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A SURVEY ON GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES 1 AND 3 IN THE SOUTH OF UKRAINE AND DEVELOPMENT OF PRIMERS FOR GLRaV-3 IDENTIFICATION

Viticulture is one of the most intensive and complex branches of Ukraine agriculture. Grapevine virus diseases are responsible for considerable economic losses to grape productivity and wine industries. One of the most notable and widespread viruses associated with vine leafroll disease is grapevine leafroll-associated viruses (GLRaV), belonging to the genus Ampelovirus, family Closteroviridae. Thus, ***the aim*** *of this study was to conduct a survey targeting two viruses involved in the grapevine leafroll, namely grapevine leafroll-associated virus 1 (GLRaV-1) and virus 3(GLRaV-3) distributed in commercial wine grapes growing in the vineyards of the Ovidiopol and Bolhrad districts of the Odesa region. For efficient and accurate virus detection, we aimed to design universal primers based on conserved nucleotide sequences.* ***Methods:*** *Virus surveys of vineyards, visual diagnosis, immunoassay (ELISA), polymerase chain reaction with reverse transcription (RT-PCR), and Sanger sequencing of partial genome sequences of GLRaV.* ***Results.*** *The results obtained indicate that grapevine leafroll disease symptoms in field-grown grapevines in the south ofы Ukraine are caused by GLRaV-3. GLRaV-1 was not detected in any of the samples tested. To confirm the presence of GLRaV-3 in the samples, specific primers were designed targeting the coat protein region (GLRaV3-7f 5'-AGTAGGGGATGCAGCACAAG-3'; GLRaV3-7r 5'- ATCCAAAGCTATTCCCTTGC-3') of the virus. A new set of primers (GLRaV3-7f / GLRaV3-7r) has been validated for sensitive detection of GLRaV-3 by RT-PCR and may be useful for routine virus detection in the laboratory as well as for large-scale testing. The partial coat protein gene of the isolate, GLRaV-3 ukr, distributed in the south of Ukraine, was sequenced, and the obtained sequence was deposited in GenBank under Acc. No. ON015835. The phylogenetic study demonstrated that GLRaV-3 ukr was closely related to isolates from Russia (MZ065372 and MZ065370).*

Keywords: *Vitis, grapevine leafroll disease, GLRaV-3, primer design, sequence analysis.*

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Viticulture is one of the most intensive and complex branches of Ukraine agriculture. Industrial viticulture in Ukraine occupies a large area with a great variety of soil and climatic conditions. The area of vineyards concentrated mainly in Odesa, Kherson, Mykolaiv, Zaporizhzhia, and Zakarpattia regions, is about 40 thousand hectares. Grapevine virus diseases are responsible for considerable economic losses to grape productivity and wine industries. Grapevines can be infected by a large number of viruses that differ in genome structure, expression strategies, and pathobiology. Grapevines grown for commercial purposes are commonly infected with a mixture of viruses and viral strains [1].

Grapevine leafroll disease (GLD) is one of the most widespread and economically important viral diseases of grapevines (*Vitis* spp.). The disease is associated with different virus species and strains known collectively as grapevine leafroll-associated viruses (GLRaV). Symptoms of GLD can vary greatly depending on the season, grape cultivar, virus combinations, and environmental factors [2]. GLD is economically the most damaging viral disease complex affecting global grape and wine production [3]. So far, six distinct species of viruses belonging to three genera of the family *Closteroviridae* are reportedly associated with GLD. These viruses are named as grapevine leafroll-associated viruses 1, 2, 3, 4, 7, and 13 (GLRaV-1, -2, -3, -4, -7, and -13). GLRaV-2 is a member of the genus *Closterovirus*; GLRaV-7 is a prototype member of the genus *Velarivirus*, while the other viruses belong to the genus *Ampelovirus* [1]. Two species, GLRaV-1 and GLRaV-3, belonging to subgroup I of genus *Ampelovirus* (*Closteroviridae* family) are more prevalent and commonly associated with severe leafroll disease [4]. GLRaV-3 is widely distributed in different regions of the world, including Europe, South and North America, Middle East, Northern and Southern Africa, Asia, and Oceania [5]. In Ukraine, the study of grapevine leafroll distribution in the grapevine growing regions and detection of viruses in clonal and ordinary materials of grapes are carried out regularly by a number of researchers [6-11]. In order to establish the distribution of GLRaV-1 and GLRaV-3 in the south of Ukraine, a survey was conducted in two grapevine-growing regions, namely the Ovidiopol and Bolhrad districts.

