Journal of Animal & Plant Sciences, 34(2): 2024, Page: 541-546
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2024.2.0740

EVALUATION OF THE ELIMINATION EFFICENCY OF APPLE STEM GROOVING VIRUS BY DIFFERENT DETECTION METHODS

G.J. Hu, Y.F. Dong*, Z.P. Zhang, X.D. Fan and F. Ren

Research Institute of Pomology of CAAS, Xingcheng, China
Corresponding author's email: yfdong@163.com

ABSTRACT

Apple stem grooving virus (ASGV) frequently occurs in apple (*Malus*) trees and shows high sequence variability. A sensitive detection is essential for effective forecast and control of this virus. The reverse transcription-duplex polymerase chain reaction (RT-dPCR) was developed after screening of primer combinations, adjustment of annealing temperature, and optimization of dosage of primer pair combination and cDNA. Then, RT-dPCR and RT-regular PCR (RT-rPCR) were used to detect ASGV in regenerated apple plants after thermotherapy and in nature growing apple trees. The results showed that the detection efficiency of RT-dPCR was the same as the total of two RT-rPCRs. Moreover, RT-dPCR was found a sensitive, rapid and simple method to detect ASGV from various apple plants. These findings might be useful in the prediction of viral disease in host plants and can also be helpful to construct the same detection assays for other viruses.

Keywords: Apple stem grooving virus; Detection efficiency; RT-duplex PCR; Sensitivity; Various apple plants; Virus detection.

Published first online January 29, 2024 Published final March 31, 2024

INTRODUCTION

Apple stem grooving virus, the type member of *Capillovirus* genus *Betaflexiviridae* family, is commonly distributed in apple and pear trees worldwide. The natural hosts of ASGV originally include apple, pear, citrus, lily and kiwifruit (Xuan *et al.*, 2022). In last decade, the virus has also been reported in heavenly bamboo (*Bambusoideae*), rose (*Rosa chinensis Jacq.*), himalayan wild cherry (*Prunuscerasoides* D. Don), fig (*Ficus palmata*), lotus (*Nelumbo nucifera*), perennial weed (*Rubus ellipticus*), Cnidium Rhizom (*Cnidium officinale*), huang jing (*Polygonatum kingianum*), and loquat (*Eriobotrya japonica*) (Canales *et al*., 2021; Lee *et al*., 2021). ASGV is symptomless in most commercial rootstocks and varieties, but it generally causes stem grooving, graft union abnormalities, vigor of the canopy reducing and an overall decline in susceptible *Malus* species. Moreover, ASGV coupled with apple chlorotic leave spot virus and apple stem pitting virus can produce top-working diseases of apple and pear on susceptible rootstocks (Kim *et al*., 2022). ASGV is mainly transmitted by grafting and no insect vectors are known, so the use of healthy or virus-free propagation material is important to control this virus.

The genomes of ASGV shows high sequence variability and contains two highly variable regions, variable region I (532-570 amino acids, aa) and II (1,583-1,868 aa) (Jia *et al*., 2022). Then, the screen of the suitable detection method was very important, which has close relationship with the detection effiencies and identification of virus-free plants. In some cases, to certify the virus infection status of apple, two primer pairs have been used to detect ASGV by PCR (Hu *et al*., 2015; Hu *et al*., 2017; Hu *et al*., 2018).

Although the use of more than one primer pair can increase the specificity of detection, it is more costly and time-consuming than using a single detection method. In order to reduce the cost and time consumption when using multiple primers for detection, this study developed a RT-duplex PCR (RT-dPCR) method to detect ASGV and compared the detection efficiencies of RT-dPCR with two RT-regular PCRs (RT-rPCRs).

MATERIALS AND METHODS

Plant material: Nine ASGV-positive apple plants were kept in the Research Institute of Pomology, Chinese Academy of Agricultural Sciences. Among them, four varieties (Yanfu no.1, Huahong, Gala, and Wanglin) were used to construct the detection system. Five varieties (Longga, Longwei, Liquanduanzhi, Jin no.18, and YD) were treated by thermotherapy as blow. Moreover, 123 apple samples were randomly collected from Shandong, Henan, Shanxi and Liaoning provinces.

