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Exogenous application of double-stranded RNA to reduce grapevine Pinot gris virus titre in *in vitro* grown *Vitis vinifera*

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Summary

A method of delivering short synthetic double-stranded RNA (dsRNA) to stimulate RNA interference (RNAi)-mediated control for grapevine Pinot gris virus (GPGV) in grapevines was developed and evaluated in this study. The dsRNA molecule targeting the RNA-dependent RNA polymerase (RdRp) gene of the GPGV genome was designed and produced by a two-step polymerase chain reaction (PCR) approach followed by *in vitro* transcription of the amplicon. A significant decrease in virus titre was observed seven days after dipping shoot tips of GPGV-infected tissue culture (TC) plantlets into a solution of GPGV-RdRp-dsRNA followed by re-introduction to TC. The effect was more pronounced in shoot tips dipped in the GPGV-RdRp-dsRNA solution for 24 hours than in tips dipped for two hours. This study represents the first successful demonstration of dsRNA-mediated control in TC plantlets for GPGV and offers a promising avenue to provide virus-free material to nurseries, contributing to the overall health and sustainability of the viticulture industry.

Keywords

RNAi, dsRNA, tissue culture, motifs, GPGV

Introduction

Destructive viruses of grapevine (*Vitis* sp.), such as *Trichovirus pinovitis* (syn. grapevine Pinot gris virus; GPGV) can reduce yield, compromise fruit quality and impact vineyard sustainability (Maree *et al.*, 2013; Saldarelli *et al.*, 2013; Wu *et al.*, 2020). Using "clean" planting material in which destructive viruses do not occur is recommended globally to establish vineyards and mitigate against the risk of viruses. The use of this material can reduce input associated with disease management and provide a significant cost benefit to growers (Fuller *et al.*, 2019; Cheon *et al.*, 2020).

Virus elimination methods used to produce clean grapevine planting material include *in vitro* culture techniques such as meristem and shoot tip culture (Maliogka *et al.*, 2009; Gualandri *et al.*, 2015; Marković *et al.*, 2021). Combining these methods with treatments such as chemotherapy (Guţă *et al.*, 2016; Komínek *et al.*, 2016), thermotherapy (Panattoni and Triolo, 2010; Wang *et al.*, 2018), and cryotherapy (Bi *et al.*, 2018) can enhance the efficiency of virus elimination in grapevines and successful production of virus-free plants may be enhanced.

RNA interference (RNAi) is a cellular mechanism that assists eukaryotic organisms, including plants, to defend against viral infections and is triggered when the plant encounters double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) intermediates of the viral genome (Tenllado *et al.*, 2004; Yang and Li, 2018). Synthetic viral dsRNA or siRNA molecules applied to the plant surface can enter the vascular system and plant cells and trigger the RNAi viral defence response (Konakalla *et al.*, 2016; Mitter *et al.*, 2017). Various RNAi exogenous application methods have been developed and demonstrated to protect plants, including grapevines, against virus infection, including (i) spraying of bacterially expressed dsRNAs (Tenllado *et al.*, 2003; Gan *et al.*, 2010); (ii) dsRNAs that are loaded onto nanoparticles (Mitter *et al.*, 2017); and (iii) mechanical inoculation of naked dsRNAs (Tenllado and Díaz-Ruíz, 2001; Tenllado *et al.*, 2004; Petrov *et al.*, 2015). In this study, a novel approach was used to investigate the potential for exogenous application of dsRNAs in inducing a defence response and reducing GPGV levels in infected TC grapevine plantlets, with the goal of producing virus-free planting material. The *in vitro* RNAi treatment in tissue culture (TC) plantlets has not been previously reported for virus elimination in any plant species.

Methods

Preparation of grapevine tissue culture plantlets

Two plants of the grapevine cultivar 'Sauvignon Blanc', originating from a single source and infected solely with *Foveavi-*

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rus rupestris (syn. grapevine rupestris stem pitting-associated virus; GRSPaV) were each grafted with five buds of the grapevine 'Grüner Veltliner', which was infected with GPGV isolate LT6 (GenBank accession: OQ199009) and GRSPaV (Kaur *et al.*, 2023). The GPGV inoculated plants were grown in a glasshouse at 24°C and 16/8 h light/dark cycle and were used to establish GPGV-infected TC plantlets. Total RNA was extracted from the leaf and shoot tissues of the glasshouse-grown grapevine 'Sauvignon Blanc' and tested to confirm GPGV infection using a GPGV-specific reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay (Bianchi *et al.*, 2015; Kaur *et al.*, 2023).

