FERNANDA PRIETO BRUCKNER

THE TRANSLATIONALLY CONTROLLED TUMOR PROTEIN IS NECESSARY FOR AN EFFICIENT POTYVIRUS REPLICATION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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SUMÁRIO

ABSTRACT

Bruckner, Fernanda Prieto, D.Sc., Univeridade Federal de Viçosa, November, 2016. **The Translationally controlled tumor protein is necessary for potyvirus replication.** Advisor: Francisco Murilo Zerbini Junior. Co-advisor: Poliane Alfenas-Zerbini.

The translationally controlled tumor protein (TCTP) is widely distributed among eukaryotes. It is involved in the regulation of basic processes such as cell cycle progression, cell growth, stress protection and apoptosis. During tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* infection by the potyvirus *Pepper yellow mosaic virus*, an increase of *TCTP* mRNA levels was observed. Plants silenced for *TCTP* accumulate fewer viruses than control plants, showing the importance of that gene for potyvirus infection. In this work, TCTP involvement in potyvirus infection was analyzed in details using the potyvirus *Turnip mosaic virus* (TuMV). *N. benthamiana* plants silenced for *TCTP* accumulated fewer viruses than non-silenced plants. In addition, plants overexpressing TCTP transiently accumulated more viruses than control plants, confirming that TCTP has a positive effect on infection by different potyviruses. To study TCTP subcellular localization in potyvirus infected plants, TCTP fused to GFP was co-expressed with TuMV/6K2:mCherry. Confocal analysis has shown that TCTP co-localizes with 6K2-tagged structures such as replicative vesicles and the perinuclear globular structure that is typically observed in potyvirus-infected cells. Cellular fractioning demonstrated that TCTP is mainly present in the soluble fraction but is also associated with membranes. The co-localization of TCTP with 6K2-tagged vesicles and its presence in cellular membranous fractions suggests a possible involvement of TCTP in virus replication. To test this hypothesis, protoplasts obtained from TCTP silenced plants were infected with TuMV and its mutant TuMV^{VNN}, which is defective for replication. The results showed that TuMV accumulation is reduced in silenced protoplasts, indicating that TCTP is necessary for replication. TCTP accumulation during infection was also analyzed. Viral infection induces *TCTP* mRNA expression, but not protein accumulation, suggesting that the *TCTP* mRNA and not the protein has a role in viral infection. To check this, we expressed a non-translatable form of TCTP RNA in plants and analyzed its effect in virus accumulation. The results showed that only the expression of a translatable RNA resulting in protein production is able to increase virus infection, indicating that the protein and/or the translation of TCTP is important for potyvirus replication.

RESUMO

Bruckner, Fernanda Prieto, D.Sc., Univeridade Federal de Viçosa, novembro de 2016. **Translationally controled tumor protein é necessária para a replicação de potyvírus.** Orientador: Francisco Murilo Zerbini Junior. Coorientadora: Poliane Alfenas-Zerbini.

Translationally controlled tumor protein (TCTP) é uma proteína amplamente distribuída em eucariotos. Ela está envolvida na regulação de processos básicos como progressão do ciclo celular, crescimento celular, proteção contra estresses e apoptose. Durante a infecção de tomateiro (*Solanum lycopersicum*) e *Nicotiana benthamiana* pelo potyvírus *Pepper yellow mosaic virus* ocorre aumento dos níveis de seu mRNA. Plantas silenciadas para *TCTP* acumulam menos vírus do que plantas selvagens, mostrando que esta proteína é importante para a infecção por potyvírus. Neste trabalho, o envolvimento da TCTP na infecção por potyvírus foi analisado detalhadamente utilizando-se o potyvírus *Turnip mosaic virus* (TuMV). Em plantas de *N. benthamiana* silenciadas para TCTP também ocorre uma diminuição no acúmulo do TuMV quando comparado com plantas não silenciadas. Além disso, plantas superexpressando TCTP de maneira transiente acumularam mais vírus do que plantas controle, confirmando o efeito positivo desta proteína na infecção por diferentes espécies de potyvírus. Para analisar a localização subcelular de TCTP no contexto da infecção, TCTP fusionada a GFP foi co-expressa com TuMV/6K2:mCherry. TCTP co-localiza-se com as vesículas replicativas e com estrutura a globular perinuclear tipicamente observada em células infectadas. O fracionamento de proteínas celulares demonstrou que TCTP está predominantemente na fração solúvel e uma pequena porção se associa com membranas, tanto em plantas sadias quanto em plantas infectadas. A co-localização com vesículas marcadas por 6K2 e a presença de TCTP em frações membranosas da célula sugerem um possível envolvimento desta proteína na replicação viral. Para verificar esta hipótese, protoplastos obtidos a partir de plantas silenciadas para TCTP foram infectados com TuMV e com o mutante TuMV^{VNN}, o qual não é capaz de replicar-se. Os resultados demonstraram que o acúmulo de TuMV é reduzido em protoplastos silenciados, indicando que TCTP é necessária para a replicação. O acúmulo da proteína TCTP durante a infecção também foi avaliado. A infecção viral induz o aumento dos níveis de mRNA mas

não de proteína, sugerindo que o mRNA que codifica TCTP atue na replicação. Desta forma, foi analisado se expressão de um RNA não traduzível de TCTP possui efeito sobre a infecção viral. Os resultados mostraram que apenas a expressão de um RNA traduzível é capaz de aumentar a infecção viral, indicando que a proteína TCTP, ou que a tradução se seu mRNA, é importante para a replicação viral.

INTRODUCTION

Viruses infect organisms in all life domains. Because of their compact genome and parasitic way of life, they depend upon host cells to perform all the necessary steps for virus multiplication and spread. Plant viruses have small genomes and encode about three to thirteen proteins (King et al., 2011). As plant pathogens, viruses may cause serious losses in important crops around the world and cause a variety of symptoms and damage, reflecting virus adaptation to a specific host and the efficiency of plant defense responses (Culver and Padmanabhan 2007).

To successfully infect a plant, the virus needs to suppress host defenses and also be able to manipulate the host cell physiology and metabolism in its favor. Therefore, during plant-virus co-evolution, complex interactions were established, involving pathogen attack and host defense mechanisms. During the infection, intimate interactions have to be established, and as consequence, alterations occur in host cell gene expression pattern, hormonal balance, morphology and physiology.

Potyviruses are single-strand positive-sense RNA viruses that belong to the genus *Potyvirus* in *Potyviridae* family. The genus contains 162 species, which together infect a wide range of plants around the world. Their genome is constituted by a single RNA strand with about 10Kb in length, linked covalently to the viral protein VPg at the 5´extremity and to a poly(A) tail at the 3´extremity (Adams et al., 2011). The nucleic acid is encapsidated in a long flexuous particle with about 680-900 nm in length and 11-13 nm of diameter (Bock & Conti, 1974).

Potyvirus genomes have two ORFs. The main ORF express a large polyprotein that is self-processed by viral proteases, producing all viral mature proteins except the protein P3N-Pipo, which results for the expression of the ORF named PIPO by transcriptional slippage (Chung et al. 2008; Vijayapalani et al. 2012; Olspert et al. 2015).

Potyvirus replication occurs in association with endoplasmic reticulum (ER) membranes. During the infection, at least two types of membranous structures induced by virus infection are observed by light microscopy, vesicle-like structures and a globular structure usually located near to the nucleus (Schaad et al., 1997; Grangeon et al., 2012). These ‘vesicles’ are believed to be the replication site and several viral and host factors involved in replication were located within them (Beauchemin et al., 2007; Dufresne et al. 2008; Thivierge et al. 2008; Cotton et al. 2009; Wei et al. 2010). The viral protein 6K2 is involved in membrane remodeling and can be used as a marker for the vesicles and for the globular structure. The globular structure role is not clear. It is composed by ER, chloroplasts and Golgi membranes, 6K2 and host proteins, and is functionally connected with replication vesicles (Grangeon et al. 2012).

In this work, we have studied the involvement of the protein TCTP in potyvirus infection. *TCTP* was identified as an up-regulated gene in a transcriptomic study that has described differentially expressed genes in tomato (*Solanum lycopersicum*) plants infected by the potyvirus *Pepper yellow mosaic virus* (Alfenas-Zerbini et al. 2009). TCTP is a multifunctional protein involved in several cellular processes such as cellular growth, development, resistance against stress responses and apoptosis (Bommer and Thiele 2004).

The importance of TCTP for virus infection was demonstrated by the observation that *N. benthamiana* and tomato plants silenced to *TCTP* accumulate fewer viruses than control plants (Bruckner et al., 2016). In the present work, we used the potyvirus *Turnip mosaic virus* (TuMV) to thoroughly analyze the TCTP involvement in virus cycle. *TCTP* silencing leads to a decrease in virus accumulation, while TCTP overexpression has the opposite effect, confirming TCTP’s positive effect on virus infection. Confocal microscopy analysis showed that in infected cells, TCTP co-localizes with 6K2-tagged vesicles and with the perinuclear globular structure. Protoplasts silenced for TCTP present a decrease in virus accumulation, suggesting an effect on replication. Protein accumulation was verified in the first days of infection, and interestingly, *TCTP* mRNA levels increase during the infection but protein levels do not. It led us to hypothesize that *TCTP* RNA has a role in virus infection. However, the overexpression of a non-translatable form of *TCTP* has no effect on virus accumulation, indicating that protein production or RNA translation is necessary for TCTP role in potyvirus replication.

LITERATURE REVIEW

The family *Potyviridae*

The family *Potyviridae* is currently composed of 195 species, distributed in eight genera, in which 162 belong to the genus *Potyvirus* and two are unassigned (http://www.ictvonline.org/virustaxonomy.asp). The members of the family are single-strand positive-sense RNA viruses that share a common genome structure, genome expression strategy, particle morphology and phylogenetic relationship. Except for the genus *Bymovirus* that has two components, all genus members have one genomic component. Viral RNA is attached covalently to the viral protein VPg at 5´ terminus and polyadenilated at the 3´terminus. The viral RNA is encapsidated in a filamentous and flexuous particle (Adams et al., 2011).

Protein expression occurs by translation of a large polyprotein that is self-cleaved by three viral proteases. A second ORF named PIPO (from Pretty Interesting *Potyviridae* ORF) is expressed in smaller amount by transcriptional slippage, producing the protein P3N-Pipo (Chung et al. 2008; Vijayapalani et al. 2012; Olspert et al. 2015). The genome strategy leads to protein overproduction. Excess of CI (from Cilindrical Inclusion) protein accumulates in the cytoplasm, forming typical pinwheel-shaped inclusions that are characteristic for the family (Edwardson, 1968). The proteins NIa and NIb (from Nuclear Inclusion A and B) accumulate in the nucleus, forming amorphous inclusions (Riedel, et al. 1998).

Genus *Potyvirus*

As described in the section above, the genus *Potyvirus* is the largest and the most studied genus in *Potyviridae* family. Most potyviruses species produce 11 mature proteins, named P1, HC-Pro, P3, P3N-Pipo, 6K1, CI, 6K2, NIa (also named VPg-Pro), VPg, NIa-Pro, NIb and CP. A group of phylogenetic related potyviruses that infects sweet potato (*Ipomea batatas*) presents an additional ORF called PISPO (from Pretty Interesting Sweet potato Potyvirus ORF) (Clark et al., 2012). It is proposed to be expressed by the same mechanism of ORF PIPO, producing the fusion protein P1N-Pispo (Mingot et al. 2016; Untiveros et al. 2016).

Most potyviral proteins are multifunctional, being involved in several processes of virus cycle. Protein P1 is the most diverse potyvirus protein. It has a protease domain and is responsible for the cleavage of its C-terminal extremity from HC-Pro protein. P1 is not strictly required for virus infection, but its expression amplifies virus replication. P1 seems to modulate viral infection, contributing for virus escaping from plant defense responses (Pasin, et al. 2014). Also, P1 seems to stimulate HC-Pro silencing suppressor activity (Rajamäki et al. 2005; Valli et al. 2006). Recently, the discovery of P1N-Pispo has indicated that both P1 and P1N-Pispo from *Sweet potato feathery mottle virus* (SPFMV) act as silencing suppressors by different mechanisms (Untiveros et al. 2016).

HC-Pro (Helper-Component Proteinase) is involved in a myriad of important viral processes. It has a papain-like cysteine proteinase domain that is responsible for the cleavage of its C-terminus from the P3 N-terminus. HC-Pro acts as a helper component in potyvirus transmission. Potyviruses are transmitted by aphids in a non-persistent manner. HC-Pro is involved in virus transmission by interacting with CP protein and the aphid stylet, connecting them (Pirone and Blanc 1996). HC-Pro is also a powerful silencing suppressor. It interacts with small RNA duplexes, and probably acts by sequestering them, impairing their incorporation in gene silencing machinery (Shiboleth et al. 2007; Lakatos et al.

2006). HC-Pro silencing suppressor activity may be also associated with its ability to interact with S-adenosyl-L-methionine synthase and S-adenosyl-L-homocysteine hydrolase. Those two enzymes are components of the methionine cycle that provides S-adenosyl-L-methionine for siRNA metilation, which is essential for siRNA incorporation in RISC (RNA-induced silencing complex). The authors also have shown that HC-Pro forms a stable complex with AGO1 in association with ribosomes, suggesting an effect in translation-repression gene silencing (Ivanov et al. 2016). There is also evidence of HC-Pro participation in cell-to-cell and systemic movement, however, the mechanisms involved remains unknown (Rojas et al. 1997; Sáenz et al. 2002).

The role of P3 in potyvirus cycle is poorly understood. It is suggested that it may be involved in virus replication, systemic infection and pathogenicity (Urcuqui-Inchima et al., 2001). The P3 for *Tobbaco etch virus* (TEV) forms punctate structures in association with Golgi that traffic along actin microfilaments and co-localize with 6K2-tagged vesicles, suggesting a role in replication and movement (Cui et al., 2010). The ORF PIPO is embedded within P3 sequence (Chung et al. 2008). It is expressed by transcriptional slippage, producing the protein P3N-Pipo, which has the N terminal region of P3 and C terminal corresponding to Pipo domain (Vijayapalani et al. 2012; Olspert et al. 2015). P3N-Pipo is essential for cell-to-cell virus movement. It localizes in plasmodesmata and interacts with CI, other potyvirus protein involved in virus movement. P3N-Pipo also interacts with the host protein PCaP1 through the Pipo domain. Virus infection in *Arabidopsis* plants knockout for PCaP1 is restrict to inoculated cells, suggesting its involvement in cell-to-cell movement, which is corroborated by the fact that the virus replicates normally in protoplasts from the same plants (Vijayapalani et al. 2012).

6K1 is a 6KDa protein predicted to be an integral membrane protein. However, when ectopically expressed, 6K1 locates at cytoplasm and nucleus and is not present in cellular membranous fraction, being a soluble protein (Jiang et al. 2015). Working with *Plum pox virus* (PPV) mutants, Cui and Wang (2016) have demonstrated that 6K1 is essential for virus infection. The release from the polyprotein by NIa cleavages and also the correct position of 6K1 sequence are essential for its function. The authors also have demonstrated that in infected plants, 6K1 forms punctate structures that co-localizes with 6K2 and NIb, suggesting a role in virus replication.

CI is a protein with multiple roles, and also multiple partners. It has a RNA helicase domain and is part of the replication complex. It is also involved in virus movement by interacting with P3N-Pipo, as described above. There is also genetic evidence of CI involvement in systemic long-distance movement, but the exact involved mechanism is unknown (reviewed by Sorel et al. 2014).

The protein 6K2 is an integral membrane protein that associates with ER membranes, inducing membrane remodeling and vesicle formation. During the infection, 6K2 is believed to act as an anchor for the replicative complex in ER membranes (Schaad et al., 1997). Moreover, it coats several kinds of membrane structures that are induced during the infection, including a large globular structure that contains ER, Golgi and chloroplasts membranes, and also components of the early secretory pathway, and small vesicles (Grangeon et al. 2012).

NIa or VPg-Pro is a protein with two domains separated by a weak self-proteolytic site, found as a single protein and also as two separated proteins VPg and NIa-Pro, which correspond to NIa protease domain. NIa is the main protease of potyvirus and it cleaves all the proteins junctions (with exception of cleavage sites for P1 and HC-Pro) with differential affinity for each site, which confers a sequential production of potyvirus proteins (Daròs and Carrington, 1997). VPg acts in the replication as a primer for the attachment of a uridine to the free hydroxyl group of a tyrosine residue (Anindya, et al. 2005). It also interacts with NIb and several host factors involved in virus infection.

NIb is the RNA-dependent RNA polymerase (RdRp) that replicates viral genome. Its catalytic active site contains the conserved GDD motif that is found in several viral RdRps (Kamer & Argos, 1984). NIb also interacts with many host factors that are usually described as part of replication complex.

The capsid protein (CP) is also a multifunctional protein. In addition to encapsidating the virus and participating in its transmission, it also has roles in cell-to-cell and long distance movement (Dolja et al., 1994). Moreover, CP was described to be involved in regulation of potyvirus translation (Hafrén et al. 2010).

Potyvirus replication

Potyvirus infectious process is a combination of several interconnected steps, resulting in a complex coordinated network of events regulated over time and space. Viral translation, RNA replication, cell-to-cell and systemic movement, encapsidation and counter-defense mechanisms, are necessary for a successful infection (reviewed by Mäkinen & Hafrén 2014). These processes are interconnected and sometimes simultaneous. Besides, as several potyvirus proteins are multifunctional, they participate on several steps of the viral cycle. In this topic, we are going to describe the current knowledge about potyvirus replication, highlighting the involvement of host proteins.

For potyvirus replication, a replicative complex is assembled anchored to ER membranes. The 6K2 portion of the still not completely processed peptide 6K2-VPg-Pro is responsible for anchoring the replicative complex and also for inducing membrane remodeling forming vesicle-like structures (Schaad et al., 1997). The VPg-Pro protein interacts with NIb protein, recruiting it to the replicative complex (Daròs et al., 1999). NIb is the RNA-dependent RNA polymerase that replicates virus genome and acts as a core of the viral replicative complex (VRC), which involves viral and host proteins. The VPg protein has a tyrosine residue that provides a free hydroxyl group that is uridilated by NIb to initiate RNA polymerization of a complementary RNA strand (Puustinen and Mäkinen 2004; Anindya, et al. 2005). The negative RNA strand is used as a template for generation of new positive strands that will constitute new RNA genomes, able to be translated again, replicated, encapsidated in new virions or to move to another cell.

Cellular membranes are remodeled during virus infection, forming the suitable structure for virus replication. These structures harbor the VRC and are also called viral factories. The membrane-associated viral protein 6K2 induces remodeling of endoplasmic reticulum and replicative structures similar to vesicles are formed (Restrepo-Hartwig and Carrington 1994; Schaad et al., 1997). These vesicles co-localize with the cellular protein complex COPII in ER exit sites (ERES). COPII is a secretory pathway component, and it is suggested that vesicles formation depends on a functional secretory pathway (Wei and Wang

2008). In *Nicotiana benthamiana* cells infected by a TuMV infectious clone that express 6K2 fused to a fluorescent protein, different 6K2-tagged structures are observed. Small vesicles harboring VRCs are found in the periphery of the cell. These vesicles can move along actin microfilaments through cortical and transvacuolar ER (Cotton et al. 2009; Grangeon et al. 2012). A large globular structure is formed during the infection, usually next to the nucleus. It involves ER, Golgi and chloroplasts membranes and also contains COPII coatamers. It seems to be functionally linked to 6K2-tagged vesicles since they move in and out of the globular structure (Grangeon et al. 2012).

Replicative vesicles harboring the VRC are emitted from the globular structure and move towards chloroplasts periphery, inducing invaginations in chloroplast membranes (Wei and Wang 2008; Wei et al. 2010). 6K2 ectopic expression induces chloroplast aggregation and the formation of tubular structures that connect the outter membrane of adjacent chloroplasts (Wei et al. 2013). The reticular SNARE (Soluble N-ethil-maleimide-sensitive-factor attachment protein receptor) proteins Syp71 and Vap27-1 are re-located to chloroplasts by 6K2. The three proteins form a complex located in the junction between two chloroplasts, being Vap27-1 responsible for interact with and connect 6K2 and Syp71. Silencing of Syp71 reduces virus accumulation in protoplasts, suggesting its involvement in virus replication (Wei et al. 2013).

In *N. benhamiana* plants infected by TuMV it was demonstrated that 6K2-tagged vesicles harboring the protein NIb and dsRNA are able to associate with the plasmodesma and move cell-to-cell (Grangeon et al. 2013). Cell-to-cell movement is essential for the establishment of a systemic infection and involves viral and host proteins. Analysis of 6K2-tagged vesicles in several cell-types has revealed that these vesicles are present in phloem sieve elements and xylem vessels. It was also demonstrated that the virus can move through the xylem, being suggested that 6K2-tagged vesicles are also involved in long-distance movement (Wan et al. 2015). Together, these studies have observed 6K2-tagged vesicles in different times and places and give us an idea about how steps of virus infection cycle are interconnected and coupled.

