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Natural infection of cape gooseberry (*Physalis peruviana*) by the potyvirus Tamarillo leaf malformation virus (TaLMV)

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Summary. – Tamarillo leaf malformation virus (TaLMV) is a potyvirus first discovered in cape gooseberry fields in Eastern, and South-western Antioquia. This virus is responsible for a very damaging disease that has resulted in significant reductions in yields and cultivated area for this crop in Colombia. Tamarillo is frequently co-cultivated with other solanaceous plants but no evidence for cross-pathogenicity of TaLMV has been found until now. In this work, we report a natural infection of cape gooseberry (*Physalis peruviana* L.) by TaLMV. Infection by TaLMV was detected by RNAseq screening of cape gooseberry fields and confirmed by RT-qPCR and Sanger sequencing. The sequenced genome is 99.3% identical to previously sequenced TaLMV isolates, and evidence suggests that it can accumulate at high loads in this new reported host. RT-qPCR analysis indicates that TaLMV is already widely distributed, can naturally infect other solanaceous hosts and may become an emerging threat to the cape gooseberry agroindustry, the second most important exotic fruit export in Colombia.

Keywords: high-throughput sequencing; plant virology; Potyviridae; RT-qPCR; Solanaceae

Introduction

Tamarillo leaf malformation virus (TaLMV) is a potyvirus that infects tamarillo (*Solanum betaceum* Bosh), a solanaceous fruit plant of Andean origin mainly cultivated in Colombia, Ecuador, India, and New Zealand (Enciso-Rodríguez *et al*., 2010; Biodiversity International, 2013). TaLMV is the most damaging virus of the tamarillo agroindustry in Colombia, where it causes a disease known as "Virosis" that is characterized by rugose mosaics, the outgrowth of veins, dark-green blisters, severe leaf deformation, immature fruits with conspicuous color breaks, and mature fruits with hardened pulp and purple patches on the skin (Tamayo, 1996; Gil *et al*., 2009; Duque *et al*., 2017). The "Virosis" of tamarillo was first reported in 1991 in Northern Antioquia and during the early 2000s was responsible for a decrease in the cultivated area of this crop estimated at approximately 50% (Mejía *et al*., 2009). TaLMV was first identified in 2010 through partial sequence analysis of the NIb-CP region, which revealed a potyvirus that was clearly distinct from other known members within the genus (Ayala *et al*., 2010), and fur-

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Abbreviations: CDV = Colombian datura virus; CP = coat protein; Ct = cycle threshold; HC = helper component protein; HTS = high-throughput sequencing; JTT = Jones-Taylor-Thornton model; NIa = nuclear inclusion protein a; NIb = nuclear inclusion protein b; ORF = open reading frame; PIPO = pretty interesting Potyviridae ORF; PVDP = Plant-Virus-Detection-Pipeline; RNAseq = RNA sequencing; RT-qPCR = reverse transcription quantitative real-time PCR; TaLMV = Tamarillo leaf malformation virus

ther work using conventional molecular and serological techniques confirmed the widespread distribution of this virus in the main tamarillo-producing regions of Antioquia such as the outskirts of Medellín (Santa Elena), La Ceja, La Unión, Marinilla, Entrerríos, Sonsón, Yarumal, Carmen de Víboral and Jardín (Ayala *et al*., 2010; Gallo-García *et al*., 2011).

The first genome sequence for TaLMV was obtained in 2014 in a high-throughput sequencing (HTS) study of tamarillo plants in La Unión (Eastern Antioquia) that exhibited mosaics, dark-green blisters, severe leaf deformation and defoliation, among other symptoms (Gutiérrez *et al*., 2015). This virus was finally recognized by the International Committee on Taxonomy of Viruses (ICTV) as a new species within the genus *Potyvirus* (the family *Potyviridae*) in 2015 and is currently listed as one of the 235 species approved within this genus (https://talk.ictvonline.org; ICTV Master Species List #3, 6 March 2021). A follow-up study sequenced a second TaLMV genome a few years later in field tamarillo samples from Santa Rosa de Osos in Northern Antioquia, thus confirming the distinct nature of this virus and its widespread distribution in this province (Duque *et al*., 2017). TaLMV has a genome size of 9.6 kb that encodes a polyprotein of 3,073 amino acids with typical cleavage sites for the P1, HC, and NIa proteases. TaLMV seems to be a well-conserved virus as sequences from the two fully sequenced TaLMV genomes (gb: NC_026615 and KR181938) share 99.3% nucleotide identity, and similar values have been observed for partial sequence comparisons (Gutierrez *et al*., 2015; Duque *et al*., 2017).