To produce TC plantlets, actively growing nodal shoot segments (2 cm long) from the glasshouse-grown GPGV-infected grapevines were sterilized and washed as previously described (Diab *et al.*, 2011), using 1.25% sodium hypochlorite as a sterilant. The shoot tips (0.5-3 mm) were excised from the sterilized nodal segment using a sterile scalpel and were placed on TC initiation media (Table S1). Culture vessels (473 ml, Austratec Pty Ltd) containing 100 ml of TC medium were used and up to five shoot tips were placed into each vessel. The TC plantlets were incubated in a controlled environment room (CER) at 21°C and 55% – 60% relative humidity with a photoperiod of 16 h and a light intensity of 280-480 lx provided by white, fluorescent tubes. Cultures were monitored and sub-cultured for six months to produce 150 plantlets for the experiments. GPGV infection was confirmed by RT-qPCR and high throughput sequencing (HTS) (Kaur *et al.*, 2023) of a single whole leaf of the TC plantlets used for further subculturing to produce infected plantlets for the experiments.

Designing the GPGV short synthetic GPGV dsRNA molecule

To design short synthetic GPGV-specific dsRNA molecule, conserved motifs were identified using amino acid sequences for the RNA-dependent RNA polymerase (RdRp), movement protein (MP) and coat protein (CP) of the six exemplar virus species in the *Trichovirus* genus, including GPGV, *Apple chlorotic leaf spot virus, Apricot pseudo-chlorotic leaf spot virus, Cherry mottle leaf virus, Peach mosaic virus* and *Grapevine berry inner necrosis virus*. The sequences were aligned using the MUSCLE alignment software in MEGA X (Kumar *et al.*, 2018) with default parameters (Table S2). The conserved motifs identified in the *Trichovirus* genus were further compared to the aligned amino acid sequences of 200 GPGV isolates (Table S3). The identity analysis of the conserved amino acid sequences was determined using BioEdit Sequence Alignment Editor (Hall, 1999). The conserved motifs identified in GPGV genomes were validated using E-RNAi-Version 3.2 (Horn and Boutros, 2010) and the genome region with the most conserved amino acid sequence motifs within GPGV was selected for the creation of the short synthetic GPGV dsRNA molecule.

Generation of short synthetic GPGV dsRNA

Short synthetic dsRNA of the target GPGV RdRp region identified from the motif analysis was produced from total RNA extracted from a single whole leaf of GPGV-infected glasshouse-grown plants (Fig S1). The GPGV complementary DNA (cDNA) containing the target gene sequence and flanking regions was synthesised from 100 ng of total RNA using the Superscript II reverse transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The cDNA reaction contained 1 µM of the T7link-GPGV-RdRp-Reverse primer (Table 1; Fig S1). The quality and concentration of cDNA produced was estimated using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

A KAPA HiFi PCR Kit (KAPA Biosystems, Roche Diagnostics) was used in a two-step PCR amplification procedure to produce GPGV-RdRp-T7 amplicon used for dsRNA transcription (Fig S1). Two microlitres of GPGV-cDNA (1871.4 $ng \cdot \mu l^{-1}$) were used as template for the first PCR amplification, which generated a 798 bp amplicon, and each reaction contained 10 µM each of the T7link-GPGV-RdRp-Forward primer and 10 µM T7link-GPGV-RdRp-Reverse primer (Table 1). The thermocycling conditions were one cycle of 95°C for 3 min, 35 cycles of 95°C for 20 s, 60°C for 15 s and 72°C for 45 s, and one cycle of 72 °C for 8 min. Both primers contain a T7 8-nucleotide linker sequence (ggggatcc) at the 5' end (Table 1) to facilitate the addition of a T7 promoter to the GPGV-RdRp-T7 amplicon during the second PCR amplification.

