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Grapevine leafroll-associated virus 2 and grapevine 'Pinot gris' virus are present in seedlings developed from seeds of infected grapevine plants

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Summary

Nearly 80 different viruses belonging to different genera and families have been identified in grapevines, but their seed transmissibility remains largely unclear. Thus, the specific objective of this work is to monitor the presence of viruses in seedlings grown from seeds of virus-infected grapevine plants. Using reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR, we showed the presence of grapevine leafroll-associated virus 2 (GLRaV-2), GLRaV-3, grapevine 'Pinot gris' virus (GPGV), grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine virus A (GVA), and grapevine fleck virus (GFkV) in all tested parts including mature cane, leaf, petiole, young shoot, flower, berry skin, and seed of infected grapevine 'Moldova' plants. Furthermore, GLRaV-2 and GPGV were found in all tested seedlings developed from seeds of 'Moldova' plants, while the other four viruses were not detected. Our results provide the first evidence that GLRaV-2 and GPGV can be transmitted to progeny seedlings from seeds of infected grapevine plants.

K e y w o r d s : grapevine viruses, seed transmission, seedling, RT-PCR, RT-qPCR.

Introduction

Grapevine is known to host a large number of viruses. Many of these viruses, usually in combinations, are responsible for serious viral diseases. Three viral diseases, namely grapevine leafroll disease (GLD), rugose wood (RW) complex, and infectious degeneration are of major economic importance worldwide (Bovey *et al*. 1980, Martelli 2014). Members of the family *Closteroviridae*, grapevine leafroll-associated viruses (e.g., GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-7, and GLRaV-13) have been reported as being related with GLD (Martelli 2014, Song *et al*. 2021). Grapevine virus A (GVA), GVB, GVD, GVE, GVF and grapevine rupestris stem pitting-associated virus (GRSPaV) in the family *Betaflexiviridae* are associated with the RW complex (Martelli 1993, 2014). Grapevine fanleaf virus (GFLV) and other members of the *Nepovirus* genus are related to infectious degeneration (Laimer *et al*. 2009). In addition, grapevine 'Pinot gris' virus (GPGV) in the genus *Trichovirus* has been reported to be associated with serious symptoms of virus disease (Giampetruzzi *et al*. 2012), and grapevine fleck virus (GFkV; family *Tymoviridae*), which is associated with the grapevine fleck disease (Martelli 2014), has a worldwide distribution.

Seed transmission is an efficient means of disseminating plant virus and viroid diseases. In general, embryo invasion is necessary for seed transmission of plant viruses (De Assis Filho and Sherwood 2000, Hull 2014). It's known that seed embryos are usually protected against invasion by viruses that affect the mother plant, and even embryo invasion does not always lead to seedling infection, because the virus in embryo may be degraded and lost its infectivity during germination (De Assis Filho and Sherwood 2000, Gasparro *et al*. 2017). About 20 % of the described plant viruses are seed-transmitted in one or more hosts (Johansen *et al*. 1994, Wang and Maule 1994, Amari *et al*. 2009, Hull 2014). The percentage of seed transmission varies from 0 to 100 % of plant viruses and viroids in different plants (Sastry 2013). In grapevine, very few viruses and viroids have been confirmed for seed transmission. For example, GRSPaV is suspected to be pollen-borne (Rowhani *et al*. 2000, Morelli *et al.* 2009) and transmitted at a low percentage by seeds (Lima *et al*. 2006, Laimer *et al*. 2009). Occasional transmission through seeds has also been reported for GFLV (Maliogka *et al*. 2015). In addition, Wah and Symons 1999) reported that grapevine yellow speckle viroid 1 and hop stunt viroid can be transmitted through seeds. However, seed transmissibility of phloem-restricted viruses associated with GLD, RW complex and Fleck disease in grapevine (Digiaro *et al*. 1999) remains largely unclear. Using reverse-transcription polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR (RT-qPCR) and DNA sequencing, this study examined the distribution of viruses in different parts of field-grown grapevine plants of cultivar 'Moldova', and the presence of these viruses in seedlings developed from seeds of 'Moldova' plants.