One characteristic of the potyvirus replication is that it may be coupled to virus translation. Cotton et al. (2009) have demonstrated that each 6K2 induced vesicle contains proteins generated from the translation of a unique TuMV genome, suggesting that the translation occurs in association with 6K2-tagged vesicles. Other interesting study has demonstrated that *Potato virus A* (PVA) CP protein may be involved in regulation of the shift between translation and replication. CP accumulation *in cis* but not *in trans* reduces virus translation (Hafrén et al. 2010). The DnaJ-like protein CPIP, which interacts with CP, and the chaperone Hsp70 are proposed to regulate CP acting in replication-associated translation (Hofius et al. 2007; Hafrén et al. 2010).

Other fact that supports the association between potyvirus translation and replication is that a number of plant translation factors interact with virus proteins within replication vesicles. The most studied is the translation factor eIF4E or its isoform eIF(iso)4E. The interaction between eIF4E and VPg protein was described years ago (Wittmann et al. 1997) and observed in several potyviruses (Leonard et al. 2000; Lellis et al. 2002; Nicaise et al. 2003). Recessive resistance genes identified in different crops were identified as codifying of one of these two isoforms. The resistant genes codify proteins with small mutations that impair the interaction with VPg, showing that this interaction is essential for virus infection (reviewed by Maule et al. 2007; Robaglia & Caranta 2006).

Recent studies have indicated that the VPg-eIF4E interaction is important for promoting virus translation and also repress host mRNAs translation. By producing eIF4E mutants, German-Retana et al. (2008) showed that the capacity of lettuce infection by the potyvirus *Lettuce mosaic virus* (LMV) does not depend on the interaction eIF4E-CAP or eIF4E-eIF4G, suggesting that the role in virus replication is different for the biological one. Transient expression of VPg protein from PVA in *N. benthamiana* causes a decrease in translation of both caped and non-caped mRNAs that do not contain the 5´non-translated region of PVA (Eskelin et al. 2011). The authors suggested that the decrease in mRNA translation and increase in virus translation depends on eIF4E, supporting the hypothesis that this protein acts promoting virus translation. Other host protein that seems to be involved in this interaction is eIF4G. It does not interact directly with VPg but VPg-eIF4E interaction increases the affinity of eIF4E-eIF4G interaction, leading to a decrease in translation of caped mRNAs (Michon et al. 2006).

Other translation factors interact with viral proteins inside replicative vesicles. The protein poly(A) binding protein (PABP) interact with VPg and NIb (Léonard et al. 2004; Dufresne et al. 2008). The biological role of PABP is to act in mRNA circularization, increasing its translation rate. PABP presence in replication complex suggests it has a role in translation or replication (Dufresne et al. 2008). The interaction between the elongation fator eEF1A and VPg-Pro and NIb was detected *in vivo* by tandem affinity purification and confirmed *in vitro* by

ELISA. In infected cells, the proteins co-localize inside replicative vesicles (Thivierge et al. 2008).

The involvement of chaperones in virus infection has been described for several viruses. For potyviruses, the involvement of Hsc70.3, a member of Hsp70 protein family was described. Hsc70.3 interacts with TuMV NIb *in vitro* and *in vivo*. During infection, it co-localizes with NIb within replicative 6K2-tagged vesicles, probably acting in replication (Dufresne et al. 2008). The involvement of Hsp70 and its co-chaperone CPIP in CP regulation was described above.

The interaction of viral proteins with cellular DEAD-box RNA helicases was described. Peach (*Prunus persicae*) protein PpDDXL interacts with the VPg from PPV *in planta* and its *Arabidopsis* homolog AtRH8 interacts with VPg from PPV and TuMV. This interaction is necessary for virus infection and all the involved proteins co-localize inside 6K2-tagged vesicles (Huang et al. 2010). Other DEAD-box RNA helicase AtRH9 interacts with TuMV NIb and co-localizes with the replication complex, suggesting that this interaction is important for virus replication (Li et al. 2016).

The dynamism and presence of several types of 6K2-tagged membranous structures observed by light microscopy show that the steps involved in the virus cycle are connected to each other, and similar membranous structures can be related to more than one process. Recently, Wan et al. (2015) described the time course of membrane remodeling induced in *N. benthamiana* by TuMV infection using transmission electronic microscopy and tomography. Three types of membrane structures were observed. First, convoluted membranes appeared connected to ER. One day later, several single membrane tubular structures harboring replicative components were observed. The single membrane structures seem to differentiate into double membrane tubular structures. This last structure was proposed to be the assembly site of virus particle, because it contains several electron-dense bodies.

Translationally controlled tumor protein

The Translationally controlled tumor protein (TCTP) is a family of highly conserved proteins among eukaryotes. It was first identified independently by three groups working with cancerous cells in the 1980s, being named P23, P21 and Q23. After the publishing of the first cDNA sequences, the name TCTP was adopted (reviewed by Bommer, 2012). *TCTP* genes are present in almost all eukaryotic species, with the exception of several species of chlorophytes (Gutiérrez-Galeano et al. 2014). They are present in a low number of copies per genome, being commonly found as a single copy gene (Hinojosa-Moya et al. 2008).

TCTP is a 20-25KDa protein with two domain signatures for the family. It is a calcium binding protein with a non-canonical binding site. Also, TCTP interacts with tubulin, acting in microtubule stabilization. Structure determination of *Schizosaccharomyces pombe* TCTP by NMR spectroscopy has revealed the presence of a conserved core structure that corresponds to a GTPase binding surface and has homology with the human Mss4 nucleotide free chaperone (Thaw et al. 2001). The aminoacids E^{12}, L^{74} and E^{134}, which correspond to the catalytic triad necessary for the interaction, are conserved in all sequences analyzed, suggesting strongly that TCTP interact with Ras GTPases.

TCTP is a multifunctional protein, involved in several cellular processes. It is related to cellular growth and mitosis, being highly expressed in proliferating and meristematic tissues, and also in tumor cells (Bommer and Thiele 2004).

Knockout mutants for *TCTP* are not viable in mouse, *Drosophila* or *Arabidopsis*, showing the essentiality of the protein. Transgenic lines with *TCTP* levels silenced by RNAi present a reduction in growing and slow development (Hsu et al., 2007; Berkowitz et al., 2008). Mammalian cells overexpressing TCTP also presents growth defects, suggesting that TCTP has a regulatory role (Gachet et al. 1999). TCTP is considered a general mitotic regulator. In Mammalia, it was demonstrated that it binds to the mitotic spindle, being released during the metaphase-anaphase transition (Gachet et al. 1999). Moreover, TCTP interact with the protein Chfr, a check point in cell cycle progression (Burgess et al. 2008). A study using *Drosophila* transgenic lines silenced to *TCTP* in specific tissues has demonstrated that *Arabidopsis* TCTP can rescue cell number reduction and partially rescue cell expansion defects, being a conserved mitotic growth integrator in plant and animals (Brioudes et al. 2010).

A protective effect of TCTP against stress and cell death was described in several organisms. TCTP expression is induced by several stress types, such as heat shock, heavy metals, oxidative and calcium stresses (Rupec et al., 1998; Sturzenbaum et al., 1998; Xu et al. 1999; Cao et al., 2010). Also, TCTP has antioxidant and anti-apoptotic properties. The protective mechanisms of TCTP seem to be diverse. For example, TCTP protects cells against apoptosis induced by calcium stress, probably by calcium sequestration. Also, TCTP acts as a molecular chaperone in heat shock conditions in *Schistosoma mansoni* (Gnanasekar, et al., 2009). In plants, it was demonstrated that TCTP overexpression has a protective effect against programed cell death (PCD) induced by Bax and Tunicamicyn (Hoepflinger et al. 2013). Also, *TCTP* silencing increases *N. benthamiana* susceptibility to hypersensitive response (HR) induced by *Pseudomonas* sp. and HR elicitors (Gupta et al. 2013). Moreover, silenced plants overproduced hydrogen peroxide (Gupta et al. 2013) and TCTP overexpression in rice reduces accumulation of hydrogen peroxide by stimulating antioxidant enzymes activity (Wang et al. 2015).

A role as a nucleotide exchange factor (GEF) of Rheb (Ras homologue enriched in brain) GTPases was suggested by Hsu et al. (2007). It is based on studies with *Drosophila* that showed that dTCTP interacts with dRheb and has GEF activity *in vitro* and *in vivo*, and is supported by the GTPase binding surface presence in TCTPs. However, the studies aiming to confirm this association in humans are controversial. Rheb is activated by GTP binding and regulates TOR (Target of Rapamicyn) signaling positively. The TOR signaling pathway is involved in the regulation of growth and cell proliferation, protein translation and homeostasis (Laplante and Sabatini 2013). *Arabidopsis* plants silenced for *TCTP* present a phenotype that resembles plants silenced for TOR, supporting that TCTP might act as a positive regulator of TOR signaling in plants (Berkowitz et al. 2008). Also, TCTP interacts with the *Arabidopsis* Ras homologs AtRABA4a, AtRABA4b, AtRABF1 and AtRABF2b, and also with *Drosophila* Rheb (Brioudes et al. 2010).

The interaction between TCTP and the elongation factors eEF1A and eEF1B was described in mammals (Cans et al. 2003; Langdon et al., 2004). In that case, TCTP may act as Guanine Dissociation Inhibitor (GDI) of eEF1B, impairing protein translation. eEF1A acts in peptide elongation, delivering aminoacilated-tRNAs into ribosomes. It depends on the activity of eEF1B, which promotes GDP-GTP exchange at eEF1A. Wu et al. (2015) have identified and characterized the structure of TCTP-eEF1B binding interfaces. The highly conserved CAR domain present in all forms of eEF1B is involved in interaction establishment. The key residues involved in this interaction are conserved in both proteins among organisms of different kingdoms, being probably a conserved interaction.

TCTP protein synthesis is regulated in the translational level (and also in transcriptional level) (Bommer and Thiele 2004). For mammalian TCTP, there are two models for translational regulation of TCTP. The presence of an oligopyrimidin tract on the 5´UTR region of its mRNA suggests that its protein synthesis may be subject to regulation by mTORC1 (Yubero et al. 2009; Bommer et al. 2015). Moreover, *TCTP* mRNA can be folded in a highly structured dsRNA conformation that is able to interact with and activate the dsRNA-dependent protein kinase (PKR) from mouse (Bommer et al. 2002). PKR is responsible for phosphorylation of the translation eukaryotic initiation factor 2 alpha subunit (eIF2α) in response to dsRNA, including virus, causing protein synthesis inhibition (reviewed by Dabo & Meurs 2012). It is believed that *TCTP* mRNA inhibits its own synthesis, regulating protein translation.

Plant TCTPs

Studies on plant TCTPs are less abundant than on animal TCTPs. The roles previously mentioned seem to be conserved. However, plant-specific roles have been described. TCTP was identified as a protein whose expression is induced by several abiotic stresses such as drought, salinity, cold, heat, aluminum and mercury (Ermolayevet al., 2003; Liao et al., 2009; Cao et al. 2010; Qin et al. 2011; Wang et al. 2015). TCTP overexpression in *Arabidopsis* increases drought resistance by a mechanism in which TCTP interacts with microtubules, resulting in faster stomata closure in a calcium dependent way (Kim et al. 2012). TCTP overexpression also increases rice tolerance against mercury by activation of anti-oxidant enzymes that reduce Hg-induced hydrogen peroxide levels (Wang et al. 2015).

A study with pumpkin (*Cucurbita maxima*) RNA binding proteins has demonstrated that CmTCTP is a phloem protein that interacts with CmPP16-1 and CmPP16-2, regulating CmPP16-1 root-ward movement (Aoki et al. 2005). Two *TCTP* genes are present in the *Arabidopsis thaliana* genome, *AtTCTP*1 and *AtTCTP2*. *AtTCTP1* was characterized by Berkowitz et al. (2008). These authors have considered *AtTCTP2* as a pseudogene because they couldn't detect its expression in any analyzed tissue. However, Toscano-Morales et al. (2014) demonstrated that *AtTCTP2* is expressed and functional. *AtTCTP2* RNA and protein are able to move through sieve tubes and can induce tobacco regeneration (Toscano-Morales et al. 2014). Based on tridimensional structure prediction, the same group proposed a division of plant TCTPs in two groups, AtTCTP1-like and CmTCTP-like. Those groups would present specialized functions, in which AtTCTP2 belongs to CmTCTP-like group (Gutiérrez-Galeano et al. 2014).

There are two studies linking TCTP to plant pathogen infection. TCTP was identified as a *Ralstonia solanacearum* responsive gene (Kiba et al. 2007) and its gene silencing results in faster HR development in *N. benthamiana* plants challenged by *R. solanacearum* 8107 and Pseudomonas sp. *TCTP* is up-regulated during the early stages of tomato infection by the potyvirus *Pepper yellow mosaic virus* (Alfenas-Zerbini et al. 2009). Tomato and *N. benthamiana* plants silenced for *TCTP* present a decrease in virus accumulation, showing that TCTP contributes for virus infection (Bruckner et al., 2016).

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Chapter 1: Translationally controlled tumor protein (TCTP) from tomato and *Nicotiana benthamiana* is necessary for a successful infection by a *Potyvirus*

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Translationally controlled tumor protein (TCTP) FROM TOMATO AND *Nicotiana benthamiana* is NECESSARY FOR SUCCESSFUL INFECTION BY A *Potyvirus*

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SUMMARY

Translationally controlled tumor 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 conserved domains of the TCTP family. To study the effects of TCTP silencing in PepYMV infection, *Nicotiana benthamiana* plants were silenced by VIGS and transgenic tomato plants silenced for TCTP were obtained. In the early stages of infection both tomato and *N. benthamiana* silenced plants accumulated fewer 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 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 favor its establishment. 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 occurs in other eukaryotes and help us to discuss possibilities of TCTP involvement in viral infection.

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 favor their establishment, and while doing so they cause cell physiological and morphological alterations that result in a multitude of symptoms (Mandadi & Scholthof, 2013). Viral infection affects expression patterns of host cell genes, leading to the up- and down-regulation of a wide variety of genes that are involved in defense responses or that are useful or even required for infection (Senthil *et al.*, 2005, Pompe-Novak *et al.*, 2006, Gandia *et al.*, 2007, Alfenas-Zerbini *et al.*, 2009).

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 mono and dicot plant species distributed around the world (Adams *et al.*, 2011). The potyvirus genome is comprised of a 10 kb single-strand of RNA linked to the viral protein VPg at the 5' terminus and with 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 a ribosomal frameshift within the coding region of the P3 protein to produce a fusion P3N-Pipo protein (Chung *et al.*, 2008, Wen & Hajimorad, 2010).

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 tumor protein (TCTP) as up-regulated (Alfenas-Zerbini et al., 2009). TCTP is a highly conserved protein present in all eukaryotes, and the mammalian homologs are the best studied due to 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 of 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 aluminum toxicity (Ermolayev *et al.* 2003, Lee and Lee 2003, Fabro *et al.* 2008, Liao *et al.* 2009, Alfenas-Zerbini *et al.* 2009, Qin *et al.* 2010). In *Arabdopsis thaliana*, there are two TCTP homologs, 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 root-ward movement of CmPP16-1 is positively affected by the presence of the two interacting proteins, while CmPP16-2 is not affected. Recently, it was demonstrated that the AtTCTP2 (initially considered a pseudogene) is expressed and functional in Arabdopsis 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.*).

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

Based on both transient (VIGS) and stable TCTP silencing (transgenic plants expressing a TCTP dsRNA), we demonstrate here that TCTP is a host factor that is necessary for the establishment of an efficient PepYMV infection in tomato and *N. 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 only cytoplasmic 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 and putative 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 homolog of translationally controlled tumor protein (SlTCTP), and its up-regulation at 72 hours post-viral inoculation was confirmed by 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 (access 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 2 casein kinases; one putative myristoylation site; and the typical signature sequence TCTP2 (Figure 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) (Figure 1).

The TCTP sequences of several plant and animal species were aligned and a strong conservation was observed among plant sequences (Figure S1). The casein kinase II phosphorylation site at the position 9 in SlTCTP is conserved in all sequences analyzed and the other three phosphorylation sites are exclusive of plant TCTPs, beeing present in all sequences except by the Casein kinase II phosphorilation site in the 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 (virus-induced gene silencing) assay was conducted in *N. benthamiana* plants to verify the effect of TCTP silencing in the infection by PepYMV. Fragments of approximately 400 nt of the genes *PDS*, *GFP* and *SlTCTP* were cloned into the RNA2 of the TRV silencing vector and, along with TRV RNA1, were agroinfiltrated into *N. benthamiana* leaves. *PDS* silencing was observed by a typical photo-bleaching phenotype and appeared approximately 8 to 10 days after agroinoculation (daa), being completely established in young leaves by 14 daa. TCTP silencing did not induce phenotypic alterations (Figure S2). At 14 daa, a leaf disc was collected from upper leaves of each plant for molecular confirmation of TCTP silencing before viral inoculation. Then, half of the plants in each treatment group were sap-inoculated with PepYMV, and leaf discs adjacent to the previously collected discs were collected 72 hours post-viral inoculation (72 hpvi) to analyze PepYMV accumulation as well as the maintenance of TCTP silencing. TRV-TCTP plants were efficiently silenced for TCTP by VIGS (Figure 2A), and the level of silencing was maintained at 72 hpvi (Figure 2B, compare TRV-TCTP mock with TRV-GFP mock). As expected, *TCTP* expression was induced by viral infection (Figure 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 detrimental to viral establishment during the early stages of PepYMV infection (Figure 2C).

We also attempted to analyze the efffects of *TCTP* silencing later in the (systemic) infection. Young (non-inoculated) leaves from all plants were collected 14 days post-viral inoculation (14 dpvi). At this time, all inoculated plants showed similar symptoms, with no differences observed between treatments and control plants (Figure S3). Viral load determined by qRT-PCR was equivalent in both TRV-GFP and TRV-TCTP inoculated plants (Figure 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, accumulation of TCTP mRNA was quantified at 14 dpvi. The pattern of expression observed at 72 hpvi was also present at 14 dpvi, except for TRV-TCTP plants inoculated with PepYMV (Figure 3B). Compared with 72 hpvi, there was an increase in *TCTP* expression in previously silenced plants when PepYMV infection was established, and the expression level became similar to that in TRV-GFP mock-inoculated plants (Figure 3B, compare TRV-GFP mock with TRV-TCTP PepYMV). Because potyviruses have a strong silencing suppressor (HC-Pro), it is possible that after viral establishment, PepYMV suppressed the TRV-mediated *TCTP* silencing. To test this hypothesis, the relative accumulation of TRV was quantified by qRT-PCR (Figure 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 suppressed VIGS.

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

Since 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). Flow cytometric analysis revealed average nuclear DNA (2C) values in regenerated plants of 1.96 pg, consistent with 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 (Figure 4A). The silencing level was variable, with transcript accumulation ranging from 3.5 to 70% in relation to WT plants (Figure 4A, upper panel). Plants TCTP5 and TCTP22 were weakly silenced, with a relative transcript accumulation of 41 and 70% of that in WT plants, respectively. Plants TCTP6, TCTP11 and TCTP26 were strongly silenced, with a relative accumulation of 12, 6,4 and 3,5%, respectively, of that in WT plants. Protein accumulation correlated with transcript *TCTP* levels quantified by qRT-PCR (Figure 4A, middle panel).

Phenotypic alterations were observed in transgenic plants TCTP11 and TCT26, with the strongest reduced 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 TCTP 11 was stunted (Figure 4B), displayed embryo necrosis (Figure 4D) and developed slower than WT plants (not shown). The clonal progeny of plant TCTP26, the strongest silenced plant, showed stunting (Figure 4C), morphologic alterations in fruit (Figure 4E), decrease in the size of seeds (Figure 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 TCTP silencing. Thus, WT plants and the weakly silenced TCTP5 and TCTP22, and TCTP 6 (strongly silenced, but not as much as TCTP11 and TCTP26) 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 (Figure 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 (Figure 6A, C). Thus, PepYMV infection did not revert TCTP silencing in the transgenic plants. Also at both time points, WT plants showed induction of *TCTP* expression due to PepYMV infection (Figure 6A, C). Analysis of the viral load by qRT-PCR showed that PepYMV accumulated to much lower levels in silenced plants compared to WT plants at both time points (Figure 6B, D). This negative effect of TCTP silencing on viral infection was directly proportional to the level of silencing. Thus, a small amount of viral RNA could be detected in the clonal progeny of plant TCTP11, while no virus could be detected in the clonal progeny of plant TCTP26 (Figure 6B, D).