The GPGV-RdRp amplicon from the first PCR was diluted (1:100) and used as a template in a second PCR amplification containing 10 mM dNTPs, 1 $U \cdot \mu L^{-1}$ KAPA HiFi Taq DNA polymerase and 10 µM of the 'T7-linker Forward and Reverse primer' (Table 1), which has the recognition site of restriction endonuclease *EcoRI* at the 5' end (upper case), T7 promoter (in bold) in the middle and the linker sequence at the 3' end (lower case) and anneals to the linker sequence of the template (Konakalla *et al.*, 2016; Melita *et al.*, 2021). These primers introduce a T7 promoter sequence at the 5' ends of both sense and antisense strands of the targeted region of the GPGV-RdRp gene for dsRNA transcription. The thermocycling conditions were one cycle

Table 1: Primer names and their sequences used in various steps of short synthetic grapevine Pinot gris virus (GPGV) dsRNA generation.

Primer name	Primer sequence	Step of short synthetic GPGV dsRNA generation
T7link-GPGV-RdRp-Forward primer	ggggatccCAAATGATGCCAATCCACAG	First step of PCR amplification
T7link-GPGV-RdRp-Reverse primer	ggggatccGCAATTTTGCAGACGACAGA	cDNA synthesis and first step of PCR amplification
T7-linker Forward and Reverse primer (T7 promoter in bold)	GAGAATTC**TAATACGACTCACTATA**ggggatcc	Second step of PCR amplification

of 95 °C for 2 min, 35 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 45 s, and one cycle of 72 °C for 8 min.

The GPGV-RdRp-T7 amplicon was gel purified using QIAquick PCR & Gel Cleanup Kit (Qiagen) following the manufacturer's instructions and 1.2 µg of the amplicon (798 bp) was used as a template for an *in vitro* transcription of short synthetic GPGV dsRNA using the T7 Ribomax™ Express large-scale RNA production system (Promega, USA) also following manufacturer's instructions with the transcription reaction performed at 37 °C for 4 h. The resulting dsRNA was treated with DNase and RNase at 37 °C for 15 min and further purified using RNeasy® Plant Mini Kit (Qiagen) following the manufacturer's instructions. The quality and concentration of first and second PCR amplicons and short synthetic GPGV dsRNA were estimated using the Nanodrop spectrophotometer (Thermo Fisher Scientific) and by 1.2% agarose gel electrophoresis imaging on a GelDoc Go Gel Imaging System (Bio-Rad). The PCR amplicons from the first and second PCR were sent to Macrogen (Seoul, Korea) for Sanger sequencing confirmation of the GPGV target region amplicons.

Exogenous application of short synthetic GPGV-RdRp-dsRNA to grapevine tissue culture plantlets

To stimulate RNAi, excised shoots or leaves of intact TC plantlets were treated with 60 µg of short synthetic GPGV-RdRp-dsRNA (1200ng·µl^{-1}). Control treatments included the application of 50 µg GPGV-RdRp-T7 amplicon solution (1000ng·µl^{-1}) and buffer control (RNase-free water). A no-treatment control was also included. Three exogenous applications were evaluated: two dipping applications which included placing the cut end of an individual TC plantlet single node shoot tip (approximately 3 cm long, with two or three leaves) into 50 µl of the GPGV-RdRp–dsRNA solution or the control solutions in individual sterile 1.5 ml centrifuge tubes, which were then sealed and incubated for two (application 1) (Fig S2a) or 24 hours (application 2) (Fig S2b). After dipping, each of the treated tips were transferred into individual culture vessels with 100 ml of growth media to initiate further growth. For the third application, 60 µg GPGV-RdRp–dsRNA solution; 50 µg GPGV-RdRp-T7 amplicon solution and the buffer control solution were exogenously applied to the upper surface of a single leaf of an individual rooted TC plantlet (Fig S2c). Each treated TC plantlet and the untreated plantlets were maintained separately in an individual culture vessel with 100 ml of media. For each treatment or control application, there were nine biological replicates resulting in a total of 108 TC plantlets (4 treatments/controls × 3 applications × 9 biological replicates = 108 plants total; Table S6)

