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Translationally controlled tumour protein (TCTP) from tomato and *Nicotiana benthamiana* is necessary for successful infection by a potyvirus

FERNANDA PRIETO BRUCKNER[1], ANDRÉ DA SILVA XAVIER[2], RENAN DE SOUZA CASCARDO[1], WAGNER CAMPOS OTONI[3], FRANCISCO MURILO ZERBINI[2] AND POLIANE ALFENAS-ZERBINI[1,*]

[1]*Departamento de Microbiologia/BIOAGRO/National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil*

[2]*Departamento de Fitopatologia/BIOAGRO/National Institute of Science and Technology in Plant-Pest Interactions, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil*

[3]*Departamento de Biologia Vegetal/BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil*

SUMMARY

Translationally controlled tumour protein (TCTP) is a ubiquitously distributed protein in eukaryotes, involved in the regulation of several processes, including cell cycle progression, cell growth, stress protection, apoptosis and maintenance of genomic integrity. Its expression is induced during the early stages of tomato (*Solanum lycopersicum*) infection by the potyvirus *Pepper yellow mosaic virus* (PepYMV, a close relative of *Potato virus Y*). Tomato TCTP is a protein of 168 amino acids, which contains all the conserved domains of the TCTP family. To study the effects of TCTP silencing in PepYMV infection, *Nicotiana benthamiana* plants were silenced by virus-induced gene silencing (VIGS) and transgenic tomato plants silenced for TCTP were obtained. In the early stages of infection, both tomato and *N. benthamiana* silenced plants accumulated less virus than control plants. Transgenic tomato plants showed a drastic reduction in symptoms and no viral accumulation at 14 days post-inoculation. Subcellular localization of TCTP was determined in healthy and systemically infected *N. benthamiana* leaves. TCTP was observed in both the nuclei and cytoplasm of non-infected cells, but only in the cytoplasm of infected cells. Our results indicate that TCTP is a growth regulator necessary for successful PepYMV infection and that its localization is altered by the virus, probably to favour the establishment of virus infection. A network with putative interactions that may occur between TCTP and *Arabidopsis thaliana* proteins was built. This network brings together experimental data of interactions that occur in other eukaryotes and helps us to discuss the possibilities of TCTP involvement in viral infection.

Keywords: host factor, *Pepper yellow mosaic virus*, potyvirus, translationally controlled tumour protein.

* *Correspondence*: Email: palfenas@ufv.br

INTRODUCTION

Viruses are intracellular parasites that infect diverse organisms in all life domains. Viruses depend on their host cells to carry out all life cycle steps, including viral protein synthesis, genome replication and dispersion to other cells. Thus, viruses need to manipulate their host cells to favour their establishment and, in so doing, cause cell physiological and morphological alterations that result in a multitude of symptoms (Mandadi & Scholthof, 2013). Viral infection affects the expression patterns of host cell genes, leading to the up- and down-regulation of a wide variety of genes that are involved in defence responses or that are useful or even required for infection (Alfenas-Zerbini *et al.*, 2009; Gandia *et al.*, 2007; Pompe-Novak *et al.*, 2006; Senthil *et al.*, 2005).

Potyvirus is one of the largest and most economically important genera of plant viruses. Its members are transmitted by aphids and collectively infect a wide range of hosts, including monocot and dicot plant species distributed around the world (Adams *et al.*, 2011). The potyvirus genome comprises a 10-kb single strand of RNA linked to VPg (viral protein genome-linked) at the 5′ terminus and to a poly-A tail at the 3′ terminus. The viral genome has two open reading frames (ORFs): a major one that encodes a polyprotein that is self-cleaved to generate 9–11 mature viral proteins and a second ORF, named PIPO, which is translated by transcriptional slippage within the coding region of the P3 protein to produce a fusion P3N-PIPO protein (Chung *et al.*, 2008; Wen & Hajimorad, 2010; Olspert *et al.*, 2015).

A transcriptome analysis of differentially expressed genes during the early stages of tomato (*Solanum lycopersicum*) infection by the potyvirus *Pepper yellow mosaic virus* (PepYMV) identified the gene that encodes the translationally controlled tumour protein (TCTP) to be up-regulated (Alfenas-Zerbini *et al.*, 2009). TCTP is a highly conserved protein present in all eukaryotes, and the mammalian homologues are the best studied because of their role in cancer development. TCTP is involved in cell cycle progression, cell growth, stress protection, maintenance of genomic integrity and apoptosis (reviewed by Bommer, 2012).

A number of structural differences between plant and animal TCTPs can be observed when their sequences are compared. Some putative phosphorylation sites are exclusive to plant TCTPs; these sites may be targets of C-type kinases and may be involved in specific functions of plant TCTPs (Thayanithy, 2005). Plant TCTP expression can be induced by distinct stress stimuli, including drought, salinity, heat, cold and aluminium toxicity (Alfenas-Zerbini *et al.*, 2009; Ermolayev *et al.*, 2003; Fabro *et al.*, 2008; Lee and Lee, 2003; Liao *et al.*, 2009; Qin *et al.*, 2010). In *Arabidopsis thaliana*, there are two TCTP homologues: AtTCTP1 (At3g16640) and AtTCTP2 (At3g05540). AtTCTP1 is involved in mitotic growth, cell division and embryogenesis (Berkowitz *et al.*, 2008; Brioudes *et al.*, 2010). A role for TCTP in the selective trafficking of macromolecules in the phloem was suggested by Aoki *et al.* (2005). These authors showed that TCTP is one of two pumpkin (*Cucurbita maxima*) phloem sap proteins that interact with two RNA-binding proteins: CmPP16-1 and CmPP16-2 (the other interacting protein is eIF5A). The rootward movement of CmPP16-1 is positively affected by the presence of the two interacting proteins, whereas CmPP16-2 is not affected. Recently, it has been demonstrated that AtTCTP2 (initially considered as a pseudogene) is expressed and functional in Arabidopsis plants. Both AtTCTP2 protein and RNA are able to move long distance across graft junctions involving transgenic and wild-type (WT) *A. thaliana* plants (Toscano-Morales *et al.*, 2014).

The AtTCTP1 structure was predicted by Berkowitz *et al.* (2008). It is similar to other TCTPs and to Mss4, a known guanine nucleotide exchange factor (GEF). The similarity between the phenotypes of *A. thaliana* silenced for TCTP and for TOR (target of rapamycin) kinase led the authors to suggest a GEF function for AtTCTP1 (Berkowitz *et al.*, 2008). AtTCTP1 can interact *in vivo* with four Arabidopsis Ras homologues (AtRABA4a, AtRABA4b, AtRABF1 and AtRABF2b) and with *Drosophila* Rheb, supporting the hypothesis that plant TCTPs act to regulate the TOR pathway in a similar manner to animal TCTPs (Brioudes *et al.*, 2010).

Based on both transient (virus-induced gene silencing, VIGS) and stable TCTP silencing (transgenic plants expressing a TCTP double-stranded RNA), we demonstrate here that TCTP is a host factor that is necessary for the establishment of an efficient PepYMV infection in tomato and *Nicotiana benthamiana*. TCTP silencing leads to decreased virus accumulation and greatly attenuated symptoms. Viral systemic infection alters the subcellular localization of TCTP from cytoplasmic and nuclear to cytoplasmic only in epidermal cells. Because TCTP is a multifunctional protein, it is difficult to ascribe a specific role for TCTP during viral infection. Therefore, we searched for proteins that interact with TCTP in different taxa and that are present in the *A. thaliana* genome. These known interactions were used to build a network from which we discuss the possibilities of TCTP involvement in viral infection.

