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ORIGINAL ARTICLE

Genetic diversity of the coat protein of olive latent virus 1 isolates

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Abstract The CP gene variability among 21 olive latent virus 1 (OLV-1) isolates obtained from different hosts and locations and at different times was assessed. Amplicons obtained by RT-PCR were cloned, and at least 10 sequences from each isolate were analyzed and compared. OLV-1 sequences available in GenBank were included. The encoded CPs consisted of 270 amino acids, except those of isolates G1S and C7 (269 aa) and G6 (271 aa). Comparison of CP genomic sequences of the isolates under study showed very low values of nucleotide diversity, 0.02, and maximum nucleotide distances between (0.087) or within isolates (0.001). Although very few nucleotide sequence differences were observed among the isolates, olive isolates exhibited lower diversity (0.012). In addition, at position 158 (157 in C7 and G1S and 159 in G6) of the deduced aa sequences, an alanine residue was found to be conserved among the olive isolates. In citrus and tulip isolates, a threonine residue was present at position 158, whereas a valine was present at this same position in tomato isolates. Phylogenetic analysis indicated that OLV-1 isolates clustered in five groups according to original host. However, G6, originally recovered from olive but repeatedly inoculated and maintained in *N. benthamiana* plants for 8 years in our laboratory, was separated from other isolates. This may be attributable to adaptation to the experimental host over time. There was no

correlation of phylogenetic grouping of isolates based on geographical location or year of collection. Strong negative selection may have contributed to the low diversity among the OLV-1 CP isolates.

Introduction

Olive latent virus 1 (OLV-1) belongs to the genus *Alphanecrovirus*, family *Tombusviridae*, and was first detected in olive (*Olea europaea* L.) trees growing in southern Italy [15]. Since then, OLV-1 has been detected in olive trees in other countries, where it either causes no symptoms or is associated with low vigor, dwarfing, bushy growth, fasciation and bifurcation of stems, or yellow and sickle-shaped leaves [1, 7–9, 25, 36, 42]. OLV-1 also has been shown to be associated with chlorotic dwarf disease of citrus in Turkey, with necrosis of tulips in Japan, and necrosis of tomato in Poland [2, 20, 26]. OLV-1 can infect a range of herbaceous hosts, causing chlorotic, necrotic or reddish local lesions [13]. OLV-1 causes systemic mosaic in *N. benthamiana* [15, 27]. The Portuguese OLV-1 isolate GM6 seldom causes systemic leaf mosaic, but co-inoculation with olive mild mosaic virus (OMMV) results in systemic invasion of *N. benthamiana* by both necroviruses [4, 12]. OLV-1 has been detected in all plant organs, from roots to seeds, as well as free in soil and plant growth substrate [10, 24, 35, 39], which are important sources for virus spread in nature.

Complete genome sequences of OLV-1 recovered from citrus [17], olive [11] and tomato [19] are available, as well as a partial sequence that includes the coat protein (CP) gene of a tulip OLV-1 isolate [20]. OLV-1 has a positive-sense single-stranded RNA genome of about 3.7 kb containing 5 ORFs. The 5'-proximal ORF1 encodes a 23-kDa

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polypeptide that is predicted to be involved in RNA replication. ORF1 RT results from readthrough of an amber stop codon, generating an 82-kDa product identified as the RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode small peptides of 8 kDa and 6 kDa, respectively, which are predicted to be involved in viral movement. The 3'-proximal gene, ORF 4, encodes the 30-kDa CP, which is essential for virus assembly and systemic spread [30].

Most CPs of plant icosahedral positive-stranded RNA viruses have four distinct structural domains: an 'R' domain involved in the interaction with RNA, a connecting arm 'a', a central shell domain 'S' and a C-terminal projecting 'P' domain. Necroviruses lack the 'P' domain [6, 28]. For OLV-1 CP, only the 'S' domain has been identified, covering positions 53 to 270 [11]. The 'S' domain in icosahedral viruses has a signature pattern, consisting of a 26 amino acid residues located at OLV-1 CP aa positions 135-160 ([FYW]-x-[PSTA]-x(7)-G-x-[LIVM]-x-[LIVM]-x-[FYWIL]-x(2)-D-x(5)-P) [13] and includes four sites likely to be involved in Ca²⁺ binding [17, 29].

RNA viruses have potential for high genetic diversity due to the lack of proofreading activity of the RdRp, conferring a high potential for adaptation and evolution. However, selective pressures imposed by certain aspects of viral biology (e.g., host type and range) may limit genetic diversity [31, 33, 34, 40, 41].

