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Genetic divergence of tomato ringspot virus

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Abstract Tomato ringspot virus (ToRSV) has been detected in Chile, causing economically important diseases in a wide range of hosts. A ToRSV isolate was obtained from raspberry cv Heritage (Rasp-CL) showing leaf yellowing and stunting. The complete genome of Rasp-CL was sequenced by deep sequencing. The Rasp-CL RNA1 sequence shared 97.4 % nucleotide sequence identity with divergent RNA1 of isolate Rasp1-2014, while Rasp-CL RNA2 showed high divergence from all four isolates available in the database, sharing only 63.9-72.7 % nucleotide sequence identity. This difference was mainly based on the X4 coding region, which has been reported to be a high-variability region. Moreover, based on differences in the X4 region, three Rasp-CL RNA2 variants of different length were identified in the same host. One putative recombination event was identified between the Rasp-CL and GYV-2014 X4 genes. Phylogenetic analysis suggested that ToRSV isolates with currently available sequences form three distinct groups. Our results suggest that, for an accurate phylogenetic classification of ToRSV, it is necessary to obtain sequences of both RNAs. This is the first report of a complete ToRSV genome sequence from South America.

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Tomato ringspot virus (ToRSV) is a member of the genus *Nepovirus* (nematode-transmitted polyhedral viruses) of the family *Secoviridae*, and this virus causes economically important diseases in a wide range of hosts. ToRSV isolates have been detected in different hosts in Chile, including *Prunus* sp., blueberry (*Vaccinium corymbosum* L.), apple (*Malus domestica* Bork), raspberry (*Rubus idaeus* L.) and tomato (*Solanum lycopersicum* L.) [1–7].

ToRSV causes variable symptoms in raspberry, mainly stunting of the bush and a general yellowing of leaves, and both fruit size and yield are reduced. By the third year of infection, 10-80 % of fruiting canes may be killed [8].

Three nepovirus subgroups have been identified: A, B, and C. ToRSV belongs to the subgroup C, characterized by an additional protein domain (X4) in their RNA2. ToRSV has a bipartite positive single-stranded RNA genome. The two monocistonic genome segments are encapsulated separately into two different icosahedral particles. The polyproteins are cleaved by the viral protease (Pro) to intermediate and mature proteins [9]. The RNA1-encoded polyprotein contains the domains for proteins likely to be involved in replication, including a putative nucleoside triphosphate binding (NTB) protein, the VPg, the 3C-like proteinase, the RNA-dependent RNA polymerase, and X1 and X2, for which the functions are unknown [10, 11]. The RNA2-encoded polyprotein contains two proteins of unknown function (X3 and X4), the movement protein (MP), and the coat protein (CP). RNA 1 and RNA 2 have a 3′untranslated region (3′UTR) of aproximately 1,500 nucleotides (nt), a hallmark of nepovirus subgroup C [12, 13]. The complete genome sequence is currently available for four isolates of ToRSV, all from the United States of America (USA): from raspberry, named Raspberry and Rasp1-2014 (accession numbers NC003840 and KM083895, respectively); grapevine (GYV-2014)

(accession number KM083893); and *Prunus* sp. (13C280) (accession number KM083890) [10, 12, 14].

In this study, using deep sequencing, we determined the complete genome sequence of a Chilean ToRSV strain detected in raspberry cv. Heritage with stunting of bush and yellowing of leaves.

Plant samples were collected in spring from commercial orchards of the Metropolitana Region of Santiago. The lithium chloride method was used for RNA extraction from leaves [15], and ToRSV was detected by reverse transcription (RT) using a specific reverse primer, followed by nested polymerase chain reaction (nested PCR) to improve sensitivity. A segment of the RNA1-encoded RdRp gene was amplified using specific pairs of primers: ToRSV-R/U1 in direct PCR and ToRSV-F/D1 in nested PCR [16, 17].

The Chilean isolate of ToRSV from raspberry was named Rasp-CL. "Nanking cherry" (*Prunus tomentosa* hybrid IR473 × IR474) seedlings, obtained from virus-free seeds, were used as indexing hosts for maintaining the ToRSV isolate. Three-month-old plants were inoculated by grafting three bark chips collected from raspberry (Rasp-CL). Three chip-inoculated seedlings were maintained in a greenhouse and used as a source for virus characterization [18].

