UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Vanja MILJANIĆ

HIGH-THROUGHPUT SEQUENCING DETECTION AND MOLECULAR CHARACTERIZATION OF VIRAL DISEASES OF GRAPEVINE (*Vitis vinifera* L.) AND THEIR ELIMINATION BY THERMOTHERAPY AND MERISTEM TISSUE CULTURE

DOCTORAL DISSERTATION

Ljubljana, 2022

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DETEKCIJA IN MOLEKULARNA KARAKTERIZACIJA VIRUSNIH BOLEZNI VINSKE TRTE (*Vitis vinifera* L.) Z VISOKO ZMOGLJIVIM SEKVENCIRANJEM TER NJIHOVA ELIMINACIJA S POMOČJO TERMOTERAPIJE IN KULTURE MERISTEMOV

DOKTORSKA DISERTACIJA

Ljubljana, 2022

Based on the Statute of the University of Ljubljana and the decision of the Biotechnical Faculty senate, as well as the decision of the Commission for Doctoral Studies of the University of Ljubljana adopted on 27th session, June 30th, 2020, it has been confirmed that the candidate meets the requirements for pursuing a PhD in the interdisciplinary doctoral programme in Biosciences, Scientific Field Biotechnology. Prof. dr. Nataša Štajner is appointed as supervisor.

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TI HIGH-THROUGHPUT SEQUENCING DETECTION AND MOLECULAR CHARACTERIZATION OF VIRAL DISEASES OF GRAPEVINE (*Vitis vinifera* L.) AND THEIR ELIMINATION BY THERMOTHERAPY AND MERISTEM TISSUE CULTURE

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AB We studied the virome of preclonal candidates obtained after mass selection of grapevines using HTS technology. Nine viruses (GFLV, GLRaV-3, GRSPaV, GFkV, GSyV-1, GRVFV, GRGV, GPGV, and RBDV) and two viroids (HSVd and GYSVd-1) were identified. GRGV, GRVFV, and GSyV-1 were detected for the first time in Slovenia. All in silico predicted viruses and viroids were validated with RT-PCR and Sanger sequencing. We obtained a comprehensive insight into genetic diversity, phylogeny and co-infections. In the second part of the dissertation, we investigated the viruses and viroids elimination efficacy by in vivo thermotherapy and in vitro meristem tip micrografting. Heat therapy was performed at 36-38 °C for at least six weeks. Meristem tips (0.1-0.2 mm) were aseptically isolated and micrografted onto the sectioned, etiolated hypocotyls of Vialla (*Vitis labrusca* × *Vitis riparia*). The overall regeneration rate was low (8.53%). The higher regeneration rate was observed in the white varieties. The regenerated plants were micropropagated several times. The sanitation status was checked with RT-PCR. All viruses were completely eliminated, while the elimination of viroids was less successful (39.2% for HSVd and 42.6% for GYSVd-1). It is important to emphasize that plant growth regulators (hormones) were not used. In the third part of the thesis, we studied the virome of samples that were not part of the clonal selection process. We detected: GLRaV-1, GLRaV-2, GLRaV-3, GFkV, GRVFV, GRSPaV, GFLV (in association with its satellite RNA), GPGV, GV-Sat, HSVd, and GYSVd-1. GV-Sat was also detected for the first time in Slovenia. We developed a protocol for high-throughput validation of in silico predicted infections, including various combinations of viruses, viroids, and satellites.

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IN DETEKCIJA IN MOLEKULARNA KARAKTERIZACIJA VIRUSNIH BOLEZNI VINSKE TRTE (*Vitis vinifera* L.) Z VISOKO ZMOGLJIVIM SEKVENCIRANJEM TER NJIHOVA ELIMINACIJA S POMOČJO TERMOTERAPIJE IN KULTURE MERISTEMOV

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JI en/sl

AI Na osnovi visokozmogljivega sekvenciranja smo preučevali virom predklonskih kandidatov (82 vzorca, 6 sorti), pridobljenih po množični selekciji vinske trte. Identificirali smo devet virusov (GFLV, GLRaV-3, GRSPaV, GFkV, GSyV-1, GRVFV, GRGV, GPGV in RBDV) in dva viroida (HSVd in GYSVd-1). Virusi GRGV, GRVFV in GSyV-1 so bili prvič odkriti v Sloveniji. Vsi in silico napovedani virusi in viroidi so bili potrjeni z RT-PCR in Sangerjevim sekvenciranjem. Na osnovi sekvenc posameznih delov virusov in viroidov smo dobili podatke o genetski raznolikosti, filogeniji in sočasnih okužbah. V drugem delu doktorske naloge smo raziskali učinkovitost eliminacije virusov in viroidov po in vivo termoterapijo vinske trte ter po mikrograftingu izoliranih meristemov v in vitro pogojih. Toplotno terapijo smo izvajali pri 36-38 °C vsaj šest tednov. Meristemsko tkivo velikosti 0,1-0,2 mm smo aseptično izolirali in cepili na etolirane hipokotile sorte Vialla (*Vitis labrusca* × *Vitis riparia*). Celotna stopnja regeneracije vzorcev iz meristemov je bila 8,53 %. Višjo stopnjo regeneracije smo opazili pri belih sortah. Regenerirane rastline smo večkrat subkultivirali in mikropropagirali. Stanje okuženosti po eliminaciji smo preverili z RT-PCR. Vsi virusi so bili odstranjeni iz vseh analiziranih vzorcev, medtem ko je bila eliminacija viroidov manj uspešna (39,2 % z HSVd in 42,6 % z GYSVd-1). Pomembno je poudariti, da pri regeneraciji in mikropropagaciji regulatorji rasti niso bili uporabljeni. V tretjem delu doktorskega dela smo proučevali virom vzorcev, ki niso bili del klonskega selekcijskega procesa. Potrdili smo prisotnost virusov GLRaV-1, GLRaV-2, GLRaV-3, GFkV, GRVFV, GRSPaV, GFLV (v povezavi s svojo satelitsko RNA), GPGV, GV-Sat, ter viroidov HSVd in GYSVd-1. GV-Sat je bil tokrat prvič potrjen v Sloveniji. Razvili smo protokol za visoko zmogljivo validacijo in silico predvidenih okužb, na osnovi hkratnega pomoževanja z RT-PCR (multiplex RT-PCR) za različne kombinacije virusov, viroidov in satelitov.

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ABBREVIATIONS AND SYMBOLS

aa	amino acid
AGO	argonaute protein
AGVd	australian grapevine viroid
AlkB	alkylation B domain
CCR	central conserved region
cDNA	complementary deoxyribonucleic acid
CEVd	citrus exocortis viroid
CP	coat protein
CTV	citrus tristeza virus
DCL	Dicer enzyme
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
GAMaV	grapevine asteroid mosaic-associated virus
GaMV	grapevine-associated marafivirus
GC	guanine-cytosine
GFDD	grapevine fanleaf degeneration disease
GFLV	grapevine fanleaf virus
GFkV	grapevine fleck virus
GHVd	hammerhead viroid-like RNA
GINV	grapevine berry inner necrosis virus
GLD	grapevine leafroll disease
GLRaV-3	grapevine leafroll-associated virus 3
GLRaV-1-9	grapevine leafroll-associated viruses 1-9
GLMD	grapevine leaf mottling and deformation
GLVd	grapevine latent viroid
GPGV	grapevine Pinot gris virus
GPoV-1	grapevine polero virus 1
GRBV	grapevine red blotch virus
GRGV	grapevine red globe virus
GRSPaV	grapevine rupestris stem pitting-associated virus

GRVFV	grapevine rupestris vein feathering virus
GSyV-1	grapevine Syrah virus-1
GVN	grapevine virus N
GVO	grapevine virus O
GV-Sat	grapevine satellite virus
GYSVd-1	grapevine yellow speckle viroid 1
GYSVd-2	grapevine yellow speckle viroid 2
Hel	helicase
HP	homing protein
HSP70h	heat shock 70 protein homologue
HSVd	hop stunt viroid
HTS	high-throughput sequencing
IC-RT-PCR	immunocapture-reverse transcription-polymerase chain reaction
ISEM	immunosorbent electron microscopy
LFIA	lateral flow immunoassay
L-Pro	papain-like leader protease
mCP	minor coat protein
miRNA	micro ribonucleic acid
MP	movement protein
mRT-PCR	multiplex reverse transcription-polymerase chain reaction
MTR	methyltransferase
nt	nucleotide
ORF	open reading frame
OTU	ovarian tumor cysteine protease
PCR	polymerase chain reaction
P-Pro	papain-like cysteine protease
Pro	protease
PTGS	post-transcriptional gene silencing
QGB	quintuple gene block
RdRp	RNA-dependent RNA polymerase
RGB	replication gene block
RISC	RNA-induced silencing complex
RITS	RNA-induced initiation of transcriptional gene silencing

RNA	ribonucleic acid
RSP	rupestris stem pitting
RT-LAMP	reverse transcription-loop-mediated isothermal amplification
RT-nPCR	reverse transcription-nested polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
satGFLV	GFLV satellite RNA
siRNA	short interfering RNA
sRNA	small RNA
sRNA-seq	small RNA sequencing
ssRNA	single-stranded RNA
TCH	terminal conserved hairpin
TCR	terminal conserved region
TGB	triple-gene block
TGS	transcriptional gene silencing
Tm	temperature melting
TMV	tobacco mosaic virus
UTR	untranslated region
VPg	viral genome-linked protein
YS	yellow speckle

1 INTRODUCTION

1.1 GRAPEVINE AND GRAPEVINE VIRAL PATHOGENS

The grape family (*Vitaceae*) consists of 16 genera and about 950 species (Wen et al., 2018). The genus *Vitis* encompasses roughly 60 species, and *Vitis vinifera* L. is the most important, with more than 10,000 varieties, and the number of new varieties is constantly growing due to grapevine breeding programs (Reynolds, 2017). It is mainly used for wine production, but also for fresh fruit, raisins, juice, spirits, seed oils, vinegar, and other products. According to the OIV (International Organization of Vine and Wine Intergovernmental Organization), 7.3 million hectares of land worldwide were planted with vines in 2020, with five countries (Spain, France, China, Italy, and Turkey) accounting for 50% of the total vineyard area. In the same year, the largest wine producers were Italy, France, Spain, the United States and Argentina. In Slovenia, the area under vines (15,075 ha) is divided into three wine-growing regions (Primorska, Podravje and Posavje) with a total of nine smaller districts (Štajerska, Prekmurje, Bizeljsko-Sremč, Dolenjska, Bela krajina, Goriška brda, Vipavska dolina, Kras, and Slovenska Istra). Among them, Primorska is the largest wine-growing region (6,428 ha), followed by Podravje (6,160 ha) and Posavje (2,487 ha). In 2021, 3,700.12 ha in Primorska were planted with white varieties and 2,727.54 ha with red varieties (Database of Ministry of Agriculture, Forestry and Food, 2021).

One of the most important limiting factors for sustainable viticulture worldwide are diseases caused by viral pathogens. In 2020, it was published that 86 viruses are infectious to grapevines (Fuchs, 2020). Since then a few new viruses have been found, such as grapevine polerovirus 1 (GPoV-1) (Chiaki and Ito, 2020), grapevine-associated marafivirus (GaMV) (Fan et al., 2021), grapevine virus O (GVO), grapevine virus N (GVN) (Read et al., 2022). Most grapevine viruses have a positive or a negative single-stranded RNA genome (ssRNA), a few have a double-stranded RNA genome (dsRNA) or DNA genome (Martelli, 2017). Four major disease complexes are infectious degeneration and decline, leafroll, rugose wood, and fleck disease complex.

In Slovenia, according to the Official Gazette of the RS N°93/05 and 101/20, all vine propagation material must be compulsorily tested on: grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV), tomato black ring virus (TBRV), raspberry ringspot virus (RpRSV), grapevine leafroll-associated virus 1 and 3 (GLRaV-1, -3), grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine virus A (GVA), grapevine virus B (GVB), and grapevine fleck virus (GFkV) (only for rootstocks). In preclonal candidates, we detected and characterized the following viruses: GFLV, GLRaV-3, GRSPaV, GFkV, grapevine red globe virus (GRGV), grapevine rupestris vein feathering virus (GRVFV), grapevine Syrah virus-1 (GSyV-1), grapevine Pinot gris virus (GPGV), and raspberry bushy dwarf virus (RBDV), as well as two viroids: hop stunt viroid (HSVd) and grapevine yellow speckle

viroid 1 (GYSVd-1). This chapter describes their taxonomic status, genome organization, symptomatology, hosts, and transmission.

1.1.1 Grapevine fanleaf virus

Grapevine fanleaf virus (GFLV) is the oldest and most important causal agent of grapevine fanleaf degeneration disease (GFDD) and one of the most detrimental grapevine viruses with worldwide distribution (Martelli, 1993, 2014; Andret-Link et al., 2004a). GFLV belongs to the genus *Nepovirus* and the family *Secoviridae*. Its particles are polyhedral and have a diameter of about 30 nm. The genome is bipartite and consists of two positive-sense (+) RNAs: RNA1 (7,326–7,342 nt) and RNA2 (3,730–3,817 nt). Both RNAs have a viral genome-linked protein (VPg) at the 5' end and a polyA tail at the 3' end. The RNA1 encodes a polyprotein (P1) containing the RNA-dependent RNA polymerase (RdRp) at the N-terminus, the cysteine protease, the genome-linked protein (VPg), the nucleotide triphosphate-binding domain (helicase; Hel), the protease cofactor, and a protein of 46 kDa at the C-terminus (Martelli, 2014; Digiaro et al., 2017). The RNA2 encodes a polyprotein (P2) that includes the homing protein (HP), the movement protein (MP), and the coat protein (CP) (Martelli, 2014; Digiaro et al., 2017). An additional GFLV satellite RNA (satGFLV) associated with some GFLV isolates, has been identified (Pinck et al., 1988; Fuchs et al., 1989; Gottula et al., 2013; Lamprecht et al., 2013; Čepin et al., 2016). Symptoms caused by GFLV vary widely depending on the virus strain, variety and environmental factors. The virus causes leaves distortion (fan shape, asymmetry, puckering with toothed margins), chromatic alterations (yellow spots, yellow mosaic, chlorotic mottling, partial or total chrome leaves yellowing, vein banding) (Figure 1a), shoots malformation (fasciation, zigzag growth, bifurcation, double nodes, shorten internodes) (Figure 1b), clusters are smaller, berries ripen irregularly, sugar content and acid concentration are altered, root is less developed, grafting success is lower (Martelli, 1993, 2014; Andret-Link et al., 2004a; Digiaro et al., 2017; Panno et al., 2021). The presence of endocellular cordons (trabeculae) in tracheids is a specific internal symptom of GFLV infection (Martelli, 1993, 2014; Andret-Link et al., 2004a; Digiaro et al., 2017). Yield losses can exceed 80% (Andret-Link et al., 2004a). GFLV was detected in herbaceous weeds (*Aristolochia clematitis*, *Lagenaria siceraria*, *Cynodon dactylon*, *Sorghum halepense*, *Melilotus* sp., *Plantago lanceolata*, *Polygonum* sp., *Rubus* sp.) that may serve as reservoirs for infection (Horvath et al., 1994; Izadpanah et al., 2003; Zaki-Aghl et al., 2015). The virus is transmitted non-circulatively and semipersistently by the ectoparasitic dagger nematode *Xiphinema index* (Nematoda: Longidoridae) (Hewitt et al., 1958; Andret-Link et al., 2004b; Demangeat et al., 2010). GFLV can be mechanically transmitted from infected vines to various herbaceous hosts (Cadman et al., 1960; Martelli, 1993, 2014; Digiaro et al., 2017). GFLV was detected in pollen and seeds, but with conflicting reports (Martelli, 1993, 2014; Digiaro et al., 2017). Over long distances, it is transmitted through infected propagating material (Martelli, 1993, 2014; Andret-Link et al., 2004a; Digiaro et al., 2017).

(a) (b)

Figure 1. Symptoms present on grapevine infected with GFLV: (a) chromatic alteration ('Refošk' variety); (b) shoots malformation ('Volovnik' variety).

1.1.2 Grapevine leafroll-associated virus 3

Grapevine leafroll-associated virus 3 is the main etiological agent of grapevine leafroll disease (GLD) (Maree et al., 2013). GLRaV-3 belongs to the genus *Ampelovirus* and the family *Closteroviridae* (Martelli et al., 2012). GLRaV-3 particles are flexuous, filamentous, 1,800 nm in length and 12 nm in diameter. The genome is a (+) ssRNA. It is capped at the 5′ end and it is not polyadenylated at the 3′ end (Maree et al., 2013). GLRaV-3 has the largest genome (18,433–18,671 nts) among plant viruses after citrus tristeza virus (CTV) (Burger et al., 2017). All known GLRaV-3 genomes have unusually long 5′ UTRs with considerable length variation and very high uracil content (Maree et al., 2008; Fei et al., 2013; Maree et al., 2013; Burger et al., 2017). Different groups of GLRaV-3 genetic variants exist. Isolates of groups I–III have 12 ORFs (ORF1a, ORF1b, ORF2-12), isolates of groups IV and V have not yet been fully sequenced, isolates of groups VI and VII have not ORF2. ORF1a and ORF1b form the replication gene block (RGB) which contains domains for MTR, Hel, and RdRp. ORF1a also contains a papain-like leader protease (L-Pro) (associated with RNA accumulation, systemic virus spread and invasiveness), and an alkylation B domain (AlkB) (repair RNA from methylation damage). The function of ORF2 is unknown but probably not important, as it is absent in some isolates. ORF3-7 are conserved among members of the *Closteroviridae* family, and they form the quintuple gene block (QGB). ORF3 encodes a small transmembrane protein. ORF4 encodes the heat shock 70 protein homolog (HSP70h), while ORF5 encodes a ~60 kDa protein that likely has a similar function to HSP70h. CP is encoded by ORF6, while minor CP (mCP) is encoded by ORF7 and is the main component

of the virion head. ORF8-12 are unique to the genus *Ampelovirus*. ORF8-10 encode proteins that potentially may be involved in suppression of the host RNA silencing defense mechanism and long-distance virus transport. Small ORF11 and ORF12 are unique and highly diverse among GLRaV-3 variants (Maree et al., 2013; Burger et al., 2017). Symptoms vary depending on season, variety, scion-rootstock combinations and environmental conditions. GLRaV-3 in red varieties causes reddening of the interveinal areas, while veins remain green (Figure 2a). White varieties show yellowing or chlorotic mottling (Figure 2b). At the end of the season (in late autumn) leaves roll downward (Figure 2c). The virus may also be latent (Maree et al., 2013; Naidu et al., 2014; Martelli, 2014; Burger et al., 2017). GLRaV-3 reduces cluster size, ripening, alters sugar content, acidity, pigments and aromatic components (Lee and Martin, 2009; Vega et al., 2011; Maree et al., 2013; Alabi et al., 2016; Burger et al., 2017), resulting in significant economic losses (Cheon et al., 2020). There is no evidence of mechanical transmission of GLRaV-3, it is mainly transmitted by propagation of infected material, and by grafting. Known vectors are soft scale insects (Homoptera: Coccidae) and mealybugs (Homoptera: Pseudococcidae) (Mahfoudhi et al., 2009; Tsai et al., 2010; Maree et al., 2013; Naidu et al., 2014; Martelli, 2014; Burger et al., 2017).

(a) (b) (c)

Figure 2. Symptoms present on grapevine infected with GLRaV-3: (a) reddening of the interveinal areas while veins remain green ('Cabernet Sauvignon' variety); (b) leaves yellowing ('Zeleni Sauvignon' variety); (c) downward rolling of leaf margins ('Merlot' variety).

In addition to GLRaV-3, grapevine leafroll-associated viruses 1-9 (GLRaV-1-9), GLRaV-Car, GLRaV-De, and GLRaV-Pr have also been associated with GLD. Further analysis revealed that GLRaV-7 is novel virus, GLRaV-8 is not viral origin, and GLRaV-4-6, -9, -Car, -De, and -Pr are different strains of GLRaV-4 (Martelli et al., 2012).

1.1.3 Grapevine rupestris stem pitting-associated virus

Grapevine rupestris stem pitting-associated virus (GRSPaV) was discovered in 1998 in association with graft-transmissible Rupestris stem pitting (RSP), one of the four causes of rugose wood disease complex (Zhang et al., 1998). GRSPaV is a member of the genus *Foveavirus* and the *Betaflexiviridae* family (Martelli and Jelkmann, 1998; Martelli, 2014; Meng and Rowhani, 2017). GRSPaV particles are flexuous, filamentous, 723 nm in length and 12 nm in diameter (Petrovic et al., 2003). The genome is a (+) ssRNA of ~8,725 nt. The 5' end is capped and the 3' end is polyadenylated. GRSPaV has five ORFs. ORF1 encodes the replicase protein: MTR, Hel, RdRp, and additionally three unique domains: papain-like cysteine protease (P-Pro), ovarian tumor cysteine protease (OTU), and AlkB domain. ORF2, ORF3 and ORF4 form a triple-gene block (TGB) encoding MPs. ORF5 encodes CP. In addition, the putative ORF, which overlaps the CP gene at the 3' end, encodes a 14 kDa protein with unknown function (Zhang et al., 1998; Meng et al., 1998; Martelli, 2014; Meng and Rowhani, 2017). The virus is restricted only to *Vitis* and its hybrids (Meng and Rowhani, 2017). GRSPaV has a wide range of genetic variants (Meng et al., 1999, 2006; Nolasco et al., 2006; Alabi et al., 2010; Terlizzi et al., 2010, 2011; Glasa et al., 2017; Hily et al., 2018; Rai et al., 2021). There are reports that GRSPaV has no major effects on grape yield and growth (Reynolds et al., 1997), or may even be beneficial to grapevine (Gambino et al., 2012). But it was also found in association with RSP (Zhang et al., 1998; Meng et al., 1998), vein necrosis disease (Bouyahia et al., 2005) (Figure 3a), and with the severe decline of 'Pinot noir' (Lima et al., 2009) and 'Syrah' varieties (Figure 3b) (Al Rwahnih et al., 2009; Beuve et al., 2013). Therefore, its actual effect on grapevines is poorly known. It is graft-transmissible, and no vectors have been identified (Martelli, 2014; Meng and Rowhani, 2017). It was detected in pollen and seeds, but the highest transmissibility to seedlings reported to date is 0.4% (Lima et al., 2006).

(a) (b)

Figure 3. Diseases associated with GRSPaV: (a) Vein necrosis on Richter 110R (Reproduced from Bouyahia et al., 2005); (b) Declining of 'Syrah' variety (necrosis of stem wood) (Reproduced from Al Rwahnih et al., 2009).

1.1.4 Grapevine fleck and fleck-similar viruses

Grapevine fleck and similar viruses are classified into two genera: grapevine fleck virus (GFkV) and grapevine red globe virus (GRGV) belong to the genus *Maculavirus*, while grapevine rupestris vein feathering virus (GRVFV), grapevine asteroid mosaic-associated virus (GAMaV), and grapevine Syrah virus-1 (GSyV-1) belong to the genus *Marafivirus* (Sabanadzovic et al., 2017). Grapevine fleck and fleck-like viruses are evolutionarily related and share similar characteristics. These viruses are phloem-limited, there is no evidence of mechanical transmission, and they spread through infected propagating material. Regarding vectors and natural spread, GSyV-1 was detected in leafhopper (*Erythroneura variabilis*), but transmissibility to grapevine has not yet been reported (Al Rwahnih et al., 2009), and there are few reports of natural field spread of GFkV, which have never been confirmed experimentally (Martelli, 2014; Sabanadzovic et al., 2017). GFkV is not seed-borne, and transmission by dodder has no epidemiological significance (Martelli, 2014; Sabanadzovic et al., 2017). Complete genomes are known for all of them and they differ slightly in the number and organization of cistrons, but they all share common features: (i) isometric particles about 30 nm in diameter, (ii) (+) ssRNA, (iii) capped 5′ end, (iv) polyadenylated 3′ end, (v) large polyprotein essential for their replication, (vi) synthesis of 3' coterminal subgenomic RNA (sgRNA) as a template for CP translation, (vii) unusually high cytidine content (Martelli et al., 2002; Sabanadzovic et al., 2017). These viruses are latent or semi-latent in most *Vitis* species and rootstock hybrids (Martelli, 2014; Sabanadzovic et al., 2017). GFkV and related viruses infect only *Vitis* species, with the exception of GSyV-1 which was also detected in wild blackberry (*Rubus* sp.) (Sabanadzovic et al., 2009). Moreover, GSyV-1 is unique among plant viruses due to the specific permutation of motifs in the RdRp gene (Sabanadzovic et al., 2009).

1.1.5 Grapevine Pinot gris virus

Another dangerous and emerging grapevine virus is the grapevine Pinot gris virus (GPGV), which belongs to the genus *Trichovirus* and the family *Betaflexiviridae* (Giampetruzzi et al., 2012). The virus is associated with grapevine leaf mottling and deformation (GLMD). It was discovered in Italy in 2012 using Illumina small RNA sequencing (Giampetruzzi et al., 2012). It is similar to grapevine berry inner necrosis virus (GINV). GPGV has a (+) ssRNA genome. The complete sequence encompasses 7,258 nt (excluding 3′ polyA tail) (Giampetruzzi et al., 2012). The genome is organized into 3 ORFs. ORF1 encodes a replication protein (MTR, Hel, RdRp) and also contains an AlkB domain; ORF2 encodes a MP; and ORF3 encodes the CP (Giampetruzzi et al., 2012). An additional putative small ORF, encoding an 11.5 kDa protein, has been identified within ORF1 which bears no similarity to known proteins (Glasa et al., 2014). Recent studies showed that GPGV particles are located in deep parenchyma cells (Tarquini et al., 2018), and it is likely that the virus originated in China (Hily et al., 2020). Many symptoms are associated with GPGV (Figure 4), such as chlorotic mottling, leaf deformation, puckering, low vigor, shortened internodes,

stunting, inner necrosis of berries, low quality, and low yield (Giampetruzzi et al., 2012; Cho et al., 2013; Mavrič Pleško et al., 2014; Saldarelli et al., 2015; Gazel et al., 2016). The presence of the virus has been observed both in asymptomatic plants and in plants with characteristic symptoms (Giampetruzzi et al., 2012; Bianchi et al., 2015; Saldarelli et al., 2015; Shvets and Vinogradova, 2022). GPGV is a graft-transmissible virus (Saldarelli et al., 2015), and the known vector is the eriophyid mite *Colomerus vitis* (Acari: Eriophyidae) (Malagnini et al., 2016). GPGV is not only infecting grapevine as Gualandri et al. (2017) reported that this virus also infects two herbaceous plants (*Chenopodium album* and *Silene latifolia*), making the epidemiology of this virus much more complex (Saldarelli et al., 2017).

(a) (b)

Figure 4. Symptoms present on grapevine infected with GPGV: (a) leaves mottling, deformation and shoot stunting ('Zeleni Sauvignon' variety); (b) uneven fruit set ('Cabernet Sauvignon' variety).

1.1.6 Raspberry bushy dwarf virus

The first report that grapevine is a natural host of raspberry bushy dwarf virus (RBDV) was found in Slovenia on two white varieties, 'Laški rizling' and 'Štajerska belina' (Mavrič et al., 2003). Later, its occurrence was reported on grapevines from Serbia (Jevremovic and Paunovic, 2011), Hungary (Mavrič Pleško et al., 2012; Czotter et al., 2018) and Russia (Navrotskaya et al., 2021). RBDV is a pollen- and seed-borne virus and belongs to the genus *Idaeovirus*. It has quasi-spherical particles about 33 nm in diameter and a bipartite genome. RNA1 encodes a polyprotein with MTR, Hel, and RdRp domains (Ziegler et al., 1992). In addition, a small overlapping ORF was observed near the 3′ end of RNA1 that encodes 12K protein (Wood et al., 2001). RNA2 encodes MP at the 5′ end and CP at the 3′ end (Natsuaki et al., 1991). The CP is expressed from the subgenomic RNA3 (Mayo et al., 1991). The virus causes yellowing of leaves and curved line patterns (Mavrič et al., 2003; Jevremovic and

Paunovic, 2011) (Figure 5). In raspberry, it infects seedlings progeny via pollen (up to 77%), but in grapevine ('Laški rizling' variety) it is not transmissible via seeds (Mavrič Pleško et al., 2009). It was detected in the nematode *Longidorus juvenilis* (Nematoda: Longidoridae), but its transmissibility to grapevine has not yet been experimentally confirmed (Mavrič Pleško et al., 2009).

Figure 5. Curved line patterns present on grapevine infected with RBDV ('Zeleni Sauvignon' variety).

1.1.7 Grapevine viroids

Viroids are small, single-stranded, circular, non-encapsidated, and nonprotein-coding RNAs. Six viroids and one viroid-like RNA have been identified in grapevines: grapevine yellow speckle viroid 1 (GYSVd-1), grapevine yellow speckle viroid 2 (GYSVd-2), grapevine latent viroid (GLVd), australian grapevine viroid (AGVd), hop stunt viroid (HSVd), citrus exocortis viroid (CEVd), and grapevine hammerhead viroid-like RNA (GHVd) (Di Serio et al., 2017). They are members of the genus: *Apscaviroid* (GYSVd-1, GYSVd-2, GLVd, and AGVd); *Hostuviroid* (HSVd), and *Pospiviroid* (CEVd), and all belong to the family *Pospiviroidae* (Di Serio et al., 2017). GHVd shares characteristics with members of the family *Avsunviroidae* (Wu et al., 2015). Members of the family *Pospiviroidae* replicate and accumulate in the nucleus, whereas members of the family *Avsunviroidae* replicate and accumulate in the chloroplast. HSVd and GYSVd-1 are distributed worldwide, and they are the only two viroids found in grapevines in Slovenia. HSVd was first detected in hop plants (Sasaki and Shikata, 1977), and since then it has been reported to infect various plants from different botanical families (Sano et al., 1985, 1989; Astruc et al., 1996; Yakoubi et al., 2007; Zhang et al., 2009; Elbeaino et al., 2012; Elleuch et al., 2013; Pirovano et al., 2014; Marquez-Molins et al., 2021). In 2012 it was published that this viroid infects hops in Europe (Radisek et al., 2012). Although HSVd is asymptomatic in grapevines it can be transmitted to hops and cause epidemics (Sano et al., 2001; Kawaguchi-Ito et al., 2009). The genome is 296-302 nt in size and assume rod-like conformation containing the typical central conserved region (CCR) and terminal conserved hairpin (TCH) (Di Serio et al., 2017). HSVd in grapevine exhibits the typical features of a

quasispecies (Sano et al., 2001). GYSVd-1 and GYSVd-2 are associated with yellow speckle (YS), a disease that causes yellow spots on leaves induced by high temperatures (Koltunow et al., 1989; Hajizadeh et al., 2015) (Figure 6a). GYSVd-1 and/or GYSVd-2 in co-infection with GFLV may cause vein banding (Figure 6b) (Hajizadeh et al., 2015). GYSVd-1 has a rod-like conformation that contains the CCR and terminal conserved region (TCR) (Di Serio et al., 2017). GYSVd-1 in grapevine exhibits characteristics of a quasispecies (Polivka et al., 1996).

Viroids have no vectors and are spread by propagating material and grafting. They can also be spread through pruning tools in the vineyard (Di Serio et al., 2017).