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 (Figure 7A). In systemically infected plants (12 dpvi), TCTP was observed only in the cytoplasm

(Figure 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 (Figure 8). A total of 51 different Arabidopsis proteins were identified, including fourteen interactions described in plants (twelve in *A. thaliana* and two in *C. maxima*), fourteen in *Drosophila*, nine in humans and seventeen 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), signaling (AtRAB4A, AtRAB4b, AtRABF1, AtRABF2b, NDPK2, NDPK3, and SAE1A), and transmembrane transport (AT5G56450, AAC2, AAC3, and AAC1), plus three proteins with unknown roles (AT2G26810, AT4G14000, and AT2G43320).

DISCUSSION

Here, we show 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 lead to reduced viral accumulation. However, in a systemic infection of PepYMV, *TCTP* silencing regressed together with an increased accumulation of TRV, probably due to 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*, was previously demonstrated (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, TCTP 11 and TCTP 26, 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 our lineages the plants showed delayed development and growth, reduced size of leaves and flowers and reduced root growth and development. After PepYMV inoculation the TCTP26 plants did not show any symptoms, and TCTP11 plants showed only small chlorotic spots. Determination of the viral load showed that these plants accumulated fewer virus than WT, extending the observations from the early infection in *N. benthamiana*. Thus, decreased TCTP expression impaired, but did not prevent, PepYMV accumulation. The 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, likely due to the multiple essential roles that this protein plays in plant development.

Due to 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 contains 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 structures, 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) demonstrated that these vesicles move cell-to-cell in *Turnip mosaic virus* (TuMV)-infected plants, suggesting that the membranous vesicles with the VRC are the entity that moves cell-to-cell and 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 compared 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 data that potentially interacts with TCTP. These proteins interact with actin and act in microfilament dynamics. TCTP may participate in the cell-to-cell movement process by interacting with profilins. Another interaction that could be related to viral movement is the one of CmTCTP with CmPP16-1 and CmPP16-2, two proteins in pumpkin phloem (Aoki et al., 2005). The authors propose that TCTP is involved in the selective transport of molecules by phloem. AtTCTP interacts with BRL2 and TTL3, two proteins that controls 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. The authors suggest 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 that are 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-alpha and its guanine nucleotide exchange factor, eEF1B-beta, were demonstrated by (Cans *et al.*, 2003). TCTP impairs GDP-GTP exchange by eEF1B-beta, favoring the inactive form of eEF1A-GDP. Interestingly, potyviral proteins interact directly with eIF4E, eEF1-alpha and PABP, all of which are involved in the translation process, and eEF1-alpha 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 (Wittmann *et al.*, 1997, Léonard *et al.*, 2000). Eskelin *et al.*, (2011) demonstrated that the interaction VPg-eIF4E 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-alpha 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-alpha-TCTP interaction were confirmed for plant TCTP, it could link TCTP to the viral factory, where it might affect the 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) suggested that TCTP controls growth by acting in the TOR pathway, based on the structural similarity of TCTPs and the fact that silencing 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 signaling was demonstrated in *Drosophila* and in humans (Hsu et al., 2007, Dong *et al.*, 2009). TCTP interacts with Rheb, a Ras GTPase that is activated by TCTP and in turn activates TOR. Plants do not have a Rheb homolog, 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 (Zoncu *et al.*, 2011, Wullschleger *et al.*, 2006). 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 remodeling (Leiber *et al.*, 2010) and mRNA translation (Deprost et al., 2007). These functions are important for an efficient viral infection, thus we speculate that TCTP could link the TOR pathway with viral infection and with the development of symptoms, since infected plants usually exhibit reduced growth.

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

In summary, we demonstrated that TCTP is an important host factor for potyviral infection and that the virus interferes with the subcellular localization of this protein, probably due to 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

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

Viral isolate

The PepYMV isolate 3 was described by (Truta *et al.*, 2004) and has been 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 greenhouse .

Viral inoculation

PepYMV was sap inoculated to *N. benthamiana* and tomato plants as described above. Viral infection was confirmed by indirect 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 nt for each gene (*TCTP*, AY642284.1; *PDS*, NM_202816.2 and *GFP*, amplified form pK7WFG2 (Karimi et al., 2002) were cloned in RNA2 of the TRV viral vector

(Liu *et al.*, 2002), resulting in 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) and then subcloned in pK7GWIWG2 (Karimi *et al.*, 2002), resulting in pK7WIWG2-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’ ACT GGA ATT CTG TTG GTT TAT CAG G 3’ and TCTP-XhoI R: 5’ ACT GCT CGA GCT AGC ACT TGA TC 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. (dna.macrogen.com) to confirm their integrity.

Agrobacterium transformation

All recombinant plasmids were initially transformed into *E. coli* DH5α by the heat shock method (Sambrook & Russel, 2001). Then, TRV1/TRV2 plasmids were separately transformed into *A. tumefaciens* strain C58C1, and pK7FWG2-TCTP/pK7WIWG2-TCTP were transformed into *A. tumefaciens* strain GV3101. All *A. tumefaciens* transformations were performed using the heat shock method described by (Brasileiro & 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 until reaching an OD_{600} of 0.6. These cultures were then centrifuged at 10000 rpm and resuspended in agroinfiltration buffer (MES 10 mM, MgCl2 10 mM and 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 leaves of *N. benthamiana* (three 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 two weeks after agroinfiltration), seven plants silenced to TCTP and three plants for the controls 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 systemicaly infected with TRV of all plants immediately before the viral inoculation.72 hours post-viral inoculation (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 14 and 21 days post-viral inoculation (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 3 drops of Tween 80 per 100 mL 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 half-strength 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) 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,). The flasks were kept in the dark for 15 d until the seeds germinated. The seedlings were then transferred to a temperature-controlled growth chamber with a 16/8 h (light/dark) light regime, an irradiance of 36 µmol m^{-2} s^{-1} (provided by fluorescent tubes, 20W) and 27 ± 2°C for 15 d. Hypocotyl segments (average 10 mm length) were used for *Agrobacterium*-mediated transformation with the construct pK7WIWG2-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’ TCA GCG CAG GGG CGC CCG GTT 3’ and NPTIIR: 5’ GCG GTC AGC CCA TTC GCC 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), according to the manufacturer’s instructions. Total RNA from tomato plans 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 Scientific) and by electrophoresis in 1% agarose gels.

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

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 LB01 buffer to release 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 RNase (Amresco) was added to each sample. Samples were incubated at 4^{o}C in the dark and examined after 1–2 h. At least 10,000 nuclei were analyzed for each sample. Analyses were performed using a flow cytometer (FACS Calibur, Becton–Dickinson) at the Institute of Biological Sciences (ICB), Federal University of Juiz de Fora (UFJF). Cytometric histograms were generated and analyzed using Cell Quest and WinMDI 2.8 software (facs.scripps.edu/software.html).

Quantitative real-time RT-PCR (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 triplicates using Fast SYBR Green Master Mix (Applied Biosystems) in a final volume of 10 µl, and the CFX96 Real-Time System (Bio-Rad). Primer sequences are listed in Table S1.

Protein extraction and Western blot

Leaf tissue of WT or transgenic tomato plants (0,3g) was grinded with liquid nitrogen and boiled with 1ml of extraction buffer (150 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol 5%, 2% SDS, 10% glycerol 10%) for 3 minutes. The extract was analyzed by 12% SDS-PAGE and transferred to PVDF membrane Immobilon-P Transfer Membrane (Millipore). The membrane was blocked with powder milk 5% in PBS-T and incubated with specific polyclonal rabbit antiserum against tomato TCTP, produced in the Laboratory of Industrial Microbiology (Universidade Federal de Viçosa). The membrane was washed in PBS-T and incubated with secondary goat anti-rabbit IgG, conjugate to alkaline phosphatase (Biorad). After that, colorimetric protein detection was performed Alkaline Phosphatase Conjugate Substrate Kit (BioRad), according to manufacturer’s instructions.

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

N. 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 until reaching an OD_{600} of 0.6, centrifuged at 10,000 rpm 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 hours after agroinfiltration using a laser confocal scanning microscope LSM 510 META (Carl Zeiss) 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 homologs, At3g04450.1 and AT3G16640.1. At3g04450.1, the most similar sequence to SlTCTP (90%), is reported as a nonfunctional pseudogene (Berkowitz *et al.* 2008). Thus, AtTCTP AT3G16640.1 (83% of similarity with SlTCTP) was used to identify putative interactions with other *A. thaliana* proteins using 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

PAZ directed the project. FMZ and PAZ designed the experimentes. FPB performed the VIGS experiments. ASX, FPB RSC and WCO performed experiments with transgenic plants. ASX and RSC performed the subcellular localization assay. FPB built the *in silico* interaction network. FPB, FMZ and PAZ wrote the paper.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Primers used for qRT-PCR analysis.

Figure S1. Plant TCTPs are strongly conserved.

Figure S2. *Nicotiana benthamiana* silencing phenotype two weeks after agroinoculation.

Figure S3. Symptoms of PepYMV systemic infection at 14 dpvi in VIGS silenced plants.

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FIGURE LEGENDS

Figure 1. Putative conserved motifs in tomato TCTP.

Putative motifs were identified using PROSITE software and are indicated by the following colors: yellow – casein kinase type II phosphorylation site; red – myristoylation site; green - kinase C phosphorylation site. The TCTP2 signature sequence is represented by the dark bar. The putative nuclear localization signal identified using cNLS is represented by a double bar, and the leucine rich NES identified using the NetNES 1.1 server is indicated in gray.

Figure 2. (a) *TCTP* expression in *Nicotiana benthamiana* plants 14 days after agroinoculation with the VIGS vector, before PepYMV inoculation. *TCTP* expression was quantified by qRT-PCR in relation to TRV-GFP plants. The bars represent the average expression level in six plants from each treatment. The expression level was 1.05 ± 0.20 in TRV-GFP plants and 0.15 ± 0.025 in TRV-TCTP plants. Error bars represent standard deviations. **(b)** Effect of *TCTP* silencing on viral accumulation at 72 hours post-viral inoculation (hpvi). TCTP expression was induced by viral infection (1.85 ± 0.27) in TRV-GFP plants as compared with mock-inoculated plants (1.003 ± 0.07). TCTP was silenced in both infected and mock-inoculated TRV-TCTP plants (0.12 ± 0.016 and 0.12 ± 0.025, respectively). **(c)** Viral accumulation decreased in *TCTP*-silenced plants. PepYMV load was 2185 ± 713 viral copies in TRV-GFP plants and 1200 ± 710 viral copies in TRV-TCTP plants. Bars represent the average of three plants from each treatment. Error bars represent standard deviations.

Figure 3. TCTP expression and viral accumulation in *N. benthamiana* plants agroinfiltrated with the VIGS vector, 14 days post-viral inoculation. **(a)** TRV-GFP and TRV-TCTP plants infected with PepYMV accumulated equivalent viral

loads (113278 ± 29330 and 113520 ± 13972 copies, respectively). Bars represent the average of three plants for each treatment. Error bars represent standard deviations. **(b)** TCTP expression increased in TRV-TCTP-infected plants, to 1.17 ± 0.19 as compared with mock-inoculated TRV-GFP plants (1.05 ± 0.21). In mock-inoculated TRV-TCTP plants, TCTP remained silenced (0.14 ± 0.04). In control TRV-GFP-infected plants, TCTP expression was strongly induced by PepYMV (3.49 ± 0.10). **(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 (12.25 ± 3.36 and 13.19 ± 4.55, respectively). In mock-inoculated plants, TCTP expression was 1.17± 0.33 in TRV-GFP and 1.63 ± 0.60 in TRV-TCTP plants. Bars represent the average of three plants for each treatment. Error bars represent standard deviations.

Figure 4. (a) *TCTP* expression in transgenic tomato plants. TCTP mRNA levels were quantified by qRT-PCR (top panel), and protein levels were quantified by Western blot analysis (middle panel). Loading controls are shown in the bottom panel. Bars represent the average of six plants for each lineage. Error bars represent standard deviations. **(b-f)** Phenotype of TCTP-silenced transgenic tomato plants. Stunting in **(b)** TCTP11 and **(c)** TCTP26 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 to seeds from wild type plants (right).

Figure 5. Symptoms of PepYMV infection in WT and in the clonal progeny of TCTP-silenced transgenic tomato plants.

Figure 6. TCTP expression level and PepYMV accumulation in WT and in the clonal progeny of TCTP-silenced transgenic tomato plants TCTP11 and TCTP26. **(a)** TCTP expression at 72 hpvi. **(b)** PepYMV accumulation at 72 hpvi**.** **(c)** TCTP expression at 14 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.

Figure 7. Subcellular localization of TCTP in **(a)** healthy and **(b)** PepYMV-infected *N. benthamiana* cells, 48 hours after agroinfiltration with a construct expressing a TCTP-GFP fusion protein. The images correspond to a single optical section.

Figure 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 colors: red, proteins related to protein biosynthesis; gray, 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 and purple proteins of plant Rab family. Other proteins are shown in light green. The network was generated using Cytoscape software.

FIGURES

Figure 1

MLVYQDLLTGDELLSDSFPYKEVENGVLWEVQGKWVVQGAVDVNIGANPSAEGGCEDEGVDDQAVRVVDIVDTFR
LQEQPAFDKKQFVTFMKRYIKNLTPKLEGETQEAFKKNIEAATKFLLQKIKDLQFFVGESMHDDGALVFAYYKEG
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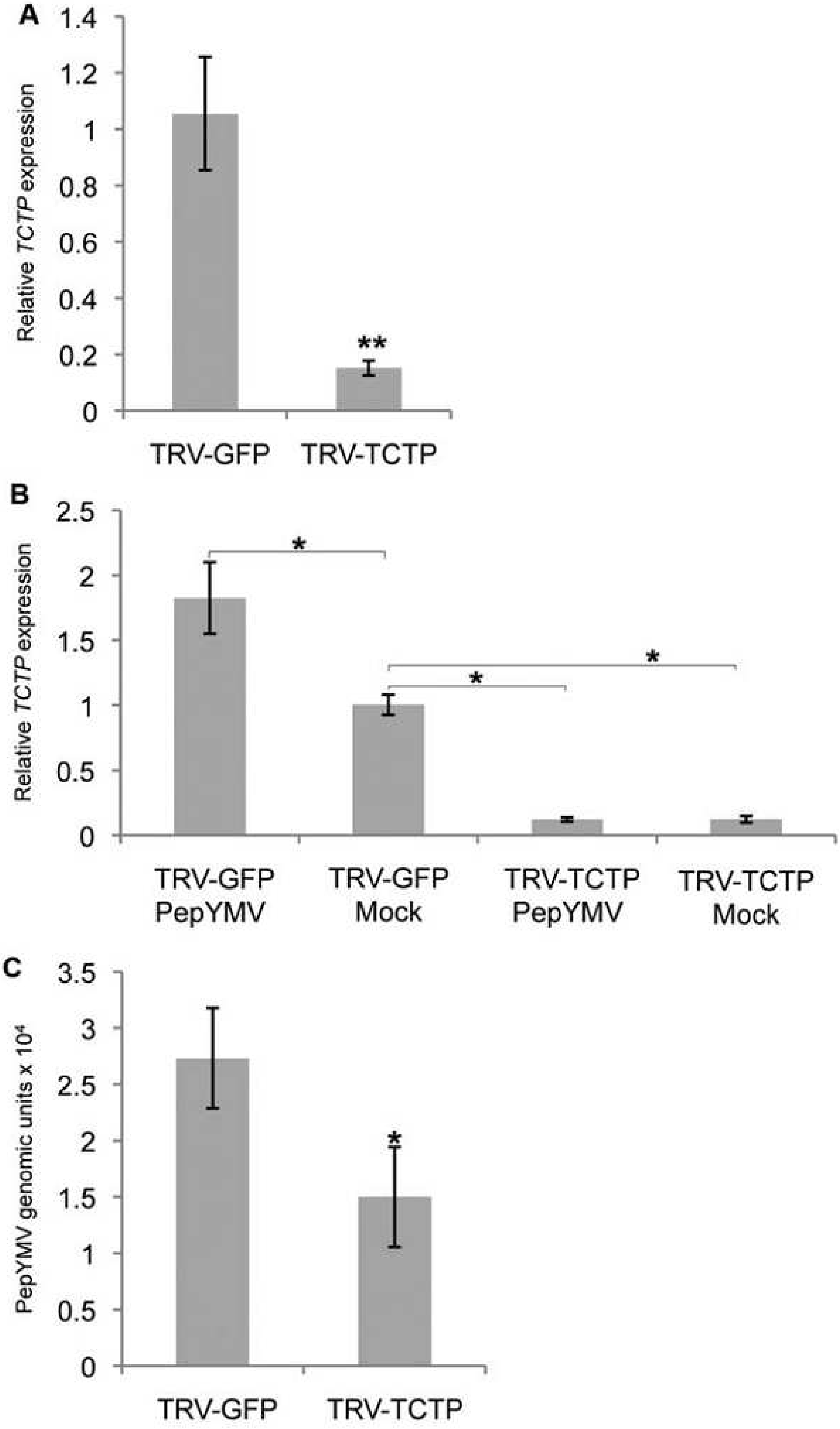
Figure 2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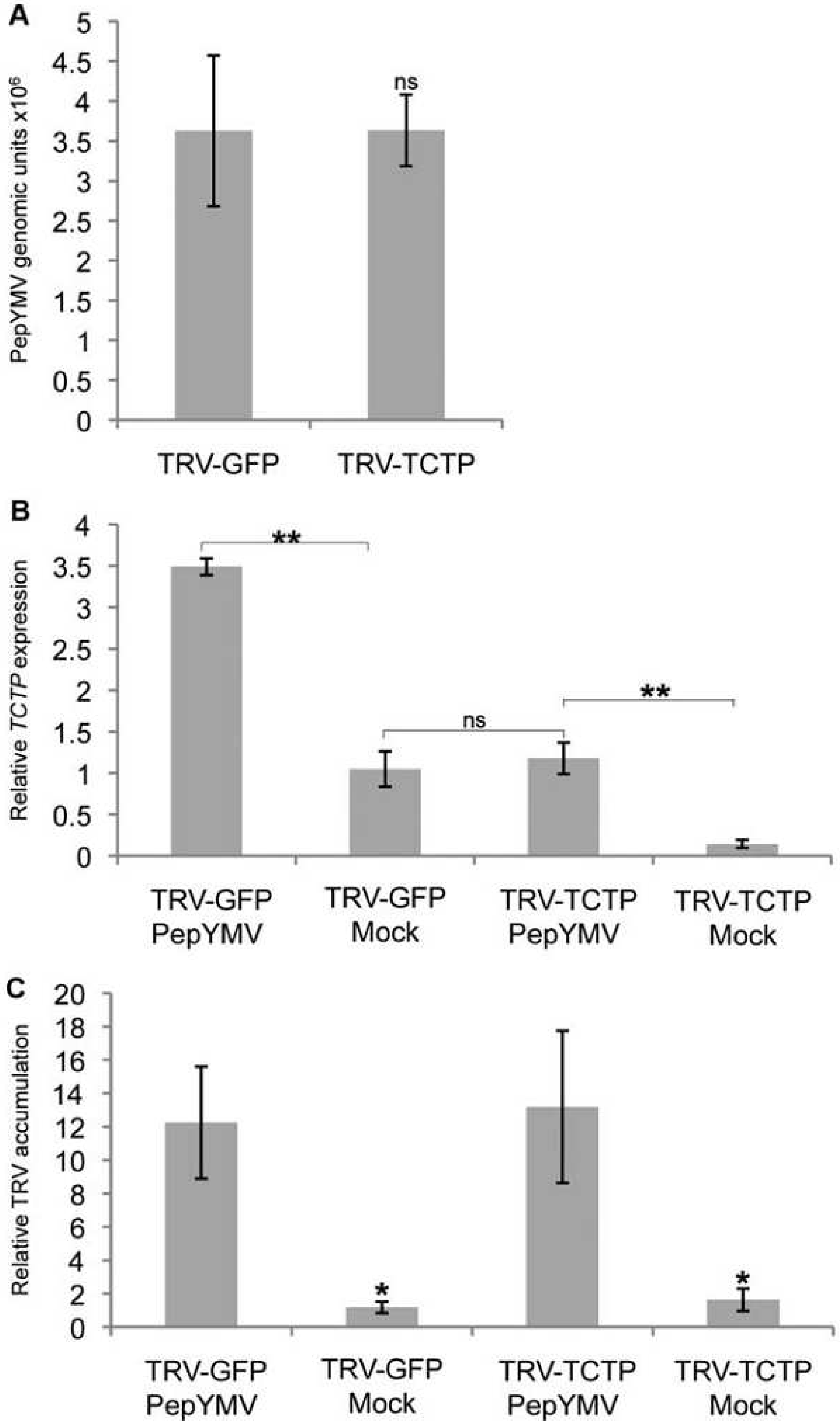
Relative TCTP expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
TRV-GFP
TRV-TCTP
B
Relative TCTP expression
2.5
2
1.5
1
0.5
0
*
*
*
TRV-GFP PepYMV
TRV-GFP Mock
TRV-TCTP PepYMV
TRV-TCTP Mock
C
PepYMV genomic units x 10⁴
3.5
3
2.5
2
1.5
1
0.5
0
*
TRV-GFP
TRV-TCTP