RESULTS

Tomato TCTP is a typical plant TCTP

In a previous study, Alfenas-Zerbini *et al.* (2009) identified several tomato genes that are induced during the early stages of PepYMV infection. One of these genes encodes the tomato homologue of TCTP (SlTCTP), and its up-regulation at 72 h post-viral inoculation (hpvi) was confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Alfenas-Zerbini *et al.*, 2009). The complete cDNA was cloned, sequenced and characterized *in silico*. Sequence analysis showed that the amino acid sequence of the up-regulated protein is identical to the SlTCTP reference sequence available in GenBank (accession no. NM_001247637.1). SlTCTP has 168 amino acids (aa) and contains all the conserved features of plant TCTPs, including four putative phosphorylation sites, two of which are phosphorylated by kinase C and two of which are phosphorylated by type II casein kinases, one putative myristoylation site and the typical signature sequence TCTP2 (Fig. 1). We also identified a putative leucine-rich nuclear export signal (NES; aa 122–130) and a nuclear localization signal (NLS; aa 89–117), suggesting that the protein shuttles between the nucleus and the cytoplasm (Kosugi *et al.*, 2009) (Fig. 1).

The TCTP sequences of several plant and animal species were aligned, and a strong conservation was observed among plant sequences (Fig. S1, see Supporting Information). The casein kinase II phosphorylation site at position 9 in SlTCTP is conserved in all sequences analysed and the other three phosphorylation sites are exclusive to plant TCTPs, being present in all sequences, except for the casein kinase II phosphorylation site in position 135 that is absent in AtTCTP1.

Transient TCTP silencing leads to decreased viral accumulation in *N. benthamiana* during the early stages of infection

A VIGS assay was conducted in *N. benthamiana* plants to verify the effect of TCTP silencing on infection by PepYMV. Fragments

MLVYQDLLTGDELLSDSFPYKEVENGVLWEVQGKWVVQGAVDVNIGANPSAEGGCEDEGVDDQAVRVVDIVDTFR
LQEQPAFDKKQFVTFMKRYIKNLTPKLEGETQEAFKKNIEAATKFLLQKIKDLQFFVGESMHDDGALVFAYYKEG
SADPPFLYIAPGLKEIKC

Fig. 1 Putative conserved motifs in tomato translationally controlled tumour protein (TCTP). Putative motifs were identified using PROSITE software and are indicated by the following colours: yellow, casein kinase type II phosphorylation site; red, myristoylation site; green, kinase C phosphorylation site. The TCTP2 signature sequence is represented by the dark underline. The putative nuclear localization signal (NLS), identified using cNLS, is represented by the double underline, and the leucine-rich nuclear export signal (NES), identified using the NetNES 1.1 server, is indicated in grey.

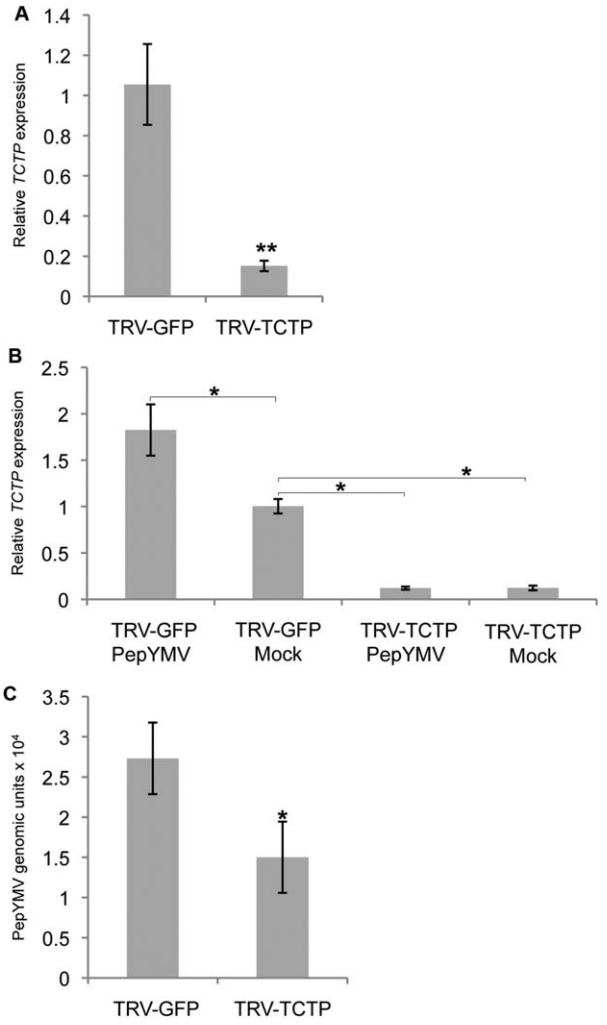

Fig. 2 (A) *TCTP* (translationally controlled tumour protein gene) expression in *Nicotiana benthamiana* plants at 14 days after agroinoculation with the virus-induced gene silencing (VIGS) vector, before *Pepper yellow mosaic virus* (PepYMV) inoculation. *TCTP* expression was quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in relation to TRV-GFP (*Tobacco rattle virus*-green fluorescent protein) plants. The bars represent the average expression level in six plants from each treatment. (B) *TCTP* silencing at 72 h post-viral inoculation (hpvi). (C) Viral accumulation decreased in *TCTP*-silenced plants. Error bars represent standard deviations. $*P < 0.05$; $**P < 0.01$; (Student's *t*-test).

of approximately 400 nucleotides of the genes *phytoene desaturase* (*PDS*), *green fluorescent protein* (*GFP*) and *SlTCTP* were cloned into RNA2 of the *Tobacco rattle virus* (TRV) silencing vector. TRV silencing vectors were infiltrated, together with TRV RNA1, into *N. benthamiana* leaves. *PDS* silencing was observed by a typical photobleaching phenotype and appeared approximately 8–10 days after agroinoculation (daa), being completely established in young leaves by 14 daa. TCTP silencing did not induce phenotypic alterations (Fig. S2, see Supporting Information). At 14 daa, a leaf disc was collected from the upper leaves of each plant for molecular confirmation, using qRT-PCR, of TCTP silencing before viral inoculation. Then, one-half of the plants in each treatment group were sap inoculated with PepYMV, and leaf discs adjacent to the previously collected discs were collected at 72 hpvi to analyse PepYMV accumulation as well as the maintenance of TCTP silencing. TRV-TCTP plants were efficiently silenced for TCTP by VIGS (Fig. 2A), and the level of silencing was maintained at 72 hpvi (Fig. 2B, compare TRV-TCTP mock with TRV-GFP mock). As expected, *TCTP* expression was induced by viral infection (Fig. 2B, compare TRV-GFP PepYMV with TRV-GFP mock), in line with our previous results (Alfenas-Zerbini *et al.*, 2009). Viral accumulation was reduced in *TCTP*-silenced plants compared with control plants, showing that a decrease in the accumulation of TCTP mRNA levels was inhibitory to viral establishment during the early stages of PepYMV infection (Fig. 2C).