Virus elimination: Five ASGV-positive apple varieties were treated at 38°C for 30 days. After thermotherapy, the 0.8-1.0 cm shoot tips were cut from treated apple plants and grafted onto the virus-free rootstocks *M. baccata* (L.) Borkh referencing Hu *et al*. (2017).

RNA isolation and cDNA synthesis: Total RNA was extracted from different materials (phloem or new leaves) of apple according to the method described previously (Hu *et al*., 2018). The cDNA was synthesized from above RNA using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA).

RT-rPCR amplification: The reaction volume: 10× PCR buffer (Mg^{2+} plus) 2.5 µl, dNTP Mixture (2.5 mmol/l each) 0.5 µl, primer 1 (P1) and P2 0.5 µl (10 µmol/l, each), r*Taq*® 0.125 µl (5 U/µl, Takara, Dalin, China), cDNA 2.0 µl, adding ddH_2O to 25 µl. Five primer pairs, ASG-F1/R1, ASG-F2/R2, ASG-F3, ASG-F4/R4 and ASG-F5/R5, were used to detect ASGV (Table 1). The thermocycling conditions was 3 min at 94 °C, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (52-57 °C, 30-45 s) and extension (72 °C, 30-45 s), and final extension for 7 min at 72 °C. Amplification was conducted in a 96-well PCR Thermal Cycler (Bio-Rad, S1000™, Hercules, CA, USA). The PCR products were separated by electrophoresis on a 1.2 % agarose gel (Sangon BiotechCo., Ltd., Shanghai, China), stained with GeneFinder™ (Biov Bio, Xiamen, China) and visualized under ultraviolet light.

Optimization of reaction conditions of RT-dPCR: Our previous results showed that the detection efficiencies of the above five primer pairs was similar (data not showed). Therefore, two of these primer pairs were usually selected for routine ASGV detection, which was more costly and time-consuming than using a single detection method. To reduce the consumption, nine prime pair combinations (ASG-F1/R1-F2/R2, ASG-F1/R1-F3/R3, ASG-F2/R2-F3/R3, ASG-F1/R1-F4/R4 ASG-F2/R2-F4/R4, ASG-F3/R3-F4/R4, ASG-F1/R1-F5/R5, ASG-F2/R2-F5/R5, and ASG-F3/R3-F5/R5) were set and the reaction volume referencing system 1 (Table 2). The thermocycling conditions was 3 min at 94 °C, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (51 °C, 45 s) and extension (72 °C, 45 s), and final extension for 7 min at 72 °C. The dosage of each primer (system 2 and system 3), dosage of cDNA (system 4 and system 5), and annealing temperature (51 °C and 60 °C) were optimized during PCR amplification, respectively (Table 2).

RESULTS

RT-dPCR amplification: The amplification results showed that three primer pair combinations ASG-F1/R1-F4/R4, ASG-F2/R2-F4/R4 and ASG-F3/R3-F4/R4 could produce two clear target band for one sample, respectively, but the products of other six combinations were easily confused with the primer dimer or had some weak nonspecific bands on the gels (Fig. 1). Among the three primer pair combinations, ASG-F3/R3-F4/R4 were better in the consistency of amplification. The intensities of the two amplified targets were uneven under the 51 °C annealing temperature, which was improved after increased to 60 °C (Fig. 2). In the optimization of dosage of the two primer pairs, the consistency and intensities of system 2 were better than system 1 and 3 (Fig. 3). In this primer pairs dosage (system 2), two clear targets could still be amplified when the dosage of cDNA was decreased to 3 µl (system 5). Therefore, the optimized reaction system for dPCR with primer pair combination ASG-F3/R3-F4/R4 was system 5 and thermal profile was 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 60 °C, 45 s at 72 °C, and final incubation at 72 °C for 7 min.

