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Molecular characterization of a grapevine leafroll-associated virus 4 from Slovenian vineyards

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Summary. – During a survey conducted in vineyards in Slovenia, variety of grapevine leafroll disease symptoms were observed. Mixed infection with grapevine leafroll-associated viruses 3 and 4 (GLRaV-3, -4) in two grapevines from a vineyard in south-western part of Slovenia was confirmed by DAS-ELISA in 2010. The 3'final 1769 nucleotides of the Slovenian GLRaV-4 isolate were assembled from amplicons obtained by IC RT-PCR. The complete coat protein (CP) and p23 gene sequences were compared with other GLRaV-4 sequences from GenBank. Results showed that CP and p23 amino acid sequences of Slovenian variant (055-SI) are 88% and 85%, respectively, identical to corresponding genes of reference sequence GLRaV-4 LR106 (GenBank Acc. No. FJ467503). Phylogenetic analyses show that Slovenian variant clusters together with other corresponding strains of GLRaV-4. The sequencing results show great variability of the N-terminal part of the CP sequence indicating that this part of the genome is not suitable for molecular detection of the virus. To our knowledge this is also the first report of GLRaV-4 in Slovenian vineyards.

Keywords: Ampelovirus; Closteroviridae; CP; GLRaV-4; p23; Slovenia

Introduction

Grapevine leafroll disease (GLD) is one of the most widespread and damaging diseases in all major grapevine-growing areas in the world. It is considered to be a major virus disease of grapevine.

Mild downward rolling and premature reddening or yellowing of the lower leaves in red and white grapevine varieties are the first symptoms of GLD. In summer and fall symptoms progress and cause downward rolling of leaves and phloem disruption. The disease affects fruit quality and leads to significant decrease in yield (Martelli and Boudon-Padieu, 2006; Moutinho-Pereira *et al.,* 2012).

GLD is caused by different grapevine leafroll-associated viruses (GLRaVs) transmitted by scale insects in semi-persistent manner. Most of these viruses belong to the genus *Ampelovirus* (GLRaV-1, -3, -4), GLRaV-2 is assigned to the genus *Closterovirus* and GLRaV-7 is a member of the new genus *Velarivirus*, all in the family *Closteroviridae* (Al Rwahnih *et al.,* 2012; Martelli *et al.,* 2012).

According to the current taxonomy, the genus *Ampelovirus* is split into two subgroups (Martelli *et al.,* 2012). Among grapevine-infecting viruses, GLRaV-1 and -3 are members of subgroup I, and GLRaV-4 is a member of subgroup II. GLRaV-5, -6, -9, -De, -Pr and -Car used to be distinct species within the genus *Ampelovirus*. After the complete genomic sequences have been obtained for different members of subgroup II, GLRaV-5, -6, -9, -De, -Car and -Pr were recognised as genetically divergent strains of GLRaV-4 (Abou Ghanem-Sabanadzovic *et al.,* 2012; Martelli *et al.,* 2012; Thompson *et al.,* 2012). The last variant was published in GenBank in 2014 and was named GLRaV-4 strain 9, already according to the new taxonomy.

GLRaV-4 has been reported from many grapevine-growing countries worldwide (Martelli, 2014). In Europe it was reported from Portugal (Esteves *et al.,* 2012), Spain (Padilla

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Abbreviations: CP = coat protein; GLRaV = grapevine leafroll-associated virus; HSP70h = heat-shock protein 70 homolog; RdRp = RNA dependent RNA polymerase

et al., 2010), France (Thompson *et al.,* 2012), Switzerland (Reynard *et al.,* 2015), Italy (Giampetruzzi *et al.,* 2011; Rizzo *et al.,* 2014), Greece (Maliogka *et al.,* 2008) and Turkey (Buzkan *et al.,* 2010). Except Switzerland, there are no reports of GLRaV-4 from Central and Eastern Europe.

In this work we report about the first finding of GLRaV-4 in Slovenia, about molecular characterisation of the 3' end of the genome and about the relationship of Slovenian isolate with other variants of GLRaV4.