Evaluation of GPGV infection and titre by quantitative RT-PCR

All treated and untreated TC plantlets (n = 108) were screened before treatment by testing a single whole leaf using the GPGV RdRp gene RT-qPCR, to confirm GPGV infection and evaluate GPGV titre (Bianchi *et al.*, 2015). A single whole leaf from all the plantlets were tested with the same assay to evaluate the presence and titre of GPGV seven days post-application (dpa) of the treatments. Prior to PCR, the quantity of the RNA extracted from each TC plantlet at both time points was measured using the Nanodrop spectrophotometer (Thermo Fisher Scientific). All RNA extracts were diluted to 33.62 ng·µl^{-1} so that the cycle threshold (Ct) values could be used as a relative measure of the effect of the treatments on virus titre. The GoTaq® one-step RT-qPCR System (Promega), containing GPgVPozRT-forward (400 nM) and reverse primers (400 nM) along with GPgVPozRT probe (100 nM) for GPGV detection, was used according to the manufacturer's instructions except that the total reaction volume was 25 µl and contained 4 µl of RNA template (Bianchi *et al.*, 2015). Each RNA extract was tested in triplicate. Statistical analysis was performed using a t-test in Microsoft Excel to compare average Ct values, representing virus titre, before and after treatment (7 dpa) with GPGV-RdRp-dsRNA, GPGV-RdRp amplicon, buffer controls, or no treatment. The statistical significance of the relative differences between the average Ct value before and after each treatment were calculated based on three RT-qPCR technical replicates for each of the nine biological replicates of the individual treatments and controls across the three applications.

Results

Grapevine plantlet production in tissue culture

The grafted glasshouse-grown grapevines 'Sauvignon Blanc' tested positive for GPGV by RT-PCR and was successfully introduced into TC. Additionally, the initial TC plantlets tested positive for GPGV by both RT-PCR and HTS. A total of 2,215,609 raw reads were generated by HTS and reduced to 2,209,873 reads after quality trimming. *De novo* assembly of reads using SPAdes resulted in 38,472 contigs from which twelve contigs matched with known viral sequences. A BLASTn (Altschul *et al.*, 1990) search of the GenBank database confirmed that ten contigs (ranging from 7196-7295 bp) matched most closely to GPGV and two contigs (ranging from 8632-8681 bp) matched with GRSPaV. The genome size of the GPGV contig was 7275 bp and GRSPaV was 8681 bp (Table S4). The percentage nucleotide sequence identity between GPGV isolate LT6 from Australia (GenBank accession: OQ199009) and TC isolate is 99.4%. The glasshouse-grown plants and TC plantlets did not display grapevine leaf mottle and deformation or other virus-like symptoms.

Choice of target sequences in the GPGV genome and dsRNA molecule synthesis

A total of 55, 15 and eight amino acid motifs in the RdRp, MP and CP regions of the GVPV genome, respectively, ranged in size from two to 12 amino acids and were conserved across the six exemplar *Trichovirus* species (Fig S3a; Table S5). Twenty-three of 55 RdRp amino acid motifs were also found and conserved amongst the 200 GPGV isolates that were analysed (Table S5). These conserved motifs mostly occurred in the region including methyltransferase, helicase, and catalytic core domains of the RdRp ORF (Fig S3a). Three GPGV RdRp regions with five, three and nine conserved amino acid mo-

tifs, respectively, were selected for further validation using the E-RNAi tool (Table S5) and a 265 amino acid region, from amino acid position 943 to 1209 of the GPGV exemplar isolate (RefSeq: NC_015782.2) was identified as the most suitable region in the GPGV genome for developing a short synthetic dsRNA molecule to stimulate RNAi. This region included most of the helicase domain and had three (FGFAGSGKS, TFESA, GDPLQA) conserved motifs (Fig S3b). The average pairwise percentage identity within the 265 amino acid target region amongst the 200 GPGV isolates was 98.3% with the percentage amino acid identity ranging from 89.9% (Japanese GPGV isolate H-JP2, GenBank Accession: BCR39159) to 100% (Australian GPGV isolate 5.6, GenBank Accession: WCZ55049). Only 6/8 of the CP motifs and none of the 15 MP motifs were conserved amongst all the 200 GPGV isolates.