Figure 3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PepYMV genomic units x10^6
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0.5
1
1.5
2
2.5
3
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4
4.5
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ns
TRV-GFP
TRV-TCTP
B
Relative *TCTP* expression
0
0.5
1
1.5
2
2.5
3
3.5
4
**
ns
**
TRV-GFP PepYMV
TRV-GFP Mock
TRV-TCTP PepYMV
TRV-TCTP Mock
C
Relative TRV accumulation
0
2
4
6
8
10
12
14
16
18
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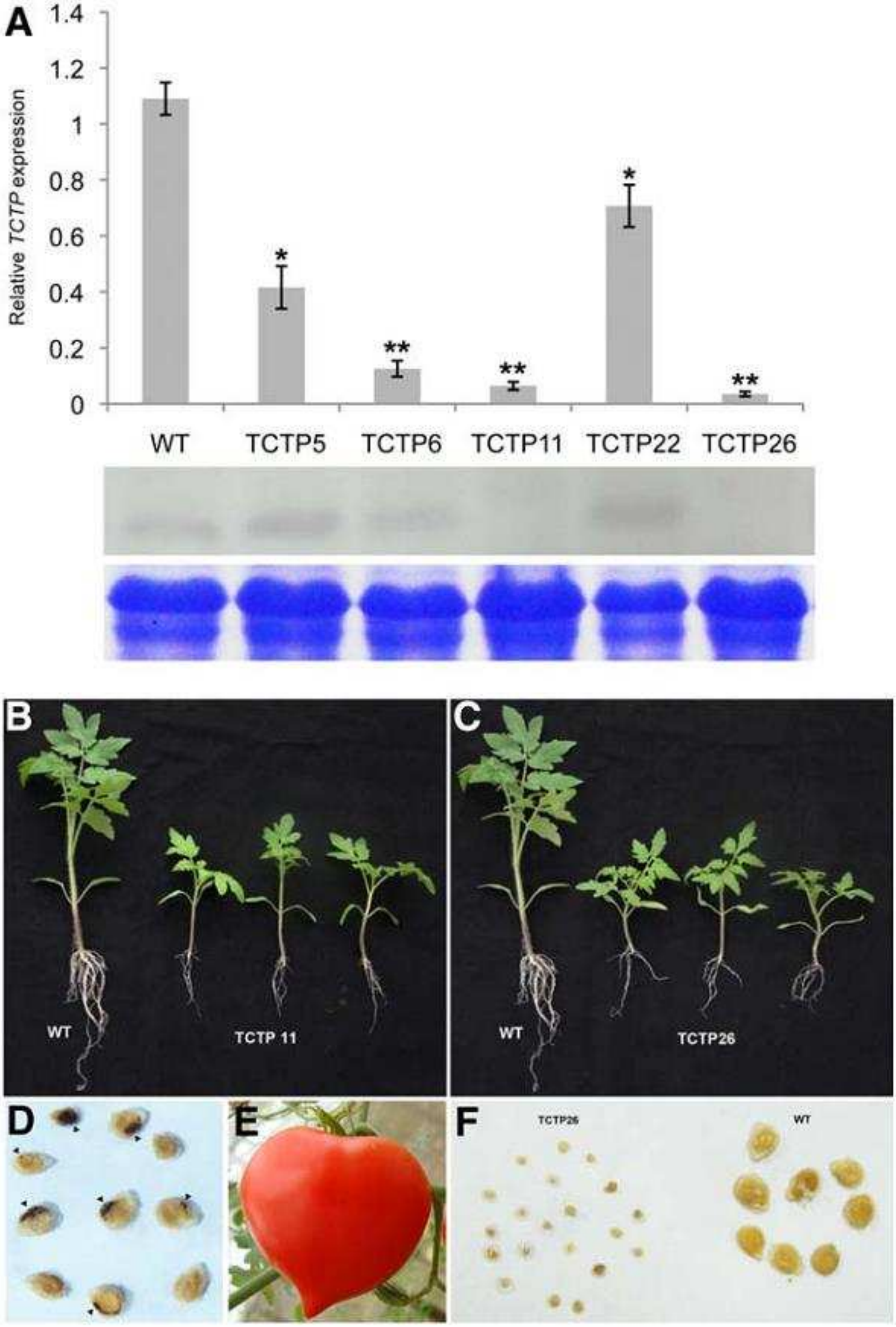
TRV-GFP PepYMV
TRV-GFP Mock
TRV-TCTP PepYMV
TRV-TCTP Mock

Figure 4


A
Relative TCTP expression
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0.6
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0.2
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*
**
**
*
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WT
TCTP5
TCTP6
TCTP11
TCTP22
TCTP26
B
WT
TCTP 11
C
WT
TCTP26
D
E
F
TCTP26
WT

Figure 5

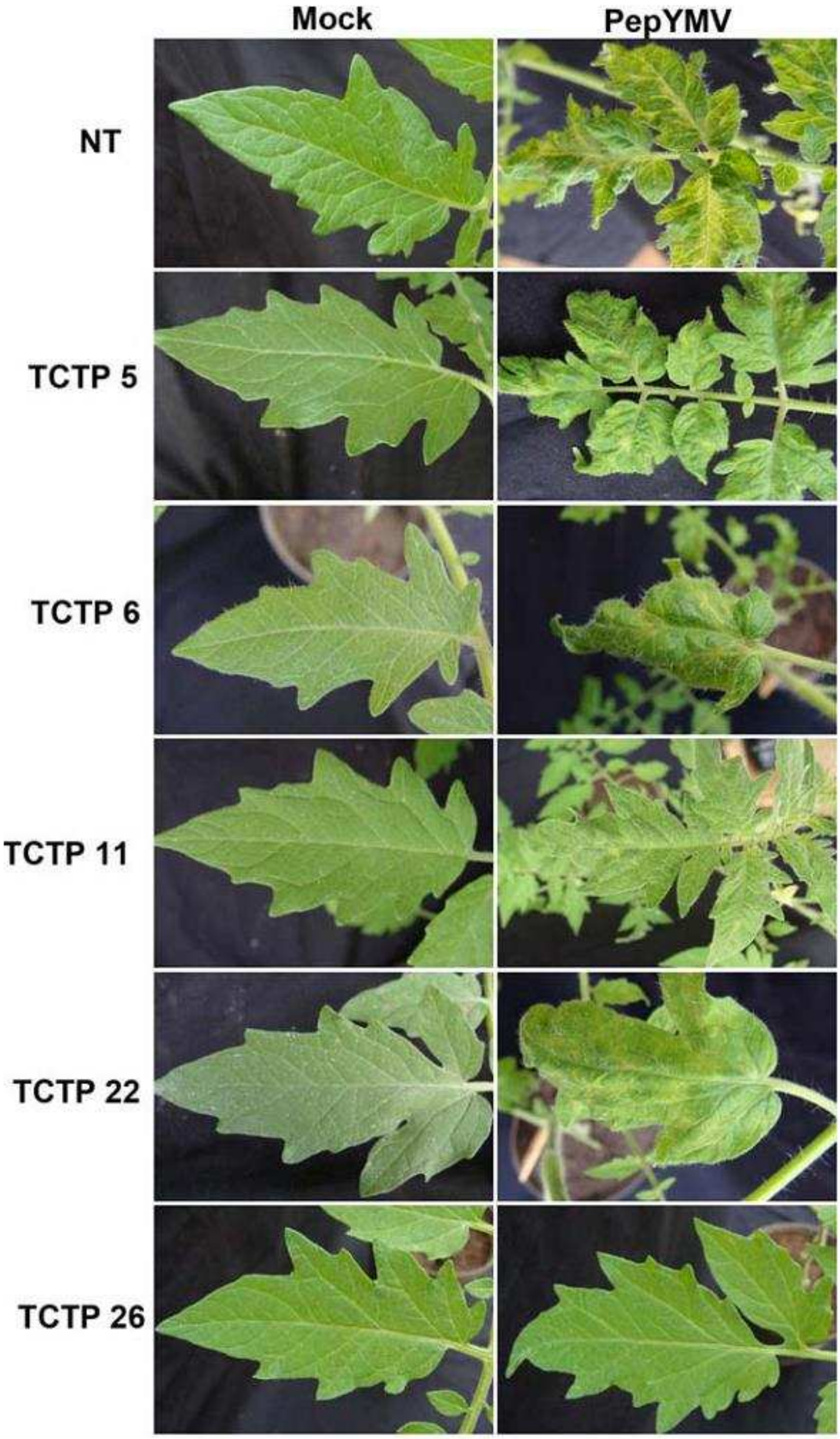
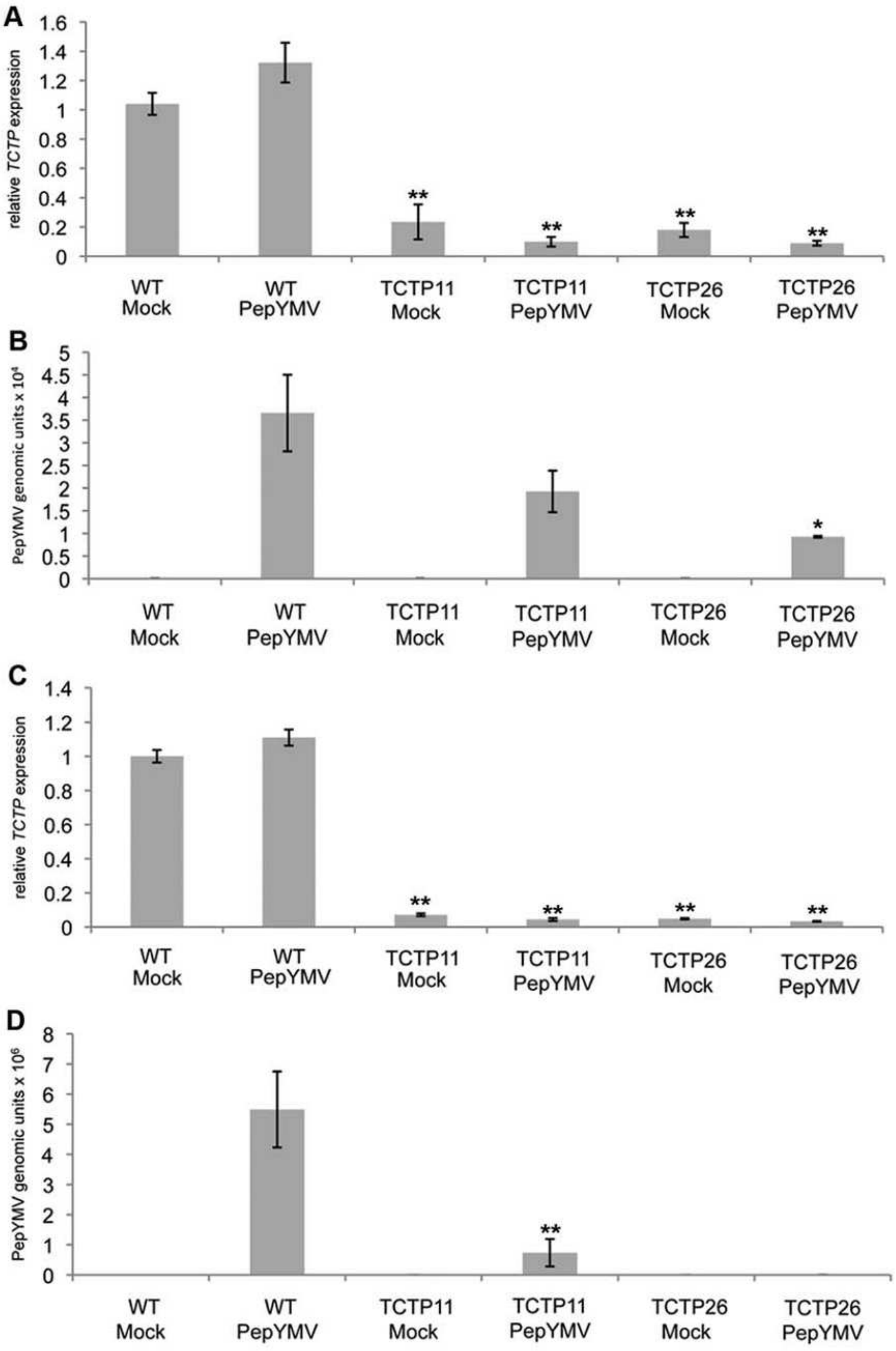


Figure 6


A
relative *TCTP* expression
1.6
1.4
1.2
1
0.8
0.6
0.4
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WT Mock
WT PepYMV
TCTP11 Mock
TCTP11 PepYMV
TCTP26 Mock
TCTP26 PepYMV
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PepYMV genomic units x 10⁴
5
4.5
4
3.5
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2.5
2
1.5
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0.5
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*
WT Mock
WT PepYMV
TCTP11 Mock
TCTP11 PepYMV
TCTP26 Mock
TCTP26 PepYMV
C
relative *TCTP* expression
1.4
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0.8
0.6
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WT Mock
WT PepYMV
TCTP11 Mock
TCTP11 PepYMV
TCTP26 Mock
TCTP26 PepYMV
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PepYMV genomic units x 10⁶
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WT Mock
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TCTP11 PepYMV
TCTP26 Mock
TCTP26 PepYMV

Figure 7

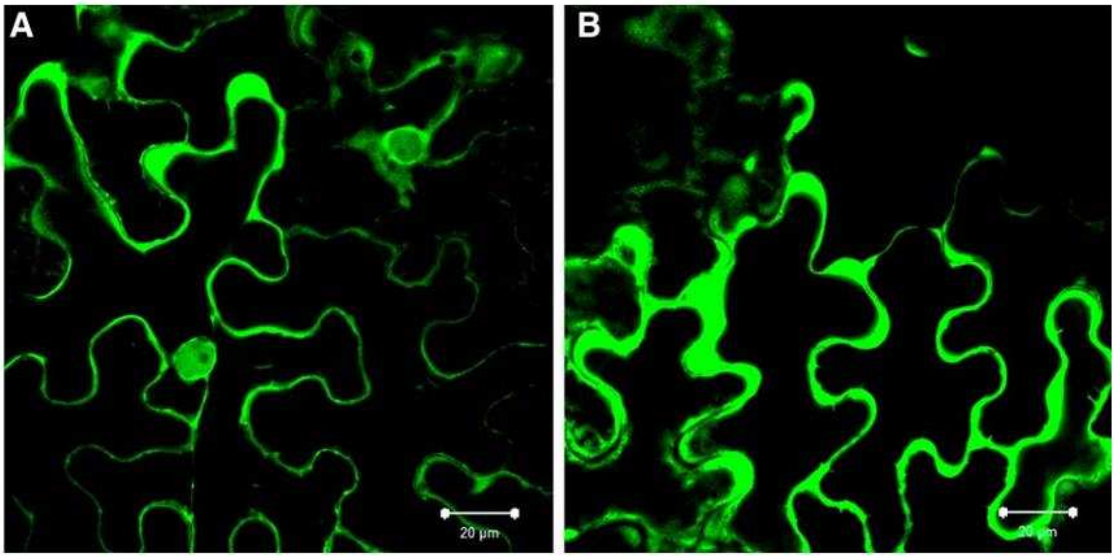
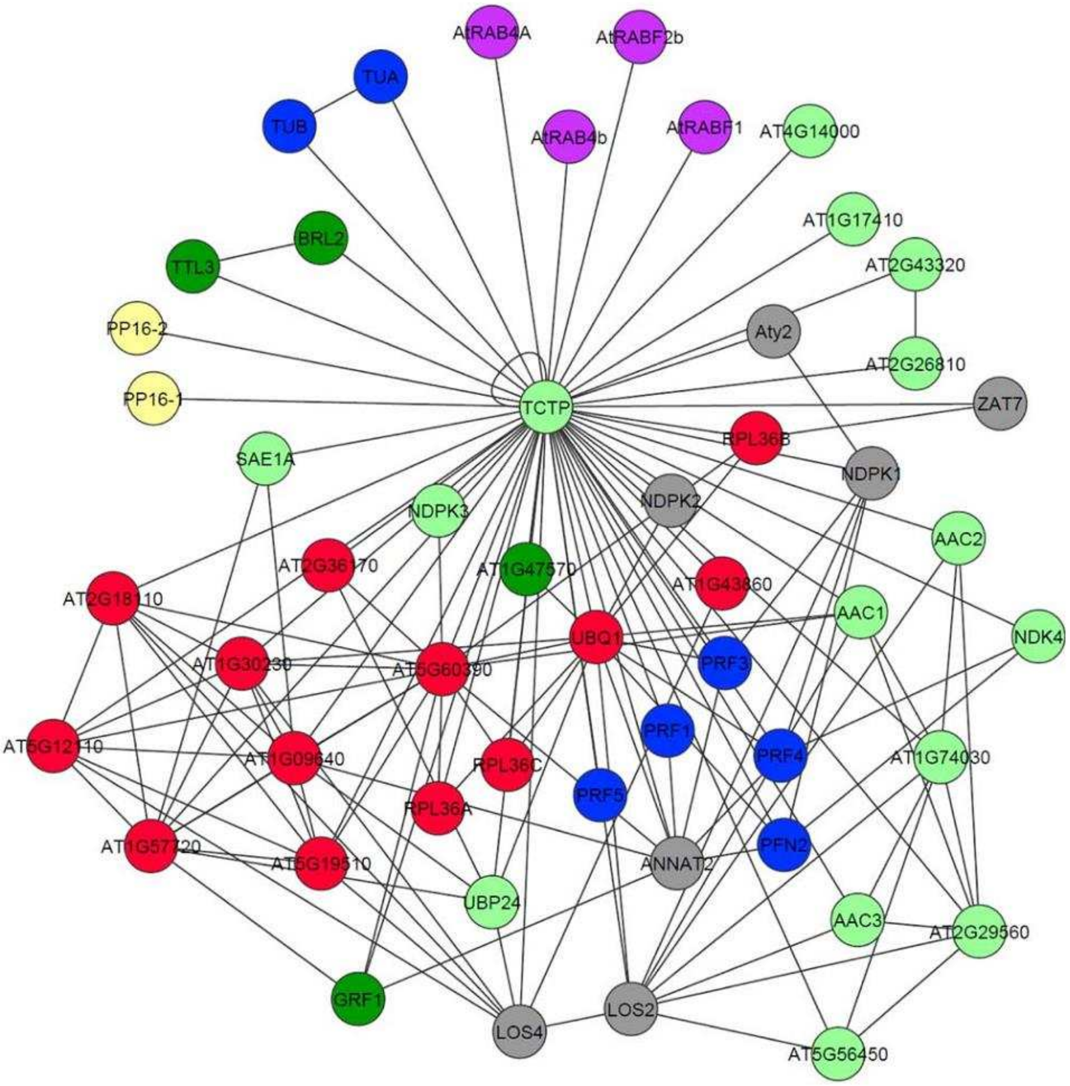


Figure 8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TUA
TUB
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PP16-2
Aty2
AT2G26810
PP16-1
TCTP
ZAT7
RPL36B
SAE1A
NDPK1
NDPK2
NDPK3
AAC2
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AT1G43860
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AAC1
NDK4
UBQ1
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PRF3
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PRF1
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AT1G57720
PFN2
ANNAT2
AT5G19510
UBP24
AAC3
AT2G29560
GRF1
LOS2
LOS4
AT5G56450

Chapter 2: The translationally controlled tumor protein (TCTP) has a positive effect on potyvirus replication

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TITLE: The translationally controlled tumor protein (TCTP) has a positive effect on potyvirus replication

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ABSTRACT

Translationally controlled tumor protein (TCTP) was previously identified as necessary for infection by the potyvirus *Pepper yellow mosaic virus*. TCTP is a multifunctional protein involved in central processes such as cell cycle progression, apoptosis and stress response, but its role in potyvirus infection remains unclear. This study analyzed the involvement of TCTP in *Turnip mosaic virus* (TuMV) infection. Silencing and overexpression of TCTP confirmed that it has a positive effect on infection. Cell-living confocal microscopy demonstrates that TCTP co-localizes with 6K2-tagged vesicles, which are believed to be replication sites, and with the globular structure induced in infected cells. Protoplasts silenced for TCTP present decreased virus accumulation, suggesting an effect on replication. Interestingly, *TCTP* mRNA increases during the infection but protein levels do not. However, the overexpression of a non-translatable form of *TCTP* has no effect on virus accumulation, indicating that the translation of TCTP is essential for potyvirus infection.

INTRODUCTION

The family *Potyviridae* is one of the most important groups of plant viruses, containing a great number of species that infect a wide range of important crops and wild plants around the word. Among them, the genus *Potyvirus* is the largest and most studied (Gibbs and Ohshima 2010).