Complete genome sequences of Rasp- CL RNA1 and RNA2 were obtained by deep sequencing (GenBank accession numbers KR911669 to KR911672). Total RNA was extracted from *P. tomentosa* leaves infected with a raspberry isolate (Rasp-CL). The quality of the RNA in each sample was verified using a Bioanalyzer instrument, and the RNA was quantified using a RiboGreen kit (Invitrogen). RNA sequencing was performed in an Ion Torrent sequencer, following manufacturer's protocol, in a next-generation sequencing facility by OMICS Solutions (Santiago, Chile). Fragment library beads were sequenced using standard chemistry for fragment libraries with a read length of 400 bp. Additional specific primers were designed in some regions of the Rasp-CL genome with low depth of coverage. The resulting PCR products were purified and cloned into pGEM-T Easy Vector (Promega) and sequenced by Macrogen (USA). These sequences were compared with available sequences in public databases [10, 12, 14]. Multiple alignments of nucleotide and deduced amino acid (aa) sequences were performed using ClustalW and BioEdit [19], and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [20]. Phylogenetic reconstructions were performed using the neighbor-joining method with 500 bootstrap replicates.

The Rasp-CL RNA1 was found to be 8207 nucleotides in length, excluding the 3′ poly (A) tail, and contained one long open reading frame (ORF) initiating at the first AUG codon (nucleotide 71) and terminating at a UGA stop codon (nucleotide 6644). The polyprotein sequence encoded by Rasp-CL RNA1 shared 97.6 % nucleotide sequence identity (98.2 % aa sequence identity) with the reference Rasp1-2014 isolate, and only 76.8, 80.5 and 81 % nt sequence identity (83.9, 87.6 and 88.3 % aa sequence identity) with the reference isolates GYV-2014, 13C280 and Raspberry, respectively. The RNA1 sequence of the Rasp-CL isolate shared high sequence identity with RNA1 of the Rasp1-2014 isolate. However, both isolates shared only 81 % nt sequence identity in the RNA1 5′UTR region (Table 1).

The Rasp-CL RNA2 variants were found to be 6866, 7064 and 7325 nucleotides in length, excluding the 3′ poly (A) tail, and they each contained a single ORF initiating at the first AUG codon (nucleotide 87) and terminating at a UGA stop codon (nucleotide 5298, 5496 and 5757, respectively, for each variant). The difference in the length of Rasp-CL RNA2 is due to variability of the X4 domain. The results of deep sequencing showed a low depth of coverage in the X4 region of Rasp-CL, and therefore, additional specific primers were designed for this region: ToRSV-X4F (5′ CTTCCATTCAAGGGGCTGT 3′) and ToRSV-X4R (5′ CGCGAGTGT ATGTCCTATCC 3′). Three different sizes of PCR products were obtained, cloned, and sequenced, and these were found to be of 1635, 1374 and 1176 nt in length. These variants of X4 were identified as X4-v1, X4-v2, and X4-v3, respectively. Three series of tandem repeats with two and eight copies were identified in the deduced amino acid sequence from the X4-v1 protein. The repeats A1 and A2 were 24 aa long, B1 to B8 were 22 aa long, and C1 and C2 were 19 aa long (Fig. 1a). The number of copies of the repeats B varied within each variant of X4. While eight copies of the repeat were present in X4-v1, only six and three were found in the X4-v2 and X4-v3 sequences, respectively. The rate of sequence identity in the X4 protein with the four ToRSV isolates available in GenBank was 29.8-40.9 % at the aa level (35.6-46.3 % at the nt level), a value significantly lower than that observed for the others protein domains (Table 1).

Jafarpour *et al*. showed that there was a high degree of sequence diversity in the X4 protein among ToRSV isolates from the USA, in part due to the insertion of multiple copies of two types of large amino acid repeats [21]. Two series of tandem repeats were identified in the deduced aa sequence of the X4 protein. The first type of repeat was a 53-aa motif, and the second type of repeat was 22 aa long. X4 protein variants of the Rasp-CL isolate did not contain the 53-aa-long repeat but did contain the 22-aa-long repeat (repeat B) (Fig.1a). The repeats A and C found in the Rasp-CL X4 protein were not present in the X4 protein of ToRSV isolates from the USA.