ASGV detection of regenerated apple plants after thermotherapy by RT-dPCR: The average survival rate of all grafted shoot tips after thermotherapy was 42.6%. The elimination efficiencies of ASGV in forty-three regenerated apple plants were analyzed by both RT-rPCR and RT-dPCR, respectively. The detection rate using the two primer ASG-F1/R1 and ASG-F2/R2 for regenerated plants of Longga, Jin 18, Meile, and Liquanduanzhi were the same, but the one more plants was detected ASGV-positive by primer ASG-F1/R1 than ASG-F2/R2 for regenerated plants of YD. Then, the total elimination rate of this variety was 36.4%, which was the same with the result of RT-dPCR. The average detection rates of ASGV using RT-rPCR primer pair ASG-F1/R1 and ASG-F2/R2 were 65.1% and 67.4%, respectively. The elimination rate of ASGV detected by RT-dPCR was 65.1% (28/43), which was the same with that detected by two RT-rPCRs (Table 3).

ASGV detection of apple plants in filed by RT-dPCR: The RT-rPCR and RT-dPCR were also used to analyze the ASGV prevalence in 123 apple samples of four regions of China. The detection rates of ASGV using RT-rPCR primer pair ASG-F1/R1 and ASG-F2/R2 were 75.0% and 73.3%, respectively, and the total rate of the two primer pairs was 75.8%. The infection rate of ASGV tested by RT-dPCR with pair combination ASG-F3/R3-F4/R4 was 75.8%. These results demonstrated that the new RT-dPCR method met the requirements of accurate detection and low cost.

Table 1. Primers used to detect ASGV.

Primers	Sequence 5′-3′	Positions (nt)[a]	Gene[b]	Size (bp)	Annealing temperatures (°C)	Reference
ASG-F1	CCCGCTGTTGGATTTGATACACCTC	5872-5896	CP	525	55	Clover *et al*. (2003)
ASG-R1	CTGCAAGACCGCGACCAAGTTT	6373-6396				
ASG-F2	CCCGCTGTTGGATTTGATACACCTC	5872-5896	CP	500	53	James (1999)
ASG-R2	GGAATTTCACACGACTCCTAACCCTCC	6345-6371				
ASG-F3	ATGAGTTTGGAAGACGTGCTTCA	5641-5663	CP	449	57	Li *et al*. (2018)
ASG-R3	CAAAGCTTYCKGAACGTACATTC	6067-6089				
ASG-F4	TCCACATCATCAAGCTTCAGA	3589-3609	Rep	274	57	This study
ASG-R4	TTCCAGTCAATGTCAGACCTTTC	3840-3862				
ASG-F5	GCAGAACTCTTTGAACGAATG	6051-6071	CP	124	52	This study
ASG-R5	GGGCCATTTCTTGTAAATGTTGGTG	6150-6174				

[a] Positions refer to nucleotides relative to ASGV genomic sequences in GenBank.
[b] CP: coat protein; Rep: replication protein

Table 2. Different reaction systems designed for RT-dPCR analysis.

PCR Reagents	System (µl)				
	1	2	3	4	5
10× PCR buffer (Mg^{2+} plus)	2.50	2.50	2.50	2.50	2.50
dNTP Mixture (2.5 mmol/l each)	1.00	1.00	1.00	1.00	1.00
Primer pair 1 F (10 µmol/l)	0.50	0.60	0.40	0.60	0.60
Primer pair 1 R (10 µmol/l)	0.50	0.60	0.40	0.60	0.60
Primer pair 2 F (10 µmol/l)	0.50	0.40	0.60	0.40	0.40
Primer pair 2 R (10 µmol/l)	0.50	0.40	0.60	0.40	0.40
r*Taq*® (5 U/µl)	0.25	0.25	0.25	0.25	0.25
cDNA	4.00	4.00	4.00	2.00	3.00
ddH_2O	15.25	15.25	15.25	17.25	16.25
Total	25.00	25.00	25.00	25.00	25.00

Table 3. Survival and elimination rate of apple plants after thermotherapy.

