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METHODS AND PROTOCOLS

Long-term preservation of potato leafroll virus, potato virus S, and potato spindle tuber viroid in cryopreserved shoot tips

Jing-Wei Li[1] · Min-Rui Wang[1] · Hai-Yan Chen[1] · Lei Zhao[1,2] · Zhen-Hua Cui[3] · Zhibo Zhang[4] · Dag-Ragnar Blystad[4] · Qiao-Chun Wang[1,3]

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Abstract

Availability of and easy access to diverse plant viruses and viroids is a prerequisite in applied and basic studies related to viruses and viroids. Long-term preservation of viruses and viroids is difficult. A protocol was described for long-term preservation of potato leafroll virus (PLRV), potato virus S (PVS), and potato spindle tuber viroid (PSTVd) in cryopreserved shoot tips of potato cv. Zihuabai. Shoot regrowth levels following cryopreservation were higher in 1.5 mm-shoot tips (58–60%) than in 0.5-mm-ones (30–38%). All shoots recovered from 0.5-mm-shoot tips were PVS- and PSTVd-preserved, but none of them were PLRV-preserved. Cryopreservation of 1.5-mm-shoot tips resulted in 35% and 100% of PLRV- and PVS- and PSTVd-preserved shoots. Studies on cell survival patterns and virus localization provided explanations to the varying PLRV-preservation frequencies produced by cryopreservation of the two sizes of shoot tips. Although micropropagation efficiencies were low after 12 weeks of subculture following cryopreservation, similar efficiencies were obtained after 16 weeks of subculture in pathogen-preserved shoots recovered from cryopreservation, compared with the diseased in vitro stock shoots (the control). Pathogen concentrations in the three pathogens-preserved shoots analyzed by qRT-PCR were similar to those in micropropagated shoots. The three pathogens cryopreserved in shoot tips were readily transmitted by grafting and mechanical inoculation to potato plants. PLRV, PVS, and PSTVd represent a diverse range of plant viruses and viroid in terms of taxonomy and infectious ability. Therefore, shoot tip cryopreservation opens a new avenue for long-term preservation of the virus and viroid.

Keywords Cryopreservation · Potato · Shoot tips · Virus · Viroid

Introduction

Serological methods have been used for a long time for plant virus detection (Hull 2002). Antigen preparation is necessary in virus detection by serological methods. Pathogen-derived resistance (PDR) has been used in genetic transformation to produce virus-resistant plants (Sudarshana et al. 2007). In the past few decades, plant-based production of vaccines provided a new strategy for the manufacture of vaccines for the prevention and treatment of human diseases (Salazar-González et al. 2015; Loh et al. 2017). Recent studies have shown plant viruses have potential applications to nanotechnology to produce nanodrugs (Lomonossoff and Evans 2011; Yang et al. 2018). Availability of and easy access to diverse plant viruses is a prerequisite in these applied studies and basic research such as origin and evolution of viruses and viroids (Hull 2002; Di Serio et al. 2017).

Plant viruses and viroids are obligate intracellular parasites that replicate only inside the living cells of the host by using the host's biochemical machinery (Hull 2002; Flores et al. 2017). Viruses and viroids do not capture or store free energy and therefore cannot live without living tissues (Hull 2002; Flores et al. 2017). Preservation of viruses and viroids has

✉ Lei Zhao
Zhaolei5105@126.com

✉ Qiao-Chun Wang
qiaochunwang@nwsuaf.edu.cn

[1] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, China

[2] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling 712100, Shaanxi, China

[3] College of Horticulture, Qingdao Agriculture University, Qingdao 266109, Shandong, People's Republic of China

[4] Division of Plant Health and Biotechnology, Norwegian Institute of Bioeconomy Research, Ås, Norway

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long received interest of scientists working on virus- and viroids-related field. So far, the information on long-term preservation of viroids is limited. Studies using RT-PCR identified peach latent mosaic viroid in the 50-year-old herbarium preserved peach leaves showing peach calico disease (Guy 2013) and apple scar skin viroid in the 10-year-old air-dried apple twig with no disease symptoms (Hadidi et al. 2016). Over the past several decades, various strategies have been developed for the preservation of plant viruses, including freezing (Fukumoto and Tochinara 1998), freeze-drying (Hollings and Stone 1970; Purcifull 1975; Fukumoto and Tochinara 1998; Yordanova et al. 2000), dehydration by physical and chemical drying (Mckinney et al. 1965; Grivell et al. 1971), and in vitro culture (Chen et al. 2003; Infante et al. 2008), among which freeze-drying was the most widely and reliable method. With this method, although cucumber mosaic virus could be preserved for up to 240 days, its infection efficiency rapidly decreased as preservation time increased, with only 7% infection frequency maintained after 240 days of preservation (Yordanova et al. 2000).

De and Suda-Bachmann (1979) reported potato virus Y (PVY) and watermelon mosaic virus (WMMV) contained in leaf powder could be cryopreserved in liquid nitrogen (LN) for 22 months for the former and 32 months for the latter, without any decrease in infectivity. Moreover, Fan et al. (2014) reported preservation of viral genomes in 700-year-old caribou feces from a subarctic ice patch. These data indicate cryopreservation of virus seems a very promising long-term preservation method for plant viruses and probably viroids.

Cryopreservation, i.e., storage of living cells, tissues, and organs in extra low temperatures, usually that of LN, is currently considered an ideal means for long-term preservation of plant genetic resources. Since Sakai (1960) reported for the first time successful plant cryopreservation, this technique has been widely applied to almost all economically important agricultural crops (Wang et al. 2009a; Feng et al. 2011; Vollmer et al. 2017), horticultural plants (Höfer 2015; Wang et al. 2018a), and forest trees (Li et al. 2017). Recently, cryobanks have been established for some vegetatively propagated crops such as potato at the International Potato Center (CIP) in Peru (Vollmer et al. 2017), apple at the Julius Kühn-Institute for Breeding Research on Fruit Crops in Germany (Höfer 2015), and garlic at the National Agrobiodiversity Center in South Korea (Kim et al. 2012).

