Serological relationships among strains of grapevine leafroll-associated virus 4 reflect the evolutive behavior of its coat protein gene

Las relaciones serológicas y la identidad molecular de variantes de grapevine leafroll-associated virus 4 reflejan el comportamiento evolutivo del gen de su proteína de cubierta

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Abstract

This research studied serological relationships and genetic diversity of Argentinean isolates of grapevine leafroll-associated virus-4 (GLRaV-4). Phylogenetic analysis of coat protein (CP) sequences from 19 local isolates revealed clustering with the previously described GLRaV-4 strain 5, strain 6, and strain 9 groups. Evolutionary sequence analysis of the obtained and database-available sequences showed evidence of recombination events. Additionally, both CP N- and C-terminal regions appeared to be under purifying selection, but the N-terminal region presented seven sites under positive selection, with a d_N/d_S ratio 5-fold greater than that of the C-terminal region. Serological reactivity against monoclonal antibodies supports a higher occurrence probability for linear epitopes in the N-terminal region, as inferred by the sequence analysis. The obtained results reflect an unusual evolutionary behavior of the CP that, together with protein serological reactivity, suggests biological significance of the observed variability.

Keywords

molecular characterization and serology • grapevine leafroll disease • ampelovirus • selection pressure • antigenic properties

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Resumen

Las relaciones serológicas y la diversidad genética de cepas argentinas de grapevine leafroll associated virus 4 (GLRaV-4) fueron analizadas. El análisis filogenético de la cápside proteica (CP) conducido sobre las secuencias obtenidas mostró un agrupamiento de las primeras con GLRaV-4 raza 5, GLRaV-4 raza 6 y GLRaV-4 raza 9. El análisis evolutivo de las secuencias locales y las disponibles en bases de datos infirió eventos de recombinación y sugirió que tanto los extremos C-terminal como N-terminal de la CP están bajo presión de selección purificante, pero la región N-terminal mostró siete sitios bajo presión de selección positiva, con una relación d_N/d_S cinco veces mayor que aquellas posiciones de la región C-terminal. La reactividad serológica contra anticuerpos monoclonales sustenta la probabilidad de ocurrencia de epitopes lineales en la región N-terminal inferida en el análisis evolutivo. Los resultados obtenidos reflejan un comportamiento evolutivo inusual de la CP y junto con la reactividad serológica de dicha proteína, permiten postular una significancia biológica de dicha variabilidad.

Palabras clave

caracterización molecular y serología • enfermedad del enrollado de la hoja de la vid • ampelovirus • presión de selección • propiedades antigénicas

Introduction

Grapevine Leafroll Disease (GLD) is one of the most widespread and deleterious viral diseases affecting grapevines with a particularly complex etiology. The development of serological reagents led to the identification of seven putative species belonging to the Closteroviridae family and generically named grapevine leafroll-associated virus (GLRaV) 1-7. However, during the 1990s and 2000s, the easier acquisition of genetic data arose the number of new putative species to 12 GLRaVs, tentatively or definitively assigned to the *Ampelovirus* genus (most of the species), to the *Closterovirus* genus (GLRaV-2) or the newly defined *Velarivirus* genus (GLRaV-7) in the *Closteroviridae* family. Generally, the main criteria establishing newly described viral isolates as new species were, first, the lack of serological reaction against previously developed monoclonal antibodies or polyclonal antiserum, and second, over 10% divergence in the sequence of taxonomic relevant genes (HSP70h and CP). The proliferation of taxonomic entities required a revision of the GLRaVs taxonomy, and considering available genetic information, it was established that most of the GLRaVs described at an early stage (GLRaV-4, -5, -6, -9, -De, -Pr and -Carn) should be considered as divergent isolates of a single species (GLRaV-4) (21).

In addition to the above-mentioned taxonomic issues, the serological relationships among the GLRaV-4 groups of isolates (previously known as different species) remain unclear. Gugerli (2009) performed an extensive review of the different serological reagents developed during the past 30 years and highlighted some of the arisen ambiguities concerning antibodies and antisera. However, scarce information followed this review, being a serological characterization of four isolates of GLRaV-4 (1) the most comprehensive work up to date. Western blot reactivity among those four isolates (two of GLRaV-4 strain 6 and one of each strain 4 and 5) against monoclonal antibodies (Mabs) raised against GLRaV-4 strain 4, 5 and 6, was clear and straight, without any cross-reaction among them. Meanwhile, all four isolates reacted against a monoclonal mix developed for generic detection of GLRaV-4 (2) and a commercial polyclonal antiserum anti-GLRaV-4 strain 5.