Materials and Methods

Plant material and RNA extraction. Leaves from 70 field-grown grapevine plants were collected in autumn 2010 in one vineyard from south western part of Slovenia. The presence of arabis mosaic virus (ArMV), cherry leaf roll virus (CLRV), GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-9, grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), grapevine virus A (GVA), raspberry bushy dwarf virus (RBDV), raspberry ringspot virus (RRSV), strawberry latent ringspot virus (SLRSV), tobacco ringspot virus (TRSV), tomato black ring virus (TBRV) and tomato ringspot virus (ToRSV) was determined by DAS-ELISA according to the manufacturer's instructions (BIOREBA AG, Switzerland). One of two GLRaV-4 positive samples was used for further molecular characterization.

Immunocapture RT. PCR reaction tubes were pre-coated with anti-GLRaV-4-9 IgG (BIOREBA AG, Switzerland), washed with ELISA washing buffer and 50 µl of plant sap were incubated overnight. RT reactions were performed in a final volume of 25 µl containing 1X M-MLV RT Buffer (Promega, USA), 2 mmol/l dNTPs, 200 U M-MLV Reverse Transcriptase (Promega, USA), 40 U RNasin (Promega, USA) and 50 pmol of reverse gene specific primer. The RT reactions were incubated at 42°C for 1 hr.

PCR, cloning, sequencing and sequence analysis. PCR reactions were performed in a final volume of 25 µl using 5X KAPA2G Buffer A (Kapa Biosystems, South Africa), 0.4 mmol/l dNTPs, 5 U KAPA2G Robust HotStart DNA polymerase (Kapa Biosystems, South Africa) and 50 pmol of each of gene-specific primers (Table 1). The primers from this work were designed using Primer3 software (Ye *et al.,* 2012) based on GenBank sequences FJ467503 (GLRaV-4 LR106) and KJ810572 (GLRaV-4 strain 9), targeting coat protein (CP) and p23 gene. The amplification conditions comprised an initial denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 56°C (or 50°C for LRamp-F/R) for 45 sec, extension at 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were visualized in 1.2% agarose gels. Before cloning, PCR products were purified from the gel using a JETQUICK Gel Extraction Spin Kit (Genomed, Germany).

Cloning of purified PCR products was carried out using a pGEM-T Easy Vector System (Promega, USA) and transformed into competent JM-109 *Escherichia coli* cells (Promega, USA) according to manufacturer's instructions. Colony PCR was used to identify colonies with inserts. Plasmids were purified from selected positive clones using a JETQUICK Plasmid Miniprep Spin Kit (Genomed, Germany). At least three clones for each amplicon were sequenced in both directions at Macrogen (Netherlands) and consensus sequences were assembled. The obtained amplicon sequences were analyzed, aligned and assembled using software BioEdit version 7.0.5.3. The assembled sequence of isolate 055-SI was deposited in the GenBank Acc. No. KM892778. A sequence database was constructed containing the GLRaV-4 sequences available in the GenBank that corresponded in length to the sequence obtained during this work. The sequence of pineapple mealybug wilt-associated virus 1 (PMWaV-1) was used as the outgroup for phylogenetic analyses (Table 2). The nucleotide and amino acid sequences were multiple aligned using CLUSTAL W and MUSCLE using default settings from MEGA6. The phylogenetic relationships for each gene dataset were determined with the maximum-likelihood algorithm (ML) of the MEGA6 with 1000 bootstrap replications for both CP and p23. A gene-specific model was chosen on the basis of the lowest BIC score of deduced amino acid sequences.