Material and Methods

S a m p l e c o l l e c t i o n : To analyze the spatial distribution of viruses, three 10-year-old grapevine plants of the same cultivar ('Moldova') were used. These plants cultivated

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in the experimental fields of China Agricultural University have previously been shown mixed infections with GLRaV-2, GLRaV-3, GFkV, GPGV, GVA, and GRSPaV. Samples were collected from different parts, *i.e.*, mature cane, leaf, petiole, young shoot, flower, and berry (further separated into skin and seed) of these three grapevine plants in May (for sampling leaf, petiole, young shoot and flower), August (berry) 2020, and October (mature cane) 2021, respectively. Seeds were extracted from berries and their coat surfaces were cleaned with tap water. For testing the presence of viruses, seeds were disinfected by being soaked in a solution of 3.5 % sodium hypochlorite for 5 min and in double-distilled H_2O (ddH_2O) for 1 min. Three samples of each plant part were collected. Each sample consists of phloem scraped from one mature cane, five petioles, three leaves, three young shoots, thirty-forty flowers, fifteen seeds, or skins obtained from twenty-five berries.

Obtaining seedlings: One hundred shade-dried grapevine seeds were placed in a refrigerator at 4 °C for stratification for about 30 days. Seeds were then treated with 250 ppm gibberelic acid solutions for 2 d, washed with sterile ddH_2O and placed on filter paper with sterilized ddH_2O at 25 °C for about 15 d. Once the seeds had germinated, they were individually sown in pots containing commercial soil and kept in an illuminating incubator (16 h light/8 h dark at 25-26 °C). When seedlings were 4 weeks old, individual seedlings were sampled for RNA extraction and detection of viruses by RT-PCR. Randomly selected samples were further analyzed by RT-qPCR and DNA sequencing. Nucleotide sequences of GLRaV-2 and GPGV amplified from one seedling sample were deposited in the GenBank under accession numbers MK894576.1 and MZ424189, respectively.

RT-PCR and RT-qPCR: The primers are listed in the Table. Primer pairs GLRaV-2F/GLRaV-2R and GLRaV-3F/GLRaV-3R used for RT-PCR analysis were designed in this study. Primer pairs qGLRaV-2F/qGLRaV-2R, and qGLRaV-3F/qGLRaV-3R used for RT-qPCR analysis, and GRSPaV-F/GRSPaV-R, GFkV-F/GFkV-R, GPGV-F/GPGV-R, and GVA-F/GVA-R used for both RT-PCR and RT-qPCR analyses were previously reported (Wang *et al*. 2020). *V. vinifera* actin (XM_002282480.4) mRNA served as internal control with the primer pair qActin-F/qActin-R (Wang *et al*. 2020).

Total genomic RNA was extracted with a Polysaccharide and polyphenol total RNA isolation kit (Bioteke Corporation, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesized (at 42 °C) with a mix of random primers using 500 ng total RNA. PCR amplification was conducted according to the procedure as follows: cDNA denaturation at 95 °C for 5 min; 34 cycles at 94 °C for 30 s, 53-60 °C (depending on specific primers used) for 30 s and 72 °C for 12-30 s (depending on specific primers used), and a final extension step at 72 °C for 5 min. Plasmid DNA containing the targeted sequence of virus or ddH_2O instead of cDNA served as positive or negative control.

RT-qPCR was conducted with the SYBR PrimeScript RT-PCR kit (TAKARA, Beijing, China) according to the manufacturer's protocols. The qPCR procedure was conducted as follows: all reactions were incubated on 96-well plates at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 58 °C for 20 s, 72 °C for 35 s, then 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. qPCR analysis was conducted in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc., USA).

Results and Discussion

Distribution of viruses in different parts of grapevine plants: Three 10-year-old grapevine plants of cultivar 'Moldova' which are known to be mix-infected with GLRaV-2, GLRaV-3, GFkV, GPGV, GVA, and GRSPaV were used. The RT-PCR amplicons of all tested samples showed a specific band of expected sizes (618, 540, 210, 340, 429, and 327 bp for GLRaV-2, GLRaV-3, GFkV, GPGV, GVA and GRSPaV, respectively), while the negative control had no specific amplified band (Fig. 1A). The RT-qPCR melting curve analysis for samples

Table

Primers used in this study

Primer name	Sequence (5'-3')	Size (bp)	Reference	Target
qGLRaV-2F	CCTGGTCATAACTGACGCCTC	124	Wang *et al*. (2020)	*CP*
qGLRaV-2R	GTAGACCGAACACCACTTCTATACCG			
GLRaV-2F	ATGAGGGTTATAGTGTCTCCTTAT	618	designed in this study	*p24*
GLRaV-2R	TTAACATTCGTCTTGGAGTTCG			
qGLRaV-3F	GTAGTGGACGAAAGGTGGCTCTTC	170	Wang *et al*., (2020)	*HEL*
qGLRaV-3R	GACAGACAACACCAATTCCTTCGTC			
GLRaV-3-F	GCCCGCATCGATATGGACCTATCGTTTATT	540	designed in this study	*p19.7*
GLRaV-3-R	GCCGTCGACTTATAGTGCTCCGCAACAAA			
GRSPaV-F	CGTCACTGCTCTGATGTTGGTAG	327	Wang *et al*. (2020)	*CP*
GRSPaV-R	AGGCGATATTAGCAACCATCTCAG			
GFkV-F	GTGTAAGCATCCATCTCCCCTTCCAG	210	Wang *et al*. (2020)	*CP*
GFkV-R	GAGTCGATGGTCCAGCAGAGGTC			