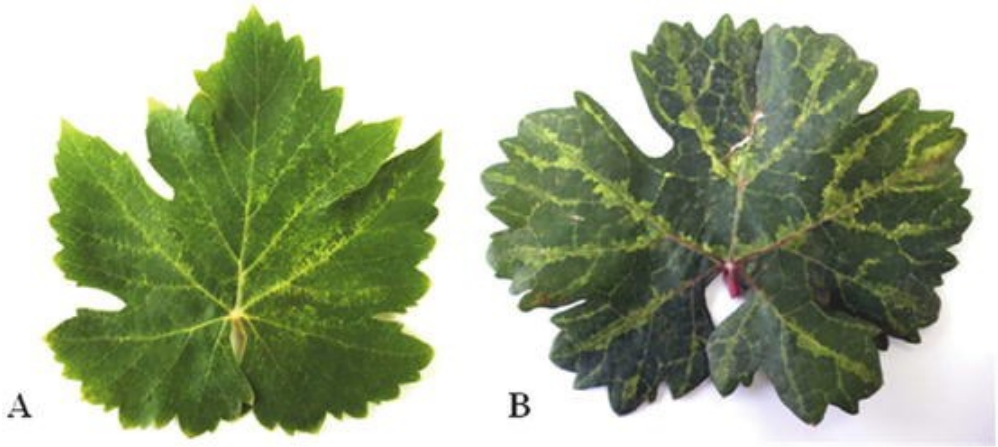

Figure 6. (a) Yellow speckle symptoms present on grapevine infected with GYSVd-1 and GYSVd-2; (b) vein-banding symptoms present on grapevine infected with GYSVd-1, GYSVd-2 in co-infection with GFLV (Reproduced from Hajizadeh et al., 2015).

In the part of the dissertation where we worked with randomly collected samples from Ampelographic collection Kromberk, that were not included in the clonal selection process, we found: GFLV (and its satellite RNA), GLRaV-1, GLRaV-2, GLRaV-3, GRSPaV, GFkV, GRVFV, GPGV, GV-Sat, HSVd, and GYSVd-1.

1.2 METHODS FOR DETECTION OF GRAPEVINE VIRAL PATHOGENS

Visual examination of symptoms is certainly the first approach to diagnosis of viral pathogens. This approach is unreliable because different abiotic and biotic factors can elicit similar symptoms. For example, leaves reddening can be caused by leafroll viruses (in red varieties), grapevine red blotch virus (GRBV) (Sudarshana et al., 2015), phytoplasmas (Grapevine flavescence dorée) (Chuche and Thiéry, 2014), mechanical injuries, pesticides, mineral nutrition disorders, etc. The expression of symptoms also depends on the virus strain, weather conditions, and viral titer. In addition, infected plants may be asymptomatic. Reliable diagnosis of viral pathogens is crucial to take measures to control their spread. Diagnostic methods can be divided into biological indexing, electron microscopy, immunodiagnosis and diagnostic methods based on nucleic acids amplification.

Biological testing is one of the oldest diagnostic methods, and takes a lot of time and space. In biological testing indicators plants are artificially inoculated with grapevine sap (mechanical inoculation) or by grafting. The inoculation technique depends on both the virus and the plant indicator. After inoculation, the indicator plants are incubated for a period ranging from a few days to several weeks or even longer (in the case of woody indicators), followed by evaluation of manifested symptoms. For example, *V. rupestris* St George is often used as an indicator plant for fanleaf, fleck, asteroid mosaic, and RSP diseases; red varieties for leafroll disease; Kober 5BB for Kober stem grooving; LN33 for LN33 stem grooving, Corky bark, and enations; *V. riparia* Glorie de Montpellier for vein mosaic; 110R for vein necrosis; Baco 22A for the stunting component of leafroll disease; *V. vinifera* Mataro or Mission for leafroll, yellow speckle; etc (Martelli et al., 1993). Herbaceous indicators are frequently from the families *Chenopodiaceae*, *Amaranthaceae*, *Solanaceae*, *Cucurbitaceae*, and *Leguminosae*. Biological tests are being replaced by faster, more accurate and less labor-intensive diagnostic methods. However, this method is still important for certification programs and testing of new viruses or new strains.

Electron microscopy (EM) is one of the most significant achievements that has contributed to the development of virology, as it allows the visibility of virus particles. The first EM was developed in 1931 by Ernst Ruska. This achievement was awarded the Nobel Prize in 1986. EM has found wide application in the study of virus morphology, size and structure. The first virus visualized using EM was tobacco mosaic virus (TMV) (Goldsmith and Miller, 2009).

Immunoassays (serological methods) rely on the binding of an antigen to specific antibodies produced against that antigen. Various immunoassays are used for the detection of grapevine viruses such as: immunosorbent electron microscopy (ISEM) (Scagliusi et al., 2002; Petrovic et al., 2003); enzyme-linked immunosorbent assay (ELISA) (Fiore et al., 2008; Cogotzi et al., 2009; Komínek, 2009; Vončina et al., 2011; Zindović et al., 2014); lateral flow immunoassays (LFIAs) (Liebenberg et al., 2009); western blot (Saldarelli et al., 2000; Maliogka et al., 2009a; Alkowni et al., 2011). The ELISA test is the most commonly used. It is performed on microtiter plates, and reagents are added in the following order: specific antibody, antigen (homogenized sample in extraction buffer), specific enzyme-labeled antibody and substrate (usually para-nitrophenylphosphate) with appropriate incubation time and washing of the plate. When the sample is infected, the color of the reaction changes. The color intensity is precisely proportional to the concentration of antigens in the plant. The results are analyzed visually and by spectrophotometric measurements at 405 nm (Clark and Adams, 1977).

The most sensitive and reliable diagnostic methods are based on the detection of nucleic acids. One such technique is the polymerase chain reaction (PCR), which was developed in 1983 and awarded the Nobel Prize in 1993. PCR is based on the amplification of complete

or part of the viral genome by the heat-stable DNA polymerase enzyme. PCR is used to detect viruses that possess a DNA genome (Maliogka et al., 2015; Čarija et al., 2022). Most grapevine viruses, as well as virus-like organisms, have an RNA genome; therefore, reverse transcription is required for the synthesis of complementary DNA (cDNA) prior to PCR amplification, and this method is called reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR is used for confirmation of serological results (Fiore et al., 2008; Mavrič Pleško et al., 2012), for screening (Fattouh et al., 2014; Glasa et al., 2015; Porotikova et al., 2021; Čarija et al., 2022), and for validation of HTS results (Diaz-Lara et al., 2018; Czotter et al., 2018; Eichmeier et al., 2019; Demian et al., 2020; Turcsan et al., 2020; Navrotskaya et al., 2021). For higher accuracy immunocapture-RT-PCR (IC-RT-PCR) which is a combination of serological and molecular methods (Wetzel et al., 2002; Mavrič et al., 2003; Koolivand et al., 2014; Kumar et al., 2015), and nested RT-PCR (RT-nPCR) in which the amplified product from the first reaction is used as a template in the second reaction (Farooq et al., 2013; Fan et al., 2015), can be used. Quantitative RT-PCR (RT-qPCR) allows quantification in addition to detection (Osman et al., 2007; Čepin et al., 2010; Bianchi et al., 2015). Multiplex RT-PCR (Nassuth et al., 2000; Gambino and Gribaudo, 2006; Digiaro et al., 2007; Hajizadeh et al., 2012; Gambino, 2015; Ahmadi et al., 2017; Komínková and Komínek, 2020) and multiplex RT-qPCR (Osman et al., 2013; López-Fabuel et al., 2013; Aloisio et al., 2018) are used to amplify multiple viral organisms in a single reaction. Other methods such as RT loop-mediated isothermal amplification (RT-LAMP) (Walsh and Pietersen, 2013) and microarrays (Engel et al., 2010) can also be used for diagnostics.

All of the above diagnostic methods require prior knowledge of the potential organisms, with the exception of high-throughput sequencing (HTS). HTS allows detection of known and novel viral pathogens in symptomatic or asymptomatic plants (Al Rwahnih et al., 2009; Fajardo et al., 2017; Massart et al., 2017; Hily et al., 2018). HTS of small virus- and viroid-derived RNAs (small RNA sequencing; sRNA-seq) relies on RNA silencing-an antiviral defense mechanism of plants. Upon viral infection, Dicer enzymes (DCL) cleave long dsRNAs and microRNA (miRNA) precursors into short interfering (si)RNA and miRNA duplexes (miRNA/miRNA*), respectively (Bernstein et al., 2001; Baulcombe, 2004; Bartel, 2004). Small RNAs (sRNAs) are loaded into Argonaute proteins (AGOs), which are core catalytic component of the RNA-induced silencing complex (RISC) or RNA-induced initiation of transcriptional gene silencing (RITS) (Parker and Barford, 2006). RISC (post-transcriptional gene silencing-PTGS) targets mRNA through complementary sequence-specific mechanisms and leads to its degradation or inhibits its translation (Hammond et al., 2001). RITS (transcriptional gene silencing-TGS) is involved in the formation of heterochromatin (Verdel et al., 2004) (Figure 7). Upon viral infection sRNAs accumulate in plants and can be detected by sRNA-seq. sRNA-seq has been shown to be highly efficient in grapevine virology (Navarro et al., 2009; Giampetruzzi et al., 2012; Czotter et al., 2018; Turcsan et al., 2020; Demian et al., 2020; Li et al., 2021; Navrotskaya et al., 2021).

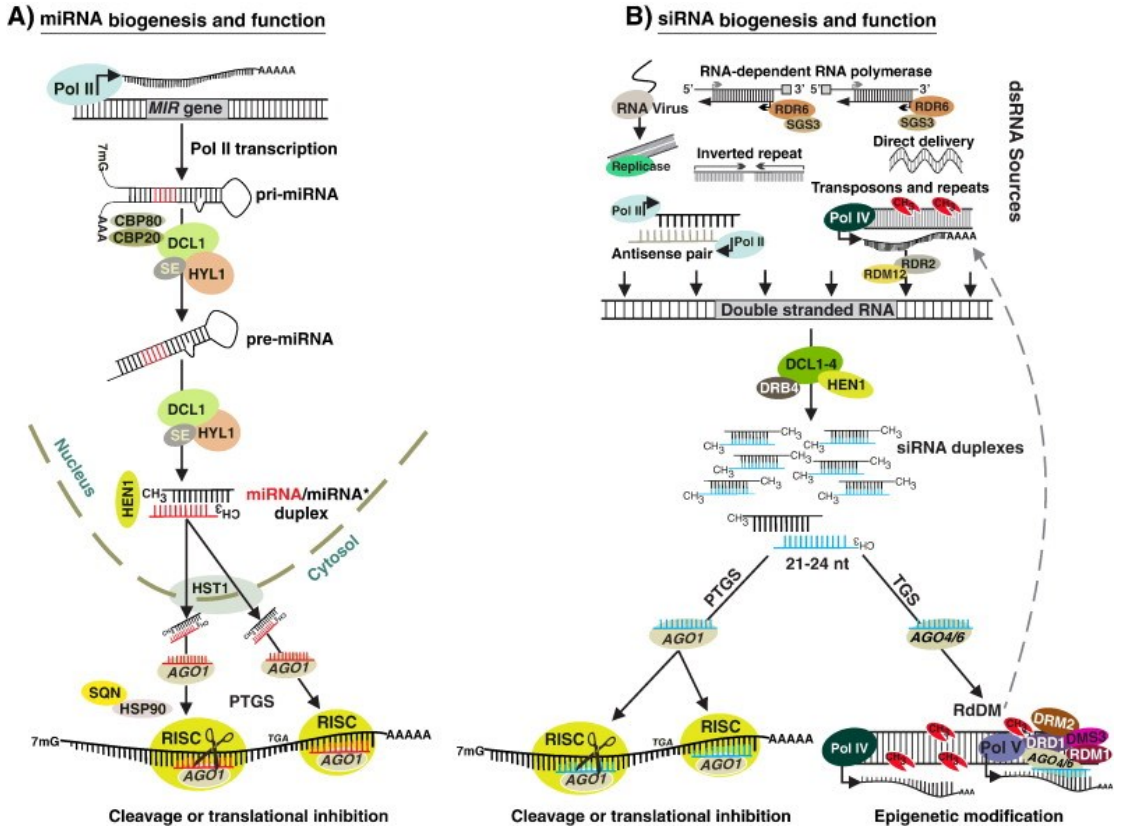

Figure 7. Biogenesis and function of: (a) miRNAs; (b) siRNAs (Reproduced from Khraiwesh et al., 2012).

1.3 METHODS FOR ELIMINATION OF GRAPEVINE VIRAL PATHOGENS

Viruses and viroids are obligate pathogens and cannot be controlled by phytopharmaceutical measures like fungi and bacteria, so planting of healthy material is essential. Selection of healthy material requires effective therapeutic methods and permanent controlling of sanitary status of vines for propagation. Various biotechnological methods for producing healthy vine material have been used experimentally and routinely.

Among them, thermotherapy/heat therapy and meristem or shoot tip tissue culture are the most commonly used. Heat therapy is a treatment in which plants are kept at an elevated temperature for a period of time, which allows the plants to survive and slows down virus replication or even degrades the virus (Panattoni et al., 2013). High temperatures trigger an RNA silencing mechanism (Szittya et al., 2003; Qu et al., 2005; Chellappan et al., 2005; Wang et al., 2008; Velázquez et al., 2010; Liu et al., 2015, 2016; Kim et al., 2021). Meristem cells are smaller and proliferate rapidly, therefore they have the ability to exclude pathogenic organisms from donor plants. In addition, shoot development from the meristem avoids the formation of callus tissue (Figure 8), which reduces the risk of off-types (Grout, 1999). To

accelerate the regeneration of plants from meristems, the technique of micrografting (placing an excised meristem/shoot tip on a seedling growing in vitro) can be used (Jonard et al., 1983; Hussain, et al., 2014).

Figure 8. Developed shoot from the meristem (no callus formation).

Other sanitation strategies are: cryotherapy, chemotherapy, somatic embryogenesis, and electrotherapy. Cryotherapy is a promising tool based on freezing shoot tips in liquid nitrogen (-196 °C) for a short period of time, usually 1 hour (Bettoni et al., 2016). Because meristem cells have less water, denser cytoplasm, and smaller vacuoles compared to other cells, they can survive in liquid nitrogen, whereas other cells die upon ice crystallization (Bettoni et al., 2016). After freezing, shoot tips are thawed and placed in an appropriate regeneration medium (Bettoni et al., 2016). Chemotherapy is a method for virus elimination with mixed success. It consists of placing plant explants in a medium containing antiviral compounds that interfere viral replication. The most common antiviral chemicals are: ribavirin, tiazofurin, oseltamivir, 6-tioguanine, and mycophenolic acid (Panattoni et al., 2011; Skiada et al., 2013; Komínek et al., 2016). Somatic embryogenesis is a strategy for virus elimination with excellent results but with a high risk of somatic variations/mutations. In this technique, plants are regenerated from somatic embryos produced directly or indirectly from floral explants (Gambino et al., 2006, 2009, 2011; Bouamama-Gzara et al., 2017; Turcsan et al., 2020). Electrotherapy is a method with limited success. It consists of exposing herbaceous cuttings or rooted plants to an electric current for a short period of time, where the electric field inactivates the viruses by heating the tissue, followed by in vitro regeneration (Guţa et al., 2010, 2019).

In addition, different elimination methods were combined to evaluate the efficacy of virus elimination, including thermotherapy with meristem tip culture (Maliogka et al., 2009b; Salami et al., 2009), thermotherapy with shoot tip culture (Maliogka et al., 2009b; Bota et

al., 2014), thermotherapy with shoot apices micrografting (Spilmont et al., 2012), thermotherapy with chemotherapy (Hu et al., 2020, 2021), and thermotherapy with somatic embryogenesis (Goussard and Wiid, 1992).

Sanitation success depends on various factors such as variety, virus/viroid species, sanitation method used, treatment conditions, etc.

1.4 AIMS AND HYPOTHESES

Given the importance of rapid and accurate detection and molecular characterization of viruses and virus-like organisms and the development of effective methods for virus elimination, the objectives of this dissertation were (i) to perform virome screening of different grapevine varieties using HTS of small RNAs; (ii) to validate in silico results; (iii) to study the genetic diversity of viruses/viroids, phylogeny and co-infections; (iv) to develop a protocol for the production of healthy plants.

We set up four research hypotheses:

1) Vines are infected with different viruses and viroids, which can be adequately determined using HTS of small RNAs.

2) Based on the sequences information of viruses and viroids obtained by the HTS approach specific primers could be designed for amplification and validation of the predicted viral pathogens by RT-PCR and Sanger sequencing.

3) Predicted infections will be confirmed with Sanger sequencing and additional information about strain-specific polymorphisms related to different host grapevines could be obtained.

4) Using thermotherapy and meristem/shoot tip culture virus-free material could be established, but the percentage of elimination will vary depending on variety and viral pathogen.

2 SCIENTIFIC WORKS

2.1 PUBLISHED SCIENTIFIC WORKS

2.1.1 Virome Status of Preclonal Candidates of Grapevine Varieties (*Vitis vinifera* L.) From the Slovenian Wine-Growing Region Primorska as Determined by High-Throughput Sequencing

Miljanić V., Jakše J., Kunej U., Rusjan D., Škvarč A., Štajner N. 2022. Virome Status of Preclonal Candidates of Grapevine Varieties (*Vitis vinifera* L.) From the Slovenian Wine-Growing Region Primorska as Determined by High-Throughput Sequencing. Frontiers in Microbiology, 13, doi: 10.3389/fmicb.2022.830866: 11 p.

Diseases caused by viruses and virus-like organisms are one of the major problems in viticulture and grapevine marketing worldwide. Therefore, rapid and accurate diagnosis and identification is crucial. In this study, we used HTS of virus- and viroid-derived small RNAs to determine the virome status of Slovenian preclonal candidates of autochthonous and local grapevine varieties (*Vitis vinifera* L.). The method applied to the studied vines revealed the presence of nine viruses and two viroids. All viral entities were validated and more than 160 Sanger sequences were generated and deposited in NCBI. In addition, a complete description into the co-infections in each plant studied was obtained. No vine was found to be virus- and viroid-free, and no vine was found to be infected with only one virus or viroid, while the highest number of viral entities in a plant was eight.

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Virome Status of Preclonal Candidates of Grapevine Varieties (*Vitis vinifera* L.) From the Slovenian Wine-Growing Region Primorska as Determined by High-Throughput Sequencing

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Diseases caused by viruses and virus-like organisms are one of the major problems in viticulture and grapevine marketing worldwide. Therefore, rapid and accurate diagnosis and identification is crucial. In this study, we used HTS of virus- and viroid-derived small RNAs to determine the virome status of Slovenian preclonal candidates of autochthonous and local grapevine varieties (*Vitis vinifera* L.). The method applied to the studied vines revealed the presence of nine viruses and two viroids. All viral entities were validated and more than 160 Sanger sequences were generated and deposited in NCBI. In addition, a complete description into the co-infections in each plant studied was obtained. No vine was found to be virus- and viroid-free, and no vine was found to be infected with only one virus or viroid, while the highest number of viral entities in a plant was eight.

Keywords: ***Vitis vinifera* L., preclonal candidates, HTS, viruses, viroids**

INTRODUCTION

Grapevine is one of the most important fruit crops by acreage and economic importance (Torregrosa et al., 2015). According to the International Organization of Vine and Wine Intergovernmental Organization (OIV), 7.4 million hectares around the world were planted with grapevines in 2018, and vineyards in Spain, China, France, Italy, and Turkey represented 50% of the total world cultivated grapevine area. According to the OIV, the world production of grapes in 2018 was 77.8 million tons. In Slovenia in 2018, grapevines occupied an area of 15,630 hectares and annual production of grapes was 126,958 tons.[1]

Grapevine may harbor more than 86 viruses and viroids, belonging to different families and genera (Fuchs, 2020). Viruses and virus-like organisms cause severe damage to grapevine production worldwide. They cause leaf degeneration, malformation, puckering, leaf rolling, chlorosis, necrosis, ringspots, line patterns, mosaic patterns, vein-banding, vein-clearing, stunting, wilting, shortened internodes, fasciation, zigzag growth, grooving, cracking, and pitting of wood

[1]https://pxweb.stat.si/SiStat/en

(Credi et al., 1981; Mavrič et al., 2003; Andret-Link et al., 2004; Bouyahia et al., 2005; Al Rwahnih et al., 2009; Lunden et al., 2010; Giampetruzzi et al., 2012; Mavrič Pleško et al., 2014; Sudarshana et al., 2015). They impact vine yield and wine quality, as viral entities delay ripening, affect grape quality, decrease sugar content, affect the content of pigments, various aromatic components and other metabolites, and increase the acidity of wines (Lee and Martin, 2009; Vega et al., 2011; Alabi et al., 2016; Girardello et al., 2020; Lee et al., 2021). Viral entities eventually lead to the death of chronically infected plants.

Therefore, rapid and accurate diagnosis and identification is very important. Most methods for detection and identification require prior knowledge of the potential pathogens (e.g., use of antibodies in serological methods or virus specific primers in PCR amplification), with the exception of the metagenomic approach called high-throughput sequencing technology (HTS). HTS is a powerful technology that enables rapid detection of viral entities in plant tissues, including unknown as well as known viruses and viroids in symptomatic and asymptomatic plants, without the need for prior knowledge (Al Rwahnih et al., 2009; Kreuze et al., 2009; Fajardo et al., 2020). HTS of small RNAs (small RNA sequencing; sRNA-seq) has been shown to be efficient in detecting plant viruses or viroids (Kreuze et al., 2009; Kashif et al., 2012; Vives et al., 2013; Jakse et al., 2015; Singh et al., 2020). This approach exploits a natural antiviral defense mechanism called RNA silencing or RNA interference (RNAi). The silencing mechanism is initiated by RNase III-like enzymes called Dicer-like enzymes (DCL) which cleave long double-stranded RNAs (dsRNAs) into short interfering (si)RNA and miRNA precursors with a hairpin or stem-loop structure into miRNA duplexes (miRNA/miRNA*) (Bernstein et al., 2001; Bartel, 2004; Baulcombe, 2004). During the process of viral infection small RNAs (sRNAs) accumulate abundantly in plants and can be detected by deep sequencing of infected plants. sRNA-seq provides a unique opportunity to easily detect and identify grapevine viruses and viroids due to the abundance of sRNAs (Navarro et al., 2009; Giampetruzzi et al., 2012; Eichmeier et al., 2016; Czotter et al., 2018; Demian et al., 2020; Li et al., 2021).

Slovenia is a traditional wine-growing country with many local and indigenous grapevine varieties revitalized in current clonal selection programs, according to the rules on the marketing of material for the vegetative propagation of vine (Official gazette, N°93/05 and 101/20) and OIV process for the clonal selection of vines (Resolution oiv-viti-564a-2017). In the past, propagation material was controlled just visually, which led to uncontrolled spread of viruses.

The aim of the presented work was to investigate the virome status of Slovenian preclonal grapevine candidates and to study their genetic diversity and co-infections using identification by sRNA-seq and confirmation by RT-PCR and Sanger sequencing.

MATERIALS AND METHODS

Plant Material

A total of 82 dormant cuttings of 6 preclonal grapevine varieties (*Vitis vinifera* L.)—2 reds, "Refošk" ("Terrano") and "Pokalca" ("Schioppettino"), and 4 whites, "Laški rizling" ("Welschriesling"), "Rebula" ("Ribolla Gialla"), "Malvazija" ("Malvasia d'Istria"), and "Zeleni Sauvignon" ("Sauvignon Vert"), were collected from the 3 vineyards [Pouzelce (P); Base (B) and Genebank (G), referenced to **Table 1**)] maintained by Centre of grapevine selection (STS Vrhpolje, Vipava) in Primorska wine-growing region in Slovenia in February 2019. After 3–4 weeks in water at room temperature one-bud cuttings started bud-bursting and the obtained leaves were collected and stored at −80°C for further analysis.

High-Throughput Sequencing of Virus- and Viroid-Derived Small RNAs

Eighty-two grapevine samples were pooled into 12 bulks/libraries, each bulk representing samples of the same variety; some varieties were represented with more than one bulk (**Table 1**). sRNAs were extracted by an enrichment procedure using a mirVana miRNA Isolation Kit (Ambion, Life Technologies) according to the manufacturer's instructions. sRNA libraries were prepared using the Ion Total RNA-Seq kit and checked for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies). Barcode-labeled cDNA libraries were sequenced on an Ion PI chip v3 using an Ion Proton Sequencer (Ion Torrent; Life Technologies) according to the manufacturer's instructions. According to the Ion Torrent sequencing pipeline, raw reads had removed adapter sequences and they were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject accession number PRJNA765925.

Analysis of High-Throughput Sequencing Data

Raw sequence reads were filtered based on quality score and read length using cutadapt tools (Martin, 2011). VirusDetect[2] (Zheng et al., 2017), an automated bioinformatics pipeline, was employed for further analysis of obtained sequences. VirusDetect is a highly sensitive and enables efficient analysis of sRNA datasets for viral identification. The software package first align sRNA reads to viral GenBank references using Burrows-Wheeler Aligner (BWA). The mapped sRNA reads are then assembled into contigs according to the viral reference. This program also maps the sRNA reads to host reference sequences to discard host-derived sRNAs. VirusDetect performs *de novo* assembly of sRNAs using Velvet with automated parameter optimization. Contigs obtained from *de novo* assemblies were aligned to the grapevine genome and all contigs with nucleotide identity greater than 90% with the grapevine genome were discarded. *De novo* assembled contigs were concatenated with reference-guided generated contigs and then all redundancies were removed, according to the employed iAssembler pipeline. The obtained contigs were then compared with the viral GenBank references for their identification. This automated pipeline used the BLASTN algorithm to compare the contigs to the reference virus nucleotide sequences and BLASTX algorithm to

[2]http://virusdetect.feilab.net

TABLE 1 | Viruses and viroids detected in 12 libraries using the VirusDetect approach.

Library labels	Samples	Detected viruses and viroids	Reference sequence	Reference length	Consensus length	Reference coverage (%)	No. of contigs	Sequencing depth
005	Laški rizling 3/34B	RBDV (RNA1)	AB948214	5,449	5,449	100	2	1556.2
	Laški rizling 3/45B	RBDV (RNA2)	AB948215	2,231	2,202	98.7	4	513.8
	Laški rizling 3/56B	GPGV	KP693444	7,172	6,957	97	8	487.4
	Laški rizling 3/64B	GRSPaV	AY881627	8,743	7,434	85	66	9.8
		GFkV	AJ309022	7,564	6,210	82.1	55	49.3
		GRVFV	KY513701	6,730	2,669	39.7	55	15.5
		GSyV-1	KP221269	334	150	44.9	3	20.9
		HSVd	KJ810551	309	309	100	3	2223.0
		GYSVd-1	AB028466	368	368	100	5	971.9
006	Refošk 9/3B	GPGV	FR877530	7,259	7,257	100	7	123.3
	Refošk 10/1B	GRSPaV	KX035004	8,743	8,666	99.1	41	16.6
	Refošk 10/2B	GRGV	KX171166	6,863	4,362	63.6	40	53.0
	Refošk 10/3B	GRVFV	KY513702	6,716	3,420	50.9	34	46.3
		HSVd	KJ810551	309	309	100	2	1856.0
		GYSVd-1	KP010010	389	389	100	3	1505.9
007	Rebula 15/1B	GPGV	FR877530	7,259	7,248	99.8	8	53.5
	Rebula 15/2B	GFkV	KT000362	7,564	5,814	76.9	60	13.2
	Rebula 15/3B	GRVFV	KY513702	6,716	2,483	37	40	14.6
	Rebula 16/1B	HSVd	KJ810551	309	309	100	3	490.5
	Rebula 16/2B	GYSVd-1	KP010010	389	389	100	3	374.2
	Rebula 16/3B							
	Rebula 19/1B							
	Rebula 19/2B							
008	Rebula 19/3B	GPGV	KY747494	7,156	7,135	99.7	9	56.5
	Rebula 20/3B	GRSPaV	KR054734	8,753	7,168	81.9	70	5.6
	Rebula 22/1B	GFkV	AJ309022	7,564	5,815	76.9	46	29.0
	Rebula 22/2B	GRVFV	KY513702	6,716	5,706	85	55	93.3
	Rebula 22/3B	HSVd	KY508372	316	314	99.4	3	650.5
	Rebula 24/2B	GYSVd-1	AB028466	368	368	100	6	298.8
	Rebula 26/1B							
	Rebula 26/2B							
	Rebula 26/3B							
009	Malvazija 32/1B	GPGV	FR877530	7,259	7,259	100	5	142.5
	Malvazija 32/2B	GRSPaV	KX035004	8,743	7,838	89.6	70	7.0
	Malvazija 32/3B	GSyV-1	KP221256	6,482	3,160	48.8	25	29.4
	Malvazija 32/9B	HSVd	KJ810551	309	309	100	4	1462.5
		GYSVd-1	KJ466324	367	367	100	5	2201.1
010	Refošk 9/3P	GPGV	FR877530	7,259	7,248	99.8	16	93.5
	Refošk 9/4P	GRSPaV	KX035004	8,743	7,595	86.9	76	6.5
	Refošk 9/5P	GLRaV-3	GQ352631	18,498	18,234	98.6	23	39.6
	Refošk 10/2P	GFkV	AJ309022	7,564	6,137	81.1	52	69.5
	Refošk 10/3P	GRGV	KX109927	6,863	3,144	45.8	36	22.9
	Refošk 10/5P	GRVFV	KY513702	6,716	4,752	70.8	90	33.1
	Refošk 11/2P	HSVd	KJ810551	309	309	100	5	889.0
	Refošk 11/3P	GYSVd-1	KP010010	389	389	100	4	694.1
	Refošk 11/4P							
011	Refošk 12/1P	GPGV	FR877530	7,259	7,246	99.8	6	83.2
	Refošk 12/3P	GRSPaV	KX274274	8,725	7,663	87.8	65	6.3
	Refošk 12/6P	GFkV	KF417610	532	177	33.3	3	6.8
	Refošk 12/18P	GRVFV	KY513701	6,730	1,852	27.5	30	5.4
	Refošk 12/19P	HSVd	KJ810551	309	309	100	3	503.7
	Refošk 12/20P	GYSVd-1	KP010010	389	389	100	2	565.2

(Continued)

TABLE 1 | (Continued)

Library labels	Samples	Detected viruses and viroids	Reference sequence	Reference length	Consensus length	Reference coverage (%)	No. of contigs	Sequencing depth
012	Zeleni Sauvignon 14/2P	GPGV	FR877530	7,259	7,240	99.7	11	119.6
	Zeleni Sauvignon 14/5P	GRSPaV	KT008379	780	486	62.3	8	6.7
	Zeleni Sauvignon 14/6P	GFkV	AJ309022	7,564	5,984	79.1	54	28.5
	Zeleni Sauvignon 14/7P	GRVFV	KY513701	6,730	2,927	43.5	47	9.0
	Zeleni Sauvignon 15/1P	HSVd	KJ810551	309	309	100	3	279.5
	Zeleni Sauvignon 15/2P	GYSVd-1	KP010010	389	389	100	2	452.0
	Zeleni Sauvignon 15/3P							
	Zeleni Sauvignon 16/1P							
	Zeleni Sauvignon 16/2P							
013	Zeleni Sauvignon 16/3P	GPGV	KY747494	7,156	7,135	99.7	14	122.9
	Zeleni Sauvignon 24/3P	GRSPaV	MG938309	8,753	4,240	48.4	63	9.5
	Zeleni Sauvignon 24/9P	GFLV (RNA1)	JX513889	7,340	6,752	92	31	149.6
	Zeleni Sauvignon 24/10P	GFLV (RNA2)	JX559643	3,769	3,417	90.7	12	214.9
	Zeleni Sauvignon 24/11P	GRVFV	MH544692	494	149	30.2	3	5.6
	Zeleni Sauvignon 26/1P	HSVd	KJ810551	309	309	100	3	412.4
	Zeleni Sauvignon 26/2P	GYSVd-1	AB028466	368	368	100	6	97.4
	Zeleni Sauvignon 26/3P							
014	Malvazija 20/2P	GPGV	FR877530	7,259	6,917	95.3	38	10.7
	Malvazija 20/6P	GFkV	AJ309022	7,564	2,302	30.4	41	5.3
	Malvazija 20/7P	GRVFV	KY513702	6,716	3,227	48	64	11.0
	Malvazija 20/46P	HSVd	KY508372	316	314	99.4	2	232.1
	Malvazija 20/47P	GYSVd-1	KP010010	389	389	100	2	394.9
	Malvazija 20/48P							
	Malvazija 20/50P							
015	Malvazija 21/6P	GPGV	FR877530	7,259	7,247	99.8	12	33.2
	Malvazija 21/7P	GRSPaV	FJ943281	780	635	81.4	9	7.3
	Malvazija 21/8P	GFkV	AJ309022	7,564	2,432	32.2	36	6.4
	Malvazija 21/20P	GRVFV	KY513701	6,730	4,364	64.8	94	20.7
	Malvazija 23/2P	HSVd	KJ810551	309	309	100	5	473.3
	Malvazija 23/3P	GYSVd-1	AB028466	368	368	100	5	394.1
	Malvazija 23/4P							
016	Pokalca 3/4P	GPGV	FR877530	7,259	7,243	99.8	12	40.7
	Pokalca 3/5P	GRSPaV	KX958435	8,743	5,728	65.5	86	5.3
	Pokalca 3/6P	GFLV (RNA1)	KX034843	7,347	5,268	71.7	21	737.3
	Pokalca 9/2G	GFLV (RNA2)	GQ332370	3,773	3,475	92.1	18	752.5
	Pokalca 9/3G	GRVFV	KY513702	6,716	4,627	68.9	84	28.9
	Pokalca 9/26G	HSVd	KJ810551	309	309	100	2	371.0
	Pokalca 9/27G	GYSVd-1	AB028466	368	368	100	4	116.2

compare the contigs to the reference virus protein sequences (Zheng et al., 2017).

