

1 **Recently Accepted**

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

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26 genomes.

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36 Abstract

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

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50 Introduction

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

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

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

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

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

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

93 To investigate the apparent multiple strains co-infecting the HU-PSTS isolate we
94 sub-inoculated sour orange seedlings and deep sequenced by high throughput sequencing
95 (HTS) technology the small RNAs produced in the bark as antiviral mechanism (Voinnet
96 2005; Margis et al., 2006). Analysis of mapped reads with several CTV reference genomes
97 enabled us to fully re-assemble the genome of a VT strain, while T68, T3 and T30 strains

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

100

101 **Materials and methods**

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103 **Bioindexing of source tree**

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

115 **Small RNAs high-throughput sequencing**

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

126 **Sequence analysis of sRNAs**

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

129 2012; Matsumura et al., 2017; Licciardello et al., 2015b). Three key mapping metrics were
130 recorded: read counts, percentage of read counts and genome fraction coverage at 30 X
131 depth. ORFs were identified using the NCBI ORF finder, and protein domains were
132 ascertained with BLASTP (Johnson et al., 2008) and search of the NCBI Conserved Domain
133 Database (Marchler-Bauer et al., 2004). Multiple sequence alignments and phylogenetic
134 analysis were performed by MEGA6 using the neighbor-joining (NJ) method with 1000
135 bootstrap replicates as the test of phylogeny (Tamura et al., 2013). Quality control of
136 mapping data in the resulting alignments was assessed by Qualimap v.2.1 (Garcı-Alcalde et
137 al., 2012).

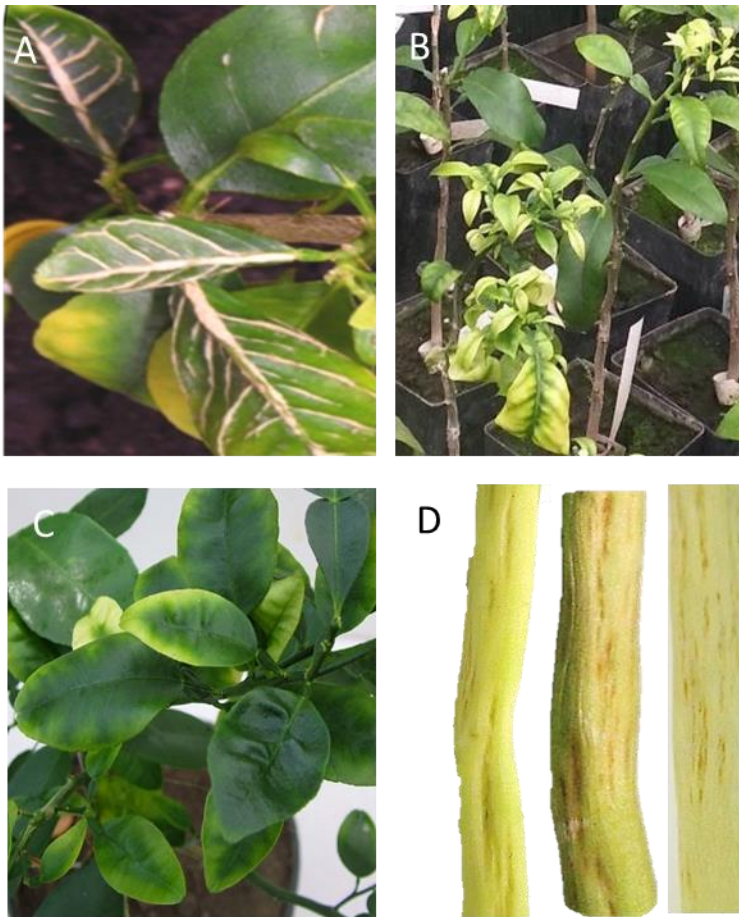
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139 **Results**