Shoot tip cryopreservation has been shown to efficiently eliminate plant pathogens including viruses (Wang and Valkonen 2009; Wang et al. 2009b, 2014a). Although shoot tip cryopreservation produced much higher frequencies of pathogen elimination than the traditional methods like meristem culture, pathogen elimination frequencies varied with types of pathogens and plants (Brison et al. 1997; Helliot et al. 2002; Wang et al. 2003, 2006; Li et al. 2016), as well as infection status and combinations of viruses and hosts (Wang and Valkonen 2008; Li et al. 2016; Kushnarenko et al. 2017). Frequently, not all plants recovered from shoot tip cryopreservation were pathogen-free, and a certain proportions of the recovered plants were still pathogen-infected, i.e., pathogen-preserved (Wang et al. 2009b, 2014a). Furthermore, shoot tip cryopreservation completely failed to eradicate viruses and viroids that can infect meristematic cells of the shoot tips, such as raspberry bushy dwarf virus (RBDV, Wang et al. 2008), apple stem grooving virus (ASGV, Li et al. 2016), potato spindle tuber viroid (PSTVd, Bai et al. 2012), and chrysanthemum stunt viroid (CSVd, Zhang et al. 2014). These data indicate that shoot tip cryopreservation may be used for long-term preservation of viruses and viroids (Wang et al. 2018b).

The present study attempted to cryopreserve potato leafroll virus (PLRV), potato virus S (PVS), and PSTVd, three major pathogens infecting potato and widely present in potato-growing regions of the world, in shoot tips of potato cv. Zihuabai. Concentrations of the cryopreserved pathogens in the pathogen-preserved shoots were quantitatively analyzed by qRT-PCR. The cryopreserved pathogens were tested for their infectious ability to infect healthy potato plants by grafting and mechanical inoculation.

Materials and methods

Plant materials

Potato cv. Zihuabai, a cultivar susceptible to the pathogens studied, was used in the present study. In vitro certified healthy (virus-free) shoots and diseased shoots infected with PLRV, PVS, or PSTVd, respectively, were available in the Plant Cryobiology Laboratory of College of Horticulture of Northwest A&F University, Yangling, China. These in vitro shoots were maintained on a basic medium (BM) composed of solid half-strength Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar (pH = 5.8), according to Li et al. (2013, 2018). Infection status of all the in vitro stock shoots was confirmed before cryopreservation using reverse transcription-polymerase chain reaction (RT-PCR), as described below. The cultures were grown at 22 ± 2 °C under a 16-h photoperiod at 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Subculturing was conducted every 3 weeks.

Virus cryopreservation

Shoot tips were cryopreserved by droplet-vitrification, as described by Wang et al. (2013, 2014b). Nodal segments (1 cm in length), each containing an axillary bud, were taken from 3-week old stock cultures and cultured on BM under the same conditions as described for in vitro stock cultures. Shoots (1–

1.5 cm in length) developed from axillary buds after 7 days of culture were transferred to a growth chamber for cold-hardening in the dark at 5 °C for 3 weeks. Two sizes of shoot tips: 0.5 mm and 1.5 mm in length containing 2–3 and 5–6 leaf primordia (LPs), respectively, were excised from the cold-hardened stock shoots and pre-cultured on BM containing 0.3 M sucrose in the dark at 5 °C for 3 days. Pre-cultured shoot tips were treated for 30 min with a loading solution containing 2 M glycerol and 0.4 M sucrose in MS medium and then dehydrated with PVS2, as defined below (Sakai et al. 1990) at 0 °C for 40 min. PVS2 consisted of 30% (*w*/*v*) glycerol, 15% (*w*/*v*) dimethyl sulfoxide (DMSO), 15% (*w*/*v*) ethylene glycol, and 0.4 M sucrose in MS medium. Dehydrated shoot tips were transferred onto 3 μL PVS2 droplets on aluminum foils, followed by a direct immersion in LN for 1 h. Frozen foil strips with shoot tips were removed out from LN and rapidly transferred into an unloading solution composed of MS supplemented with 1.2 M sucrose at 25 °C for 20 min.

Post-culture for shoot recovery of cryopreserved shoot tips

Cryopreserved shoot tips were post-cultured on a shoot recovery medium composed of BM supplemented with 0.05 mg L^{-1} GA_3, for shoot regrowth. The cultures were grown kept in the dark at 22 ± 2 °C for 3 days and then transferred into the light condition, as used for the in vitro stock shoots. Shoot regrowth was defined as percentage of the total number of shoot tips regenerating into normal shoots (≥ 5 mm) 6 weeks after post-culture. Subculturing was conducted every 3 weeks.

Detection of PLRV, PVS, and PSTVd by RT-PCR

Detection of PLRV, PVS, and PSTVd was made on the in vitro stock shoots before cryopreservation, to confirm their infection status. Detection of PLRV, PVS, and PSTVd was repeated again on the shoots recovered from cryopreservation after 6 weeks of post-culture following cryopreservation. For PVS and PSTVd, since all samples tested this time were positive, they were considered to be pathogen-preserved and thus used for micropropagation. PLRV positive samples were considered to be virus-preserved and used for micropropagation. Negative samples were considered to be virus-free and cultured for further growth. After 18 weeks of post-culture, pathogen-positive samples were transferred to soil in a net-proof greenhouse. Their virus status was tested again after 3 months of growth.

