OPEN ACCESS Freely available online

Journal of Citrus Pathology

Recently Accepted

High throughput sequencing of a stem pitting citrus tristeza virus isolate from Hunan Province P.R. China.

G. Licciardello[1,2*], G. Scuderi[1,2], R. Ferraro[1], M. Russo[1,2], S. M. Dai[3], A. Catara[1], Z. N.Deng[3]

[1]Science and Technology Park of Sicily, z.i. Blocco Palma I, Stradale Lancia 57, 95121 Catania, Italy
[2]Agrobiotech Soc. Coop. z.i. Blocco Palma I, Stradale Lancia 57, 95121 Catania, Italy
[3]National Center for Citrus Improvement (Changsha), Hunan Agricultural University, Hunan 410128, P.R. China.

Corresponding author: grazia.licciardello@crea.gov.it

***Present address:** Council for Agricultural Research and Economics, Research Centre for Olive, Citrus and Fruit Trees (CREA-OFA), Acireale (Catania), Italy

Citation: Licciardello, G., Scuderi, G., Ferraro, R., Russo, M., Dai, S., Catara, A. F, & Deng, Z. N. (2023). High Throughput Sequencing of a stem pitting Citrus Tristeza Virous isolate from Hunan Province P.R. China. *Journal of Citrus Pathology*, 10. http://dx.doi.org/10.5070/C410150834 Retrieved from https://escholarship.org/uc/item/99g3b9vw

Keywords: Citrus tristeza virus, next generation sequencing, small RNAs, reference genomes.

Abstract

A stem-pitting isolate of citrus tristeza virus (CTV), spreading in Hunan province of China (HU-PSTS), assessed as a complex isolate based on the molecular marker and CE-SSCP testing, was sequenced and indexed on indicator plants. Biological assays showed that HU-PSTS is an aggressive stem pitting isolate belonging to biotype 5. Viral small RNAs (18-26 nt) of the isolate were deep sequenced by Illumina technology and the reads mapped with 17 CTV reference genomes. The high percentage of mapped reads (47-41%) and genome coverage (98-100%) obtained with SG29, T318A, CT11A, Nuaga and AT-1 reference genomes enabled to re-assemble the full genome of a VT strain. T68, T30 and T3 genomes were less represented with a coverage above 80%. Alignments with genomes belonging to T36 and RB strains revealed small percentage of mapped reads (10-12%) and genome coverage (52-57%), thus excluding the presence of these strains. To our knowledges, this is the first sequenced genome of a CTV isolate from Hunan province.

Introduction

Citrus tristeza virus (CTV) is a *Closterovirus* transmitted worldwide by propagation material and vectors. Different variants and strains coexist in an area and may co-infect a single tree. Two main phenotypes cause substantial damage to the citrus industry in terms of decline and stem pitting. Quick or slow 'decline' (CTV-D) is responsible for destructive epidemics killing millions of sweet orange trees grafted on sour orange rootstock (Moreno and Garnsey 2010). Stem pitting affects grapefruit and/or sweet orange scions (CTV-SP), regardless of rootstock. Some strains causing decline may (or may not) induce seedling yellows (CTV-SY) on specific hosts (Moreno, 2008).

With more than 320,000 ha of citrus trees, Hunan Province provides 15% of the total production in China and is one of the most important production areas in the world (Spreen et al., 2012). The use of CTV-tolerant rootstocks has long protected its citrus industry from the devastating effects caused by the CTV-D isolates, allowing a fast development of citriculture. However, stem pitting is spreading and significant damage to the citrus production is feared (Zhou et al., 2007). Moreover, isolates inducing seedling yellows (SY) have been recorded during extensive bioindexing (Rizza et al., 2010; Licciardello et al., 2015a).

Within the framework of a research project between China and Italy, in 2016 the genetic structure of local CTV isolates was investigated along with the feasibility of finding ways to protect the local citrus industry (Costa et al., 1980; Roistacher et al., 2010), starting from the knowledge of the genetic structure of the virus population (Scott et al., 2012).

Old data on the genetic and phenotypic diversity of CTV strains in China were confused. Many years before, Hilf et al. (2005) reported on the diversity of 22 Chinese isolates based on multiple molecular marker (MMM) analysis and found a relatively low occurrence of mixed infection by multiple genotypes. Using biological indexing, p25/Hinf I restriction fragment length polymorphism (RFLP), multiple molecular markers, and bidirectional RT-PCR assay, Zhou et al. (2007) found that a mixture of severe stem pitting isolates was dominant in the field, mostly associated with a mixture of T30 and VT genotypes. Jiang et al. (2008) found two mild isolates showing a high identity with the isolates T30 (Florida) and T385 (Spain).