The complete genome sequencing of different GLRaV-4 strains allowed clarifying the taxonomy of this virus. Nevertheless, the significance of CP antigenic properties of the different GLRaV-4 isolates remains unclear.

The present work presents a sequence analysis of the CP gene of Argentinean GLRaV-4 isolates, and an additional serological analysis of purified virions, aiming to establish significance levels of such genetic and serological variability.

Material and Methods

Virus Isolates

One hundred forty-one grapevine plants of different varieties exhibiting mild to severe symptoms of grapevine leafroll disease (GLD) were selected from the ampelographic collection located in the Mendoza Research Station of the National Institute of Agronomic Technology (EEA Mendoza, INTA). In order to determine symptomatic nature, these plants were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) using commercial reagents for GLRaV-1, -2, -3, -4, -4 strain 6, and -7. The study included positive samples for GLRaV-4 or GLRaV-4 strain 6 (24 samples) and those negative for all tested viruses (13 samples).

RNA extraction, RT-PCR, cloning, and sequencing

Double-stranded RNA (dsRNA) was extracted from cambial scrapings of mature grapevine canes, reverse-transcribed, and subsequently amplified by Polymerase Chain Reaction (PCR) using a proofreading polymerase (DeepVent DNA Polymerase, New England Biolabs, USA). The complete Coat Protein (CP) Open Reading Frame (ORF) of GLRaV-4 was amplified using specific primers (ACPF 5'-GCTGGATAGGTTYAGRTCNAAAGAYACYCC-3' and ACPR 5'-TAACCTCCATATTTTCAAACG-3') designed over the upstream and downstream sequences of such ORF (p60 and p23, respectively) based on database available nucleotide sequences of GLRaV-4. The resulting PCR products were resolved by agarose gel electrophoresis. The occurrence of multiple infections with GLRaV-4 genetic variants in a single plant was investigated by RT-PCR-Restriction Fragment Length Polymorphism (RFLP), digesting the resulting PCR products with both AluI and HinfI restriction enzymes. Restriction fragments were resolved by electrophoresis on a 2% agarose gel. Undigested PCR products were cloned into the pGEM-T Easy Vector System I, and the resulting clones were sequenced. After blue/white screening, 19 white colonies from each transformation were selected, amplified with ACPF and ACPR, and restricted as mentioned for accurate identification of clones of different genetic variants. Three colonies belonging to each restriction pattern by sample were randomly selected and sequenced using both pUC/M13 reverse and forward sequencing primers at Macrogen Inc. (Korea).

Sequence analysis

Sequences from each clone were assembled and edited obtaining the coding sequence of the CP ORF. Codon multiple sequence alignment was performed using the aligned codons from these sequences, together with all the GLRaV-4 CP sequences available in the NCBI GenBank database. Using the HyPhy software package, evolutionary and phylogenetic analyses evaluated whether selection pressure affects viral strain evolution (16). Recombination analysis was performed using Single Breakpoint (SBP), Genetic Algorithm Recombination Detection (GARD), and confirmed by RDP software (19). Three methods, namely Single Likelihood Ancestor Counting (SLAC), Random Effects Likelihood (REL), and Fixed Effects Likelihood (FEL), allowed the identification of selection pressure. Results were integrated by integrative selection analysis. The aligned codons (with additional CP sequence of PMWaV-1 (AF414119) as outgroup) were subjected to preliminary phylogenetic reconstruction by Maximum Likelihood (ML) analysis using the PAUP software package (28) and heuristic search, with random addition sequences considering one hundred replicates. Group support was estimated by Garli program (31) generating 1000 replicates to obtain bootstrap values. Branches with bootstrap values under 70% were collapsed. From the phylogenetic tree inferred, seven clusters were defined. Sequences belonging to the different strains of GLRaV-4 allowed a clear identification of such groups: GLRaV-4 strain 5 (AF233934), strain 9 (AY297819), strain 6 (FJ467504), strain 4 (FJ467503), strain Ob (AB720874), strain Carn (FJ907331), and strain Pr (FM244690). The genetic distance within and among groups was estimated using the Tamura-Nei model of MEGA5 software by estimating the standard error from a bootstrap of 1000 replicates. Overall distance of GLRaV-4, and genetic distances for all the available CP sequences of GLRaV-1, -2 and -3 were also estimated.