Total RNA was extracted from shoots with leaves (0.5 g) using the Trizol Reagent (Invitrogen Ltd., Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized, according to Li et al. (2013, 2018). The primers used for the three pathogens are listed in Table 1. The PCR reaction was performed as described by Li et al. (2013, 2018). Programs used for PLRV, PVS, and PSTVd were as follows: for PLRV: initial denaturation step at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 56 °C for 20 s, and 72 °C for 30 s, followed by the final extension step at 72 °C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 M EDTA, pH, 8.0), stained with ethidium bromide, and visualized and photographed under ultraviolet light.

Quantitative analysis by RT-qPCR of relative mRNA expression levels of pathogens in pathogen-preserved shoots recovered from cryopreservation

Relative mRNA expression levels of the pathogens were analyzed by qRT-PCR in the pathogen-preserved shoots recovered from cryopreservation at 6 and 18 weeks of subculture. qRT-PCR was performed, according to Wang et al. (2018b). Total RNA extraction and cDNA reverse transcription was described as above. The qRT-PCR was performed using a CFX1000 (Bio-Rad, Shanghai, China) instrument and a SYBR Premix ExTaq II Kit (Takara, Dalian, China) reagent. Since *EF-1α* was stably expressed in samples, *EF-1α* was used as the reference gene. The relative mRNA expression levels of ASGV were expressed as Cq values. All primers and the reference gene used for qRT-PCR are listed in Table 1. The relative expression levels of each gene were normalized to the expression of *EF-1a* gene (Wang et al. 2018b). The relative mRNA expression levels of PLRV, PVS, and PSTVd were expressed as Cq values (Wang et al. 2018b).

Histological observations on cell survival patterns and PLRV localization in shoot tips

In order to understand why frequencies of PLRV cryopreservation varied with different size of shoot tips, cell survival patterns in cryopreserved shoot tips and virus localization in the PLRV-infected in vitro stock shoots were conducted, according to Wang et al. (2014b) and Li et al. (2016), respectively. For histological observations on cell survival patterns, cryopreserved shoot tips of PLRV-infected shoots were collected 1 day after post-culture, fixed in formalin-acetic-alcohol (FAA) (ethanol:formalin:acetic acid = 18:1:1) for 24 h, and dehydrated through an incremental ethanol series (70, 85, 90, 95, and 100% ethanol). After embedding in paraffin, sections (5 μm thick) were cut with a microtome (Leica 2235, Wetzlar, Germany) and stained with 0.01% toluidine blue (TB) (Sakai 1973). The stained sections were observed under a light microscope (Leica DM2000, Wetzlar, Germany). Shoot-tips that were freshly excised from stock shoots served as positive controls, while those that were freshly excised, directly immersed in LN served as negative controls. Both

Table 1 Names, sequences, and amplified bands of primers and reference used for detection of PLRV, PVS and PSTVd by RT-PCR and real time RT-qPCR in potato cv. Zihuabai

Primer name	Sequence(5′-3′)	Amplified band (bp)	Ref
PLRV-F	CCCACTGGAAGAGGGATGTAACT	155	Designed in this study
PLRV-R	CTTCGGATGCTTCCCGCTCTA		
PVS-F	CAGATGTGCCCAGAGCCAAGT	137	Designed in this study
PVS-R	GCCAGACCCAGATTACCAAAA		
PSTVd-F	ATCGATGAGGAGCGCTTCAGGGATC	224	Designed in this study
PSTVd-R	GTCGACGGAGCTTCAGTTGTTTCC		
EF-1a-F*	ATTGGAAACGGATATGCTCCA	101	Wang et al. 2018b
EF-1a-R	TCCTTACCTGAACGCCTGTCA		

**EF-1a*-F and *EF-1a*-R were used only in RT-qPCR

positive and negative controls received the same histological processes as described above.

For PLRV localization, shoot tips were harvested from the in vitro PLRV-infected stock shoots. Samples of the healthy in vitro shoots were used as negative controls. Cross sections were obtained, as described above. Virus localization was conducted, as described by Li et al. (2016). The sections were treated with phosphate buffered saline (PBS) containing 4% bovine serum albumin (BSA) for 30 min, followed by overnight incubation at 5 °C with coat protein (rabbit polyclonal antibodies to PLRV) (dilution 1:500 with PBS). After washing with PBS three times, the samples were incubated with alkaline phosphatase-conjugated antibodies (mouse anti-rabbit monoclonal antibodies) (dilution 1:500 with PBS) for 30 min at room temperature. After washing again three times with PBS, samples were stained using a freshly prepared Fuchsin substrate solution. The sections were observed with a light microscope (Leica DM 2235).

Micropropagation of pathogen-infected shoots recovered from cryopreservation

After 6 weeks of post-culture for shoot regrowth, shoots recovered from cryopreservation were transferred onto BM and cultured under the same light conditions as used for in vitro stock shoots, for micropropagation. Pathogen-infected in vitro shoots without cryopreservation were used as controls. Subculturing was conducted every 3 weeks. Shoot length and node number, the two major parameters determining micropropagation efficiency in potato, were recorded every 6 weeks of subculture.

Establishment of the diseased plants recovered from cryopreservation in soil

After 18 weeks of subculture, plantlets with well-developed roots were transferred into soil and grown in a net-proof greenhouse, with regular irrigation and fertilization, according to practical managements. The plants were used for the pathogen transmission by grafting and mechanical inoculation, as described below.