As far as it concerns Hunan province, using markers for the p23 gene, MMMs, and sequence analysis of the three RNA silencing suppressor genes (p20, p23 and p25), Xiao et al. (2016) demonstrated that the CTV population structure in Hunan is extremely complex. The severe VT and T3 strains appeared to be predominantly associated with field SP isolates, while the mild T30 and RB strains were related to asymptomatic samples. Overall, only two full genome sequences of Chinese CTV isolates were available, CT11A (from the municipality of Chongqing) and AT1 (from Hubei province), and the related papers are not published.

Our previous investigation in Hunan province, based on MMM, revealed the prevalence of VT and T3 genotypes, either individually or in combination. One isolate (HU-PSTS) showed a mixture of three genotypes (VT + T30 + T36), whereas capillary-electrophoresis-single strand conformation polymorphism (CE-SSCP) analysis and further sequencing of *p25* gene revealed a multiple strains profile with phylogenetic proximity with recombinant and VT strains (Licciardello et al., 2012; Licciardello et al., 2015a).

To investigate the apparent multiple strains co-infecting the HU-PSTS isolate we sub-inoculated sour orange seedlings and deep sequenced by high throughput sequencing (HTS) technology the small RNAs produced in the bark as antiviral mechanism (Voinnet 2005; Margis et al., 2006). Analysis of mapped reads with several CTV reference genomes enabled us to fully re-assemble the genome of a VT strain, while T68, T3 and T30 strains

were qualified as potential minor component. Preliminary results were presented at the XX International Conference of Citrus Virologist (Licciardello et al., 2016).

Materials and methods

Bioindexing of source tree

The source plant used for this work was originated from a survey on citrus virus and viroid diseases in Hunan province, China (Rizza et al., 2010; Licciardello et al., 2015a). The selected source, named HU-PSTS, was collected in Chenzou county, from an asymptomatic sweet orange grafted on *Poncirus trifoliata* Raf. and transferred on sour orange seedlings by bark inoculation. Biological indexing was carried out in a safe greenhouse with heat regulation, located near Catania (Sicily, Italy), lat. 37°30'4"68 N, long.15°4'27"12 E, by bark inoculation of eight-month-old seedlings of sour orange, 'Duncan' grapefruit, Mexican lime and alemow, and budlings of 'Hamlin' sweet orange grafted onto sour orange. Three plants were inoculated for each indicator and one more was used as control. Visual assessment of symptoms was made after ELISA positive tests and periodically over a two-year period (Garnsey et al., 2005).

Small RNAs high-throughput sequencing

Two hundred mg of young bark tissue were harvested from one inoculated sour orange seedling showing seedling yellows 15-mo post inoculation with the isolate HU-PSTS. Bark was ground to a fine powder in liquid nitrogen and small RNA fraction extracted using mirPremier® microRNA isolation kit (Sigma Aldrich) according to manufacture instructions and used as input for library preparation using NEXT flex Small RNA Sequencing kit (Bioo Scientific, USA). The library was then multiplexed, clustered, and sequenced on an Illumina HiSeq 2000 (TruSeq v3 chemistry) with a single-read 50 cycles sequencing protocol. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.2), with demultiplexing based on sample-specific barcodes. Small RNA adapters were removed using the "Trim sequences" option of the CLC Genomics Workbench (v 6.0.4).

Sequence analysis of sRNAs

Unpaired reads were mapped with a set of 17 references genomes of CTV (Table 1) using Bowtie2-build program v 2.1.0 using default parameters (Langmead and Salzberg

2012; Matsumura et al., 2017; Licciardello et al., 2015b). Three key mapping metrics were recorded: read counts, percentage of read counts and genome fraction coverage at 30 X depth. ORFs were identified using the NCBI ORF finder, and protein domains were ascertained with BLASTP (Johnson et al., 2008) and search of the NCBI Conserved Domain Database (Marchler-Bauer et al., 2004). Multiple sequence alignments and phylogenetic analysis were performed by MEGA6 using the neighbor-joining (NJ) method with 1000 bootstrap replicates as the test of phylogeny (Tamura et al., 2013). Quality control of mapping data in the resulting alignments was assessed by Qualimap v.2.1 (Garci-Alcalde et al., 2012).

