef1Δ* or *ccc2Δ* yeast strains will likely experience higher exposure to cytosolic copper ions, even if these Cu²⁺ ions are not free (bound to glutathione and small metal-binding proteins).

also studied the effect of an iron chelator, which causes iron starvation of yeast cells, on TBSV replication. The obtained data supported a limited role, if any, of iron starvation in TBSV replication. This is somewhat surprising, since it is known that several viruses depend on or even actively influence cellular iron homeostasis to facilitate viral infections. For example, several plant viruses carry genes that encode proteins with the AlkB domain that require iron for activity, and these proteins might be involved in maintaining the integrity of the viral genomic RNA (76). Also, several human viruses, such as hepatitis C virus, can also actively influence iron homeostasis. Accordingly, application of iron chelators seems to be a beneficial approach to fight against HIV-1, human cytomegalovirus, vaccinia virus, herpes simplex virus 1, and hepatitis B virus (19).

On the basis of the known regulatory function of Gef1p in copper, iron, and pH homeostasis and the inhibitory effect of Cu²⁺ ions on the assembly of the tombusvirus replicase and on the activity of viral RdRp *in vitro*, we propose that the proper function of the Gef1p proton-chloride exchanger is required for efficient TBSV replication in yeast. We predict a similar role for plant CIC proteins in TBSV replication. As summarized in our model (Fig. 12), we suggest that the open state of the Gef1p proton-chloride exchanger supports proper subcellular distribution of Cu²⁺ ions, thus allowing highly efficient TBSV replication in the cytosol. In

contrast, blocking the function of Gef1p, or deletion of *GEF1* and *CCC2* copper pump, leads to increased Cu²⁺ ion accumulation in the cytosol that “poisons” the viral polymerase, and overall hinders the synthesis of new viral RNA.

Copper ions mainly exist in protein-bound forms in the cytosol, forming a complex with glutathione (GSH) or thiol-containing metallothioneins and other copper-binding proteins in the cytoplasm (77). It is possible that the TBSV replication proteins are also among these proteins binding copper ions directly when disturbance of cytosolic copper homeostasis occurs in *gef1Δ* or *ccc2Δ* yeasts. Alternatively, the tombusvirus replication proteins might obtain the copper ions from GSH and other chelators in the cytoplasm in *gef1Δ* or *ccc2Δ* yeasts. Since Ccc2p and Gef1p are clearly implicated in copper sequestration to the endosomal lumen (28), it is likely that the corresponding deletion strains will experience higher exposure to cytosolic copper ions, even if these Cu²⁺ ions are not free. Indeed, transcription activator protein Ace1p reacts to even modest extracellular additions of copper, suggesting that copper ions are accessible to proteins in the yeast cell (78).

Interestingly, we previously found a stimulatory role for Mn²⁺ ions in TBSV replication and recombination (15, 79). Pmr1p Ca²⁺/Mn²⁺ ion pump was shown to regulate the cytosolic Mn²⁺ ion level, which, in turn, affects the template activity and template

switching by the tombusvirus replicase (79). Thus, it seems that regulation of intracellular ion homeostasis is rather critical during tombusvirus replication.

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