Validation of Predicted Infections by RT-PCR, Direct Sanger Sequencing and Cloning, Sequence Analysis and Phylogenetic Studies

Validation of HTS results of predicted infections was performed by RT-PCR and Sanger sequencing. Total RNAs for all individual samples were extracted from 70 to 100 mg of frozen leaves using Monarch RNA Total Miniprep Kit (New England Biolabs) following recommended instructions. The RNAs concentration and purity were assessed with NanoVue Plus spectrophotometer (GE Healthcare Life Sciences). Due to the low RNA concentration and purity, three samples (Malvazija 20/2P, Malvazija 21/7P and Malvazija 23/4P) were excluded from further analysis. RT−PCRs were performed using a two−step protocol, where total RNAs were first reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions followed by PCR with specific primers (**Supplementary Table 2**). The PCR reaction mixture (20 μL total) contained 10.7 μL nuclease-free water, 4 μL 5 × PCR buffer (Promega), 1.6 μL $MgCl_2$ (Kapa Biosystems), 1.6 μL dNTP mix (10 mM each of the 4 dNTPs) (Promega), 0.5 μL of each primer, 0.1 μL KAPA Taq DNA polymerase (Kapa Biosystems), and 1 μL of cDNA. RT-PCR products were analyzed by electrophoresis on a 1.4% agarose gel,

stained with ethidium bromide, and visualized under a UV transilluminator and remaining reaction was cleaned by Exo-Sap treatment. RT-PCR products of a predicted sizes were sequenced directly in both directions for all viruses and viroids, except for *Grapevine rupestris stem pitting-associated virus* (GRSPaV), where RT-PCR products were ligated into the pGEM-Teasy Vector Systems cloning kit (Promega) and then transformed into *Escherichia coli* DH-5α competent cells. Blue/white screening was performed on the LB/carbenicillin/IPTG/X-gal/agar plates. The positive clones were randomly picked and then colony PCR was performed using specific primers (RSP 52/RSP 53) (**Supplementary Table 2**). After purification, direct RT-PCR or cloned products were sequenced using an Applied Biosystems 3130 Genetic Analyzer. After sequencing, the forward and reverse traces were trimmed and assembled using CodonCode Aligner 9.0.1 (CodonCode Corporation). All sequences generated in this study were deposited in the NCBI GenBank database.[3] The generated virus and viroid sequences were compared using the ClustalW program (Thompson et al., 1994) implemented in MEGA X software (Kumar et al., 2018). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) divergence sequence analysis. Phylogenetic trees were constructed using MEGA X software. The Modeltest implemented in MEGA X was applied to investigate the best-fitting model of nt substitution. The reliability of the obtained trees was evaluated using the bootstrap method based on 1,000 replicates and bootstrap values lower than 50% were omitted.

RESULTS AND DISCUSSION

Viruses and Viroids Detected by High-Throughput Sequencing of Virus- and Viroid-Derived Small RNAs

sRNA-seq from the pooled grapevine samples yielded 4,206,135–17,668,261 reads. In VirusDetect pipeline 3,643,531–13,905,492 reads per pool were processed (**Supplementary Table 1**). Additional results of the detection pipeline are presented in **Supplementary Table 1**. Using the described approach, nine viruses: *Raspberry bushy dwarf virus* (RBDV), *Grapevine Pinot gris virus* (GPGV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine fleck virus* (GFkV), *Grapevine Red Globe virus* (GRGV), *Grapevine rupestris vein feathering virus* (GRVFV), *Grapevine Syrah virus-1* (GSyV-1), and two viroids: *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid- 1* (GYSVd-1) were identified. GRGV, GRVFV, and GSyV-1 were detected for the first time in Slovenia (paper in review).

The highest number of viral entities in a library was eight (in libraries 005 and 010), the lowest number was five (in libraries 007, 009, 014), while the remaining libraries contained six viral entities. The genomes of RBDV and GFLV are bipartite and consist of two single-stranded positive-sense RNAs (RNA1 and RNA2), therefore the consensus length, reference coverage, number of contigs and sequencing depth for both, RNA1 and RNA2 are shown (**Table 1**). GPGV, HSVd, and GYSVd-1 were detected in all analyzed libraries. RBDV and GLRaV-3 were detected in only one library, 005 ("Laški rizling" variety) and 010 ("Refošk" variety), respectively (**Table 1**).

Validation of Predicted Infections by RT-PCR, Direct Sanger Sequencing and Cloning, Sequence Analysis and Phylogenetic Studies

Raspberry Bushy Dwarf Virus

The natural occurrence of RBDV in grapevine was first confirmed in Slovenia in "Laški rizling" and "Štajerska belina" using DAS-ELISA and IC-RT-PCR (Mavrič et al., 2003). Reports that this virus infecting grapevines are rare, except in Slovenia it has also been reported in neighboring Sebria and Hungary (Jevremovic and Paunovic, 2011; Pleško et al., 2012; Czotter et al., 2018). In our study, RBDV was found only in "Laški rizling" variety (005 library). It was found in this variety in all Slovenian wine-growing regions (Mavrič Pleško et al., 2009). Complete or almost complete reference coverage of both RNA1 (100%) and RNA2 (98.7%) was obtained (**Table 1**). All four samples were confirmed positive with RT-PCR and partial RNAs2 were Sanger sequenced and deposited in NCBI (GenBank accession no. OK139039-OK139042). The MP sequences of our isolates shared 100% nt identity (100% aa identity), while in the CP gene region they shared 98.18–99.55% nt identities (97.95–100% aa identities). Considering phylogenetic analysis of partial sequences of the CP gene (438 bp), our isolates clustered among other isolates of *Vitis* sp. retrieved from NCBI and they were clearly separated from isolates of *Rubus* sp. (**Supplementary Figure 1**), which was also reported in other studies (Mavrič Pleško et al., 2009, 2020; Valasevich et al., 2011).

Grapevine Pinot Gris Virus

GPGV is an emerging virus associated with grapevine leaf mottling and deformation (GLMD) disease (Giampetruzzi et al., 2012), but has not yet been included in certification programs in Europe. In Slovenia, the first symptoms (shortened internodes, mottling, deformation, and poor leaf development) were observed in 2001, and samples were tested for eight viruses (ArMV, CLRV, GFLV, RBDV, SLRSV, TBRV, ToRSV, and TRSV), but none was confirmed by DAS-ELISA (Mavrič Pleško et al., 2014). In 2012, GPGV was discovered in Italy using sRNA-seq (Giampetruzzi et al., 2012), and in 2014 its occurrence was reported also from Slovenia (Mavrič Pleško et al., 2014). These authors reported that GPGV seems to be widespread in the Primorska wine-growing region, but they also observed it in different parts of Slovenia. In addition to Italy, the virus seems to be widespread in other neighboring countries, Hungary (17 out of 18 libraries) and Croatia (61.97%) (Czotter et al., 2018; Hančević et al., 2021). Based on HTS data, GPGV was the most prevalent virus in our study. It was found in all 12 libraries (95.3–100% coverage of complete reference sequences) (**Table 1**). Seventy-two out of 79 samples were positive (91.14%) (**Figure 1**). Forty

[3]www.ncbi.nlm.nih.gov

sequences were generated and deposited in NCBI (GenBank accession no. OK139043-OK139082). A polymorphism showing C/T variation introducing a premature termination codon was observed in the MP sequence. The C/T polymorphism was observed in 13 samples making MP shorter by 18 nt (6 aa). This polymorphism was also observed for isolates analyzed in some other studies (Reynard, 2015; Saldarelli et al., 2015; Czotter et al., 2018; Abou Kubaa et al., 2020). The MP sequences of 40 Slovenian isolates shared nt identities of 93.94–100% (87.79–100% aa identities). 40 Slovenian CP sequences shared pairwise nt identities of 94.53–100% (97.86–100% aa identities). The phylogenetic tree was constructed using part of the sequences of the MP gene and CP gene (718 nt) and it showed partitioning of our isolates into two clades with isolates from geographically relatively close countries (**Supplementary Figure 2**).

Grapevine Rupestris Stem Pitting-Associated Virus

Using the HTS approach, GRSPaV (member of the rugose wood complex) was detected in 10 libraries (**Table 1**). In eight libraries complete reference sequences were covered 48.4–99.1%, while in other two libraries, 012 ("Zeleni Sauvignon" variety) and 015 ("Malvazija" variety), only partial genome sequences were covered 62.3 and 81.4%, respectively. In all libraries GRSPaV had a low sequencing depth (5.3X–16.6X). It was confirmed in all libraries where it was predicted with RT-PCR, and even in two other libraries (007 and 014), where it was not detected by sRNA-seq/VirusDetect pipeline. In library 007 ("Rebula" variety) all eight samples were infected, while in library 014 ("Malvazija" variety) only two samples were infected. In Hungary, using sRNA-seq approach, the same contradictory results were reported by three independent studies (Czotter et al., 2018; Demian et al., 2020; Turcsan et al., 2020). The authors indicated that this could be due to technical issues, the possibility that concentrations were under detection threshold due to the bulk sequencing strategy or deeper biological aspects, as a possible long coexistence between grapevine and GRSPaV resulted in mutual adaptation (Gambino et al., 2012), and potentially the plant immune response was not activated. The reason why GRSPaV was not detected by sRNA-seq in some libraries in different studies conducted in different countries requires further studies. Overall, 70 out of 79 samples were tested positive (88.61%), making GRSPaV the second most abundant virus (**Figure 1**). The selected RT-PCR products were directly sequenced, but due to different genetic variants in the same sample, we were not able to generate high quality sequences, therefore cloning was performed. One RT-PCR product was selected from each variety. Three white colonies were randomly picked from each variety, and after colony PCR, three products were obtained for Laški rizling 3/45B, Rebula 16/1B, Zeleni Sauvignon 14/2P, and Malvazija 20/48P, and one product for Refošk 10/3B and Pokalca 9/27G. In total, 14 products were sequenced (GenBank accession no. OK138921–OK138934), and all were different from each other, even when originating from the same plant. The highest overall mean distance was revealed for three variants of Laški rizling 3/45B (17.14%), and the lowest overall mean distance for Malvazija 20/48P (6.32%). While the overall mean distance for all 14 sequenced variants was 14.06%. It can be concluded that at least three genetic variants exist in the selected samples which differ extensively in the analyzed genome region. The high genetic diversity could be due to the lack of proofreading activity of RdRp, errors in genome replication, frequent recombination, and grafting of individual plants onto differentially infected rootstocks and scions (Glasa et al., 2017). The phylogenetic tree also showed that the genetic variants, even if from the same plant, clustered in different clades (**Supplementary Figure 3**).

Grapevine Fanleaf Virus

GFLV, responsible for a fanleaf degeneration disease and one of the viruses causing the most significant damages on vines (Andret-Link et al., 2004), was detected in two of our libraries, 013 ("Zeleni Sauvignon" variety) and 016 ("Pokalca" variety) (**Table 1**). RNA1 was covered 92 and 71.7%, respectively, while RNA2 was covered 90.7 and 92.1%, respectively. Validation by RT-PCR using published primers resulted in one sample positive from bulk 013 and three samples from bulk 016. All positive samples were sequenced (GenBank accession no. OK139035–OK139038). Three isolates from "Pokalca" variety shared 99.67 or 99.84% nt identities (99.5 or 100% aa identities), whereas the isolate Zeleni Sauvignon 16/3P differed greatly from the Pokalca isolates with 87.27 or 87.44% nt identities (96.49 or 96.99% aa identities). In addition to the differences between our isolates they also differed from the isolates deposited in NCBI. Pokalca isolates shared the highest identity, 90.62 or 90.79%, with the isolate from France (MG731624), while Zeleni Sauvignon 16/3P shared the highest identity 91.29% with the isolate from Switzerland (MG731616). High sequence variation between GFLV isolates of partial or complete RNA2 ($2A^{HP}$, $2B^{MP}$, and $2C^{CP}$) has been reported in several studies (Naraghi-Arani et al., 2001; Fattouch et al., 2005; Pompe-Novak et al., 2007; Elbeaino et al., 2014). This virus does not possess proofreading activity of RdRp and the large genetic variability indicates that the GFLV genome consists of quasispecies populations (Naraghi-Arani et al., 2001). Phylogenetic analysis showed that our isolates differed in the region of partial RNA2 from isolates retrieved from NCBI, even from the previously characterized isolates from Slovenia (Pompe-Novak et al., 2007), but they were the closest to the isolates from Italy and France (**Supplementary Figure 4**). Due to the differences observed among sequences, we designed new primers based on our sequencing data and repeated analysis. Positive amplifications were observed for additional two samples from bulk 013 and for four samples from bulk 016.

Grapevine Leafroll-Associated Virus 3

GLRaV-3 is the major causal agent of one of the most detrimental grapevine diseases named as grapevine leafroll disease (GLD) (Maree et al., 2013). GLRaV-3 was detected only in library 010 ("Refošk" variety). The reference sequence GQ352631 was 98.6% covered and a sequencing depth of 39.6X (**Table 1**). With the primer pair amplifying the CP gene region, only one sample (Refošk 11/4P) was positive, therefore we used the primer pair amplifying the HSP70h gene region and the same result was obtained. The sequencing of CP gene region of Refošk 11/4P (GenBank accession no. OK138920) showed the highest nt identity (99.76%) with 15 sequences originating

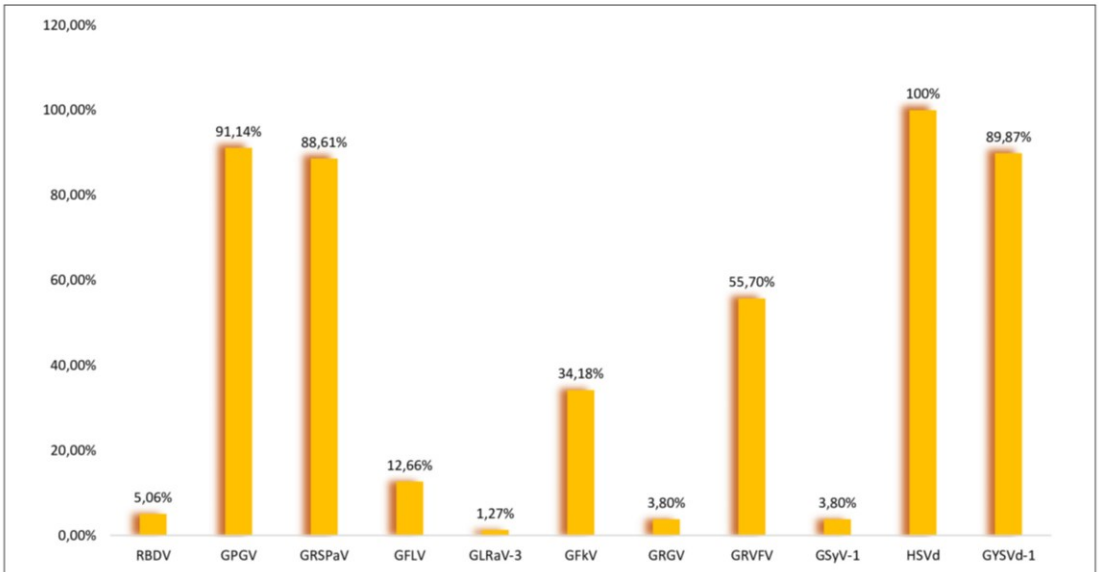

FIGURE 1 | Prevalence of nine viruses and two viroids in analyzed samples.

from Greece, Portugal, United States, Canada and Pakistan and 3 sequences of unknown origin. Phylogenetic analysis showed that our isolate clustered together with the isolate from Portugal (**Supplementary Figure 5**). GLRaV-3 was the least prevalent virus in our sample set. Other grapevine leafroll-associated viruses, members of *Ampelovirus* or *Closterovirus* genus, were not detected. The main reason for the lack of detection of leafroll-associated viruses can be explained by the fact that after the mass selection, all selected vines (potential preclonal vines—ELITE) were screened with ELISA tests, which have a fairly good detection for viruses of GLD, therefore at that step, all infected vines were excluded by further selections and propagation. We are aware that serological tests can have quite large deviations in the detection of viruses, but in this case, it seems that we have been quite successful in the leafroll-associated viruses detection with the ELISA test.

Grapevine Fleck Virus

GFkV was detected in eight libraries. In five libraries (005, 007, 008, 010, and 012) complete genome reference sequences (AJ309022 or KT000362) were covered with 76.9–82.1%, while in two libraries of "Malvazija" variety (014 and 015), reference sequence AJ309022 was covered only with 30.4 and 32.2%, respectively, and in library 011 partial sequence KF417610 was covered with 33.3%. GFkV was validated in all predicted libraries and for 34 samples we got positive RT-PCR result. All samples were sequenced, but the results showed that seven products belonged to GRVFV, which is consistent with reports from Czotter et al. (2018) from Hungary, indicating high similarity between these two viruses and possible cross-amplification with primers. Sequences of two samples, Laški rizling 3/56B and 3/64B had lower quality, therefore they were excluded from further analysis. Twenty-five GFkV sequences were generated and deposited in NCBI (GenBank accession no. OK139010–OK139034). They shared nt identities of 91.6–100% (93.7–100% aa identities). GFkV is phloem-limited, not mechanically transmissible, and spreads by grafting and infected propagating material (Sabanadzovic et al., 2017). Our isolates shared the highest nt identities with isolates from Bosnia and Herzegovina, North Macedonia, Hungary and the United States. Phylogenetic studies showed that the sequenced isolates clustered in different clades depending on the variety, except "Laški rizling," with isolates from neighboring countries, while all samples of "Refošk" variety cluster together with isolate from the United States (**Supplementary Figure 6**). A few decades ago the predecessors of our samples were grafted onto untested rootstocks imported from neighboring countries and from Davis University in California (Hrček, 1977). It seems that with the rootstocks GFkV was imported, but also a lot of grafts produced in Slovenia were exported in neighboring countries, especially in former Yugoslavia.

In addition to GFkV, three fleck-like viruses (GRGV, GRVFV, and GSyV-1) were detected for the first time in Slovenia (paper in review).

Hop Stunt Viroid

HSVd has a wide natural host range from different botanical families. Slovenia is one of the major hop producers, and the first report that HSVd infects hops in Slovenia was published in 2012 (Radisek et al., 2012), while it was recently confirmed

on grapevines in co-infection with GV-Sat, GLRaV-1, GLRaV-2, GRSPaV, GPGV, and GYSVd-1 (Miljanić et al., 2021). According to the HTS results, HSVd was present in all libraries (**Table 1**). In ten libraries the reference sequence KJ810551 was covered 100%, while in the other two libraries (008 and 014) the reference sequence KY508372 was covered 99.4% (**Table 1**). It was validated by RT-PCR and all samples were positive. Forty complete genome sequences were generated and deposited in NCBI (GenBank accession no. OK138935–OK138974). Thirty-eight isolates were 100% identical to each other, while two isolates (Pokalca 3/4P

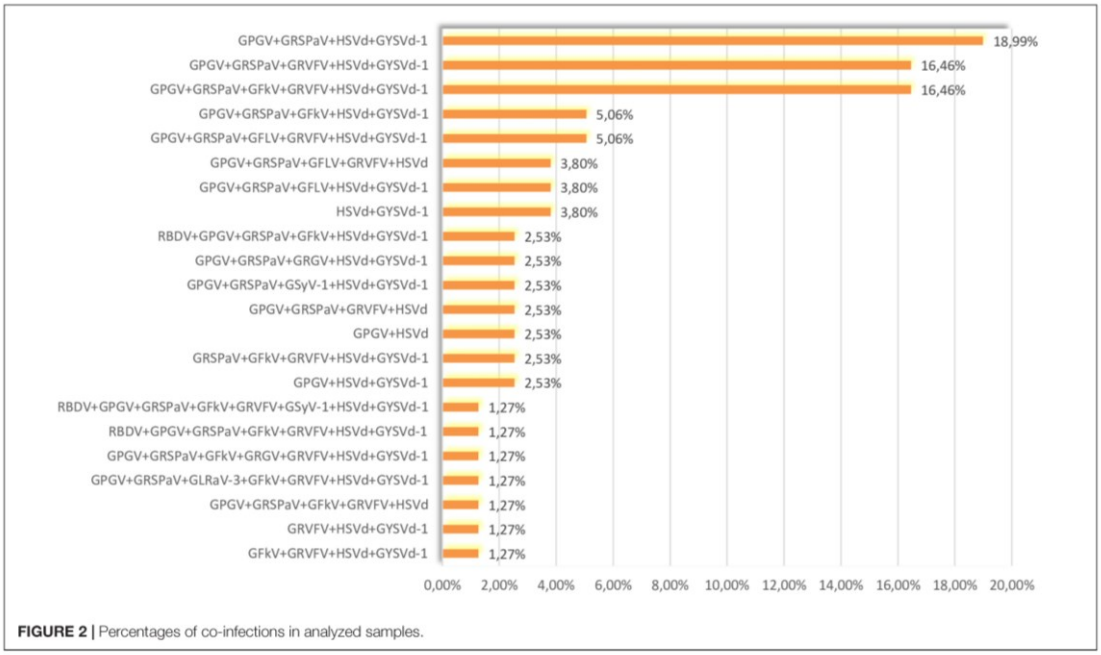

FIGURE 2 | Percentages of co-infections in analyzed samples.

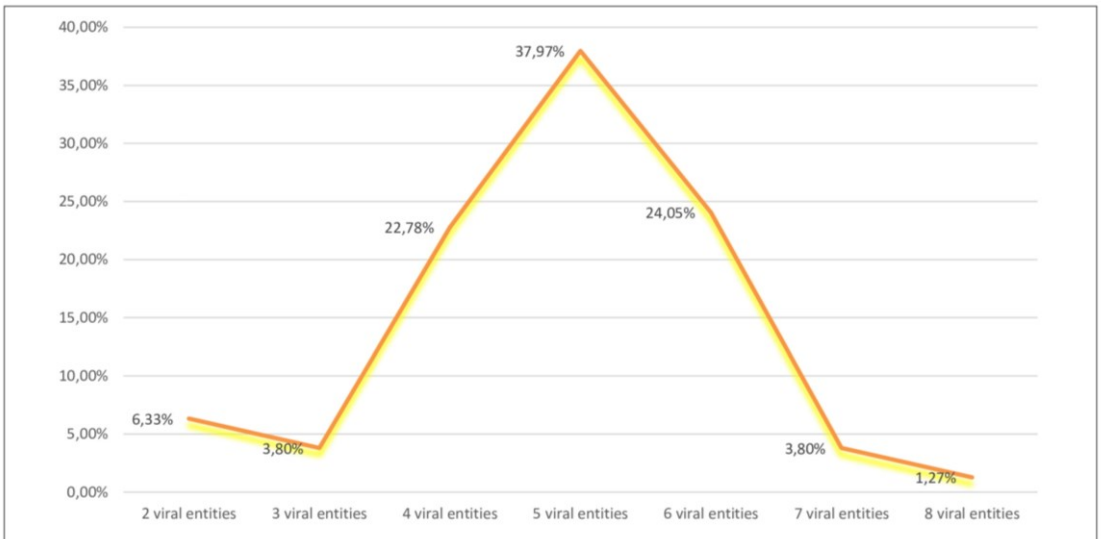

FIGURE 3 | Percentages of mixed infections in analyzed samples.

and Pokalca 3/6P) were identical and shared 98% identities with other isolates. In the genome of Pokalca 3/4P and 3/6P isolates, insertions were observed at positions 123 and 257, while SNPs were observed at positions 171, 172, 238, 244, 259, and 260 (**Supplementary Figure 9**). Also, phylogenetic tree showed that this two isolates clustered completely different from other isolates (**Supplementary Figure 7**).

Grapevine Yellow Speckle Viroid-1

According to our analysis GYSVd-1 was found in all analyzed libraries with 100% references coverage (**Table 1**). According to RT-PCR, 71 samples were positive (89.87%) (**Figure 1**). To our knowledge, nine GYSVd-1 sequences of Slovenian autochthonous grapevine varieties have been deposited in NCBI so far (Štajner et al., 2019). In this study, 35 complete genome sequences were generated and deposited in NCBI (GenBank accession no. OK138975–OK139009). GYSVd-1 was less prevalent and showed higher genetic diversity than HSVd. Slovenian GYSVd-1 sequences had 95.35–100% nt identities. Multiple alignment with ClustalW revealed InDel mutations at four positions in the genome (63, 92, 163, and 287) (**Supplementary Figure 10**). Phylogenetic analysis showed that analyzed GYSVd-1 isolates clustered in different clades regardless of variety (**Supplementary Figure 8**).

Co-infections in Analyzed Samples

GPGV, GRSPaV, HSVd, and GYSVd-1 were the most prevalent in our sample set (**Figure 1**) and their co-infection were the most common (18.99%) (**Figure 2**). The second most prevalent co-infections were GPGV + GRSPaV + GRVFV + HSVd + GYSVd-1 and GPGV + GRSPaV + GFkV + GRVFV + HSVd + GYSVd-1 (16.46%) (**Figure 2**). There were no vines that were free of viruses or viroids, and there were no vines that were infected with only one viral entity. The highest number of tested plants were infected with five viral entities (37.97%), followed by six (24.05%) and four viral entities (22.78%), while one sample (Laški rizling 3/45B) was infected with eight viruses/viroids (**Figure 3**).

CONCLUSION

The main advantage of using the HTS approach is the complete insight into virome of the analyzed samples (Czotter et al., 2018). When screening the virome status of selected plants the HTS approach is considered method of choice. The HTS approach used for virome screening is mainly based on bulked samples, which is cost effective, because in the analysis usually a lot of samples are included, and the main limitation is the possibility that due to the bulk sequencing strategy viral concentrations may be under detection threshold in some cases. In our study all individual samples were tested with RT-PCR for each HTS predicted infection, and all obtained results were consistent, except for GRSPaV, but due to the fact that similar results related to inconsistent detection of GRSPaV were obtained in different studies, it may have deeper biological aspect and required further analysis which are discussed.

The present study gives us a detailed insight into the virome status of preclonal candidates of autochthonous and local grapevine varieties in the Primorska wine-growing region of Slovenia. In this study significant number of sequences were generated for different viral pathogens and could further improve their routine diagnostics, which is especially important as they cannot be controlled by conventional plant protection methods.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

VM: HTS and bioinformatics analysis, validation of HTS data, data analysis, and writing the original draft. UK: HTS and bioinformatics analysis. DR: provided the plant material, review and editing of the original draft. AŠ: provided the plant material. JJ and NŠ: experimental design, review and editing of the original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.830866/full#supplementary-material

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2.1.2 First Report of Grapevine Red Globe Virus, Grapevine Rupestris Vein Feathering Virus, and Grapevine Syrah Virus-1 Infecting Grapevine in Slovenia

Miljanić V., Jakše J., Kunej U., Rusjan D., Škvarč A., Štajner N. 2022. First Report of Grapevine Red Globe Virus, Grapevine Rupestris Vein Feathering Virus, and Grapevine Syrah Virus-1 Infecting Grapevine in Slovenia. Plant Disease, 106, 9: 2538

The study of the virome of preclonal candidates of six grapevine varieties revealed three viruses: grapevine rupestris vein feathering virus (GRVFV), grapevine red globe virus (GRGV), and grapevine Syrah virus-1 (GSyV-1), which were not previously confirmed in Slovenia. GRVFV was the most widespread, being detected in 11 out of 12 libraries. GRGV was detected in two libraries of the variety 'Refošk', and GSyV-1 was also detected in two libraries of the varieties 'Laški rizling' and 'Malvazija'. In silico results were validated with RT-PCR and Sanger sequencing. Forty-four samples were infected with GRVFV and 3 with GRGV and GSyV-1. Twenty-eight sequences were generated and deposited in NCBI (Acc. numbers MW446914-MW446941).