Cultivars	No. of treated plants	No. of grafted shoot tips	No. of survival plants (%)#	Elimination rate (%)*			
				rPCR			dPCR
				ASG-F1/R1	ASG-F2/R2	Total	ASG-F3/R3-F4/R4
Longga	8	18	5 (27.8)	80.0 (4/5)	80.0 (4/5)	80.0 (4/5)	80.0 (4/5)
Jin 18	11	21	6 (28.6)	100.0 (6/6)	100.0 (6/6)	100.0 (6/6)	100.0 (6/6)
YD	16	35	11 (31.4)	36.4 (4/11)	45.5 (5/11)	36.4 (4/11)	36.4 (4/11)
Meile	2	15	11 (73.3)	100.0 (11/11)	100.0 (11/11)	100.0 11/11)	100.0 (11/11)
Liquanduanzhi	12	12	10 (83.3)	30.0 (3/10)	30.0 (3/10)	30.0 (3/10)	30.0 (3/10)
Total	49	101	43 (42.6)	65.1 (28/43)	67.4 (29/43)	65.1 (28/43)	65.1 (28/43)

The numbers outside the parentheses represent the number of survival plants, and the numbers inside represent the percentage of survival plants / cut shoot tips; *The numbers outside the parentheses represent the percentage of virus-free plants, and the numbers inside represent the number of virus-free plants / detected plants.

Figures

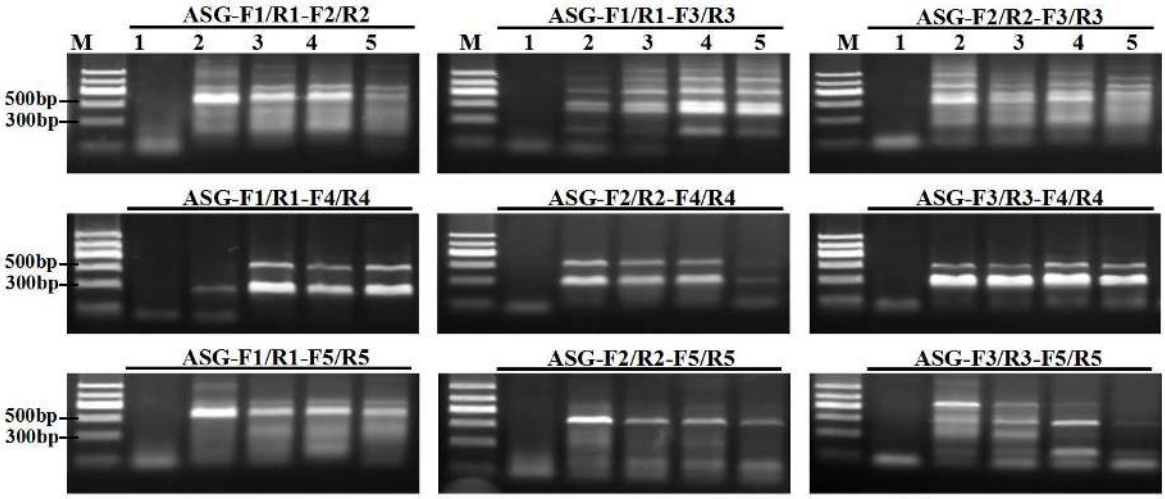

Figure 1. Gel electrophoresis of duplex PCR (dPCR) products of apple stem grooving virus (ASGV) amplified by different primer pair combination. M: DNA marker II (TianGen, China); Lane 1: negative control; Lanes 2–5: ASGV-positive apple samples (Yanfu no.1, Huahong, Gala, and Wanglin).

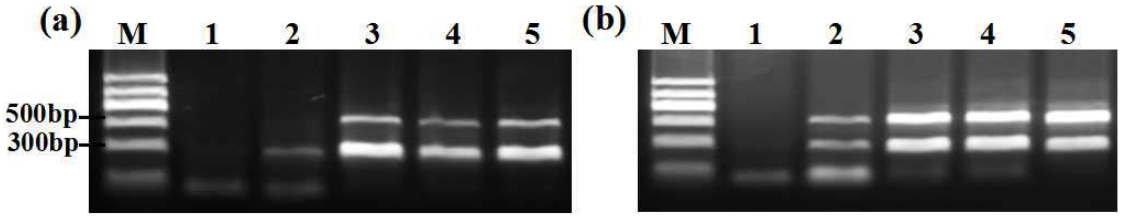

Figure 2. Gel electrophoresis of RT-dPCR products of ASGV amplified under annealing temperature 51 °C (a) and 60 °C (b) . M: DNA marker II; Lane 1: negative control; Lane 2-5: ASGV-positive apple samples as above.