Potyviruses have a positive-sense single-strand RNA genome with about 10 Kb in length, being covalently linked to the viral protein VPg on 5´ and polyadenilated at 3´ extremities. Their genome is expressed by translation of a large polyprotein that is self-processed by three viral proteases, producing about 9-11 mature peptides and by the expression the ORF PIPO by transcriptional slippage, resulting in the translation of P3N-Pipo, a fusion of N-terminal region of the protein P3 and the ORF PIPO (Adams et al., 2011; Vijayapalani et al., 2012; Olspert et al., 2015).

Potyvirus infectious process is a combination of several interconnected steps, resulting in a complex coordinated network of events regulated in time and space. Viral translation, RNA replication, cell-to-cell and systemic movement, encapsidation and counter-defense mechanisms, are necessary for a successful infection (Reviewed by Mäkinen & Hafrén 2014). Host factors participate of every step. In the last decades, several host proteins involved in potyvirus infection have being identified and characterized (Leonard et al., 2000; Beauchemin et al., 2007; Thivierge et al., 2008; Li et al., 2016). However, there are many plant proteins associated with potyvirus infection whose exact role in virus cycle remains indeterminated.

Potyviruses replicate in association with membranous structures induced by the potyviral protein 6K2 during infection (Restrepo-Hartwig and Carrington

1994; Schaad et al. 1997). In plants infected by a TuMV infectious clone that expresses 6K2 as a fluorescent protein, two types of 6K2-tagged structures can be observed by confocal microscopy: motile vesicles originated from the ER and a globular structure, usually observed around the nucleus, that contains ER and Golgi membranes, chloroplasts and COPII coatamers (Grangeon et al. 2012). 6K2-tagged vesicles co-localize with dsRNA (a marker for virus replication), the replication-associated viral proteins VPg-Pro, RdRp and CI, and host factors that are considered part of the viral replication complex (VRC), being assumed to be the viral factories (Beauchemin et al. 2007; Dufresne et al. 2008a; Dufresne et al. 2008b; Thivierge et al. 2008; Cotton et al. 2009; Huang et al. 2010; Li et al. 2016).

Besides harboring the VRC, 6K2-tagged vesicles can move around the cytoplasm along actin microfilaments (Cotton et al. 2009). Grangeon et al. (2013) have demonstrated that vesicles containing viral RNA (vRNA) and the RdRp are able to reach the plasmodesmata (PD) and move to adjacent cells, probably carrying an active VRC. In addition, 6K2-tagged vesicles containing vRNA and RdRp were observed in phloem sieve elements and xylem vessels of systemically infected plants, suggesting that TuMV can move systemically through the plant as membrane-associated ribonucleoprotein complexes (Wan, et al. 2015). Viral proteins that are not part of viral factories are necessary for virus movement. For example, the protein P3N-Pipo is capable to move itself cell-to-cell and is necessary for virus movement together with P3, CI, CP and HC-Pro (Wei et al. 2010; Vijayapalani et al. 2012). A few host factors involved in potyvirus movement have been identified. The host proteins PCaP1 and NbDREPP interact with P3N-Pipo, and are necessary for cell-to-cell movement (Vijayapalani et al. 2012; Geng et al. 2015).

Recently, the protein Translationally controlled tumor protein (TCTP) was described as a host factor necessary for infection of tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* by the potyvirus *Pepper yellow mosaic virus* (PepYMV). Its mRNA expression is induced by virus infection and its downregulation reduces virus accumulation (Alfenas-Zerbini et al., 2009; Bruckner et al., 2016). Plants silenced for *TCTP* expression accumulate fewer viruses than control plants, and transgenic tomatoes with a great level of *TCTP* silencing show very weak or no symptoms in systemic infection (Bruckner et al., 2016).

TCTP is a multifunctional protein involved in a wide range of cellular processes. It has a great level of conservation among eukaryotes, being present in almost all eukaryotic taxa (Bommer and Thiele 2004). In plants, TCTP was described to be involved in cell cycle progression, cellular growth, control of stomata closure, response to drought stress, apoptosis and programed cell death, making it difficult to find out the manner in which it is involved in potyvirus cycle (Berkowitz et al. 2008; Brioudes et al. 2010; Kim et al. 2012; Gupta et al. 2013; Hoepflinger et al. 2013).

Biochemically, it has proprieties to bind microtubules, calcium and it may acts as a guanine nucleotide exchange factor (GEF) that interacts with and regulates Ras GTPases (Thaw et al. 2001; Kim et al. 2012; Hoepflinger et al. 2013). Hsu et al. (2007) described TCTP from *Drosophila* as a GEF that positively regulates the protein Ras homologue enriched in brain (Rheb), a positive regulator of the mammalian target of rapamycin complex 1 (mTORC1) pathway. In plants, the role of TCTP as a positive regulator of TOR signalization was suggested by Berkowitz et al. (2008), who have demonstrated that *Arabidopsis thaliana* silenced for *TCTP* have a phenotype that resembles the one observed in plants silenced for TOR kinase. Moreover, Brioudes et al (2010) have demonstrated that *Arabidopsis* TCTP interacts with four Rab GTPases *in planta*, reinforcing the idea that plant TCTP regulates TOR signaling.

This study demonstrates the involvement of TCTP in potyvirus replication. The importance of TCTP for TuMV infection was confirmed by measuring viral accumulation in silenced and transiently overexpressing plants. Also, co-localization of TCTP with 6K2-tagged vesicles and the perinuclear globular structure were observed in epidermal cells. Measuring of TuMV relative accumulation in protoplasts silenced to TCTP showed a great decrease in relation to accumulation in non-silenced protoplasts, indicating an involvement of TCTP in virus replication. It was also demonstrated that the increase in *TCTP* mRNA level induced by the virus is not followed by any increase in protein level. It led us to hypothesize that the RNA might be responsible for the positive effect in virus accumulation in plants overexpressing TCTP. However, the overexpression of a non-translatable *TCTP* RNA has no effect on virus accumulation, while protein overexpression increases virus accumulation. Taking together, our data demostrate that the protein TCTP is involved in potyvirus replication.

RESULTS

TCTP is necessary for TuMV infection

In previous studies, Bruckner et al. (2016) have shown that TCTP expression is necessary for PepYMV successful infection. To validate TCTP as a host factor involved in infection by other potyviruses, *TCTP* was silenced by virus induced gene silencing (VIGS) in *N. benthamiana* plants and its effect on TuMV accumulation was analyzed. As expected, silenced plants accumulate fewer viruses than control plants at 4 days after TuMV agroinfiltration (Figure 1A).

Additionally, the effect of TCTP transient overexpression in potyvirus infection was verified. *N. benthamiana* plants had their leaves completely agroinfiltrated to overexpress RFP-TCTP or with the empty vector pK7WGR2. After 24 hours, the same leaves were agroinoculated with pCambia-TuNOS and virus accumulation was analyzed. Tomato TCTP was transiently expressed fused to RFP on N-terminal to make it possible to distinguish it from endogenous TCTP on western blot analysis by molecular mass, using the antiserum against TCTP. Western blot analysis showed a strong overexpression of RFP-TCTP, together with a stronger accumulation of TuMV CP in RFP-TCTP samples, when compared to control samples (Figure 1B). Moreover, the level of endogenous TCTP was not affected by RFP-TCTP overexpression (Figure 1B). These results confirm that TCTP expression plays an important role in potyvirus infection.

TCTP co-localizes with 6K2 induced structures

It is assumed that potyvirus infection occurs within membranous structures observed as vesicles by confocal microscopy. These vesicles are induced by 6K2 in endoplasmic reticulum membranes and harbor viral and host components that are part of the replication machinery, regarded as viral factories. To find a clue on the role of TCTP in TuMV infection cycle, TCTP-GFP was co-expressed with 6K2:mCherry or TuMV/6K2:mCherry and analyzed by confocal microscopy. In healthy plants, TCTP-GFP localizes in both nucleus and cytoplasm (not shown), as previously described (Bruckner et al., 2016). When co-expressed with 6K2:mCherry, TCTP co-localizes with 6K2 induced vesicles (Figure 2A). In plants infected with TuMV/6K2:mCherry, besides the co-localization with 6K2 induced vesicles (Figure 2B), TCTP-GFP also co-localizes with TuMV induced perinuclear structures (Figure 2C).

TCTP association with membranes

Since TCTP co-localizes with 6K2 induced vesicles, it could be part or be associated with it. To verify this possibility, total protein of infected and healthy *N. benthamiana* plants were fractioned by differential centrifugation and the presence of TCTP in each fraction was checked by western blot. In both, TCTP was observed mainly in soluble but also in membranous fraction (Figure 3), which is corroborated by the fact that TCTP is visualized in cytoplasm by confocal microscopy as a strong signal.

TCTP has a role in potyvirus replication

TCTP co-localization with 6K2-tagged vesicles and its presence in membranous fraction in infection cells suggests that TCTP may be part of the vesicles, which are involved in replication and cell-to-cell movement. To find out which type of process TCTP is affecting during TuMV infection, protoplasts from plants silenced by VIGS were obtained and the effect of *TCTP* knockdown in virus replication was verified. The infectious clones 35S-TuMV and 35S-

TuMV^{VNN}, which is defective in replication, were transfected to protoplasts and *TCTP* silencing level and virus accumulation were analyzed by relative qRT-PCR after 24 hours. *TCTP* mRNA level decreases considerably in TRV-TCTP plants when compared to control plants, showing a high level of gene silencing in protoplasts (Figure 4A).

Virus accumulation in protoplasts was measured by qRT-PCR, using specific primers for the CP coding region to amplify both TuMV and TuMV^{VNN} cDNAs. Since the infectious clones are both expressed by 35S promoter, there is a viral RNA accumulation that is consequence of plasmid transcription and not of virus replication. To separate the RNA accumulation originated of transcription from that derived of replication, TuMV^{VNN} Ct values were used to normalize TuMV replication in each preparation of protoplasts. Comparison of TuMV replication in silenced and not silenced plants showed a reduction of about 95% due to *TCTP* gene silencing (Figure 4B), indicating that the positive effect of TCTP is due its involvement in potyvirus replication.

TuMV infection does not lead to increase in TCTP accumulation

TCTP has been found to have its mRNA accumulation induced by PepYMV infection in tomato up to as many as three-fold when compared with no infected plants (Alfenas-Zerbini et al. 2009; Bruckner et al., 2016). However, protein level exchange during potyvirus infection was not analyzed. To verify what happens with protein level during infection, *N. benthamiana* leaves were agroinoculated with pCambia-TuNOS or pCambia0390 (Mock), and collected 48, 72 and 96 hours after agroinoculation. Total protein was extracted and TCTP accumulation was verified by western blot. No apparent difference was found in protein accumulation and statistical analysis shows that there are no significant differences among treatments (Figure 5A). *TCTP* mRNA expression was checked by qRT-PCR for samples collected 72 hours after virus agroinoculation. As for PepYMV, *TCTP* mRNA expression was induced by virus infection, becoming about as twice as the expression in mock inoculated plants (Figure 5B). These results suggest that the mRNA induction does not result in the same increase in protein levels. However, both VIGS and overexpression experiments indicate its importance to virus accumulation, and somehow this induction contributes to virus infection.

Effect of TCTP RNA on virus infection

In mammal, *TCTP* mRNA was observed to be highly structured, being folded into dsRNA strands that are able to interact with and activate the dsRNA-dependent protein kinase (PKR) of mouse (Bommer et al. 2002). PKR is responsible for phosphorylation of the alpha subunit of the translation eukaryotic initiation factor 2 (eIF2α) in response to dsRNA, including virus, causing protein synthesis inhibition (Dabo and Meurs, 2012). For mammalian TCTP, it is believed that *TCTP* mRNA inhibits its own synthesis, regulating protein translation. Since no clear increase of protein level in infected plants was identified by Western blot, the possibility of the involvement of *TCTP* mRNA on virus infection was checked.

First, the secondary structure was predicted for the coding region of *TCTP* RNA using RNA Folding Form software (Zuker, 2003). Several similar and stable structures were predicted, with ΔG among -189.02 and -179.66, showing a high probability for their formation. The most stable structure was chosen for analysis (Figure 6A).

To test the effect of *TCTP* RNA in virus infection, a clone that expresses *TCTP* RNA without protein synthesis was constructed. Because RFP-TCTP expression is able to increase TuMV infection, the initiation codon of this construction was replaced by the stop codon TGA. This new construction was termed RFP-TCTPstop. The secondary structures for this RNA were also predicted and compared to that predicted for TCTP. The most stable is identical to that predicted for *TCTP* RNA, with the same value for ΔG (Figure 6B), in which this small mutation is not likely to alter any functional activity of *TCTP* RNA.

To check if RFP-TCTPstop was able to express a non-translatable TCTP RNA, RFP-TCTP, RFP-TCTPstop and pK7WGR2 were expressed in *N. benthamiana* plants and the presence of tomato *TCTP* RNA was checked by RT-PCR using specific primers that can distinguish tomato from *N. benthamiana* TCTP (Figure 6C, bottom line. The absence of the RFP-TCTPstop expression was verified by Western blot analysis (Figure 6C, top line).

To check if *TCTP* RNA rather than protein could be important for potyvirus replication, *N. benthamiana* plants were agroinoculated with RFP-TCTP, RFP-TCTPstop and pK7WGR2. After 24 hours, the same leaves were agroinoculated with pCambia-TuNOS. After four days, virus accumulation was checked by Western blot (Figure 6C, second line) and qRT-PCR (Figure 6D). Both analyses have shown that TuMV accumulation increases when RFP-TCTP, but not RFP-TCTPstop, is expressed. To verify whether RFP-TCTP or RFP-TCTPstop overexpression may alter endogenous *TCTP* mRNA accumulation, both tomato and *N. benthamiana* RNAs were quantified by qRT-PCR using specific primers. The overexpression of both constructs led to a decrease in *N. benthamiana TCTP* mRNA to about a half of the accumulation observed in control plants (Figure 6E, gray columns). However, the accumulation of tomato *TCTP* RNA was about as many as three times higher than that observed for endogenous TCTP in control plants agroinfiltrated with the empty vector (Figure 6E compares the gray column of ‘pK7WGR2’ and light gray columns), showing that the final result was an increase in *TCTP* RNA accumulation in RFP-TCTP and RFP-TCTPstop overexpressing plants. Taken together, the results indicate that the translation of *TCTP* is essential for potyvirus infection.

DISCUSSION

TCTP is a multifunctional protein with several molecular partners. Most studies that have characterized TCTP were performed using animal models, especially mammals. Some TCTP proprieties, such as binding microtubules and calcium, and a role as mitotic regulator are known to be conserved among plants and animals (Reviewed by Bommer 2012). However, specific functions of mammalian and plant TCTP have been described. For example, mammalian TCTP interacts and antagonizes the pro-apoptotic and tumor suppressor protein P53, which is absent in plants. On the other hand, TCTP is involved in ABA-mediated stomata closure, a specific role in plants (Amson et al. 2011; Kim et al. 2012).

Since TCTP is a regulator of central processes and has multiple functions, there is a myriad of possibilities by how it could be affecting virus cycle (Bruckner et al., 2016). In this study, we have demonstrated that *TCTP* gene silencing reduces potyvirus accumulation in protoplasts, suggesting an involvement in virus replication (translation and/or RNA synthesis). First, the importance of TCTP, previously demonstrated for PepYMV, was validated for TuMV infection by VIGS experiments. Additionally, it was demonstrated that TCTP overexpression increases TuMV accumulation, evidencing the positive effect of this host factor on potyvirus infection. TCTP-GFP subcellular localization was observed in plants infected by TuMV/6K2:mCherry and it showed that TCTP co-localizes with 6K2-tagged vesicles and with the perinuclear globular structure. It is interesting that our study has linked TCTP to virus replication and TCTP co-localizes with 6K2-tagged vesicles that are believed to be the viral factories (Grangeon et al. 2012).

Potyviruses VCRs are composed of vRNA, viral and host factors involved in potyvirus replication. Among host proteins, the translation factors eIF4A, PABP and eEF1A, DEAD-box helicases such as AtRH8 and AtRH9 and the heat-shock protein Hsc70-3 interact with the viral proteins VPg-Pro and/or RdRp within 6K2-tagged vesicles (Beauchemin et al. 2007; Thivierge et al. 2008; Dufresne et al. 2008a; Dufresne et al. 2008b; Huang et al. 2010; Li et al. 2016). It is interesting to notice that the interaction between human TCTP and eEF1A and its GEF eEF1B has been demonstrated (Cans et al. 2003). eEF1A in its GTP-bounding form is responsible for delivering aminoacilated-tRNAs into ribosomes for elongation of the nascent polypeptide. In this process, GTP is hydrolyzed to GDP, and the exchange for GTP depends on the action of GEF eEF1B. TCTP was described to act as a Guanine Dissociation Inhibitor of eEF1B, leading to the permanence of eEF1A-GDP and impairing translation (Cans et al. 2003; Langdon et al. 2004). Wu et al. (2015) have identified and characterized the structure of the binding interfaces between TCTP and eEF1B. The interaction involves the highly conserved CAR domain present in all forms of eEF1B. The key residues for the interaction are conserved in both proteins among different kingdoms, which may be a conserved interaction. The interaction among eEF1A, VPg-Pro and RdRp was described by Thivierge et al. (2008). In spite of the co-localization with 6K2-tagged vesicles, the exact role of eEF1A in potyvirus infection remains unknown. TCTP may establish a direct interaction with eEF1A and also regulates its current state by suppression of eEF1B. The accumulation of eEF1A in its inactive stage might contribute for translational repression of host proteins that is induced by potyvirus infection (Khan et al. 2008; Eskelin et al. 2011). Whether these interactions really occur and their exact role in virus cycle remains to be elucidated.

Membrane fractioning has demonstrated that TCTP is mainly cytoplasmic, but a small fraction is membrane-associated, being possible for TCTP to be part of 6K2-tagged vesicles. Other host proteins such as PABP2, eEF1A and Hsc70.3, which are recognized to be within 6K2-tagged vesicles are present, even weakly, in membranous fraction on infected cells (Beauchemin and Laliberté 2007; Dufresne et al. 2008a; Thivierge et al. 2008). The knowledge of the complete nature of 6K2-associated vesicles and the host factors that are part of them is a current challenge. In a recent study, Lõhmus et al. (2016) have purified 6K2-tagged membranes by combining sucrose gradient centrifugation and affinity chromatography and identified viral and host proteins by mass spectrometry. The authors have published a list of 94 host proteins that were identified with high coverage in more than one biological experiment. TCTP is not present in the final list of proteins but supplemental information shows that it was identified in one biological experiment. Therefore, whether TCTP is part of VCRs, within or associated with 6K2-tagged vesicles is not clear, but it might be part of it by interactions with host or viral proteins which haven’t been described yet.

One interesting finding of this work is that *TCTP* mRNA up-regulation does not result in protein increase. This is intriguing because TCTP is clearly necessary for virus infection, which led us to hypothesize that *TCTP* RNA rather than the protein could be the necessary host factor for the infection. However, our experiments have shown that protein expression is essential for the virus increasing observed during TCTP transient overexpression. As the name implies, TCTP protein synthesis is regulated at the translational level (and also at the transcriptional level) (Bommer and Thiele 2004). For mammalian TCTP, there are two models for translational regulation of TCTP. The presence of an oligopyrimidin tract on the 5´UTR region of its mRNA suggests that its protein synthesis may be target of regulation by mTORC1 (Yubero et al. 2009; Bommer et al. 2015). Also, *TCTP* mRNA can be folded in a highly structured dsRNA conformation that is able to interact with and activate mouse PKR (Bommer et al. 2002). PKR inhibits protein synthesis by phosphorylation of eIF2α, and may be involved in a mechanism by which *TCTP* mRNA regulates its own synthesis by protein inhibition. All these studies have been carried out using animal models, but the mechanisms described might be conserved. It is likely that *TCTP* transcription is induced by the virus as a mechanism to ensure that the cell will have enough protein for virus replication. *TCTP* mRNA not associated with polyribosomes can be maintained stable in the mammal cell and it may act as a reservoir for protein translation (Yenofsky et al. 1982). It is also possible that infection results in a slight increase of protein that cannot be detected by western blot analysis.

Taken together, our results show that TCTP is necessary for virus replication, and it may be part of viral factories or act in central signaling pathways. Furthers studies are necessary to determine the relation of TCTP with the VRC and the way its roles in central signaling pathways interfere and support virus replication.

EXPERIMENTAL PROCEDURES

Plasmids, molecular cloning and site directed mutagenesis

TuMV infectious clones used in this work were previously described. Clone 35S-TuNOS expresses complete TuMV UK1 genome under 35S promoter control (Sanchez et al. 1998). The pCambia-TuNOS, pCambia-TuMV/6K2:mCherry that express 6K2 protein fused to mCherry fluorescent protein and pCambia-6K2:mCherry were described by Cotton el al. (2009). pCambia-TuMV/6K2:GFP that expresses 6K2 protein fused to GFP fluorescent protein and pCambia-6K2:GFP were described by Thivierge et al. (2008). Clone 35S-TuMV^{VNN} (Grangeon et al. 2013) has the GDD motif of the RdRp mutated to VNN, resulting in a replication defective mutant of TuMV.