Disease Note

Diseases Caused by Viruses

First Report of Grapevine Red Globe Virus, Grapevine Rupestris Vein Feathering Virus, and Grapevine Syrah Virus-1 Infecting Grapevine in Slovenia

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To investigate the virome status of preclonal grapevine candidates, 82 dormant cuttings of six cultivars (Laški rizling, Refošk, Rebula, Malvazija, Zeleni Sauvignon, and Pokalca) were taken in 2019 from vines in the second cycle of clonal selection in Vipavska dolina, Primorska. Samples were pooled in 12 groups (1× Laški rizling, 1× Pokalca, 2× Rebula, 2× Zeleni Sauvignon, 3× Malvazija, and 3× Refošk) from which small RNA was isolated using a mirVana miRNA Isolation Kit (Ambion, Life Technologies); 12 cDNA libraries were constructed and sequenced on an Ion Proton Sequencer (Ion Torrent, Life Technologies). A VirusDetect pipeline (Zheng et al. 2017) was used for bioinformatics analysis and 3.6 to 13.9 million reads/library were processed. According to the pipeline, grapevine red globe virus (GRGV; *Maculavirus*, *Tymoviridae*) was found in two Refošk libraries. In one, GRGV showed highest coverage of 63.6% and nt identity of 92.71% with GRGV isolate Graciano-T101 (KX171166) from Spain and was represented by 40 contigs with sequence depth of 53.0×. In the other, GRGV showed highest coverage of 45.8% and nt identity of 94.49% with GRGV isolate Graciano-T90 (KX109927) from Spain and was represented by 36 contigs with sequence depth of 22.9×. Grapevine rupestris vein feathering virus (GRVFV; *Marafivirus*, *Tymoviridae*) was found in 11 libraries from six cultivars. In six libraries, GRVFV showed highest coverage of 37 to 85% and nt identity of 88.53 to 94.05% with GRVFV isolate CHASS (KY513702) from Switzerland, and was represented by 34 to 90 contigs with sequence depth of 11.0 to 93.3×; in four, GRVFV showed highest coverage of 27.5 to 64.8% and nt identity of 92.36 to 93.87% with GRVFV isolate Mauzac (KY513701) from France, and was represented by 30 to 94 contigs with sequence depth of 5.4 to 20.7×; in one, GRVFV showed highest coverage of 30.2% and nt identity of 95.30% with GRVFV isolate SK925 (MH544692) from Slovakia, and was represented by three contigs with sequence depth of 5.6×. Grapevine Syrah virus-1 (GSyV-1; *Marafivirus*, *Tymoviridae*) was found in two libraries. In the Laški rizling library, GSyV-1 showed highest coverage of 44.9% and nt identity of 95.36% with GSyV-1 isolate SK351 (KP221269) from Slovakia and was represented by three contigs with sequence depth of 20.9×. In a Malvazija library, GSyV-1 showed highest coverage of 48.8% and nt identity 92.06% with GSyV-1 isolate SK30 (KP221256) from Slovakia and was represented by 25 contigs with sequence depth of 29.4×. Results of high-throughput sequencing were confirmed in individual samples using reverse transcription PCR (RT-PCR). GRGV was amplified using specific primer pair RG6061F: 5′-CCGAGCTTCTCTCCAAGATCA-3′ / RG6801R: 5′-ACTTAA CGTAGGCCACTGGGT-3′ (Cretazzo and Velasco 2017). GRVFV was amplified using specific primer pair GRVFV_6090F: 5′-CATCGTTCTGATCCT CAGCC-3′ / GRVFV_6605R: 5′-AGAGACGCTGACCATGCCAC-3′ (Glasa et al. 2019). GSyV-1 was amplified using specific primer pair SY5922F: 5′-CC AATGGGTCGCACTTGTTG-3′ / SY6295R: 5′- ACTTCATGGTGGTGCCG GTG -3′ (Glasa et al. 2015). Three of 13 Refošk samples tested for GRGV, 44 of 78 samples tested for GRVFV (2 Laški rizling, 12 Refošk, 9 Rebula, 7 Malvazija, 8 Zeleni Sauvignon, and 6 Pokalca), and three of eight samples tested for GSyV-1 (one Laški rizling and two Malvazija) were positive. Further amplicon verification was done by bidirectional Sanger sequencing. Sequences were submitted to GenBank: GRGV (MW446914 to MW446916), GRVFV (MW446917 to MW446938), and GSyV-1 (MW446939 to MW446941). Phylogenetic analysis showed that Slovenian GRGV isolates clustered separately from others in NCBI, that some Slovenian GRVFV isolates clustered with those from France or Slovakia and some clustered separately, and that Slovenian GSyV-1 isolates clustered with those from Hungary and Slovakia. This is the first report of GRGV, GRVFV, and GSyV-1 infecting grapevine in Slovenia. Further work is needed on their prevalence in Slovenian vineyards and their impact on production.

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Keywords: grapevine, HTS detection, viruses

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2.1.3 Elimination of Eight Viruses and Two Viroids from Preclonal Candidates of Six Grapevine Varieties (*Vitis vinifera* L.) through In Vivo Thermotherapy and In Vitro Meristem Tip Micrografting

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Viruses and virus-like organisms are a major problem in viticulture worldwide. They cannot be controlled by standard plant protection measures, and once infected, plants remain infected throughout their life; therefore, the propagation of healthy vegetative material is crucial. In vivo thermotherapy at 36–38 °C for at least six weeks, followed by meristem tip micrografting (0.1–0.2 mm) onto in vitro-growing seedling rootstocks of Vialla (*Vitis labrusca* × *Vitis riparia*), was successfully used to eliminate eight viruses (grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine Pinot gris virus (GPGV), grapevine fanleaf virus (GFLV), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine fleck virus (GFkV), grapevine rupestris vein feathering virus (GRVFV), grapevine Syrah virus-1 (GSyV-1), and raspberry bushy dwarf virus (RBDV)), as well as two viroids (hop stunt viroid (HSVd) and grapevine yellow speckle viroid 1 (GYSVd-1)) from preclonal candidates of six grapevine varieties (Vitis vinifera L.). A half-strength MS medium including vitamins supplemented with 30 g/L of sucrose and solidified with 8 g/L of agar, without plant growth regulators, was used for the growth and root development of micrografts and the subsequently micropropagated plants; no callus formation, hyperhydricity, or necrosis of shoot tips was observed. Although the overall regeneration was low (higher in white than in red varieties), a 100% elimination was achieved for all eight viruses, whereas the elimination level for viroids was lower, reaching only 39.2% of HSVd-free and 42.6% GYSVd-1-free vines. To the best of our knowledge, this is the first report of GPGV, GRVFV, GSyV-1, HSVd, and GYSVd-1 elimination through combining in vivo thermotherapy and in vitro meristem tip micrografting, and the first report of RBDV elimination from grapevines. The virus-free vines were successfully acclimatized in rockwool plugs and then transferred to soil.

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Elimination of Eight Viruses and Two Viroids from Preclonal Candidates of Six Grapevine Varieties (*Vitis vinifera* L.) through In Vivo Thermotherapy and In Vitro Meristem Tip Micrografting

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Abstract: Viruses and virus-like organisms are a major problem in viticulture worldwide. They cannot be controlled by standard plant protection measures, and once infected, plants remain infected throughout their life; therefore, the propagation of healthy vegetative material is crucial. In vivo thermotherapy at 36–38 °C for at least six weeks, followed by meristem tip micrografting (0.1–0.2 mm) onto in vitro-growing seedling rootstocks of Vialla (*Vitis labrusca* × *Vitis riparia*), was successfully used to eliminate eight viruses (grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine Pinot gris virus (GPGV), grapevine fanleaf virus (GFLV), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine fleck virus (GFkV), grapevine rupestris vein feathering virus (GRVFV), grapevine Syrah virus-1 (GSyV-1), and raspberry bushy dwarf virus (RBDV)), as well as two viroids (hop stunt viroid (HSVd) and grapevine yellow speckle viroid 1 (GYSVd-1)) from preclonal candidates of six grapevine varieties (*Vitis vinifera* L.). A half-strength MS medium including vitamins supplemented with 30 g/L of sucrose and solidified with 8 g/L of agar, without plant growth regulators, was used for the growth and root development of micrografts and the subsequently micropropagated plants; no callus formation, hyperhydricity, or necrosis of shoot tips was observed. Although the overall regeneration was low (higher in white than in red varieties), a 100% elimination was achieved for all eight viruses, whereas the elimination level for viroids was lower, reaching only 39.2% of HSVd-free and 42.6% GYSVd-1-free vines. To the best of our knowledge, this is the first report of GPGV, GRVFV, GSyV-1, HSVd, and GYSVd-1 elimination through combining in vivo thermotherapy and in vitro meristem tip micrografting, and the first report of RBDV elimination from grapevines. The virus-free vines were successfully acclimatized in rockwool plugs and then transferred to soil.

Keywords: *Vitis vinifera* L.; grapevine viruses and viroids; thermotherapy; micrografting

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1. Introduction

Viruses and virus-like organisms can cause severe economic problems in vitiviniculture due to (i) yield reduction, (ii) alterations of grape and wine chemical and sensorial quality, (iii) various developmental and morphological malformations in vine organs, and (iv) shortened vine life [1–4]. They cannot be controlled by conventional plant protection measures; thus, planting healthy vegetative propagation material is crucial for sustainable vine cultivation. The presence of viruses and viroids is particularly common in local, domesticated, and indigenous grapevine varieties. These are usually grown in limited areas and, due to the high concentration of the same variety cultivated on the same vineyard lands for decades, or even centuries, vine infections are usually increased, causing additional problems for clonal selection in these varieties. This is one of the main reasons why clonal

selection is very difficult. Various measures for producing virus- and viroid-free grapevine material have been used, including thermotherapy [5,6], meristem tissue culture [7–10], micrografting, chemotherapy [11–14], cryotherapy [15], somaclonal embryogenesis [10,16–19], electrotherapy [20], and various combinations of these treatments [21–24]. Thermotherapy in combination with a meristem tissue culture is a widely used method. Thermotherapy is a treatment in which plants are exposed to high temperatures for a specific period of time [25]. High temperatures can inhibit virus replication or cause virus RNA degradation [26,27]. Furthermore, thermotherapy is associated with an antiviral immune defense mechanism, termed RNA silencing [27–34]. Elevated temperatures induce virus-derived small interfering RNA (vsiRNA) biogenesis and inhibit viral RNA accumulation, whereas key genes in the RNA silencing pathway were up-regulated in pear shoot meristem tips infected with apple stem grooving virus (ASGV) [33], and in pepper plants infected with tobacco mosaic virus pathotype P0 (TMV-P0) [34]. It was found that miRNAs were differentially expressed at high temperatures and miRNA-mediated target genes related to disease defense and hormone signal transduction were up-regulated in pear shoot meristem tips infected with ASGV, leading to a reduction in viral titer [32]. To increase virus elimination efficiency, thermotherapy is often combined with a meristem culture. Meristem tips consist of the apical dome and a limited number of leaf primordia, and they exclude differentiated vascular tissue [35]. The main advantages of the use of meristems are the ability to exclude pathogens present in mother plants and genetic stability [35]. The regeneration of woody plants directly from meristems is difficult; another technique that can speed up this process is micrografting. Micrografting corresponds to the placement of a meristem or shoot tip explant onto a decapitated rootstock grown under in vitro conditions [36,37]. Thermotherapy combined with meristem/shoot tip micrografting has been used to eliminate citrus viruses [38,39] and major grapevine viruses [22]. The meristem size plays an important role in the efficiency of viral entity elimination, because smaller meristems have lower survival rates but the highest virus elimination efficiency. Sanitation success also depends on the grapevine variety, virus/viroid species, their localization and interaction with plants, and treatment conditions.

Slovenia is a traditional wine-growing country, with 15,075 hectares of vineyards in 2021 (Database of Ministry of Agriculture, Forestry and Food). The Primorska winegrowing region represents 40.6% of the total Slovenian vineyard area, where a successful program of clonal selection, especially of indigenous, domesticated, and local grapevine varieties, has been taking place for decades. According to the Official Gazette of the RS N°93/05 and 101/20, all propagated vine material must undergo mandatory testing on: arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), raspberry ringspot virus (RpRSV), tomato black ring virus (TBRV), grapevine virus A (GVA), grapevine virus B (GVB), grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine leafroll-associated virus 1 and 3 (GLRaV-1, -3), and grapevine fleck virus (GFkV) (only for rootstocks). Testing on grapevine leafroll-associated virus 2 and 4–9 (GLRaV-2, -4–9) is not obligatory but just recommended. In our previous study [40], preclonal candidates were screened for viruses and viroids using high-throughput sequencing (HTS) technology, and nine viruses and two viroids were detected. In addition to viruses with obligatory testing—GRSPaV, GFLV, GLRaV-3, and GFkV (for rootstocks)—we detected grapevine Pinot gris virus (GPGV), raspberry bushy dwarf virus (RBDV), and three grapevine fleck-similar viruses: grapevine red globe virus (GRGV), grapevine rupestris vein feathering virus (GRVFV), and grapevine Syrah virus-1 (GSyV-1). Two viroids, hop stunt viroid (HSVd) and grapevine yellow speckle viroid 1 (GYSVd-1) were also detected.

Thus, in the present study, we report on the efficiency of in vivo thermotherapy followed by in vitro meristem tip micrografting in the elimination of the eight above-listed viruses (except GRGV) and two viroids from preclonal candidates of six grapevine varieties.

2. Results

2.1. Plant Regeneration

A total of 598 meristems were isolated and micrografted, from which 51 plants were regenerated (Table 1; Figure 1a,b). To increase their number, the regenerated plants were micropropagated several times, during which period callus formation, hyperhydricity, or necrosis were never observed (Figure 1c).

Table 1. Number of isolated and micrografted meristems, number of regenerated plants, and regeneration rate (%) per individual preclonal candidate of grapevine varieties (*Vitis vinifera* L.).

Sample Name	No. of Isolated and Micrografted Meristems	No. of Regenerated Plants	Regeneration Rate (%)
Laški rizling 3/34B	26	2	7.7
Laški rizling 3/45B	28	0	-
Laški rizling 3/64B	27	4	14.8
Laški rizling 3/56B	22	5	22.7
Rebula 15/3B	13	3	23.1
Rebula 16/1B	22	1	4.5
Rebula 19/2B	12	3	25.0
Rebula 22/3B	19	4	21.1
Zeleni Sauvignon 14/2P	18	1	5.6
Zeleni Sauvignon 14/5P	17	3	17.6
Zeleni Sauvignon 14/7P	25	2	8.0
Zeleni Sauvignon 15/2P	17	1	5.9
Zeleni Sauvignon 15/3P	26	4	15.4
Malvazija 32/1B	27	3	11.1
Malvazija 32/2B	12	1	8.3
Malvazija 32/3B	25	1	4.0
Malvazija 20/47P	13	1	7.7
Malvazija 21/8P	24	1	4.2
Malvazija 23/2P	25	1	4.0
Malvazija 23/3P	19	3	15.8
Refošk 11/4P	28	2	7.1
Refošk 12/3P	18	0	-
Refošk 12/6P	27	0	-
Refošk 12/18P	25	2	8.0
Refošk 12/19P	22	1	4.5
Pokalca 3/4P	20	1	5.0
Pokalca 3/6P	22	1	4.5
Pokalca 9/2G	19	0	-

Higher regeneration rates were observed in white varieties compared with red varieties (Table 1; Figure 2). Only one sample of the white variety, 'Laški rizling' (3/45B), infected with eight viral entities and with at least three genetic variants of GRSPaV, did not regenerate. Among the reds, one sample of 'Pokalca' (9/2G) and two samples of 'Refošk' (12/3P and 12/6P) did not regenerate (Table 1). 'Rebula' had the highest regeneration rate (16.7%), followed by 'Laški rizling' and 'Zeleni Sauvignon' (10.7%). Although 'Rebula' had the highest regeneration rate, 'Zeleni Sauvignon' regenerated and grew much faster during micropropagation. However, 'Pokalca' had the lowest regeneration rate (3.3%) (Figure 2).

2.2. Virus and Viroid Elimination and Vine Acclimatization

The efficiency of the elimination of viruses and viroids from regenerated plants grown in vitro for seven months was analyzed using RT-PCR. A 100% elimination rate was achieved for all viruses. However, the elimination of viroids HSVd and GYSVd-1 was significantly lower at 39.2% and 42.6%, respectively (Table 2; Figure S1). Specific RT-PCR products of the positive controls were obtained in all cases, whereas no amplicons were generated in the negative controls (Figure S1). Virus-free plants grown in vitro were successfully acclimatized in rockwool plugs, which proved to be excellent for growth and root

development (Figure 3). Plants were kept in a mini greenhouse in the growth chamber (Figure 3), and then transplanted into pots (Figure 4). All the virus-free preclonal candidates will be retested after approximately three years before being officially established as certified clones.

(**a**) (**b**) (**c**)

Figure 1. (**a**) Micrograft at the beginning of shoot and root development; (**b**) well-developed micrograft; (**c**) micropropagated grapevine separated from rootstock.

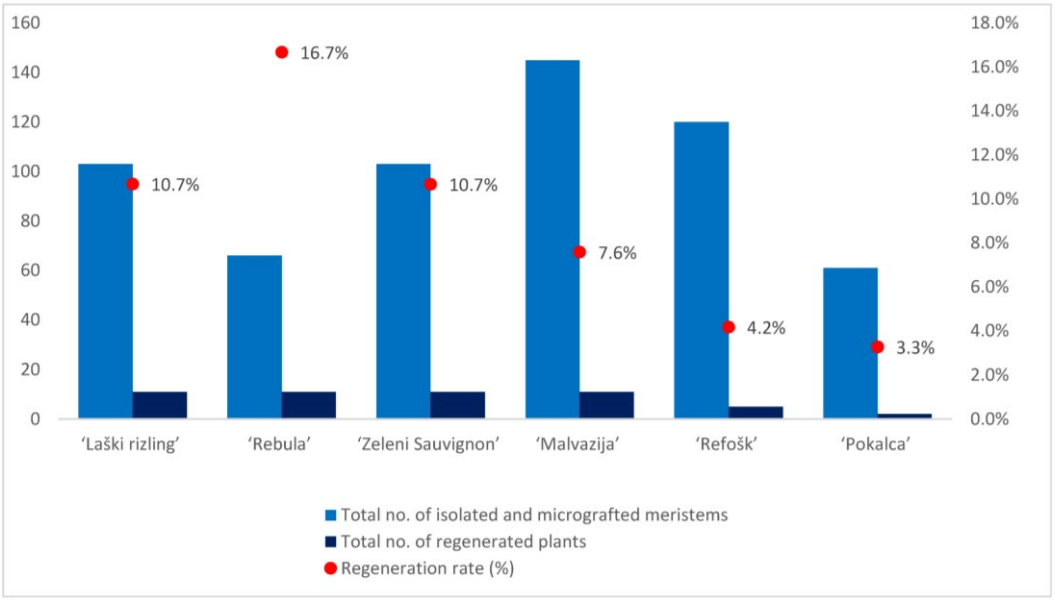


Figure 2. Number of isolated and micrografted meristems, number of regenerated plants, and regeneration rate per variety.

Table 2. Number of infected preclonal candidates before the sanitation process, number of tested vines after the sanitation process, number of virus/viroid-free vines, and elimination rate (%) per individual virus/viroid.

Virus/Viroid	No. of Infected Preclonal Candidates before the Sanitation Process	No. of Tested Vines after the Sanitation Process	No. of Virus/ Viroid-Free Vines	Elimination Rate (%)
GRSPaV	26	49	49	100
GPGV	26	49	49	100
GFLV	3	2	2	100
GLRaV-3	1	2	2	100
GFkV	13	26	26	100
GRVFV	19	33	33	100
GSyV-1	3	2	2	100
RBDV	4	11	11	100
HSVd	28	51	20	39.2
GYSVd-1	27	47	20	42.6

(**a**) (**b**)

Figure 3. Acclimatization of virus-free plants: (**a**) in rockwool plugs; (**b**) in mini greenhouses maintained in a growth chamber.

Figure 4. Acclimatized plants cultivated in pots in the greenhouse.

3. Discussion

In this study, 28 preclonal candidates from 6 grapevine varieties (*Vitis vinifera* L.), infected with various viruses and viroids, were included into the elimination process by in vivo thermotherapy followed by in vitro meristem tip micrografting.

According to Křižan et al. [5], in vivo thermotherapy is more advisable than in vitro thermotherapy because it is less labor-intensive and provides more apical segments, whereas the shorter duration of in vitro cultivation reduces the risk of somaclonal variability. The regeneration of grapevines directly from the meristem is often difficult [41]. To improve and accelerate this process, the micrografting technique was used.

The elimination of phloem-limited viruses through the meristem tip culture is particularly effective, whereas thermotherapy, which hampers virus replication and promotes virus RNA degradation [26,27], is desirable for the elimination of other viruses [42]. In order to increase the elimination efficiency, these methods should be combined, especially in cases of mixed infections.

GLRaV-3, the main causal agent of one of the most severe grapevine diseases, grapevine leafroll disease (GLD), is phloem-limited [3]. This virus was the least prevalent in our preclonal candidate set and only one preclonal candidate (Refošk 11/4P) was found infected. Twenty-eight meristems were isolated and micrografted, and only two (7.1%) regenerated and were found to be free of GLRaV-3. The complete eradication of GLRaV-3 was achieved in several studies by thermotherapy combined with shoot apices micrografting [22], somatic embryogenesis [16,19], and cryotherapy [15]. Different efforts for GLRaV-3 elimination had thermotherapy [6,11,23] and different chemotherapeutics in combination with or not with thermotherapy [11,23]. GFkV is the causative agent of fleck disease and is also phloem-limited [43]. In Slovenia, testing for GFkV is only obligatory for rootstocks. Panattoni and Triolo [6] reported that thermotherapy had no impact on the elimination of this virus in the rootstock Kober 5BB. Bota et al. [44] reported that the combination of either a high temperature during summer in the field, or thermotherapy in the growth chamber with shoot tip culture (1–3 mm), resulted in 25% and 20% GFkV-free plants, respectively, in the 'Manto Negro' variety. GFkV elimination efficiency for meristem tip culture from dormant buds (0.3 mm) was 100%, whereas larger meristems (0.8 mm)

resulted in a 50% lower elimination rate [9]. When thermotherapy was combined with shoot apices micrografting, complete elimination of the virus was achieved [22]. In our study, 13 GFkV-infected preclonal candidates were included into the sanitation process and 26 regenerants were obtained, which were all free of GFkV. Complete elimination was also achieved with somatic embryogenesis [10], repeated ribavirin treatment [13,45], the combination of ribavirin and oseltamivir [14], and ribavirin combined with thermotherapy [24]. Two fleck-similar viruses, GRVFV and GSyV-1, were detected for the first time in Slovenia; GRVFV was significantly more abundant [46]. GRVFV has been described to cause the mild chlorotic discoloration of leaf veins upon grafting on *V. rupestris* [43,47], whereas GSyV-1 was discovered in 2009 in an attempt to study viruses associated with decline symptoms in the 'Syrah' variety [48]. Although both viruses have been known for more than a decade, only one report on their elimination using a meristem tip culture and/or somatic embryogenesis has been published [10], reporting a 100% elimination, which is in accordance with our results. GFLV is the main causal agent of grapevine fanleaf disease, and is one of the most damaging grapevine viruses [1]. It is not phloem-limited and it is susceptible to heat treatment at approximately 37 °C in several rootstocks [5,6]. A meristem tip culture without thermotherapy also resulted in a high number of GFLV-free plants [8,21]. Salami et al. [21] obtained the best results when thermotherapy was combined with a meristem tip culture (0.3–0.5 mm). Thermotherapy followed by shoot apices micrografting resulted in 81% GFLV/ArMV-free plants [22]. In our study, three GFLV-infected vines of the 'Pokalca' variety were selected for therapy. The 'Pokalca' variety showed the lowest regeneration rate; only two regenerated plants were obtained. The successful elimination of GFLV had previously been achieved with somatic embryogenesis [17]. In contrast, Goussard and Wiid [49] reported that GFLV-free plants were obtained only when somatic embryogenesis was combined with thermotherapy. Chemotherapeutic agents (ribavirin and oseltamivir, independently or in a mixture) were unsuccessful in GFLV elimination from the 'Valerien' variety [14]. In contrast, Weiland et al. [50] reported high ribavirin efficiency (94%) in the 'Zalema' variety. GPGV is associated with grapevine leaf mottling and deformation disease (GLMD), which was discovered in Italy in 2012 [2]; two years later, its occurrence was reported in Slovenia [51]. It is an emerging virus in viticulture, but it has not yet been included in EU certification programs. Cytological analysis revealed the presence of GPGV particles in deep parenchyma cells [52]. Gualandri et al. [53] reported the successful sanitation of GPGV-infected vines by meristem tip culture with or without thermotherapy, whereas Turcsan et al. [10] reported that the virus elimination rates in nine vines of 'Trilla' and 'Sziren' varieties were 60% and 50%, respectively, when the meristem tissue culture was used without thermotherapy. In our previous study [40], GPGV was the most prevalent virus with 91.14% of infected vines included in the study, for which 26 samples were selected for therapy and all 49 regenerated plants were GPGV-free. Successful elimination has also been achieved through somatic embryogenesis [10] and repeated treatment with ribavirin [13]. Previous studies [27,42,54] indicated that GRSPaV and RBDV are difficult to eliminate in grapevines and raspberries, respectively, whether by thermotherapy, a meristem/shoot tip culture, or their combination, because they are presumed to infect meristematic tissues. Maliogka et al. [41] reported that successes in GRSPaV elimination by thermotherapy and shoot tip culture were significantly different in two Greek cultivars (39.62% and 92.85%), suggesting that virus elimination depends on genotype. In our study, 26 out of 28 samples were infected with this virus. Thermotherapy at 36–38 °C for at least 6 weeks and the isolation of smaller meristems, followed by micrografting to accelerate the regeneration process, resulted in the complete elimination of GRSPaV from all regenerated plants. Somatic embryogenesis was also very efficient in GRSPaV eradication [10,16,19,42], although Turcsan et al. [10] reported that when the same procedure was applied as in the other varieties with 100% virus eradication, the highest elimination rate for the 'Sziren' variety was 54%. Different proportions of GRSPaV-free plants were obtained with chemotherapy and its combination with other methods, such as thermotherapy and shoot tip culture [12,13,24,55]. The first report of natural infection

of vines with RBDV was published in Slovenia in 2003 [56]. Outside Slovenia, there have been few reports of grapevine infections by this virus [57–60]. Although several studies have reported RBDV elimination from raspberries using different methods [27,61–63], to the best of our knowledge, there are no reports of RBDV elimination from grapevines. In our sample set, four preclonal candidates of the 'Laški rizling' variety were found to be infected with RBDV; all of them were included in the therapy process, but the candidate Laški rizling 3/45B did not regenerate at all. A total of 11 regenerated 'Laški rizling' vines were obtained, all of which were RBDV-free.

Although the complete elimination of all eight viruses was achieved, sanitation rates for the widely distributed viroids HSVd and GYSVd-1 were much lower in our study, i.e., 39.2% and 42.6%, respectively, compared with virus elimination. Viroids accumulate at higher titers upon experiencing a high temperature; therefore, thermotherapy alone was unsuccessful in their elimination [18]. Different elimination rates for HSVd and GYSVd-1 by meristem tissue culture have been reported [7,10]. Treatment with ribavirin was unsuccessful [45]. Somatic embryogenesis completely eliminated both viroids from four Italian varieties [18]. Turcsan et al. [10] reported that somatic embryogenesis was more efficient in eradicating HSVd than GYSVd-1.

It can be concluded that the elimination success of viruses and viroids from vines depends on several factors. The generally high elimination rates and low regeneration rates found in our study could be because smaller meristems have low survival rates but higher efficiency in virus elimination. The low regeneration rate could be also linked to the fact that meristem isolation and micrografting techniques are difficult to handle and require a high level of expertise and rapid handling to avoid explant drying and oxidation problems. Overall, it is sufficient to obtain one virus-free, regenerated plant per candidate that can be further micropropagated.

With somatic embryogenesis, high risks of somaclonal variation exist, whereas chemotherapeutics may prove highly phytotoxic. In our study, limiting the duration of the in vitro cultivation phase through in vivo thermotherapy and plant regeneration from meristems reduced the risk of genetic instability. However, regenerated plants will be carefully monitored. In addition, the elimination success rate using combined thermotherapy and meristem tip micrografting is encouraging. It remains to be seen whether our virus-free preclonal candidates will remain negative for up to about three years, when they will be retested prior to their official certification.

4. Materials and Methods

4.1. Plant Material

Eighty-two woody cuttings of preclonal candidates, without exhibiting any visual morphological symptoms regarding viruses and viroids infections, were collected of the following grapevine varieties (*Vitis vinifera* L.): two reds, 'Refošk' ('Terrano') and 'Pokalca' ('Schioppettino'), and four whites, 'Laški rizling' ('Welschriesling'), 'Rebula' ('Ribolla Gialla'), 'Malvazija' ('Malvasia d'Istria'), and 'Zeleni Sauvignon' ('Sauvignon vert'). They were collected in February 2019, in the three vineyards (B, Baza; P, Pouzelce; G, Genebank) of the clonal center of Vrhpolje (STS; Vipava Valley, Primorska wine-growing region) where the clonal selection was conducted. However, preclonal candidates were selected in various vineyards in the Primorska region according to the rules on the marketing of material for the vegetative propagation of vines (Official Gazette of the RS N°93/05 and 101/20) and the OIV process for the clonal selection of vines (Resolution oiv-viti-564a-2017). Later, after vegetative propagation, they were planted in the vineyards of STS (45°50′02.2″ N 13°56′18.8″ E). One-bud cuttings were forced to bud burst and root in common tap water with no additional nutrients at room temperature (21–22 °C) at the Biotechnical Faculty, University of Ljubljana. After one month of rooting in water, well-rooted cuttings were planted into pots and transferred to the greenhouse, from which 28 plants were randomly selected for the sanitation study.

4.2. Virome Status of the Preclonal Candidates

Twenty-eight grapevine preclonal candidates were included in the virus/viroid elimination process (Table S1). The viromes of preclonal candidates were investigated using the HTS of virus- and viroid-derived small RNAs and were validated with RT-PCR and Sanger sequencing [40]. All candidates harbored mixed infections. A total of 26 out of 28 candidates were found to be infected with GRSPaV and GPGV. Moreover, 13 candidates were infected with GFkV, whereas 19 and 3 candidates were infected with two fleck-similar viruses, GRVFV and GSyV-1, respectively. GLRaV-3, the least prevalent virus, was only detected in Refošk 11/4P. Three candidates representing the 'Pokalca' variety were infected with GFLV. RBDV was present only in candidates representing the 'Laški rizling' variety. All candidates were found to be infected with HSVd, whereas GYSVd-1 was also present in all candidates, except for Zeleni Sauvignon 15/3P. The highest number of viral infections per candidate was eight, in Laški rizling 3/45B, and the lowest was three, in Malvazija 23/2P (Table S1).

4.3. Rootstock Source

Vialla seeds (*Vitis labrusca* × *Vitis riparia*) were provided by INRAE, Montpellier, France. Seeds were surface-disinfected in a laminar flow hood with a 1.66% solution of sodium dichloroisocyanurate (Sigma-Aldrich, St. Louis, MO, USA), supplemented with three drops of surfactant, Tween 20 (Duchefa Biochemie, Haarlem, The Netherlands), for 15 min with constant agitation. They were then washed three times with sterile, distilled water and plated (five seeds per plate) (Figure 5a) on a 1/4 MS basal salt medium [64], supplemented with 20 g/L of sucrose and solidified with 8 g/L of agar (all chemicals were obtained from Duchefa Biochemie). The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The sterile seeds were stored at 4 °C for at least two months to break dormancy. After stratification, seeds were allowed to germinate in their Petri dish in a growth chamber (LTH, Slovenia) at 25 °C, in the dark. The resulting etiolated hypocotyls (Figure 5b) were sectioned into 4–5 segments, each of which served as a rootstock (Figure 5c).