Here, we studied the diversity of OLV-1 by molecular analysis of the CP gene of isolates obtained from different hosts and locations. We believe this will allow a deeper knowledge of virus populations and facilitate understanding of the epidemiology of this widely disseminated virus. The study of virus diversity also will contribute to design of more efficient and durable diagnostic methods.

Materials and methods

Virus isolates

Twenty-one OLV-1 isolates were characterized in this study: 16 from Portugal, two from Poland, two from Croatia and one from Italy (Table 1). Most of the isolates were from olive (18), but two were isolated from tomato, and one isolate was recovered from citrus. Four additional OLV-1 CP gene sequences were retrieved from GenBank: original host and locations were Turkey (citrus), Portugal (olive), Poland (tomato) and Japan (tulip) (Table 1). OLV-1 isolate G6 is the result of repeated passage of isolate GM6 through the experimental host *N. benthamiana* for the last eight years. All isolates were maintained in *N. benthamiana* plants following mechanical inoculation of olive fruit extracts in the presence of 0.05 M sodium phosphate

buffer, with the exception of those from Poland and Croatia and OlivMitra (Portugal), which were recovered from 2-year olive stem scrapings and used directly as a source for nucleic acid extraction.

Nucleic acid extraction

Total RNA was extracted from 100 mg of symptomatic leaves of *N. benthamiana* plants macerated in liquid nitrogen, using an RNeasy Plant Mini Kit (QIAGEN) following manufacturer's instructions. Double-stranded (ds) RNA was extracted from 10 g of olive stem scrapings essentially as described by Valverde et al. [38], denatured for 5 min at 100 °C and then placed on ice for 15 min prior to RT-PCR reactions.

RT-PCR

For cDNA synthesis, 1 µg of total RNA or 1 µg of denatured dsRNA was used in a 20-µL reaction with 200 U of M-MLV reverse transcriptase (Invitrogen) in the presence of random hexamers (Promega) and 1x first-strand buffer (Invitrogen), in accordance with the manufacturer's instructions. For PCR, specific primers encompassing the CP ORF (OLV1coat5', GACATTTTCGCAACTCTCT, and OLV1coat3', CACAACGATGGGTGAGTTGC) were designed based on OLV-1 genomic sequences available in GenBank. One microliter of cDNA was used in a 50-µL reaction with 2.5 U of Fidelity TaqDNA Polymerase (USB Corporation) performed in 10 mM Tris HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs and 0.3 µM of each primer. Amplifications were carried out in a Thermal Cycler (Bio-Rad) following initial denaturation at 94 °C for 1 min, 35 cycles at 94 °C for 30 seconds, 53 °C for 1 min and 68 °C for 1 min and 30 seconds, and a final extension step of 68 °C for 5 min. The use of these primers in RT-PCR assays produces a fragment of ≈857 nt.

Cloning and sequence analysis

RT-PCR products were purified using a GFX PCR DNA Purification kit (GE Healthcare Biosciences) and cloned into pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Plasmid DNA was extracted from *Escherichia coli* cells using a GenElute™ HP Plasmid Miniprep Kit (Sigma) following manufacturer's instructions after growing cells (overnight, 37 °C, 175 rpm) in low-salt LB medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, pH 7.5) supplemented with 100 µg of ampicillin per mL. DNA sequencing reactions were performed on both strands by Macrogen (The Netherlands). Sequences of at least ten clones of each isolate were determined. These, as well as four OLV-1 CP gene sequences available

Table 1 Olive latent virus 1 isolates and OLV-1 sequences retrieved from GenBank

Isolate	Origin	Crop/cultivar	Year of collection	Accession number
Oliv Mitra	South Portugal	Olive Galega vulgar	2012	KF804065
G1PB			1995	KF804051
G1S				KF804052
G3S				KF804053
G6				KF804054
G1A				KF804056
G2A				KF804057
G5A				KF804058
G8 M				KF804059
V6		Verdeal alentejana		KF804062
V10				KF804063
C1VM		Cordovil		KF804055
C7				KF804061
A4P2	North Portugal	Santulhana		KF804064
A1P2				KF804066
Negr		Negrinha de Freixo		KF804060
Croatia1	Croatia	Rosinjola	2012	KF804067
Croatia2		Buza Puntoza		KF804068
OLV1it	Turkey	Citrus	1996	KF804048
Poltom	Poland	Tomato	2009	KF804049
Polbenth				KF804050
GM6	South Portugal	Olive Galega vulgar	1995	DQ083996
OLV1c	Turkey	Citrus	1996	NC_001721
CM1	Poland	Tomato	2010	GU326337
Pare-P	Japan	Tulip	2001	AB061815