Materials and methods. The survey was performed in 5 vineyards of wine-growing regions of South Ukraine in the Ovidiopol and Bolhrad districts of Odesa region from 2019 to 2020. Vineyards with an area of over 70 hectares in the Ovidiopol district (Joint-stock agricultural company (J-sAC) «Ukragro», Limited Liability Company (LLC) «Technologist», LLC «Shustov-Agro», LLC «Vitis-Agro») and 430 hectares in the Bolhrad district (State Enterprise (SE) «Experimental Economy Named after O.V. Suvorov») were surveyed in the Odesa region. Leaves and green shoots (scraped phloem) of plants showing the symptoms of virus diseases as well as asymptomatic ones were collected from 500 ha plantation area and immediately frozen in liquid nitrogen for further analysis by enzyme-linked-immunosorbent assay (ELISA) or the reverse transcription polymerase chain reaction (RT-PCR). The samples were assayed for the presence of GLRaV-1 and GLRaV-3. Totally 650 samples were obtained from the grape varieties such as Cabernet Sauvignon, Merlot, Rhine Riesling, Chardonnai in the Ovidiopol district and 580 samples from the grape varieties such as Cabernet Sauvignon, Rhine Riesling, Chardonnai, and Sukholiman white in the Bolhrad district.

Double antibody sandwich ELISA (DAS-ELISA). Commercial ELISA kits (Agritest, Italy) against GLRaV-3 and GLRaV-1 were used in the DAS-ELISA method according to the manufacturer's guidelines [12]. Absorbance was measured at 405 nm with a microplate reader (Dynatech ELISA reader, USA). A result was considered positive if the OD value was ≥ 0.2.

RNA extraction and reverse transcription. RNA was extracted from the virus-infected plant ma-

terial using the RIBO-sorb kits (AmpliSens, russia). The reverse transcription (RT) was performed with Reverta kits (AmpliSens, russia), according to the manufacturer's instruction.

Polymerase chain reaction. For PCR, 2 μL of the template genomic DNA was amplified in a 25-μL total volume containing 1× reaction buffer, 5 pmol of each primer, 0.3 mM dNTPs, 1.25 U of TaqDNA polymerase, and nuclease-free water.

Reactions were performed under the following conditions: 3 min denaturation at 95 °C, thermal cycling for 35 cycles (1 min at 94 °C, 1 min at 60 °C and 1 min 30 s at 72 °C), ending with the final extension at 72 °C for 5 min.

Primers. PCR primers capable of amplifying GLRaV-3 DNA templates were designed using Primer3 primer-design software (http://primer3.ut.ee/). All sequences referenced in the study were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/nuccore). The multiple sequence alignment of CP genes was carried out using the MultAline software (http://multalin.toulouse.inra.fr/multalin).

Gel electrophoresis. 10 mL from each PCR reaction was subjected to electrophoresis in a 1.5% agarose gel using 0.5 x TBE (0.045 M tris-borate, 1 mM EDTA, pH 8.0) running buffer. Products were stained with ethidium bromide and visualized by a UV transilluminator.