Transmission of the cryopreserved pathogens to potato plants by grafting and mechanical inoculation

For grafting transmission, PLRV-, PVS,- and PSTVd-preserved plants that recovered from cryopreservation and had grown for 6 weeks in soil in net-proof greenhouse were used as inocula materials. The healthy plants of potato cv. Zihuabai grown in the same net-proof greenhouse were used as rootstocks. Shoot segments (2.0–2.5 cm), each containing 2–3 well-developed leaves, were excised from middle to low parts of the pathogen-preserved plants and used as scions. A "V" shape (approximately 0.5 cm in length) was cut at the base of the scions. The healthy rootstocks were decapitated approximately 5.0 cm above the soil. A vertical cut (approximately 0.6 cm in length) was made at the top of the rootstocks. Grafting was performed by inserting the "V"shape of scions into the vertical cut of rootstocks, and then parafilm was used to fix the graft union. Sanitary status of the rootstocks was tested by RT-PCR after 4 weeks of grafting, as described above.

For mechanical transmission, since PLRV is only transmitted by an insect vector and grafting and cannot be mechanically transmitted, it was excluded in this experiment. PVS- and PSTVd-preserved plants that recovered from cryopreservation and had been grown in soil in net-proof greenhouse for 3 months were used for mechanical inoculation to transmit PVS and PSTVd to potato "Zihuabai" and *Solanum jasminoides* plants, respectively. *S. jasminoides* plants are available in the Plant Virology laboratory of the College of Plant Protection of the Northwest A&F University, Yangling, China. All plants were grown in a net-proof greenhouse at 22 ± 2 °C with 16-h photoperiod. Mechanical transmission of PVS was performed as described by Li et al. (2015). Briefly, leaves positioning between five and seven nodes (counting

from shoot terminal downward) were taken from PVS-preserved plants and ground with PBS (1 g/5 ml) contained in a plastic bag (Li et al. 2015). The freshly prepared virus inoculum was maintained on ice until use. The first 2–3 fully-opened leaves (counting from shoot terminal downward) of the inoculum plants that had been slightly dusted with carborundum were inoculated by rubbing gently with a cotton-swab soaked in the virus solution. Plants inoculated with the inoculation buffer served as negative controls. Inoculated plants were grown in a growth chamber at 25 °C under the light conditions. Leaves were taken from the inoculated plants after 4 weeks of inoculation and used for virus detection by RT-PCR, as described above.

Mechanical transmission of PSTVd was performed, according to Verhoeven et al. (2010). Briefly, leaves positioning between five and seven nodes (counting from shoot terminal downward) were taken from PSTVd-preserved plants and ground with SPB (1 g/10 ml), as described above. The freshly prepared viroid inocula were maintained on ice until use. The first 2–3 fully-opened leaves (counting from shoot terminal downward) of the healthy plants were inoculated with PSTVd-contaminated razor blades. Plants inoculated with PBS served as negative controls. Inoculated plants were grown in a growth chamber at 25 °C under light conditions. Leaves were taken from the inoculated plants after 4 weeks of inoculation and used for viroid detection by RT-PCR, as described above.

Experimental design and data analysis

For experiments of shoot tip cryopreservation and micropropagation, ten samples were included in each treatment of three replicates. All experiments were conducted twice. Data were presented as means with their standard errors and analyzed using one-directional ANOVA and Students' t test. Significant differences were calculated at $P < 0.05$. Twenty samples were included in histological observations on cell survival patterns, PLRV localization, and virus transmission by grafting and mechanical inoculation. Five biological replicates were used in analyses of RT-PCR and qRT-PCR.

Results

Effects of size of shoot tips on shoot regrowth and pathogen preservation following cryopreservation

The size of shoot tips significantly affected shoot regrowth levels in cryopreserved shoot tips. Larger shoot tips (1.5 mm) produced significantly higher shoot regrowth levels (52–60%) than small ones (0.5 mm, 30–38%) among the three pathogen-infected shoots (Table 2). No significant differences were found in shoot regrowth levels produced in the same size of shoot tips infected with different pathogens (Table 1). For PLRV, cryopreservation of 0.5-mm shoot tips produced no virus-preserved shoots and 1.5-mm shoot tips resulted in production of 35% of virus-preserved shoots (Table 2). For PVS and PSTVd, 100% of shoots recovered from cryopreservation were pathogen-preserved, regardless of their sizes of shoot tips (Table 2).

Detection of PLRV, PVS, and PSTVd by RT-PCR

At the beginning of the cryopreservation experiments, specific bands of 155 bp for PLRV, 137 bp for PVS, and 224 bp for PSTVd were detected in all in vitro stock shoots infected with the corresponding pathogens, whereas no such bands were found in the healthy ones (Fig. 1a), thus ensuring infection status of the in vitro stock shoots used in this study. When RT-PCR was applied for the detection of pathogens in shoots recovered from cryopreservation after 18 weeks of subculture, specific bands of about 155 bp for PLRV, 137 bp for PVS, and 224 bp for PSTVd were detected in PLRV-, PSV-, and PSTVd-preserved shoots, respectively (Fig. 1a). For PLRV, the results of virus detection were identical in shoots recovered from cryopreservation after 6 weeks of post-culture and in plants grown in soil in the net-proof greenhouse for 3 months.

Quantitative analysis by qRT-PCR of relative mRNA expression of PLRV, PVS, and PSTVd in pathogen-preserved shoots recovered from cryopreservation

Stable and similar values were obtained in the virus- and viroid-infected shoots recovered from cryopreservation when the reference gene *EF-1α* was used (Table 3, Fig. 2), indicating that the qRT-PCR method used here was reliable. Patterns of relative mRNA expression levels of virus and viroid were similar in the three pathogen-preserved shoots recovered from cryopreservation and subcultured for different times (Table 3). The relative mRNA expression levels were low in the pathogen-preserved shoots after 6 weeks of subculture, significantly increased as subculture times increased and reached similar levels after 18 weeks of subculture, compared with those of the in vitro diseased stock shoots without cryopreservation (Table 3).