We also attempted to analyse the effects of *TCTP* silencing later during the (systemic) infection. Young (non-inoculated) leaves from all plants were collected at 14 days post-viral inoculation (dpvi). At this time, all inoculated plants showed similar symptoms, with no differences observed between treatments and control plants (Fig. S3, see Supporting Information). Viral load determined by qRT-PCR was equivalent in both TRV-GFP and TRV-TCTP inoculated plants (Fig. 3A). This result indicated that either *TCTP* silencing was unable to prevent virus establishment, or that PepYMV infection was able to suppress TCTP silencing. To test this, the accumulation of TCTP mRNA was quantified at 14 dpvi. The pattern of expression observed at 72 hpvi (Fig. 2B) was also present at 14 dpvi, except for TRV-TCTP plants inoculated with PepYMV (Fig. 3B). Compared with 72 hpvi, there was an increase in *TCTP* expression in silenced plants when PepYMV infection was established, and the expression level was similar to that in TRV-GFP mock-inoculated plants (Fig. 3B, compare TRV-GFP mock with TRV-TCTP PepYMV). Because potyviruses have a strong silencing suppressor (helper component proteinase, HC-Pro), it is possible that, after viral establishment, PepYMV suppresses TRV-mediated *TCTP* silencing. To test this hypothesis, the relative accumulation of TRV was quantified by qRT-PCR (Fig. 3C). TRV accumulation increased 8–10-fold in both TRV-TCTP and TRV-GFP plants when infected by PepYMV, supporting the hypothesis that PepYMV infection suppresses VIGS.

Stably *TCTP*-silenced transgenic tomato plants have abnormal phenotypes and are not systemically infected by PepYMV

As it was not possible to study the effect of *TCTP* silencing during a systemic infection by VIGS, tomato plants (*Solanum lycopersicum* cv. Moneymaker) were transformed with a TCTP construct to induce stable TCTP silencing. T-DNA integration was confirmed by PCR in five plants (TCTP5, TCTP6, TCTP11, TCTP22 and TCTP26).

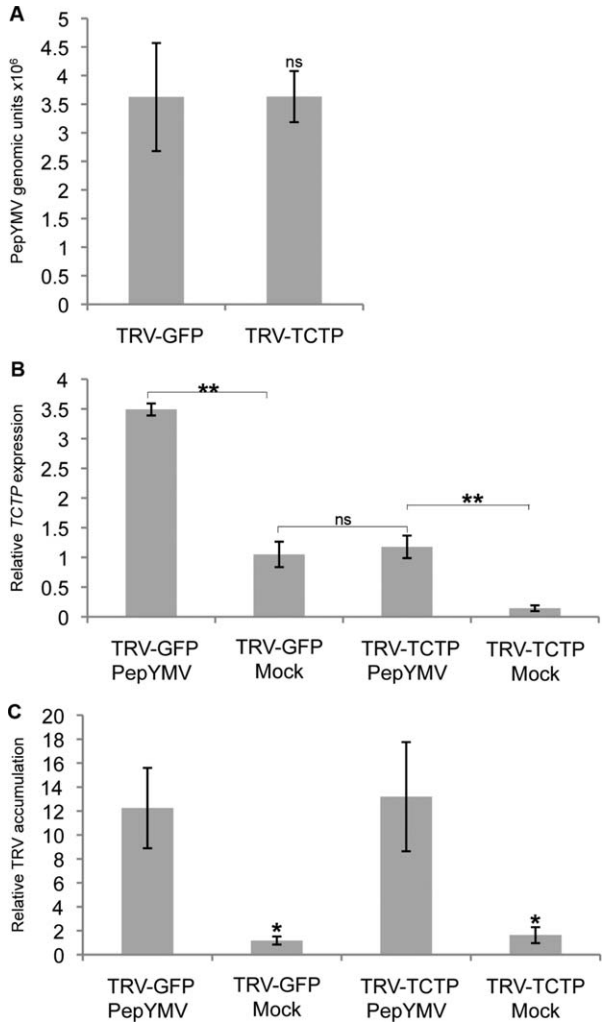

Fig. 3 Translationally controlled tumour protein (TCTP) expression and viral accumulation in *Nicotiana benthamiana* plants agroinfiltrated with the virus-induced gene silencing (VIGS) vector at 14 days post-viral inoculation (dpvi). (A) TRV-GFP (*Tobacco rattle virus*-green fluorescent protein) and TRV-TCTP plants infected with *Pepper yellow mosaic virus* (PepYMV) accumulated equivalent viral loads. (B) TCTP expression increased in PepYMV-infected TRV-GFP plants relative to mock-inoculated TRV-GFP plants. In PepYMV-infected TRV-TCTP plants, TCTP expression was also induced relative to mock-inoculated TRV-TCTP plants. (C) Relative accumulation of TRV in infected and mock-inoculated plants at 14 dpvi. TRV accumulation increased compared with mock-inoculated plants in both TRV-GFP and TRV-TCTP plants following PepYMV infection, and was equivalent in these plants. Bars represent the average of three plants for each treatment. Error bars represent standard deviations. $*P < 0.05$; $**P < 0.01$; ns, not significant (Student's *t*-test).

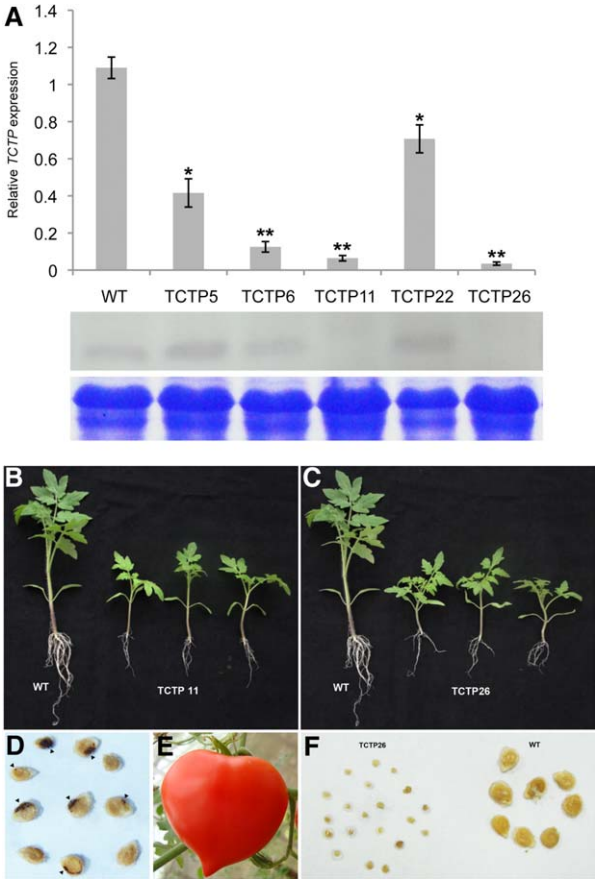

Fig. 4 (A) *TCTP* (translationally controlled tumour protein gene) expression in transgenic tomato plants. TCTP mRNA levels were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (top panel), and protein levels were quantified by Western blot analysis (middle panel). Loading controls are shown in the bottom panel. (B–F) Phenotype of TCTP-silenced transgenic tomato plants. Stunting in TCTP11 (B) and TCTP26 (C) plants. (D) Embryo necrosis (black arrows) in seeds of plant TCTP11. (E) Heart-shaped fruit morphology in plant TCTP26. (F) Seeds from plant TCTP26 showing a strong reduction in size (left) compared with seeds from wild-type plants (right). $*P < 0.05$; $**P < 0.01$; (Student's *t*-test).

Flow cytometric analysis revealed average nuclear DNA (2C) values in regenerated plants of 1.96 pg, consistent with the DNA amounts of seed-derived 'Moneymaker' controls (1.92 pg) and with those from the standard variety 'Stupike' used as internal control (1.93 pg). The relative TCTP expression level in these five plants was quantified by qRT-PCR, and protein accumulation was verified by Western blot analysis (Fig. 4A). The silencing level was variable, with transcript accumulation ranging from 3.5% to 70% in relation to WT plants (Fig. 4A, top panel). Plants TCTP5 and TCTP22 were weakly silenced, with relative transcript accumulations of 41% and 70%, respectively of that in WT plants. Plants TCTP6, TCTP11 and TCTP26 were strongly silenced, with relative accumulations of 12%, 6.4% and 3.5%, respectively, of that in WT plants. Protein accumulation correlated with transcript *TCTP* levels quantified by qRT-PCR (Fig. 4A, middle panel).