140 **Bioindexing**

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

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

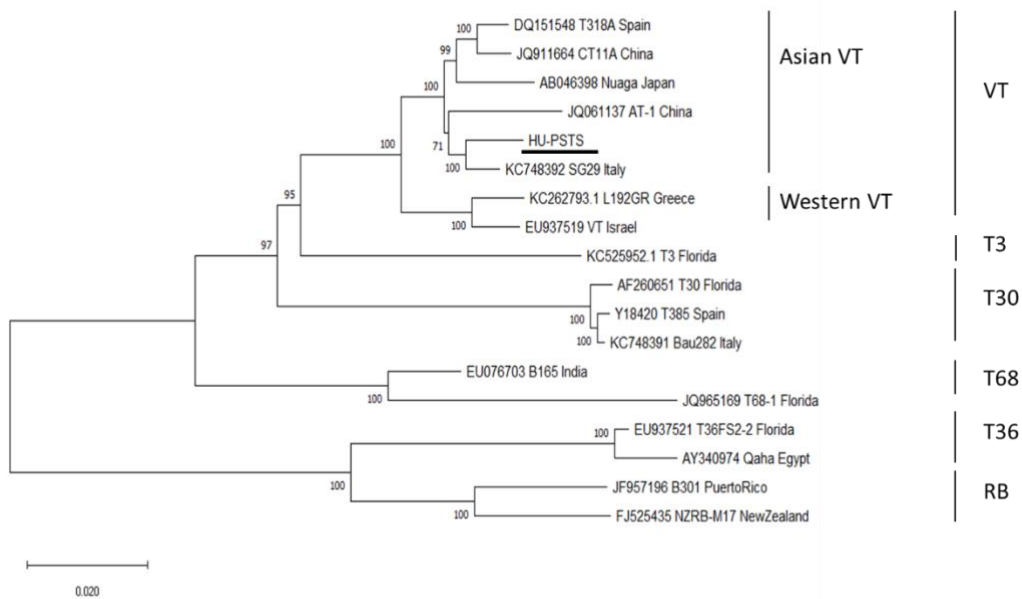
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155 **Analysis of small RNA data set**

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

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

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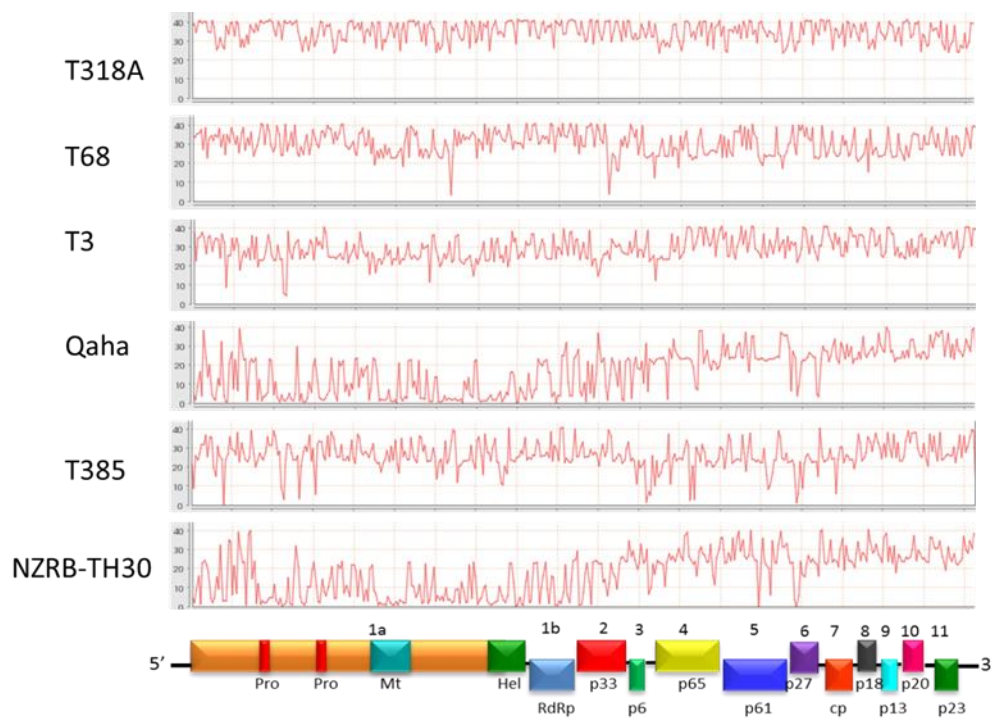


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Fig. 2. Neighbor-joining phylogenetic tree obtained by full genome analysis of HU-PSTS (KU720382) and reference *Citrus tristeza virus* isolates. Bootstrap values (1000 replicates) are presented near the tree nodes. The scale bar represents 0.02 nucleotide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

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

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

202

203 **Table 1.** Citrus tristeza virus genomes used in the read alignments of the HU-PSTS isolate,
 204 listed according to read count and relative percentage and genome fraction coverage at 30X
 205 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

206

207 **Discussion**

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

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

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

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

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

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

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

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

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

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260 **Conflict of interest**

261 The authors declare that they have no conflict of interest.

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