Cell survival patterns

Living cells in the positive control showed dense TB-stained and well-preserved cytoplasm and clearly visible nucleolus enclosed in the nucleus (Fig. 3a). Damaged or dead cells in the negative control showed reduced levels of TB-stained cytoplasm and the nuclei were heavily condensed (Fig. 3b). In

Table 2 Effects of shoot tip size on shoot regrowth levels and frequencies of pathogen preservation in diseased in vitro shoots following cryopreservation in potato cv. Zihuabai

Virus infection status of stock shoots	Size of shoot tips	Shoot regrowth (%)	Virus-free shoots (%)	Virus-preserved shoots (%)
PLRV	0.5 mm, 2–3 LPs	35 ± 5b	100 (0/20)	0 (0/20)
	1.5 mm, 5–6 LPs	60 ± 5a	65 (7/20)	35 (7/20)
PVS	0.5 mm, 2–3 LPs	38 ± 5b	0 (20/20)	100 (20/20)
	1.5 mm, 5–6 LPs	58 ± 5a	0 (20/20)	100 (20/20)
PSTVd	0.5 mm, 2–3 LPs	30 ± 5b	0 (20/20)	100 (20/20)
	1.5 mm, 5–6 LPs	52 ± 5a	0 (20/20)	100 (20/20)

Size of shoot tips was defined as length (mm) + number of leaf primordium (LP)

Numbers in parentheses indicate positive reactions to PLRV, PVS and PSTVd/total samples tested by RT-PCR

Data of shoot regrowth are presented as means ± SE and followed by different letters indicate significant differences at $P < 0.05$ analyzed by Student's t test

cryopreserved shoot tips of PLRV-infected shoots, surviving cells were found in the upper part of apical dome (AD) (Fig. 3c) and leaf primordia (LPs) 1–3 (Fig. 3d–f). Surviving cells were occasionally found in LP 4 (Fig. 3g). Among the 20 shoot tips tested, 6 shoot tips showed this survival patterns, accounting for 30%. Cells in LPs 5–6 (Fig. 3h, i), and other older tissues were damaged or killed.

Virus localization

With the histoimmunological virus localization protocol used in the present study, tissue infected with PLRV showed specific purple color reaction, while the healthy tissue did not show such color reaction (Fig. 4a). PLRV was not detected in AD (Fig. 4b) and LPs 1–3 (Fig. 4b),

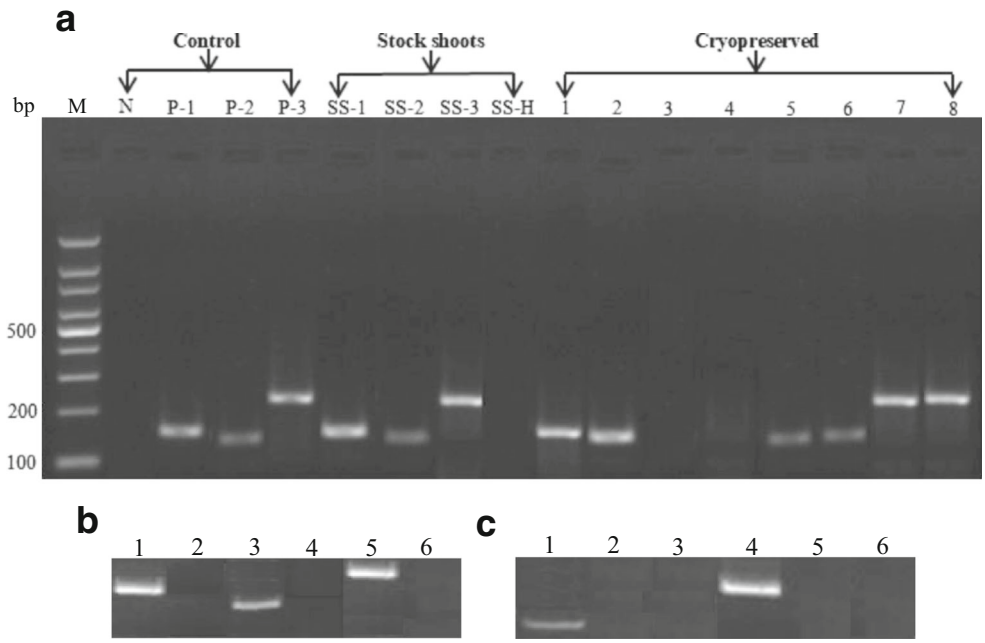

Fig. 1 Detection by RT-PCR of potato leafroll virus (PLRV), potato virus S (PVS), and potato spindle tuber viroid (PSTVd) in in vitro stock shoots before cryopreservation and shoots recovered after cryopreservation in potato cv. Zihuabai (**a**), in the healthy rootstocks grafted upon the virus- and virus-preserved rootstocks in potato cv. Zihuabai (**b**), and in the healthy potato cv. Zihuabai and *Solanum jasminoides* plants inoculated with PVS- and PSTVd-preserved shoots, respectively (**c**). **a**: M, molecular marker; N, negative control; P1, positive control of PLRV, P2, positive control of PVS; P3, positive control of PSTVd; SS1, PLRV-infected stock shoots; SS2, PVS-infected stock shoots; SS3, PSTVd-infected stock shoots; SS-H, healthy stock shoots; Lanes 1–2, shoots recovered from cryopreserved shoots of PLRV-infected stock shoots; Lanes 3–4, shoots recovered from cryopreserved shoots of PLRV-infected stock shoots; Lanes 5–6, shoots recovered from cryopreserved shoots of PVS-infected stock shoots; Lanes 7–8, shoots recovered from cryopreserved shoots of PSTVd-infected stock shoots. **b** lane 1, grafted with PLRV-preserved scions; lane 2, grafted with the healthy scions; lane 3, grafted with PVS-preserved scions; lane 4, grafted with the healthy scions; lane 5, grafted with PSTVd-preserved scions; lane 6, grafted with the healthy scions. **c** lane 1, inoculated with PVS-preserved samples; lane 2, mock inoculation for PVS; lane 3, inoculated with the healthy samples; lane 4, inoculated with PSTVd-preserved samples; lane 5, mock inoculation for PSTVd; lane 6, inoculated with the healthy samples