Phenotypic alterations were observed in transgenic plants TCTP11 and TCT26, with the lowest levels of TCTP transcript and protein. The vegetatively propagated (clonal) progeny of plants TCTP5, TCTP6 and TCTP22 displayed no phenotypic alterations in relation to WT plants. The clonal progeny of plant TCTP11 were

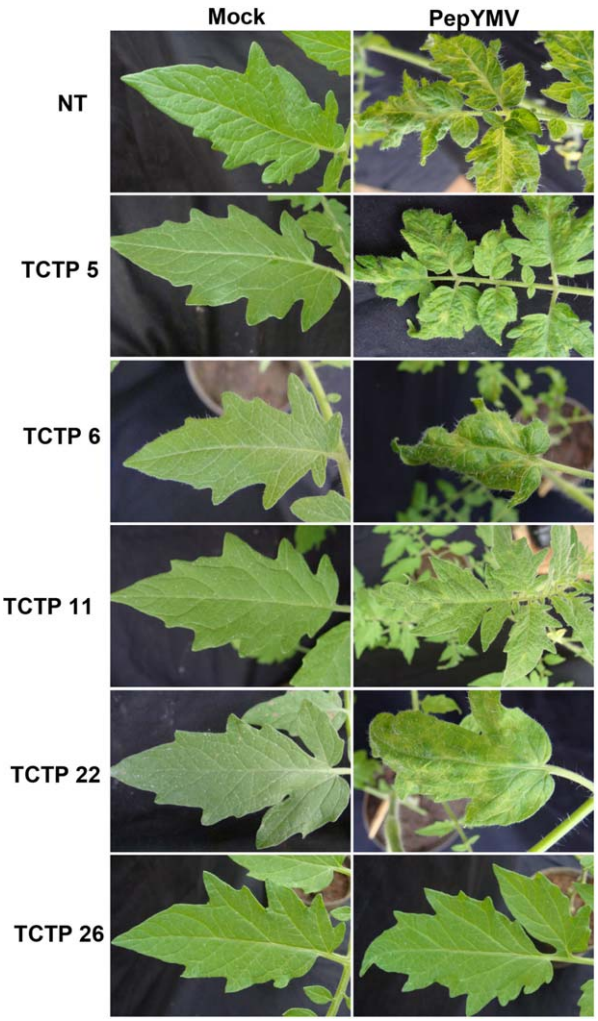

Fig. 5 Symptoms of *Pepper yellow mosaic virus* (PepYMV) infection in wild-type (WT) tomato plants and in the clonal progeny of translationally controlled tumour protein (TCTP)-silenced transgenic tomato plants.

stunted (Fig. 4B), displayed embryo necrosis (Fig. 4D) and developed more slowly than WT plants (not shown). The clonal progeny of plant TCTP26, the strongest silenced plant, showed stunting (Fig. 4C), morphological alterations in fruit (Fig. 4E), decrease in the size of seeds (Fig. 4F), changes in inflorescence habit and increased number of leaflets per leaf (not shown).

The clonal progeny of plants TCTP5, TCTP6, TCTP11, TCTP22 and TCTP26, as well as WT tomato plants, were sap inoculated with PepYMV. Symptom severity was negatively correlated with the level of TCTP silencing. Thus, WT plants and TCTP5, TCTP22 and TCTP6 plants showed typical symptoms of PepYMV infection; in contrast, the strongly silenced TCTP11 plants developed only small chlorotic points, and TCTP26 plants, with the strongest level of TCTP silencing, did not develop any visible symptoms (Fig. 5).

TCTP expression in the clonal progeny of TCTP11 and TCTP26 plants was quantified by qRT-PCR at 72 hpvi and 14 dpvi. *TCTP* remained efficiently silenced at both time points (Fig. 6A,C). Thus, PepYMV infection did not revert TCTP silencing in the transgenic plants. In addition, at both time points, WT plants showed induction of *TCTP* expression as a result of PepYMV infection (Fig. 6A,C). Analysis of the viral load by qRT-PCR showed that PepYMV accumulated to much lower levels in silenced plants relative to WT plants at both time points (Fig. 6B,D). This negative effect of TCTP silencing on viral infection was directly proportional to the level of silencing and stronger in systemic than in inoculated leaves. Thus, a small amount of viral RNA could be detected in the clonal progeny of plant TCTP11, in both inoculated leaves (Fig. 6B) and in leaves infected systemically (Fig. 6D), whereas, in the clonal progeny of plant TCTP26, the viral load was very low in inoculated leaves (Fig. 6B) and no virus could be detected in systemic infection (Fig. 6D).

Subcellular localization of *TCTP* in healthy and PepYMV-infected plants

SlTCTP fused to GFP was expressed transiently in *N. benthamiana* to verify whether PepYMV infection alters TCTP subcellular localization. In healthy plants, TCTP was observed in both the nucleus and the cytoplasm (Fig. 7A). In systemically infected plants (12 dpvi), TCTP was observed only in the cytoplasm (Fig. 7B). These results indicate that PepYMV infection alters the subcellular localization of TCTP.

Putative interactions of TCTP with other plant proteins

TCTP is a multifunctional protein that is involved in the regulation of fundamental cellular processes, and several proteins have been described in the literature that interact with TCTP in different organisms. To identify possible processes that could be affected by PepYMV infection, we searched for *Arabidopsis* proteins that could interact with AtTCTP, and built a network (Fig. 8). A total of 51 different Arabidopsis proteins was identified, including 14 interactions described in plants (12 in *A. thaliana* and two in *C. maxima*), 14 in *Drosophila*, nine in humans and 17 in yeast (with two shared by the latter two taxa). These proteins were clustered in groups according to the information available in the TAIR database: proteins involved in growth and development (GRF1, TTL3, BRL2 and AT1G47570), stress responses (ZAT7, NDPK1, LOS2, ANATT2, NDPK2 and LOS4), vascular movement (PP16-1, PP16-2 and BRL2), protein biosynthesis (AT5G60390, AT5G19510, AT5G12110, AT2G18110, AT1G30230, UBQ1, AT2G36170, AT1G57720, AT1G09640, AT1G43860, RPL36C, RPL36B and RPL36A), cytoskeleton organization (TUA, TUB, PFN2, PRF4, PRF3, PRF5 and PRF1), metabolism (ANNAT2, AT1G74030, AT2G29560, NDPK2, NDK4, NDPK3, AT1G17410 and UBP24), signalling (AtRAB4A, AtRAB4b, AtRABF1, AtRABF2b, NDPK2, NDPK3 and SAE1A) and transmembrane transport (AT5G56450, AAC2, AAC3