Despite frequent co-cultivation of tamarillo with other solanaceous crops such as tomato (*S. lycopersicum*), bell pepper (*C. annuum*), lulo (*S. quitoense*), potato (*S. tuberosum* and *S. phureja*), and cape gooseberry (*P. peruviana*), TaLMV has only been reported to infect *S. betaceum*. This is an unexpected result, as tamarillo can be infected by several of the viruses affecting these crops, and cross-pathogenicity among these hosts is well-documented in the region (Jaramillo *et al*., 2011; Henao-Díaz *et al*., 2013; Alvarez *et al*., 2017; Gallo *et al*., 2020, 2021). Here, we present the first report of TaLMV infection in a plant host different than tamarillo: cape gooseberry. Cape gooseberry is the second most important exotic fruit export in Colombia with annual production estimated at 12,152 tons, comprising about 976 ha of cultivated area (Procolombia, 2020). Within Colombia, the province of Antioquia is currently the most efficient producer with an average yield of 18.1 t/ha surpassing the national average of 12.45 t/ha (Agronet, 2021). Unfortunately, cape gooseberry is highly susceptible to a wide variety of viruses that are starting to have a serious impact on productivity (Gallo *et al*., 2018, 2020; Corrales-Cabra *et al*., 2021). As part of a local initiative describing the viruses affecting the cape gooseberry crop in South-western Antioquia using RNAseq, we have identified and assembled a third TaLMV genome from commercial plots. The prevalence of TaLMV in different municipalities was further investigated using RT-qPCR and Sanger sequencing. These results suggest that TaLMV can infect other plant hosts and may become an emerging threat to the cape gooseberry agroindustry.

Materials and Methods

Plant material. Cape gooseberry samples were collected at commercial plots in the municipalities of Jardín (5.5988° N, 75.8198° W), Urrao (6.3139° N, 76.1318° W), La Unión (5.9340° N, 75.3645° W), Sonsón (5.7120° N, 75.3098° W), San Vicente (6.3033° N, 75.3179° W) and Rionegro (6.1409° N, 75.4112° W) in Southwestern, and Eastern Antioquia (Colombia) (Fig. 1a). A total of twenty samples were obtained from adult plants and each sample consisted of twenty leaves collected at random from different commercial *P. peruviana* plots. For RT-qPCR, each sample was tested individually, while for RNAseq, the analyte consisted of a bulked sample comprising leaves from three samples collected in the municipality of Urrao (PP92, PP94, and PP95), which were selected due to the presence of some plants showing symptoms of a distinctive rugose mosaic, leaf distortion, green islands, and necrotic veins (Fig. 1b-c).

RNAseq. High-throughput sequencing was performed on total RNA extracted using the Trizol method from 100 mg of leaf tissue finely ground into a powder with a pestle and a mortar using liquid nitrogen (Chomczynski and Sacchi, 1987). The quantity and quality of RNA were determined with a 2100 Bioanalyzer (Agilent Technologies, USA). cDNA libraries were synthesized with the TruSeq Stranded Total RNA LT Sample kit (Illumina, USA); ribosomal RNA (rRNA) was removed with the Ribo-Zero Plant kit (Illumina). Sequencing was performed with the Illumina NovaSeq system service provided by Macrogen (South Korea), which resulted in a paired-end library of 12,391,139 reads of 101 nt with an average quality Phred score of 37. Preliminary detection of TaLMV was achieved with the Plant Virus Detection Pipeline (Gutiérrez *et al*., 2021). The *P. peruviana* TaLMV isolate (TaLMV-Physalis) was assembled *de novo* with rnaSPAdes (Bushmanova *et al*., 2019), and the resulting contig was manually inspected for errors and inconsistencies using the Integrative Genomics Viewer program (https://software.broadinstitute.org/software/) after mapping reads with Magic-BLAST (ncbi.github.io/magicblast/). The ORF encoding the potyviral polyprotein was identified with ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder) and BLASTX (Gish and States, 1993). P3N-PIPO, and potyboxes A and B, were identified by sequence comparison with the available TaLMV genomes. The final curated genome was submitted to the NCBI nucleotide database under accession code OL355097.

