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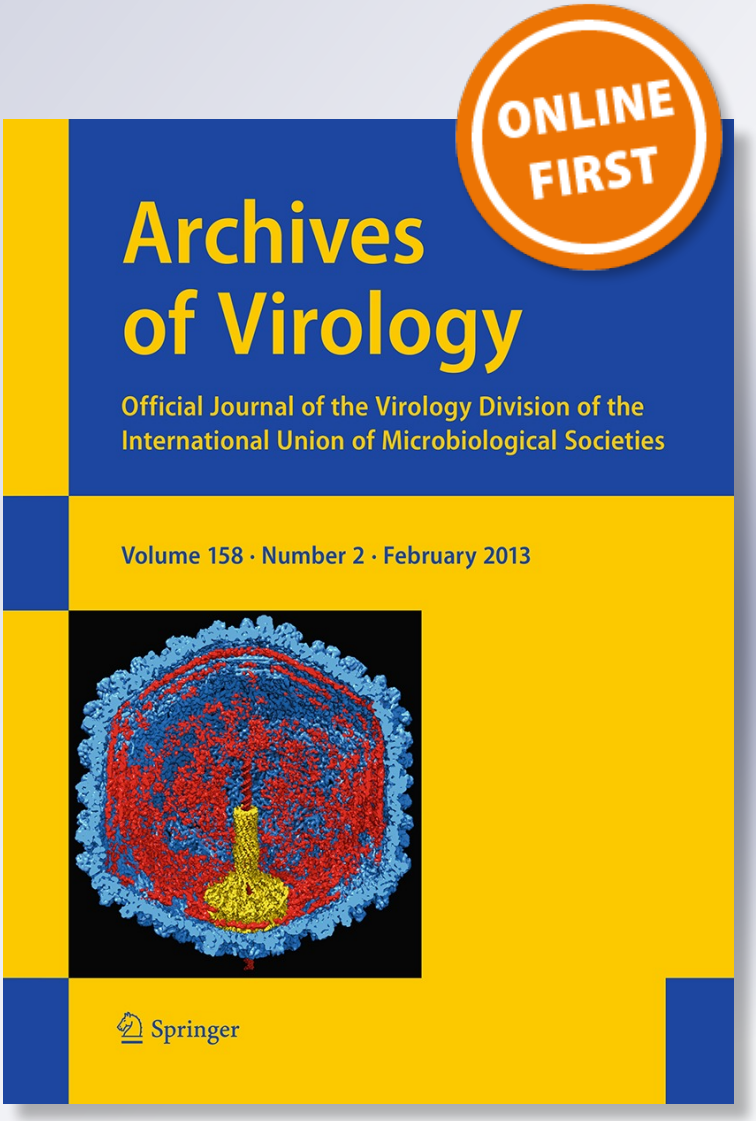

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BRIEF REPORT

Striking differences in the biological and molecular properties of onion and garlic isolates of onion yellow dwarf virus

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Abstract Complete nucleotide (nt) and deduced amino acid sequences of two onion yellow dwarf virus (OYDV) isolates showing mild and severe symptoms in onion but being unable to infect garlic were determined. The genomes consisted of 10,459 and 10,461 nt (without the 3' poly(A) tail) and were 92.2 % identical. Comparison of their whole genomes, polyproteins and P1, HC-Pro, P3, CI, VPg and NIa-Pro regions with those of garlic isolates previously identified as OYDV gave percentage values below that proposed as the molecular threshold for potyvirus species demarcation. This and the striking differences in host range between onion and garlic isolates suggest that they represent different virus species.

Introduction

Onion yellow dwarf virus (OYDV) was originally detected in onions (*Allium cepa* L.), causing dwarfing, yellow striping, and crinkling of the leaves [5]. Later, a potyvirus was detected in garlic (*Allium sativum* L.) and identified as OYDV based on strong reactions with OYDV antisera and high sequence similarities in the genome fragment that encodes the coat protein (CP) [7, 10, 12, 15, 23]. It is one of the most common garlic viruses worldwide [6, 23, 24], responsible for yield reductions ranging from 24 to 60 % [9, 14].

Chen et al. [6] determined the first complete sequence of a garlic isolate of OYDV from China and found that its predicted P3 protein (530 aa) was larger than that of other potyviruses sequenced. Three years later, analysis of the complete genomes of two OYDV isolates causing attenuated and severe symptoms in garlic in Japan revealed differences in the HC-Pro-encoding region, with the attenuated isolate having a deletion of 276 nucleotides (nt) [20].