Table 3 Relative mRNA expressions levels (Cq values) of PLRV, PVS and PSTVd analyzed by RT-qPCR in pathogen-preserved shoots recovered from cryopreservation in different culture durations after shoot regrowth in potato cv. Zihuabai

Types of shoots	Culture durations (weeks) after shoot regrowth		
	9	18	21
PLRV			
Virus-preserved shoots	35.2 ± 2.7Aa	30.1 ± 1.8Ba	24.5 ± 0.9Ca
Infected in vitro stock shoots	23.1 ± 1.1Ab	22.5 ± 1.0Ab	22.3 ± 0.9Aa
Cq value of reference gene *EF-1α*	18.4 ± 0.7	17.9 ± 0.6	18.1 ± 0.8
PVS			
Virus-preserved shoots	33.2 ± 2.6Aa	30.3 ± 1.5Ba	20.4 ± 0.8Ca
Infected in vitro stock shoots	20.5 ± 0.7Ab	20.8 ± 0.9Ab	20.0 ± 0.8Aa
Cq value of reference gene *EF-1α*	17.8 ± 0.7	18.1 ± 0.8	17.9 ± 0.7
PSTVd			
Viroid-preserved shoots	32.6± 2.1Aa	28.4 ± 1.8Ba	21.5 ± 0.7Ca
Infected in vitro stock shoots	21.4 ± 0.8Ab	20.5 ± 0.7Ab	20.8 ± 0.8Aa
Cq value of reference gene *EF-1α*	18.2 ± 0.8	18.5 ± 0.8	17.8 ± 0.7

Results are presented as means ± SE. Data followed by upper-case letters in the same lines and by low-case letters in the same column of the same pathogen indicate significant differences at $P < 0.05$ by Student's *t* test

while it was found in LP 4 and older tissues (Fig. 4)b. Close reviews showed PLRV was phloem-limited (Fig. 4c, d).

Micropropagation of pathogen-preserved shoots recovered from cryopreservation

Patterns of micropropagation of the three pathogen-preserved shoots recovered from cryopreservation were similar (Table 4, Fig. 5a, b, c). Shoot length and number of nodes were significantly short and few in pathogen-preserved shoots recovered from cryopreservation after 12 weeks of subculture, significantly increased as the subculture times increased, and reached similar levels after 18 weeks of subculture, compared with pathogen-infected and healthy in vitro stock shoots without cryopreservation (Table 4, Fig. 5d–i). The pathogen-infected and the healthy in vitro stock shoots without cryopreservation produced the similar data of shoot length and number of nodes following 6, 12, and 18 weeks of subculture (Table 4).

Detection by RT-PCR of PLRV, PVS, and PSTVd in grafting and mechanically inoculated plants

Specific bands of 155 bp for PLRV, 137 bp for PVS, and 224 bp for PSTVd were readily detected in the healthy rootstocks grafted with the corresponding pathogen-preserved scions after 4 weeks of grafting (Fig. 1b). The same was true in the healthy plants mechanically inoculated with cryopreserved PVS and PSTVd after 4 weeks of mechanical inoculation (Fig. 1c).

Discussion

Most of the methods reported so far for preservation of plant viruses used dried materials (Mckinney et al. 1965; Grivell et al. 1971; Hollings and Stone 1970; Purcifull 1975; Fukumoto and Tochinara 1998; Yordanova et al. 2000) and only in vitro tissue culture preserved viruses in living tissue (Chen et al. 2003; Infante et al. 2008). When preserved in dried materials, some viruses were not stable and their infection decreased as time durations of preservation increased (Hollings and Stone

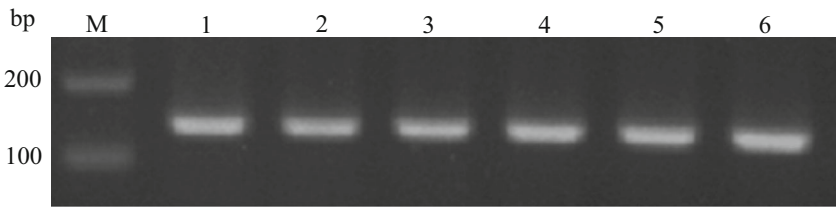

Fig. 2 Analysis of PCR products of the reference gene *EF-1α* in PLRV-, PVS-, and PSTVd-cryopreserved shoots of potato cv. Zihuabai. M, molecular marker; lanes 1–2, PLRV-cryopreserved shoots; lanes 3–4, PVS-cryopreserved shoots; lanes 5–6, PSTVd-cryopreserved shoots