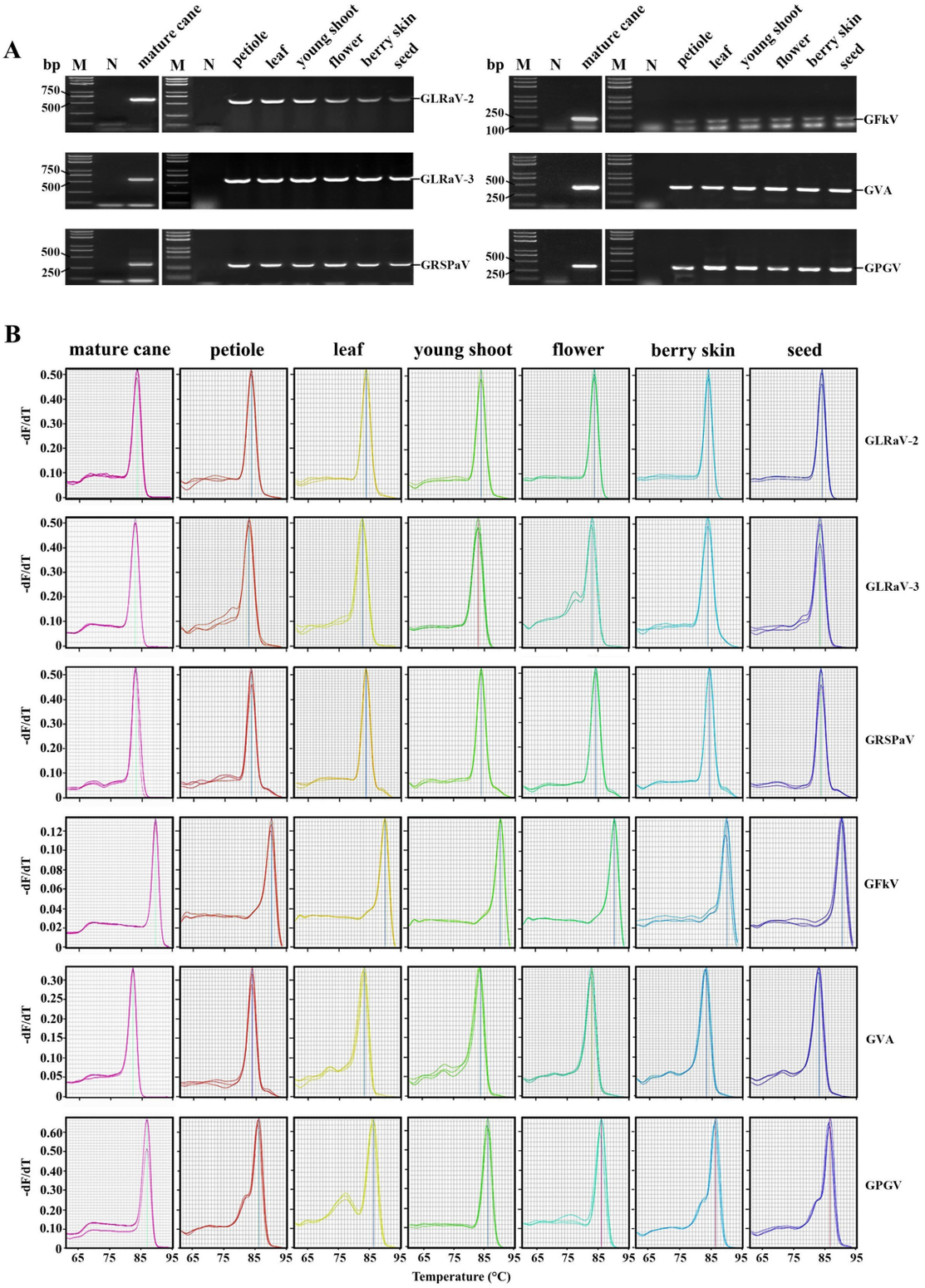

Fig. 1: Spatial distributions of GLRaV-2, GLRaV-3, GVA, GRSPaV, GFkV and GPGV in field-grown grapevine 'Moldova' plants. (**A**) RT-PCR detection of GLRaV-2, GLRaV-3, GVA, GRSPaV, GFkV and GPGV in different plant parts. M: DNA marker. N: negative control with ddH_2O instead of cDNA. (**B**) Detection of GLRaV-2, GLRaV-3, GVA, GRSPaV, GFkV and GPGV in different plant parts by RT-qPCR melting curve analysis. -dF/dT: the negative derivative of fluorescence with respect to temperature.

from different parts of plants all showed a similar single melting curve peak (Fig. 1B), which was specific to the targeted virus as confirmed by gel electrophoretic analysis of the qPCR products. Therefore, these results indicated that GLRaV-2, GLRaV-3, GFkV, GPGV, GVA and GRSPaV were distributed in all tested plant parts *i.e.