The highly conserved region of the RdRp, with three conserved amino acid motifs (FGFAGSGKS, TFESA, GDPLQA), represents a 798-nucleotide sequence, from positions 2607 – 3404 bp of the GPGV reference genome sequence (NC_015782.2). This region was used in a two-step PCR and *in vitro* dsRNA transcription method to synthesise the short synthetic GPGV RdRp-specific dsRNA (GPGV-RdRp-dsRNA) for exogenous application to induce an RNAi response (Fig S3b). The short synthetic GPGV-RdRp–dsRNA product was successfully produced after the two-step PCR process and *in vitro* transcription (Fig S4).

Induced resistance by exogenous application of dsRNA targeting the RdRp genome sequence of GPGV to inhibit replication in plant material from tissue culture

Prior to the exogenous dsRNA treatment, the average Ct value of TC plantlets for GPGV, ranged from 19.7 – 21.8, with standard deviations ranging from 0.1 – 0.4 (Table 2, for detailed values, see Table S6). For application 1, the average difference in Ct values between pre-treatment and post-treatment at 7 dpa for shoot tips dipped in the GPGV-RdRp–dsRNA solution for 2 hours was 2.8 ± 0.9 ($P < 0.0001$), indicating a reduction in the GPGV-titre. The effects of the controls (GPGV-RdRp-T7 amplicon, RNase-free water and no application) were not statistically significant. For application 2, the average difference in Ct values between pre-treatment and post-treatment TC plantlets at 7 dpa for shoot tips dipped in the GPGV-RdRp-dsRNA solution for 24 hours was 5.1 ± 1.2 PCR cycles ($P < 0.0001$). However, other control treatments did not show any significant changes. In application 3, where dsRNA was directly applied into TC vessels, there was no significant difference in Ct values between pre-treatment and post-treatment at 7 dpa for the direct application of GPGV-RdRp–dsRNA and control treatments to leaves of TC plantlets (Table 2).

The average Ct values for the three applications of short synthetic GPGV-RdRp-dsRNA treatment were compared with other controls (GPGV-RdRp-T7 amplicon, RNase-free

Table 2: The average cycle threshold (Ct) values with standard deviation before and after seven days post application of a targeted short synthetic grapevine pinot gris virus RNA-dependent RNA polymerase double-stranded RNA (GPGV-RdRp-dsRNA) treatment, or control treatments, by either dipping tissue culture grapevine shoot tips for two or 24 hours prior to reintroduction into tissue culture, or by directly applying treatments to the leaves of plantlets in tissue culture. Control treatments were the application of GPGV-RdRp-T7 amplicon used to prepare the GPGV-RdRp-dsRNA, a buffer control (RNase-free water) and a no application control.

Treatment	Ct Before application		Ct After 7 days post application		Difference in Ct*		Significance (P value)
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation	
Application 1- Dipping excised shoot tips for 2 hours and then introduce in TC plant culture vessels							
GPGV-RdRp-dsRNA	21.1	0.1	23.9	0.9	+2.8	0.9	P < 0.0001
GPGV-RdRp-T7 amplicon	19.7	0.1	20.2	0.5	+0.5	0.5	0.10 > P > 0.05
RNase-free water	20.8	0.1	21.2	0.6	+0.4	0.5	0.10 > P > 0.05
No application	21.8	0.1	21.8	0.8	+0.0	0.8	P > 0.10
Application 2- Dipping excised shoot tips for 24 hours and then introduce in TC plant culture vessels							
GPGV-RdRp-dsRNA	19.8	0.1	24.9	1.2	+5.1	1.2	P < 0.0001
GPGV-RdRp-T7 amplicon	21.5	0.2	21.7	0.6	+0.2	0.6	P > 0.10
RNase-free water	19.4	0.4	19.8	0.5	+0.4	0.5	0.10 > P > 0.05
No application	20.8	0.2	20.9	0.5	+0.1	0.5	P > 0.10
Application 3- Direct application of dsRNA in TC plant culture vessels							
GPGV-RdRp-dsRNA	20.0	0.1	21.0	1.1	+1.0	1.1	P < 0.05
GPGV-RdRp-T7 amplicon	19.6	0.2	19.7	0.4	+0.1	0.4	P > 0.10
RNase-free water	20.2	0.2	20.6	0.6	+0.4	0.6	0.10 > P > 0.05
No application	19.8	0.2	20.2	0.4	+0.4	0.4	P > 0.10