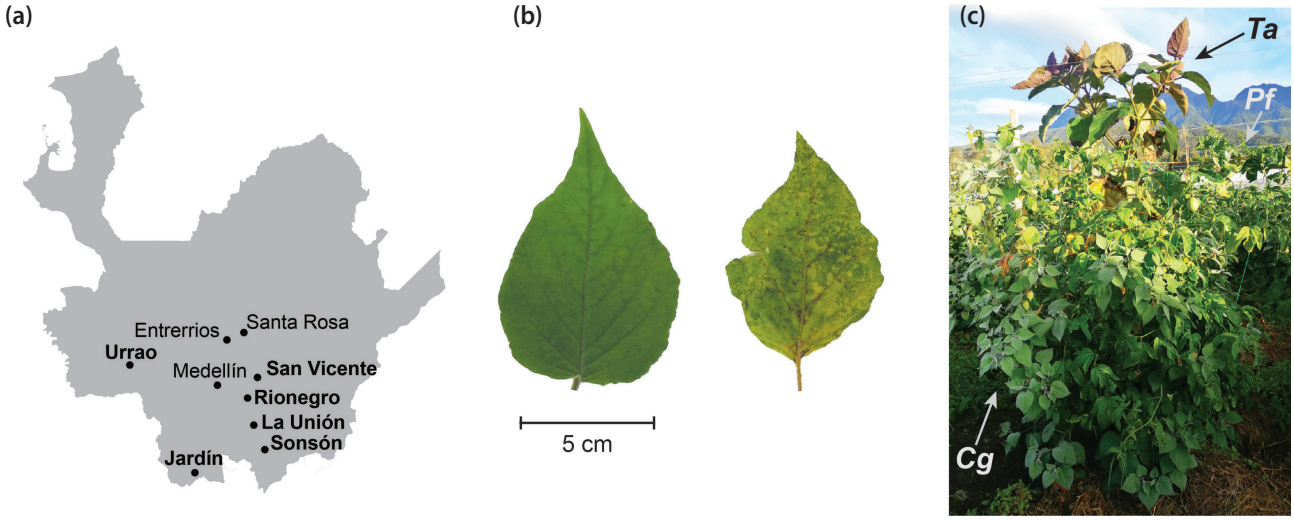

Fig. 1

Collection of cape gooseberry samples

(a) Cape gooseberry samples were collected from the municipalities in South-western (Urrao, and Jardín), and Eastern Antioquia (San Vicente, Rionegro, La Unión, and Sonsón) shown in bold. Other regions where TaLMV has been detected are shown in normal case. **(b)** Asymptomatic cape gooseberry leaf with characteristic pointed heart shape of about 6–15 cm long and 4–10 cm wide (left); collected leaf sample exhibited symptoms typical of viral infection, which included rugose mosaic, leaf distortion, green islands, and necrotic veins (right). **(c)** Cape gooseberry (Cg) is an herbaceous plant of about 0.9–1.6 m in height that is typically co-cultivated with other crops such as tamarillo (Ta) and purple passion fruit (Pf).

Phylogenetic analysis. Sequence alignments were performed with the online version of MUSCLE (Edgar, 2004), available at the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/muscle/) using default parameters; columns containing missing or ambiguous residues were removed with MEGAX (Kumar *et al.*, 2018). The phylogenetic reconstructions were made using the Neighbor-Joining method (Saitou and Nei, 1987) with 1,000 bootstrap replicates in MEGAX. For the polyprotein analysis, evolutionary distances were calculated with the JTT model (Jones *et al.*, 1992) using a gamma distribution with a shape parameter of 0.7; for the partial CP sequences, distances were calculated using the Tamura-3 parameter model (Tamura, 1992) using a gamma distribution with a shape parameter of 2.3. The Tamura-3 parameter was selected based on the Akaike information criterion estimated with Modeltest (Posada and Crandall, 1998); the JTT model was selected as it is the one used by the ICTV to demarcate species within the genus *Potyvirus* (https://talk.ictvonline.org/).