Table 1. **Vineyard survey results in the Odesa region**

Farm	Variety	N of examined vine bushes	N of examined vines with symptoms	Infected by *GLRaV-3*	
				N	%
Ovidiopol district					
J-sAC «Ukragro»	Cabernet Sauvignon	2222	120	18	0.81
	Merlot	560	30	5	0.89
	Rhine Riesling	430	30	2	0.47
	Chardonnay	1570	30	7	0.44
LLC «Technologist»	Cabernet Sauvignon	2210	110	21	0.95
	Merlot	420	30	4	0.95
	Rhine Riesling	1370	30	8	0.58
	Chardonnay	1500	30	5	0.33
LLC «Shustov-Agro»	Cabernet Sauvignon	2239	120	15	0.67
	Merlot	1490	30	10	0.67
	Rhine Riesling	1400	30	7	0.5
	Chardonnay	750	30	2	0.27
LLC «Vitis-Agro»	Cabernet Sauvignon	2220	30	10	0.45
			650		
Bolgrad district					
SE «Experimental economy named after O.V. Suvorov»	Cabernet Sauvignon	1800	150	17	0.94
	Rhine Riesling	1500	140	10	0.66
	Chardonnay	2500	150	18	0.72
	Sukholiman white	2700	140	19	0.70
			580		
Totally		26883		167	11.0

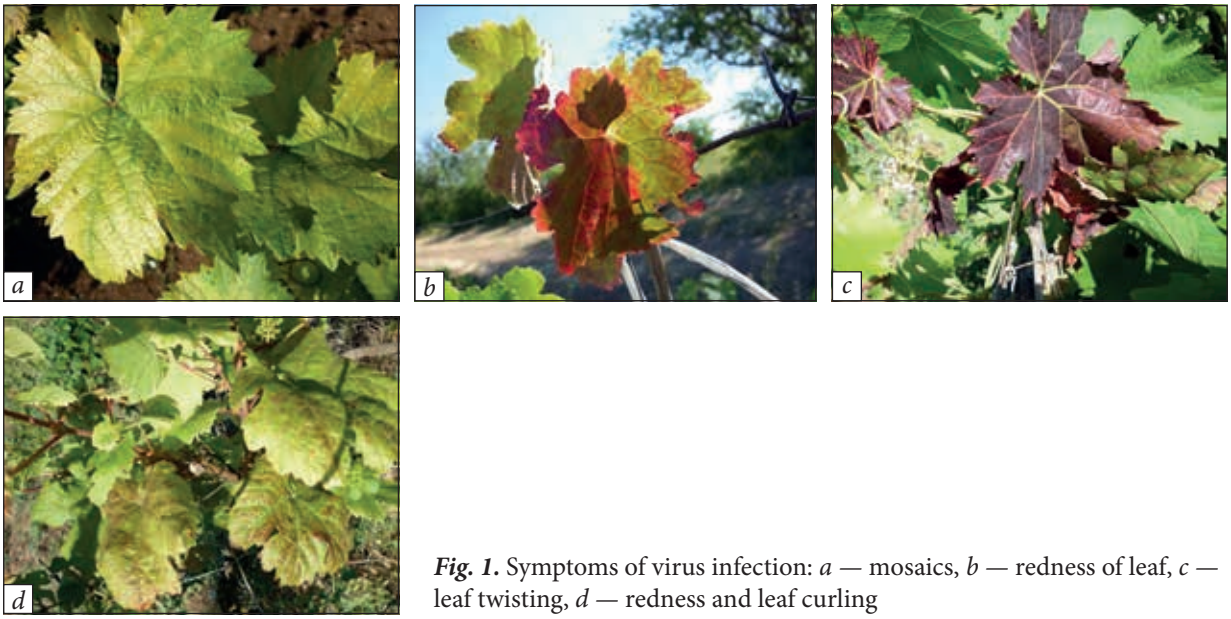

Fig. 1. Symptoms of virus infection: *a* — mosaics, *b* — redness of leaf, *c* — leaf twisting, *d* — redness and leaf curling

Sequence analysis. The amplified DNAs were sequenced by the Sanger dideoxy sequencing method using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). The samples were run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic Analysis. Molecular phylogenetic analysis of Ukrainian GLRaV-3 was carried out based on partial nucleotide sequences of CP compared 21 reference sequences from Russia, Greece, Australia, USA, Brazil, and Canada. Sequences were aligned with those available in the GeneBank by ClustalW [13]. Phylogenetic studies were performed by MEGA version X [14]. Bootstrap analysis, based on 1000 permutations, was used to assess the confidence of the topologies. Bootstrap values lower than 50% were cut off.