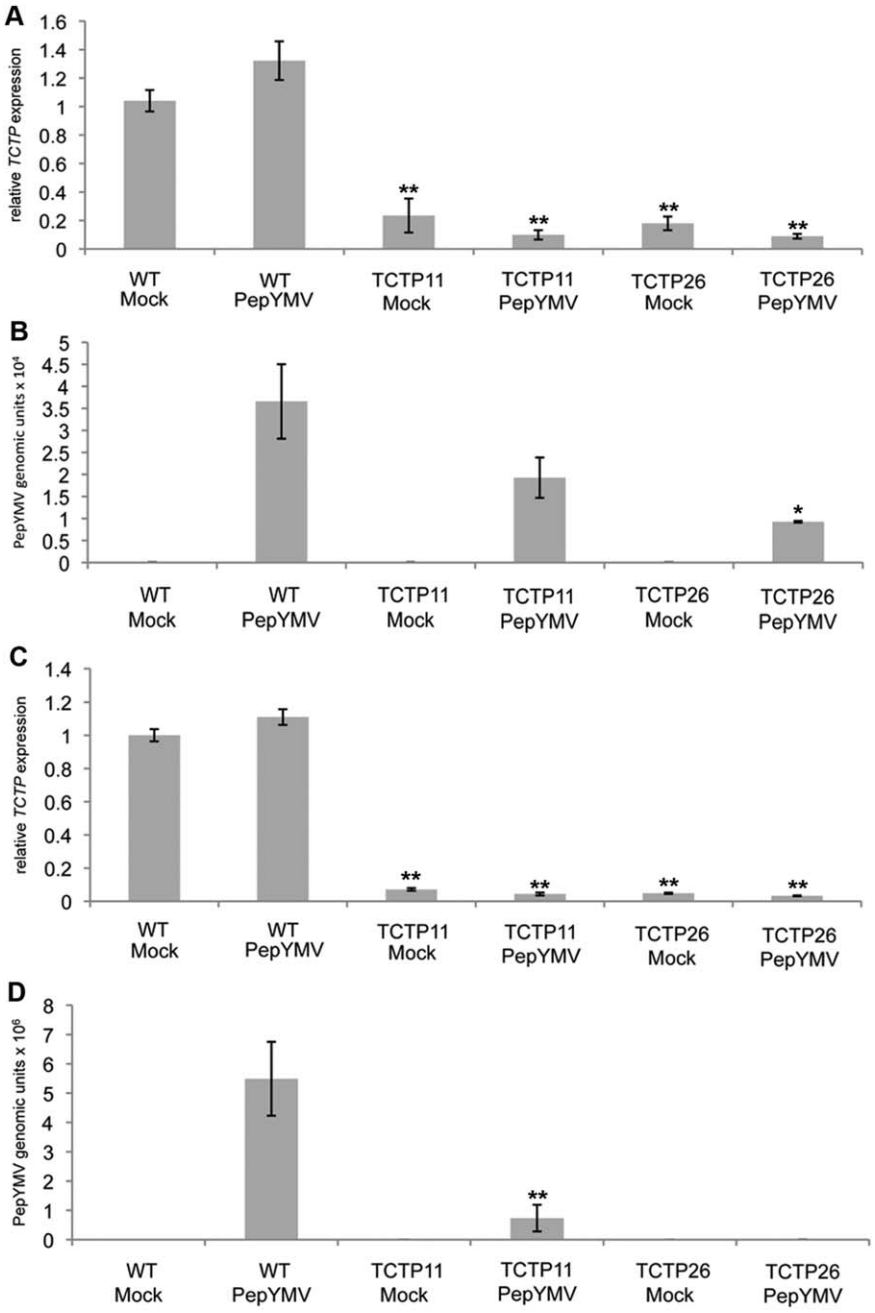

Fig. 6 Translationally controlled tumour protein (TCTP) expression level and *Pepper yellow mosaic virus* (PepYMV) accumulation in wild-type (WT) tomato plants and in the clonal progeny of TCTP-silenced transgenic tomato plants TCTP11 and TCTP26. (A) TCTP expression at 72 h post-viral inoculation (hpvi). (B) PepYMV accumulation at 72 hpvi. (C) TCTP expression at 14 days post-viral inoculation (dpvi). (D) PepYMV accumulation at 14 dpvi. Mock, mock-inoculated plants. PepYMV, plants infected by PepYMV. Each bar represents the average of three plants. Error bars represent standard deviations. $*P < 0.05$; $**P < 0.01$; (Student's *t*-test).

and AAC1), plus three proteins with unknown roles (AT2G26810, AT4G14000 and AT2G43320).

DISCUSSION

Here, we have shown that TCTP is a susceptibility factor that acts to promote PepYMV infection in tomato and *N. benthamiana*. *TCTP* was silenced in *N. benthamiana* by VIGS, which allows an analysis of the effect of this gene during the early stages of viral infection. At this point, the decrease in TCTP levels leads to reduced viral accumulation. However, in a systemic infection of PepYMV, *TCTP* silencing regressed, together with an increased accumulation of TRV, probably because of the effect of the PepYMV silencing suppressor HC-Pro. A similar suppression of TRV-induced gene silencing by 126K, the viral silencing suppressor of *Tobacco mosaic virus*, has been demonstrated previously (Harries *et al.*, 2008).

In transgenic tomato plants, TCTP silencing was stable in both local and systemically infected leaves and was not affected by viral infection. The most strongly silenced lineages, TCTP11 and

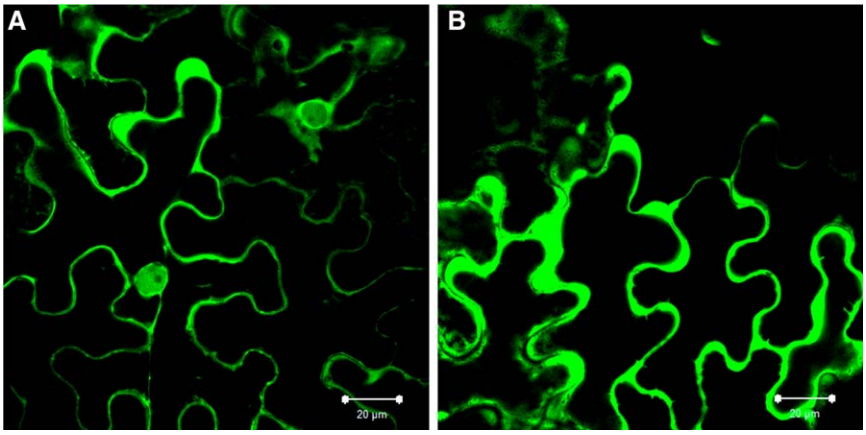

Fig. 7 Subcellular localization of translationally controlled tumour protein (TCTP) in healthy (A) and *Pepper yellow mosaic virus* (PepYMV)-infected (B) *Nicotiana benthamiana* cells, 48 h after agroinfiltration with a construct expressing a TCTP-GFP fusion protein. The images correspond to a single optical section. GFP, green fluorescent protein.

TCTP26, developed phenotypic alterations compared with WT plants, and this finding is in agreement with the previously reported phenotype of RNAi TCTP-silenced lines of *A. thaliana* (Berkowitz *et al.*, 2008). In both of our lineages, the plants showed delayed development and growth, reduced size of leaves and flowers, and reduced root growth and development. After PepYMV inoculation, TCTP26 plants did not show any symptoms, and TCTP11 plants showed only small chlorotic spots. The determination of the viral load showed that these plants accumulated a lower virus titre than WT, extending the observations from the early infection in *N. benthamiana*. Thus, decreased TCTP expression impaired, but did not prevent, PepYMV accumulation. The