Table 1. GLRaV-4-specific primers used in this study

Target region[a]	Primer name	Sequence 5'–3'	Genome position[b]	Product size (bp)	Reference
CP	LR4-IR-F1	TCCGRSCCAYATGAACTTCGA	11920-12921	1001	This work
	LR-9-CP-R	TGAGGCGTCGTAACCGAACAATCT			Jerugula *et al.,* 2008
	LR4ms-CP-F	CGTCTGGGAAACTGGAAGTGA	12800-13070	270	This work
	LR4ms-CP-R	ACGAAAATATGAGAAGTCACAGAC			
CP, p23	LR4-56-MS4-F	GTGGCCATAAACAAACCGTC	12946-13370	424	This work
	LR4-56-MS4-R	GGAAAAGGTACCGAACGTTT			
p23	LRamp-F	ATTTAGGTAATGTTGTAGCTAC	13274-13758	484	Abou Ghanem-Sabanadzovic *et al.,* 2012
	LRamp-R	TATCCTCAGWGAGGAARCGG			

[a]CP = coat protein, p23 = open reading frame 6, [b]Positions of the primers in the genome of GLRaV-4 sequence FJ467503.

Table 2. GLRaV-4, its strains and their GenBank Acc. Nos of coat protein and p23 gene sequences used in this study

Virus/Strain	Isolate	GenBank Acc. No.	Country	Reference
GLRaV-4	LR106	FJ467503	USA	Abou Ghanem-Sabanadzovic *et al.*, 2012
GLRaV-4	Y252-IL[a]	AM176759	Israel	Saldarelli *et al.*, 2006
GLRaV-4	Y253-TK[a]	AM162279	Turkey	Saldarelli *et al.*, 2006
GLRaV-4	055-SI	KM892778	Slovenia	This study
GLRaV-4	Ru1	AB720874	Japan	Ito *et al.*, 2013
GLRaV-4	Ru2	AB720875	Japan	Ito *et al.*, 2013
GLRaV-4 strain 5	GLRaV-5	AF233934		Good & Monis, 2001
GLRaV-4 strain 5	1050-02	JX513893	Canada	Unpublished
GLRaV-4 strain 5	Y217	FR822696	France	Thompson *et al.*, 2012
GLRaV-4 strain 6	Estellat	FJ467504	USA	Abou Ghanem-Sabanadzovic *et al.*, 2012
GLRaV-4 strain 9	GLRaV-9	AY297819		Unpublished
GLRaV-4 strain 9	strain 9	KJ810572	Spain	Unpublished
GLRaV-4 strain Car		FJ907331	USA	Abou Ghanem-Sabanadzovic *et al.*, 2010
GLRaV-4 strain De		AM494935	Greece	Maliogka *et al.*, 2008
GLRaV-4 strain Pr		AM182328	Greece	Maliogka *et al.*, 2008
PMWaV-1		AF414119		Melzer *et al.*, 2008

[a]Sequence of p23 gene is not available in GenBank.

Table 3. Nucleotide and amino acid sequence identity (%) of the coat protein (below and above the diagonal, respectively) between Slovenian variant and corresponding sequences of GLRaV-4

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
LR106	**1**		94	94	88	74	76	83	82	83	81	81	81	77	77	80	51
Y252-IL	**2**	93		99	88	75	76	81	82	81	81	81	82	78	79	80	50
Y253-TK	**3**	90	95		88	74	75	81	82	81	81	80	81	78	78	80	51
055-SI	**4**	81	81	82		76	78	79	80	79	81	81	81	76	77	81	50
Ru1	**5**	68	68	67	69		93	74	73	74	72	71	73	74	73	74	53
Ru2	**6**	70	70	70	70	88		75	75	75	74	73	74	74	75	75	53
GLRaV-5	**7**	71	73	73	72	67	68		96	99	85	87	85	78	77	86	52
1050-02	**8**	72	74	73	73	68	69	94		96	85	86	85	78	77	85	52
Y217	**9**	71	73	73	73	67	68	99	94		85	87	85	78	77	86	52
-6Est	**10**	73	72	72	72	68	68	74	74	75		84	83	78	75	91	51
GLRaV-9	**11**	72	72	72	74	66	67	80	79	80	76		97	78	77	86	50
strain 9	**12**	72	72	72	74	66	68	81	79	81	77	97		78	76	85	51
GLRaV-Car	**13**	70	69	69	68	67	68	68	69	68	69	68	68		75	75	52
GLRaV-Pr	**14**	70	70	71	68	64	67	69	68	69	69	67	68	68		75	51
-De	**15**	71	72	72	73	68	69	75	74	75	85	75	76	69	68		51
PMWaV-1	**16**	55	55	55	56	57	56	56	56	56	55	55	55	60	58	56	