Results. ***Visual observation.*** Grape plants showing symptoms such as striking interveinal, reddish-purple discoloration of the foliage and pronounced downward leaf curling and leaf twisting, and in some cases — symptoms similar to short-knot and grape wood furrow complex were collected from grape plantations from 2018 to 2020 (Fig. 1).

Collected samples of grapevines were tested for the presence of GLRaV-1 and GLRaV-3 using DAS-ELISA. The presence of GLRaV-3 was confirmed in all symptomatic grape plants of Cabernet Sauvignon, Rhine Riesling, and Chardonnay varieties in both surveyed regions. Sukholiman white and Merlot grape varieties located in the Bolhrad and Ovidiopol districts were also affected by the virus. In total, 18 vines from 178 symptomatic plants were infected with this virus, which was 11% of the total number of plants (Table 1). The incidence of GLRaV-3 was insignificant and only 0.27—0.95% of the samples were infected with the virus. Also, all samples were tested for the presence of GLRaV-1 by ELISA, but none of them turned out to be positive. Mixed infection with GLRaV-1 and GLRaV-3 was not detected in any of the samples.

Available results of viral distribution in Ukraine vines mostly rely on serological detection tools so in this study we developed a primer set for routine detection of GLRaV by RT-PCR. ELISA-positive samples were used as a positive control in the primer development and validation as well as in the determination of the efficiency of RT-PCR and optimization of PCR conditions.