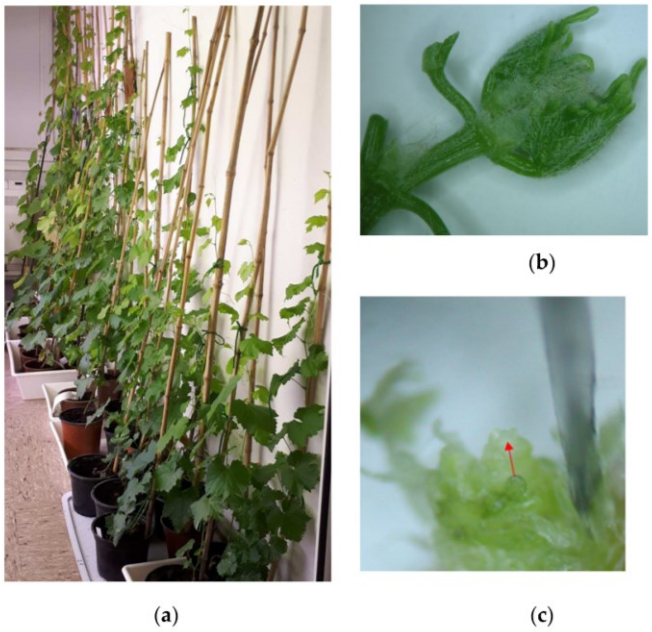
(**a**) (**b**) (**c**)

Figure 5. (**a**) Vialla seeds (*Vitis labrusca* × *Vitis riparia*); (**b**) etiolated hypocotyls of Vialla (*Vitis labrusca* × *Vitis riparia*); (**c**) sectioned hypocotyls into segments.

4.4. In Vivo Thermotherapy and In Vitro Meristem Tip Micrografting

Thermotherapy was performed in a growth chamber (Kambič, Slovenia) for a minimum of six weeks to a maximum of three months at a temperature of 36–38 °C, and a photoperiod of 16 h of light and 8 h of dark (Figure 6a). After heat treatment, the apical and axillary segments were sampled (Figure 6b) and surface-disinfected according to the following protocol. First, they were rinsed under tap water and immersed in 70% ethanol for 30 s and washed in sterile, distilled water. After the ethanol was removed, the plant material was treated in a 1.66% solution of sodium dichloroisocyanurate (Sigma-Aldrich), supplemented with three drops of the surfactant Tween 20 (Duchefa Biochemie) for 10 min

with constant agitation, and then rinsed three times with sterile, distilled water. Meristem tips (0.1–0.2 mm) were aseptically excised from infected buds under 10–50× magnification using a stereomicroscope (Nikon C-LEDS, Japan) (Figure 6c). The isolated meristem tips were immediately aseptically micrografted onto the sectioned hypocotyls (Figure 5c) under a stereomicroscope and inoculated on a half-strength MS medium including vitamins [64], supplemented with 30 g/L of sucrose and 8 g/L of agar (all chemicals were obtained from Duchefa Biochemie). The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The micrografts were incubated at 25 °C under a light intensity of 40 µmol/m^2/s in the growth chamber (LTH, Slovenia). The plant material obtained was micropropagated several times on a fresh medium with the same components.



(**a**) (**b**) (**c**)

Figure 6. (**a**) In vivo thermotherapy; (**b**) segment prepared after in vivo thermotherapy for meristem isolation; (**c**) grapevine meristem.

4.5. Verification of Virus and Viroid Elimination

Virus and viroid elimination rates were determined by examining the tissues of plants maintained in vitro for seven months. Total RNA was extracted from all regenerated plants using the Monarch RNA Total Miniprep Kit (New England Biolabs). RNA concentration, quality, and purity were checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences, MA, USA). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions. The PCR was performed in a 20 µL reaction volume containing 10.7 µL of nuclease-free water, 4 µL of 5× PCR buffer (Promega, Madison, WI, USA), 1.6 µL of $MgCl_2$ (Kapa Biosystems, Cape Town, South Africa), 1.6 µL of dNTP mix (10 mM of each of the 4 dNTPs) (Promega), 0.5 µL of each primer, 0.1 µL of KAPA Taq DNA polymerase (Kapa Biosystems), and 1 µL of cDNA. The primers used for testing after the sanitation experiment are listed in Table S2. Positive and negative (nuclease-free water) controls were used for each virus and viroid. Amplification was performed in a thermal cycler (Applied Biosystems, Waltham, MA, USA). Results were analyzed by electrophoresis on a 1.4% agarose gel in a 1× TBE buffer and visualized with UV light after staining with ethidium bromide.

4.6. Acclimatization

A few virus-free and well-developed in vitro plants per preclonal candidate were taken out of the tissue culture jars and washed with sterilized water to remove any adherent medium. They were then transferred to mini greenhouses in rockwool plugs with added perlite (Plagron) and kept in a growth chamber (Kambič, Slovenia) for two months at 25 °C with a photoperiod of 16 h of light and 8 h of darkness. During acclimatization, the plants were irrigated with MS including vitamins (Duchefa Biochemie). The vents on the mini greenhouse covers were gradually opened. The acclimatized plants were later transplanted into pots and cultivated under greenhouse conditions at the STS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11081064/s1. Figure S1: Agarose gels of eight viruses and two viroids testing after sanitation process; Table S1: Virome status of mother plants; Table S2: List of primers used for RT-PCR detection.

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2.1.4 Small RNA Sequencing and Multiplex RT-PCR for Diagnostics of Grapevine Viruses and Virus-like Organisms

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Metagenomic approaches used for virus diagnostics allow for rapid and accurate detection of all viral pathogens in the plants. In order to investigate the occurrence of viruses and virus-like organisms infecting grapevine from the Ampelographic collection Kromberk in Slovenia, we used Ion Torrent small RNA sequencing (sRNA-seq) and the VirusDetect pipeline to analyze the sRNA-seq data. The used method revealed the presence of: *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3*), Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fanleaf virus* (GFLV) and its satellite RNA (satGFLV), *Grapevine fleck virus* (GFkV), *Grapevine rupestris vein feathering virus* (GRVFV), *Grapevine Pinot gris virus* (GPGV), *Grapevine satellite virus* (GV-Sat), *Hop stunt viroid* (HSVd), and *Grapevine yellow speckle viroid 1* (GYSVd-1). Multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for validation of sRNA-seq predicted infections, including various combinations of viruses or viroids and satellite RNA. mRT-PCR could further be used for rapid and cost-effective routine molecular diagnosis, including widespread, emerging, and seemingly rare viruses, as well as viroids which testing is usually overlooked.

viruses

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Small RNA Sequencing and Multiplex RT-PCR for Diagnostics of Grapevine Viruses and Virus-like Organisms

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Abstract: Metagenomic approaches used for virus diagnostics allow for rapid and accurate detection of all viral pathogens in the plants. In order to investigate the occurrence of viruses and virus-like organisms infecting grapevine from the Ampelographic collection Kromberk in Slovenia, we used Ion Torrent small RNA sequencing (sRNA-seq) and the VirusDetect pipeline to analyze the sRNA-seq data. The used method revealed the presence of: *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fanleaf virus* (GFLV) and its satellite RNA (satGFLV), *Grapevine fleck virus* (GFkV), *Grapevine rupestris vein feathering virus* (GRVFV), *Grapevine Pinot gris virus* (GPGV), *Grapevine satellite virus* (GV-Sat), *Hop stunt viroid* (HSVd), and *Grapevine yellow speckle viroid 1* (GYSVd-1). Multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for validation of sRNA-seq predicted infections, including various combinations of viruses or viroids and satellite RNA. mRT-PCR could further be used for rapid and cost-effective routine molecular diagnosis, including widespread, emerging, and seemingly rare viruses, as well as viroids which testing is usually overlooked.

Keywords: *Vitis vinifera* L.; virome; sRNA-seq; mRT-PCR

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1. Introduction

Grapevine is one of the most susceptible plants to viral infections. More than 86 viruses belonging to different families and genera have been reported to infect grapevine [1], and their number is constantly growing. Recently, two novel members of the genus *Vitivirus* have been identified in South Africa [2].

Most grapevine viruses have an RNA genome, including viruses associated with four major and widespread disease complexes (infectious degeneration and decline, leafroll, rugose wood, and fleck disease complex) [3]. Viruses with a DNA genome have also been identified in grapevine, and they are associated with vein-clearing and vine decline syndrome [4], red blotch disease [5,6], roditis leaf discoloration [7], and fruit tree decline syndrome [8].

Viral pathogens are spread over long distances by infected material (nursery productions), whereas infections within a vineyard or an area are transmitted mechanically and by insects, mites, or nematodes [3]. Viruses and virus-like organisms can cause severe developmental and morphological malformations, affect grapevine physiological activity and metabolism, reduce yield, decrease quality of grapes and wines, and shorten vineyard life, resulting in high economic losses [9–12]. For example, estimated economic losses caused by *Grapevine leafroll-associated virus 3* (GLRaV-3) in California are more than USD 90 million annually [13]. Therefore, rapid, effective, and reliable detection is crucial to limit their spread.

High-throughput sequencing technology (HTS), which targets all nucleic acid types, enables rapid and accurate detection, including previously described and novel viruses and virus-like organisms [14–17]. An approach that enables virus discovery through HTS technology and assembly of small RNAs (small RNA sequencing, sRNA-seq) has proven to be highly efficient in the detection of new RNA and DNA grapevine viruses [4,7,18,19], virome studies [20–23], and to evaluate the efficacy of different elimination methods such as chemotherapy, somatic embryogenesis, and meristem tissue culture [24,25]. All in silico predicted grapevine viral infections are most commonly validated using RT-PCR [21–26]. Several other molecular diagnostic methods as well as immunological detection methods, and biological indexing are used in plant virology [27]. However, most routine diagnostic assays can only be used for detection of one target virus/virus-like organism. Multiplex RT-PCR (mRT-PCR)/multiplex PCR (mPCR), which enables simultaneous amplification of several viral entities in a single reaction, is less labor intensive, time saving and cost-effective, especially when a large number of samples needs to be tested for mixed infections. To date, mRT-PCR/mPCR has been used to detect various herbaceous and woody plant-infecting viruses and viroids [28–42], including those infecting grapevine [43–49]. However, mRT-PCR/mPCR has not been used for validation of HTS-predicted viral infections in grapevines thus far.

The aim of the presented work was to perform sRNA-seq for the diagnosis of grapevine viral pathogens in six grapevine varieties from the Ampelographic collection Kromberk, Slovenia, and to develop an mRT-PCR assay for the validation of sRNA-seq data that could be further used for rapid and cost-effective routine molecular diagnosis in large-scale surveys.

2. Materials and Methods

2.1. Plant Material

A total of 13 cuttings from six grapevine varieties, two red, 'Cipro' ('Rosenmuscateller') and 'Pokalca' ('Schioppettino'), and four white, 'Malvazija' ('Malvasia d'Istria'), 'Volovnik' ('Vela pergola'), 'Rebula' ('Ribolla gialla'), and 'Poljšakica', were collected from the Ampelographic collection Kromberk near Nova Gorica, Slovenia, in 2017 (Figure 1). Cuttings were sprouted in water at room temperature (21 °C) at the Biotechnical Faculty, University of Ljubljana. Developed young leaves were sampled and stored at –80 °C for further analysis.

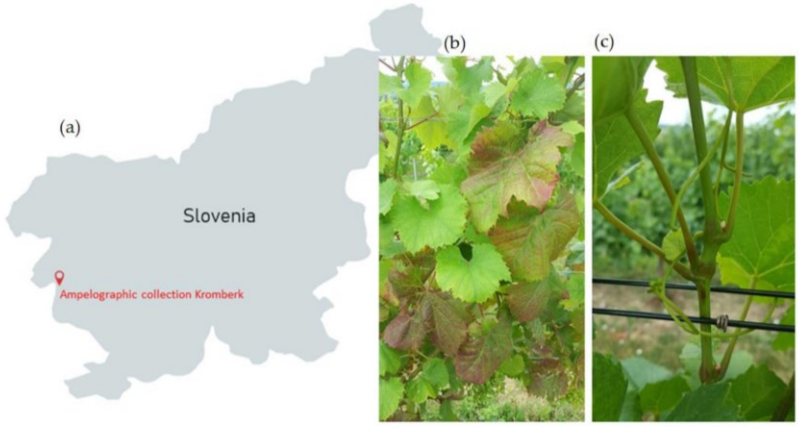

Figure 1. (**a**) Location of the Ampelographic collection Kromberk 45°57′40.8″ N 13°39′44.7″ E; (**b**) redding of the interveinal areas caused by GLRaV-3 on the 'Pokalca' variety; (**c**) shoot malformation (shorten internodes) caused by GFLV on the 'Rebula' variety.

2.2. Small RNA Isolation, Library Construction, sRNA-Seq and Bioinformatics Analysis

The selected samples were pooled together into four pools representing either samples of the same variety (L1, L2, and L3) or of different varieties (L4). Small RNAs (sRNAs) were isolated using mirVana™ miRNA Isolation Kit (Ambion, Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions for the enrichment of sRNAs. The quantity and quality of sRNAs were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Libraries of sRNAs were constructed using the Ion Total RNA-Seq Kit v2 (Ion Torrent™, Waltham, MA, USA) and were barcoded using the Xpress™ RNA-Seq Barcode 1–16 Kit (Ion Torrent™, Waltham, MA, USA) according to the manufacturer's instructions. The yield and size distribution of the amplified cDNA libraries were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Libraries were pooled at equimolar concentrations and prepared for sequencing using the Ion PI™ Hi-Q™ OT2 200 Kit and Ion PI™ Hi-Q™ Sequencing 200 Kit (Ion Torrent™, Waltham, MA, USA) according to the manufacturer's instructions. Sequencing was performed on Ion PI™ chips v3 using an Ion Proton™ System (Ion Torrent™, Waltham, MA, USA), according to the manufacturer's instructions. Raw sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject number PRJNA667593, BioSamples: SAMN16378719-SAMN16378722. The sRNA-seq data were analyzed using the VirusDetect pipeline with default parameters [50]. The pipeline performs reference-guided assembly using the Burrows–Wheeler Aligner (BWA) and de novo assembly using the Velvet Genomic Assembler. The plant virus database was used as reference, and the grapevine genome was selected to subtract host sRNAs.

2.3. mRT-PCR for Validation of sRNA-Seq Predicted Viral Pathogens

Confirmation of sRNA-seq-predicted infections was performed by mRT-PCR. Total RNA was extracted from 100 mg of frozen leaves using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA) according to the manufacturer's instructions. mRT-PCR was performed using the KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems, Wilmington, MA, USA). The reaction mixture was prepared using 12.5 µL of KAPA2G Fast Multiplex Mix (KAPA2G Fast HotStart DNA Polymerase, KAPA2G Buffer A, 0.2 mM of each dNTP, 3 mM $MgCl_2$, and stabilizers), 0.2 µL (0.1 µL for GRVFV) of each 10 µM forward and reverse primer (final concentration 0.08 µM; for GRVFV 0.04 µM), 1 µL of pooled cDNA, and nuclease-free water up to 25 µL. Primers are listed in Table 1. Amplification was performed in a thermal cycler (Applied Biosystems™, Waltham, MA, USA) under the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles consisting of a denaturation step at 95 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 1 min. The amplified products were analyzed by electrophoresis on 1.2% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator. Amplicons sizes were determined by comparison with the GeneRuler™ 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. List of primers used for mRT-PCR detection.

Viral Pathogen	Primer Name	Primer Sequence (5′-3′)	Product Size *	Tm *	GC % *	Amplified Region	Reference
GLRaV-3	LR3-8504V LR3-9445C	ATGGCATTTGAACTGAAATT CTACTTCTTTTGCAATAGTT	942 bp	51.81 48.91	30.00 30.00	CP	[51]
GLRaV-2	LRaV-2 (1) LRaV-2 (2)	AGGCGGATCGACGAATAC ATCCTGTCCGGCGCTGTG	821 bp	56.64 62.46	55.56 66.67	hsp70-like protein, p63	[52]
GPGV	Pg-Mer-F1 Pg-Mer-R1	GGAGTTGCCTTCGTTTACGA GTACTTGATTCGCCTCGCTCA	770 bp	58.21 60.47	50.00 52.38	MP/CP	[53]
GRVFV	GRVFV_6090F GRVFV_6605R	CATCGTTCTGATCCTCAGCC AGAGACGCTGACCATGCCAC	516 bp	58.14 62.51	55.00 60.00	polyprotein	[54]

Table 1. *Cont.*

Viral Pathogen	Primer Name	Primer Sequence (5′-3′)	Product Size *	Tm *	GC % *	Amplified Region	Reference
GFLV	GFLV_13_16_F GFLV_13_16_R	TGACACGTGCCTTTATTGGA CTCAAGTTGGGGAAGGTCAA	488 bp	57.45 57.34	45.00 50.00	polyprotein, segment RNA2	[23]
GLRaV-1	CPd2/F CPd2/R	GTTACGGCCCTTTGTTTATTATGG CGACCCCTTTATTGTTTGAGTATG	398 bp	58.42 57.88	41.67 41.67	CPd2	[55]
GRSPaV	RSP 48 RSP 49	AGCTGGGATTATAAGGGAGGT CCAGCCGTTCCACCACTAAT	330 bp	57.63 60.04	47.62 55.00	CP	[56]
GV-Sat	GV-Sat_for GV-Sat_rev	CCCGGACTCACATTAAGTCAA GCACAAGCGAGATAACAGCA	305 bp	57.67 58.92	47.62 50.00	ORF1, ORF2, 3′UTR	[57]
GFkV	GFkVf GFkVr	TGACCAGCCTGCTGTCTCTA TGGACAGGGAGGTGTAGGAG	179 bp	60.25 59.96	55.00 60.00	CP	[44]
satGFLV	FP3-F RP-R	GTGGSCCCGCRAGTGT TAAWGAGCAACCAAAATCCCA	870 bp	degenerative primer pair		hypothetical protein	[58]
HSVd	HSV-78P HSV-83M	AACCCGGGGCAACTCTTCTC AACCCGGGGCTCCTTTCTCA	~300 bp	62.13 63.34	60.00 60.00	complete genome	[59]
GYSVd-1	- -	TGTGGTTCCTGTGGTTTCAC ACCACAAGCAAGAAGATCCG	~368 bp	58.24 58.19	50.00 50.00	complete genome	[60]

* Determined with Primer-BLAST.

3. Results

3.1. Viruses and Virus-like Organisms Detected by sRNA-Seq

sRNA-seq of pooled grapevine samples resulted in 17,195,263–18,713,942 reads per pool (Table 2). Of the total reads, 50.12–67.22% were mapped to the grapevine genome, while 3.06–11.98% were mapped to viral genomes (Table 2). After concatenating unique reference-guided contigs and unique de novo assembled contigs and after removing redundancies, 461–1102 unique viral contigs were generated (Table 2). The total number of reference viral sequences identified by BLASTN search per library is presented in Table 2, while for each viral pathogen, it is presented in Table S1.

Table 2. Summary of results obtained with VirusDetect.

Library Label	Samples	BioSample ID	Total No. of Reads	Viral Mapping	Grapevine Mapping	Final Unique Viral Contigs	References Identified by BLASTN Search
L1	3 'Cipro'	SAMN16378719	17,398,590	1,835,271 (10.55%)	10,437,476 (59.99%)	461	62
L2	3 'Malvazija'	SAMN16378720	17,594,842	2,108,476 (11.98%)	8,818,062 (50.12%)	699	128
L3	3 'Volovnik'	SAMN16378722	18,713,942	571,865 (3.06%)	12,578,899 (67.22%)	882	98
L4	2 'Rebula' 1 'Pokalca' 1 'Poljšakica'	SAMN16378721	17,195,263	756,769 (4.40%)	10,508,279 (61.11%)	1102	203

Out of identified references, we selected one complete genome sequence per viral pathogen in each library that had the highest coverage. The used method revealed the presence of: *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fanleaf virus* (GFLV) and its satellite RNA (satGFLV), *Grapevine fleck virus* (GFkV), *Grapevine rupestris vein feathering virus* (GRVFV), *Grapevine Pinot gris virus* (GPGV), *Grapevine satellite virus* (GV-Sat), *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1). The highest number of viral entities (nine) was found in the library that was a mixture of three different varieties (L4). Eight viral pathogens were detected in the library of variety 'Cipro' (L1), while in the other two libraries (L2 and L3), the number of identified viral pathogens was seven (Table 3). The coverage with references from the database was between 61.99% (GRVFV, L1) and 99.96% (GLRaV-3, L2) (Table 3; Figure 2), with a sequencing depth between 7X (GRSPaV, L1) and 5313.2 X (satGFLV, L2) (Table 3). GLRaV-1, GLRaV-2, and GV-Sat were present only in one library (L1). GFkV was detected in L3 and L4, and GFLV and satGFLV were detected in L2 and L4. GFLV possesses a bipartite genome; thus, the sRNA-seq data for RNA1 and RNA2 are shown in Table 3. GLRaV-3 was detected in three libraries (L2, L3, and L4) and had the highest coverage (99.80–99.96%) among viruses in all three libraries. GRSPaV, GPGV, and GRVFV were

detected in all libraries. GRSPaV had low sequencing depth in all libraries (7X, 8.9X, 10.4X, and 9.7X, respectively). GRVFV had the lowest references coverage in all libraries (61.99%, 70.24%, 68.47%, and 70.21%, respectively). Considering viroids, HSVd was detected in all libraries, while GYSVd-1 was absent only in L2 (Table 3).

Table 3. Viruses and virus-like organisms detected with BLASTN search (VirusDetect pipeline).

Library Label	Detected Viral Pathogens	Reference Sequence	Reference Origin	Reference Length	Consensus Length	Reference Coverage (%)	No. of Contigs	Sequencing Depth	Nucleotide Identity (%)
L1	GLRaV-1	MG925332	France	18,863	18,608	98.65	36	343.8	94.22
	GLRaV-2	FJ436234	USA	16,486	16,463	99.86	8	1254.3	99.43
	GRSPaV	KX035004	France	8743	6058	69.29	64	7	95.84
	GPGV	KP693444	Czech Republic	7172	7089	98.84	12	586.3	95.96
	GRVFV	KY513702	Switzerland	6716	4163	61.99	85	21.4	92.6
	GV-Sat	KC149510	USA	1060	969	91.42	6	1567	95.74
	HSVd	KJ810551	Taiwan	309	309	100	4	1257.4	93.93
	GYSVd-1	KP010010	Thailand	389	389	100	4	1951.3	96.92
L2	GLRaV-3	MH814482	unknown	18,580	18,572	99.96	11	142.3	99.55
	GRSPaV	KX035004	France	8743	7978	91.25	52	8.9	98.05
	GPGV	MN458445	France	7269	7254	99.79	4	134.1	97.63
	GFLV (RNA1)	JX513889	Canada	7340	7302	99.48	127	897.8	90.47
	GFLV (RNA2)	MN496418	France	3743	3521	94.07	55	2738	90.77
	satGFLV	KR014543	Slovenia	989	933	94.34	13	5313.2	92.96
	GRVFV	MF000326	New Zealand	6701	4707	70.24	62	224	87.97
	HSVd	KY508372	Mexico	316	314	99.37	5	1645.3	93.26
L3	GLRaV-3	MH814485	unknown	18,656	18,618	99.8	8	253.9	98.5
	GRSPaV	JQ922417	USA	8758	8462	96.62	60	10.4	96.28
	GPGV	MN458445	France	7269	7254	99.79	5	114.4	96.79
	GFkV	AJ309022	Italy	7564	6654	87.97	83	113.8	94.28
	GRVFV	KY513701	France	6730	4608	68.47	111	50.1	90.54
	HSVd	KJ810551	Taiwan	309	309	100	3	1233.8	95.19
	GYSVd-1	KP010010	Thailand	389	389	100	2	1931.4	97.62
L4	GLRaV-3	MH814482	unknown	18,580	18,565	99.92	19	45.3	99.46
	GRSPaV	KX035004	France	8743	8427	96.39	67	9.7	96.4
	GPGV	MN458445	France	7269	7257	99.83	11	189.7	97.43
	GFLV (RNA1)	KX034843	France	7347	6957	94.69	100	411.7	89.95
	GFLV (RNA2)	MG418840	France	3777	3517	93.12	54	989.7	91.03
	satGFLV	KR014587	Slovenia	863	617	71.49	4	21.8	97.64
	GFkV	AJ309022	Italy	7564	6454	85.33	47	82.2	95.67
	GRVFV	KY513702	Switzerland	6716	4715	70.21	125	37.3	93.05
	HSVd	KJ810551	Taiwan	309	309	100	4	2036.3	94.34
	GYSVd-1	MF510389	Hungary	368	368	100	3	942.2	97.46

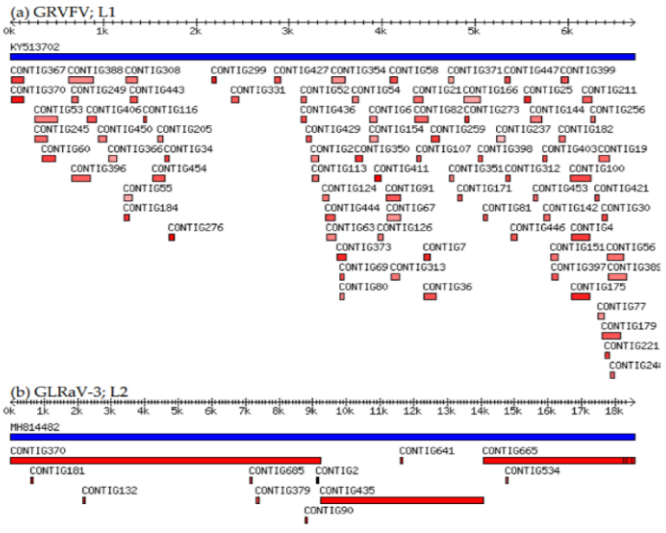

Figure 2. Virus-assembled contigs (red bars) mapped to complete reference genome sequence (blue bars): (**a**) GRVFV, virus with the lowest reference genome coverage (61.99%; L1); (**b**) GLRaV-3, virus with the highest reference genome coverage (99.96%; L2).

3.2. mRT-PCR for Validation of sRNA-Seq Predicted Viral Pathogens

Primer combinations with different expected amplified fragments were chosen for mRT-PCR to allow for differentiation on the agarose gel. All primers corresponded to those found in the literature (Table 1). The primers for GV-Sat and GFLV had been designed in our previous studies [23,57]. Several parameters such as primer concentration (0.04–0.2 µM), annealing temperature (55–60 °C), number of cycles (30–35), and amount of cDNA (1 µL and 2 µL) were optimized to determine the best conditions for simultaneous amplification of the predicted infections. As under-amplified amplicons were obtained with a higher primers concentration (0.2 µM), it was reduced to 0.08 µM. With this primers concentration (0.08 µM) and an annealing temperature of 55 °C, all predicted viruses were amplified in all libraries, although nonspecific banding patterns of approximately 250 bp were also observed. In an effort to reduce these background bands, the annealing temperature was increased to 58 °C, and the concentration of the primer pair (GRVFV_6090F/GRVFV_6605R) amplifying 516 bp of GRVFV polyprotein product (Table 1) was decreased to 0.04 µM. Better results were obtained with a lower amount of cDNA (1 µL), compared with 2 µL (data not shown). Under these conditions (primer concentration 0.08 µM and 0.04 µM for GRVFV, annealing temperature 58 °C, 35 cycles and 1 µL of cDNA), specific RT-PCR amplification products of the expected sizes were obtained for all viral pathogens in all libraries. Different combinations of viruses were amplified simultaneously in all four libraries: L1 (GV-Sat, GRSPaV, GLRaV-1, GRVFV, GPGV, and GLRaV-2); L2 (GRSPaV, GFLV, GRVFV, GPGV, GLRaV-3); L3 (GFkV, GRSPaV, GRVFV, GPGV, GLRaV-3); L4 (GFkV, GRSPaV, GFLV, GRVFV, GPGV, GLRaV-3) (Figure 3). In addition, different combinations of viroids/satGFLV were amplified simultaneously: L1 and L3 (HSVd, GYSVd-1), L2 (HSVd, satGFLV), L4 (HSVd, GYSVd-1, satGFLV) (Figure 3).

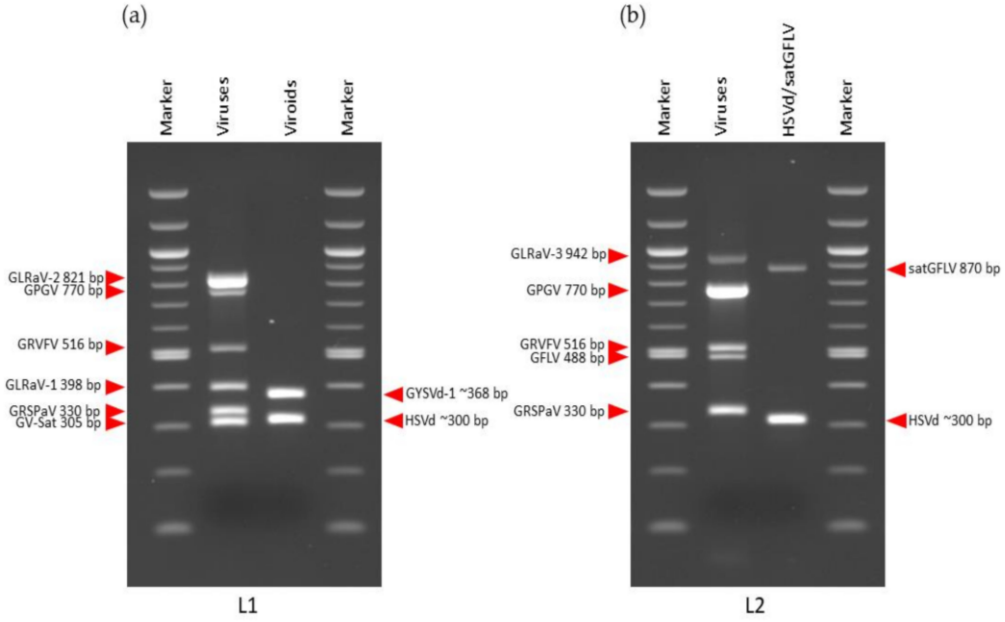

Figure 3. *Cont.*

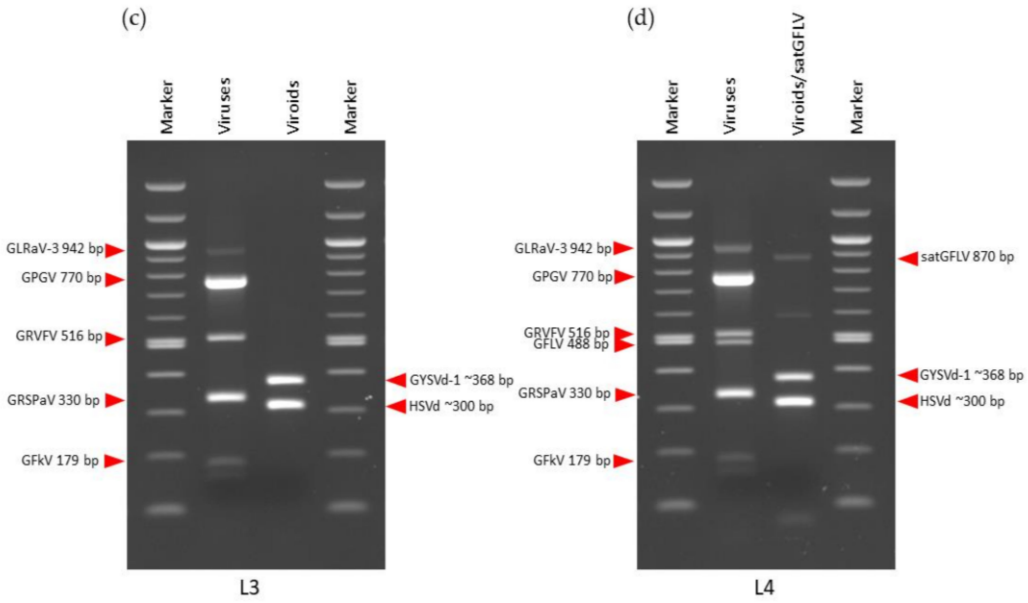

Figure 3. Validation of sRNA-seq-predicted viruses and virus-like organisms with mRT-PCR: (**a**) L1; (**b**) L2; (**c**) L3; (**d**) L4.