RT-qPCR and RT-PCR. Total RNA was extracted using the Trizol method from 100 mg of leaf tissue finely ground into a powder with a pestle and a mortar with liquid nitrogen (Chomczynski and Sacchi, 1987). RNA was eluted in 40 µl of DEPC-treated water and preserved at -20°C until further use. RNA concentration and purity for each sample were determined using the A260/280 ratio measured in a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized using 200 U of RevertAid Reverse Transcriptase (RT) (Thermo Fisher Scientific), RT buffer (1X), dNTPs at 0.5 mM, 20 U of RiboLock RNase Inhibitor, 100 ng of total RNA and 100 pmoles of Oligo-(dT)18 in a reaction volume of 20 µl at 42°C for 1 h. The real-time PCR (qPCR) step was performed in a final volume of 14 µl with 6.25 µl of the Maxima SYBR Green/ROX kit (Thermo Fisher Scientific) using 50–100 ng of cDNA and 0.3 µM of primers TaLMV_F_CP (5'-GCT GAT AAA CTT GAT GCC GGA GC-3'), and qTaLMV_R_CP (5'-GAT GAA CCA GCA TCG ACA TCC C-3') targeting the CP region of TaLMV (Duque *et al.*, 2017). Amplifications were carried out in a Rotor-Gene Q-5plex Real-time PCR cycler (Qiagen, Germany) with 10 min at 95°C, following 35 cycles of 15 s at 95°C, and 60 s at 52°C. The specificity of the RT-qPCR results was confirmed by high resolution melting (HRM) in the 50-99°C range being the expected Tm range of 79 ± 1°C (Duque *et al.*, 2017). Additionally, cDNA from three of the samples that resulted positive for TaLMV was used as a template for conventional PCR in a total volume of 25 µl containing 1X enzyme buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Thermo Fisher Scientific), 50–100 ng of cDNA, 0.2 µM of primers TaLMV_F_CP and TaLMV_R_CP (5'-AGG TTG TGC ATG CCA CGG-3') (Duque *et al.*, 2017) and 17.8 µl H_2O. The PCR program had an initial denaturation step at 95°C for 3 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min and extension at 72°C for 1 min; a final extension step at 72°C for 5 min was included at the end of reactions. Amplicon sizes were verified in a 1.5% agarose gel electrophoresis containing GelRed 1X (Biotium, USA) and visualized in a ChemiDoc MP Imaging System (BioRad, USA). Positive and negative controls were included in each test and consisted of infected tamarillo

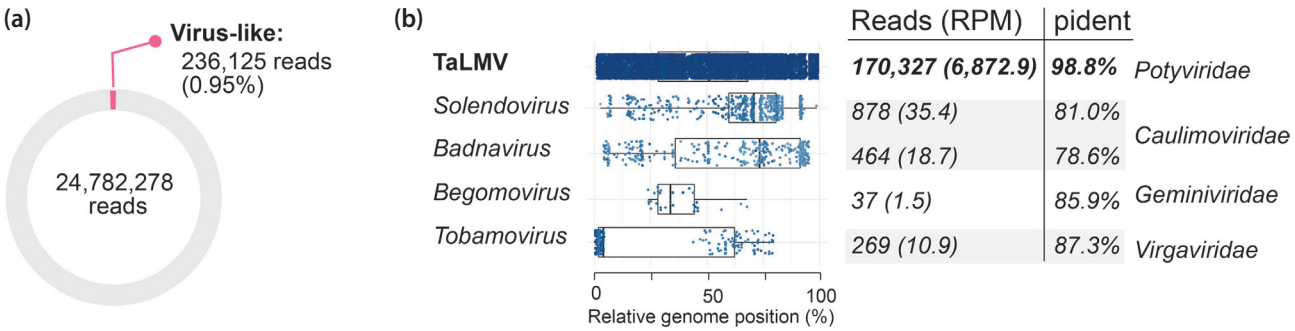

Fig. 2

Detection by RNAseq of TaLMV in *P. peruviana*

Evidence for TaLMV infecting *P. peruviana* was obtained from the analysis of a bulked RNA sample of leaves using the PVDP software. **(a)** Approximately 0.95% of virus-like sequences exhibited sequence similarity to viruses within the families *Potyviridae*, *Geminiviridae*, *Caulimoviridae*, and *Virgaviridae*. **(b)** According to this analysis, TaLMV was the dominant virus representing 72.1% of viral sequences sharing an average of 98.8% nucleotide sequence identity with respect to reference TaLMV sequences.

and virus-free samples. For five RT-qPCR and the three RT-PCR amplicons, identity was confirmed by gel electrophoresis, and Sanger sequencing after purification with the GeneJET Gel Extraction kit (Thermo Fisher Scientific). Sanger sequencing was performed at Macrogen (South Korea) using an Applied Biosystems 3730XL sequencer.