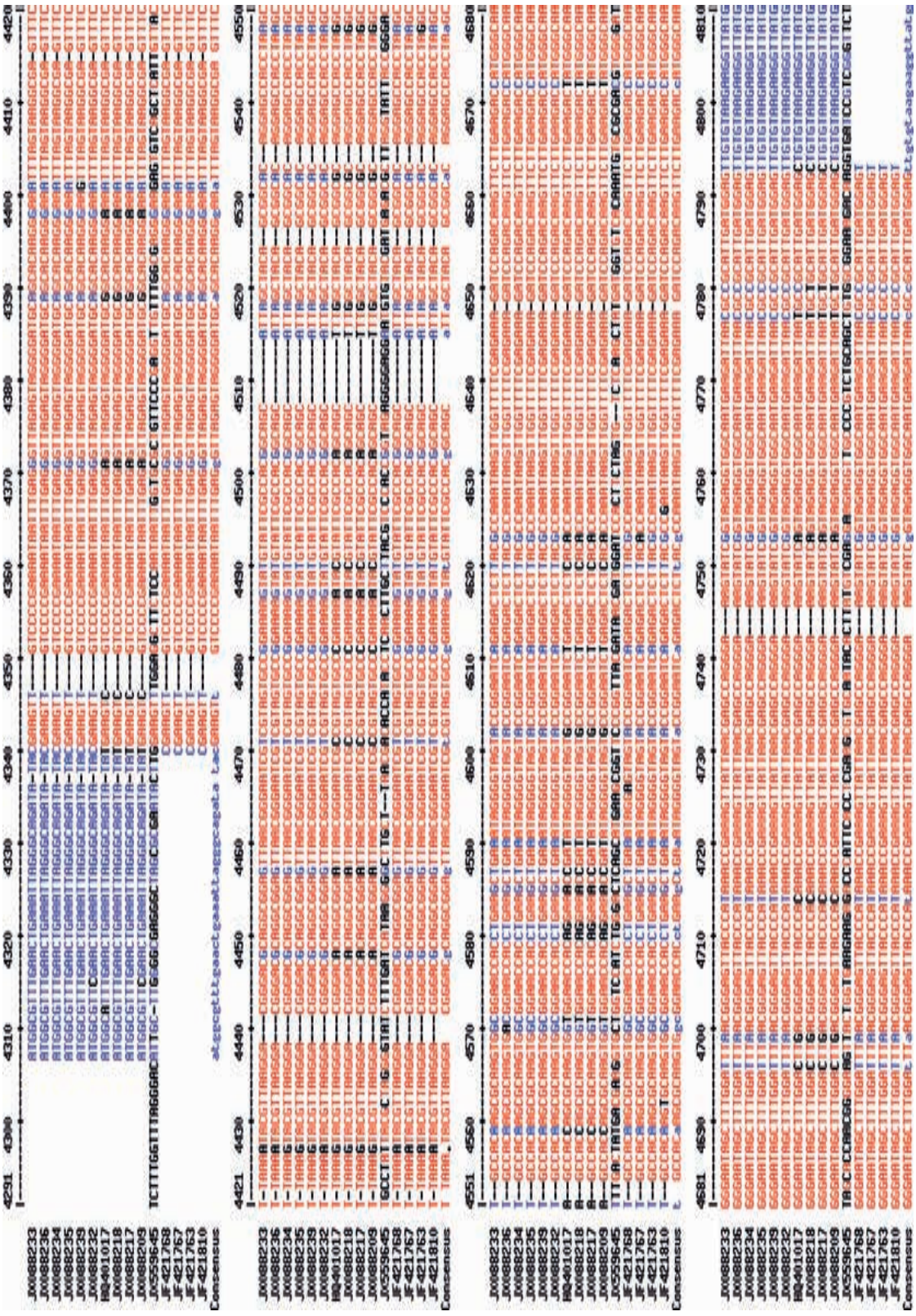

Fig. 2. Multiple sequence alignment of CP genes to identify a conserved region among the GRLaV-3 isolates. Left column corresponds to GenBank accession numbers: JX559645 Canada, JX088233 China, JX088232 China, JX088236 China, JX088234 China, HQ401017 Portugal, JX088235 China, JF421768 USA, JF421767 USA, JF421763 USA, JX088239 China, JF421788 China, JF4217182 China, and JF4217882 USA

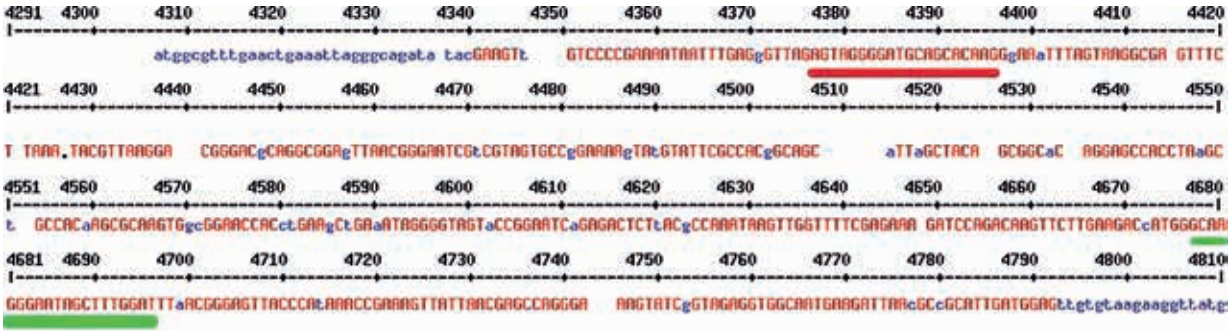

Fig. 3. GLRaV3-7f / GLRaV3-7r priming positions on the DNA matrix of the consensus sequence

Table 2. **Primer sets for the identification of GRLaV-3**

No.*	Primer sequence (5'—3')	Sequence length, bp	Melting temperature (Tm), °C	GC-content, %	Product size, bp
1f	agagtaggggatgcagcaca	20	60.82	55.00	199
1r	tttcagcttcaggtggttcc	20	60.23	50.00	
2f	gggttagagtaggggatgcag	21	59.97	57.14	204
2r	tttcagcttcaggtggttcc	20	60.23	50.00	
3f	agtaggggatgcagcacaag	20	60.28	55.00	318
3r	tgggtaactcccgttaaatcc	21	60.06	47.62	
4f	gagttaacgggaatcgtcgt	20	59.06	50.00	300
4r	cttcattgccacctctaccg	20	60.65	55.00	
5f	agtaggggatgcagcacaag	20	60.28	55.00	372
5r	ttcattgccacctctaccg	19	59.66	52.63	
6f	agtaggggatgcagcacaag	20	60.28	55.00	355
6r	cgatactttccctggctcgt	20	61.52	55.00	
7f	**agtaggggatgcagcacaag**	**20**	**60.28**	**55.00**	**301**
7r	**atccaaagctattcccttgc**	**20**	**58.28**	**45.00**	
8f	agtaggggatgcagcacaag	20	60.28	55.00	312
8r	actcccgttaaatccaaagc	20	58.19	45.00	
9f	agagtaggggatgcagcaca	20	60.82	55.00	304
9r	aatccaaagctattcccttgc	21	59.58	42.86	
10f	gagtaggggatgcagcaca	19	59.80	57.89	312
10r	gggtaactcccgttaaatcca	21	60.06	47.62	