Plasmid TRV2-TCTP, used for TCTP virus induced gene silencing, was previously described by Bruckner et al. (2016). Empty silenced vectors TRV1 and TRV2 gateway were described by Liu et al. (2002).

To express TCTP fused to RFP at the N-terminal extremity, the complete sequence of *Solanum lycopersicum* TCTP previously cloned in pENTR11 (Bruckner et al. 2016) was transferred to pK7WGR2 (Karimi et al. 2002) by gateway recombination system, using LR clonase II enzyme mix (Thermo Fischer) according to the manufacturer´s instructions, resulting in the plasmid RFP-TCTP. The plasmid that expresses TCTP fused to GFP at the C-terminal extremity, TCTP-GFP, was previously described (Bruckner et al. 2016).

To express *TCTP* RNA with no protein translation, site directed mutagenesis was performed to replace the initiation codon TAG for the stop codon TGA. For this, the complementary primers TCTPstopF: 5 ´ACT GGA TCC GGT ACC GAC TTC TGA TTG GTT TAT CAG G 3´ and TCTPstopR: 5´CCT

GAT AAA CCA ATC AGA AGT CGG TAC CGG ATC CAG T 3´, containing the mutation, were used for amplification of the whole plasmid pENTR11-TCTP using Phusion High-Fidelity DNA polymerase (New England Biolabs). The PCR product was treated with enzyme DpnI (Jena Bioscience) for 4 hours at 37°C and transformed into *E. coli* DH5α by electroporation using standard procedures. The identity of the clones was verified by restriction pattern and sequencing (Macrogen). A correctly mutated clone was transferred to pK7WGR2 by gateway recombination system, using LR clonase II enzyme mix (Invitrogen) according to the manufacturer´s instructions, resulting in the plasmid RFP-TCTPstop.

Virus-induced gene silencing (VIGS)

To obtain *N. benthamiana* plants silenced to *TCTP*, TRV2-TCTP construction was co-agroinoculated with TRV1 silencing vector in *N. benthamiana* plants at about 3 weeks of age as described by Bruckner et al. (2016). As control, the empty vectors TRV1 and TRV2 gateway were co-agroinoculated. After two-three weeks, when a higher level of silencing is achieved, the plants were used for subsequent experiments.

Analysis of *TCTP* silencing effect on TuMV infection

Plants previously silenced by VIGS had their leaves completely agroinoculated with pCambia0390 (negative control) or pCambia-TuMV/6K2:GFP. The cultures were grown until exponential phase, centrifuged and ressuspended in agroinoculation buffer (10 mM MgCl2; 150 µM acetosseringone) adjusting OD600 to 0.05. After four days, agroinfiltrated leaves of four plants per treatment were collected, pulled together and used to total protein extraction. *TCTP* silencing level and TuMV CP accumulation were verified by Western blot using specific rabbit polyclonal antiserum for each protein (Cotton et al., 2009; Bruckner et al., 2016).

Protein and RNA transient expression

To analyze the effect of TCTP or TCTPstop overexpression in TuMV infection, *N. benthamiana* leaves were completely agroinfiltrated with RFP-TCTP, RFP-TCTPstop or pK7WGR2 (negative control) and the silencing suppressor P19. After 24 hours, the same leaves were completely agroinoculated with pCambia-TuNOS. On day 4 after TuMV agroinoculation, total protein was extracted and TuMV and TCTP accumulation were verified by Western blot using specific rabbit polyclonal antiserum. The expression of *TCTP* RNA was verified by PCR using the enzyme Gotaq (Promega) and tomato TCTP specific primers described by Alfenas-Zerbini et al. (2009). PCR products were analyzed by electrophoresis in 2% agarose gels.

For localization experiments, TCTP-GFP and P19 were co-agroinfiltrated with pCambia-TuMV/6K2:mCherry or pCambia-6K2:mCherry in equal amounts (OD_{600} adjusted to 0,3 for each culture) in *N. benthamiana* leaves. Four days after agroinfiltration, the leaves were sectioned and analyzed using a Zeiss LSM780 inverted confocal microscope. GFP was excited using Argon laser at 488nm and the emitted light between 490 and 550 nm was captured. mCherry was excited using HeNe laser at 561nm and the emitted light between 580nm and 630nm was captured. The images were acquired with a 40X oil immersion objective. Image processing was performed using ZEN2.1 software (Carl Zeiss).

Cellular fractionation

Fractioning of membranous and soluble proteins was performed as described by Thivierge et al. (2008). Leaves of *N. benthamiana* from TuMV infected or healthy plants were collected, grounded in liquid nitrogen and 4g of leaf tissue were homogenized in buffer Q [50mM Tris-HCl pH 7.4; 15 mM MgCl2; 10 mMKCl; 20% glycerol; 0.1% β-mercaptoethanol and 1 mM PMSF]. The homogenate was centrifuged twice at 3000 ×g at 4°C for 10 min for removal of cellular debris, cell wall, nuclei and chloroplasts. The supernatant (S3) was recovered and centrifuged at 30,000 ×g at 4°C for 30 min, resulting in soluble (S30) and membrane (P30) fractions. The P30 pellet was ressuspended in 1 ml of buffer Q. The presence of TCTP on fractions S3, S30 and P30 was analyzed by Western blot.

Protoplasts obtaining and transfection

Protoplasts were obtained from *N. benthamiana* plants previously silenced to *TCTP* by VIGS and transfected as described by Yoo et al. (2007). Briefly, leaves of silenced or control plants were cut in small pieces and incubated with enzyme solution [1.5% cellulase R10, 0.2% macerozyme R10, 0.4M mannitol, 20mM KCl, 20mM MES (pH 5.7), 10mM CaCl2, 0.1% BSA] for four hours under vacuum in the dark. After that, protoplast were filtrated with 41-µm Nylon Net Filter (Millipore) and diluted with equal volume of W5 solution [2mM MES (pH 5.7), 154mM NaCl, 125mM CaCl2, 5mM KCl]. The mix was centrifuged at 100×g for 5min and the pellet was carefully washed twice in W5 solution, keeping it in ice for 30 minutes before the last centrifugation at 100×g for 5min. The supernatant was discarded and the pellet was resuspended in MMg solution [4mM MES (pH 5.7), 0.4M mannitol, 15mM MgCl2]. The protoplasts of silenced or control plants were used to transfection mediated by PEG4000 with 4µg of plasmids 35S-TuNOS or 35S- TuMV^{VNN}, as decribed by Yoo et al., (2007). They were incubated in 12-well tissue culture plate at room temperature and recovered after 24 hours. The collected protoplasts were used for RNA extraction and qRT-PCR, as described below.

RNA extraction, cDNA synthesis and quantitative PCR

Total RNA from protoplasts or leaf tissue was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer´s instructions. RNA quality was evaluated by electrophoresis in agarose gel and quantified by spectrophotometry using NanoDrop (Thermo Scientific). One microgram of total RNA was treated with DNAse I (Promega) according to the manufacturer´s instructions and used for cDNA synthesis using SuperScriptIII reverse transcriptase (Invitrogen) according to manufacturer’s instructions, and oligo-dT as primer. *TCTP* mRNA level was quantified by comparative cycle threshold method (ΔΔCT) (Livak and Schmittgen 2001) using specific primers for *N. benthamiana* TCTP and for the normalizer histone 2B (H2b), as described by Bruckner et al. (2016).

For TuMV absolute quantification, the specific primers qTuMV F (5' CCG AAC ATA AAC GGA ATG TGG GTG A 3') and qTuMV R (5' TGG CGT GGT CAA TGA GCG GTT 3') were designed based in TuMV CP sequence. Specificity of the amplicons was verified by analysis of melting curve and by electrophoresis in 2.0% agarose gel. The standard curve was obtained from serial dilution of $3x10^6$ copies of 35S-TuNOS plasmid with dilution factor of 10, until $3x10^2$ plasmids. Viral accumulation was determined by interpolation of the Ct values of each tested sample within the standard curve (Rutledge and Côté 2003).

Quantification of TuMV replication in protoplasts was performed using the comparative cycle threshold method (ΔΔCT). Both TuMV and TuMV^{VNN} were amplified using the specific primers qTuMV F and qTuMV R and Ct values of TuMV^{VNN} were used to normalize TuMV accumulation due to virus replication in each preparation of protoplasts.

All reactions were performed in triplicates using Fast SYBR Green Master Mix (Applied Biosystems) in a final volume of 10 µl, and the CFX96 Real-Time System (Bio-Rad).

Western blot

SDS-PAGE and western blot were performed as standard procedures. Specific rabbit polyclonal antiserum against TuMV CP (Cotton et al., 2009) was diluted 2,500 times and that against TCTP (Bruckner et al., 2016) was diluted 10,000 times. The secondary antibody Anti-Rabbit IgG (H+L), Peroxidase labeled (KPL) was used according to the manufacturer´s instruction. Membrane revelation was performed using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer´s instruction. Band intensity measurement was estimated using Image J software (Scheneider et al., 2012).

RNA structure prediction

TCTP RNA secondary structures were predicted using RNA Folding Form (version 2.3 energies) software from mFold RNA server (Zuker, 2003) with standard parameters except for temperature, which was 25°C. Since it was expressed under control of 35S promoter and terminator, only the coding sequence (or its correspondent) was used. The most stable structures were chosen for analysis.

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FIGURE LEGENDS

Figure 1: TCTP is involved in TuMV infection. A) Effect of TCTP gene silencing in TuMV accumulation *in planta*. CP accumulation is on the top, TCTP accumulation in the middle and loading control stained by comassie blue is at the bottom. B) Effect of transient TCTP overexpression in TuMV accumulation. For the western blot panels, CP accumulation is on the top, RFP-TCTP expression in the middle and endogenous TCTP expression is at the bottom. Loading control stained by comassie blue is below western blot panels. Western blot was performed using specific rabbit-polyclonal antiserum. All experiments were performed at least three times.

Figure 2: TCTP co-localizes with 6K2 induced structures. TCTP-GFP was co-expressed with 6K2:mCherry (A) or TuMV/6K2:mCherry (B and C) and confocal microscopy was performed. In both, TCTP is widely distributed on the cytoplasm and co-localizes with some 6K2 induced vesicles (arrows). In infected plants, it is also observed in the perinuclear globular structure (asterisk).

Figure 3: TCTP is cytoplasm and membrane associated. TCTP is present in both soluble and membranous cellular extracts of mock and infected plants. S3 – Total proteins; S30 – Soluble proteins; P30 – Membrane associated proteins.

Figure 4: *TCTP* silencing affects TuMV replication in protoplasts. Protoplasts were obtained from *N. benthamiana* plants silenced to TCTP by VIGS (TCTP) or control plants (TRV). A) Relative quantification of TCTP mRNA in control and silenced plants. B) Relative quantification of TuMV replication in control and silenced plants. Graphics represents the average of three independent experiments. Error bars represent standard deviation. ** P value ≤ 0.01 (Student´s t test).

Figure 5: TCTP level does not increase in TuMV infected plants. A) Western blot analysis of TCTP induction by TuMV over time. The samples were collected 48, 72 and 96 hours after agroinfiltration. TCTP accumulation is on the top panel, TuMV CP on the middle and loading control colored by ponceau is at the bottom. The graphic above represents the average of band intensity of three experiments, measured by image J software. Analysis of variance (ANOVA) was performed and no statistical difference was detected. B) TCTP mRNA expression is induced by TuMV infection 72 hours after virus inoculation. Quantitative RT-PCR revels that TCTP mRNA is induced by TuMV infection. The bars represent the average of three experiments. Errors bars represent standard deviation. * P value ≤ 0.05 (Student´s t test).

Figure 6: Effect of *TCTP* RNA expression on virus replication. A) Secondary structures predicted for *TCTP* by RNA Folding Form (version 2.3 energies) software (mFold RNA server). B) Secondary structures predicted for TCTPstop RNA by RNA Folding Form (version 2.3 energies) software (mFold RNA server). C) Effect of TCTPstop expression on virus accumulation: The two first panels show western blot analysis for RFP-TCTP expression (on the top) and TuMV CP accumulation (second panel), using specific antibodies to TCTP and CP, respectively. The third panel shows loading control colored by ponceau. Bottom panel is PCR amplification of tomato TCTP from cDNA, showing that tomato TCTP RNA is being overexpressed. D) Absolute quantification of TuMV viral load by qPCR. E) Relative quantification of *TCTP* RNA from *N. benthamiana* (gray bars) and tomato (light gray bars). The bars represent the average of three experiments. Error bars represent standard deviation. Analysis of variance was performed comparing endogenous expression of *TCTP’s* RNA in different

treatments and comparing tomato *TCTP* RNA expression with endogenous *TCTP* in control plants. Both analysis are show in the same graphic and were statistically significant at P value ≤ 0.05. Different letters represents different values by Tukey´s test.

FIGURES

Figure 1

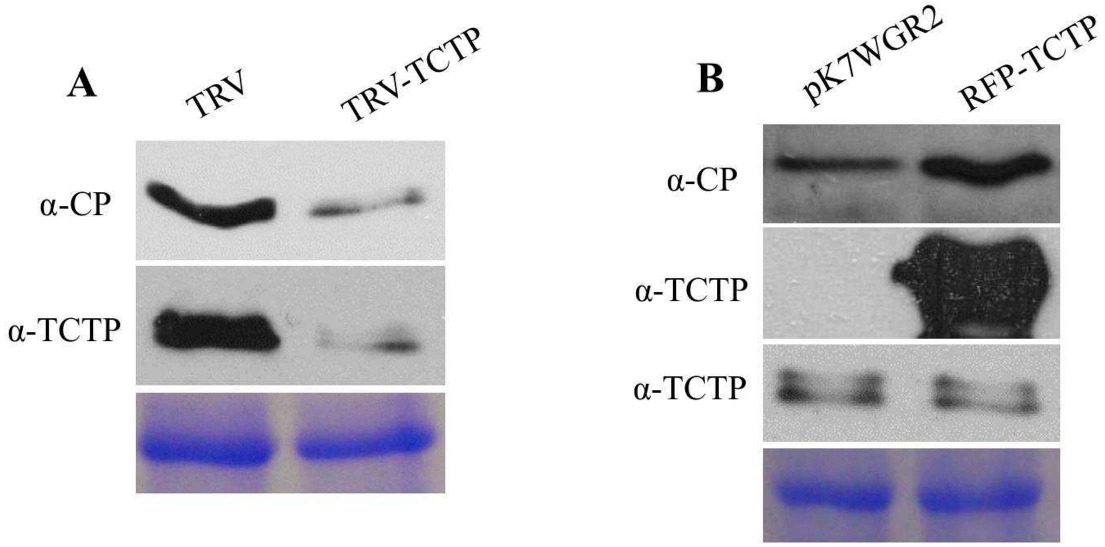
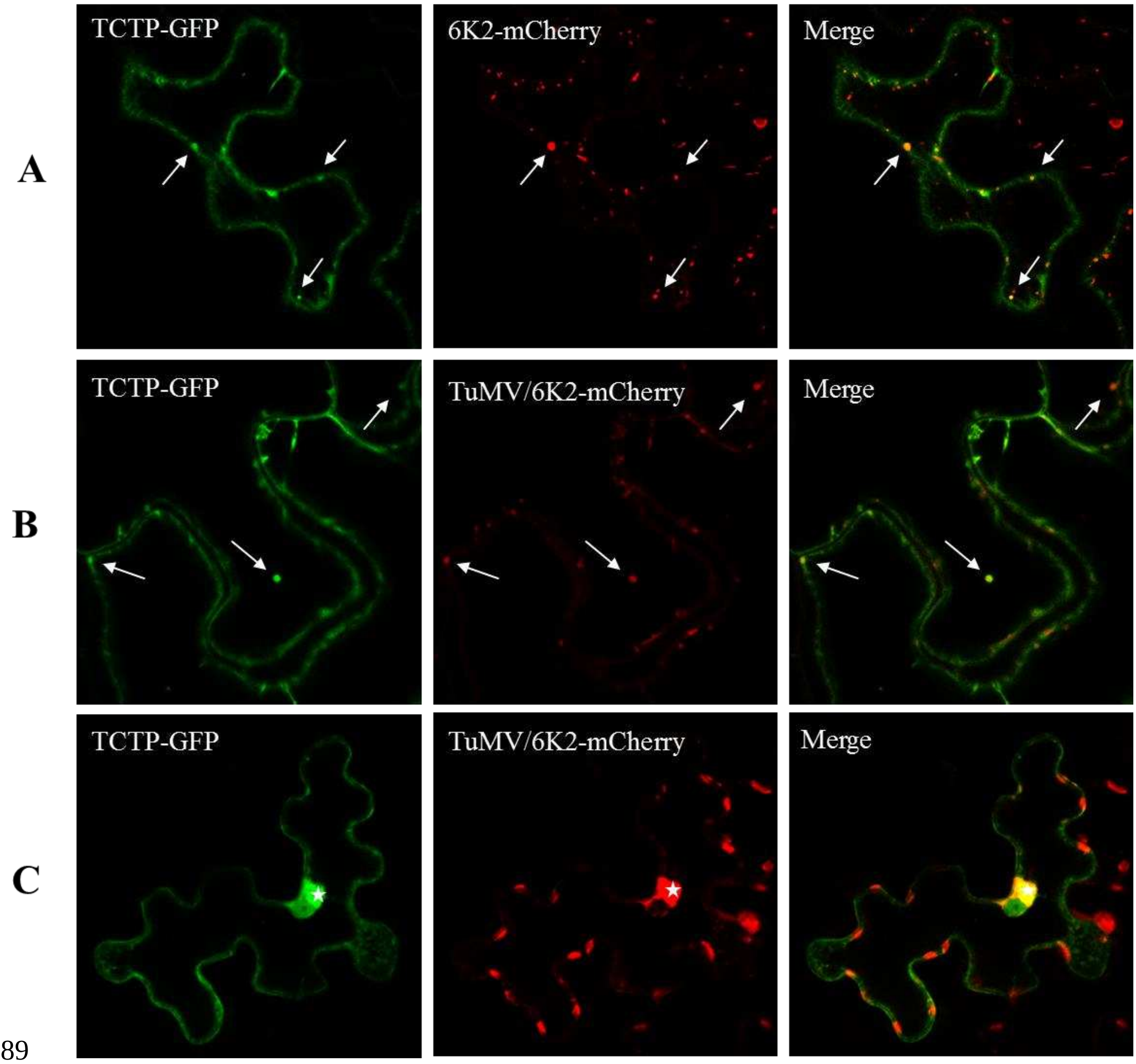


Figure 2


A
TCTP-GFP
6K2-mCherry
Merge
B
TCTP-GFP
TuMV/6K2-mCherry
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C
TCTP-GFP
TuMV/6K2-mCherry
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Figure 3