Results and Discussion

RNAseq analysis

TaLMV was first detected in a bulked sample of diseased cape gooseberry leaves collected from three commercial plots in Urrao showing symptoms that included rugose mosaics, dark green islands, and necrotic veins (Fig. 1b). The suspect sample was processed for RNAseq analysis and automated detection of viruses with the software PVDP (Gutiérrez *et al.*, 2021) revealed TaLMV as the dominant virus at 6,873 reads per million (170,327 reads). Besides TaLMV, a smaller fraction of reads shared moderate sequence similarities (78.6–87.3%) with viruses in the families *Caulimoviridae* (1,342 reads), *Virgaviridae* (269 reads) and *Geminiviridae* (37 reads); these sequences were not investigated further in this work but probably represent virus-like sequences derived from the host plant (*Caulimoviridae*) or viruses present at low titer (Fig. 2a,b). *De novo* assembly of the TaLMV genome from *P. peruviana* (TaLMV-Physalis) resulted in a contig of 9,661 nt sharing 99.3% nucleotide sequence identity with respect to the complete genomes of TaLMV isolate A (NC_026615) from La Unión (Eastern Antioquia), and Dic4 (KR181938) from Santa Rosa de Osos (Northern Antioquia). The TaLMV-Physalis genome was assembled at 1,769x coverage and contained all molecular signatures typical of potyviruses: a large ORF encoding a polyprotein of 3,073 amino acids at nucleotide positions 140–9,361; a segment encoding the slippage product P3N-PIPO at positions 2,441–3,150, which includes the slippage sequence motif GA_7T at position 2,900; and Potyboxes A (TACAACAT) and B (TTTCAAGC) at positions 38–45 and 68–75 (Fig. 3a). The polyprotein contained protein domains consistent with the function of potyviral proteins and putative protease cleavage sites were identical to those described for TaLMV (Adams *et al.*, 2005) (Fig. 3a).

A phylogenetic analysis of complete polyproteins placed TaLMV-Physalis within a clade that includes TaLMV isolates A and Dic4 (100% bootstrap) and sister to the Colombian datura virus (CDV) clade (Fig. 3c). The high number of TaLMV reads detected in the RNAseq sample suggests that this virus is well-adapted to this new host and only 60 and 62 nucleotide substitutions were found in the genome with respect to isolates A and Dic4, respectively; these differences are slightly higher than the 43 changes observed between TaLMV isolates infecting *S. betaceum* (Fig. 3b,d). At the amino acid level, 17 changes were observed with respect to isolates A and Dic4, twelve of which were unique to TaLMV-Physalis. Four changes mapped to the P1 (L77S, P137S, M138R, and G235S); one to HC-Pro (Q397R); two to P3 (A873V, and S963R); two to cylindrical inclusion (I1275V, and I1673V); two to NIb (I2295V, K2763R), and one to CP (A2944T) (Fig. 3d). To better understand the epidemiology and economic impact of TaLMV in solanaceous crops, these results should be complemented in the future with a more thorough HTS screening of potential TaLMV hosts and vectors in different geographical regions cultivating *P. peruviana*, and *S. betaceum*.