Here, we present the first complete genome sequences of two OYDV isolates, one causing mild, and the other, severe symptoms in onion, and compare them with the known sequences of garlic isolates.

Materials and methods

Two OYDV isolates from onion were studied. A German isolate showing mild mosaic symptoms with few or no yellow stripes and some mild twisting on older leaves was kindly provided by Dr. D.-E. Lesemann (Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany) and is referred to here as OYDV-Mi. An Argentine isolate showing severe mosaic, yellow mottling, striping, pronounced blistering, downward curling, flattening, crinkling and reduced plant growth originated from onion in Bahía Blanca (Buenos Aires, Argentina) and is referred to here as OYDV-Se.

OYDV-Mi- and OYDV-Se-infected plant leaves were ground in 0.05 M borate buffer, pH 8.1 (1:5 w/v). The sap extracted from each OYDV isolate was rubbed onto 15

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onion seedling plants, previously dusted with 300 mesh carborundum, from each of the following cultivars: Chata colorada, Valenciana, Cobriza INTA, Grano de Oro, Valcatorce INTA, White Wing, Navideña INTA, Valuno INTA and a selection "Roja de Emilio Rodríguez". In addition, 20 *A. schoenoprasum* plants (chive), 10 *A. porrum* plants (leek), and 10 virus-free garlic plants of cv. San Valentin were obtained as described previously [9, 11]. Negative controls of each species and cultivar were inoculated with sap from healthy plants. Plants were maintained in a greenhouse under controlled conditions at 20-27 °C and checked daily for virus symptoms. Plant infection was checked 30 days after transmission by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using OYDV antiserum (BIOREBA SRL Latin America, Mar del Plata), according to the manufacturer's instructions.

Total RNA was extracted from the leaves of infected onion plants using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA), and 20 µg was submitted to INDEAR (Genomics and Bioinformatics Platform, INDEAR Inc., Rosario, Argentina), where the mRNA was first fragmented by addition of RNA fragmentation solution (0.1 M Tris-HCl, pH 7.0 and 0.1 M zinc chloride). The cDNA libraries were constructed according to the manufacturer's protocol (Roche). One sequencing run was performed on the GS-FLX system following the manufacturer's procedure (Roche). The sequence reads were quality filtered and assembled into contigs using the Newbler Assembly v2.5.3 software (Roche). The isotigs and singletons were generated with a normalized script to establish the levels of expression with a 90 % identity threshold (software CD-HIT, [17]). Then, an assembly was performed against a reference using GS Mapper, the OYDV-Mi reads vs. longest isotig of the OYDV-Se.

The isotigs and singletons were annotated using the BEST HIT of blastn vs. nt and blastx vs. non-redundant sequence (default parameters). The reference virus was selected according to the results (score and value): |AJ510223| onion yellow dwarf virus, complete genome. The blast was repeated (blastn and tblastn), but using the virus chosen as query and the isotigs/singletons as a database. The blast result was reconstructed manually.

To obtain overlapping PCR products and therefore be able to construct the remainder of the genome, specific primers were designed based on those sequences. Primer sequences are available by request from the corresponding author. The 5' end of the genome was amplified using rapid amplification of cDNA ends with the 5' RACE system kit, following the manufacturer's protocols (Invitrogen, USA).

Sequence assembly and analysis were performed using the Lasergene 8.0.2 software package (DNASTAR, Inc., Madison, WI, USA). The EMBOSS Pairwise Alignment Algorithms program (http://www.ebi.ac.uk) was used to assess the degree of nt and aa sequence diversity in the polyprotein, non-coding and coding regions, using the Needleman-Wunsch alignment algorithm. Comparisons were made between the OYDV-Se and OYDV-Mi sequences from onions, and each one of these with an available complete sequence of the most closely related potyvirus was chosen based on previous analysis (data not shown). These included four garlic OYDV sequences available in the GenBank database (AJ510223, AB219833, AB219834, HQ258894).

Putative cleavage sites of each protein were identified by sequence comparison with those of known potyviruses.

Possible recombination events between the onion isolates and other potyvirus isolates that infect members of the *Alliaceae* were examined using the Recombination Detection Program (RDP3) available from http://www.darwin.uvigo.es/rdp/rdp.html [18].