Results

Bioindexing

The sour orange seedlings inoculated with bark showed smaller leaves, a shortening of internodes and an overall stunting typical of the presence of a seedling yellow isolate of CTV. Mexican lime reacted with water-soaked leaf veinlet, vein clearing and corking, leaf cupping and stem pitting. Alemow showed mild leaf vein clearing and stem pitting. Duncan grapefruit and 'Hamlin' sweet orange, showed small yellowing leaves, short internodes and stem pitting typical of CTV isolates belonging to biotype 5 (Garnsey et al., 2005) (Fig.1). Stem pitting reaction of the three indicators showed differences in terms of number, size, and morphology.

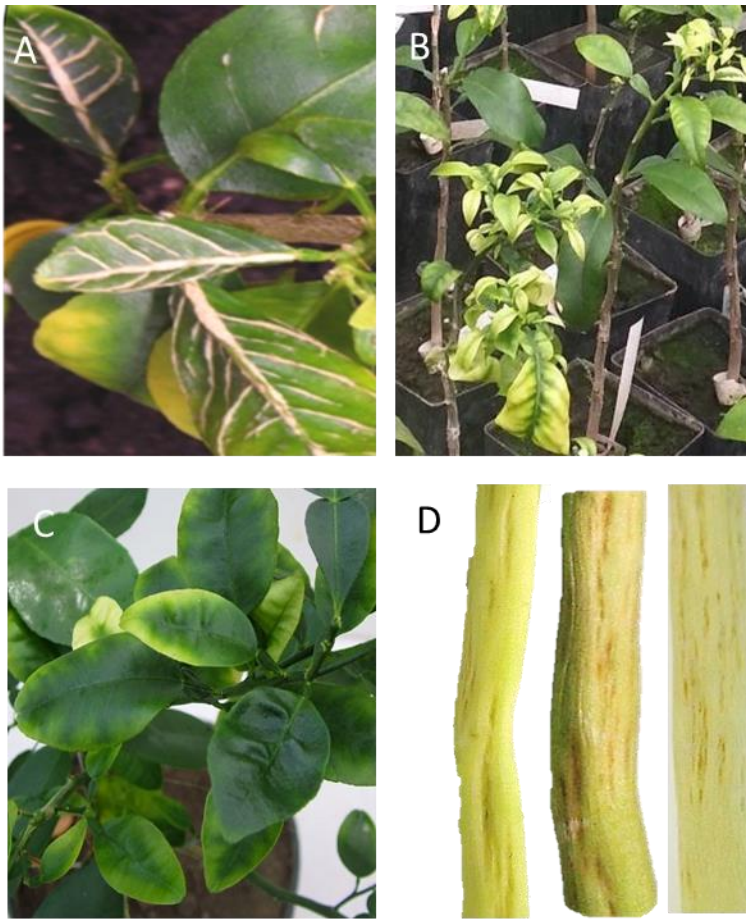

Fig. 1. Symptomatic reactions of indicator plants after bark inoculation of HU-PSTS: vein corking on Mexican lime (A); seedling yellow on sour orange (B) and Duncan grapefruit (C) seedlings; stem pitting on Duncan grapefruit (left), Hamlin sweet orange (middle) and alemow (right) (D).

Analysis of small RNA data set

The small RNA (sRNA) fraction isolated from sour orange bark infected by the HU-PSTS isolate was analyzed by high-throughput Illumina sequencing. The library generated a total of 38,718,417 reads, approximately 9 M and 11 M of which were 21nt and 22nt, respectively. The sRNA reads were aligned to a set of 17 reference sequences of CTV isolates (Table 1), representative of the genotypes described by Harper (2013). Alignments of mapped reads were also analyzed by Qualimap 2.1 to evaluate the co-presence of multiple strains in the sample focusing on the number of reads mapped per reference sequence and the percentage genome coverage (GFC) at 30X depth. Genomes of VT strain showed the highest mapped read count, ranging from 17.6 M to 11.8 M (47%-36% of the entire library), and up

to 100% genome coverage (Table 1). A hundred percentage coverage was obtained with T318A, CT11A and SG29 reference sequences, followed by 96-98% with L192GR, VT, NuaGA, AT-1, thus unequivocally supporting the presence of VT strain as a major component. The consensus sequence generated after the alignment of 17,604,200 reads, representing 45% of the entire library, with CTV T318A genome, was deposited in the GenBank database under accession number KU720382. The full genome sequence is 19,252 nt in length and is predicted to encode 12 ORFs, typical of the CTV genome. Phylogenetic analysis revealed that the HU-PSTS isolate clustered within the VT-Asian subgroup. (Fig. 2).