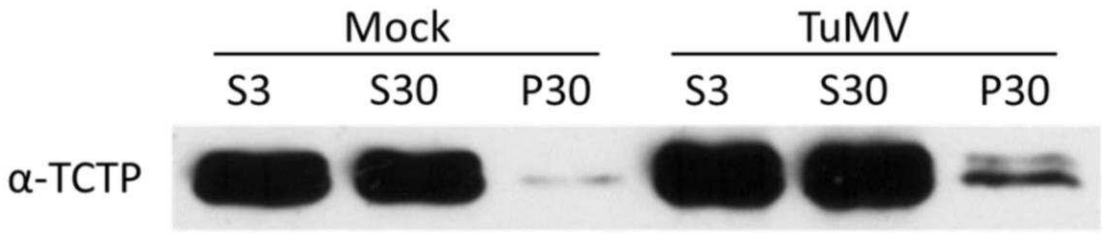


Figure 4

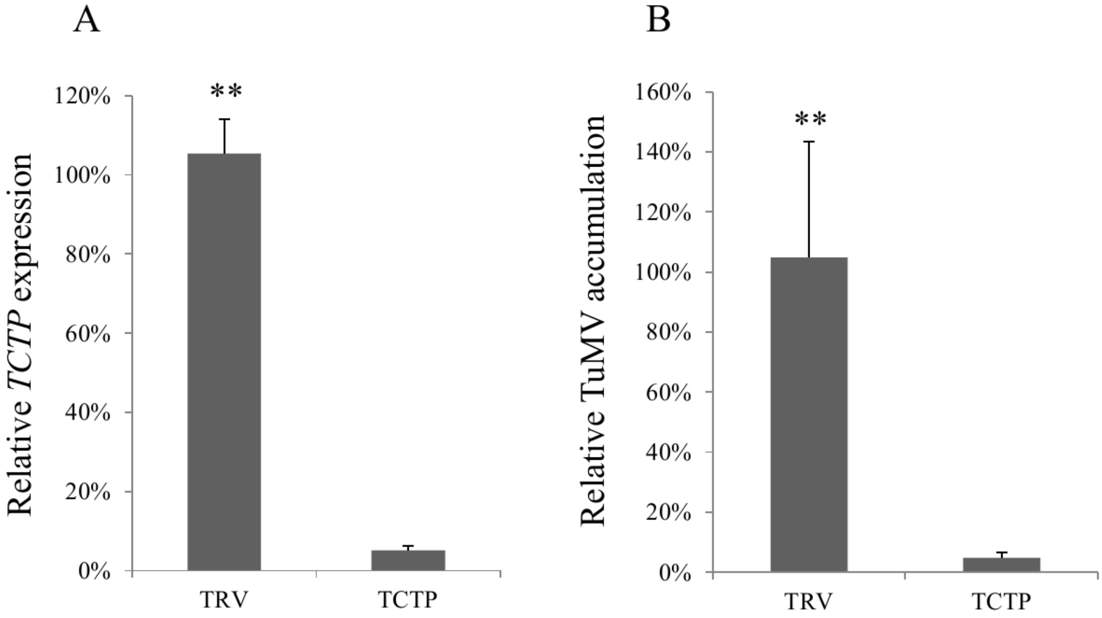


Figure 5

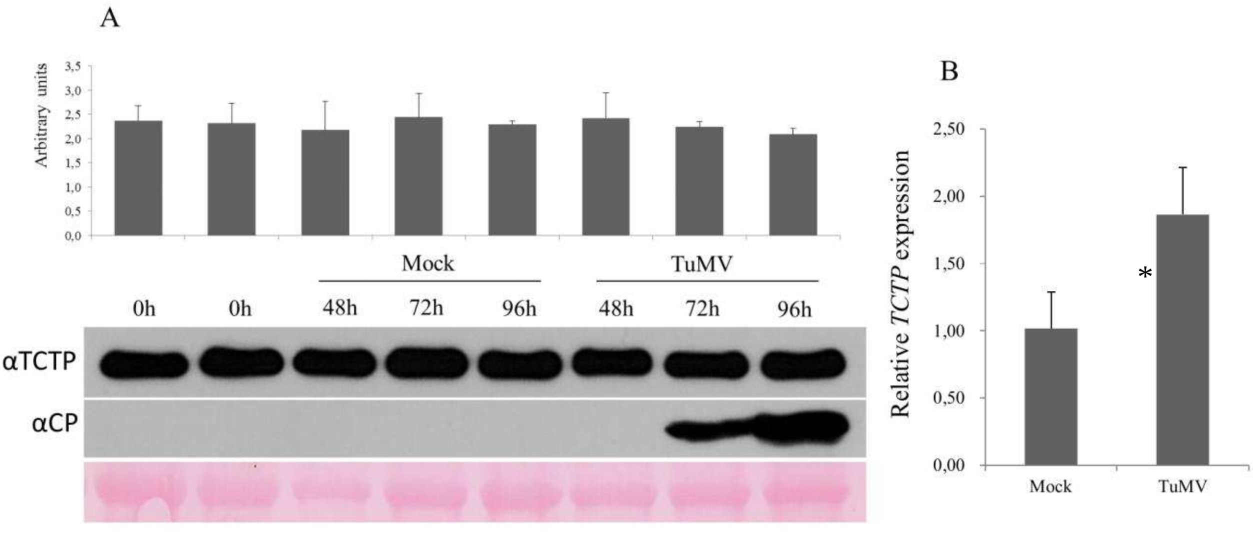
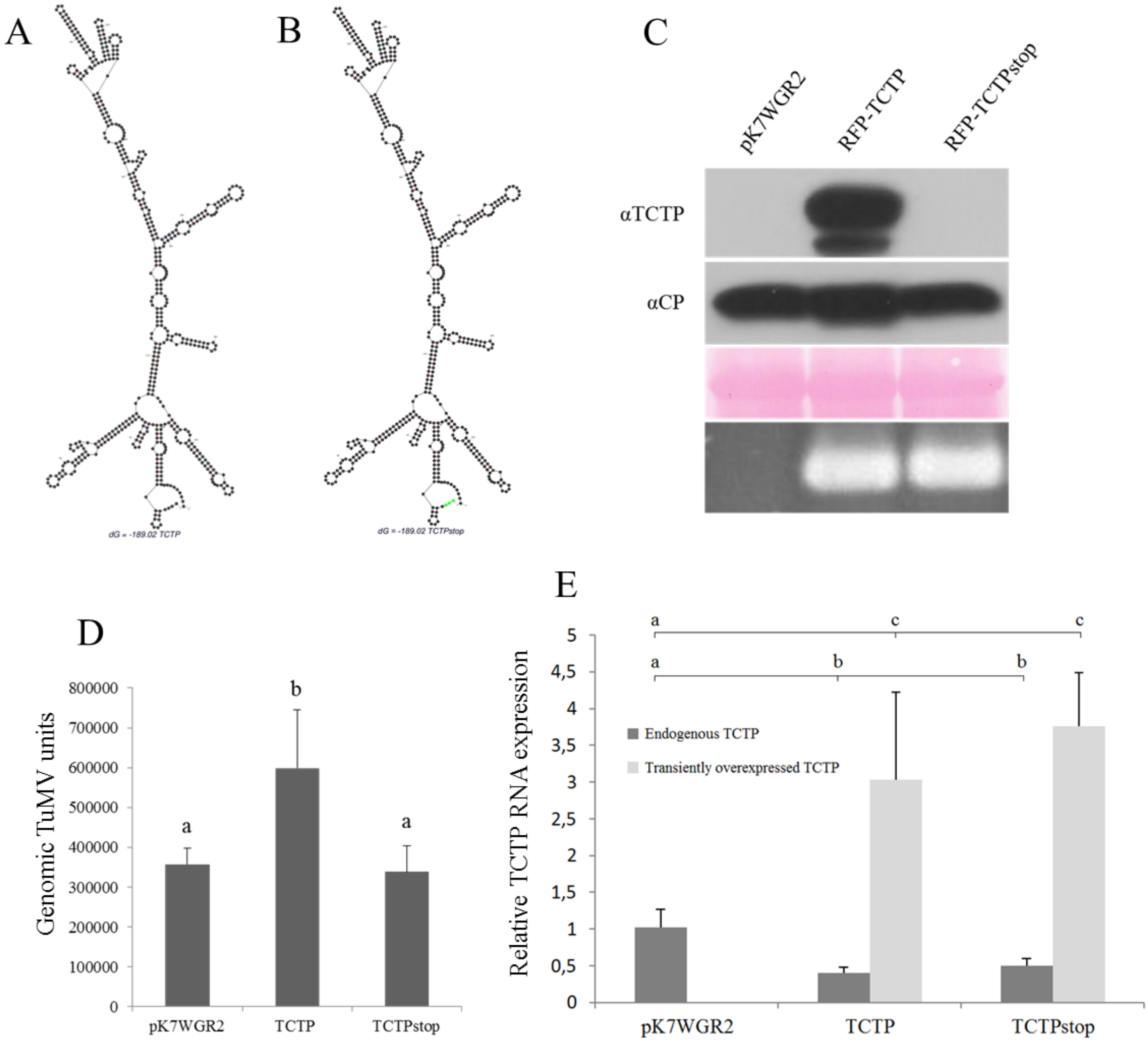


Figure 6


A
B
C
dG = -189.02 TCTP
dG = -189.02 TCTPstop
pK7WGR2
RFP-TCTP
RFP-TCTPstop
αTCTP
αCP
D
Genomic TuMV units
800000
700000
600000
500000
400000
300000
200000
100000
0
a
b
a
pK7WGR2
TCTP
TCTPstop
E
Relative TCTP RNA expression
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
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a
c
c
a
b
b
Endogenous TCTP
Transiently overexpressed TCTP
pK7WGR2
TCTP
TCTPstop

Chapter 3: Additional Information

ADDITIONAL INFORMATION

During development of the study that has resulted in the manuscript presented as the first chapter, several experiments were performed as an attempt to understand the role of TCTP in virus infection. Here, we are going to present some additional data that were not explored sufficiently or not considered suitable to be part of the manuscript. Nevertheless, they are relevant and contribute to our current understanding of the involvement of TCTP in virus replication.

ADITIONAL RESULTS

TCTP immunolocalization in stem cells

Cellular localization of TCTP-GFP overexpressed in *N. benthamiana* cells of both healthy and infected plants was described in the manuscript (Chapter 2, Figure 2). Besides the observation of TCTP transient overexpression, the localization of endogenous TCTP was observed in healthy and infected cells. Stems of *N. benthamiana* plants were cryosectioned into cross and longitudinal sections and TCTP was immunolabeled. In parequimal cells, TCTP was observed as a weak and diffuse signal in the cytoplasm and in the nucleus (Figure S1 A and D). In infected cells, besides the same distribution observed in healthy cells, TCTP co-localizes with large conglomerates of 6K2 induced vesicles and with the perinuclear globular structure (Figure S1 B, C, E and F). Because of the weakness of TCTP signal, the pictures are presented in duplication, with and without the GFP signal. The location observed is the same evidenced by transient expression and confocal microscopy.

In TuMV infected stems, xylem cells were shown to carry conglomerates of 6K2 induced vesicles containing viral and host factors involved in the replication, which suggests that they harbor the complete VCR of TuMV (Wan et al. 2015). To find out whether TCTP is part of these vesicles, images of xylem cells were analyzed. Both in healthy and infected plants, TCTP was not visualized inside the vessels but it was present in adjacent cells (Figure S1 G, H and I).

TCTP does not co-immunoprecipitate with 6K2

Because TCTP co-localizes with 6K2-tagged structures and is weakly associated with membranes, it might interact with 6K2. To verify this hypothesis, 6K2-GFP was immunoprecipitated in *N. benthamiana* plants infected with TuMV/6K2:GFP and the presence of TCTP was checked in the precipitate (Figure S2). TCTP presence was strong in Input samples, but it was absent in the precipitate, showing that it does not interact with 6K2.

Effect of TCTP mutants overexpression

After confirming TCTP effect in TuMV infection, we have tried to construct negative dominants to endorse TCTP importance in potyvirus replication and also to find out essential features of the protein that are involved in supporting potyvirus infection. TCTP is multifunctional, so obtaining a dominant negative would reveal a protein essential role or motif that is directly involved in virus support, contributing for the understanding of how TCTP is helping the virus.

Two point mutations in aminoacid sequence were chosen. The aminoacids E^{12} L^{85} and E^{152} compose a catalytic triad that is essential for TCTP interaction with GTPses and for its role as a GEF (Hsu et al. 2007). To impair this role, E^{12} was muted to A, resulting in TCTPE12A sequence. The second mutation was done in a putative myristoilation site, previously identified (Bruckner et al., 2016). In the previously presented manuscript, TCTP was found to be weakly associated with membranes, but it does not have any transmembrane domain or identified membrane binding motif. If the myristoilation motif is functional, the disruption will lead to impairing of membrane association, resulting in a negative dominant. To check the importance of this site for virus infection, the aminoacid G^{46} was muted to A, resulting in TCTPG46A sequence. After sequence confirmation, TCTP mutants were fused to RFP and overexpressed by agroinfiltration in *N.*

benthamiana plants. After 24h, the same leaves were infiltrated with TuMV/6K2:GFP and the effect in virus infection was observed.

The expression of TCTP mutants was observed by confocal microscopy in healthy and infected plants. Comparing with WT TCTP, no difference was observed in TCTP or virus location when any of the mutants was expressed (Figures S3 and S4). Moreover, virus accumulation was analyzed by Western blot. The results have shown that the overexpression of both mutants results in the same effect than that observed for WT TCTP, increasing virus accumulation (Figure S5).

The finding that virus infection increases considerably TCTP RNA level, but not protein accumulation, leads us to predict the RNA structure corresponding to the codifying region for both mutants using RNA Folding Form software. The structure was basically the same for both mutants and for the WT and TCTPstop (Figure S6), with a slight change in a small hairpin in TCTPE12A (Figure S6, circulated with blue lines) and the formation of two additional hairpins in TCTPG46A that results in a slight shortening of the longitudinal axis of the structure (Figure S6, circulated with orange and red and indicated in purple, respectively). This small change does not disturb the predicted general structure, and some RNA effect would not be impaired for these mutations. However, the results presented in figure 6 of the manuscript have shown that protein expression is necessary to increase virus accumulation

.

EXPERIMENTAL PROCEDURES

Plasmids, molecular cloning and site directed mutagenesis

All plasmids used in the experiments described here were also described in the manuscript, except TCTP mutants RFP-TCTPE12A and RFP-TCTP46A. Briefly, TuMV/6K2:GFP expresses TuMV genome and 6K2 fused to GFP (Thivierge et al. 2008). RFP-TCTP and RFP-TCTPstop express TCTP wild type sequence or the non-translatable form of TCTP, respectively, fused to RFP fluorescent protein at N terminal extremity.

The designing of TCTP mutations were performed according to the aminoacidic sequence for tomato TCTP (Bruckner et al., 2016). A mutation to disrupt the catalytic triad E^{12} L^{85} and E^{152}, necessary for the interaction with GTPases, was performed by substituting glutamic acid (E) on position 12 for an alanine (A), resulting in the sequence TCTPE12A. The second mutation was performed by disruption of the putative myristoylation site GANPSA by substituting the glycine (G) at the position 46 for an alanine, resulting in the sequence TCTPG46A. For obtaining TCTP mutants clones, specific complementary primers were designed to introduce the desired mutation and insert a restriction site without exchange in the aminoacidic sequence, allowing the selection of clones by restriction analysis. The primers used were TCTP E12AF: 5’ CCT CAC CGG TGA TGC ATT GCT CTC TGA CTC 3’, TCTP E12AR: 5’ GAG TCA GAG AGC AAT GCA TCA CCG GTG AGG 3’, TCTP G46AF: 5’ CCG TCG ATG TAA ATA TTG CGG CAA ATC CTT CTG CT 3’ and TCTP G46AR 5’ AGC AGA AGG ATT TGC CGC AAT ATT TAC ATC GAC GG 3’. Each pair of primers were used for amplification of the whole plasmid pENTR11-TCTP using Pwo DNA polymerase (Roche). The PCR product was treated with the enzyme DpnI (New England Biolabs) for 4 hours at 37°C and transformed into *E. coli* DH5α by heat chock using standard procedures. Cloning was verified by restriction pattern and sequencing (McGill University and Génome Québec Innovation Center). A correctly mutated clone was transferred to pK7WGR2 by gateway recombination system, using LR clonase II enzyme mix (Invitrogen) according to the manufacturer´s instructions, resulting in the plasmids RFP-TCTPE12A and RFP-TCTPG46E.

Histological preparation and confocal microscopy

The stem of *N. benthamiana* plants systemically infected with TuMV/6K2:GFP or of healthy plants were fixed and cryossectioned transversally and longitudinally as described by Wan et al. (2015). TCTP was immunolabeled by using of specific rabbit polyclonal antiserum as primary antibody and goat anti-rabbit conjugated to Alexa fluor 568 secondary antibodies (Molecular Probes). Cellular wall was labeled by the cell wall dye Fluorescent Brightener 28. The samples were observed by confocal microscopy using Zeiss LSM780 inverted confocal microscope. For excitation of Fluorescent Brightener 28, GFP and Alexa Fluor 568, lasers Argon 405nm, Argon 488nm and HeNe 561 were used and the emitted lights between 410-440nm, 500-535nm and 585-620nm were captured, respectively. The images were acquired with a 40X oil immersion objective. Image processing was performed using ZEN2.1 software (Carl Zeiss).

Immunoprecipitation

Plants were agroinoculated with pCambia-TuMV/6K2:GFP. After 5 days, 4 g of leaf tissue of each sample was grounded in liquid nitrogen and homogenized in lyses buffer [50mM Tris-HCl pH7,5; 150mM NaCl; 10%

Glycerol; 0,1% NP-40; 5mM DTT and 1X Complete Protease Inhibitor (Roche)]. Cell debris was removed by centrifugation at 20000g for 15 min at 4°C. The supernatant was incubated with GFP-Trap resin (Chromotek) under slow mixing at 4°C for two hours. After that, the sample was centrifuged at 2500 g for 2 min at 4°C and the pellet was washed in washing buffer [10mM Tris-HCl pH 7,5; 150mM NaCl; 0,5mM EDTA pH8,0] 4 times at 2500 g for 2 min at 4°C. For elution and western blot, loading protein buffer was added to the pellet and boiled for 10 minutes.

Protein transient expression and confocal microscopy

Agrobacterium tumefaciens strain AGL1 harboring pK7WGR2, RFP-TCTP, RFP-TCTPE12A, RFP-TCTPG46A or the P19 silencing suppressor were grown until exponential phase and then, centrifuged. After discarding the medium, the cultures were resuspended in agroinfiltration buffer (MES 10mM; MgCl2 10mM and Acetosseringone 200µM), adjusting the OD_{600} to 0.3. Leaves of *N. benthamiana* plants were completely agroinfiltrated with a 1:1 mix of each construction and P19 and after 24h, TuMV/6K2:GFP was agroinfiltrated using an OD_{600} adjusted to 0.003. The samples were imaged four days after virus inoculation using Zeiss LSM780 inverted confocal microscope or used by protein extraction and western blot, as described below. The images were acquired with a 40X oil immersion objective. Image processing was performed using ZEN2.1 software (Carl Zeiss).

Protein extraction and Western blot

Collected *N. benthamiana* leaves were grounded in liquid nitrogen and total protein was extracted using protein extraction buffer (Sodium citrate 50mM;

SDS 5%; BSA 0,01%; NaCl 150mM; β-mercaptoethanol 2%) in a proportion of 2 ml of buffer per gram of leaf tissue. The mix was boiled for ten minutes and the supernatant used for further analysis. SDS-PAGE and western blot were performed using standard procedures. Specific rabbit polyclonal antiserum against TuMV CP (Cotton et al. 2009) was diluted 2500 times and that against TCTP (Bruckner et al., 2016) was diluted 10000 times. The secondary antibody Anti-Rabbit IgG (H+L), Peroxidase labeled (KPL) was used according to the manufacturer´s instruction. Membrane revelation was performed using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer´s instruction.

RNA structure prediction

The codifying sequence of TCTPE12A and TCTPG46A were used for RNA structure prediction using RNA Folding Form (version 2.3 energies) software from mFold RNA server (Zuker, 2003) with standard parameters, except for temperature, which was 25°C. The structures with higher ΔG were chosen for analysis.

DISCUSSION

The present data support the results presented on the manuscript. The confocal images of cryossectioned samples confirm the localization observed by transient TCTP overexpression. It gives more reliability for the result, since protein overexpression sometimes can lead to the relocation of protein to cellular compartments where it does not actually belongs. The co-localization of TCTP with 6K2 in the perinuclear globular structure and vesicles was confirmed. In addition, TCTP localization in xylem was analyzed. The idea of the analysis in xylem vessels comes because xylem is a ‘clean’ environment, in comparison with other cellular types. Therefore, proteins that co-localize with 6K2-tagged vesicles in xylem must be part of long-distance movement vesicles. TCTP was not observed in the xylem vessels, neither in infected nor in healthy plants. Moreover, the co-immunoprecipitation experiments had shown that TCTP does not interact with 6K2. However, there are different kinds of vesicles and they must be specialized in specific roles during virus infection. Recently, the observation of TuMV induced membrane remodeling by electronic microscopy and tomography has demonstrated that three kinds of vesicle-like structures are observed over the infection. Convoluted membranes are suggested to be sites of protein translation, single membrane vesicles-like are suggested to be replication sites and double membrane vesicles-like are suggested to be virus assembling sites (Wan et al. 2015). In this context, TCTP probably is not part of the vesicles that move long distance through the xylem, but it might be associated with other types of vesicles in the cytoplasm of epidermal and parenquimal cells.

Acquisition of dominant negative mutants was not successful. During the designing of this experiment, we had to assume which characteristic of TCTP would be essential to support virus replication. We target the GEF activity (by mutation of the catalytic triad necessary for interaction with GTPases) because several studies have considered TCTP as a positive regulator of TOR signaling (Hsu et al. 2007; Berkowitz et al. 2008). This triad is highly conserved among eukaryotes (Thaw et al. 2001) and Hsu et al. (2007) have shown that the mutation of the glutamic acid E^{12} to a Valine (V) abolishes the GEF activity of TCTP from *Drosophila*. Also, the interaction with Ras GTPases was demonstrated for AtTCTP (Brioudes et al. 2010). An active TOR signaling was described as important for potyvirus infection with different requirements according to virus species. The infection of *Arabidopsis* by *Watermelon mosaic virus* is severely affected by TOR kinase gene silencing, while TuMV accumulation suffers a slight decrease in the same TOR silenced transgenic lines (Ouibrahim et al. 2015). Despite the uncertainty about the involvement of plant TCTP with TOR signaling, our results suggest that it is not the way TCTP is involved in virus replication, since the overexpression of a TCTP mutant with an inactive GEF site can increase virus accumulation at the same levels of WT protein.