Results and discussion

Each of the onion isolates of OYDV was successfully transmitted to all nine of the onion cultivars inoculated, as shown by symptom development and strong DAS-ELISA reaction 30 days after inoculation. OYDV-Se was transmitted to 128 (95 %) and OYDV-Mi to 15 (11 %) of the 135 onion plants inoculated with each isolate (Table 1). OYDV-Se produced the typical severe symptoms, and OYDV-Mi, the typical mild symptoms, in all the cultivars. This means that the two isolates differ in aggressiveness and virulence on the onion genotypes tested, but not in host range. Neither of the isolates could infect garlic, chives or leek. This agrees

Table 1 Results of inoculation trials with onion yellow dwarf virus mild (OYDV-Mi) and severe (OYDV-Se) isolates on *Allium* crops

Inoculated host	OYDV-Mi[a]	OYDV-Se[a]
Onion Chata Colorada	1/15	15/15
Onion Cobriza	2/15	14/15
Onion Grano de Oro	1/15	15/15
Onion Navideña	2/15	13/15
Onion Roja de E. Rodríguez	2/15	14/15
Onion Valcatorce	3/15	15/15
Onion Valenciana	2/15	15/15
Onion Valuno	1/15	14/15
Onion White Wing	1/15	13/15
Chive	0/20	0/20
Leek	0/10	0/10
Garlic San Valentín	0/10	0/10

[a] OYDV-antiserum-positive plants/inoculated plants

with earlier findings that onion isolates only infect onion and garlic isolates only infect garlic [10, 24].

After sequencing of the two samples using the GS-FLX system, 253,268 sequences reads, totalling 95,282,355 nt and large numbers of individual fragments of sequence data for OYDV were generated. For the isolate OYDV-Se, 94,503 sequence reads, totalling 36,165,474, nt were produced with de novo Assembler Software (version 1.1.03); 304 isotigs were generated, leaving behind 7,667 unassembled reads. BLAST analysis indicated that three isotigs (162 reads) were genomes of OYDV. The results of the BLAST analysis confirmed that the onion plants (OYDV-Se and -Mi) used for RNA extraction were infected only with OYDV.

For both isolates, the individual sequence reads and contigs were spread across the genome and not clustered in any particular part of the genome sequence. The dispersed distribution enabled PCR to be used effectively for closing the gaps in the sequence (Fig. 1).

The complete genomes of OYDV-Se and OYDV-Mi were 10,461 nt and 10,459 nt long (excluding the poly (A) tail) and assigned the GenBank accession numbers JX433019 and JX433020, respectively. In both isolates, the AUG initiation codon and the stop codon (UGA) are likely to be located at nt position 109-111 and 10252-10254, respectively. Therefore, the predicted open reading frame (ORF) for each isolate was 10,143 nt, encoding a polyprotein of 3,381 aa.

The polyprotein amino acid (aa) sequence was aligned with those of the other completely sequenced potyviruses, and the characteristic proteolytic cleavage sites and known functional motifs of the 10 mature potyvirus proteins were identified. The observed cleavage sites of OYDV-Mi and OYDV-Se were perfectly consistent with the known sites of potyviruses [1]. Eight of the nine cleavage sites were identical for both isolates; the only difference was the junction 6K1/CI with the motif VQYQ/A for OYDV-Mi and VHYQ/A for OYDV-Se.

Pairwise alignments of the complete nt sequence of the two onion isolates revealed an identity of 92.2 %, while the polyprotein aa sequence identity was 94.5 % (Table 2). The potyvirus polyprotein typically gives rise to 10 proteins. Here, the OYDV-Se and OYDV-Mi had the same molecular mass for proteins P1 (48.51 kDa), HC-Pro (51.06 kDa), P3 (58.72 kDa), 6K1 (5.77 kDa), CI (70.70 kDa), 6K2 (5.88 kDa), VPg (21.76 kDa), NIa-Pro (26.86 kDa), NIb (57.50 kDa), and CP (28.53 kDa). Comparison of the individual protein regions of the two onion isolates showed that the CP-encoding region was the most conserved (nt and aa sequence identities of 95.8 % and 97.7 %, respectively). In the entire CP sequence (257 aa), there were six aa changes, four of which were located in the C-terminal region. The DAG motif that is involved in transmission by aphids [4] was found in the N-terminal region of both isolates at the same position (3,150-3,152). By contrast, the P1-encoding regions were the least conserved, sharing only 86.2 % nt identity of. P1 was the most variable protein (80.8 % identity), with 84 aa changes being distributed along the protein and corresponding to 45 % of all changes in the whole polyprotein. Lee and Wong [16] suggested that P1 would affect the development of symptoms, which was also proposed by Shi et al. [19], who reported that the P1N-terminus of soybean mosaic virus interacts with the Rieske Fe/S protein. This suggests that P1 might be related to the symptomatic differences observed in plants.