* f — forward; r — reverse

Detailed bioinformatic analysis was done on primer sequences to determine their specificity and ability to detect GLRaV-3. PCR primers were designed using Primer3 primer-design software (PE Applied Biosystems, Foster City, USA) based on sequences published in GenBank: GRLaV-3 JX559645 Canada, JX088233 China, JX088232 China, JX088236 China, JX088234 China, HQ401017 Portugal, JX088235 China, JF421768, JF421767 USA, JF421763 USA, JX088239 China, JF421788 China, JF4217182 China, and JF4217882 USA. Multiple sequences were aligned using MultAline, and the conserved regions within the coat protein (CP) gene (indicated in red color) of the viruses were examined to design GRLaV-3 primers (Fig. 2). The

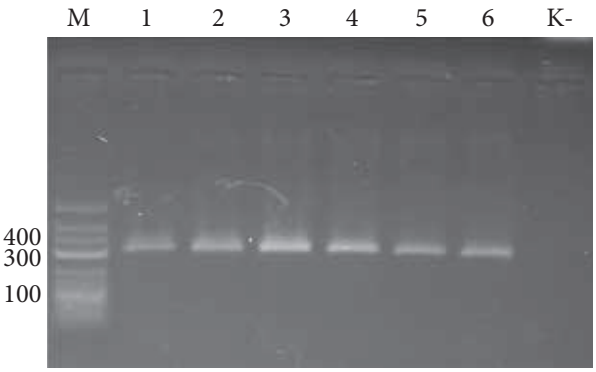

Fig. 4. Results of RT-PCR using GLRaV3-7f / GLRaV3-7r primers: M—100-kb DNA ladder; 1, 2 — Rhine Riesling; 2, 3 — Chardonnai; 5, 6 — Sukholiman white; 1, 3, 5 — Ovidiopol district; 2, 4, 6 — Bolhrad district

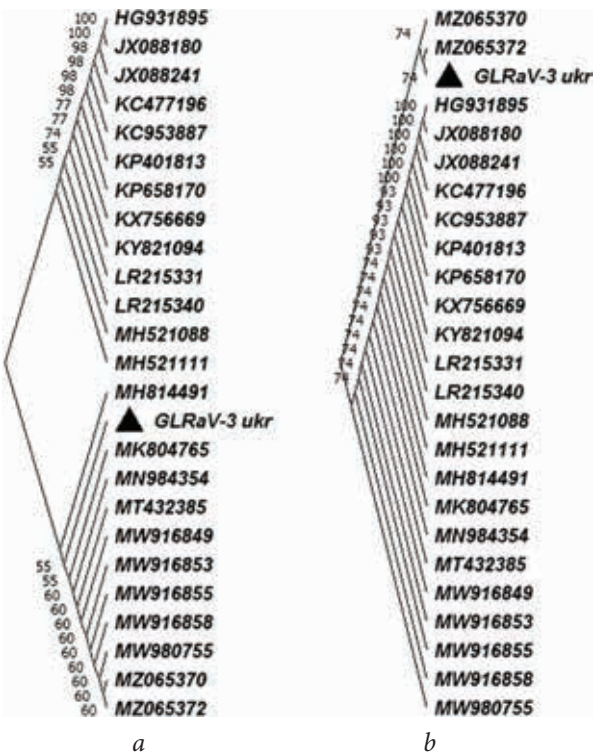

Fig. 5. Phylogenetic tree generated on the basis of nucleotide (*a*) and amino acid (*b*) partial sequences of CP. The tree was constructed by the neighbor-joining (NJ) method with 1000 bootstrap replicate

specific antisense and sense primers were selected manually basing on the melting temperature (Tm) and GC content. We identified conservative fragments in CP genes and compiled a list of candidate PCR primers (10 sets) with the size of 19—21 bases (Table 2). Conserved motifs were appropriate and long enough to generate PCR primers. Annealing temperatures were calculated by Primer3 software, and then optimal annealing temperature values were experimentally adjusted for each pair of primers.

