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Research Paper

Elimination of *Grapevine rupestris stem pitting-associated virus* from *Vitis vinifera* 'Kyoho' by an antiviral agent combined with shoot tip culture

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ABSTRACT

We evaluated the ability of different concentrations of Ribavirin (15 and 25 μg/mL, R15 and R25) to eliminate *Grapevine rupestris stem pitting-associated virus* (GRSPaV) from *in vitro*-cultured grapevine plants. *Vitis vinifera* 'Kyoho' plants infected by GRSPaV were used as the original plant materials. All of the plants survived in the treatment with R15 and R25 for 85 days, but all Ribavirin-treated plants exhibited phytotoxicity symptoms after 25 days. To ensure the accuracy of detection, optimal primer pair combinations (cpF1R2-cpF2R1 and rep1F1R2-rep1F2R1) were used to assess the eradication efficiency of GRSPaV from regenerated grapevine plants with a nested reverse transcription polymerase chain reaction. The results showed that the efficiency of the GRSPaV eradication was associated to virus titer of regenerated plant in different detection period. All surviving re-generated plants were tested for two rounds and the average elimination rates of GRSPaV in the two periods showed more different, 74.8% (113/151) and 52.3% (79/151), respectively. In addition, the efficiency of the GRSPaV eradication also depended on the concentrations and treatment duration of Ribavirin. The average elimination rates for the regenerated R25-treated and R15-treated plants in the second round detection were 54.1 and 50.6%, respectively. Moreover, the elimination rates for the R25-treated plants were 21.4, 37.5, and 77.8% after 45, 65, and 85 days of treatment, respectively. Combination of shoot tip culture with chemotherapy may greatly increase the efficiency of viral eradications.

1. Introduction

Grapevine is widely cultivated for its fruit, which is important for the production of juices and wines. In China, grapevine is cultivated on 799,000 hm^2, with annual yields of 13,669,900 t (China Agriculture Research System Statistical Data 2015). Genetic resources are required to generate new grapevine cultivars *via* traditional breeding techniques or genetic engineering. However, viruses are particularly problematic in vegetatively propagated crops because they are transmitted from generation to generation. Several diseases caused by viruses, viroids, and phytoplasmas can severely inhibit grapevine growth and development in the most important grapevine-growing regions worldwide, ultimately resulting in considerable decreases in fruit yields (Martelli, 2012). The rugose wood complex is a group of graft-transmissible and damaging grapevine diseases with a worldwide distribution, and Rupestris Stem Pitting (RSP) represents the most common component of this complex (Zhang et al., 1998).

Although the etiology of these diseases has not been characterized, there is evidence that *Grapevine rupestris stem pitting-associated virus* (GRSPaV) is closely associated with RSP (Zhang et al., 1998; Meng et al., 1999; Nakaune et al., 2008). Previous studies revealed that GRSPaV is a member of the genus *Foveavirus* (Martelli and Jelkmann, 1998) within the new viral family Betaflexiviridae (Adams et al., 2004). Additionally, GRSPaV is perhaps the most prevalent virus affecting grapevines and is commonly detected in cultivated grapevines around the world (Meng and Gonsalves, 2003). This virus is restricted to grapevines, is not mechanically transmissible (Martelli and Jelkmann, 1998), and is not known to spread naturally. Some of its strains are reportedly very closely associated with vein necrosis disease (Bouyahia et al., 2005; Morelli et al., 2011). This virus has also been associated with Syrah decline (Lima et al., 2006; Al Rwahnih et al., 2009), and may influence other disorders (Nakaune et al., 2008; Lunden et al., 2010). Although GRSPaV has been detected in pollen grains (Rowhani et al., 2000) and seeds (Stewart and Nassuth, 2001), these infected materials do not produce infected seedlings (Meng et al., 2003). Moreover, there are a diverse range of GRSPaV sequence variants (Lima et al., 2006; Meng et al., 2006; Nolasco et al., 2006; Alabi et al., 2010; Terlizzi et al., 2010).

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Table 1
Primers for analysis of *Grapevine rupestris stem pitting-associated virus* in *in vitro* grapevine plants.

Primers	Positions (nt)[a]	Genes[b]	Sequences (5′–3′)	Size (bp)	References
Regular PCR					
RSP52F	7709–7726	CP	TGAAGGCTTTAGGGGTTAG	905	Alabi et al. (2010)
RSP53R	8594–8613		CTTAACCCAGCCTTGAAAT		
Nested PCR					
cpF1	7624–7643	CP	AGGGCCACTGGAGAGTCAAT		This study
cpF2	7709–7728		TGAAGGCTTTAGGGGTTAGCC		This study
cpR1	8592–8611		TAACCCAGCCTTGAAATCGG		This study
cpR2	8614–8637		TTAGTACGGTATTCCAGCGAACAGG		This study
rep1F1	689–708	Rep	GAAGTGCTGGTTGGCTCTCC		This study
rep1F2	773–795		GATGGCAACTGGAATGAGATGTA		This study
rep1R1	1188–1209		GGCATAAGCAAAGAGCCACTCC		This study
rep1R2	1220–1246		CGGCAGAAGAATGATATGACCAACTT		This study
qPCR					
Y-cpf1	8092–8114	CP	GCACGTCACTGCTCTGATGTTGG	170	This study
Y-cpr1	8236–8261		GTCTCCAGATGGATGTTCCACACGAT		
Vivi-18Sf			AAGCCCGATCCAGCAATA	176	Wang et al. (2012)
Vivi-18Sr			GCCCTTTACGCCCAGTCA		

[a] Positions refer to the nucleotide, relative to all GRSPaV genomic sequences available in GenBank.
[b] CP: coat protein; Rep: replica polyprotein.