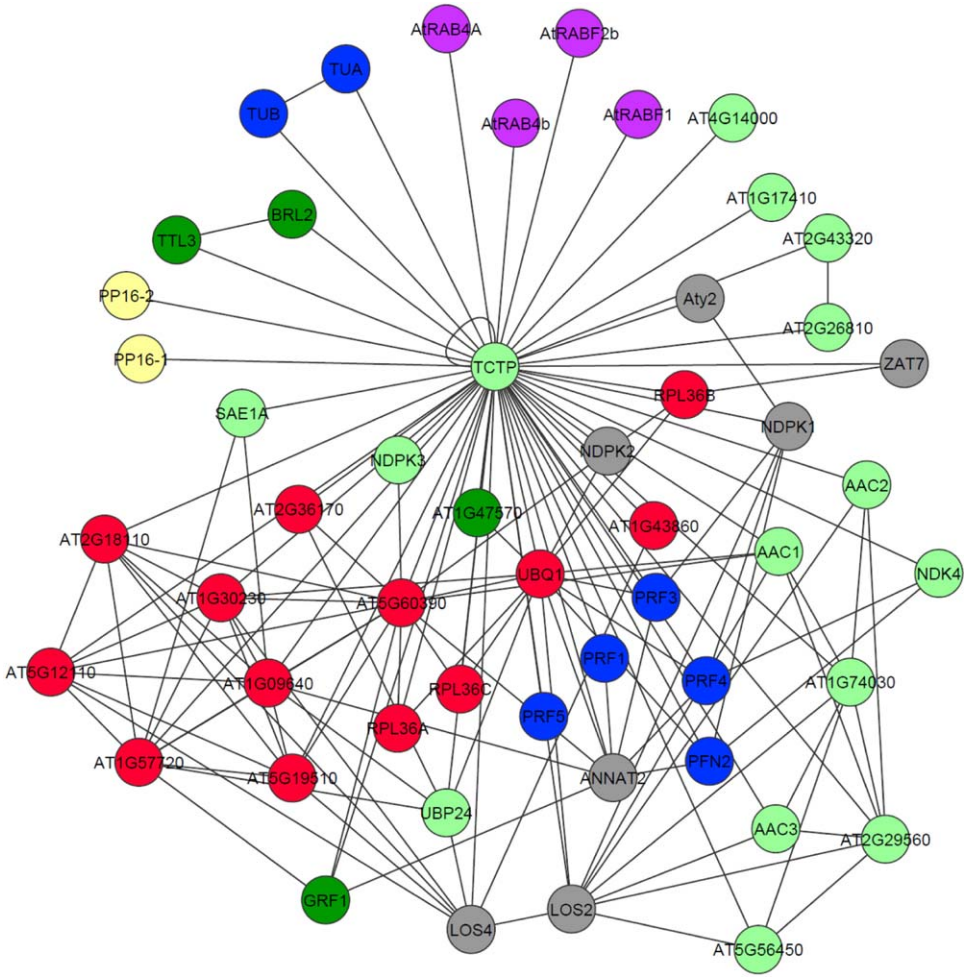

Fig. 8 Network of described and putative interactions between AtTCTP and *A. thaliana* proteins. Circles represent *A. thaliana* proteins, and lines represent the interactions indicated by the String Database or described in the literature. Groups of proteins discussed in the text are highlighted in different colours: red, proteins related to protein biosynthesis; grey, proteins related to stress responses; green, proteins related to growth and development; yellow, proteins involved in vascular transport; blue, proteins related to cytoskeleton composition and organization; purple, proteins of the plant Rab family. Other proteins are shown in light green. The network was generated using Cytoscape software.

low level of TCTP expression may have sufficiently supported low levels of viral infection. It is important to point out that it was not possible to silence TCTP completely, probably because of the multiple essential roles of this protein in plant development.

As a result of the different processes involving TCTP, it is difficult to determine the exact role of this protein during potyviral infection. Potyviruses replicate inside membranous structures, named viral replication factories, which contain the viral replication complex (VRC) formed by viral RNA, a number of viral proteins and host factors. VRCs are induced by the viral protein 6K2 (Schaad *et al.*, 1997; Wei & Wang, 2008) and are composed of the membranes of the endoplasmic reticulum, Golgi and chloroplast, and COPII coatomers (Grangeon *et al.*, 2012). There are two types of induced structure: the perinuclear globular structure and motile cortical vesicular structures (Grangeon *et al.*, 2012). The second is derived from the first and moves within the cell through actin microfilaments (Cotton *et al.*, 2009). Recently, Grangeon *et al.* (2013) have demonstrated that these vesicles move cell to cell in *Turnip mosaic virus* (TuMV)-infected plants, suggesting that the membranous vesicles within the VRC are the entities that move cell to cell, connecting replication and movement to the same structure in the cell.

In TCTP-silenced plants, we observed lower viral RNA accumulation in systemically infected leaves relative to inoculated leaves, suggesting that TCTP could be involved in viral movement. Some proteins that interact with TCTP could be associated with viral movement. Five profilins (PFN2, PRF1, PRF3, PRF4 and PRF5) have been identified in humans, *Drosophila* and yeast (Burgess *et al.*, 2008; Cans *et al.*, 2003; Guruharsha *et al.*, 2011; Ziv *et al.*, 2011) that potentially interact with TCTP. These proteins interact with actin and act in microfilament dynamics. Therefore, it is reasonable to speculate that, in plants, TCTP may participate in the viral cell-to-cell movement process by interacting with profilins. Another interaction that could be related to viral movement is that of CmTCTP with CmPP16-1 and CmPP16-2, two proteins in pumpkin phloem (Aoki *et al.*, 2005). It has been proposed that TCTP is involved in the selective transport of molecules in the phloem. AtTCTP interacts with BRL2 and TTL3, two proteins that control vascular differentiation (Ceserani *et al.*, 2009). BRL2 is a membrane receptor-like kinase, and TTL3 is an adaptor protein that interacts with its cytoplasmic portion. It has been suggested that these proteins may be involved in vesicular trafficking during vascular development (Ceserani *et al.*, 2009).

Another possibility is that decreased viral RNA accumulation in systemically infected leaves is a consequence of defective viral replication/translation in TCTP-silenced plants. In our search for TCTP interactors, we identified 13 proteins involved in translation. AT1G43860 is a transcriptional factor that is involved in rRNA synthesis. UBQ1, AT2G36170 (UBQ2), RPL36C, RPL36B and RPL36A are constituents of the large ribosomal subunit, and AT5G60390, AT5G19510, AT5G12110, AT2G18110, AT1G30230, AT1G57720 and AT1G09640 are translational elongation factors. The interactions of human TCTP with eEF1-α and its guanine nucleotide exchange factor, eEF1B-β, have been demonstrated by Cans *et al.* (2003). TCTP impairs GDP–GTP exchange by eEF1B-β, favouring the inactive form of eEF1A-GDP. Interestingly, potyviral proteins interact directly with eIF4E, eEF1-α and PABP, all of which are involved in the translation process, and eEF1-α is a partner of TCTP. The interaction of VPg with eIF4E or its isoform eIF(iso)4E is necessary for infection in several potyvirus–host pathosystems (Léonard *et al.*, 2000; Wittmann *et al.*, 1997). Eskelin *et al.* (2011) have demonstrated that the VPg–eIF4E interaction reduces the translation of capped RNAs and increases viral RNA translation. It also increases the affinity of eIF4G for eIF4E, further reducing capped RNA translation (Michon *et al.*, 2006). eEF1-α interacts with NIa and NIb of TuMV (Thivierge *et al.*, 2008) and co-localizes with PABP, eIF4E and HSC-70 in replication vesicles. If the eEF1-α–TCTP interaction was confirmed for plant TCTP, it could link TCTP to the viral factory, where it might affect translation or replication process.