4. Discussion

Thirteen grapevines of six important autochthonous and local varieties were screened for viruses and virus-like organisms with sRNA-seq. A total of 70,902,637 reads were generated, and 5,272,381 (7.44%) were mapped to viral reference sequences, while 42,342,716 (59.72%) originated from grapevine. The BLASTN search of the unique viral-assembled contigs revealed the presence of widespread viruses associated with four major disease complexes, emerging virus, GV-Sat (first report in Slovenia) [57], as well as worldwide-distributed viroids. A high number of contigs and their short length were observed for GRSPaV, GFLV, GRVFV and GFkV, which is in accordance with our previous study [23], and may be related with their high genetic variability. For example, GFLV (RNA1) reference sequence (JX513889), which is 7340 nt long, was covered with 127 contigs (Figure S1). In contrast, GLRaV-2 reference sequence (FJ436234), which is 16,486 nt long, was covered with only eight contigs, from which one was long enough to cover 99.86% of the references (Figure S2).

Additionally, in this study, we described the application of the mRT-PCR approach for validation of the sRNA-seq data. Simultaneous amplifications of different combinations of nine viruses or two viroids and satGFLV were performed. According to the KAPA2G Fast Multiplex Kit protocol, employed primers should have a similar temperature melting (Tm) and GC content of 40–60%. In our study, the Tm of primers used for virus amplification was not similar; the lowest Tm had a primer pair for GLRaV-3 amplification (51.81 °C for forward and 48.91 °C for reverse primer) (Table 1). Considering GC content, according to the protocol primers, a GC content higher than 60% may require higher and/or longer denaturation temperature and time, while a GC content lower than 40% may require increased primer concentrations, additional $MgCl_2$ and/or annealing temperature lower than 60 °C (KAPA2G Fast Multiplex PCR Kit, https://www.n-genetics.com/products/1104/1023/12664.pdf, accessed on 1 April 2022). In this study, the lowest GC content had again primers for GLRaV-3 amplification (30%), while all other primers for amplification of predicted viruses in L2, L3, and L4 had GC content in the range of 40–60%. In L1,

all primers for virus amplification had a GC content of 40–60%, except for the reverse primer of GLRaV-2 (66.67%) (Table 1). Although the primers in our study had differences in Tm and GC content in all cases, successful amplifications were obtained (Figure 3). Thus far, the highest number of grapevine viral pathogens amplified using mRT-PCR was nine (ArMV, GFLV, GVA, GVB, GRSPaV, GFkV, GLRaV-1, GLRaV-2, and GLRaV-3) [44,47]. Nassuth et al. (2000) [43] reported simultaneous detection of ArMV, GRSPaV, and malate dehydrogenase mRNA for GLRaV-3, GVA, GVB and RubiscoL mRNA. Simultaneous detection of grapevine-infecting viruses belonging to the *Nepovirus* genus were reported by Digiaro et al. (2007) [45]. Hajizadeh et al. (2012) [46] developed mRT-PCR for simultaneous detection of five grapevine viroids. Simultaneous amplification of viruses and viroids have also been reported: GFLV, GYSVd-1, and GYSVd-2, in addition, HSVd was included instead of plant internal control [48], and for GPGV, GFkV, HSVd and GYSVd-1 [49]. In this study, a cumulative number of viral pathogens was minimum 7 and maximum 9 per library. Considering that viroids may form dimers or even multimers that are also visible on agarose gel, the viroids were separately amplified. Some studies found that mRT-PCR is less sensitive compared to singleplex RT-PCR, which specifically targets one viral pathogen [61,62]. Lower detection sensitivity has also been reported when more than five primer pairs were used in a single reaction to detect stone fruit viruses [36].

However, in this study, we showed that mRT-PCR is highly effective, reliable, and sensitive, enabling validation of all viral pathogens predicted with sRNA-seq.

High-throughput screening and high-throughput validation of viral entities for some important old grapevine varieties from the Ampelographic collection Kromberk, Slovenia, was performed. The mRT-PCR protocol described herein provides a simple, time-saving, cost-efficient method for the rapid and reliable validation of sRNA-seq data and successful detection of viral pathogens belonging to different families and genera.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14050921/s1. Table S1: Number of reference viral sequences identified by BLASTN search (VirusDetect pipeline); Figure S1: GFLV (RNA1)-assembled contigs (red bars) mapped to reference sequence (JX513889) (blue bars); Figure S2: GLRaV-2-assembled contigs (red bars) mapped to reference sequence (FJ436234) (blue bars).

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2.1.5 First report of grapevine satellite virus in Slovenia

Miljanić V., Jakše J., Beber A., Rusjan D., Škvarč A., Štajner N. 2021. First report of grapevine satellite virus in Slovenia. Journal of Plant Pathology, 103: 1329–1330

The study of the virome of 13 samples of six grapevine varieties not included in the clonal selection process, revealed the presence of grapevine satellite virus, which was not previously confirmed in Slovenia. In silico results were validated with RT-PCR and Sanger sequencing. The infection was confirmed in three samples of variety 'Cipro'. The sequences were deposited in NCBI under the numbers MW446942-MW446944.

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DISEASE NOTE

First report of grapevine satellite virus in Slovenia

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Grapevine satellite virus (GV-Sat) is a member of the genus *Virtovirus*. It was first identified in California with the application of high-throughput sequencing. The reported presence of GV-Sat was 3% for a set of 346 analysed samples (Rwahnih et al. 2013). Recently, GV-Sat was reported on cv. 'Askeri' from Iran that was being held in the INRA collection in France (Candresse et al. 2017), and HTS of virus-derived small RNAs uncovered GV-Sat in two of 18 libraries for Hungarian grapevine samples that were collected from 14 vineyards (Czotter et al. 2018). Its genome encodes two open reading frames (ORFs) that may be translationally coupled through overlapping start and stop codons. Candresse et al. (2017) demonstrated the existence of multimeric forms of GV-Sat.

Thirteen grapevine samples representing six Slovenian traditional cultivars (cvs. 3 'Cipro', 3 'Malvazija', 3 'Volovnik', 2 'Rebula', 1 'Pokalca', and 1 'Poljšakica') were randomly collected from the ampelographic collection in Kromberk near Nova Gorica, Slovenia. Samples were analysed by deep sequencing of virus derived small RNAs. Small RNAs were isolated by enrichment procedure using the mirVana miRNA Isolation Kit (Ambion, Life Technolgies). cDNA libraries were constructed using an Ion Total RNA-Seq kit, followed by sequencing on the Ion Torrent platform. Bioinformatic evaluation was done using the bioinformatics pipeline VirusDetect (Zheng et al. 2017). According to the VirusDetect pipeline, GV-Sat was detected only in cv. 'Cipro' and was represented by six contigs and an average sequence depth of 1567X (no. of aligned reads was 55,410). It showed the highest representation of the reference (91.4%) and nt identity (95.74%) with the American complete genome sequence of the AUD46129 isolate (KC149510). Confirmation of HTS results of predicted GV-Sat infection was performed using RT-PCR. The presence of GV-Sat was confirmed in all three analysed samples of cultivar 'Cipro' (Cipro_AV1, Cipro_AV2, and Cipro_AV3) using primer pair GV-Sat_for (5′-CCCGGACTCACATTA AGTCAA-3′) and GV-Sat_rev (5′-GCACAAGCGAGA TAACAGCA-3′) designed in this study, targeting the end of ORF1, complete ORF2, and beginning of 3′UTR. Expected fragments of 305 bp were obtained and further bidirectionally Sanger sequenced. All three GV-Sat sequences were identical (GenBank MW446942-MW446944). They shared the highest nt identities of 99.65% with the MH802035 isolate of unknown origin. The helper virus of GV-Sat was not identified, but previous studies reported co-infection of this virus with vitiviruses and GLRaVs, which potentially indicates that these viruses have helper virus function for GV-Sat. In our study, in addition to GV-Sat, grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine Pinot gris virus (GPGV), hop stunt viroid (HSVd), and grapevine yellow speckle viroid-1 (GYSVd-1) were also identified. Our samples were infected with GLRaVs, but they were not infected with vitiviruses, which may suggest that they do not have a helper function for GV-Sat.

To our knowledge, this is the first report of grapevine satellite virus infecting grapevine in Slovenia. The results present new evidence of rare infections detected in this region and will help us to further define the research necessary to determine the distribution of this virus and develop management measures in this country.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All authors declare no conflict of interest.

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3 DISCUSSION AND CONCLUSIONS

3.1 DISCUSSION

Preclonal candidates (the 'best' vines with certain characteristics), obtained after a mass selection of grapevines that did not show visible symptoms associated with the diseases, were analyzed in the first part of the dissertation using HTS technology. HTS is a powerful metagenomic approach that allows the detection of all viral entities even in asymptomatic plants, and even if they are present in low titer. sRNA-seq of 12 libraries yielded 112,647,551 reads. In the VirusDetect pipeline, 107,016,121 reads were processed. Of the total reads, 2.24% mapped to viral genomes. Nine viruses: GFLV, GLRaV-3, GRSPaV, GFkV, GSyV-1, GRVFV, GRGV, GPGV, RBDV, and two viroids: HSVd and GYSVd-1 were detected by the method used. Considering that a considerable number of viral entities were detected, including those that require mandatory testing in Slovenia, in the second part of the dissertation we developed a protocol for the generation of healthy vines by in vivo thermotherapy and in vitro meristem tip micrografting. Meristem tip culture is effective for viruses restricted to the phloem, whereas thermotherapy is desirable for eliminating viruses that can infect various tissues. In preclonal candidates we detected both (phloem and non-phloem-limited viruses), therefore to increase the efficiency of elimination, we combined both methods. Here we present HTS results on preclonal candidates, genetic diversity studies, potential introduction of the detected viruses and viroids into Slovenia, co-infections, and elimination rates achieved with the chosen biotechnological approach.

GFLV, one of the most detrimental viruses, was detected in library 013 ('Zeleni Sauvignon' variety) and library 016 ('Pokalca' variety). In library 013, VirusDetect (BLASTN search) identified 11 complete RNA1 segment sequences, which were 78.7-92% covered with 23-31 contigs and sequencing depth of 149.6X-155.7X; and 4 complete RNA2 segment sequences, which were 77.4-90.7% covered with 11 or 12 contigs and sequencing depth of 211.9-218.2X. In library 016, the VirusDetect identified 7 complete and 1 partial RNA1 sequences which were 58.8-71.7% covered with 13-21 contigs and a sequencing depth of 719.8X-785.8X; and 16 complete RNA2 segments that were 73-92.1% covered with 13-20 contigs and a sequencing depth of 752.5X-890.3X. Predicted infections were validated using the primer pair C2647/H2042 targeting a 606 bp long fragment of RNA2 containing a partial CP gene (Fattouch et al., 2001). One sample from library 013 (Zeleni Sauvignon 16/3P) and three samples from library 016 (Pokalca 3/4P, 3/5P, 3/6P) were infected. Each sample infected with GFLV was sequenced, and nt differences between red and white varieties were more than 12%. Analyzing the same genome fragment as we did, Fattouch et al. (2005) reported that the differences between sequences were more than 11%. Naraghi-Arani et al. (2001) found that genetic diversity in the 1557 bp genome region of RNA2 in 14 isolates were from 11 to 13%. While Elbeaino et al. (2014) found that sequence variability in the HP was as high as 41% among isolates. In Slovenia, high genetic variability was found within the RNA2 segment of nine samples of the variety 'Volovnik' (Pompe-Novak et al., 2007).

The high genetic variability indicates the quasispecies nature of the genome (Naraghi-Arani et al., 2001). In addition, we found that the sequenced fragments were longer than the expected 606 bp, which is consistent with the results of Fattouch et al. (2005). The authors reported that this may indicate that RT-PCR products represented a population structure with more restrictotypes. Considering that the generated sequences showed large differences among varieties and also with the sequences from GenBank database, a novel primer pair was designed in this study, also in the RNA2 segment, and additional two samples from library 013 (Zeleni Sauvignon 26/1P and 26/2P) and four samples from library 016 (Pokalca 9/2P, 9/3P, 9/26G, 9/27G) were confirmed as infected. Phylogenetic analysis showed that the GFLV isolates were grouped into two clusters, consistent with the results of Panno et al. (2021). The Slovenian isolates generated in this study grouped into major clade in 2 subclades (one subclade 'Zeleni Sauvignon'; the other 'Pokalca'). Moreover, the phylogenetic tree showed certain degree of variability between our isolates and those from GenBank, and our isolates were closer to those from Italy and France than to those from Slovenia that had been previously characterized (Pompe-Novak et al., 2007). Testing on GFLV is obligatory in all certification programs. Therefore, various methods have been used for its elimination from grapevines: thermotherapy (Křižan et al., 2009; Salami et al., 2009; Panattoni and Triolo, 2010), meristem tissue culture (Youssef et al., 2009; Salami et al., 2009), combination of thermotherapy and meristem tissue culture (Salami et al., 2009), combination of thermotherapy with shoot apices micrografting (Spilmont et al., 2012), chemotherapy (Weiland et al., 2004; Guţa et al., 2017), somatic embryogenesis (Gambino et al., 2009), and combination of thermotherapy and somatic embryogenesis (Goussard and Wiid, 1992). In our study, 3 preclonal candidates of the 'Pokalca' variety were included in the elimination process (Pokalca 3/4P, 3/6B, and 9/2G). Pokalca 9/2G did not regenerate. Pokalca 3/4P and 3/6P, yielded one meristem per candidate, and both regenerated plants were GFLV-free. In addition, the 'Pokalca' variety had the lowest regeneration rate (3.3%) compared to the other varieties.

Among the preclonal candidates, GLRaV-3, a representative virus of the genus *Ampelovirus*, was detected only in library 010 ('Refošk' variety). VirusDetect (BLASTN search) identified only one complete genome sequence (GQ352631) from South Africa. The reference was 98.6% covered with 23 contigs and a sequencing depth of 39.6X. ORF2 is known to be absent in some isolates (Burger et al., 2017). In the 010 library, a unique viral contig was observed at position 9243-9811 that corresponded to ORF2. The presence of the virus was validated with RT-PCR using two primer pairs LR3-8504V/LR3-9445C (Fajardo et al., 2007) amplifying the CP gene and LC1/LC2 (Turturo et al., 2005) amplifying the HSP70h gene, and only Refošk 11/4P was infected. It was found in co-infection with GRSPaV, GPGV, GFkV, GRVFV, HSVd, and GYSVd-1. RT-PCR product obtained with LR3-8504V/LR3-9445C was Sanger sequenced. The 822 nt sequence (partial CP) was compared with isolates available in the database and it showed 99.76% nt identity with isolates from Europe (Portugal and Greece), North America (United States and Canada), Asia (Pakistan),

and three isolates of unknown origin. The phylogenetic tree was constructed based on the CP sequences and included our isolate and 32 isolates from GenBank. The phylogenetic tree showed that the isolate Refošk 11/4P belonged to the major phylogroup and was the closest to the isolate from Portugal. CP gene is commonly used in phylogenetic studies (Turturo et al., 2005; Jooste et al., 2010; Sharma et al., 2011; Wang et al., 2011; Gouveia et al., 2011; Kumar et al., 2012; Bester et al., 2012; Liu et al., 2013; Lehad et al., 2015; Crnogorac et al., 2021), as well as HSP70h (Turturo et al., 2005; Fuchs et al., 2009; Jooste et al., 2010; Kumar et al., 2012; Lehad et al., 2015; Čarija et al., 2022). Considering that GLRaV-3 is one of the most economically important viruses, numerous studies have been published on its elimination, including: thermotherapy (Panattoni and Triolo, 2010; Panattoni et al., 2011; Hu et al., 2020), combination of thermotherapy and shoot apices micrografting (Spilmont et al., 2012), cryotherapy (Bi et al., 2018), somatic embryogenesis (Gambino et al., 2006; Bouamama-Gzara et al., 2017), chemotherapy (Panattoni et al., 2011; Hu et al., 2020), and combination of thermotherapy and chemotherapy (Hu et al., 2020). In our study, 28 meristems were isolated from the infected mother plant (Refošk 11/4P) and micrografted; 2 plants were regenerated and were both free of GLRaV-3.

In preclonal candidates, GRSPaV was detected in 10 libraries based on sRNA-seq data. In 8 libraries, VirusDetect identified 4 (008 library; 'Rebula' variety) to 40 (005 library; 'Laški rizling' variety) complete and partial (mainly CP gene) reference sequences per library. The highest coverage of complete reference sequences was 48.4% (013 library; 'Zeleni Sauvignon' variety) - 99.1% (006 library; 'Refošk' variety). They were covered with a high number of contigs (41-86), and a low sequencing depth (5.3X-16.6X). Only partial and/or complete CP gene sequences were identified in libraries 012 ('Zeleni Sauvignon' variety), and 015 ('Malvazija' variety). The virus was not detected in libraries 007 ('Rebula' variety) and 014 ('Malvazija' variety). A primer pair targeting the highly conserved CP gene (RSP 52/RSP 53) was used to validate GRSPaV (Nolasco et al., 2000). GRSPaV was confirmed in 10 libraries where it was predicted and additionally in two libraries where it was not predicted. Problems in detecting this virus with sRNA-seq technology have also been reported in several studies (Czotter et al., 2018; Turcsan et al., 2020; Demian et al., 2020). The possibility that its concentration was low was ruled out because each sample in library 007 was infected. The reason why GRSPaV was not detected with sequencing of sRNAs may have a deeper biological background, because it is known that GRSPaV can be beneficial to grapevine and that mutual adaptation exists (Gambino et al., 2012). Overall, 88.61% of the preclonal candidates were infected with GRSPaV. Direct sequencing of the RT-PCR products was not possible due to the presence of different genetic variants, so the RT-PCR products were ligated into the vector and transformed into bacterial competent cells. GRSPaV exhibits high heterogeneity and has a wide range of sequence variants (Meng et al., 1999, 2006; Nolasco et al., 2006; Glasa et al., 2017). In our study, the results showed that at least 3 genetic variants were present in the same sample. The same result was obtained by Glasa et al. (2017). Laški rizling 3/45B had the highest genetic variability (17.14%) and

Malvazija 20/48P (6.32%) had the lowest. Interestingly, VirusDetect identified the highest number (40) of sequences from the database in the 'Laški rizling' library, while in one 'Malvazija' library (014) GRSPaV was not detected with sRNA-seq and in the other 'Malvazija' library (015) only one CP gene sequence was identified. Although the virus was mainly studied in the highly conserved Hel domain or CP, several groups of sequence variants were found. Additionally, new variants were found in the RdRp domain (Meng and Rowhani, 2017). Our phylogenetic analysis showed differential clustering of genetic variants even when they originated from the same sample, and no clustering by geographic origin was observed, which is consistent with other studies (Nolasco et al., 2006; Alabi et al., 2010). In plant virology, GRSPaV is thought to be benign and may also be beneficial to grapevine. However, because GRSPaV has high genetic variability, and was found in association with RSP (Zhang et al., 1998; Meng et al., 1998), vein necrosis disease (Bouyahia et al., 2005), and the severe decline of 'Pinot noir' (Lima et al., 2009) and 'Syrah' varieties (Al Rwahnih et al., 2009; Beuve et al., 2013), its real impact on grapevine is not yet known, and testing is therefore mandatory in all certification programs. Previous studies indicated that GRSPaV is difficult to eliminate, because it is presumed that this virus is able to infects meristems (Gribaudo et al., 2006; Meng and Rowhani, 2017; Hu et al., 2021). However, it should be noted that its elimination also strongly depends on the variety. For example, the combination of thermotherapy and shoot tip culture resulted in 39.62% and 92.85% GRSPaV-free plants in two Greek varieties, 'Mantilaria' and 'Prevezaniko', respectively (Maliogka et al. 2009b). Other methods used for its elimination so far are: somatic embryogenesis (Gambino et al., 2006; Gribaudo et al., 2006; Bouamama-Gzara et al., 2017; Turcsan et al., 2020), chemotherapy (Skiada et al., 2013; Komínek et al., 2016; Hu et al., 2021), combination of thermotherapy and chemotherapy (Hu et al., 2021), and combination of chemotherapy and shoot tip culture (Hu et al., 2018). In our study, 26 of 28 samples included in the elimination process were infected with GRSPaV, and by in vivo thermotherapy (36-38 °C) and in vitro meristem tip micrografting (0.1-0.2 mm), all regenerated plants (49) were GRSPaV-free.

GFkV was predicted in eight libraries. In seven libraries, VirusDetect identified 4-10 reference sequences from the database per library. In library 011 ('Refošk' variety) only one partial replicase gene was identified, with a coverage of 33.3%. For the complete reference genome sequences, the lowest coverage was in the 'Malvazija' variety (libraries 014 and 015), 30.4% and 32.2%, respectively, while coverage in the other libraries was 76.9-82.1%. Many contigs (36-60) and their short length were observed in all libraries. The HTS results were validated with RT-PCR using the primer pair GFkV-U279/GFkV-L630 targeting the replicase gene (Shi et al., 2003). Thirty-four products were obtained and Sanger sequenced but 7 products belonged to fleck-similar virus (GRVFV), which is consistent with the results reported by Czotter et al. (2018). Phylogenetic analysis, performed on the partial replicase gene sequences of 25 Slovenian isolates and 18 reference isolates from GenBank, showed clustering into two groups. The Slovenian isolates were within the both groups. Interestingly, the isolates clustered according to variety, with the exception of 'Laški rizling'. GFkV is

graft-transmissible; the vectors are unknown (Sabanadzovic et al., 2017). Isolates analyzed in this study had the highest nt identity and clustered with isolates from neighboring countries and the United States, from which untested rootstocks were imported (Hrček, 1977) and predecessors of our isolates were grafted onto these rootstocks, suggesting that infection occurred via propagation material and grafting. In addition, propagation material produced in Slovenia was exported to geographically nearby countries. In Slovenia, testing on GFkV is obligatory only for rootstocks. GFkV is a phloem-limited virus, and with shoot/meristem tissue culture different eliminations efficacy have been reported depending mainly on the size of the shoots/meristems. Shoots (1-3 mm) resulted in 20% or 25% of GFkV-free plants (in combination with thermotherapy in the growth chamber and thermotherapy during summer in the field, respectively) (Bota et al., 2014); meristems (0.8 mm) resulted in 50% GFkV-free plants, while meristems (0.3 mm) resulted in 100% GFkV elimination (Kim et al., 2017). Complete eradication was also achieved by combining thermotherapy and shoot apices micrografting (Spilmont et al., 2012). Thermotherapy alone (Panattoni and Triolo, 2010; Hu et al., 2021), somatic embryogenesis (Turcsan et al., 2020), chemotherapy (ribavirin, repeated ribavirin treatment, combination of ribavirin and oseltamivir) (Komínek et al., 2016; Guţa et al., 2017; Hu et al., 2021), and ribavirin in combination with thermotherapy (Hu et al., 2021) have also been used for its elimination.

GRGV was discovered in 'Red Globe' variety from Southern Italy and in two samples of an unknown variety from Albania, and the virus was studied in the domains RdRp and MTR (Sabanadzovic et al., 2000). Recently, the whole genome of GRGV was obtained (Cretazzo and Velasco, 2017). Comparing the whole GRGV genome sequences with the available RdRp and MTR domains, Cretazzo and Velasco (2017) found that the RdRp gene region corresponded to GRGV, while the MTR gene corresponded better to GRVFV, suggesting that the same samples were simultaneously infected with both viruses. This virus is less studied because it is asymptomatic in *V. vinifera* and *V. rupestris* (Cretazzo and Velasco, 2017). When our analysis was performed, only four complete or nearly complete GRGV sequences were available in NCBI, three from Spain (KX109927, KX171167, and NC_030693) and one from Brazil (KX828704). In this study, GRGV was detected in two libraries of 'Refošk' variety (006 and 010). In both libraries, unique viral contigs were compared with these four sequences; the highest coverage in library 006 was 63.6% (KX171166) and in library 010 was 45.8% (KX109927). In addition, the MTR domain (AJ249360) was identified in library 010, which is indeed the domain of GRVFV (Cretazzo and Velasco, 2017). HTS results were validated with primers RG6061F/RG6801R (Cretazzo and Velasco, 2017) and 2 samples from library 006 (Refošk 10/2B and Refošk 10/3B) and 1 sample from library 010 (Refošk 9/5P) were infected. The virus was amplified at 40 cycles. Partial CP gene was Sanger sequenced and phylogenetic analysis showed that our isolates clustered separately, probably due to the few sequences deposited in NCBI. No studies regarding its elimination have been published.

El Beaino et al. (2001) reported that an unknown pathogen in Greek vines, that caused unusual symptoms reminiscent of asteroid mosaic upon grafting onto *V. rupestris*, was related to grapevine fleck virus-like viruses, but it was different enough which required further studies. The Greek virus was later characterized at the 3′ end and its name - GRVFV was proposed (Abou Ghanem-Sabanadzovic et al., 2003). In our study, GRVFV was detected in eleven libraries, being absent only in library 009 ('Malvazija' variety). The VirusDetect (BLASTN search) identified between 5 and 25 complete and partial reference sequences per library, with the exception of library 013, where only one partial reference was identified with a coverage of 30.2%. Complete reference sequences were covered with 30-94 contigs and sequencing depth of 5.4X-93.3X. The low genome coverage (61%) and low sequencing depth (13X) for this virus was also obtained in the study by Saldarelli et al. (2015) using sRNA-seq. The authors reported that the data indicated limited GRVFV replication in the analyzed tissue. The presence of the virus was validated in all predicted libraries. GRVFV was confirmed in the 44 samples by RT-PCR, and all of them were Sanger sequenced. Twenty-two sequences were of lower quality, likely due to the presence of different genetic variants in the same samples (a similar situation was also observed for GRSPaV), and were excluded from further analysis. The other 22 high-quality sequences were deposited in NCBI. The overall average divergence between the 22 Slovenian GRVFV polyprotein partial sequences was 10.9% ± 0.9%. High average divergence was also reported for Slovak GRVFV isolates (11.9% ± 0.9%) (Glasa et al., 2019). Phylogenetic analysis of 22 partial sequences from GenBank and 22 Slovenian isolates suggested the existence of two molecular groups, consistent with the results reported by Glasa et al. (2019), and some of our isolates clustered with isolates from Slovakia and France, but the majority clustered separately. Although GRVFV has been known for two decades, only one report of its elimination by somatic embryogenesis in 'Muscat Ottonel' variety has been published recently (Turcsan et al., 2020). In our study, 19 candidates infected with GRVFV were included in therapy process, 33 regenerated plants were obtained and all were GRVFV-free.

GSyV-1 was discovered in California in 2009 during the study of severe decline of 'Syrah' variety using HTS (Al Rwahnih et al., 2009). In a contemporary study conducted in Southeastern United States, the same virus named as grapevine virus Q (GVQ) was discovered in asymptomatic muscadine grape (*V. rotundifolia* Michx.) (Sabanadzovic et al., 2009). The same study reported that this virus is also infective for summer grape (*V. aestivalis*) and wild blackberry (*Rubus* sp.). Two complete genome sequences derived from the above studies, FJ436028 (GSyV-1) and FJ977041 (GVQ), deposited in NCBI, have 99.09% nt identity (99% query coverage), confirming that these two viruses are the same species. In addition, GSyV-1 exhibits permuted and non-canonical organization of RdRp motifs (C → A → B), a feature not previously reported in plant virology, as this feature is associated with animal viruses (Sabanadzovic et al., 2009). GSyV-1 was found in North America and has been detected in only nine European countries (Italy, France, Hungary, Slovakia, Czech Republic, Spain, Turkey, Croatia, and Russia) (Giampetruzzi et al., 2012;

Beuve et al., 2013; Czotter et al., 2015; Glasa et al., 2015; Ruiz-García et al., 2017; Caglayan et al., 2017; Vončina et al., 2017; Navrotskaya et al., 2021). However, knowledge about the distribution and genome organization of GSyV-1 on the European continent is still very limited. GSyV-1 was found in two of our libraries: library 005 ('Laški rizling' variety) and library 009 ('Malvazija' variety). In library 005, VirusDetect (BLASTN search) identified only one partial polyprotein reference sequence (KP221269) with a length of 334 nt, which was 44.9% covered by 3 contigs (69 nt, 35 nt and 47 nt) and a sequencing depth of 20.9X. Four reference sequences (3 complete and 1 partial) were identified in library 009. The complete genome sequence from Slovakia (KP221256) had the highest coverage of 48.8%. Validation of the predicted GSyV-1 was performed with primers SY5922F/SY6295R (Glasa et al., 2015) targeting a fragment of the CP gene. One sample from the 005 library (Laški rizling 3/45B) and two samples from the 009 library (Malvazija 32/2B and Malvazija 32/3B) were infected. The virus was amplified at 40 cycles, the same as GRGV, which may indicate that the concentration of these two viruses was very low, which could explain the results of HTS (scarce genome coverage and low sequencing depth). In Laški rizling 3/45B, GSyV-1 was found in co-infection with RBDV, GRSPaV, GPGV, GRVFV, GFkV, HSVd, and GYSVd-1; while in Malvazija 32/2B and 32/3B it was found in co-infection with GRSPaV, GPGV, HSVd, and GYSVd-1. Phylogenetic analysis of the partial CP gene showed that GSyV-1 isolates clustered into two clades, and the major clade consisting of two major lineages, consistent with the results of Glasa et al. (2015). Our isolates clustered together with isolates from Hungary and Slovakia. All three samples were included in the elimination process, Laški rizling 3/45B did not regenerate. From Malvazija 32/2B and 32/3B, only two meristems regenerated (one meristem per candidate), and both regenerated plants were free of GSyV-1. To date, only one study considering GSyV-1 elimination by meristem tissue culture and somatic embryogenesis has been published with an elimination success of 100% (Turcsan et al., 2020).