Results and Discussion

Grapevine leafroll disease is known to be present in Slovenia. It is mostly caused by GLRaV-1 and GLRaV-3 (Mavrič Pleško *et al.,* 2011; Štrukelj *et al.,* 2013). Of other GLRaVs, GLRaV-2 was found by ELISA and electron microscopy in 9 out of 210 grapevines from selection vineyards in southwestern part of Slovenia (Tomažič *et al.,* 2008). Also GLRaV-6 was occasionally found by ELISA but its presence has never been confirmed by other methods (Čepin, 2011).

In this study, GLRaV-4 was detected in mixed infection with GLRaV-3 in two out of 70 grapevine leaf samples by DAS-ELISA. ArMV, CLRV, GFkV, GFLV, GLRaV-1, GLRaV-2, GVA, RBDV, RRSV, SLRSV, TBRV, ToRSV and TRSV tested negative by DAS-ELISA. One of GLRaV-4-positive samples was used for further molecular characterization and the immunocapture RT-PCR was used to ensure specific detection of GLRaV-4.

Evolutionary relationships in the family *Closteroviridae* are studied on the basis of the molecular divergence

of RNA-dependent RNA polymerase (RdRp), heat-shock protein 70 homolog (HSP70h) and coat protein (CP) gene (Dolja *et al.,* 2006). Species demarcation criteria within the genus *Ampelovirus* are at least 75% amino acid identity of RdRp, HSP70h and CP (Martelli *et al.,* 2011). The assembled sequence of Slovenian GLRaV-4 isolate 055-SI covered the 1769 nucleotides of 3'-end of the genome containing part of p60, whole CP and p23 gene and 42 nucleotides of 3' noncoding region. The CP and p23 were compared with 16 CP sequences (Table 3) and 13 sequences of p23 (Table 4) of subgroup II ampeloviruses.

The CP sequence of 055-SI consists of 789 nucleotides. It shows the highest identity with the CP sequences of LR106, Y235-TK and Y252-IL (81%–82%). Identity with other sequences is 69%–74% (Ru1, Ru2, GLRaV-5, 1050-02, Y217, -6Est, GLRaV-9, strain 9 and -De) and 68% (-Car and -Pr) (Table 3). The deduced amino acid sequence identity of CP (262 aa) is 88% (LR106, Y235-TK and Y252-IL), 79%–81% (GLRaV-5, 1050-02, Y217, -6Est, GLRaV-9, strain 9 and -De) and 76%–78% (Ru1, Ru2, -Car and -Pr) (Table 3). Comparison of deduced amino acid sequence shows that CP of 055-SI is 10 aa shorter than three most closely related GLRaV-4 CP sequences. Furthermore, comparison of the 234 aa of the C-terminal part of the CP shows that 055-SI shares 95%–97% identity with them and 82%–89% with all other sequences (data not shown). Most of the mismatches are located in the N-terminal part of the CP, which is 29–39 amino acids long, depending on the isolate. The sequence identity for the N-terminal part of CP among isolates ranges between 13% (Ru1 and GLRaV-9) and 97% (strain 5; GLRaV-5 and Y217). On that part of CP, identity of 055-SI comparing with others is 42% (Y235-TK and Y252-IL) and 24% (GLRaV-5 and Y217) (data not shown). Saldarelli *et al.* (2006) already reported that the N-terminal part of the CP is the most heterogeneous part of the CP of compared variants and only few differences can be found in the rest of the CP. Similar to them we had problems amplifying the 5'-end of the CP sequence using different primer pairs. The comparison of these primers with the obtained sequence of 055-SI showed 3 to 7 mismatches, which prevented the amplification of the target product.