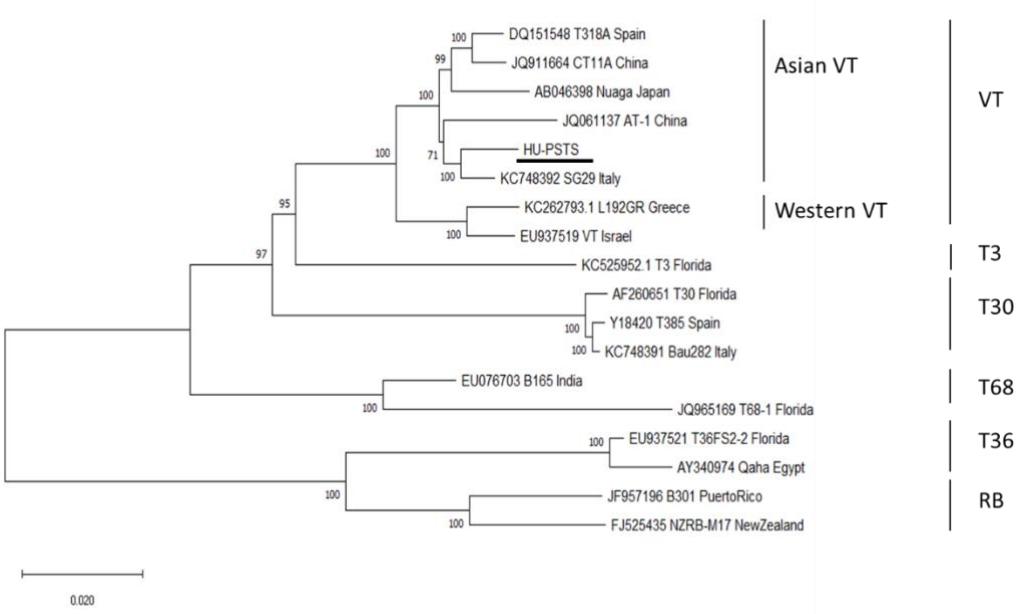

Fig. 2. Neighbor-joining phylogenetic tree obtained by full genome analysis of HU-PSTS (KU720382) and reference *Citrus tristeza virus* isolates. Bootstrap values (1000 replicates) are presented near the tree nodes. The scale bar represents 0.02 nucleotide substitutions per site.

A considerable read count, ranging from 7 M to 11 M reads (about 20-28% of the entire library), was obtained with reference genomes of T3, T68 and T30 strains. The relative percentages of genome coverage, ranging from 88% to 84%, below a cutoff of 90% assumed as positive call, qualify a potential presence of these additional strains in the HU-PSTS sample.. The coverage value obtained for T68-1 (70%), was highly different from the companion B165. On the contrary, the strains T36 and RB showed a low coverage (52-57%) and should be qualified as not present. Figure 3 shows a comparative representation of mapping quality obtained for each base call along the entire genomes of representative reference sequences.