The interactions of TCTP with proteins that are related to growth and development are compatible with its previously described role in growth control. Berkowitz *et al.* (2008) have suggested that TCTP controls growth by acting in the TOR pathway, based on the structural similarity of TCTPs and the fact that silencing of TCTP and TOR in *A. thaliana* leads to very similar phenotypes (Berkowitz *et al.*, 2008; Deprost *et al.*, 2007). The role of TCTP in TOR signalling has been demonstrated in *Drosophila* and humans (Dong *et al.*, 2009; Hsu *et al.*, 2007). TCTP interacts with Rheb, a Ras GTPase that is activated by TCTP and, in turn, activates TOR. Plants do not have a Rheb homologue, but other small RAS GTPases (AtRABA4a, AtRABA4b, AtRABF1 and AtRABF2b) that interact with TCTP have been identified (Brioudes *et al.*, 2010). TOR is a kinase that acts as a central regulator and integrator of signals, such as nutrient and sugar availability, stress and hormones (Wullschleger *et al.*, 2006; Zoncu *et al.*, 2011). It has been well characterized in mammals and regulates cellular growth, protein synthesis and ribosome biogenesis (Wang & Proud, 2006). In plants, TOR is implicated in cell wall remodelling (Leiber *et al.*, 2010) and mRNA translation (Deprost *et al.*, 2007). These functions are important for efficient viral infection, and thus we speculate that TCTP could link the TOR pathway with viral infection and with the development of symptoms, as infected plants usually exhibit reduced growth.

The subcellular localization of TCTP was analysed in healthy and both local and systemically infected leaves of *N. benthamiana*. In healthy plants, TCTP was present in the nucleus and cytoplasm of epidermal cells, and this pattern remained in locally infected leaves. In systemically infected plants, TCTP was redirected completely to the cytoplasm, and aciculate projections were observed. In plants, AtTCTP localization was determined to be

exclusively cytoplasmic in epidermal cells of seedlings (Hoepflinger *et al.*, 2013). However, TCTP localization in human cells is dynamic. In (cancerous) HeLa cells, TCTP localizes predominantly in the nucleus (Li *et al.*, 2001) or in both the nucleus and cytoplasm (Ma & Zhu, 2010). TCTP localization in PWR-IE cells, a non-neoplastic human prostate epithelial cell line, is cytoplasmic (Arcuri *et al.*, 2004). Zhang *et al.* (2012) showed that TCTP expression can be induced in normal AG1522 human fibroblasts by irradiation and that, in this case, TCTP accumulates in the nucleus, where it aids in DNA damage repair. In eosinophilic granuloma cells, TCTP nuclear localization is controlled by sumoylation. Mutations at the sumoylation site impair nuclear localization and the ability of TCTP to protect against oxidation stress (Munirathinam & Ramaswamy, 2013).

Analysis of the SlTCTP sequence reveals the presence of a putative NLS that is absent in human TCTP, and the absence of the sumoylation site described by Munirathinam & Ramaswamy (2013). Furthermore, a nuclear export site is present. Post-translational modifications or protein interactions may control TCTP localization in plants in a different manner than in humans, and localization may be associated with the different roles of TCTP in each cellular compartment. During infection, the virus may redirect TCTP to the cytoplasm, where it promotes viral infection.

In summary, we have demonstrated that TCTP is an important host factor for potyviral infection and that the virus interferes with the subcellular localization of this protein, probably as a result of the involvement of TCTP in some crucial stage of the infection process. Further studies should be performed to elucidate the exact role of TCTP during PepYMV infection.

EXPERIMENTAL PROCEDURES

Plant material

Nicotiana benthamiana plants were germinated and, after 2 weeks, transferred to growth chambers with a controlled temperature (22°C) and photoperiod (14 h/10 h light/dark). Plants were maintained under these conditions for at least 5 days before being agroinoculated and until the end of the experiments. Tomato (*Solanum lycopersicum* cv. Moneymaker) seeds were disinfected and germinated *in vitro*, and hypocotyl segments were used for transformation.

Viral isolate

The PepYMV isolate 3 has been described by Truta *et al.* (2004) and was maintained in *Nicotiana debneyi* plants by successive sap inoculations in potassium phosphate buffer (0.2 M, pH 7.2) with 1% sodium bisulfite. Infected *N. debneyi* plants were maintained in a glasshouse.

Viral inoculation

PepYMV was sap inoculated to *N. benthamiana* and tomato plants as described above. Viral infection was confirmed by indirect enzyme-linked immunosorbent assay (ELISA) with a specific polyclonal antibody at 14 or 21 days post-inoculation, as described by Truta *et al.* (2004).

Cloning and sequencing

Plasmids were constructed for VIGS, protein expression and plant transformation. To induce silencing, cDNA fragments of approximately 400 nucleotides for each gene [*TCTP*, AY642284.1; *PDS*, NM_202816.2; *GFP*, amplified from pK7WFG2 (Karimi *et al.*, 2002)] were cloned in RNA2 of the TRV viral vector (Liu *et al.*, 2002), resulting in the constructs TRV2-TCTP, TRV2-PDS and TRV2-GFP. For tomato transformation, a 285-nucleotide fragment of the TCTP gene, corresponding to the central region of the mRNA, was cloned into the Gateway entry plasmid pENTR11 (Invitrogen, Carlsbad, CA, USA) and then subcloned into pK7GWIWG2 (Karimi *et al.*, 2002), resulting in pK7GWIWG2-TCTP. This construct contains two inverted copies of the TCTP sequence and expresses an RNA that forms a hairpin. For the transient expression of the TCTP-GFP fusion, the complete TCTP coding sequence was amplified from tomato cDNA by PCR with specific primers (TCTP-EcoRI F, 5′-ACTGGAATTCTGTTGGTTTATCAGG-3′; TCTP-XhoI R, 5′-ACTGCTCGAGCTAGCACTTGATC-3′) and cloned into pENTR11. Then, the sequence was transferred to the binary vector pK7FWG2 (Karimi *et al.*, 2002), resulting in the plasmid pK7FWG2-TCTP. All plasmid constructs were sequenced at Macrogen Inc. (Seoul, Korea) to confirm their integrity.

Agrobacterium transformation

All recombinant plasmids were initially transformed into *Escherichia coli* DH5α by the heat shock method (Sambrook & Russel, 2001). Then, TRV1/TRV2 plasmids were separately transformed into *Agrobacterium tumefaciens* strain C58C1, and pK7FWG2-TCTP/pK7GWIWG2-TCTP were transformed into *A. tumefaciens* strain GV3101. All *A. tumefaciens* transformations were performed using the heat shock method described by Brasileiro and Carneiro (1998).

Virus-induced gene silencing (VIGS)

To induce transient gene silencing, cultures of *A. tumefaciens* C58C1 that had been transformed with the appropriate constructs were grown to an optical density at 600 nm (OD_{600}) of 0.6. These cultures were then centrifuged at 9 000 g and resuspended in agroinfiltration buffer [2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM; $MgCl_2$, 10 mM; acetosyringone, 200 μM], adjusting the OD_{600} to 1.2. Equal volumes of suspensions of agrobacteria transformed with TRV RNA1 and RNA2 were mixed and infiltrated in the abaxial surfaces of the leaves of *N. benthamiana* (3 weeks post-germination) using sterile syringes without needles. Ten plants were agroinfiltrated with TRV1 + TRV2-TCTP, six with TRV1 + TRV2-PDS and six with TRV1 + TRV2-GFP. Six plants were agroinfiltrated with non-transformed agrobacteria.

After the establishment of the silencing phenotype (approximately 2 weeks after agroinfiltration), seven plants silenced to TCTP and three plants for the control treatments (TRV1 + TRV2-PDS or TRV1 + TRV2-GFP) were sap inoculated with PepYMV (as described above), and three plants were mock inoculated with inoculation buffer only. Leaf discs (1.5 cm) were collected from the young leaves systemically infected with TRV immediately before viral inoculation. At 72 hpvi, leaf discs were

collected from PepYMV-inoculated leaves. These discs were collected from the same leaf and were located next to each other. Systemic young leaves were collected at 14 and 21 dpvi. The collected material was immediately frozen in liquid nitrogen and stored at −80°C. The experiments were repeated at least twice.