from GenBank (Table 1) were compared. The search for homologous sequences was done using BLAST. A multiple sequence alignment was generated using BioEdit 7.1.3.0 [18] and CLUSTAL W in MEGA 5.1 software [37]. The best-fit nucleotide substitution model for these data was the Kimura 2-parameter model in the MEGA 5.1 software, showing the lowest Bayesian information criterion (BIC) score. This model was used to estimate nucleotide distance, diversity and phylogenetic relationships, which were inferred using the neighbor-joining (NJ) method. All sequences, including repeated ones, were used for estimation of genetic diversity. To confirm the results of phylogenetic analysis using the NJ method, trees were produced using the Minimum Evolution, Maximum Parsimony and Maximum Likelihood methods in the MEGA 5.1 software. Bootstrap analyses with 1000 replicates were performed to evaluate the significance of nodes. Phylogenetic analysis was performed using a consensus sequence for each isolate based on sequences derived from at least 10 clones. Recombination events were evaluated by RDP, GENCONV, Bootscan, Chimaera, 3Seq and SiSCAN in the RDP4.18 software, using default settings and a Bonferroni-corrected highest *P*-value of 0.05. To identify specific amino acid sites under selective constraints, the difference

between nonsynonymous (d_N) and synonymous (d_S) substitution rates was estimated for each position in the alignments using the REL method as implemented in the HYPHY server (<http://www.datamonkey.org>) [21].

Results

Amplified RT-PCR products were of the expected size (≈ 857 bp). The CP sequences of 21 OLV-1 isolates (11, 12, 19 and 21 clones from G1A, G5A, G6 and G3S, respectively, and 10 clones from the other isolates) and the four OLV-1 CP gene sequences available from GenBank (totaling 237 sequences) were compared.

All sequences encoding the OLV-1 CP encoded 270 aa, with the exception of isolates G1S and C7 (269 aa) and isolate G6 (271 aa). Pairwise distances for all OLV-1 sequences ranged from 0.000 to 0.086. A lower pairwise distance range (0.000 – 0.037) was observed among isolates originating from olive. Isolates that showed the lowest pairwise distance (0.000) were OlivMitra and G3S; highest pairwise distances were observed among isolates from different hosts, G6 and Poltom or Polbenth, and G6 and OLV-1c. The genetic distance values within isolates were

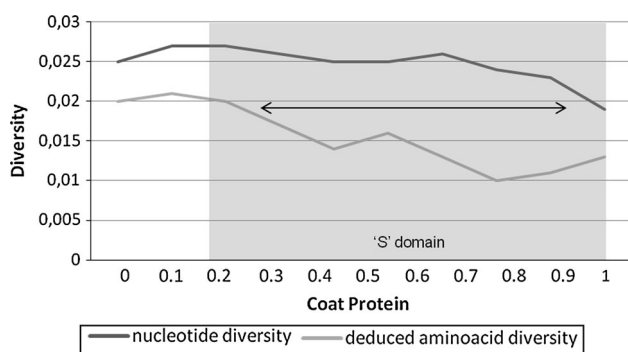


Fig. 1 Nucleotide and deduced amino acid sequence diversity along the OLV-1 CP gene. Nucleotide sequence diversity was examined in successive windows of 81 nt, and amino acid sequence diversity was examined in successive windows of 26 aa. The shaded region represents the ‘S’ domain. The arrow indicates a decrease in nucleotide and amino acid sequence diversity

very low, averaging 0.001: 0.007 (isolates G2A, G1 PB, G3S), 0.004 (isolates G1A, G1S, G6, C1VM) and 0.000 (all others). Diversity between isolates also was very low, with total diversity mostly due to differences between isolates and not within isolates, as observed by the high coefficient of differentiation of 0.98 (S.E. 0.004). There was not a distinct variable region along the encoded CP ORF of OLV-1 isolate sequences, although a slight decrease in the nucleotide diversity in the ‘S’ domain region was noted (Fig. 1).