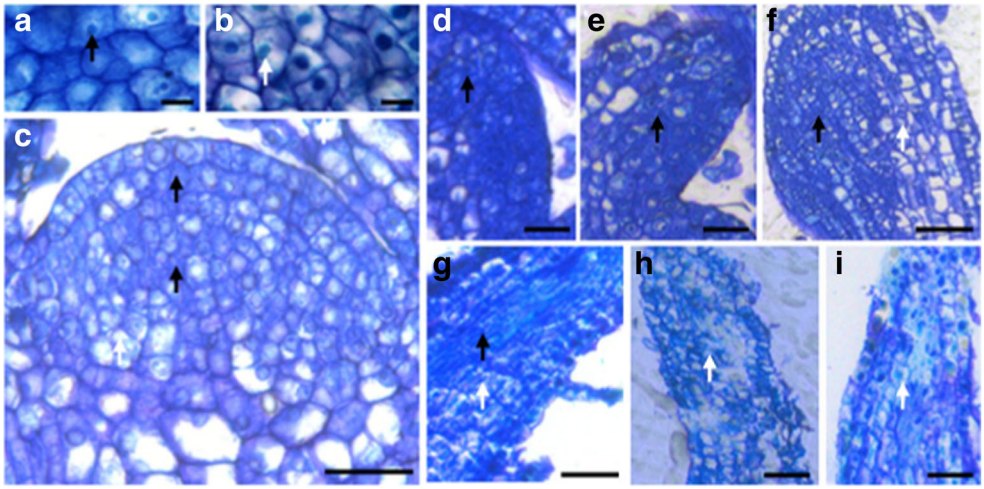

Fig. 3 Cell survival patterns in cryopreserved shoot tips of PLRV-infected shoots of potato cv. Zihuabai. Positive (**a**) and negative (**b**) control. Apical dome of cryopreserved shoot tips (**c**). Leaf primordia 1 (**d**), 2 (**e**), 3 (**f**), 4 (**g**), 5 (**h**), and 6 (**i**). Living cells and damage or dead cells are indicated by black arrows and white arrows, respectively. Scale bars in **a** and **b** = 10 μm, and in **c**–**i** = 20 μm

1970; Grivell et al. 1971; Yordanova et al. 2000). For example, infection frequencies of CMV preserved by freeze-drying were 95% and only 7% after 15 and 240 days of preservation, respectively (Yordanova et al. 2000). Following preservation, the virus can be transmitted only by mechanical inoculation to the target host (Mckinney et al. 1965; Hollings and Stone 1970; Grivell et al. 1971; De and Suda-Bachmann 1979; Yordanova et al. 2000). Since a number of plant viruses cannot be transmitted by mechanical inoculation (Hull 2002), such preservation methods largely limited applications of the virus preservation. In in vitro culture for virus preservation, virus-infected tissues have to be periodically subcultured (Chen et al. 2003; Infante et al. 2008). Subculture has risks of contamination, which may result in total loss of the stored materials. In addition, in vitro culture can be used only for medium-term virus preservation.

In the present study, PLRV, PVS, and PSTVd were successfully cryopreserved in living shoot tips of potato. PLRV is the type member of the genus *Polerovirus* and PVS is a member of the genus *Carlavirus*, and both viruses infect a wide range of plant species (Valkonen 2007). PSTVd belongs to the genus *Pospiviroid* and the family

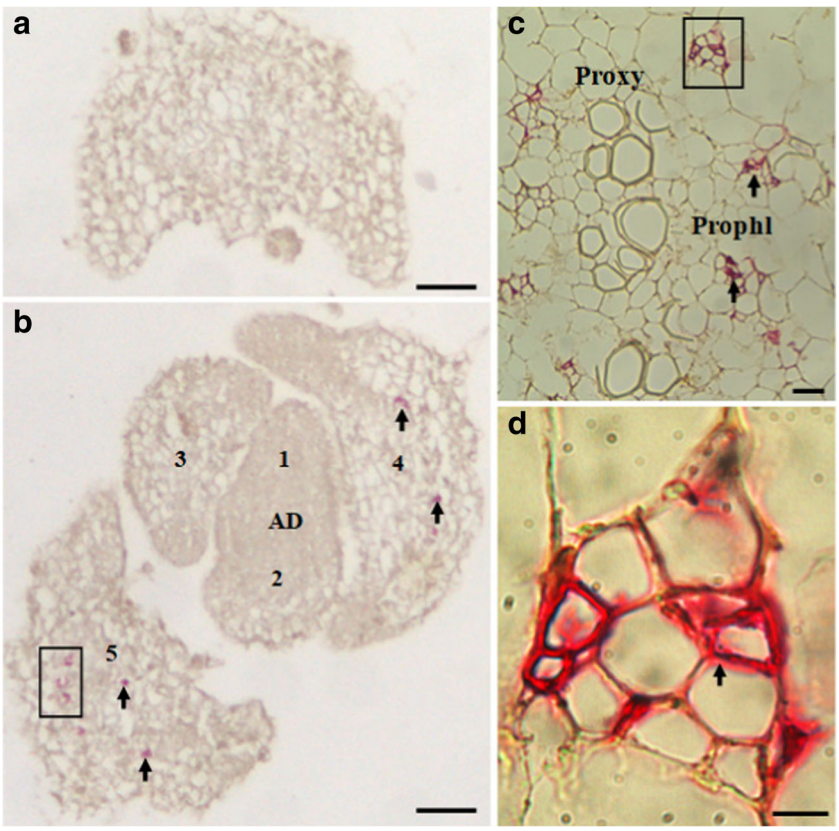

Fig. 4 Histoimmunological localization of potato leafroll virus (PLRV) in the diseased in vitro shoot tips of potato cv. Zihuabai. **a** Cross section of healthy tissues. **b** Cross section of virus-infected shoot tips. **c** Close view of the black square in (**b**). **d** Close view of the black square in (**c**). PLRV-infected tissues gave purple color reaction, as indicated by black arrows, while healthy tissues did not give such reaction. Virus AP, apical dome; 1, 2, 3, 4, and 5, leaf primordia 1, 2, 3, 4 and 5, respectively; Prophl, prophloem; Proxy, proxylem. Scale bars in **a** and **b** = 50 μm, and in **c** and **d** = 10 μm

Table 4 Micropropagation of pathogen-preserved shoots recovered from cryopreservation in different culture durations after shoot regrowth in potato cv. Zihuabai