GPGV was detected in all libraries. The lowest reference genome coverage (95.3%), the lowest sequencing depth (10.7X), and the highest number of contigs (38) were observed in library 014 ('Malvazija' variety); while in the other 11 libraries the reference genomes were 97-100% covered with 5-16 contigs and sequencing depth ranging from 33.2X-487.4X. The sRNA-seq results were validated with primers targeting partial MP and partial CP genes. Among the preclonal candidates, GPGV was the most abundant, and 91.14% of the tested plants were infected. High incidence of GPGV was also found in neighboring countries: Italy (Saldarelli et al., 2015; Bianchi et al., 2015), Hungary (Czotter et al., 2018), Croatia (Hančević et al., 2021). In addition, it was detected in all countries of the former Yugoslavia (Montenegro, Serbia, Bosnia and Herzegovina, and North Macedonia) (Bertazzon et al., 2016). Forty RT-PCR products were sequenced. MP sequences obtained from 40 Slovenian preclonal GPGV isolates showed a specific C/T polymorphism at position 6,685, that introduced the premature stop codon. The C/T polymorphism was also found in GPGV survey in different countries. Studies from Italy (Saldarelli et al., 2015; Marra et al., 2020)

and Switzerland (Reynard, 2015) showed that GPGV isolates from asymptomatic vines had 18 extra nt (6 aa), while a study from Hungary (Czotter et al., 2018) showed that asymptomatic isolates had 18 nt (6 aa) shorter MP due to this polymorphism. In our study, all preclonal candidates were asymptomatic, and the 18 nt (6 aa) shorter MP was found in 13 vines, while the MP with these 18 nt (6 aa) residues was observed in 27 isolates. The results of several studies indicate that the C/T polymorphism in the stop codon is most likely not responsible for symptom expression. Interestingly, it seems to be a silent mutation in the CP gene, since the same polymorphism is also part of the overlapping CP gene. In addition, a mutation responsible for 5 aa shortening of MP has been found in Spanish and Russian isolates (Morán et al., 2018; Shvets and Vinogradova, 2022). Using sRNA-seq, Saldarelli et al. (2015), examined viromes from symptomatic and asymptomatic grapevines. The authors constructed two sRNA libraries (one symptomatic and one asymptomatic vine) and found that GPGV was co-infected with two viruses (GRSPaV and GRVFV), and two viroids (HSVd and GYSVd-1). GYSVd-1 was present only in asymptomatic sample while GRVFV was present only in symptomatic sample. To investigate the possible involvement of GRVFV in symptomatology, asymptomatic and symptomatic samples were tested with RT-PCR. GRVFV was confirmed in symptomatic and asymptomatic samples, which ruled out an association of this virus with symptoms. Bianchi et al. (2015) reported that three viruses (GRSPaV, GRVFV, and GSyV-1), and two viroids (HSVd and GYSVd-1) were present in plants with or without symptoms, although GRVFV and GSyV-1 were present in a lower percentage. Bertazzon et al. (2017) reported that most samples with or without symptoms were infected with the same viruses. In our study we found GPGV only in co-infection with HSVd (Rebula 24/2B, Rebula 26/3B), but also in Laški rizling 3/45B with five viruses (RBDV, GRSPaV, GFkV, GSyV-1, GRVFV) and two viroids (HSVd and GYSVd-1). In most cases (18.99%) it was found in co-infection with GRSPaV, HSVd, and GYSVd-1. Therefore, we also did not find any correlation between the expression of symptoms and co-infection of GPGV with other viral pathogens. In addition, Saldarelli et al. (2015) performed biological assay for better understanding the role of GPGV in the etiology of GLMD, and suggested that different GPGV strains exist with diverse biological traits. Bianchi et al. (2015) and Bertazzon et al. (2017) also disclosed that symptomatic plants had significantly higher GPGV concentration than asymptomatic plants, whereas Morán et al. (2018) and Shvets and Vinogradova (2022) found no association between symptoms and virus titer. The above facts suggest that GPGV has a complex epidemiology. Therefore, additional analysis of symptom expression and general epidemiology of the disease are needed. The MP/CP region is commonly used in published GPGV research to infer phylogenetic relationships, although some authors have also included sequences of RdRp. For example, Bertazzon et al. (2017) and Al Rwahnih et al. (2021) performed a phylogenetic analysis with partial region spanning MP and CP genes which was also analyzed in our study. Eichmeier et al. (2017, 2018) and Saldarelli et al. (2015) included besides MP/CP sequences also RdRp region. Phylogenetic analysis showed that our isolates clustered into two clades, although all were asymptomatic. Isolates with shorter MP clustered in the same clade, whereas isolates with

longer MP clustered in both clades. Our isolates grouped with those from countries that are relatively close geographically, which may suggest that infection spreads geographically through the dissemination of infected propagation material, but the high prevalence also suggests that the vector plays an important role. Testing the grapevine propagation materials on GPGV is not mandatory. However, based on the complex etiology and epidemiology of the disease and its impact on grapevine production, testing on GPGV should be mandatory. To date, three studies have been carried out considering its elimination through meristem tip culture with and without thermotherapy, chemotherapy, and somatic embryogenesis (Gualandri et al., 2015; Komínek et al., 2016; Turcsan et al., 2020). In our study, 26 out of 28 samples included into sanitation process were GPGV-infected. Forty-nine regenerated plants were obtained and all were GPGV-free.

In 2001 and 2002, unusual symptoms (curved line patterns and leaf yellowing) were observed on the variety 'Laški rizling' (Mavrič et al., 2003). Samples were negative on nepoviruses, but were positive on RBDV. Later, RBDV was detected in 'Štajerska belina' variety (Mavrič et al., 2003). Since then, it was detected in all Slovenian viticulture regions in the period between 2003 and 2005, but only in white varieties (Mavrič Pleško et al., 2009). In 2006, it was detected in the red variety 'Pinot Noir' in the Podravje region (Mavrič Pleško et al., 2009). A more recent study showed that the lowest incidence of RBDV was found in the Primorska viticultural region (5.1%) compared to Podravje and Posavje (Mavrič Pleško et al., 2020). Infection of grapevine with RBDV has also been reported in Serbia (Jevremovic and Paunovic, 2011), Hungary (Mavrič Pleško et al., 2012; Czotter et al., 2018), and recently in Russia (Navrotskaya et al., 2021). In our study, RBDV was predicted only in library 005 ('Laški rizling' variety). Although the complete genome of RBDV isolates from grapevine has not yet been obtained, the assembled contigs were compared with the reference isolate J1 from *Rubus idaeus*. RNA1 was 100% covered with two contigs (one of which was sufficient to cover the complete RNA1 segment) and a sequencing depth of 1556.2X; the RNA2 segment was 98.7% covered with four contigs and a sequencing depth of 513.8X. The bulk samples showed nt identity of 93.40% and 96.10% with the reference RNA1 and RNA2, respectively. A 5' end (941 bp) of a Serbian isolate from grapevine showed 93.62% identity at the nt level with isolate R15 from raspberry (Jevremovic and Paunovic, 2011). The presence of the virus was validated using RT-PCR and all 4 samples (Laški rizling 3/34B, 3/45B, 3/54B, and 3/64B) were infected. Partial RNA2 (partial MP and partial CP) was Sanger sequenced and a phylogenetic tree was constructed based on the partial CP nucleotide sequences (438 bp) of our 4 and 29 RBDV isolates from grapevine and raspberry from the GenBank database. The RNA2 region is commonly used in studies of the phylogenetic relationship of RBDV, although some authors have also included sequences from RNA1. Mavrič Pleško et al. (2009) performed a phylogenetic analysis based on MP and CP amino acid sequences, and later on nucleotide sequences of the almost complete RNA2 segment and also only for the CP gene (Mavrič Pleško et al., 2020). These two Slovenian studies (Mavrič Pleško et al., 2009, 2020), as well as studies from Hungary

(Czotter et al., 2018), Belarus and Sweden (Valasevich et al., 2011) showed that grapevine isolates were clearly separated from *Rubus* sp. isolates, which is in agreement with our results. In addition, phylogenetic studies in Hungary showed that partial RNA1 from grapevine was also clustered separately from RNA1 sequences from *Rubus* sp. (Czotter et al., 2018). With serological methods, using monoclonal antibodies it is also possible to distinguish isolates from grapevine and raspberry (Mavrič Pleško et al., 2009). In various studies, using different elimination methods such as thermotherapy, meristem tissue culture, chemotherapy, cryotherapy, and their different combinations, it was reported that elimination of RBDV from raspberry is very difficult (Murant et al., 1974; Theiler-Hedtrich and Baumann, 1989; Wang et al., 2008; Mathew et al., 2021). It is presumed that this pollen-transmitted virus infects meristematic tissues except for the least differentiated cells of the apical dome (Wang et al., 2008). To our knowledge, this is the first report of elimination of this virus from grapevine. 103 meristems were isolated, 11 regenerated (2 from 3/34B, 5 from 3/56B, and 4 from 3/64B), all of which were free of RBDV.

Six viroids and one viroid-like RNA have been reported from grapevine (Di Serio et al., 2017). HSVd and GYSVd-1 are globally distributed and are the only two viroids known to occur in grapevine in Slovenia. In our study using sRNA-seq, HSVd was detected in all libraries. In all libraries, VirusDetect (BLASTN search) identified only one reference isolate per library (KJ810551 or KY508372). HSVd was validated in all libraries using HSV-78P/HSV-83M primers that amplified the entire genome (Sano et al., 2001), and all (79) samples were infected. Considering that HSVd is latent in grapevines, and that testing is usually overlooked, this contributed to its high prevalence in many countries. Forty RT-PCR products were selected and Sanger sequenced. Interestingly, 38 sequences were 100% identical. Two other sequences (Pokalca 3/4P and Pokalca 3/6P) were identical and had 98% identity with the other 38 isolates. These two isolates clustered separately from the other 38 sequences from this study and 29 sequences from different hosts from the GenBank database (*Vitis* sp., *Humulus lupulus*, *Ficus carica*, *Morus alba*, *Citrus* sp., *Prunus* sp.). HSVd is latent in grapevines but can be transmitted to hop plants and cause epidemics (Sano et al., 2001; Kawaguchi-Ito et al., 2009). This is particularly important because Slovenia is one of the largest hop producers. GYSVd-1 was also detected in all libraries, and only one reference sequence was identified per library (AB028466, KP010010, or KJ466324). GYSVd-1 predicted by sRNA-seq was validated in all libraries using primers that amplified the whole genome (Ward et al., 2011), and 71 samples were positive (89.87%). It should be noted that in order to validate GYSVd-1, the number of amplification cycles for all libraries was 40. The increased number of amplification cycles (45) in order to validate sRNA-seq predicted GYSVd-1, was reported from Russia (Navrotskaya et al., 2021). Thirty-five RT-PCR products were selected and Sanger sequenced. GYSVd-1 had higher genetic diversity in comparison with the HSVd. InDel mutations were also observed. GYSVd-1 and/or GYSVd-2 in co-infection with GFLV may elicit vein banding symptoms (Hajizadeh et al., 2015). In our study GYSVd-1 in co-infection with GFLV was found in two samples of 'Zeleni

Sauvignon' variety (26/1P and 26/2P), and five samples of 'Pokalca' variety (3/4P, 3/5P, 3/6P, 9/2G, and 9/3G), but symptoms were not observed. Simultaneous infection with GYSVd-1 and GFLV also did not induce visible symptoms of vein banding or yellow speckles in two varieties of *V. vinifera* and one variety of *V. labrusca* in Brazil (Fajardo et al., 2016). Phylogenetic analysis of 35 Slovenian GYSVd-1 isolates generated in this study and 28 sequences from database showed that our isolates clustered in different phylogroups, independently of variety or geographic distribution. Testing of material for clonal selection and vegetative propagation on viroids is not obligatory, therefore they are often overlooked. Three methods for viroids eradication from grapevines have been used so far: thermotherapy (Gambino et al., 2011), meristem tissue culture (Duran-Vila et al., 1988; Turcsan et al., 2020), and somatic embryogenesis (Gambino et al., 2011; Turcsan et al., 2020). Thermotherapy alone (Gambino et al., 2011) and treatment with ribavirin (Eichmeier et al., 2019) were unsuccessful. Several studies reported different elimination success with meristem tissue culture (Duran-Vila et al., 1988; Turcsan et al., 2020). The best results were obtained with somatic embryogenesis (Gambino et al., 2011; Turcsan et al., 2020). In our study, 28 samples infected with HSVd and 27 infected with GYSVd-1 were included in the elimination process. Fifty-one and 47 regenerated plants were tested on HSVd and GYSVd-1, respectively. Elimination of viroids was lower because heat therapy induced their replication; 39.2% of HSVd-free, and 42.6% of GYSVd-1-free plants were obtained.

Considering that nine viruses and two viroids were detected and validated in the asymptomatic preclonal samples, in the third part of the dissertation we wanted to investigate the virome of samples not included in clonal selection programs. Thirteen samples from six grapevine varieties ('Malvazija', 'Rebula', 'Pokalca', 'Cipro', 'Volovnik', and 'Poljšakica') were analyzed. Four libraries were constructed and sequenced. A total of 70,902,637 reads were generated, of which 7.44% mapped to viral reference sequences. The method used revealed the presence of: GLRaV-1, GLRaV-2, GLRaV-3, GFLV, satGFLV, GRSPaV, GPGV, GFkV, GRVFV, GV-Sat, HSVd, and GYSVd-1. Among the preclonal candidates, GLRaV-3 was detected in only one library and only one sample was infected, while other viruses belonging to the leafroll disease complex were not detected. In these samples, GLRaV-3 was more prevalent as it was detected in three libraries (L2, L3, and L4). In addition, two viruses from the leafroll complex, GLRaV-1 and GLRaV-2, were detected in the variety 'Cipro' (L1). GFLV was found in two libraries (L2 and L4) and its satellite RNA was also detected in both. GPGV, the emerging and most abundant virus in preclonal candidates, was also found here in all libraries. Two viruses (GRGV and GSyV-1), found for the first time in Slovenia in preclonal candidates, were not detected in these samples, while the third (GRVFV) was found in all libraries. In preclonal candidates, the viroids were detected in all libraries, while RT-PCR showed that GYSVd-1 was less abundant. In these sample set, HSVd was detected in all libraries, while GYSVd-1 was absent in one library (L2). GRSPaV was also detected in all libraries, whereas it was not detected in preclonal candidates with sRNA-seq in 2 out of 12 libraries, although it was confirmed in all libraries

with RT-PCR. In addition, GV-Sat was detected, which is the first report in Slovenia. GV-Sat was detected in a very low percentage (3%; 346 samples were analyzed) in California with HTS of dsRNAs (Al Rwahnih et al., 2013). Four years later, it was reported on Iranian variety 'Askeri' held in the INRA collection in France (Candresse et al. 2017). In European vineyards has been found only in Hungary (Czotter et al., 2018). To date, only eleven sequences have been deposited in NCBI, including three generated in this study. The stop codon of ORF1 and the start codon of ORF2 overlap, and multimeric forms exist (Candresse et al., 2017). GV-Sat require the help of another virus(es) to replicate in plants. The helper virus is still unknown, but in published studies (Al Rwahnih et al., 2013; Czotter et al., 2018) it was found in co-infections with vitiviruses and leafroll-associated viruses. In the 'Cipro' library, we detected two viruses belonging to the leafroll complex (GLRaV-1, GLRaV-2), but we did not detect vitiviruses. This result may suggest that GLRaVs play a role in amplification, which could also explain why GV-Sat was not detected in the preclonal candidates. In addition, GRSPaV, GPGV, GRVFV, HSVd, and GYSVd-1 were detected in the same library. As we expected, more viruses with mandatory and recommended tests were detected in samples that were not included in certification programs. Therefore, our goal was to develop a multiplex RT-PCR for validation of sRNA-seq data that could be used for rapid and cheaper routine diagnostics. The KAPA2G Fast Multiplex PCR Kit was used. According to the protocol, the primers used should have a similar melting temperature (Tm) and a guanine-cytosine content (GC) of 40-60%. We chose primer pares with different amplicon sizes that allowed differentiation on the agarose gel. Although the selected primers had different Tm and some primers did not have optimal GC content, successful amplification was achieved in all cases. mRT-PCR has been used in several studies to detect grapevine viruses and viroids (Nassuth et al., 2000; Gambino and Gribaudo, 2006; Digiaro et al., 2007; Hajizadeh et al., 2012; Gambino, 2015; Ahmadi et al., 2017; Komínková and Komínek, 2020).

3.2 CONCLUSIONS

Viral pathogens are one of the major obstacles in grapevine production. Rapid and accurate detection, molecular characterization, and implementation of viral elimination methods are crucial for viticulture worldwide.

In this doctoral dissertation, we aimed to study the virome of different grapevine varieties (included or not in the clonal selection process) using HTS of virus- and viroid-derived small RNAs, and to validate all predicted pathogens with RT-PCR and Sanger sequencing. We also aimed to study their genetic diversity, phylogeny, and co-infections, as well as to investigate the efficiency of their elimination by thermotherapy and meristem tip micrografting.

We set up four research hypotheses:

1) Vines are infected with different viruses and viroids, which can be adequately determined using HTS of small RNAs.

The virome status of 82 preclonal candidates and 13 samples not included in the clonal selection was examined by high-throughput sequencing of small RNAs. For the preclonal candidates, 12 libraries were constructed, and for the samples not included in the clonal selection, 4 libraries were constructed. Sequencing was performed using the IonTorrent System. In total, 12 viruses were detected: GRSPaV, GFLV (in association with its satellite RNA), GLRaV-1, GLRaV-2, GLRaV-3, GPGV, GFkV, GSyV-1, GRVFV, GRGV, RBDV and GV-Sat, as well as two viroids: HSVd and GYSVd-1. GRGV, GRVFV, GSyV-1 and GV-Sat have been found for the first time in Slovenia.

2) Based on the sequences information of viruses and viroids obtained by the HTS approach specific primers could be designed for amplification and validation of the predicted viral pathogens by RT-PCR and Sanger sequencing.

Based on the sequences obtained by the HTS approach, primers were selected for validation of the predicted viral pathogens. The primers corresponded to those found in the literature or were newly designed in this study. The predicted viral pathogens in the preclonal candidates were validated using RT-PCR and Sanger sequencing. The predicted viral pathogens in samples not included in the clonal selection were validated using the multiplex RT-PCR developed in this study.

3) Predicted infections will be confirmed with Sanger sequencing and additional information about strain-specific polymorphisms related to different host grapevines could be obtained.

In preclonal candidates, 191 Sanger sequences were generated. Genetic diversity was studied in different viral genomic regions. Detailed insights into genetic diversity, phylogeny, and co-infections were obtained.

4) Using thermotherapy and meristem/shoot tip culture virus-free material could be established, but the percentage of elimination will vary depending on variety and viral pathogen.

Twenty-eight preclonal candidates infected with eight viruses and two viroids were selected for elimination experiment by in vivo thermotherapy and in vitro meristem tip micrografting. Efficient protocols were established for hypocotyl production (used as rootstocks), micrografts and micropropagated plants separated from rootstocks. The overall regeneration rate was 8.53%, the elimination rate for viruses was 100%, while it was lower for viroids (39.2% for HSVd and 42.6% for GYSVd-1). The regenerated plants were successfully acclimatized.

4 SUMMARY (POVZETEK)

4.1 SUMMARY

In the PhD thesis, we investigated the virome of grapevine (*Vitis vinifera* L.) using high-throughput sequencing technology, studied the genetic diversity of viruses and viroids, their phylogeny, and co-infections, developed a protocol for healthy vines production by in vivo thermotherapy and in vitro meristem tip micrografting, and developed a multiplex RT-PCR for validation of sRNA-seq data, that could be used for rapid, reliable, sensitive and cheaper diagnostics.

Grapes are considered one of the most widely grown fruit crops in the world (7.3 million hectares). Viruses are one of the major obstacles to sustainable viticulture. Viral pathogens can be latent, but also can cause severe symptoms and high economic losses. Therefore, rapid and accurate detection, characterization and application of biotechnological approaches for viral elimination are of great importance.

The aim of the first part of our study was to investigate the virome of preclonal candidates in the Primorska viticultural region, where clonal selection has been carried out for decades. Eighty-two samples of six grape varieties (2 red and 4 white) were analyzed using small RNA sequencing technology. Micro RNAs were isolated, twelve libraries were constructed, and then sequenced using the Ion Proton™ System. VirusDetect was used for data analysis. Nine viruses: GFLV, GLRaV-3, GRSPaV, GFkV, GSyV-1, GRVFV, GRGV, GPGV, and RBDV, and two viroids: HSVd and GYSVd-1 were identified. Three viruses (GRGV, GRVFV, GSyV-1) have never been reported in Slovenia before. In silico results were validated by RT-PCR and Sanger sequencing. Total RNA from all samples (82) was extracted and reverse transcribed, followed by amplification with specific primers selected based on sequences obtained by sRNA-seq. The amplification products were analyzed by gel electrophoresis, and the remaining reactions were sequenced in both directions after purification (Exo-Sap treatment). The sequences were trimmed and assembled. A total of 191 sequences were generated and analyzed. All generated Sanger sequences were deposited in NCBI under the accession numbers: GSyV-1 (MW446939-MW446941), GRVFV (MW446917-MW446938), GRGV (MW446914-MW446916), GLRaV-3 (OK138920), GRSPaV (OK138921-OK138934), HSVd (OK138935-OK138974), GYSVd-1 (OK138975-OK139009), GFkV (OK139010-OK139034), GFLV (OK139035-OK139038), RBDV (OK139039-OK139042), GPGV (OK139043-OK139082).

GLRaV-3 was predicted only in library 010 ('Refošk' variety). The reference sequence was an isolate from South Africa (GQ352631). A unique viral contig corresponding to ORF2 (known to be absent in some isolates) was observed. The predicted virus was validated with two primer pairs that amplified two different genomic regions (CP and HSP70h), and only one sample was infected. Prior to our analysis, all preclonal vines were tested by ELISA on

viruses from the leafroll complex, and all infected vines were excluded from further analysis, which explains why GLRaV-3 was detected in only one sample and why other leafroll-associated viruses were not detected. CP gene of Refošk 11/4P was sequenced and was 99.76% identical with 18 sequences originating from different continents, but phylogenetic study showed that our isolate was clustered with a Portuguese isolate.

RBDV was also detected only in one library ('Laški rizling'). Reference RNA1 was 100% covered, while RNA2 was 98.7% covered. All four samples were infected. Part of the MP and part of the CP gene were sequenced. The phylogenetic tree was constructed based on 438 bp of the partial CP gene, and the sequences of grapevine were clearly separated from those of raspberry.

Four viruses from the grapevine fleck complex were detected, three of which (GSyV-1, GRVFV, GRGV) were detected for the first time in Slovenian vineyards. GFkV was predicted in eight libraries. In seven libraries, VirusDetect identified 4-10 reference sequences from the GenBank database per library, while in one library (011; 'Refošk' variety) only one partial replicase gene was identified, with 33.3% coverage. Many contigs and their short length were observed in all libraries. GFkV was validated in all predicted libraries. Genetic diversity was examined in the replicase gene. Interestingly, phylogenetic studies showed that isolates clustered according to variety, the only exception was 'Laški rizling'. Based on nt identity and phylogenetic studies, we concluded that GFkV infection occurred through the exchange of infected propagation material. Among the viruses detected for the first time in Slovenia, GRVFV was more prevalent than the other two. GRVFV was detected in 11 libraries. In all libraries, mainly scarce reference genomes coverage with many short contigs were obtained. The virus was validated in all libraries where it was predicted. Forty-four RT-PCR products were sequenced (partial polyprotein), and 22 sequences were of lower quality, probably due to the presence of different genetic variants in the same sample, but these sequences were not cloned; only the high quality ones were further analyzed. We found high genetic variability between sequences (10.9% ± 0.9%) and their partitioning into two molecular groups, in different subclades. GRGV was detected in two libraries of 'Refošk' variety. In both libraries, references from Spain had the highest genome coverage. GRGV was validated in both libraries, and a total of 3 samples were infected. Due to the limited number of sequences deposited in NCBI, we could not conclude the possible introduction of this virus in Slovenia. GSyV-1 was also detected in two libraries, library 005 ('Laški rizling' variety) and library 009 ('Malvazija' variety) with very scarce reference genomes coverage. GSyV-1 was validated in both libraries. Phylogenetic analysis (partial CP gene of our sequences and those retrieved from NCBI) showed that isolates clustered into two clades, and our three isolates belonged to the major clade and clustered together with isolates from Hungary and Slovakia. It should be noted that for validation of GRGV and GSyV-1, the number of amplification cycles for all libraries was 40, which could explain the HTS results (very low coverage of the reference genomes and very low sequencing depth).

From the infectious degeneration and decline disease complex, GFLV was detected. GFLV was predicted in library 013 ('Zeleni Sauvignon' variety) and library 016 ('Pokalca' variety). In both libraries, VirusDetect (BLASTN search) identified more sequences from the database for both the RNA1 and RNA2 segments. The virus was validated in both predicted libraries, and four samples were positive with primers amplifying the 606 bp RNA2 region, including the partial CP gene. Sequence analysis revealed high genetic diversity (more than 12%) between red and white varieties. Based on the high genetic diversity between our isolates and isolates from GenBank, a new primer pair was designed, and an additional six samples were found infected. The phylogenetic tree showed that the isolates included in the analysis clustered into two clades; our isolates belonged to the major clade and were closer to the isolates from Italy and France than to the previously characterized isolates from Slovenia (variety 'Volovnik').

From the rugose wood disease complex, only GRSPaV was detected. GRSPaV was detected in ten libraries. In eight libraries 4 to 40 complete and partial (mainly CP gene) reference sequences were identified. The highest coverage of complete reference sequences ranged from 48.4% to 99.1%. They were covered by a high number of contigs (41-86) and a low sequencing depth (5.3X-16.6X). Only partial sequences were identified in two libraries (012 and 015). The virus was not detected in libraries 007 and 014. The virus was validated in all libraries where it was predicted and additionally in both libraries where it was not predicted. This may have a profound biological background and requires further investigation, as the hypothesis that this occurred due to low concentration and bulk sequencing strategy was ruled out. Overall, 88.61% of the samples were infected. Sequencing of the whole RT-PCR product was not possible, so cloning was performed. At least three genetic variants were found to be present in the same sample, and the mean distance between the 14 sequences was 14.06%. The variety 'Laški rizling' showed the highest diversity (17.14%), interestingly, VirusDetect identified the highest number of reference sequences from the database in the 'Laški rizling' variety (40). Phylogenetic analysis showed that the genetic variants clustered differently even if they were from the same sample, and that there was no clustering by geographic origin.

GPGV is an emerging virus that may be latent or cause severe damage. It is of concern that this virus was predicted in all libraries and that 72 samples (91.14%) were infected. Genetic diversity was examined in 40 partial MP and partial CP sequences. A specific C/T polymorphism at position 6,685 was observed in 13 samples, which caused premature stop codon, and shorter MP by 18 nt (6 aa). The data obtained here indicate that the C/T polymorphism is not responsible for the expression of symptoms. Synergism with different viruses/viroids is also not responsible for symptomatology, as we found GPGV only in co-infection with HSVd, but also with up to five viruses (GRSPaV, GFkV, GSyV-1, GRVFV, and RBDV) and two viroids (HSVd and GYSVd-1). Phylogenetic analysis of the partial sequences of the MP/CP genes showed that our isolates clustered into two clades, although all were asymptomatic. Our isolates clustered mainly with those from countries relatively

close geographically, which may suggest that infections spread through the dissemination of infected propagation material, but the high prevalence also suggests that vectors play an important role. Testing on GPGV is only recommended but should be mandatory due to the complex etiology and epidemiology of the disease and its impact on vine production.

Considering viroids, HSVd and GYSVd-1 are distributed worldwide, and are the only two viroids known to occur on grapevines in Slovenia. HSVd was detected and validated in all libraries. With RT-PCR, HSVd was detected in all samples. Forty RT-PCR products were selected and Sanger sequenced. Interestingly, 38 sequences were identical, while two sequences showed 98% identity with other isolates but clustered separately in the phylogenetic tree from other isolates generated in this study and from other isolates retrieved from NCBI. Considering that HSVd is latent in grapevines and was found in all samples, and that isolates from grapevines have been shown to cause epidemics in hops, this viroid requires close attention.

GYSVd-1 was also detected in all libraries, and only one reference sequence per library was also identified. Seventy-one samples were positive (89.87%). Thirty-five RT-PCR products were selected and Sanger sequenced. Sequences were 95.35-100% identical, and InDel mutations were also observed. Overall, it was more genetically diverse than HSVd. Phylogenetic analysis showed that our isolates clustered in different phylogroups regardless of variety or geographic distribution.

Overall, we obtained a complete insight into the virome of the preclonal candidates. GLRaV-3 was the rarest (1.27%), followed by GRGV and GSyV-1 (3.80%), RBDV (5.06%), GFLV (12.66%), GFkV (34.18%), GRVFV (55.70%), GRSPaV (88.61%), GYSVd-1 (89.87%), GPGV (91.14%), HSVd (100%). Most samples were simultaneously infected with five viral pathogens, while eight viral pathogens were detected in one sample. Infections with GRSPaV, GPGV, HSVd and GYSVd-1 were the most frequent (18.99%).

Considering that the preclonal candidates were infected with nine viruses and two viroids, the objective of the second part of this dissertation was to develop a protocol for the production of healthy vines. Twenty-eight preclonal candidates from six varieties infected with the viral pathogens predicted and validated above (except GRGV) were selected for virus/viroid elimination. Heat therapy was performed from six weeks to three months at 36-38 °C. After in vivo thermotherapy and surface disinfection of apical and axillary segments, meristem tips (0.1-0.2 mm) were isolated and immediately micrografted onto etiolated and sectioned hypocotyls of Vialla (*Vitis labrusca* × *Vitis riparia*). The overall regeneration rate was very low (8.53%). Four preclonal candidates did not regenerate. A higher regeneration rate was observed in white varieties. 'Rebula' had the highest regeneration rate (16.7%), and 'Pokalca' had the lowest (3.3%). The regenerated plants were micropropagated several times to increase their number and to have enough material for testing, acclimatization and in vitro storage. The in vitro plants were tested after seven months using RT-PCR. A 100%

elimination rate was achieved for eight viruses. Elimination rates for viroids were lower (39.2% for HSVd and 42.6% for GYSVd-1), probably because the high temperature promoted viroid replication and accumulation. Although this method is difficult, labor-intensive, and time-consuming, it has many advantages over other biotechnological approaches for viral elimination, such as chemotherapy, which can cause severe phytotoxicity, or somatic embryogenesis, which carries a high risk of mutations. In our study, we reduced the risk of genetic instability by in vivo thermotherapy (shortened duration of in vitro cultivation), meristem isolation (no callus formation), micrografting (accelerated regeneration), and the use of a medium without plant growth regulators/hormones.

The aim of the third part of this dissertation was to study the virome of symptomatic samples not included in clonal selection programs. Thirteen samples were analyzed. Four libraries were constructed and sequenced. GLRaV-3 was more prevalent (compared to preclonal candidates) and was detected in three libraries. In addition to GLRaV-3, two viruses from the leafroll disease complex (GLRaV-1 and GLRaV-2) were identified. Other viruses with obligatory testing (GRSPaV, GFLV, GFkV) as well as GPGV, GRVFV and GV-Sat were also detected. GV-Sat is the first report in Slovenia, the second in Europe and the fourth in the world. We also developed a multiplex RT-PCR for validation of sRNA-seq data (viruses, viroids and satellites).

In the first part of the dissertation, we studied the virome of 82 preclonal candidates of six grapevine varieties. 191 sequences were generated, genetic diversity and phylogenetic studies were performed. In the second part of the dissertation, the efficacy of virus and viroid elimination rates of six grapevine varieties was studied using in vivo thermotherapy and in vitro micrografting of meristem tips. In the third part of the dissertation, the virome of grapevine samples not involved in clonal selection was studied and a multiplex RT-PCR was developed for efficient validation of HTS predicted organisms.