*, mature cane, leaf, petiole, young shoot, flower, berry skin and seed of 'Moldova' grapevine plants. Our findings are similar to previous reports, where authors observed the presence of GLRaV-1, GLRaV-3, GVA, and GRSPaV in flowers (Gam-

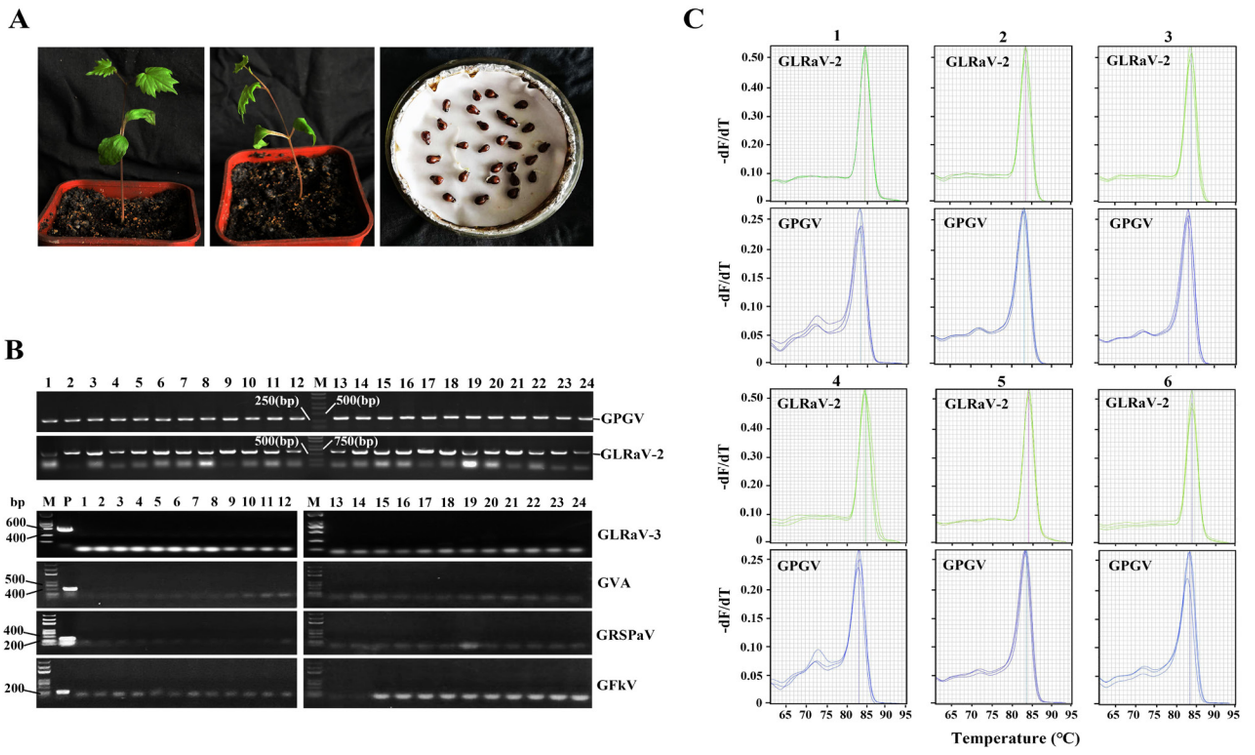

Fig. 2: Detection of GLRaV-2, GLRaV-3, GVA, GRSPaV, GFkV and GPGV in seedlings grown from seeds of infected grapevine 'Moldova' plants. (**A**) Potted seedlings (n = 24) were propagated from seeds (right panel). (**B**) The presence of GLRaV-2 and GPGV in seedlings revealed by RT-PCR. M: DNA marker. P: positive control with plasmid DNA containing the targeted sequence of virus instead of cDNA. Numbers 1-24: denote samples from twenty-four seedlings. (**C**) Detection of GLRaV-2 and GPGV in six randomly selected seedling samples by RT-qPCR melting curve analysis. Numbers 1-6: denote six seedling samples. -dF/dT: the negative derivative of fluorescence with respect to temperature.