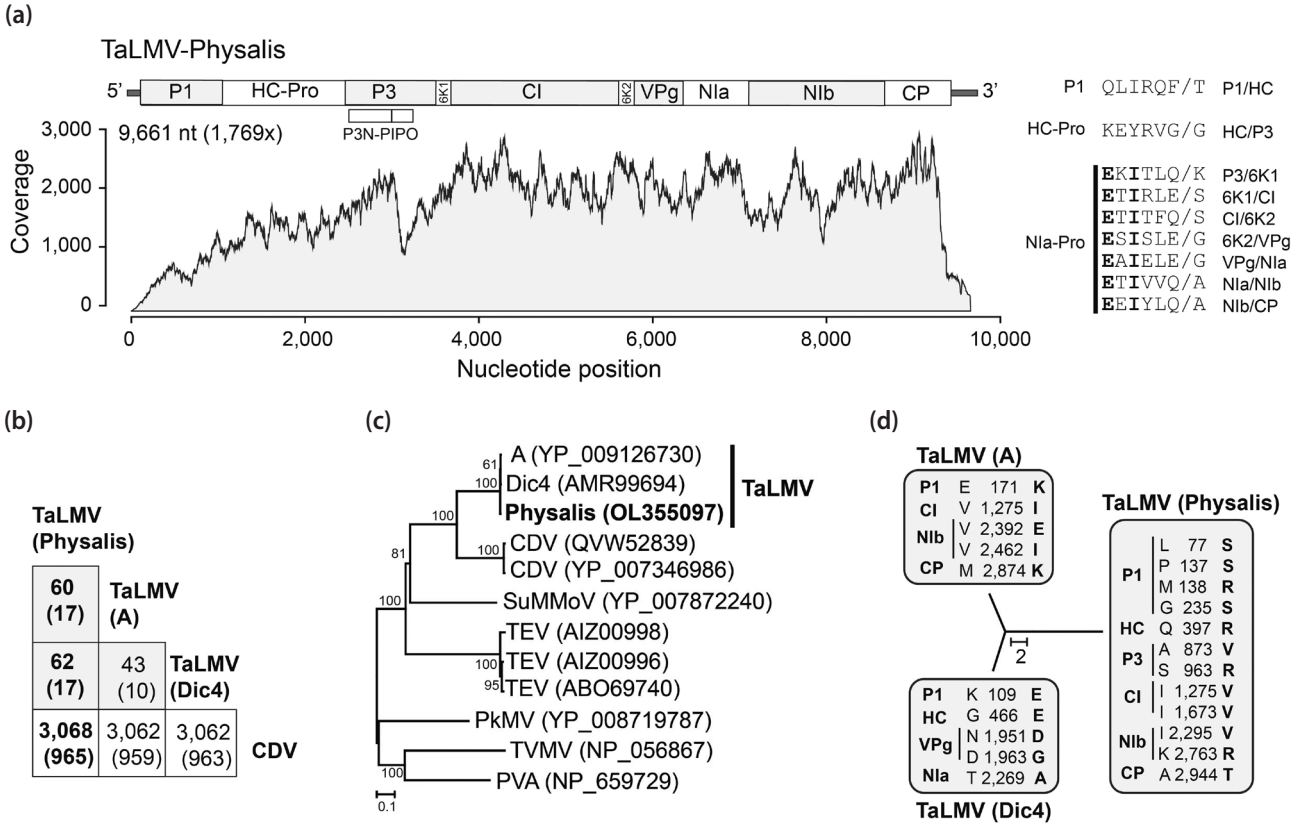

Fig. 3

Genome description of TaLMV-Physalis

(a) The TaLMV-Physalis was assembled as a contig of 9,661 nt with a sequence depth of 1,769x and encoded a polyprotein of 3,073 amino acids that is processed into ten mature proteins, in addition to slippage product P3N-PIPO. Predicted cleavage sites for the P1, HC, and NIa protease are shown to the right. **(b)** Nucleotide and amino acid changes of TaLMV-Physalis with respect to available TaLMV sequences, CDV was included as an outgroup. **(c)** Neighbor-joining tree of complete polyprotein sequences confirms TaLMV-Physalis as a new TaLMV isolate. **(d)** Dendrogram illustrating the amino acid differences between completely sequenced TaLMV isolates. TaLMV-Physalis exhibits twelve unique amino acid changes (bold), mostly within the P1 protein, that might be involved in adaptation to this new host.