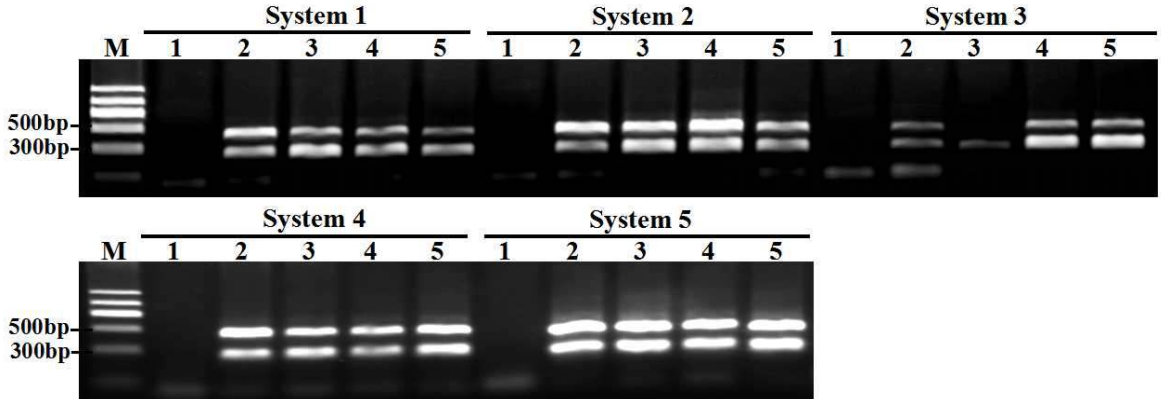

Figure 3. Gel electrophoresis of RT-dPCR products of ASGV amplified under different reaction system. See Table 3 for details of systems 1, 2, 3, 4 and 5. M: DNA marker II; Lane 1: negative control; Lane 2-5: ASGV-positive apple samples as above.

DISCUSSION

In this study, the RT-dPCR method was successfully used to detect one apple virus, ASGV and the detection efficiencies of the RT-dPCR was the total of two RT-rPCRs. ASGV is widely distributed in apple and pear trees throughout China (Hu *et al*., 2017). The establishment of sensitive, reliable, fast, and inexpensive methods to detect this virus is very important for certification and quarantine programs for apple and pear plants and germplasm (Huang *et al*., 2023). Different RNA viruses may infect a single host, so sensitive

detection is needed to ensure the exclusive propagation of virus-free plant materials (Nabi *et al*., 2022; Reddy *et al*., 2022). The RT multiplex PCR and RT-dPCR methods are very useful in plant virology. Using these methods, the cost and number of samples might be further decreased (Miljanić *et al*., 2022). Similar assays have been developed and widely used to detect and identify viruses in pear, apple and other fruit trees (Huang *et al*., 2014; Miljanić *et al*., 2022; Nongsiang *et al*., 2022).

The key factors affecting the virus detection efficiency include the sensitivity of the primer pair, the concentration of the virus in the materials and the sampling period (Hu *et al*., 2015). The genetic diversity of the virus contributes to the complexity of the virus population, which can affect virus detection and reduce the effectiveness of virus control measures. The relatively high divergence of viral genomes can result in the failure of PCR and lead to false negative results (Kwon *et al*., 2022; Xu and Ming, 2022). To address this problem, we developed the RT-dPCR assay to simultaneously detect two genomic regions of ASGV. In virus detection, selection of the two primer pairs for RT-dPCR was important.