The presence of putative linear epitopes over the deduced amino acid CP sequence was evaluated by BepiPred software (18). Complementary to epitope detection and through the SomeNA and SNAP2 tools implemented in the Predict Protein server, a structure prediction analysis identified a putative nucleic acid binding motif and the functional effect of a point mutation in CP (30).

Serological characterization

After the ELISA-positive samples for GLRaV-4 or GLRaV-4 strain 6, nineteen samples were selected for further serological assays based on the restriction pattern of observed CP. Viral particles were purified from cortical scrapings of mature grapevine canes as described by Savino (1993). The resulting extracts were analyzed to determine serological characteristics of the GLRaV-4 variants. The purified virions were resolved over 30 mm wide lanes into a 14%/4% SDS PAGE, electroblotted to nitrocellulose membrane. After blocking the membrane, ten individual longitudinal strips from each lane were excised. Each strip (3 mm wide) was probed with each of seven monoclonal antibodies: Mab 36-117, Mab3-1, Mab8-2, Mab43-1, Mab3-3, Mab6-3, Mab 15-5 (12, 13) and three polyclonal antisera: ASGLRaV-5 from Biorad (Hercules, CA, USA), AS GLRaV-4 strain-6 from Bioreba AG (Switzerland) and AS GLRaV-4 I252-IL (provided by Dr. Boscia, 2006). The strips were revealed after incubation with Goat-AntiMouse AP conjugated or Goat-AntiRabbit AP conjugated (Sigma, MI, USA).

Results

Out of the 141 ELISA-tested samples exhibiting leafroll disease symptoms, 13 samples reacted with none of the tested reagents. When considering reactions against GLRaV-4 and GLRaV-4 strain 6 reagents, 10 samples resulted positive with both antibodies, 12 only reacted with the GLRaV-4 reagents, and 2 samples only reacted with the GLRaV-4 strain 6 reagents. These 37 samples were examined by RT-PCR using the primers described above. No product was amplified from the 13 ELISA-negative samples, while a product of the expected size (1,100 bp) was generated from all 24 ELISA-positive samples.

Restriction of the RT-PCR products yielded several fragments in all cases (Supplementary Figure 1). In some cases, band number and size indicated presence of a single genetic variant, while in other cases, the digestion of multiple PCR products had the same size but different sequence. Cloning and screening of these RT-PCR products using PCR and restriction over the white colonies allowed to identify the genetic variants in the original sample. When two samples shared the same restriction pattern and serological behavior, only one was sequenced. Three colonies corresponding to each restriction pattern were sequenced from samples with multiple patterns, considering a total of 90 clones sequenced from 19 different plant samples. Generally, sequences of clones sharing the same restriction pattern obtained from the same plant were identical and considered a single sequence. Consequently, this study generated 30 sequences (Supplementary Table 1). One single ORF was identified in each sequence, sizing according to GLRaV-4 CP. Most sequences produced a 269-amino acid translation product, but some sequences exhibited minor size divergences (individual sequences of 265, 268, 271, and 272 amino acids). Codon multiple alignment revealed deletions in the first 40 amino acids of the protein (shorter sequences) or a mutation in the stop codon leading to size differences.

Sequence analysis

The 164 sequences of GLRaV-4 used considered 30 CP sequences obtained in this study and 134 sequences available in NCBI Genbank database. The phylogenetic tree inferred by ML analysis discriminated seven monophyletic groups supported by high bootstrap values (Supplementary Figure 2). The seven GLRaV-4 described strains (strain 4, strain 5, strain 6, strain 9, strain Ob, strain Pr, strain Carn) represent the seven groups. This phylogeny agrees with the previously reported phylogeny of the HSP70 of GLRaV-4 (20). Most of the local sequences obtained in this study clustered with the reference sequences of GLRaV-4 strain 5 and GLRaV-4 strain 6, while only one sequence grouped with GLRaV-4 strain 9. No local sequence clustered with GLRaV-4 strain 4, strain Pr, strain Ob or strain Carn.