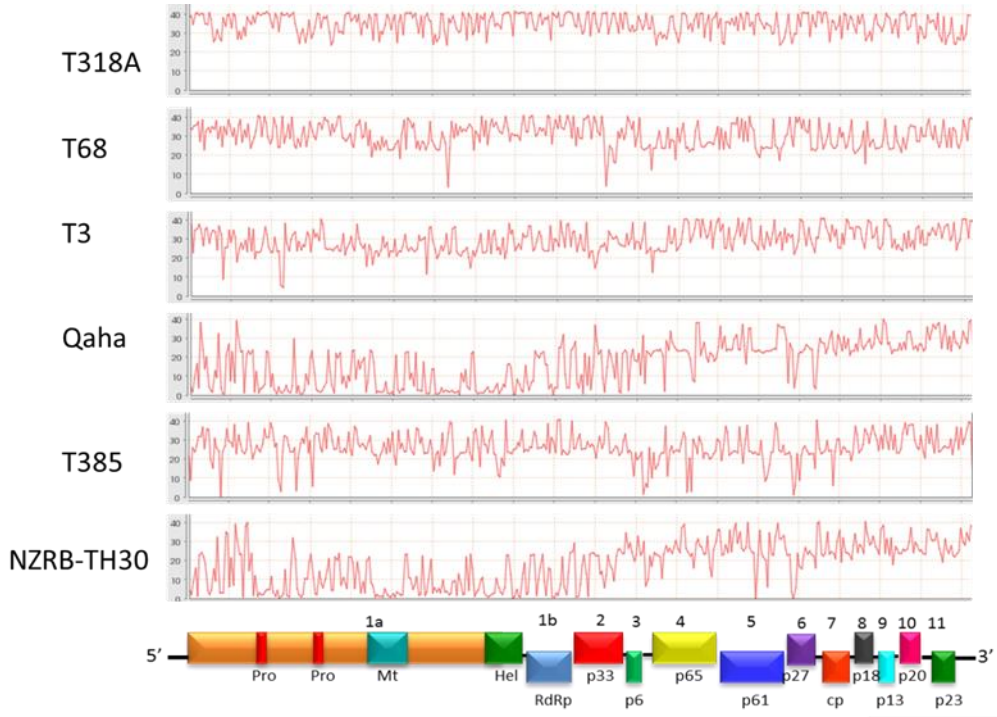

Fig. 3. Comparative mapping quality representations generated after alignments of the HU-PSTS library with six reference genomes representative of the main CTV genotypes by Qualimap v.2.1. The axis shows the full-length genome and the abscissa the value of mapping quality.

Table 1. Citrus tristeza virus genomes used in the read alignments of the HU-PSTS isolate, listed according to read count and relative percentage and genome fraction coverage at 30X depth.

Strain	Isolate	GenBank	Country	Mapped reads (%)	Read count (RC)	GFC 30X (%)
VT	SG29	KC748392	Italy	47.1	18,236,057	100
	T318A	DQ151548	Spain	45.47	17,604,200	100
	CT11A	JQ911664	China	45.39	17,574,250	100
	NuaGA	AB046398	Japan	42.53	16,465,833	97
	AT-1	JQ061137	China	41.04	15,889,949	98
	VT	CTU56902	Israel	36.91	14,290,915	96
	L192GR	KC262793	Greece	36,87	14,254,645	96
T68	B165	EU076703	India	28.58	11,064,522	82
T3	T3	KC525952	Florida	23.95	9,272,166	88
T68	T68-1	JQ965169	Florida	23.75	9,196,194	70
T30	T385	Y18420	Spain	21.07	8,159,789	85
	Bau282	KC748391	Italy	20.38	8,004,737	85
	T30	AF260651	Florida	20.58	7,967,373	84
RB	NZRB-M17	FJ525435	New Zealand	12.26	4,862,776	57
	B301	JF957196	Puerto Rico	12.51	4,841,741	57
T36	FS2-2	EU937521	Florida	11.22	4.278.101	55
	Qaha	AY340974	Egypt	10.76	4,166,126	52

Discussion

The study of genetic and phenotypic diversity of CTV isolates in China is quite complex because of the long history and the extensiveness of the citriculture in the country. In Hunan province, where citrus tristeza is widespread, most of the infections are associated to multiple CTV isolates that fall into different genotype groups, with some discrepancies attributed to the different methodologies used for the investigation (Licciardello et al., 2015a; Xiao et al., 2016). The study on the CTV profile of the HU-PSTS isolate was undertaken to clarify by using the better performant HTS technology some discrepant results previously investigated obtained by CE-SSCP and MMM (Licciardello et al., 2015a).

The sensitive small RNA deep sequencing of the isolate revealed the clear prevalence of a VT strain, well positioned as principal component. The highest output of mapped reads was shared with VT strains T318A from Spain, SG29 from Italy, CT11A from Chongqing, and AT-1 from Wuhan (Hubei). Whereas T3, T68 and T30 might be considered as potential

minor components, with a GFC 30X above 80% and appreciable quality data of alignment. Phylogenetic analysis showed that the full genome sequence HU-PSTS is positioned within the Asian-VT subgroup (Harper, 2013), very close to SG29. Interesting enough is the fact that SG29 was found also very close to a CTV isolate found in Brazil associated to citrus sudden death (Matsumura et al., 2017).