In this study, six of the primer pair combinations did not produce reliable results. Analysis of the position of these primer pairs, we found that the amplified fragments of primer pair ASG-F1/R1, ASG-F2/R2, ASG-F3/R3 and ASG-F5/R5 were all in coat protein gene and ASG-F3/R3 was in replication protein. The amplicons of ASG-F1/R1 and ASG-F3/R3 had an overlapping sequence (5872–6089 nt) and ASG-F2/R2 and ASG-F5/R5 was inside of ASG-F1/R1. The best results were obtained from primer pair combination ASG-F3/R3-F4/R4, whose amplified fragments were in different positions of the virus genome and did not contain overlapping sequences. That is, the primers should amplify regions of different genes. Moreover, our results also showed that the RT-dPCR method was not material-specific, so it should be effective for detecting ASGV from different hosts.

The RT-dPCR method was as good as the sum of the two conventional methods, whether it was to detect field samples or regenerated plants after elimination treatment. In addition, its application effect was consistent with that of other apple viruses (Hu *et al*., 2019a; 2019b). The quality of RNA is very important in the detection of apple viruses. RT-dPCR performed two amplification during one reaction, so it need more high quality RNA. Apple had high levels of polyphenols and polysaccharides in plant tissues. To meet requirement of viral detection, it is important to use more reliable RNA extraction method suitable for vast woody plant types. In addition, in order to further improve the efficiency of detection, RT-dPCR detection of multiple viruses can be attempted in the future.

Acknowledgments: This work was financially supported by Applied Basic Research Program of Liaoning Province (2022JH2/101300189) and the Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences.

REFERENCES

Canales, C., F. Morá, A. Olmos, and A.B. Ruiz-García (2021). First detection and molecular characterization of apple stem grooving virus, apple chlorotic leaf spot virus, and apple hammerhead viroid in Loquat in Spain. Plants 10:2293. doi: 10.3390/plants10112293

Clover, G.R.G., M.N. Pearson, D.R. Elliott, Z. Tang, T.E. Smales, and B.J.R. Alexander (2003). Characterization of a strain of apple stem grooving virus in *Actinidia chinensis* from China. Plant Pathol. 52:371-378. doi: org/10.1046/j.1365-3059.2003.00857.x

Hu, G.J., Y.F. Dong, Z.P. Zhang, X.D. Fan, F. Ren, and J. Zhou (2015). Virus elimination from in vitro apple by thermotherapy combined with chemotherapy. Plant Cell Tiss. Org. Cult. 121:435-443. doi: 10.1007/s11240-015-0714-6

Hu, G.J., Y.F. Dong, Z.P. Zhang, X.D. Fan, F. Ren, and Z.N. Li (2017). Efficacy of virus elimination from apple by thermotherapy coupled with *in vivo* shoot-tip grafting and *in vitro* meristem culture. J. Phytopathol. 165:701-706. doi: 10.1051/fruits:2008050

Hu, G.J., Y.F. Dong, Z.P. Zhang, X.D. Fan, F. Ren, and Z.N. Li (2018). Effect of pre-culture on virus elimination from *in vitro* apple by thermotherapy coupled with shoot tip culture. J. Integr. Agric. 17:2015-2023. doi: 10.1016/S2095-3119(18)61913-6

Hu, G.J., Y.F. Dong, Z.P. Zhang, X.D. Fan, and F. Ren (2019a). Establishment and application of duplex primer pair reverse transcript-PCR detection method for apple chlorotic leaf virus. J. Plant Prot. 46(5): 1155-1156. doi: 10.13926/j.cnki.apps.000375

Hu, G.J., Y.F. Dong, Z.P. Zhang, X.D. Fan, F. Ren, and M.Y. Zhang (2019b). Establishment and application of apple stem pitting virus detection system using dual RT-PCR. Acta Phytopathologica Sinica 49 (3):428-432. doi: 10.13802/j.cnki.zwbhxb.2019.2018166

Huang, A.J., P.P. Gu, Y. Wang, J.L. Li, Z.X. Yang, L. Yi (2023) Simultaneous detection and differentiation of four viruses in passion fruit plants by a multiplex RT-PCR. Trop. Plant Pathol. 48 (1):23-29. doi: 10.1007/s40858-022-00538-5