* Difference in Ct calculated = values after application minus before application

water and no application). At 7 dpa, the average difference in Ct values between shoot tips treated with GPGV-RdRp–dsRNA for 2 hours (application 1; 2.1 – 3.7 cycles lower) or 24 hours (application 2; 3.2 – 4.0 cycles lower) was significantly different ($P < 0.0001$) from shoot tips treated with control solutions and the no treatment control. There was a significant difference observed between the direct application of GPGV-RdRp–dsRNA (application 3; 1.3 cycles lower) and GPGV-RdRp-T7 amplicon ($P < 0.001$). However, no significant differences were observed with direct application of any of the treatment control solutions ($P > 0.10$; 0.4 – 0.8 cycles lower) (Table S7).

The average Ct values of short synthetic GPGV-RdRp-dsRNA treatment in the three applications were compared amongst each other after 7dpa. The difference in average Ct values for shoot tips dipped in the GPGV-RdRp–dsRNA for 2 hours and 24 hours is moderately significant ($0.10 > P > 0.05$) and both are significantly different ($P < 0.0001$) from the average Ct of TC plantlets to which the dsRNA was applied directly to TC plantlets (Table S8).

Discussion

This study demonstrated that exogenous application of short synthetic GPGV-RdRp-dsRNA molecules to shoot tips of GPGV-infected TC plantlets by dipping induced an RNAi-based antiviral defence response which led to a statistically significant ($p<0.05$) reduction in GPGV titre over a seven-day period. Whilst this methodology has demonstrated similar success in reducing titre to control disease in *ex vitro* plants (Tenllado and Díaz-Ruíz, 2001; Holeva *et al.*, 2007; Yin *et al.*, 2009; Gan *et al.*, 2010; Kaldis *et al.*, 2018), this research represents the first application of a dsRNA treatment in TC plantlets. The method developed in this study has the potential to improve outcomes for the production of virus-free planting material and avoid the negative impact of treatments for the production of virus-free plants such as thermotherapy and chemotherapy. The lower GPGV titre at 7dpa of dsRNA suggests a systemic reduction in GPGV accumulation. Additional research is required to determine if the effect is durable and capable of further reducing titre with time. There is also a need to determine if the treatment led to differential virus accumulation throughout the TC plantlet to inform subculturing methods, including tissue types, for the production of virus-free plants.

Of the three short synthetic GPGV-RdRp-dsRNA applications that were tried, only the dipping applications reduced the virus titre. The longer application (24 hours) resulted in a larger reduction in virus titre compared to 2 hour application, although neither application reduced the titre to below detectable levels. Both dipping applications used the same concentration of short synthetic GPGV-RdRp-dsRNA, therefore the larger reduction in virus titre, which was observed after dipping shoots in solution for 24 hours suggests a dose-dependent response associated with length of exposure to the GPGV-RdRp-dsRNA treatment. This could be related to the uptake of more dsRNA over time by the treated GPGV-infected shoot tips, and this hypothesis is supported by other studies that also observed a similar dose response after exogenous applications to leaves (Tenllado and Díaz-Ruíz, 2001; Rego-Machado *et al.*, 2020).