The CP ‘S’ domain of OLV-1 starts at aa 53 and ends at aa 270, except for those of isolates G1S and C7 and isolate G6, in which the S domain spans aa 52 to 269 and 54 to 271 aa, respectively. The typical signature pattern for this domain ([FYW]-x-[PSTA]-x(7)-G-x-[LIVM]-x-[LIVM]-x-[FYWIL]-X(2)-D-x(5)-P) was detected at 135 to 160 aa (134 to 159 and 136 to 161 aa, for isolates G1S and C7 and isolate G6, respectively). Within the signature pattern, an alanine residue was conserved at position 158 (157 and 159 in isolates G1S and C7 and isolate G6, respectively) of all olive isolates except that of G6. In citrus and tulip isolates, position 158 is occupied by threonine, and in tomato isolates a valine residue is present. In the G6 olive isolate, which was repeatedly passaged in *N. benthamiana*, valine occurred at position 158, similarly to that observed in OLV-1 tomato isolates. This observation suggests that the valine substitution reflects adaptation to solanaceous hosts, which requires further confirmation. No isolates showed variation in the four amino acids predicted to be involved in Ca^{2+} binding (two residues of aspartic acid [D], one of threonine [T] and one of asparagine [N]) [17, 29].

The CP genes of the OLV-1 isolates under study did not show distinct recombination sites when examined using the RDP software (data not shown), suggesting that recombination had not occurred.

The phylogenetic tree deduced from the alignment of CP consensus sequences (Fig. 2) revealed segregation of the isolates under study into five clusters. As expected from the matrix of sequence identity, OLV-1 isolates were grouped according to host origin, with the exception of G6.

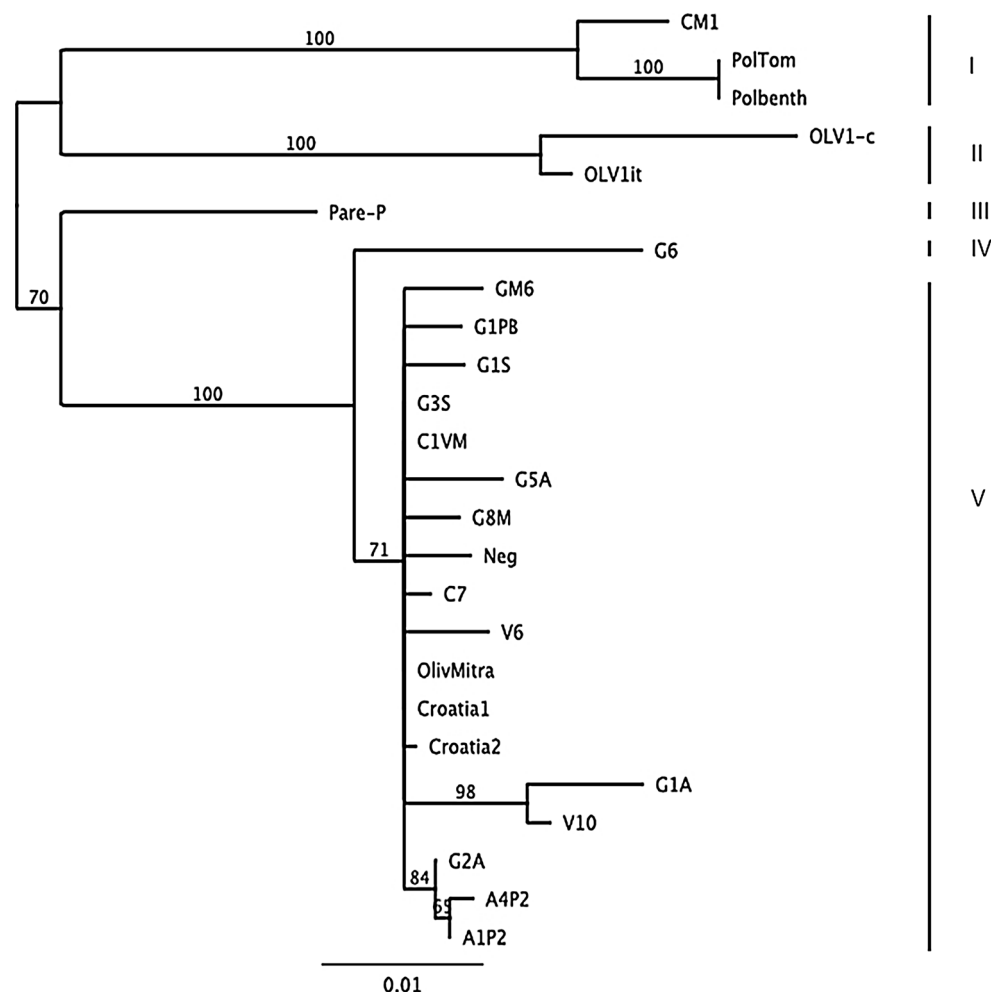
The difference between d_N and d_S at each individual codon was statistically tested by the REL method to determine if negative selection affected genetic variability. No positively selected codon was detected, whereas 87 codons were under negative selection (data not shown), suggesting strong negative or purifying selection.