James, D (1999). A simple and reliable protocol for the detection of apple stem grooving virus by RT‐PCR and in a multiplex PCR assay. J. Virol. Methods 83:1-9. doi: 10.1016/S0166-0934(99)00078-6

Jia, X.J., Z.P. Zhang, X.D. Fan, F. Ren, Y.F. Dong, and G.J. Hu (2022). Detection and genetic variation analysis of apple chlorotic leaf spot virus and apple stem grooving virus in wild apple (*Malus sieversii*) from Xinjiang, China. Forest Pathol. 52: e12771. doi: 10.1111/efp.12771

Kim, S.W., H.J. Lee, K.H. Cho, and R.D. Jeong (2022). Detection and quantification of apple stem grooving virus in micropropagated apple plantlets using reverse-transcription droplet digital PCR. Plant Pathol. J. 38:417-422. doi: 10.5423/PPJ.NT.05.2022.0072

Kwon, S.J., Y.E. Cho, H.S. Byun, H.R. Kwak, and J.K. Seo (2022). A multiplex RT-PCR assay for detection of emergent pepper Tsw resistance-breaking variants of tomato spotted wilt virus in South Korea. Mol. Cell Probe. 61:101792. doi: 10.1016/j.mcp.2022.101792

Lee, D.S., H.K. Lee, S.Y. Kim, B.R. Kwon, H.J. Yang, C.Y. Park, and S.H. Lee (2023). Complete genome sequences of two apple stem grooving viruses in *Cnidium officinale* in Korea. Microbiol. Resour. Announc. 12: e0090222. doi: 10.1128/mra.00902-22

Li, Z.N., S.N. Zhang, Z.P. Zhang, X.D. Fan, F. Ren, G.J. Hu, and Y.F. Dong (2018). Analysis of the complete genome of apple stem grooving virus isolate Jilin-shaguo. Acta Hort. Sin 45:641-649. doi: 10.16420/j.issn.0513-353x.2017-0578

Miljanić, V., J. Jakše, D. Rusjan, A. Škvarč, and N. Štajner (2022). Small RNA sequencing and multiplex RT-PCR for diagnostics of grapevine viruses and virus-like organisms. Viruses 14:921. doi: 10.3390/v14050921

Nabi, S.U., G.S. Madhu, G.P. Rao, and V.K. Baranwal (2022). Development of multiplex RT-PCR assay for simultaneous detection of four viruses infecting apple (*Malus domestica*). Lett. Appl. Microbiol. 74:586-592. doi: 10.1111/lam.13643

Nongsiang, A., and M.C. Das (2022). Simultaneous detection of odontoglossum ringspot virus (ORSV) and cymbidium mosaic virus (CymMV) infecting orchids in Meghalaya, India by multiplex RT-PCR. S. Afr. J. Bot. 151:774-780. doi: 10.1016/j.sajb.2022.10.036

Reddy, M.G., and G.P. Rao (2022). Duplex PCR assay for detection of chickpea chlorotic dwarf virus and peanut witches' broom phytoplasma in chickpea. 3 Biotech. 12:23. doi: 10.1007/s13205-021-03094-y

Xu, L., and J. Ming, (2022). Development of a multiplex RT-PCR assay for simultaneous detection of lily symptomless virus, lily mottle virus, cucumber mosaic virus, and plantago asiatica mosaic virus in lilies. Virol. J. 19:219. doi: 10.1186/s12985-022-01947-3

Xuan, Z.Y., S. Zhang, P. Li, F.Y. Yang, H.M. Chen, K.H. Liu, Y. Zhou, Z.A. Li, C.Y. Zhou, and M.J. Cao (2022). Apple stem grooving virus is associated with leaf yellow mottle mosaic disease on *Citrus grandis* cv. Huangjinmiyou in China. J. Integr. Agri. 21: 2031-2041. doi: 10.1016/S2095-3119(21)63823-6.