These results differ from those previously obtained by MMM which indicated the presence of VT, T30 and T36, and from those obtained by CE-SSCP of p25 gene, which revealed a multiple strains profile with phylogenetic proximity with recombinant and VT strains (Licciardello 2015a). Differences in genotyping detection can be attributed to the ~~study~~ the small target regions covered by MMM, not reflective of information given by the entire genome analysis contributing to a misleading information in case of mixed isolates(Harper, 2013).

Biological indexing showed that the HU-PSTS is a stem pitting isolate inducing severe symptoms on Mexican lime, alemow, grapefruit and sweet orange, therefore qualified as belonging to biogroup 5 (Garnsey et al., 2005). The inoculation of sour orange allowed to detect the seedling yellow reaction which was not shown on sweet orange. Moreover, we cannot exclude that this passage on sour orange may have caused loss of part of the CTV population or may have altered the original field profile. In such respect it should be also considered that none of the reference genome sequences in GenBank was originated from a sour orange source.

The HU-PSTS isolate is the first fully sequenced CTV genome from Hunan province. The small RNA deep sequencing to detect multiple infections, associated to bioindexing, helped in redirect previous biological and molecular results (Licciardello et al., 2015a), increasing knowledge on the genomic structure of CTV in Hunan province.

This situation would not interfere with the potential application of the mechanism of super infection exclusion (SIE) to cross-protect local citriculture from CTV-SP damage (Folimonova et., 2010), which inspired the cooperation program between China and Italy. In fact, the phenomenon is described effective also in presence of additional genotypes in the same host and should have an important role also in field conditions in presence of multiple infections (Bergua et al., 2016).

In this regard, to discriminate between different genotypes and isolates of CTV co-infecting a tree, the full phenotypic and genomic profiles of a larger number of samples

should be analyzed by bioindexing and sequencing. Thanks to its reliability, rapidity and sensitivity, integration with sRNA deep sequencing would be helpful and cost-effective.

Acknowledgements

This work was initially supported by the project IT-Citrus genomics PON 01_1623, funded by MIUR and MISE and co-funded by EU, and the 'Lotta al virus della tristezza degli agrumi' project funded by Assessorato delle Risorse Agricole ed Agroalimentari, Regione Siciliana.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Bergua M, Kang SH, Folimonova SY. 2016. Understanding superinfection exclusion by complex populations of Citrus tristeza virus. Virology 499: 331–339.

Costa AS, Müller G. 1980. Tristeza control by cross-protection; a U.S.-Brazil cooperative success. Plant Dis. 64: 538–541.

García-Alcalde F, Okonechnikov K, Carbonell J, Cruz LM, Götz S, Tarazona S, Dopazo J, Meyer TF, Conesa A. 2012. Qualimap: evaluating next-generation sequencing alignment data. Bioinformatics. 15:2678-2679.

Garnsey SM, Civerolo EL, Gumpf DJ, Paul C, Hilf ME, Lee RF, Brlansky RH, Yokomi RK, Hartung JS. 2005. Biological characterization of an International collection of Citrus tristeza virus (CTV) isolates. In: Hilf ME, Duran-Vila N, Rocha-Peña MA, editors. Proceedings of the 16th Conference of the International Organization of Citrus Virologists. IOCV, Riverside (CA): IOCV p. 75-93.

Harper SJ. 2013. Citrus tristeza virus: evolution of complex and varied genotypic groups. Front Microbiol. 4: 93. doi: 10.3389/fmicb.2013.00093.

Hilf ME, Mavrodieva VA, Garnsey SM. 2005. Genetic marker analysis of a global collection of isolates of Citrus tristeza virus: characterization and distribution of CTV genotypes and association with symptoms. Phytopathology 95: 909–917.

Folimonova SY, Robertson CJ, Shilts T, Folimonov AS, Hilf ME, Garnsey SM, Dawson WO. 2010. Infection with strains of Citrus tristeza virus does not exclude superinfection by other strains of the virus. J Virol. 84: 1314–1325.

Jiang B, Hong N, Wang GP, Hu J, Zhang JK, Wang CX, Liu Y, Fan XD. 2008. Characterization of citrus tristeza virus strains from southern China based on analysis of restriction patterns and sequences of their coat protein genes. Virus Genes 37:185–192.

Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden T.L. 2008. NCBI BLAST: a better web interface. Nucleic Acids Res. 36: W5-9.