RT-qPCR and RT-PCR

The presence of TaLMV in the RNAseq sample (BPP15) was further confirmed by RT-qPCR of the bulked RNA, and each of the individual samples used in the bulk. The bulked sample tested positive for TaLMV with a Ct of 14.6 that suggests high viral loads and is consistent with the high sequence coverage found by RNAseq. All three samples used in the bulk (PP92, PP94, and PP95) also tested positive for TaLMV with Ct values in the 13.1–22.5 range. The RT-qPCR amplicons had the expected size of 139 bp and Tm values were consistent with the reported range for amplification of the CP region of TaLMV (Duque *et al.*, 2017) (Fig. 4a,b); Sanger sequencing further confirmed the identity of the virus (not shown). The presence of the TaLMV was further investigated in sixteen leaf samples from Cape gooseberry crops at the municipalities of Jardín, Urrao, La Unión, Sonsón, San Vicente, and Rionegro (Fig. 4d). Infection of *P. peruviana* by TaLMV was detected in 60% of the samples with Ct values in the 8.7 (sample PP50 from Rionegro) to 29.3 (sample PP26 from Sonsón) range. TaLMV was detected in all samples from Rionegro and was not detected in any of the samples from La Unión or San Vicente (Fig. 4d). Conventional RT-PCR using primers TaLMV_F_CP and TaLMV_R_CP also resulted in fragments with the expected size of 805 bp (Fig. 4b), and sequencing of selected products confirmed the identity of the template as TaLMV (Accession number OL355098), which clustered in a clade with 100% bootstrap comprising all available partial TaLMV CP sequences (Fig. 4c).

TaLMV is a very damaging potyvirus in tamarillo crops in Colombia, for which no other natural hosts have been reported prior to this work (Ayala *et al.*, 2010; Gallo-García *et al.*, 2011; Gutiérrez *et al.*, 2015; Duque *et al.*, 2017). In Antioquia, the TaLMV epidemic in tamarillo is an important factor explaining the decrease in yields from