Gene p23 is separated by 33 nt from the CP, the same as in all other GLRaV-4 strains. The p23 gene of 055-SI consists of 624 nt and shows 87% identity with LR106, 79%–81% with GLRaV-5, 1050-02, Y217, -6Est, GLRaV-9 and strain 9, 70% with -Pr and 61%–62% with Ru1, Ru2 and -Car (Table 4). The identity of deduced protein sequence of 207 aa is 85% with LR106, 75%–76% with GLRaV-5, 1050-02, Y217, -6Est, GLRaV-9 and strain 9, 65% with -Pr and 56%–57% with Ru1, Ru2 and -Car (Table 4).

Phylogenetic analyses of ampeloviruses have mainly been made on the HSP70h and CP genes (Abou Ghanem-Sabanadzovic *et al.,* 2012; Martelli *et al.,* 2012; Ito *et al.,* 2013) but phylogenetic trees obtained from six different ampeloviruses proteins (Mt/Hel, RdRp, HSP70h, CP, p60 and p23) showed similar topologies (Thompson *et al.,* 2012). The CP- and p23-based phylogenetic trees made during our study show five clusters corresponding to five strains of GLRaV-4 (-4, -5, -6, -9 and -De), -Car and -Pr. 055-SI is grouped together with three GLRaV-4 isolates (LR106, Y252-IL and Y253-TK). In both phylogenetic trees, Ru1 and Ru2 grouped together, -Car and -Pr appear to be the most diverse members of subgroup II ampeloviruses (Fig. 1 and 2). They indicate a wide and complex genetic diversity of GLRaV-4. Results of our study are in agreement with previously published studies on complete or partial sequences of these two genes (Abou Ghanem-Sabanadzovic *et al.,* 2012; Martelli *et al.,* 2012; Thompson *et al.,* 2012; Ito *et al.,* 2013).

Table 4. Nucleotide and amino acid sequence identity (%) of the p23 gene (below and above the diagonal, respectively) between Slovenian variant and corresponding sequences of GLRaV-4

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
LR106	**1**		85	52	52	71	70	71	71	70	70	52	62	37
055-SI	**2**	87		57	56	76	75	76	76	75	75	57	65	38
Ru1	**3**	60	61		87	53	52	53	54	54	53	55	56	38
Ru2	**4**	61	61	88		52	53	52	53	54	52	55	55	38
GLRaV-5	**5**	79	80	58	60		94	100	83	92	93	56	62	40
1050-02	**6**	79	81	58	59	96		94	83	89	90	56	61	39
Y217	**7**	79	81	58	59	99	96		83	92	93	56	62	40
-6Est	**8**	77	79	58	59	85	85	85		86	85	59	62	38
GLRaV-9	**9**	79	80	59	59	91	89	90	85		97	57	62	40
strain 9	**10**	78	80	58	58	92	90	92	85	97		56	63	39
GLRaV-Car	**11**	60	62	60	59	61	61	61	63	62	61		50	37
GLRaV-Pr	**12**	69	70	61	59	68	68	68	70	67	69	59		38
PMWaV-1	**13**	46	46	46	45	46	45	46	46	45	45	48	50	