BINO *et al*. 2006, 2009), GLRaV-1 and GLRaV-3 in berries (SHABANIAN *et al*. 2020), and GRSPaV in seeds (STEWART and NASSUTH 2001, LIMA *et al*. 2006) of infected plants.

Detection of viruses in seedlings developed from seeds of infected plants: A total of 24 grapevine seedlings were obtained from one hundred seeds of 'Moldova' plants. GLRaV-3, GFkV, GVA and GRSPaV, which were present in seeds (Fig.1), were not detected in any of twenty-four seedlings analyzed. Our results are similar to those reported by GASPARRO *et al*. (2017) that GFkV, GVA and GRSPaV cannot be seed transmitted. However, our findings are based on the small number of grapevine seedlings included. An extensive survey of the presence of these viruses in grapevine seedlings is required to get more solid data. It should be noted that there are inconsistent reports regarding GRSPaV: seed transmission did not occur in our study and in literature (GASPARRO *et al*. 2017), where authors monitored the virological condition of seedlings obtained by crosses between GRSPaV-infected parents ('Almeria' x 'Supernova', 'Ceresa' x 'Carati', and 'Red Globe' x 'Regal'), whereas LIMA *et al*. (2006) observed that seedlings grown from seeds of GRSPaV-infected cultivar 'Cabernet Sauvignon' demonstrated a 0.4 % infection rate. However, LIMA *et al*. (2006) also reported that GRSPaV was not transmitted through the seed from GRSPaV-positive grapevine cultivar 'Pinot Noir' and 'Muscadelle'. Different varieties of the same host species often vary widely in the rate at which seed transmission of a particular virus occurs (HULL 2014). The findings of these studies suggest that seed transmission of GRSPaV may be cultivar dependent in grapevine.

Surprisingly, PCR products of all tested seedling samples showed a specific single band of expected size for GLRaV-2 (618 bp) and GPGV (340 bp), respectively. RT-qPCR melting curve analyses of six randomly selected seedling samples all displayed a similar single melting curve, which was specific to GLRaV-2 and GPGV, respectively. In addition, the presence of GLRaV-2 and GPGV was further confirmed by DNA sequencing of PCR products of one seedling sample. Thus, these results demonstrated that GLRaV-2 and GPGV are present in all tested seedlings grown from seeds of infected 'Moldova' plants. Our results provide the first evidence that GLRaV-2 and GPGV can be transmitted *via* seeds, indicating the two viruses may be at high risk for seed transmission in grapevine. Our findings are in contrast with the previous report showing that GLRaVs cannot be transmitted *via* seeds (GASPARRO *et al*. 2017). The methodological difference may be responsible for the inconsistency. Seedlings used in this study are derived from infected seeds of the same plant, while those in GASPARRO *et al*'s (2017) study were obtained by crosses between infected parents. Alternatively, the inconsistency may be due to the different grapevine cultivars used in two studies. It is suggested that seed transmission is mainly achieved by virus invasion of the embryo (HULL 2014). Future research is needed to investigate seed transmissibility of GLRaV-2 and GPGV in

different grapevine cultivars and the distribution of the two viruses in seed tissues.

Grapevine is not seed-propagated in commercial viticultural practices, thus vegetative propagation by cutting and grafting contribute to the worldwide spread of grapevine viruses. However, seedlings grown from *Vitis amurensis* seeds are widely used as cold-resistant grape rootstocks in Northeast China. In this case, the potential risk of seed transmission of GLRaV-2 and GPGV should not be ignored.

Conclusion

In conclusion, our results demonstrate that GLRaV-2, GLRaV-3, GVA, GRSPaV, GFkV and GPGV can be detected in all tested parts including seeds of infected grapevine 'Moldova' plants, and GLRaV-2 and GPGV are present in all seedlings developed from virus-contaminated seeds. Our results provide the first evidence for seed transmission of GLRaV-2 and GPGV in grapevine, which could be used to improve the current practices and regulatory measures.

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