4.2 POVZETEK

V doktorski disertaciji smo raziskali virom vinske trte (*Vitis vinifera* L.), na osnovi tehnologije visokozmogljivega sekvenciranja, proučevali genetsko raznolikost virusov in viroidov, njihovo filogenijo in sočasne okužbe. Poleg tega smo tudi razvili protokol za eliminacijo virusov in viroidov vinske trte in vivo s termoterapijo in in vitro z mikrograftingom meristemov ter razvili hkratni RT-PCR (multipleksni) za validacijo podatkov sRNA-seq (sekvenciranje malih RNA), za namen hitre, zanesljive in cenejše diagnostike.

Vinska trta velja za eno najbolj razširjenih kulturnih rastlin na svetu (goji se na površinah, ki obsegajo 7,3 milijona hektarjev). Ena izmed glavnih ovir za trajnostno vinogradništvo so okužbe z virusi in z njimi povezana obolenja. Virusni patogeni so lahko latentni, a vendar lahko povzročijo tudi hude simptome in veliko gospodarsko izgubo. Zato je velikega pomena hitro in natančno odkrivanje virusov, karakterizacija ter uporaba metod za zatiranje virusov.

Cilj prvega dela naše raziskave je bil raziskati virom predklonskih kandidatov v vinogradniški regiji Primorska, kjer se klonska selekcija izvaja že desetletja. Analizirali smo 82 vzorcev šestih sort vinske trte (2 rdeči in 4 bele sorte) z uporabo tehnologije sekvenciranja malih RNA. Izolirali smo male RNA iz 12 združenih vzorcev, v katerih so bili zbrani predklonski kandidati posamezne sorte, pripravili knjižnice in jim določili nukleotidno zaporedje s sistemom Ion Proton; v primeru, da smo imeli znotraj sorte veliko število predklonskih kandidatov, smo za isto sorto naredili dva ali tri združena vzorca. Za analizo podatkov smo uporabili sklop orodij programskega cevovoda VirusDetect, ki so prosto dostopna na spletu. Odkrili smo devet virusov: GFLV, GLRaV-3, GRSPaV, GFkV, GSyV-1, GRVFV, GRGV, GPGV, RBDV in dva viroida: HSVd in GYSVd-1 (Poglavje 2.1.1; Tabela 1). Trije virusi (GSyV-1, GRVFV, GRGV) v Sloveniji pred tem še niso bili odkriti. In silico rezultati so bili potrjeni z RT-PCR in sekvenciranjem po Sangerju. V nadaljevanju smo izolirali celokupno RNA tudi iz vseh posameznih vzorcev. Najprej smo celotno RNA reverzno prepisali in jo nato v reakciji PCR pomnožili s specifičnimi začetnimi oligonukleotidi, ki smo jih izbrali glede na sekvence pridobljene z sRNA-seq. Pomnožke RT-PCR smo analizirali z elektroforezo in jih po čiščenju (tretiranje z Exo-Sap) sekvencirali v obe smeri (sekvenciranje po Sangerju). Pridobljena zaporedja smo obrezali in sestavili, tako da smo dobili 191 sekvenc, ki smo jih analizirali. Vse generirane sekvence smo deponirali v NCBI pod sledečimi pristopnimi številkami: GSyV-1 (MW446939-MW446941), GRVFV (MW446917-MW446938), GRGV (MW446914-MW446916), GLRaV-3 (OK138920), GRSPaV (OK138921-OK138934), HSVd (OK138935-OK138974), GYSVd-1 (OK138975-OK139009), GFkV (OK139010-OK139034), GFLV (OK139035-OK139038), RBDV (OK139039-OK139042), GPGV (OK139043-OK139082).

Virus GLRaV-3, pripadnik rodu *Ampelovirus*, smo odkrili le v knjižnici 010 (sorta 'Refošk') (Poglavje 2.1.1; Tabela 1). Preko aplikacij VirusDetect (BLASTN) smo zanj identificirali samo eno celotno sekvenco genoma (GQ352631), ki izhaja iz Južne Afrike. Naš genom se je z referenčnim genomom ujemal v 98,6 %, s 23 soseskami in 39,6 kratno (X) globino sekvenciranja. Opazili smo eno virusno sosesko, ki ustreza ORF2 (za katerega je znano, da ga v nekaterih izolatih ni). Predvideni virus smo potrdili z dvema paroma začetnih oligonukleotidov, ki sta pomnožila dve različni genomski regiji (CP in HSP70h). Ugotovili smo, da je bil z virusom GLRaV-3 okužen le en vzorec. Pred tem, so bili vsi vzorci testirani s testom ELISA na prisotnost virusov ki povzročajo zvijanje listov vinske trte, in vse okužene trte so bile izključene iz nadaljnjih analiz. To pojasnjuje, zakaj je bil GLRaV-3 odkrit samo v enem vzorcu in zakaj nismo zaznali drugih virusov ki so povezani z zvijanjem listov. Sekvencirali smo genomsko regijo CP vzorca Refošk 11/4P, pri čemer smo ugotovili 99,76 % nukleotidno podobnost z 18 sekvencami, ki izhajajo iz različnih celin. Vendar je filogenetska študija pokazala, da je naš izolat najbolj podoben izolatu s Portugalske (Poglavje 2.1.1; Slika 5 v prilogi).

Virus RBDV smo odkrili v knjižnici 005 ('Laški rizling') (Poglavje 2.1.1; Tabela 1), kar je zanimivo, saj je bil v Sloveniji prvič odkrit pred dvajsetimi leti pri isti sorti. Pri sekvenciranju smo potrdili visoko pokritost segmentov RNA1 in RNA2. Čeprav celoten genom izolatov RBDV iz vinske trte še ni na razpolago, so sestavljeni soseski primerjani z izolatom J1 iz maline (*Rubus idaeus*). RNA1 se je z referenco 100 % ujemal v 2 soseskah in 1556,2 X globino sekvenciranja; segment RNA2 se je ujemal v 98,7 % s 4 soseskami in 513,8 X globino sekvenciranja. Podobnost sekvenc združenih vzorcev glede na referenco RNA1 je bila 93,40 % in glede na RNA2 96,10 %. Vsi štirje vzorci iz knjižnice 005 so bili okuženi. Sekvencirali smo del gena MP in del gena CP. Sekvence gena MP naših izolatov so si 100 % podobne, medtem ko so imele sekvence regije CP 98,18–99,55 % podobnost (97,95–100 % aminoksilinska podobnost). Filogenetsko drevo smo narisali na podlagi dela gena CP dolgega 438 bp, pri čemer so bile sekvence izolatov iz vinske trte jasno ločene od sekvenc izolatov iz malin (Poglavje 2.1.1; Slika 1 v prilogi).

Iz kompleksa virusov, ki povzročajo bolezen (kompleks bolezni) imenovano 'fleck' smo potrdili štiri viruse (GFkV, GSyV-1, GRVFV in GRGV) (Poglavje 2.1.1; Tabela 1), od tega so bili trije (GSyV-1, GRVFV in GRGV) v Sloveniji odkriti prvič. Virus marmoriranosti vinske trte (GFkV) smo odkrili v osmih knjižnicah (Poglavje 2.1.1; Tabela 1). V sedmih knjižnicah smo z orodjem VirusDetect na posamezno knjižnico identificirali 4-10 referenčnih nukleotidnih zaporedij. V knjižnici 011 (sorta 'Refošk') je bila s 33,3 % pokritostjo določena le ena delna sekvenca gena ki kodira replikazo. Pokritost celotnih referenčnih sekvenc je bila 76,9-82,1 %. Izjema sta bili dve knjižnici sorte 'Malvazija', katerih je bila pokritost sekvenc le 30,4 % oziroma 32,2 %. V vseh knjižnicah smo opazili veliko sosesk (36-60) s kratko dolžino. Z RT-PCR smo pomnožili del sekvence za replikazo virusa GFkV. Z reakcijo PCR smo namnožili 34 produktov, ki smo jih analizirali s sekvenciranjem po Sangerju, pri čemer je 7 produktov pripadalo virusu GRVFV. Dve

sekvenci sta bili slabše kakovosti in sta bili izključeni iz nadaljnje analize. Potrjenih pa je bilo petindvajset sekvenc GFkV, katerih nukleotidna podobnost je bila 91,6–100 % (93,7–100 % aminokislinska podobnost). Filogenetska analiza, ki smo jo opravili na delu nukleotidnega zaporedja gena za replikazo, je 25 slovenskih izolatov in 18 referenčnih izolatov virusa iz GenBank, razvrstila v dve skupini. Slovenski izolati so bili prisotni v obeh skupinah. Zanimivo je, da so se, z izjemo sorte 'Laški rizling', izolati združili glede na sorto (Poglavje 2.1.1; Slika 6 v prilogi). Naši izolati so se združili z izolati iz sosednjih držav in iz ZDA, iz katerih so bile uvožene podlage in na katere so bili cepljene gostiteljske trte. Predvidevamo, da je do širjenja virusnih okužb prišlo preko razmnoževanega materiala in cepljenja na okužene podlage. Poleg tega je bilo veliko cepljenk nato iz Slovenije izvoženih v sosednje države. Med virusi, ki so bili prvič odkriti v Sloveniji, je bil virus GRVFV bolj razširjen kot druga dva. Virus GRVFV smo odkrili v 11 knjižnicah (Poglavje 2.1.1; Tabela 1). V posamezni knjižnici smo z orodjem VirusDetect identificirali od 5 do 25 popolnih in delnih referenčnih sekvenc, z izjemo knjižnice 013, kjer je bila identificirana samo ena delna referenca. Največja pokritost celotnega referenčnega genoma je bila v knjižnici 008 (KY513702; 85 %), medtem ko je bila pokritost delne referenčne sekvence v knjižnici 013 (MH544692) 30,2 %. Virus smo potrdili v vseh predvidenih knjižnicah. Vsem produktom RT-PCR (44) smo določili nukleotidno zaporedje (delni poliprotein), pri čemer je bilo 22 zaporedij nižje kakovosti, verjetno zaradi prisotnosti različnih genetskih variant v istem vzorcu. Za nadaljnje analize smo uporabili le tiste z visoko kakovostjo. Ugotovili smo visoko genetsko variabilnost med nukleotidnimi zaporedji (10,9 % ± 0,9 %), pri čemer so se razdelili v dve molekularni skupini z različnimi podskupinami. Virus GRGV je bil potrjen v dveh knjižnicah sorte 'Refošk' (006 in 010) in največja pokritost in identičnost je bila z izolati iz Španije; 63,6 % v knjižnici 006 (KX171166) in 45,8 % v knjižnici 010 (KX109927) (Poglavje 2.1.1; Tabela 1). GRGV smo potrdili v obeh knjižnicah, pri čemer so bili okuženi 3 vzorci. Zaradi relativno majhnega števila sekvenc, deponiranih v NCBI, nismo mogli sklepati o morebitni vnosu tega virusa v Slovenijo. V dveh knjižnicah smo odkrili tudi virus GSyV-1, in sicer v knjižnici 005 (sorta 'Laški rizling') in knjižnici 009 (sorta 'Malvazija'), vendar je bila pokritost genoma zelo nizka (Poglavje 2.1.1; Tabela 1). V knjižnici 005 je bila identificirana samo ena delna poliproteinska referenčna sekvenca (KP221269) z dolžino 334 nt, ki je imela pokritost 44,9 % s 3 kontigi in globino sekvenciranja 20,9 X, medtem ko so bile v knjižnici 009 določene štiri referenčne sekvence (3 popolne in 1 delna). Pri tem je največjo pokritost (48,8 %) imelo zaporedje iz Slovaške (KP221256). Virus GSyV-1 smo potrdili v obeh knjižnicah. Filogenetska analiza na osnovi dela gena CP je izolate združila v dve skupini, kjer so trije slovenski izolati pripadali glavni skupini, združenimi z izolati iz Madžarske in Slovaške. Naj še omenimo, da smo za validacijo virusov GRGV in GSyV-1 za vse knjižnice uporabili 40 ciklov pomnoževanja (za ostale viruse pa le 35 ciklov), kar sovpada z zelo nizko pokritostjo referenčnega genoma in zelo nizko globino sekvenciranja.

Virus pahljačavosti listov vinske trte (GFLV) je bil potrjen v knjižnici 013 (sorta 'Zeleni Sauvignon') in 016 (sorta 'Pokalca') (Poglavje 2.1.1; Tabela 1). V knjižnici 013 smo

identificiranih 11 zaporedij segmenta RNA1. Poleg njih smo določili 4 popolne sekvence segmenta RNA2. V knjižnici 016 smo identificirali 7 popolnih in 1 delno sekvenco segmenta RNA1, v tej knjižnici smo določili tudi 16 popolnih sekvenc segmenta RNA2. Virus smo potrdili v obeh predvidenih knjižnicah. Pri pomnoževanju 606 bp dolge regije RNA2 z vključenim delom gena CP, smo dobili pozitiven rezultat pri 4 vzorci. Trije izolati sorte 'Pokalca' so imeli 99,67-99,84 % nukleotidno podobnost (99,5 ali 100 % aminokislinsko podobnost), medtem ko se je izolat Zeleni Sauvignon 16/3P močno razlikoval od izolatov 'Pokalca', s 87,27-87,44 % podobnostjo (96,49 ali 96,99 % aminokislinska podobnost). Poleg razlik med našimi izolati, smo potrdili tudi razlike z izolati, ki so dostopni v bazi podatkov NCBI. Zaradi velikih sekvenčnih razlik med našimi izolati in izolati iz NCBI baze podatkov smo oblikovali nov par začetnih oligonukleotidov, specifičen za zaporedja naših izolatov in z RT-PCR analizo potrdili okužbo pri dodatnih šestih vzorcih. Na osnovi filogenetske analize so se naši izolati in izolati iz baze podatkov NCBI razvrstila v dve skupini. Naši izolati so bili razvrščeni v glavno skupino in so bili bližje izolatom iz Italije in Francije, kakor že predhodno analiziranim izolatom iz Slovenije (sorta 'Volovnik') (Poglavje 2.1.1; Slika 4 v prilogi).

Virus razbrazdanja lesa vinske trte (GRSPaV) je bil odkrit v desetih knjižnicah (Poglavje 2.1.1; Tabela 1). V osmih knjižnicah smo z orodjem VirusDetect identificirali od 4 do 40 popolnih in delnih referenčnih sekvenc (večinoma gen CP). Pokritost referenčnih sekvenc je bila 48,4-99,1 %, pri čemer smo dobili veliko število sosesk (41-86) in nizko globino pokritosti (5,3 X-16,6 X). V dveh knjižnicah, 012 (sorta 'Zeleni Sauvignon') in 015 (sorta 'Malvazija') smo identificirali samo delne in/ali popolne sekvence gena CP. V knjižnicah 007 (sorta 'Rebula') in 014 (sorta 'Malvazija') virusa nismo zaznali. Za potrditev rezultatov sRNA-seq smo izbrali par začetnih oligonukleotidov, ki pomnožujejo visoko ohranjeno regijo CP gena. Virus smo potrdili v vseh knjižnicah, kjer je bil predviden, in tudi v dveh knjižnicah, kjer ni bil predviden. Predvidevamo, da do tega ni prišlo zaradi nizke koncentracije in strategije sekvenciranja združenih vzorcev, ampak, da je razlog za to kompleksno ozadje biologije razmnoževanja virusa in okuževanja, kar zahteva nadaljnje študije. Skupno je bilo okuženih 88,61 % vzorcev (Poglavje 2.1.1; Slika 1). Direktno sekvenciranje produkta RT-PCR ni bilo možno izvesti, zato smo produkte reakcije RT-PCR ligirali v vektor in transformirali v kompetentne bakterijske celice. Skupno smo sekvencirali 14 produktov. Najvišja skupna povprečna genetska razdalja je bila odkrita med tremi različicami Laškega rizlinga 3/45B (17,14 %), najnižja pa za Malvazijo 20/48P (6,32 %). Povprečna skupna genetska razdalja med vsemi 14 sekvenciranimi različicami je bila 14,06 %. Pri sorti 'Laški rizling', kjer smo potrdili največjo pestrost je bilo preko VirusDetect aplikacije poravnanih z našimi zaporedij tudi največje število referenčnih sekvenc iz baze (40). Na osnovi filogenetske analize so bile genetske različice razvrščene v različne skupine, kljub temu, da so izvirale iz istega vzorca, prav tako se niso združili glede na geografski izvor (Poglavje 2.1.1; Slika 3 v prilogi).

Virus GPGV je nedavno odkrit virus, ki je lahko latenten ali zelo škodljiv. Zaskrbljujoče je, da smo ta virus odkrili v vseh knjižnicah (95,3–100 % pokritost celotnih referenčnih sekvenc) (Poglavje 2.1.1; Tabela 1) in da je bilo z njim okuženih 72 vzorcev (91,14 %) (Poglavje 2.1.1; Slika 1). Genetsko raznolikost smo preučevali v 40 vzorcih zaporedja delov gena MP in CP. Podobnost nukleotidnega zaporedja gena MP je bila pri 40 slovenskih izolatih 93,94-100 % (87,79-100 % aminokislinska podobnost), podobnost gena CP pa 94,53-100 % (97,86-100 % aminokislinska podobnost). V 13 vzorcih smo ugotovili, da je na mestu 6,685 specifičen polimorfizem C/T, in polimorfno zaporedje kodira prezgodnji stop kodon, zato je v teh primerih gen MP 18 nt (6 ak) krajši. Pridobljeni podatki kažejo, da polimorfizem C/T ni odgovoren za spremembo izražanja simptomov oziroma, da na izražanje simptomov poleg tega polimorfizma vplivajo še nekateri drugi dejavniki. Sinergizem z različnimi virusi/viroidi prav tako ni odgovoren za nastanek simptomov, saj smo virus GPGV potrdili pri sočasni okužbi s samo enim viroidom (HSVd), kot tudi s kar petimi virusi (RBDV, GRSPaV, GFkV, GRVFV, GSyV-1) in dvema viroidoma (HSVd in GYSVd-1) in so bili vsi vzorci asimptomatični (Poglavje 2.1.1; Slika 2). Na osnovi filogenetske analize nukleotidnih zaporedij dela genov MP/CP so se naši izolati razdelili v dve skupini, čeprav so bili vsi asimptomatični. Izolati s krajšim genom MP so bili združeni v isto skupino, medtem ko so bili izolati z daljšim genom MP uvrščeni v obe skupini. Naši izolati so bili združeni predvsem s tistimi iz držav, ki so geografsko relativno blizu (Poglavje 2.1.1; Slika 2 v prilogi), kar lahko nakazuje, da se okužba širi z širjenjem okuženega sadilnega materiala, vendar visoka razširjenost kaže tudi na pomembno vlogo vektorjev. Testiranje na prisotnost virusa GPGV je samo priporočljivo, vendar ugotavljamo, da bi zaradi kompleksne epidemiologije in etiologije bolezni ter njenega vpliva na pridelavo vina ter velike razširjenosti in velikega števila latentnih oblik virusa, testiranje na virus GPGV moralo biti obvezno.

Glede viroidov sta HSVd in GYSVd-1 razširjena po vsem svetu in sta edina znana viroida, ki se pojavljata na vinski trti v Sloveniji. Viroid HSVd smo odkrili v vseh knjižnicah, in sicer smo v posamezni knjižnici identificirali le eno referenčno sekvenco (Poglavje 2.1.1; Tabela 1). Napovedan viroid HSVd smo potrdili v vseh knjižnicah, pri čemer so bile z RT-PCR potrjene okužbe pri vseh vzorcih. Izbrali smo 40 produktov RT-PCR in jim določili zaporedje s sekvenciranjem po Sangerju. Zanimivo je, da je bilo 38 nukleotidnih zaporedij identičnih, medtem ko sta bili dve zaporedji (Pokalca 3/4P in Pokalca 3/6P) enaki in sta pokazali 98-odstotno podobnost z drugimi izolati (Poglavje 2.1.1; Slika 9 v prilogi). Glede na to, da smo viroid HSVd odkrili v vseh vzorcih in je latenten v vinski trti ter da je dokazano, da lahko izolati iz vinske trte okužujejo hmelj, je potrebno temu viroidu posvetiti veliko pozornost.

Viroid GYSVd-1 smo z sRNA-seq metodo prav tako odkrili v vseh knjižnicah in tudi identificirali samo eno referenčno sekvenco na posamezno knjižnico (Poglavje 2.1.1; Tabela 1). Z RT-PCR smo potrdili prisotnost viroida GYSVd-1 v vseh knjižnicah, pri čemer je bilo pozitivnih 71 vzorcev (89,87 %) (Poglavje 2.1.1; Slika 1). 35 produktom RT-PCR smo

določili nukleotidna zaporedja s sekvenciranjem po Sangerju. Zaporedja so si bila 95,35-100 % podobna. Opazili smo tudi mutacije tipa indel na 4 mestih v genomu (63, 92, 163 in 287) (Poglavje 2.1.1; Slika 10 v prilogi). Na splošno je ta viroid genetsko bolj raznolik kot HSVd. Filogenetska analiza je naše izolate razvrstila v različne skupine, ne glede na sorto ali geografsko razširjenost (Poglavje 2.1.1; Slika 8 v prilogi).

Na splošno smo dobili popoln vpogled v virom predklonskih kandidatov. Najmanj razširjen virus je bil GLRaV-3 (1,27 %), sledila sta mu GRGV in GSyV-1 (3,80 %), RBDV (5,06 %), GFLV (12,66 %), GFkV (34,18 %), GRVFV (55,70 %), GRSPaV (88,61 %), GYSVd-1 (89,87 %), GPGV (91,14 %) in HSVd (100 %) (Poglavje 2.1.1; Slika 1). Večina vzorcev je bila hkrati okužena s petimi, en vzorec (Laški rizling 3/45B) pa z osmimi virusnimi patogeni (Poglavje 2.1.1; Slika 3). Najpogostejše so bile sookužbe z GPGV, GRSPaV, HSVd in GYSVd-1 (18,99 %) (Poglavje 2.1.1; Slika 2).

Cilj drugega dela naše študije je bil razviti protokol in preučiti učinkovitost eliminacije virusov in viroidov in vivo s termoterapijo ter in vitro z izolacijo mersitemov in mikrograftingom. V postopek eliminacije smo vključili 28 predklonskih kandidatov šestih različnih sort, ki so bili okuženi z napovedanimi in potrjenimi virusnimi patogeni, katere smo navedli v zgornjem odstavku (razen GRGV) (Poglavje 2.1.3; Tabela S1 v prilogi). Toplotno terapijo smo izvajali od 6 tednov do 3 mesece pri temperaturi 36-38 °C (Poglavje 2.1.3; Slika 6a). Po termoterapiji in vivo smo vzorčili apikalne in aksilarne segmente rastlin (Poglavje 2.1.3; Slika 6b), jih površinsko razkužili (z 1,66 % raztopino natrijevega dikloroizocianurata), meristeme (0,1-0,2 mm) aseptično izolirali pod stereomikroskopom (Poglavje 2.1.3; Slika 6c) ter jih takoj nacepili na etiolirane hipokotile vinske trte Vialla (*Vitis labrusca* × *Vitis riparia*) (Poglavje 2.1.3; Slika 5c). Skupno smo izolirali in nacepili 598 meristemov, od skupno se je regeneriralo 51 rastlin (8,53 %) (Poglavje 2.1.3; Tabela 1). Da bi povečali njihovo število, smo regenerante večkrat mikropropagirali, pri čemer nismo nikoli opazili tvorjenja kalusa, vitrifikacije ali nekroze. Pri belih sortah je bila stopnja regeneracije večja kot pa pri rdečih (Poglavje 2.1.3; Tabela 1, Slika 2). Samo en vzorec bele sorte 'Laški rizling' (3/45B), ki je bil okužen z osmimi virusnimi patogeni in z vsaj tremi genetskimi različicami virusa GRSPaV, se ni regeneriral. Med rdečimi sortami se ni regeneriral vzorec 'Pokalca' (9/2G) in dva vzorca 'Refoška' (12/3P in 12/6P). Najvišjo stopnjo regeneracije je imela 'Rebula' (16,7 %), sledita 'Laški rizling' in 'Zeleni Sauvignon' (10,7 %) (Poglavje 2.1.3; Slika 2). Čeprav je imela 'Rebula' najvišjo stopnjo regeneracije, se je 'Zeleni Sauvignon' med mikropropagacijo veliko hitreje obnavljal in rasel. Najnižjo stopnjo regeneracije je imela 'Pokalca' (3,3 %) (Poglavje 2.1.3; Slika 2). Učinkovitost eliminacije virusov in viroidov iz regeneriranih rastlin, ki smo jih gojili 7 mesecev v in vitro pogojih, smo določili z RT-PCR. Za vse viruse je bila dosežena 100 % eliminacija. Kar pa ne drži za viroide, saj je bila eliminacija viroidov HSVd in GYSVd-1 bistveno nižja, in sicer 39,2 % za HSVd in 42,6 % za GYSVd-1 (Poglavje 2.1.3; Tabela 2), verjetno zato, ker visoka temperatur (termoterapija) spodbuja replikacijo in kopičenje viroidov. Rastline brez virusov, vzgojene v in vitro pogojih, so bile uspešno aklimatizirane v kockah iz kamene volne, ki so

se izkazale kot odličen substrat za rast in razvoj korenin (Poglavje 2.1.3; Slika 3). Rastline smo gojili v mini rastlinjaku v rastni komori, nato pa jih presadili v lončke (Poglavje 2.1.3; Slika 4). Vsi predklonski kandidati brez virusov bodo ponovno testirani po približno treh letih, preden bodo tudi uradno uveljavljeni kot certificirani kloni. Čeprav je ta metoda zahtevna, delovno intenzivna in dolgotrajna, ima veliko prednosti pred drugimi biotehnološkimi pristopi za eliminacijo virusov, kot je na primer kemoterapija, ki lahko povzroči hudo fitotoksičnost, ali tudi somatska embriogeneza, pri kateri obstaja veliko tveganje na pojav mutacij. V naši raziskavi smo zmanjšali tveganje za genetsko nestabilnost z in vivo termoterapijo (skrajšali čas gojenja in vitro), izolacijo meristema (brez tvorbe kalusa), mikrograftingom (pospešili regeneracijo) in uporabili gojišče, ki ne vsebuje regulatorjev rasti za rastline.

Glede na to, da smo pri predklonskih kandidatih, ki niso imeli vidnih simptomov, odkrili devet virusov in dva viroida, je bil cilj tretjega dela naše študije, raziskati virom vzorcev, ki niso vključeni v programe klonske selekcije. Analizirali smo 13 vzorcev in pripravili ter sekvencirali štiri knjižnice (Poglavje 2.1.4; Tabela 2 in 3). Virus GLRaV-3 je bil bolj razširjen, saj smo ga odkrili v treh knjižnicah. Poleg GLRaV-3 sta bila identificirana še dva virusa iz kompleksa bolezni zvijanja listov vinske trte (GLRaV-1 in GLRaV-2). Odkrili smo tudi druge viruse, ki so na seznamu za obvezno testiranje (GRSPaV, GFLV, GFkV), pa tudi GPGV, GRVFV in GV-Sat. To je tudi prvo poročilo o virusu GV-Sat v Sloveniji, drugo v Evropi in četrto v svetu. Zaznali smo ga samo v kultivarju 'Cipro'. Najvišja pokritost (91,4 %) in podobnost na nukleotidnem nivoju (95,74 %) je bila z zaporedjem genoma ameriškega izolata AUD46129 (KC149510). V knjižnici 'Cipro' smo zaznali tudi viruse GLRaV-1, GLRaV-2, GRSPaV, GPGV, GRVFV in viroida HSVd ter GYSVd-1. Razvili smo tudi hkratni RT-PCR (multipleks) za validacijo podatkov sRNA-seq, ki vključuje različne kombinacije virusov, viroidov in satelitov, ki bi jih lahko uporabili za stroškovno učinkovito, visoko zmogljivo in hitro diagnostiko, kot je to potrebno pri analiziranju velikega števila vzorcev z mešanimi okužbami. Za mRT-PCR smo izbrali kombinacije začetnih oligonukleotidov, ki pomnožujejo fragmente različnih dolžin, kar omogoča določitev razlik na agaroznem gelu. Da bi lahko hkrati določili več različnih virusov in viroidov, smo za vzpostavitev najboljših pogojev PCR reakcije, optimizirali več parametrov, kot je koncentracija začetnih oligonukleotidov (0,04–0,2 µM), temperatura prileganja (55–60 °C), število ciklov (30–35) in količina vhodne cDNA (1 µL in 2 µL). Za najboljše so se izkazali naslednji pogoji: 0,08 µM koncentracija začetnih oligonukleotidov (0,04 µM samo za virus GRVFV), temperatura prileganja 58 °C, 35 ciklov in 1 µL cDNA. Različne kombinacije virusov smo hkrati pomnožili v vseh štirih knjižnicah: L1 (GLRaV-1, GLRaV-2, GRSPaV, GPGV, GRVFV in GV-Sat); L2 (GLRaV-3, GFLV, GRSPaV, GPGV in GRVFV); L3 (GLRaV-3, GRSPaV, GPGV, GFkV in GRVFV); L4 (GLRaV-3, GFLV, GRSPaV, GPGV, GFkV in GRVFV) (Poglavje 2.1.4; Slika 3). Poleg tega smo istočasno pomnožili tudi različne kombinacije viroidov/satGFLV: L1 in L3 (HSVd, GYSVd-1), L2 (satGFLV, HSVd), L4 (satGFLV, HSVd in GYSVd-1) (Poglavje 2.1.4; Slika 3).

V prvem delu disertacije smo dobili popoln vpogled v virom 82 predklonskih kandidatov šestih sort vinske trte. Pridobili smo veliko število sekvenc, opravili filogenetske analize in analize genetske raznolikosti. V drugem delu disertacije smo raziskali učinkovitost eliminacije virusov in viroidov pri šestih sortah vinske trte, kjer smo uporabili in vivo termoterapijo ter in vitro mikrografting meristemov. V tretjem delu disertacije smo preučili virom sort vinske trte, ki niso vključene v program klonske selekcije in razvili multipleks RT-PCR za učinkovito validacijo virusov, viroidov in satelitov, ki so bili napovedani na osnovi visoko zmogljivega sekvenciranja malih RNA.

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ANNEX A

Statement on publisher permissions for the inclusion of own published articles in the printed and electronic versions of the doctoral thesis:

- Miljanić V., Jakše J., Kunej U., Rusjan D., Škvarč A., Štajner N. 2022. Virome Status of Preclonal Candidates of Grapevine Varieties (*Vitis vinifera* L.) From the Slovenian Wine-Growing Region Primorska as Determined by High-Throughput Sequencing. Frontiers in microbiology, 13, 830866, doi: 10.3389/fmicb.2022.830866: 11 p.

- Miljanić V., Jakše J., Kunej U., Rusjan D., Škvarč A., Štajner N. 2022. First Report of Grapevine Red Globe Virus, Grapevine Rupestris Vein Feathering Virus, and Grapevine Syrah Virus-1 Infecting Grapevine in Slovenia. Plant Disease, 106, 9: 2538

- Miljanić V., Rusjan D., Škvarč A., Chatelet P., Štajner N. 2022. Elimination of Eight Viruses and Two Viroids from Preclonal Candidates of Six Grapevine Varieties (Vitis vinifera L.) through In Vivo Thermotherapy and In Vitro Meristem Tip Micrografting. Plants, 11, 8: 1064, doi: 10.3390/plants11081064: 14 p.

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- Miljanić V., Jakše J., Beber A., Rusjan D., Škvarč A., Štajner N. 2021. First report of grapevine satellite virus in Slovenia. Journal of Plant Pathology, 103: 1329–1330

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