Rasp-CL RNA2 was divergent from all currently available isolates, sharing only 63.9-72.7 % nt sequence

Table 1 Pairwise sequence comparisons among Rasp-CL and completely sequenced ToRSV isolates

Genome region	ToRSV isolate			
	Raspberry	Rasp1-2014	13C280	GYV-2014
RNA1	(80.9)	(97.4)	(80.0)	(74.1)
5′UTR	(64.0)	(81.0)	(65.7)	(48.2)
P1 polyprotein	88.3 (81.0)	98.2 (97.6)	87.6 (80.5)	83.9 (76.8)
X1	80.0 (80.5)	94.8 (95.8)	77.8 (77.8)	77.8 (75.1)
X2	78.1 (73.7)	98.9 (98.1)	77.6 (73.6)	74.6 (70.2)
NTB	89.3 (79.5)	99.6 (98.1)	89.0 (79.6)	85.6 (75.6)
VPg	96.2 (80.2)	100 (97.5)	96.2 (81.4)	96.2 (79.0)
Pro	90.6 (81.9)	98.7 (97.9)	90.6 (82.4)	86.2 (79.8)
Pol	94.2 (84.2)	98.7 (98.0)	93.9 (84.0)	89.1 (79.6)
3′UTR	(79.9)	(96.3)	(78.1)	(63.4)
RNA2	(72.7)	(69.8)	(72.1)	(63.9)
5′UTR	(66.6)	(66.6)	(68.0)	(50.6)
P2 polyprotein	72.0 (70.3)	68.4 (67.1)	71.7 (70.6)	68.2 (64.2)
X3	81.0 (82.8)	81.7 (83.8)	81.7 (84.0)	79.8 (80.8)
X4-v1	34.7 (44.5)	29.8 (38.4)	34.5 (44.0)	38.5 (43.2)
X4-v2	35.9 (45.2)	30.7 (38.5)	35.8 (44.5)	40.9 (46.3)
X4-v3	35.2 (41.8)	30.0 (35.6)	35.0 (41.3)	39.7 (45.6)
MP	93.5 (84.7)	94.0 (84.7)	92.4 (84.4)	86.7 (74.1)
CP	94.3 (84.2)	93.7 (84.3)	93.5 (84.2)	84.1 (74.0)
3′UTR	(80.3)	(79.5)	(78.0)	(63.3)

For each region of the genome, the first number indicates the percentage of sequence identity in the deduced amino acid sequence (when applicable), and the second number (in parentheses) indicates the percentage of sequence identity in the nucleotide sequence

identity with other isolates over the entire RNA2, and 68.2-72.0 % aa sequence identity over the entire RNA2 polyprotein (Table 1). The 5′UTRs of Rasp-CL RNA1 and RNA2 were 71 and 87 nt long, and the 3′UTRs were 1563 and 1568 nt long, respectively. A high level of sequence identity was found between the untranslated regions of RNA1 and RNA2.

Recombination events in the RNA2 sequence were analysed using the RDP4 program [22]. One recombination event, with statistically significant support in five of ten methods (RDP, GENECONV, BootScan, MaxChi and Chimaera), was predicted in the 5′ region of the X4-v1 domain in Rasp-CL when compared with the Raspberry and GYV-2014 isolates (Fig. 1b). The parent for the recombinant fragment was predicted to be related to the GYV-2014 isolate. This information, together with the close relationship of the 3′UTR of both RNAs of the Chilean isolate to the Rasp1-2014 RNA1 3′UTR (data not shown), is consistent with the position of Rasp-CL isolate in the phylogenetic analysis.

Phylogenetic studies of ToRSV Rasp1-2014 have suggested that this isolate is the result of a reassortment between RNA1 and RNA2 components of different origins [14]. In our work, we found a perfect concordance in the topology of the two phylogenetic trees built with complete or partial (390 nt) nucleotide sequences of RNA1 (Figure and Table S1), in which Rasp-CL always grouped with Rasp1-2014. On the other hand, in trees constructed with RNA2 sequences, Rasp-CL never grouped with Rasp1-2014 (Figure and Table S1). This topology was maintained even when the phylogenetic analysis was performed on 5′ and 3′UTR nucleotide sequences (data not shown), confirming the hybrid nature of the Rasp1-2014 isolate.

For an accurate characterization of ToRSV isolates, it is advisable perform phylogenetic analysis using partial nucleotide sequences of polymerase (a fragment of 390 nt in RNA1) and coat protein genes (region of 347 nts, in RNA2) [23]. Based upon these analyses, is possible propose three phylogenetic groups of ToRSV: Raspberry, which includes the reference strain from raspberry (accession numbers NC003839 and NC003840) and the isolate 13C280 (accession numbers KM083890 and KM083891); Rasp-CL, reference strain from raspberry (accession numbers KR911669 to KR911672); and GYV-2014, reference strain from grapevine (accession number KM083892 and KM083893). Rasp1-2014, reference strain from raspberry (accession numbers KM083894 and KM083895) should be considered a hybrid isolate (in