The highest degree of conservation was shown for primer pair 7 (GLRaV3-7f/GLRaV3-7r), which was therefore used as a template for hybridization. The priming positions with the DNA matrix of the consensus sequence (4377-4396 and 4677-4696 for forward and reverse primer, respectively) are shown in Fig. 3. According to the GC content and annealing temperature of the left and right primers, this pair of primers was the most optimal and therefore was used for subsequent studies. To establish the identity of the primers with the CP genes of different GRLaV-3 isolates and with the genetic sequences of other organisms, the primers were analyzed by BLAST, and possible primer dimer and hairpin loop formation were checked with an OligoCalc calculator (http://www.bio.bsu.by/molbiol/oligocalc.html). GLRaV3-7f/GLRaV3-7r were specific for the GRLaV-3 isolates; no matches with the nucleotide sequences of other organisms or the synthesis of a 301 bp PCR product were found.

RT-PCRs were carried out to confirm the specificity of the GLRaV3-7f/GLRaV3-7r primers. The reaction components and cycling parameters were optimized using a total RNA extracted from GLRaV-3 infected tissues, and a conventional RT-PCR procedure was established. Such parameters as primer concentration, annealing temperature, Mg^{2+} concentration, and elongation duration, were tested to optimize conditions. The concentrations of viral sense and antisense primers were 1 pM; the op-

timal annealing temperature was 60 °C, and the elongation duration was 120 s. No non-specific reaction products were synthesized using a Mg^{2+} concentration of 1.5 mM and an annealing temperature of 60 °C, and the number of amplified fragments was sufficient for clear visualization of the amplicon band in the agar gel. The results of PCR showed that the primers were target specific and under optimal conditions successfully amplified fragments of expected length (Fig. 4).

To assess the specificity of the primers, the amplicon obtained in RT-PCR was sequenced. The sequence analysis confirmed the results of PCR and ELISA regarding the belonging of the isolate found in the south of Ukraine to GLRaV-3.

Comparison of the viral sequence showed that the GLRaV-3 isolate found in Ukraine and named as GLRaV-3 ukr shared a 99.6 % nucleotide (nt) identity with the other sequences obtained by BLAST analysis. Phylogenetic analyses of the GLRaV-3 ukr at the nucleotide level (Fig. 5a) showed that the isolate examined in the study was clustered together in one clade with isolates from Crimea (MW916853 MW916858 MW916855 MW916849), Australia (MN984354), Brazil (MK804765), Canada (MH814491), Italy (MT432385), Turkey (MW980755), and Russia (MZ065372, MZ065370). The CP amino acid alignment showed the maximum relationship of GLRaV-3 ukr with the Russian isolates V429 and V1609 (MZ065372 and MZ065370, respectively) obtained in the Krasnodar region (Fig. 5b).

Discussion. Grapevine leafroll-associated virus 3 is the main pathogen contributing to the disease and one of the most severe of the leafroll viruses [4]. Many grapevines affected with GLRaV-3 have reduced yields, delayed or variable ripening, and produce lower quality wine. GLRaV-3 infection can also reduce the quality of wood for propagation. Some GLRaV-3 infected vines may remain symptomless, acting as reservoirs for virus spread. Up to 10 sequence variants of GLRaV-3 with various symptom intensities have been identified. GLRaV-3 is considered the major agent of GLD.