Table 1 shows the amino acid sequence identity level within and among these seven groups. Identity among sequences inside all these groups, except GLRaV-4 strain 6, exceeded 90%. Sequences belonging to GLRaV-4 strain Ob and GLRaV-4 strain Pr were the most divergent, sharing identities under the proposed 25% divergence threshold (21), with sequences from other groups. However, when the alignment was arbitrarily split into two (first 40 residues, and from residue 40 to the end of the protein), identity levels among sequences changed substantially. The C-terminal region was conserved among all analyzed sequences. Identity level in this region within the seven groups was over 95%, while identity level among sequences of the different groups was always over 80% (table 2). Variability was considerably high in the N-terminal region of CP with identity difference levels between pairs of sequences as high as 43% within the GLRaV-4 strain 5 group, and 53% within the GLRaV-4 strain 6 group (table 2). Average genetic distances estimated for the 128 CP sequences of GLRaV-4 was 0.127, higher than GLRaV-3 but in line with GLRaV-2 (0.118) and the estimated 0.106 for GLRaV-1 (7). Both GLRaV-1 and GLRaV-2 are considered highly variable viral species.

Table 1. Estimates of evolutionary divergence between coat protein sequences.

Tabla 1. Estimaciones de divergencia evolutiva entre secuencias de proteínas de cápside proteica.

Range of aminoacidic identities per site between GLRaV4 sequences of the seven groups identified and defined according to the inferred phylogeny.

Rango de identidades aminocídicas por sitio entre secuencias de GLRaV-4 de los siete grupos identificados y definidos según la filogenia inferida.

	GLRaV-4 strain Pr	GLRaV-4 strain Carn	GLRaV-4 strain 9	GLRaV-4 strain 6	GLRaV-4 strain 5	GLRaV-4 strain Ob	GLRaV-4 strain 4
GLRaV-4 strain 4	0.75 - 0.79	0.77 - 0.78	0.80 - 0.81	0.79 - 0.81	0.79 - 0.84	0.73 - 0.76	0.94 - 0.99
GLRaV-4 strain Ob	0.72 - 0.75	0.75 - 0.75	0.73 - 0.75	0.73 - 0.77	0.73 - 0.78	0.93 - 0.93	
GLRaV-4 strain 5	0.75 - 0.78	0.76 - 0.79	0.84 - 0.87	0.82 - 0.87	0.92 - 1		
GLRaV-4 strain 6	0.71 - 0.75	0.76 - 0.79	0.84 - 0.87	0.90 - 1			
GLRaV-4 strain 9	0.75 - 0.77	0.78 - 0.78	0.97 - 0.99				
GLRaV-4 strain Carn	0.75 - 0.75	- - -					
GLRaV-4 strain Pr	0.97 - 0.97						

Table 2. Estimates of evolutionary divergence between coat protein sequences considering amino-terminal and carboxyl-terminal regions.

Tabla 2. Estimaciones de divergencia evolutiva entre las secuencias de proteínas de cápside considerando las regiones amino-terminal y carboxi-terminal.

Range of aminoacidic identities per site between GLRaV4 sequences of the seven groups identified and defined according to the phylogeny inferred. Above the diagonal, comparison of the first 40 aminoacids. Below the diagonal, comparison from residue 41 to the end of the sequences.

Rango de identidades aminocídicas por sitio entre secuencias de GLRaV-4 de los siete grupos identificados y definidos en base a la filogenia inferida. Por encima de la diagonal, la comparación de los primeros 40 aminoácidos. Por debajo de la diagonal, comparación desde el residuo 41 hasta el final de la secuencia proteica.