The use of healthy propagating material is an important measure for the control of grapevine viruses. Meristem or shoot tip culture, often combined with thermotherapy is one of the most widely applied methods for eliminating viruses from infected grapevine clones (Gribaudo et al., 2006; Maliogka et al., 2009; Panattoni and Triolo, 2010). Unfortunately, removing GRSPaV from propagating materials using the meristem tip culture or *in vivo* and *in vitro* thermotherapy is particularly difficult (Gribaudo et al., 2006; Skiada et al., 2009), likely because of the ability of the virus to readily invade meristematic cells (Rowhani et al., 2000). Alternatively, controlling plant viral diseases with chemicals shows considerable promise for agriculturally and economically important crops. However, developing effective chemicals that eliminate phytoviruses or substantially inhibit viral replication has been problematic (Panattoni et al., 2007). To date, Ribavirin (1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide; commercial name: Virazole) combined with *in vitro* culturing has been used to eliminate specific viral pathogens from grapevine plants, with varying degrees of success (Weiland et al., 2004; Panattoni et al., 2007; Guta and Buciumeanu, 2011). Ribavirin is a potent chemotherapeutic agent with a multifaceted mode of action, which inhibits the capping and elongation of viral mRNA. In particular, it prevents the accumulation of guanosine 5′-phosphate and the methylation associated with the synthesized mRNA cap (Smith, 1984; Lerch, 1987), which ultimately inhibits the synthesis of viral nucleic acids.

We previously conducted *in vitro* experiments that investigated the consequences of Ribavirin treatments on viruses infecting pear and apple plants (Hu et al., 2012, 2015). However, the effects of Ribavirin on grapevine viruses remain unclear. In the present study, we evaluated the effects of combining shoot tip culture with chemotherapy treatments on the elimination of viruses from grapevine plants. We analyzed the efficiency of the combined treatments at different time points.

2. Materials and methods

2.1. Plant materials

Vitis vinifera 'Kyoho' plants, which are widely cultivated in China, were grown at the Research Institute of Pomology, Chinese Academy of Agriculture Sciences. The selected plants had been previously acquired from a thermotherapy-coupled meristem culture system and after testing for *Grapevine leafroll associated virus* (GLRaV)-1 to −7, *Grapevine fleck maculavirus* (GFKV), *Grapevine fanleave virus* (GFLV), *Grapevine vitivirus A* (GVA), *Grapevine vitivirus B* (GVB), *Grapevine vitivirus E* (GVE) by reverse transcription-polymerase chain reaction (RT-PCR), only GRSPaV was detected in this cultivar.

2.2. In vitro cultures

Plants were cultured on modified half-strength Murashige and Skoog (MS) medium supplemented with 0.5 mg/L indole-3-acetic acid, 30 g/L sucrose, and 5.6 g/L agar. The pH was adjusted to 5.8 with 1 M NaOH or HCl before the medium was autoclaved at 121 °C for 15 min. All cultures were incubated in a standard growth room at 24 ± 1 °C under a 16-h light (2000 lx)/8-h dark photoperiod. Viable cultures were transferred to fresh basic half-strength MS medium at 45-day intervals, and the presence of GRSPaV was re-assessed by RT-PCR.

2.3. Isolation of RNA

Total RNA was extracted from grapevine samples as described by Hu et al. (2015). Plant samples (0.1 g) were homogenized in 1 mL grinding buffer. Then, 150 μL 10% N-lauroyl sarkosyl was added to the clarified homogenate, and the mixture was incubated at 72 °C for 10 min and then on ice for 5 min. After a centrifugation, 300 μL supernatant was mixed with 300 μL 6 M NaI, 150 μL ethanol, and 25 μL autoclaved silica powder suspension. After washing the pellet in half-strength washing buffer, RNA was eluted by re-suspending the washed pellet in 100 μL nuclease-free water and then incubating at 72 °C for 4 min. After a centrifugation, the RNA-containing supernatants were stored at −72 °C.

2.4. RT-PCR

First-strand cDNA was synthesized in a 20-μL reaction mixture containing about 1 μg RNA, 4 μL M-MLV RT 5 × buffer, 1 μL 10 mM dNTP mix (TaKaRa, Dalian, China), 1 μL 0.1 mM random hexamer primers (Sangon Biotech, Shanghai, China), and 100 U M-MLV reverse transcriptase (Promega, Madison, WI, USA). The mixtures were incubated at 37 °C for 10 min, 42 °C for 1 h, and then 72 °C for 3 min.

2.4.1. Regular PCR amplification

The PCR was completed in a 25-μL mixture consisting of 2.5 μL 10 × PCR buffer with Mg^{2+} (15 mM), 0.5 μL 10 mM dNTP mix, 0.5 μL each primer (final concentration 1 μM), 0.2 μL 5 U/μL r*Taq* DNA polymerase (TaKaRa), 2 μL template cDNA, and 18.8 μL sterile water.