Langmead B, Salzberg S. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods, 9:357-359.

Licciardello G, Raspagliesi D, Bar Joseph M, Catara A. 2012. Characterization of isolates of Citrus tristeza virus by sequential analyses of enzyme immunoassays and capillary electrophoresis single-strand conformation polymorphisms. J Virol Meth. 181: 139–147.

Licciardello G, Xiao C, Russo M, Dai SM, Daden M, Deng ZN, Catara AF. 2015a. Genetic structure of Citrus tristeza virus in Hunan province (P.R. CHINA). Acta Hortic. 1065: 781-790.

Licciardello G, Scuderi G, Ferraro R, Giampetruzzi A, Russo M, Lombardo A. 2015b. Deep sequencing and analysis of small RNAs in sweet orange grafted on sour orange infected with two Citrus tristeza virus isolates prevalent in Sicily. Arch Virol. 160: 2583–2589.

Licciardello G, Scuderi G, Ferraro R, Russo M, DAI SM, Catara A, Deng ZN. 2016. Genome sequencing through viral small RNAs of a Citrus tristeza virus isolate from Hunan province reveals the presence of multiple stem pitting strains. IOCV-XX-Abstracts of Presentations at the 20th Conference of the International Organization of Citrus Virologists, China, 2016, J. Citrus Pathol. 3(2): 10-11.

Marchler-Bauer A, Bryant SH. 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 1: W327-31.

Margis R, Fusaro AF, Smith NA, Curtin SJ, Watson JM. 2006. The evolution and diversification of Dicers in plants. FEBS Letters. 580: 2442–2450.

Matsumura EE, Coletta-Filho HD, Nouri S, Falk BW, Nerva L, Oliveira TS, Dorta SO, Machado MA. 2017. Deep sequencing analysis of RNAs from citrus plants grown in a Citrus Sudden Death-affected area reveals diverse known and putative novel viruses. Viruses 9(4). doi: 10.3390/v9040092.

Moreno P, Ambros S, Albiach-Marti MR, Guerri J, Pena L. 2008. Citrus tristeza virus: a pathogen that changed the course of the citrus industry. Mol Plant Pathol. 9: 251–268.

Moreno P, Garnsey SM. 2010. Citrus tristeza diseases: a worldwide perspective. In: Karasev AV, Hilf ME, editors. Citrus tristeza virus Complex and Tristeza Diseases. The American Phytopathological Society, St Paul (MN) p. 27-49.

Pirovano W, Miozzi L, Boetzer M, Pantaleo V. 2015. Bioinformatics approaches for viral metagenomics in plants using short RNAs: model case of study and application to a Cicer arietinum population. Front Microbiol. 5: 790.

Rizza S, Ma XF, Bella P, Han J, Dai SM, Deng ZN, Catara A. 2010. Update on the most destructive citrus diseases in Hunan, China. Proceedings of the XI International Citrus Congress of the International Soc. Citricult. Wuhan (China). p. 1183-1184.

Roistacher CN, da Graça JV, Müller GW. 2010. Cross Protection against Citrus tristeza virus. A Review. In: Hilf ME, Timmer LW, Milne RG, da Graça, JV. editors. Proceedings of the 17th Conference of the International Organization of Citrus Virologists. IOCV, Riverside (CA): IOCV. p. 1–27.

Scott KA, Hlela Q, Zablocki O, Read DA, Van Vuuren S, Pietersen, G. 2012. Genotype composition of populations of grapefruit-cross-protecting Citrus tristeza virus strain GFMS12 in different host plants and aphid-transmitted sub-isolates. Arch Virol. 158:27 – 37.

Spreen TH, Zhifeng G, Gmitter F, Norberg R. 2012. An overview of the Citrus industry of China. Proceedings of the Florida State Horticultural Society. 125:119–121.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30: 2725–2729.

Voinnet O. 2005. Non-cell autonomous RNA silencing. FEBS Letters. 579: 5858–5871.

Xiao C, Yao RX, Li F, Dai SM, Licciardello G, Catara A, Gentile A, Deng ZN. 2016. Population structure and diversity of Citrus tristeza virus (CTV) isolates in Hunan province, China. Arch Virol.162: 409-423.

Zhou Y, Zhou CY, Song Z, Liu KH, Yang FY. 2007. Characterization of citrus tristeza virus isolates by indicators and molecular biology methods. Agr Sci China 6:573–579.