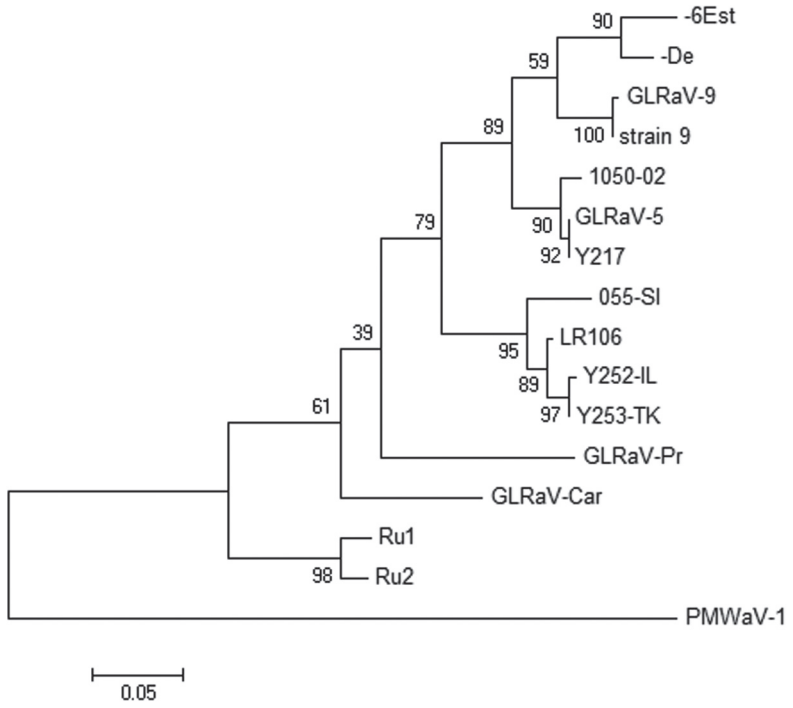

Fig. 1

Phylogenetic tree showing the relationship between GLRaV-4 strains based on homologous amino acid sequences corresponding to the CP datasets
PMWaV-1 was used as an out-group. The tree was constructed by the maximum-likelihood (ML) method. Bootstrap values of 1000 replicates are shown on the nodes. The bar represents 0.05 amino acid changes per site and branch lengths are proportional to the genetic distances.

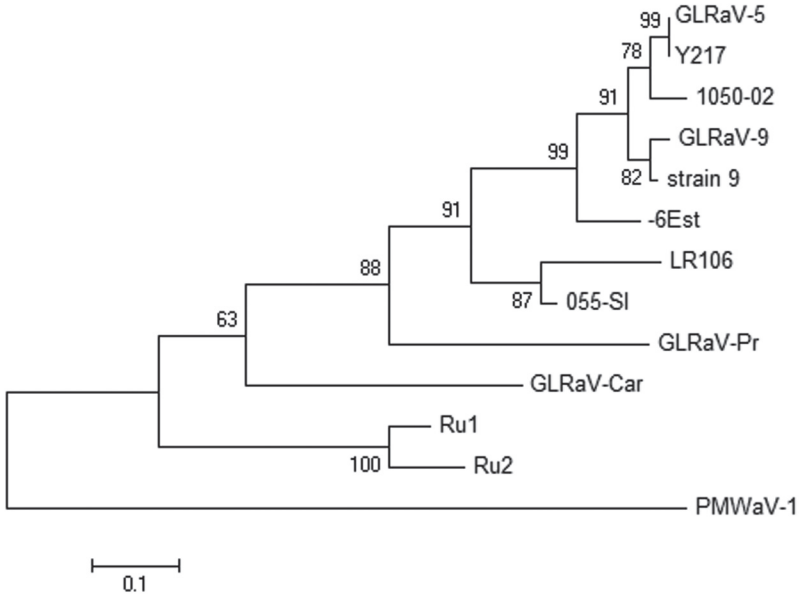

Fig. 2

Phylogenetic tree showing the relationship between GLRaV-4 strains based on homologous amino acid sequences corresponding to the p23 gene datasets
PMWaV-1 was used as an out-group. The tree was constructed by the maximum-likelihood (ML) method. Bootstrap values of 1000 replicates are shown on the nodes. The bar represents 0.1 amino acid changes per site and branch lengths are proportional to the genetic distances.

In this paper we reported about the first finding and partial molecular characterization of GLRaV-4 in Slovenia. The CP sequence of our isolate indicates a great variability of N-terminal part of the CP as was already observed by Saldarelli *et al.* (2006). These differences did not interfere with the serological detection of the virus, but caused difficulties in molecular characterization. The information about the great variability in the N-terminal region of the CP is important for further development of molecular diagnostic tests of GLRaV-4.

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