		GLRaV-4 strain 4	GLRaV-4 strain Ob	GLRaV-4 strain 5	GLRaV-4 strain 6	GLRaV-4 strain 9	GLRaV-4 strain Carn	GLRaV-4 strain Pr
		0.63 - 0.92	0.21 - 0.29	0.29 - 0.47	0.29 - 0.45	0.34 - 0.39	0.39 - 0.47	0.21 - 0.33
GLRaV-4 strain 4	0.98 - 0.96		0.63 - 0.63	0.17 - 0.39	0.14 - 0.37	0.23 - 0.31	0.27 - 0.27	0.21 - 0.31
GLRaV-4 strain Ob	0.82 - 0.84	0.97 - 0.97		0.57 - 1	0.28 - 0.60	0.31 - 0.54	0.26 - 0.34	0.26 - 0.44
GLRaV-4 strain 5	0.87 - 0.90	0.81 - 0.84	0.95 - 1		0.47 - 1	0.36 - 0.51	0.25 - 0.43	0.18 - 0.36
GLRaV-4 strain 6	0.86 - 0.89	0.80 - 0.84	0.88 - 0.93	0.95 - 1		0.85 - 0.94	0.37 - 0.40	0.36 - 0.38
GLRaV-4 strain 9	0.88 - 0.89	0.81 - 0.82	0.91 - 0.94	0.91 - 0.94	0.99 - 1		- - -	0.28 - 0.33
GLRaV-4 strain Carn	0.84 - 0.85	0.82 - 0.82	0.84 - 0.86	0.82 - 0.85	0.85 - 0.85	- - -		0.87 - 0.87
GLRaV-4 strain Pr	0.84 - 0.86	0.82 - 0.83	0.83 - 0.86	0.80 - 0.83	0.82 - 0.83	0.83 - 0.84	0.98 - 0.98	

Evolutionary analysis

The recombination analysis conducted by SBP inferred a putative recombination event over the multiple sequence alignment. Such event was also detected by using the RDP software, suggesting that GLRaV-4 strain 9 sequences were recombinants between GLRaV-4 strain 5 and GLRaV-4 strain 6. This same event was also identified when the recombination analysis was performed with complete genomic sequences of GLRaV-4 strain 5, 6 and 9 available in the database. However, GARD was unable to detect the recombination event among these three complete sequences. In consequence and to avoid a biased analysis due to the recombination effect in the selection pressure analysis, the complete alignment was split into two datasets according to the inferred breakpoint (from position 1 to 120, and from 121 to the end of the codon alignment).

Consequently, selection pressure analysis was performed for two datasets. Results of overall d_N/d_S ratios obtained by SLAC and REL were consistently different for both datasets (table 3), being the mean d_N/d_S ratio estimated by SLAC 5 times greater in the N-terminal region. Although all obtained ratios were lower than 1, indicating a negative or purifying selection, the C-terminal region (the most conserved region) was subjected to heavier purifying selection than the N-terminal region. When the d_N/d_S ratio was analyzed for the entire CP as a single dataset, the value fell in between the partial values obtained, similar to those reported by Maliogka *et al.* (2008) (mean of 0.085 by FEL). The site-by-site analysis integrating the three individual analyses (REL, FEL, and SLAC) was different between both datasets. In the first dataset, seven sites were significantly inferred as being under positive selection by REL (two of them also identified by FEL and SLAC, Supplementary Figure 3), and 23 of the 33 remaining sites resulted under negative selection pressure by at least one method. For the second dataset (position 121-end), both methods failed to detect positive selection pressure, whereas 198 sites (over 236 codons) were inferred as being under negative selection by FEL and 190 codons by SLAC. REL could not be performed due to alignment size restrictions. In general, except for the negatively selected sites at positions 1, 5, 12, 14 20 and 21, the high prevalence of negatively selected sites begins at position 24 of the multiple codon alignment (Supplementary figure 3).

Table 3. Estimates of selection pressure on the coat protein gene of GLRaV-4.

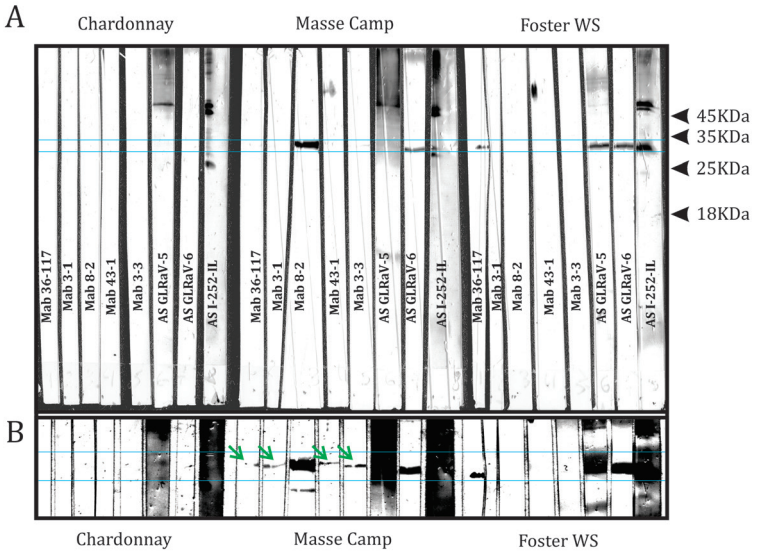
Tabla 3. Estimación de la presión de selección actuante en el gen de la cápside proteica de GLRaV-4.