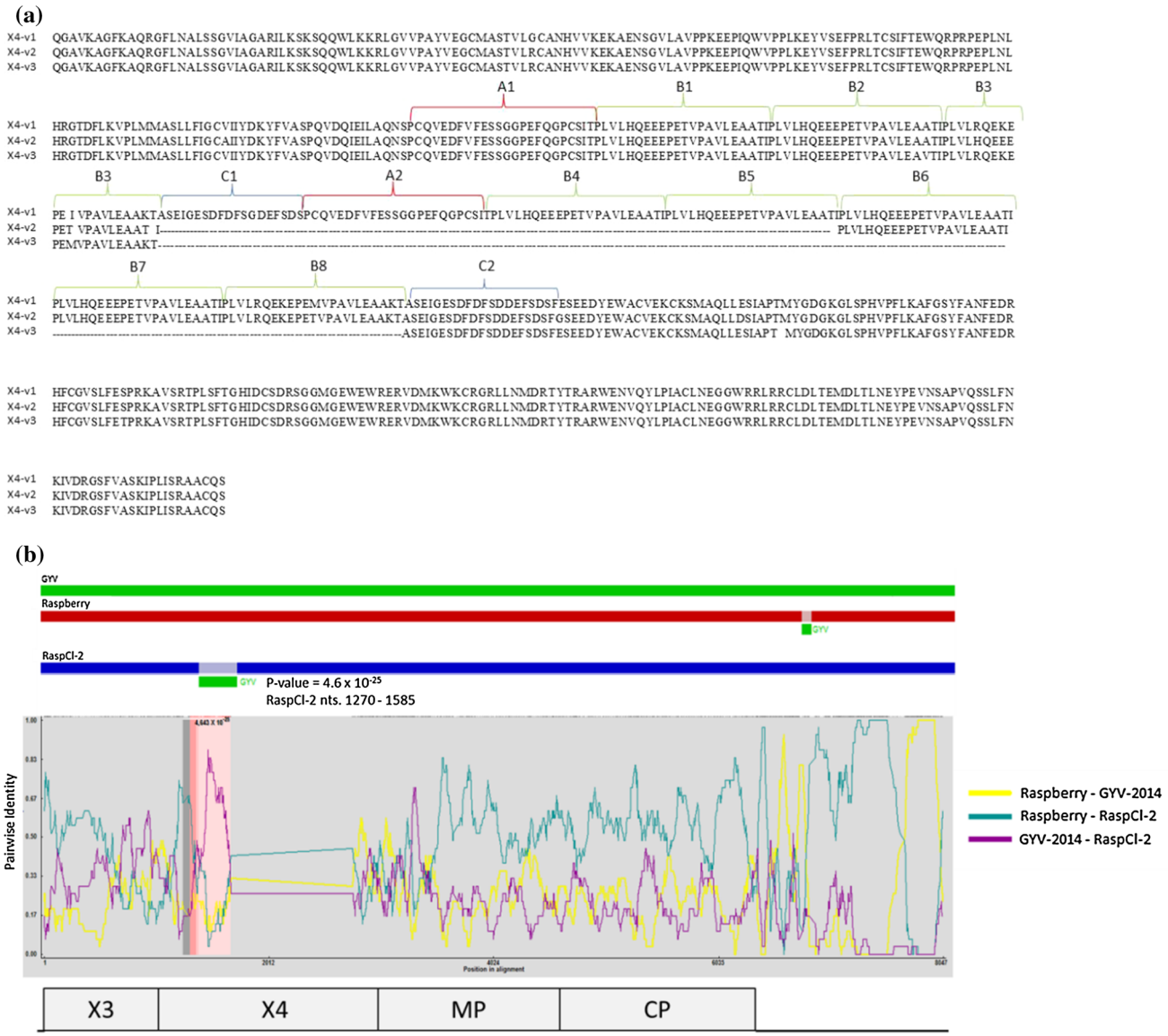

Fig. 1 X4 protein domain (RNA2) in ToRSV. (a) Alignment of the deduced amino acid sequences of the three variants of X4 protein from Rasp-CL. Amino acid sequence repeats are indicated by brackets above the sequence. (b) Recombination events predicted using the RDP4 program. Potential recombinant fragments are shown with the *p*-value and the nt numbers. The graph shows pairwise identity comparisons for the three isolates used to identify the recombination events

RNA1 grouped with Rasp-CL, while in RNA2 with Raspberry).

From the epidemiological and evolutionary standpoint, it is important to understand the role of the X4 protein in the life cycle of ToRSV, as well as the reason(s) behind the presence of three variants of the X4 gene in the single infected plant.

To our knowledge, this is the first report of the complete genome sequence of a ToRSV isolate from South America.

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