When RDP3 was used for recombination analysis, we were unable to detect any recombination events between the sequences of OYDV-Se and Mi and those of other potyviruses infecting members of the *Alliaceae* (AB194621, AB194622, AB194623, AJ307057, HQ258895, AJ865076, AM267479, NC_007433, NC_002509).

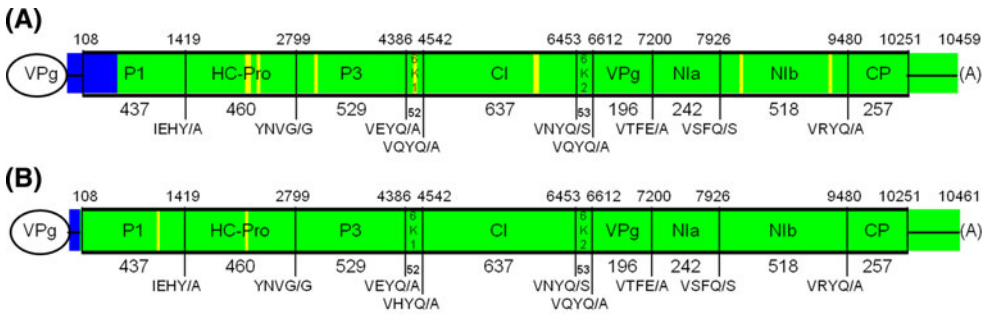

Fig. 1 Schematic representation of the genome organization of the onion yellow dwarf virus (OYDV) isolates Mi (**A**) and Se (**B**), with the mature proteins separated by vertical bars. The size of each protein is indicated below the genome as the number of amino acids, whereas the numbers above the genome indicate the nucleotide position where each region starts. The putative proteinase cleavage sites in the polyproteins of the isolates Mi and Se are indicated below each vertical bar. The illustrated sequence was generated using different methods. The sequence determined by next-generation sequencing is shown in green, the sequence obtained following PCR amplification using 5' RACE is depicted in blue, and the sequence generated following specific PCR is shown in yellow (color figure online)

Table 2 Comparison of nucleotide and amino acid sequence identities in complete genomes and individual genome regions of onion and garlic isolates of OYDV

Genomic region	Between onion isolates of OYDV[a]		Between garlic isolates of OYDV[b]	Between onion and garlic isolates of OYDV[a, b]	Nucleotide identity threshold (%) proposed for species discrimination [2]
	Nucleotide identity (%)	Amino acid identity (%)	Range of nucleotide identity (%)	Range of nucleotide identity (%)	
Whole genome	92.2	–[c]	78.6-100	67.5-70.7	–
Polyprotein	92.1	94.5	81.7-91.2	68.8-70.6	77.1
5' UTR	91.7	–	64.6-88.9	42.7-57.1	–
P1	86.2	80.8	71.3-81.7	52.5-55.8	58.0
HC-Pro	94.3	96.5	82.3-89.7	59.4-73.5	76.0
P3	93.0	94.1	79.2-92.7	64.2-66.0	74.0
6K1	94.9	98.1	82.7-100	76.9-80.1	–
CI	94.2	98.0	84.2-94.9	75.7-77.0	78.3
6K2	94.3	96.2	81.8-91.8	73.5-78.8	–
VPg	93.7	98.0	82.3-94.0	71.0-73.1	76.0
NIa-Pro	93.8	98.8	83.2-99.9	73.5-76.2	76.5
NIb	88.2	95.0	83.0-99.7	73.5-75.8	75.0
CP	95.8	97.7	88.3-99.9	79.9-81.7	76.0
3'UTR	96.6	–	89.6-99.3	82.4-87.0	76.0

[a] OYDV from onion: JX433019 and JX433020

[b] OYDV from garlic: AJ510223, AB219833, AB219834 and HQ258894

[c] No data (–)

Alignment of the sequences of both onions isolates studied here with those of garlic isolates of OYDV available from GenBank revealed surprisingly low levels of nt sequence identity (67.5-70.7 %) across the whole genome (Table 2). According to the species demarcation criteria for the family *Potyviridae* as stated in the current (9th) ICTV Report [3], "different species have a CP aa sequence identity less than about 80 %; and nt sequence identity less than 76 % either in the CP or over the whole genome. There are also differences in polyprotein cleavage sites". In previous work, one of the potyviruses detected in garlic was identified as OYDV because the percentage of CP aa identity with onion isolates of OYDV was higher than 80 % [7, 10, 15, 23]. However, in the present work, comparison of the complete genomes of the onion isolates with those of the garlic isolates showed striking differences in genetic properties (less than 76 % identity).