		Entire dataset (276 codons)	**Splitted dataset: N-terminal region (40 codons)**	**Splitted dataset: C-terminal region (236 codons)**
SLAC	Mean d_N/d_S	0.146	0.524	0.092
	Positively selected sites	4	2	0
	Negatively selected sites	206	12	190
REL	Mean d_N/d_S	Analysis not performed due to size restriction	0.641	Analysis not performed due to size restriction
	Positively selected sites		7	
	Negatively selected sites		23	
FEL	Positively selected sites	2	2	0
	Negatively selected sites	218	14	198

The linear epitope prediction analysis of all concerned sequences performed by BepiPred revealed the highest probability of occurrence of a B-cell linear epitope in the first 40 amino acids, in agreement with previous reports (6, 20). Noteworthy is that in the same positions, most of the sites under positive selection were inferred, and as previously observed, the region was the most CP variable (Supplementary Figure 3). In every protein sequence, a single polynucleotide binding site was inferred by SomeNA. All these predicted sites were located between the 175 to 182 CP residues (Supplementary Figure 3).

Serological analysis.

Western blot analysis of purified extracts from nineteen GLRaV-4 infected plants and a virus-free accession revealed variable specificity from the different Mabs and AS used. Two of the three AS used (AS-GLRaV-5 and AS-I-252-IL) showed nonspecific reactions. Several bands were observed in all the analyzed samples, even in the virus-free Chardonnay. However, the 35KDa GLRaV-4 CP band was clearly identified. The three AS reacted with the GLRaV-4 CP of the nineteen analyzed extracts, but the Sangiovesse Fiano sample only faintly reacted with AS-I252-IL. Two Mabs (6-3 and 15-5) did not react with any western blot sample. The five remaining Mabs showed variable reactions with the tested samples, from clear to faint bands (figure 1). Western blot results are summarized in Supplementary Table 1.



A) Reaction of three samples against five Mabs and three PAS. Healthy controls clearly show nonspecific reactions against two PAS. B) Overexposure of the same membrane reveals faint reaction against four Mabs.

A) Reacción de tres muestras contra cinco anticuerpos monoclonales y tres antisueros policlonales. El control sano muestra reacción no específica contra dos antisueros. B) Sobre exposición de la misma membrana muestra reacción débil contra cuatro anticuerpos.

Figure 1. Nitrocellulose membrane after western blot analysis.

Figura 1. Membrana de nitrocelulosa revelada tras western blot.

Discussion

Despite the taxonomic controversy during the early ampelovirus history, today GLRaV-4 is considered a single viral species composed of several genetic variants. In the present study, 30 CP sequences of GLRaV-4 were obtained from 19 leafroll-affected grapevine plants. All plants reacted with one or both ELISA reagents for GLRaV-4 and GLRaV-4 strain 6. The RT-PCR analysis of dsRNA extract from these plants allowed amplifying a 1.100 bp fragment in all cases, containing the entire CP ORF. Sequence analysis led to sequence identification of GLRaV-4 strain 5, strain 6 and strain 9. Serological analysis showed specific reactions of such samples against Mabs for GLRaV-4 strain 5 (Mab 8-2, 43-1, 3-3) and -6 (Mab 36-117), whereas no clear reaction was obtained against GLRaV-4-specific Mab (Mab 3-1). Mab 15-5 and 6-3 did not react against any sample in western blot (as previously recorded with the same extract used for mice immunization) (2). As these two Mabs compose the GLRaV-4 DAS ELISA reagent set used, and give positive reaction with the tested samples, both Mabs are obviously directed against a conformational epitope, dissociated during the denaturing SDS-PAGE.

Both molecular and serological analysis revealed the occurence of mixed infections, an usual behavior in grapevine-infecting closterovirids, also reported in other host species (8).