The survey for this virus conducted over 2019–2020 indicated that GLRaV-3 widely occurred in grapevines with high infection frequencies in the south of Ukraine. Cabernet Sauvignon, Rhine Riesling, Chardonnai, Sukholiman white samples of grape varieties tested in this study 18 scored positive with antiserum to GLRaV-3. ELISA results were confirmed by RT-PCR with a set of primers developed in this work. This study was conducted as part of the annual monitoring of GLRaV-1 and GLRaV-3, which is carried out to understand the epidemiology of these viruses and the phytosanitary condition of vineyards in the south Ukraine. Results of phylogenetic analysis indicate that GLRaV-3 ukr isolate belongs to the GLRaV-3 widely distributed in different geographic regions.

Samples of plants infected with GLRaV-3 were used as a positive control during the verification of the RT-PCR we developed for virus detection. The results obtained show that the primer pair GLRaV-3-7f / GLRaV-3-7r we designed was able to detect GLRaV-3 and can be used as a streamlined, sensitive, and cost-effective alternative to ELISA for investigating the epidemiology of GLRaV-3 infecting grape. RT-PCR primer pairs developed can also be routinely used for GLRaV-3 detection and for detecting screenings for viruses of propagating stocks and for control management strategies of the spread of GLRaV-3 in vineyards.

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ВИЯВЛЕННЯ ВІРУСІВ СКРУЧУВАННЯ ЛИСТЯ ВИНОГРАДУ 3 і 1 НА ПІВДНІ УКРАЇНИ ТА РОЗРОБКА ПРАЙМЕРІВ ДЛЯ ІДЕНТИФІКАЦІЇ GLRaV-3

Виноградарство є однією з найбільш інтенсивних і комплексних галузей сільського господарства України. Вірусні захворювання винограду призводять до значного зниження врожайності культури, чим завдають суттєвих економічних збитків виноградарству та виноробній промисловості. Найбільш поширеними і шкодочинними збудниками хвороби винограду, відомої як скручування листя винограду, є віруси скручуванням листя винограду (*Grapevine leafroll-associated virus*, GLRaV), що належать до роду *Ampelovirus*, родини *Closteroviridae*. З огляду на важливе значення зазначених вірусів у виноградарстві та виноробстві, метою даного дослідження було виявити та ідентифікувати віруси GLRaV-1 та GLRaV-3, які причетні до виникнення хвороби винограду на виноградниках Овідіопольського та Болградського районів Одеської області. Для ефективного виявлення ізолятів GLRaV-3, що циркулюють в Україні, ми мали на меті розробити специфічні праймери на основі консервативних нуклеотидних послідовностей вірусу. **Методи.** Обстеження виноградників, візуальна діагностика, імуноферментний аналіз (ІФА), полімеразна ланцюгова реакція зі

зворотною транскрипцією (ЗТ-ПЛР), сиквенування ділянки гена білка оболонки GLRaV-3. **Результати.** Результати дослідження свідчать про циркуляцію GLRaV-3 у виноградниках відкритого ґрунту на півдні України і симптоми скручування листя винограду, спричинені саме цим вірусом. GLRaV-1 не було виявлено в жодному з досліджуваних зразків. Результати серологічних досліджень свідчать про циркуляцію GLRaV-3 у виноградарських господарствах півдня України. Наявність GLRaV-3 у відібраних зразках підтверджено ЗТ-ПЛР з використанням специфічних праймерів (GLRaV3-7f 5'-AGTAGGGGATGCAGCACAAG-3'; GLRaV3-7r 5'- ATCCAAAGCTATTCCCTTGC-3'), розроблених нами для ампліфікації ділянки гена білка оболонки вірусу. Розроблені праймери (GLRaV3-7f / GLRaV3-7r) верифіковано та рекомендовано для рутинного використання як під час діагностики вірусів в умовах лабораторії, так і в процесі широкомасштабних тестувань. Проведено сиквенування ділянки гена білка оболонки ізоляту GLRaV-3 ukr, поширеного на півдні України, та проаналізовано його еволюційні взаємовідносини. Філогенетичний аналіз показав, що GLRaV-3 ukr близькоспоріднений до ізолятів, що циркулюють в росії (MZ065372 та MZ065370). Послідовність була депонована в GenBank за номером доступу Acc. No ON015835.

Ключові слова: *виноград, скручування листя винограду, GLRaV-3, дизайн праймерів, аналіз сиквенсів*