Discussion

We compared CP sequences of 21 OLV-1 isolates originating from different hosts, sites and years. At least 10 complete CP sequences of each isolate were obtained. Analysis of CP nt and aa sequences revealed that overall diversity of OLV-1 isolates was low between and within isolates and that total diversity was mostly due to diversity between isolates, as shown by the high coefficient of differentiation. The nucleotide sequence variability within OLV-1 isolates was very low, <0.7 %; 0.1 % on average.

Sequence conservation among the CPs of OLV-1 isolates observed in this study (nt pairwise distance < 0.086 and genetic diversity 0.02) was extremely high. Low CP sequence diversity (0.03 – 0.10) has been observed for other RNA viruses [16]. Similar studies on other members of the family *Tombusviridae*, (carnation mottle virus [3] and pelargonium flower break virus [31]) have measured diversity values for CP sequences of ≈ 0.03 . The fact that in OLV-1, as well as in other members of the genus *Alphanecrovirus*, the ‘S’ domain is the only CP domain found, contrary to other viruses of the *Tombusviridae*, may help to explain the higher stability found. The ‘S’ domain has been shown to be the most conserved region in the CP of small plant viruses, suggesting that it is the region where more functional or structural constraints are located [31]. This is confirmed in the present work, where we evaluated selective constraints by comparing rates of synonymous and nonsynonymous substitutions across codon sites. No positively selected codons were detected, whereas 87 codons were under negative selection, suggesting that aa changes would result in functional or structural disadvantages, thus indicating strong negative or purifying selection. Negative constraints to which viral CPs are subjected may be due to multiple functions, including genome protection, cell-to-cell movement, transmission between plants, interactions with the host and/or vector, etc. In addition, Chare and Holmes [5] analyzed selection pressures in the capsid genes of plant RNA viruses and found

Fig. 2 Phylogenetic tree analysis of OLV-1 isolates based on CP nt consensus sequences. An NJ tree was constructed from the sequence alignment of ≈ 857 nt of the OLV-1 CP coding region from the 21 isolates studied here and from four retrieved from the GenBank database. Eleven, 12, 19 and 21 clones from G1A, G5A, G6 and G3S isolates, respectively, and ten clones from each one of the other isolates were sequenced and used to determine the consensus sequence for each isolate. Phylogenetic analysis included 21 consensus sequences, plus four sequences retrieved from GenBank. Multiple sequence alignments were generated using MEGA 5.1, and a phylogenetic tree was constructed by the NJ algorithm, based on calculations from pairwise nt sequence distances for gene nt analysis. Bootstrap analysis was done with 1000 replicates. Numbers above the lines indicate bootstrap scores out of 1000 replicates



that vector-borne viruses are subjected to a greater selection than non-vectored viruses. However, OLV-1 CP does not seem to be subjected to particularly strong negative constraints from transmission via biological (arthropod or nematode) vectors, nor from the host, as OLV-1 infects a wide range of plant species. A possible explanation for the high stability found may be that these isolates have evolved from a single original sequence in an original host, possibly olive. Genetic drift may also have contributed to the low variability after bottlenecks that virus populations undergo, such as systemic movement or transmission between plants.

Analysis of OLV-1 aa sequences revealed that at position 157 (or 156 or 158 in certain isolates) an alanine residue was found in olive isolates, a threonine in citrus and tulip isolates, and a valine in tomato isolates. The isolate G6 seems to be an exception and, contrary to the original GM6, also has a valine in that position. This may be due to the multiple passages of this isolate in *N. benthamiana*. Tomato and *N. benthamiana* both belong to the *Solanaceae*, and this mutation may be the result of host adaptation [22, 31].

The hypothesis of host adaptation is reinforced by analysis of the phylogenetic tree, which showed OLV-1 isolates segregating in five subgroups (Fig. 2) related to the host of origin. Segregation seems to be related to the host of origin. Clade I is composed of tomato isolates, clade II is composed of citrus isolates, clade III is composed of the tulip isolate, clade IV is composed of the G6 isolate, and clade V is composed of all other olive OLV-1 isolates used in this study. No relationship was found among isolates according to collection time or spatial separation. These results agree with those of other authors who have reported a high spatial and temporal genetic stability for several RNA viruses, suggesting that selective pressures to preserve biological functions are stronger than speciation due to geographic separation [14, 23, 32, 34, 40].

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