CP sequence variability of GLRaV-4 was considerably high. In fact, some CP sequences (such as GLRaV-4 strains Ob and Pr) showed higher divergence than the proposed threshold of 25% as criteria for species discrimination in the *Ampelovirus* genus (21). Given these variability levels, the proposed divergence threshold of 25% over the CP aminoacidic sequence should be closely revised and raised to 30% considering the currently available sequences belonging to GLRaV-4. A closer analysis of such variability showed an asymmetrical distribution, with the CP N-terminal region (the first 40 residues) much more variable than the C-terminal region. Besides the nucleotide substitutions leading to amino-acid changes, all insertions or deletions were concentrated in the first 120 nucleotides of the multiple sequence alignment. Furthermore, a segmented analysis of the observed variability evidenced that all available CP sequences of GLRaV-4 shared more than 80% identity over the C-terminal region (233 to 236 residues). In all cases, pair-wise similarity among sequences of the referred C-terminal region was over 90%, reflecting a high conservation degree.

The ML analysis performed on the complete CP sequences herein generated and on the ones available in the database revealed strong clustering in seven genetic groups. Most viral strains obtained from local vineyards clustered together with GLRaV-4 strains 5 and 6, while one of the obtained sequences exhibited a close relationship with GLRaV-4 strain 9. The seven groups of sequences were compared by a genetic distance study conducted by using observed cluster distribution in the ML tree. Intragroup level of genetic distance (≤ 0.1) was different from intergroup distance (over 0.14 for the closest GLRaV-4 strain 5 and 9, and exceeding 0.2 for the remaining groups). These results are consistent with the amino acid sequence identity levels between groups (table 1, page 108). When GLRaV-4 was considered as a single species, the average genetic distance in the group was 0.127, barely higher than that in GLRaV-1 and -2, members of *Ampelovirus* and *Closterovirus* respectively, and reported as highly variable species.

In the first evolutionary analysis, a putative recombination event was inferred as related to GLRaV-4 strains 9, 5 and 6, producing a topological incongruence between the N- and C-terminal regions of CP in the phylogenetic analysis. This difference was already observed in the HSP70h and CP inferred phylogeny conducted by Maliogka *et al.* (2008), even though the authors did not record any recombination evidence. Recombination is one main force driving evolutionary history of plant viruses, with a significant impact in the *Closteroviridae* family, as observed for CTV (*Closterovirus*), GLRaV-3 and GLRaV-4 strain 5 (*Ampelovirus*). Considering GLRaV-4, the restricted host range, long host lifespan, high sequence similarity and high occurrence of mixed infections could provide a favorable environment for increasing effective recombinants. However, the recombination signal identified in GLRaV-4 could not constitute a true genetic exchange among donor and recipient viruses, but a variable rate of mutation among the different genes of the virus (29). A more detailed study with more GLRaV-4 strain 9 sequences could confirm this event.

After splitting the dataset according to putative recombination, a significant difference was revealed in the selection pressure over CP during GLRaV-4 evolution history. In fact, the entire CP sequence was subjected to a strong negative selection as indicated by the global d_N/d_S value of 0.144 (in concordance with most plant viral CP), but the variable N-terminal region appeared to present sites subjected to positive selection (fairly unusual in CP of plant virus). So far, the occurrence of positively selected sites has been reported only on a few plant viruses (for instance GLRaV-1 and GLRaV-4 strain 5) but absent positively selected sites over the CP are more frequent. In fact, the comprehensive study of Chare and Holmes (2004) showed only three of 36 plant virus species with low number of sites under positive selection into the CP gene. High conservation levels of most CP (where up to 198 of 233 C-terminal residues were inferred to be under negative selection) reflect a strong purifying selection, probably maintaining some CP functions. For instance, a putative nucleotide binding site was inferred in all the analyzed sequences in the 175 to 182 positions, typically saturated of sites under strong negative selection pressure. This selective behavior may be a consequence of structural requirements.

In addition to structural functions, CP of plant viruses is involved in vector specificity. The virion-vector interaction of lettuce infectious yellow virus (LIYV, *Crinivirus*) has been thoroughly studied, and the minor coat protein (CPm) determined virions to