The mutation in the putative myristoylation site was chosen because of the lack of evidence about how TCTP goes to the membrane. Whether the site was functional and TCTP was directly associated with the membranous part of the vesicles, the impairing of association with the membrane could result in reduction in virus replication. However, the effect of the mutation in TCTP membrane localization was not analyzed and since no alterations in protein location were observed by confocal microscopy, the site is likely not functional. The absence of a negative effect on the expression of this mutant indicates that this aminoacid is not important for TCTP’s role in virus infection. Since TCTP is multifunctional, regulatory and presents many interactors, such as calcium or tubulin, there are still many features of the protein to be explored to find out how TCTP is contributing for virus infection. Mutations cause small modifications in RNA structure. It gave us support for the hypothesis about the effect of *TCTP* RNA. However, the results have shown the necessity of *TCTP* translation, suggesting that the protein is important. Further studies have to be performed to go deeply in the understanding of TCTP role in virus replication.

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Figure legends

Figure 1: Confocal images of TCTP immunodetection in stem cells. A and D) Cross-section of non-infected parenquimal cells. TCTP (in yellow) is observed in cytoplasm (A and D) and nucleus (D). B, C, E and F) Cross-section of parenquimal cells infected by TuMV. TCTP is observed in cytoplasm, nucleus and co-localizes with 6K2:GFP induced vesicles (in green). G, H and I) Longitudinal-section of healthy (G) and TuMV infected (H and I) xylem vessels. TCTP is not observed inside any xylem vessels, but it is present in parenquimal adjacent cells. All images of infected cells were duplicated and are shown with and without 6K2:GFP signal, to make it easier to visualize TCTP weak signal. Fluorescent brightener 28-stained cellular wall is colored in blue.

Figure 2: TCTP does not immunoprecipitate with 6K2. TCTP and 6K2-GFP were detected in Input. 6K2-GFP but no TCTP was detected in 6K2-GFP precipitated. The experiment was performed twice.

Figure 3: Localization of TCTP mutants in healthy cells. RFP-TCTP, RFP-TCTPE12A and RFP-TCTPG46A were expressed in *N. benthamiana* leaves. Confocal images demonstrate that the mutations cause no apparent changes in TCTP cellular localization.

Figure 4: Localization of TCTP mutants in TuMV infected cells. WT TCTP and TCTP mutants were overexpressed with TuMV/6K2:GFP. In all cases, TCTP co-localizes with 6K2 in the perinuclear globular structure (stars) and in some small 6K2-tagged vesicles (arrows). A) Overexpression of RFP-TCTP and TuMV/6K2:GFP. B) Overexpression of RFP-TCTPE12A and TuMV/6K2:GFP.

C) Overexpression of RFP-TCTPG46A and TuMV/6K2-GFP. GFP and RFP fluorescence were digitally colored in green and red, respectively.

Figure 5: Effect of TCTP mutants on virus infection. The top panel corresponds to virus accumulation measured by western blot using polyclonal antiserum against TuMV CP. From the top to the bottom, the second panel corresponds to the overexpression of WT and TCTP mutants fused to RFP and the third panel corresponds to the measurement of endogenous TCTP, both detected using polyclonal antiserum against TCTP. The bottom panel corresponds to loading control stained with comassie blue.

Figure 6: RNA structure prediction for TCTP mutants. The structures predicted for TCTP, TCTPstop, TCTPE12A and TCTPG46A, and free energy associated with each structure are demonstrated. The mutated aminoacids are highlighted in green. The hairpins and axis commented in the text are highlighted, as follows: hairpin 1 circulated in blue, central axis 2 length indicated in purple, additional hairpins present only in TCTPG46A structure 3 and 4 circulated in orange and red, respectively.

FIGURES

Figure S1

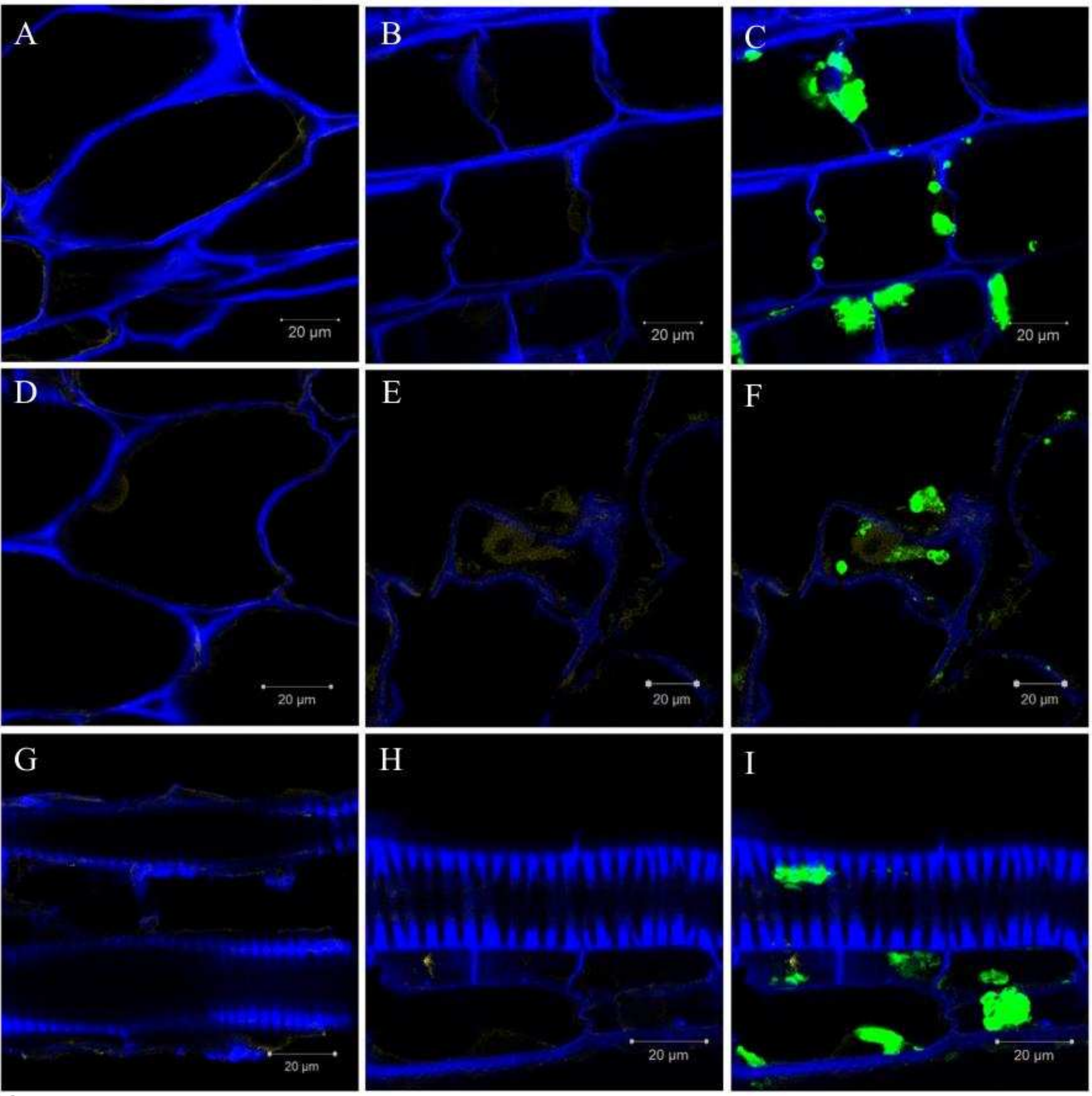
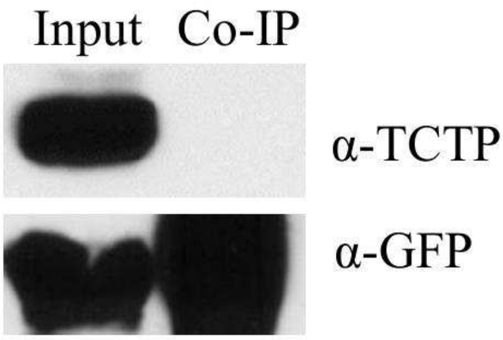


Figure S2



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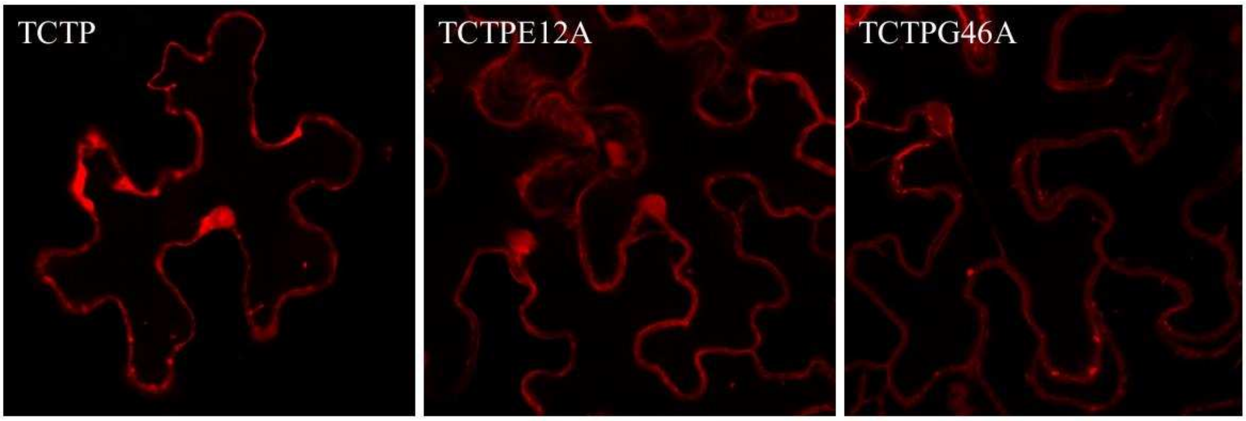

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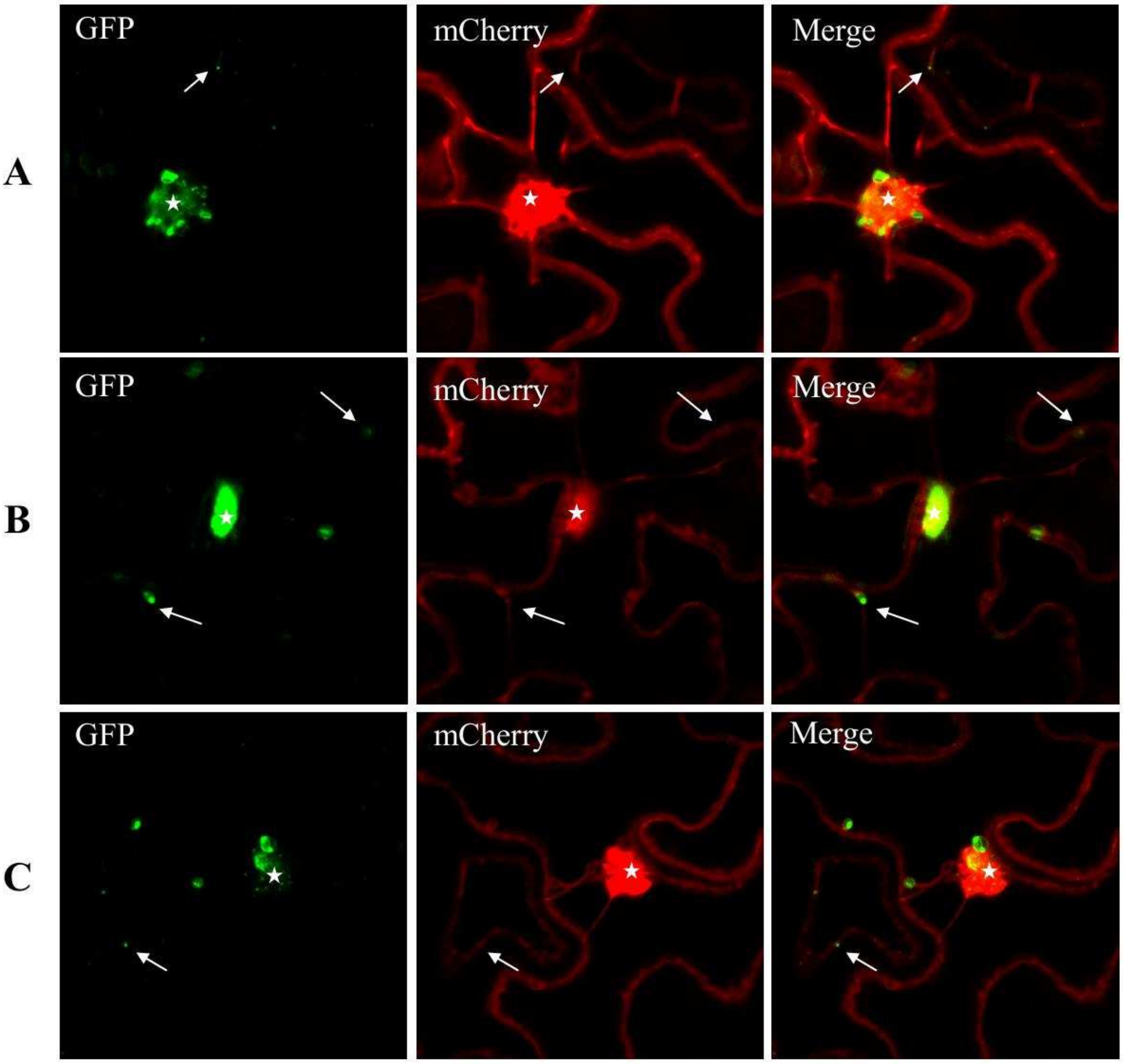


Figure S5

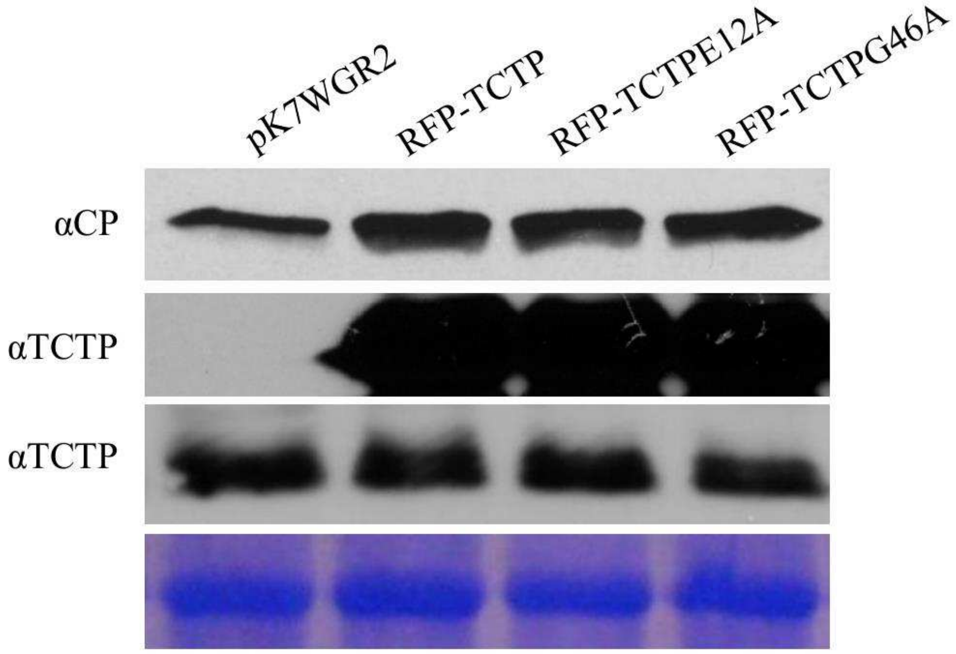
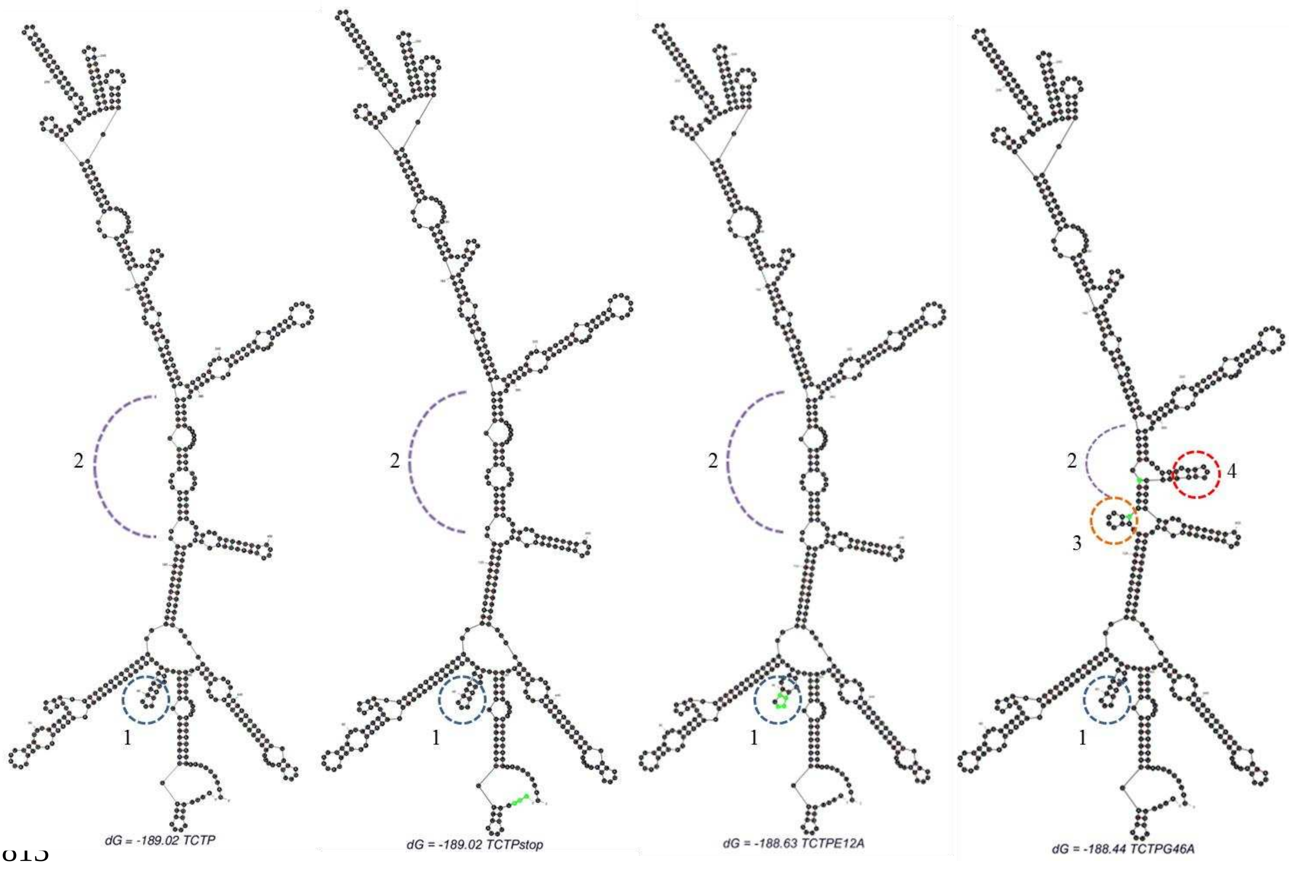


Figure S6:



GENERAL CONCLUSIONS

The translationally controlled tumor protein (TCTP) is a host factor necessary for plant infection by potyviruses. Its gene silencing affects virus replication in protoplasts, and its transient overexpression increases virus accumulation.

TCTP co-localizes with vesicles induced by the potyviral protein 6K2 that may be the replicative vesicles, and also with a 6K2-tagged perinuclear globular structure whose function is still unknown. However, TCTP does not co-immunoprecipitate with 6K2, suggesting the two proteins do not interact directly.

TCTP mRNA expression is induced by potyvirus infection, but no increase in TCTP protein leves was detected during the first steps of potyvirus infection. TCTP locates mainly in soluble cellular fraction of healthy and infected cells, and a small portion is membrane associated, which suggests it might be part of replicative vesicles.

TCTP is a regulatory and multifunctional protein, and has multiples molecular partners that could be involved in virus infection. Point mutations aiming to disrupt TCTP activity as a nucleotide exchange factor (GEF) and a putative miristoilation site do not affect TCTP positive effect in virus replication. A non translatable mutant of *TCTP* RNA was overexpressed to check if *TCTP* RNA contributes for virus infection. Its expression has no effect in virus accumulation, indicating that the protein and/or the translation of *TCTP* RNA are important for potyvirus replication.