Comparison of the nt sequences of the individual genome regions of the OYDV isolates from onion and garlic with the values provided by Adams et al. [2] as molecular criteria for potyvirus species discrimination revealed that only the CP values (79.9-80.9 %) and 3'UTR (82.4-87.0 %) were always higher than the proposed threshold (76 %). However, the nt sequence for the complete polyprotein and for the regions P1, HC-Pro, P3, VPg, NIa-Pro and CI had lower identity values than those typically observed for isolates of the same species [2]. The NIb had lower identity in three of four garlic isolations compared to those of the onion isolates. Only when the sequence AJ510223 from garlic [6] was compared with the onion isolates were the values obtained for NIb (75.8 %) higher than the threshold value (75 %) proposed for species discrimination by Adams et al. [2]. According to the same authors, comparisons of the CI gene most accurately reflected those for the complete ORF, and this region would be the best for diagnostic and taxonomic studies if only a sub-portion of the genome were sequenced, rather than the usually used CP. In this work, the CI regions of the onion and garlic isolates shared identities of 75.7-77 %, which is lower than the threshold value (78.3 %) used for species demarcation.

Of the nine characteristic proteolytic cleavage sites of potyviruses [1], only three (OYDV-Se: YNVG/G, VNYQ/S, VRYQ/A) and four sites (OYDV-Mi: YNVG/G, VQYQ/A, VNYQ/S, VRYQ/A) of the onion isolates were identical to those of the garlic isolates (GenBank accession no. AJ510223, AB219833, AB219834 and HQ258894). The GxSG motif located in the P1 protein was found at position 385-388 in the onion isolates, whereas that of the garlic isolates was at position 406-409 [6]. The same authors indicated that this motif is the active site of a serine protease that cleaves the protein downstream of the final Y (or F) residue.

One of the potyviruses detected in garlic was identified as OYDV on the basis of a high aa sequence identity in the

CP; however, it is different biologically from the virus originally detected in onion. In this work, onion isolates of OYDV were not transmitted by mechanical inoculation from onion to garlic, an observation that is in agreement with results of previous studies [10, 24].

Differences in host ranges have been cited for isolates of other potyvirus species, such as *Bean yellow mosaic virus* [8], *Plum pox virus*, [21], and *Papaya ringspot virus* [22]. Although this allowed separating them into different strains, comparison of the complete genomes of these viruses, in all of the cases, revealed sequence identity higher than 76 % (data not shown). However, the garlic and onion strains of OYDV differ not only in their host ranges, but also in the sequences of their genomes, having less than 76 % identity and many differences in the polyprotein cleavage sites. All of these characters have been proposed as species demarcation criteria by the ICTV. In addition, when comparing the different genome regions of the onion and garlic isolates, seven of the nine regions shared nt sequence identity below those recommended for species demarcation by Adams et al. [2] (Table 2).

In previous work, the high CP aa identity suggested that the viruses detected in garlic and onion belonged to the same species [7, 10, 15, 23]. In addition, a close serological relationship between isolates from onion and garlic was observed [10, 15, 23, 24]. This is a very predictable result considering that purified virions predominantly consisting of the CP are typically used for antiserum production. CP aa sequence identities were originally used for virus taxonomy, and this led to the establishment of the first molecular criterion to distinguish virus species and strains. In view of the fact that the potyvirus CP is encoded by less than 10 % of the viral genome, the CP similarity has been questioned as a universally useful taxonomical criterion [2, 13].

The availability of a greater number of complete sequences for garlic and onion isolates of OYDV would provide a higher level of certainty for classifying these onion and garlic isolates. However, the striking genetic differences between onion and garlic isolates of OYDV as presented here suggest that they might represent two different virus species. For reasons of priority, we propose that (i) the name "onion yellow dwarf virus" be reserved for onion isolates of OYDV and (ii) the name "garlic common stripe virus" (GaCSV) be given to garlic isolates of the virus hitherto referred to as OYDV.

Here, we present the first complete sequences of onion isolates of OYDV, represented by two isolates, an Argentinean one causing severe symptoms, and a German one causing mild symptoms. Although they showed a high level of genome identity, they produced significant differences in the severity of symptoms. It remains to be studied whether the genetic differences between the two onion isolates are associated with their geographic origin and/or their biological properties, e.g. aggressiveness in onion.

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