vector binding (5). GLRaV-4, in opposite to most *Closteroviridae* family members, lacks a CPm homologue, and the viral particle appears to be completely covered by CP, as a homologous antibody uniformly decorates the entire viral particle, whereas GLRaV-2 left an undecorated tail (1). In consequence, the CP replacing the absent CPm should constitute the vector binding determinant. Generally, plant viruses are considered host generalists and vector-specific. Nearly 60% of plant virus species are transmitted by a single vector, but less than 10% of viral species infect one single host (24). GLRaV-1, -3 and -4 showed a particular behavior, given they naturally infect only *Vitis spp*, whereas they are transmitted by up to eleven different mealybugs and soft-scale insect species (9). Different isolates of GLRaV-4 have been reported as transmissible with variable efficiency, or even not transmitted by six pseudococids species belonging to two families. This biological behavior is somewhat similar to the serological reactivity against Mabs previously reported (11). In this work, we confronted serological reactivity of local isolates of GLRaV-4 to a wide panel of Mabs proved to be highly specific. Furthermore, the heterologous reaction observed may be caused by multiple strain infection, rather than *sensu stricto* heterologous serological reaction. Considering the variability and antigenicity observed across the CP of GLRaV-4, we postulate that linear epitopes reactive to Mab36-117, Mab 8-2, Mab3-1 and Mab3-3 are located in the highly immunogenic N-terminal region of the protein. Previous research demonstrated that for cucumber mosaic virus, a short epitope of five residues exposed on the surface of the virion reacts with a Mab, essential for virus transmissibility, as single residue mutations abolish both transmissibility and reactivity against Mab (3, 19). However, one single mutation can provide vector affinity advantages. If such mutation increased transmission efficiency or augmented the number of vector species having affinity for the virion, it would confer an impressive ecological advantage compared with the wild-type population. This may explain the unusual occurrence of positively selected sites in the CP of GLRaV-4. Conversely, the highly conserved C-terminal region may be the result of structural conformation of CP or strong CP interaction requirements in the virus replication cycle in the plant. The SNAP2 analysis revealed that the region comprising the first 40 residues was mostly composed of amino acids whose substitution led to a neutral function effect, whereas mutations in amino acids located around the putative polynucleotide binding site (located in the C-terminal region) could have a functional effect (Supplementary Figure 3). This suggested biological significance of the observed sequence conservation.

The same evolutionary behavior described (a variable N-terminal region with positively selected sites) has been reported for bean yellow mosaic virus (Potyvirus). In that species, Parella and Lanave demonstrated that one of the positively selected sites identified belonged to a motif involved in CP-vector interaction, crucial for transmissibility (23).

The significance behind these observations in ampeloviruses can be assessed by an exhaustive study of the transmission efficiency of different genetic variants in the presence of different mealybug species. Recently, Rivadeneira *et al.* (2022) reported differential incidence of GLRaV -3 and -4, suggesting higher levels of GLRaV-3 linked to the occurrence of *Planococcus ficus.* However, surprisingly in the presence of mealybugs, GLRaV-4 incidence remains quite low. In contrast, GLRaV-3 incidence is lower in Mendoza Province (10, 17). Consequently, assertive identification of viral strains should be considered for disease impact, like in modelization approaches of vigour components in grapevine (14, 15).

Epitope prediction conducted using BepiPred revealed high occurrence probability of a linear B-cell epitope in the N-terminal region of CP, consistent with previous observations (6, 20). In addition to the implication in the abovementioned transmissibility, some important immunological issues need discussion. Considering that most available serological reagents for characterizing GLRaV-4 isolates are monoclonal antibodies (11) with good reactivity against denatured CP in Western blots, they might be directed against a linear epitope. Moreover, given viral particles were applied in the native form during immunization, these epitopes may be located on the virion surface. Considering the most immunogenic region as a linear epitope (the most variable region), monoclonal antibodies targeting these epitopes will not be useful for taxonomic purposes at species level. This statement considers the identity level found in the present study for GLRaV-4 strain 5 in the N-terminal region ranged from 57% to 100% while for GLRaV-4 strain 6, it ranged from 47% to 100%. However, these antibodies remain useful for strain discrimination.

Conversely, the antibodies present in the commercially available reagent set for GLRaV-4 (2) appeared to target a conformational epitope (as they are nonreactive against the denatured CP in Western blots) highly conserved and probably located in the C-terminal region of CP. Since no available systems predict conformational epitopes from the primary structure of the proteins, this issue remains unresolved and warrants further research.

Conclusions

This work first reports a linkage among the distinctive evolutionary behavior of the coat protein of GLRaV-4 and biological properties of such protein, providing an alternative point of view in the study of virus-vector interactions, transmissibility and ecology.

Supplementary tables and figures

https://docs.google.com/document/d/1KVV5AW4Pqq7VqANYI1G5sIkCMR8B9fmA/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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