Plant transformation

Seeds were surface sterilized by immersion (1 min) in 70% (v/v) ethanol, followed by immersion (20 min) in 2.5% (v/v) sodium hypochlorite with three drops of Tween-80 per 100 mL of solution, and rinsed four times in ultrapure autoclaved water. The seeds were subsequently transferred to 250-mL culture flasks (30 flasks with 10 seeds per flask) containing 60 mL of half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 1.5% (w/v) sucrose and 0.60% (w/v) Agargel (Sigma-Aldrich, St. Louis, MO, USA), and adjusted to pH 5.8 ± 0.1. Unless otherwise stated, media were sterilized by autoclaving at 121°C and 1.1 Pa for 15 min. The flasks were sealed with rigid polypropylene closures with two orifices (10 mm) that were covered with 0.45-μm (pore size) adhesive membranes (Milliseal AVS-045, Air Vent, Tokyo, Japan). The flasks were kept in the dark for 15 days until the seeds had germinated. The seedlings were then transferred to a temperature-controlled growth chamber with a 16-h/8-h (light/dark) regime, an irradiance of 36 μmol/m^2/s (provided by fluorescent tubes, 20 W) and 27 ± 2°C for 15 days. Hypocotyl segments (average of 10 mm in length) were used for *Agrobacterium*-mediated transformation with the construct pK7GWIWG2-TCTP, as described by Otoni *et al.* (2003).

Total DNA of transformed plants was extracted according to Doyle & Doyle (1987) and used as a template for PCR with specific primers for the *npt*II gene (NPTIIF, 5′-TCAGCGCAGGGGCGCCCGGTT-3′; NPTIIR, 5′-GCGGTCAGCCCATTCGCC-3′). Amplicon size was checked by electrophoresis in 0.7% agarose gels.

RNA extraction and cDNA synthesis

Total RNA from *N. benthamiana* plants was extracted using the RNeasy Plant Mini Kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. Total RNA from tomato plants was extracted from approximately 100 mg of leaf tissue using the Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and quantity were measured with a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and by electrophoresis in 1% agarose gels.

One microgram of total RNA was treated with DNase I (Promega, Madson, WI, USA), using twice as much enzyme as indicated by the manufacturer (2 units of enzyme per microgram of RNA). The treated RNA was employed for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions, and oligo-dT as a primer.

qRT-PCR

TCTP expression levels in transgenic tomato and VIGS *N. benthamiana* plants were quantified by qRT-PCR using the comparative cycle threshold method (ΔΔCt) (Livak & Schmittgen, 2001). Absolute quantification was performed to determine viral accumulation. A standard curve was obtained by regression of Ct values, using 10^0 to 10^6 copies of a plasmid containing the PepYMV coat protein (CP) coding region. Viral accumulation was determined by interpolation of the Ct values of each tested sample within the standard curve (Rutledge & Cote, 2003).

All reactions were performed in triplicate using Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) in a final volume of 10 μL, and the CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Primer sequences are listed in Table S1 (see Supporting Information).

Flow cytometry analysis

Approximately 30 mg of fresh leaf tissue from fully acclimatized plants was finely chopped with a disposable steel razor blade in 1 mL of LB01 buffer to release the nuclei (Dolezel & Bartos, 2005). *Solanum lycopersicon* 'Stupike' (2C DNA content = 1.93 pg) was used as an internal reference standard (Dolezel *et al.*, 2007). Previously macerated tissues were aspirated through two layers of cheesecloth with a plastic pipette, filtered through a 50-μm nylon mesh and collected in a polystyrene tube. The suspension was stained with 25 μL of 1 mg/mL propidium iodide (Sigma), and 5 μL of RNase (Amresco, Solon, OH, USA) was added to each sample. Samples were incubated at 4 °C in the dark and examined after 1–2 h. At least 10 000 nuclei were analysed for each sample. Analyses were performed with a flow cytometer (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ, USA) at the Institute of Biological Sciences (ICB), Federal University of Juiz de Fora (UFJF), Brazil. Cytometric histograms were generated and analysed using Cell Quest and WinMDI 2.8 software (facs.scripps.edu/software.html).

Protein extraction and Western blot

Leaf tissue of WT or transgenic tomato plants (0.3 g) was ground with liquid nitrogen and boiled with 1 mL of extraction buffer [150 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% sodium dodecylsulfate (SDS), 10% glycerol] for 3 min. The extract was analysed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore, Billerica, MA, USA). The membrane was blocked with powdered milk 5% (w/v) in PBS-T (Phosohate buffered saline - tween20) and incubated with specific polyclonal rabbit antiserum against tomato TCTP, produced in the Laboratory of Industrial Microbiology (Universidade Federal de Viçosa, Viçosa, Brazil). The membrane was washed in PBS-T and incubated with secondary goat anti-rabbit immunoglobulin G (IgG), conjugated to alkaline phosphatase (Bio-Rad). Then, colorimetric protein detection was performed with an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad), according to the manufacturer's instructions.

Subcellular localization of TCTP in healthy and PepYMV-infected *N. benthamiana*

Nicotiana benthamiana plants at approximately 40 days after germination, which had been maintained at 22°C, were inoculated with PepYMV or mock inoculated with buffer only as described above. Ten days later, the plants were infiltrated with a suspension of *A. tumefaciens* GV3101 transformed with pK7WGF2-TCTP to express a SlTCTP-GFP fusion protein. The transformed bacteria were grown in medium with the appropriate antibiotics to an OD_{600} of 0.6, centrifuged at 9 000 g and resuspended in agroinfiltration buffer to adjust the OD_{600} to 0.1. Agroinfiltration was

performed as described above for the VIGS experiments. Confocal images were acquired 48 h after agroinfiltration using a laser confocal scanning microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany) and processed with the software LSM Image Browser 4 (Carl Zeiss).

Construction of a TCTP-based *in silico* interaction network

The *A. thaliana* genome encodes two TCTP homologues: At3g04450.1 and AT3G16640.1. At3g04450.1, the most similar sequence to SlTCTP (90%), has been reported to be a non-functional pseudogene (Berkowitz *et al.*, 2008). Thus, AtTCTP AT3G16640.1 (83% similarity to SlTCTP) was used to identify putative interactions with other *A. thaliana* proteins employing the String Database online software (Franceschini *et al.*, 2013). Only the prediction methods 'Experiments', 'Database' and 'Text mining' were used during the search, which was performed with a score of 0.150 (low confidence). Each putative interaction was checked in the original reference, and only validated information was added to the network. Known interaction partners of TCTPs from other plant and animal species that did not appear in the String data were added manually. The connections were compiled and used to construct the network using Cytoscape 3.0.2 software.

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AUTHOR CONTRIBUTIONS

PA-Z directed the project. FMZ and PA-Z designed the experiments. FPB performed the VIGS experiments. AdSX, FPB, RdSC and WCO performed the experiments with transgenic plants. AdSX and RdSC performed the subcellular localization assay. FPB built the *in silico* interaction network. FPB, FMZ and PA-Z wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Primers used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

Fig. S1 Plant tomato translationally controlled tumour proteins (TCTPs) are strongly conserved.

Fig. S2 *Nicotiana benthamiana* silencing phenotype 2 weeks after agroinoculation.

Fig. S3 Symptoms of *Pepper yellow mosaic virus* (PepYMV) systemic infection at 14 days post-viral inoculation (dpvi) in virus-induced gene-silenced plants.