The exogenous application of the short synthetic GPGV-RdRp-dsRNA onto the surface of the leaves of infected grapevine plantlets in TC did not elicit a change in GPGV titre, although virus titre reductions in various crops have been observed after the exogenous application of dsRNAs to leaves of plants maintained under in the glasshouse and field conditions (Komínek *et al.*, 2016; Kaldis *et al.*, 2018; Delgado-Martín *et al.*, 2022). The mechanism of naked dsRNA uptake by plants when applied to leaves is uncertain but entry points might include direct cellular uptake via absorbance, wounding and/or stomata (Das and Sherif, 2020). Physical barriers such as the leaf cuticle, cell walls and cell membranes preventing entry may have prevented the uptake of directly applied GPGV-RdRp-dsRNA in this study, although TC (*in vitro*) plantlets have thin cuticles compared to *ex-vitro* plants (Ali-Ahmad *et al.*, 1998; Bennett *et al.*, 2020; Hoang *et al.*, 2022). Additionally, the GPGV-RdRp-dsRNA was applied without abrasion onto the adaxial surface of the TC plantlet leaves. Stomata are located mainly on the abaxial leaf surface in grapevines (Konlechner and Sauer, 2016) and therefore, there were unlikely to be entry points in the form of wounds or stomata on the adaxial leaf surface to facilitate uptake of dsRNA and an RNAi response in this experiment.

When designing a short synthetic dsRNA molecule for virus control, a conserved viral genomic region for an RNAi target is crucial as it ensures efficient and specific gene silencing along with broader applicability across different strains of a species. The short synthetic GPGV-RdRp-dsRNA molecule designed in this study to stimulate RNAi against GPGV specifically targets the RdRp helicase region of trichoviruses and is conserved amongst the *Trichovirus* species exemplar isolates and 200 GPGV isolates that were analysed compared to other regions. The helicase is essential for unwinding guide strand RNA from its complementary target mRNA, a pivotal step in the RNAi process (Ambrus and Frolov, 2009). Due to this critical function, mutations may be less likely to accumulate over time compared to other regions of the viral genome (Kadaré and Haenni, 1997; Li *et al.*, 2001) and are therefore hypothesised to provide long-lasting dsRNA-mediated protection from GPGV. This study also identified two other *Trichovirus* RdRp regions and a region in the CP as potential targets for RNAi induction against GPGV (Table S5). Interestingly, it has been found that the CP suppresses antiviral RNA silencing in *Nicotiana benthamiana* plants infected with a GPGV infectious clone, suggesting that it may not be a useful target to develop dsRNA for the production of GPGV-free plants (Tarquini *et al.*, 2021). Nevertheless, further work to evaluate the RNAi efficacy of these regions against GPGV is warranted as they may prove to initiate a stronger RNAi response against GPGV.

In Australia, more than 90% of grapevines are infected with GRSPaV (Habili, 2015). Therefore, tissue culture plantlets used for evaluating the dsRNA treatment were infected with both GPGV and GRSPaV, as material without GRSPaV is difficult to find. It is unknown if the presence of GRSPaV affected the efficacy of the treatment against GPGV and the effect of the treatment on GRSPaV was also not measured. Further research is required to investigate these questions.

The present work serves as a proof of concept showing that the exogenous application by dipping grapevine shoot tips in dsRNA molecules, which are re-introduced into TC, holds promise as a novel approach and an additional alternative *in vitro* method to thermotherapy, chemotherapy, and cryotherapy to produce virus-free planting material. Traditional methods of apical meristem culture or shoot tip culture could be combined with the methodology developed in this chapter to enhance the production of virus-free grapevines. The advantage of combining dsRNA treatment for virus control and TC is that the treatment can be tailored to a particular pathogen or group of pathogens, while TC can rapidly multiply virus-free TC plantlets that can be used to establish virus-free stocks of grapevine germplasm.

Supplementary material

Supplementary material to this article can be found in the Supplementary material file at: DOI: 10.5073/vitis.2024.63.06.

Conflicts of interest

The authors declare that they do not have any conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author contributions

All authors have read and agreed to the published version of the manuscript. Conceptualization, K.P.K. and F.C.; methodology, K.P.K. and F.C.; formal analysis, K.P.K.; investigation, K.P.K.; resources, K.P.K. and F.C.; data curation, K.P.K. and F.C.; writing—original draft preparation, K.P.K.; writing—review and editing, K.P.K. A.R., B.R and F.C.; visualization, K.P.K.; supervision, F.C., A.R. and B.R.; project administration, F.C..; funding acquisition, F.C. and B.R.

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