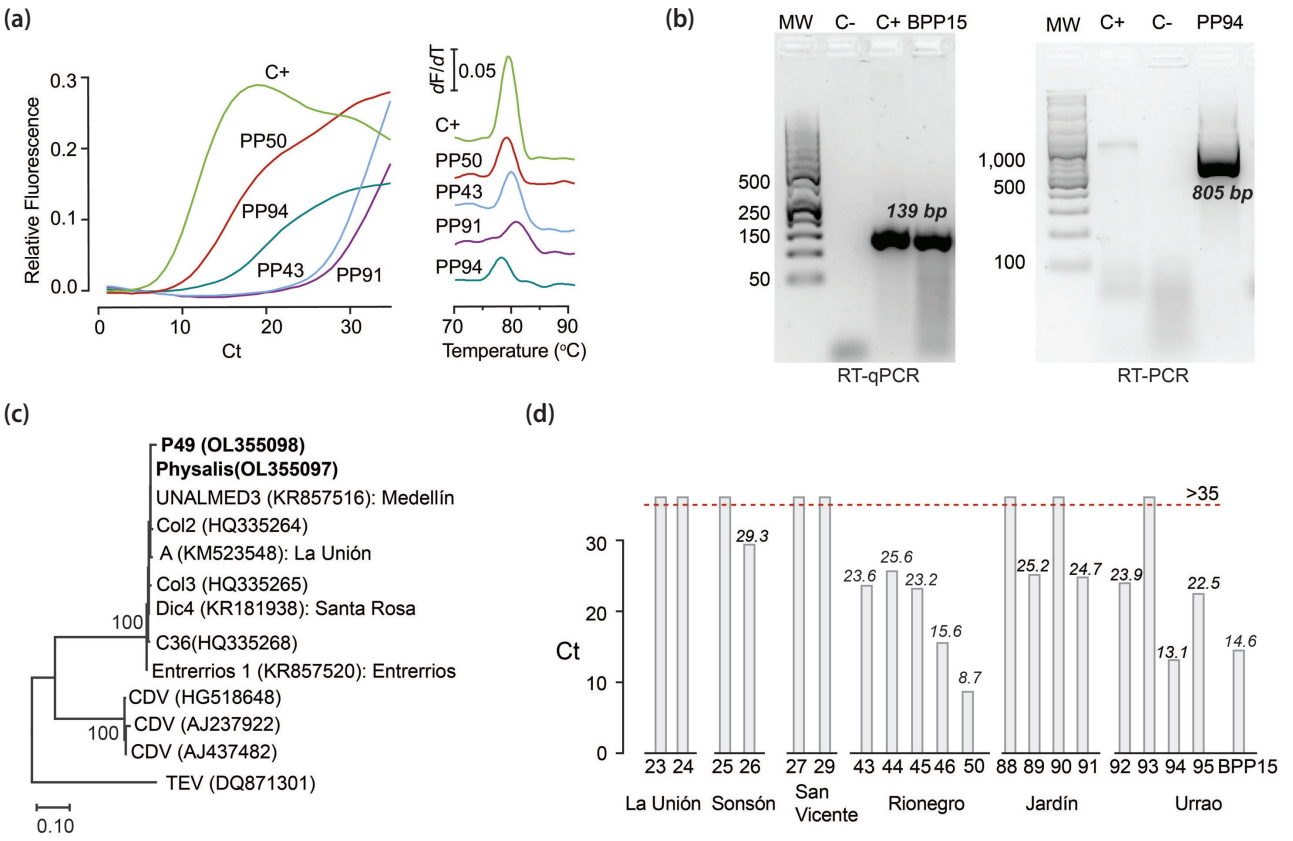

Fig. 4

Detection of TaLMV in cape gooseberry by RT-PCR

(a) Representative RT-qPCR amplification of cape gooseberry samples from different regions in Antioquia. Melting curves are shown to the right. **(b)** Agarose gel electrophoresis of RT-qPCR and RT-PCR amplicons of TaLMV in cape gooseberry showing bands with the expected size. **(c)** Phylogenetic analysis of the CP region of TaLMV detected in cape gooseberry further confirmed the identity of the virus. **(d)** RT-qPCR detection of TaLMV in leaf samples from different municipalities in Antioquia confirms the widespread distribution of this virus in this province.

40.36 t/ha in 2017 to 34.98 t/ha in 2021, and the 900 ha decrease in the cultivated area during the last five years (Agronet, 2021). In tamarillo, TaLMV-infected plants exhibit a gradual decrease in yields and fruit quality, and increased defoliation (Ayala *et al.*, 2010). Unfortunately, the lack of knowledge about viral diseases in tamarillo has resulted in farmers paying little attention to this disease, not removing infected plants from the fields, and even using them as tutors for climbing plant crops such as cape gooseberry and purple passion fruit (*Passiflora edulis* f. edulis) (Fig. 1c). It is likely that these factors have facilitated the cross transmission of TaLMV from tamarillo to cape gosseberry, probably by mechanical means and/or aphid vectors, which are the most common transmission mechanisms of potyviruses (Revers and García, 2015). Detection of TaLMV in cape gooseberry is alarming as this Andean crop has become one of the most important Colombian exports in the exotic fruit market (Procolombia, 2020). *P. peruviana* has been shown to be highly susceptible to viral diseases, which are becoming an increasing threat for local cape gooseberry producers (Gallo *et al.*, 2018, 2020; Corrales-Cabra *et al.*, 2021). Due to the economic impact of emerging viral diseases on cape gooseberry, several causal agents have been identified in Colombia based on complete and/or partial genome information, including an Eurasian potato virus X (PVX) strain (Gutierrez *et al.*, 2015), the potato virus Y (PVY) strain N; the potato virus V (PVV) strain Phu (Alvarez *et al.*, 2018), the new ilarvirus species cape gooseberry ilarvirus 1 (CGIV-1) (Gallo *et al.*, 2018), the southern tomato virus (STV) and the potato viruses: potato mop-top virus (PMTV), potato yellow vein virus (PYVV), potato yellowing virus (PYV) and potato virus S (PVS) (Gallo *et al.*, 2020). More recently, three new virus species tentatively named: Physalis vein necrosis virus (PhyVNV, Nepovirus), Physalis torradovirus (PhyTV, Torradovirus), and Physalis virus X

(PhyVX, Potexvirus) were found in a cape gooseberry HTS study from Eastern Antioquia using dsRNA preparations (Corrales-Cabra *et al*., 2021). Unfortunately, the present study shows that TaLMV should also be included as part of the *P. peruviana* virome and reinforce the idea that this virome is still largely unexplored (Gutiérrez *et al*., 2015; Gallo *et al*., 2018, 2020; Corrales-Cabra *et al*., 2021). TaLMV is a very damaging virus in tamarillo, and it is likely that it may have a similar impact in cape gooseberry; urgent measures should be implemented to avoid dispersion of this virus to other provinces of Colombia and countries where this fruit is cultivated. A good starting point would be the implementation of a planting material certification program of seeds and plantlets; together with field surveys using available PCR-based tools, as well as previously described polyclonal antibodies targeting the variable N-terminal region of TaLMV (Gallo-García *et al*., 2011). In conclusion, in this work we have shown natural infection of cape gooseberry by TaLMV in the province of Antioquia (Colombia) using RT-qPCR and HTS, which is the first report for this damaging virus in a host different than tamarillo.

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