Types of shoots	Culture durations (weeks) after shoot regrowth					
	6		12		18	
	Shoot length (cm)	Node number	Shoot length (cm)	Node number	Shoot length (cm)	Node number
PLRV						
Virus-preserved shoots	0.7 ± 0.2Cb	1.5 ± 0.2Cb	3.6 ± 0.4Bb	4.5 ± 0.5Bb	6.8 ± 0.4Aa	7.4 ± 0.7Aa
Virus-infected in vitro stock shoots	6.9 ± 0.4Aa	7.9 ± 0.7Aa	7.1 ± 0.6Aa	8.0 ± 0.8Aa	7.4 ± 0.5Aa	8.4 ± 0.9Aa
PVS						
Virus-preserved shoots	0.6 ± 0.2Cb	1.4 ± 0.2Cb	3.4 ± 0.4Bb	4.1 ± 0.3Bb	6.5 ± 0.5Aa	7.3 ± 0.6Aa
Virus-infected in vitro stock shoots	6.6 ± 0.5Aa	7.8 ± 0.7Aa	7.2 ± 0.7Aa	8.1 ± 0.7Aa	7.0 ± 0.5Aa	8.0 ± 0.8Aa
PSTVd						
Viroid-preserved shoots	0.6 ± 0.1Cb	1.4.0 ± 0.2Cb	3.3 ± 0.3Bb	4.0 ± 0.4Bb	6.5 ± 0.5Aa	7.1 ± 0.6Aa
Viroid-infected in vitro stock shoots	6.5 ± 0.5Aa	7.8 ± 0.6Aa	7.1 ± 0.8Aa	8.1 ± 0.7Aa	7.0 ± 0.5Aa	8.2 ± 0.7Aa
Healthy (virus-free)	7.4 ± 0.6Aa	8.1 ± 0.7Aa	7.2 ± 0.7Aa	8.2 ± 0.6Aa	7.4 ± 0.6Aa	8.2 ± 0.8Aa

Results are presented as means ± SE. Data followed by upper-case letters in the same lines of the same parameters and by low-case letters in the same column of the same pathogen indicate significant differences at $P < 0.05$ by Student's t test

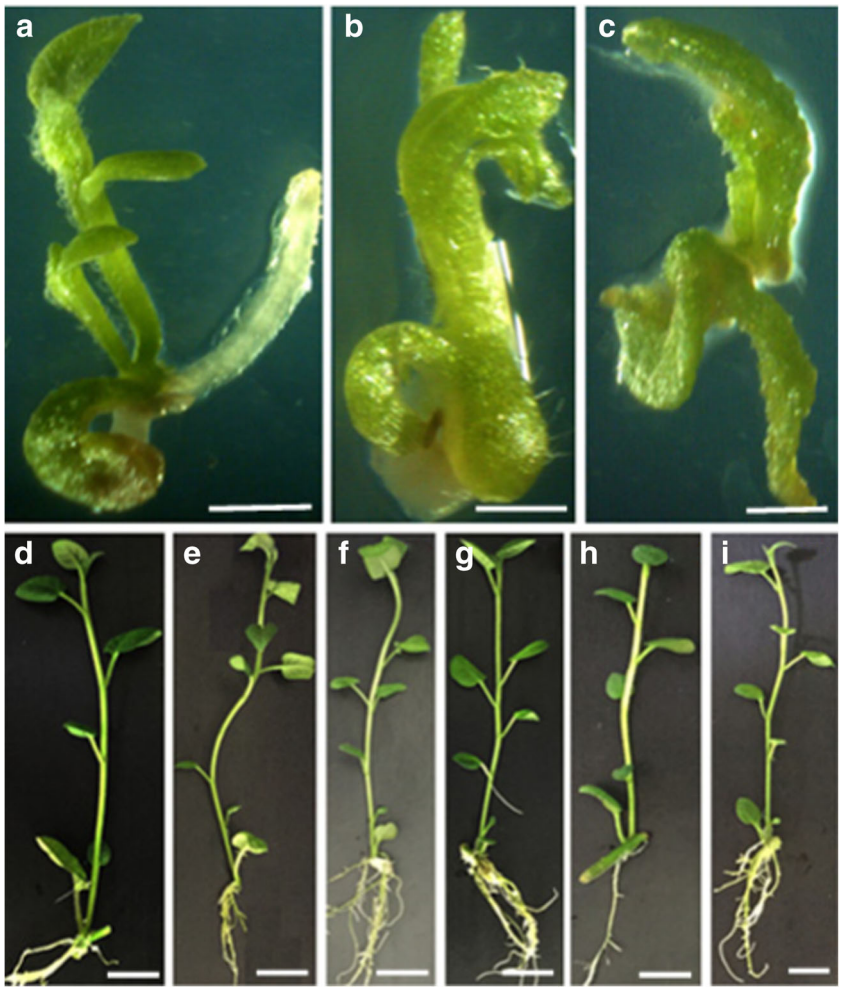

Fig. 5 Shoot regrowth and micropropagation of PLRV-, PVS- and PSTVd-cryopreserved shoots following cryopreservation in potato cv. Zihuabai. Shoot regrowth from cryopreserved shoot tips of **a** PLRV-, **b** PVS-, and **c** PSTVd-infected shoots after 6 weeks of subculture. Micropropagated shoots from **d** PLRV-, **f** PVS-, and **h** PSTVd-cryopreserved shoots after 18 weeks of subculture. Micropropagated shoots from in vitro stock shoots infected with **e** PLRV-, **g** PVS-, and **i** PSTVd-infected shoots after 27 weeks of subculture. Scale bars in **a**, **b**, and **c** = 0.5 cm, and in **d**–**i** = 1.0 cm

Pospiviroidae (Owens et al. 2012) and infects *Solanum* plants and a diverse array of ornamental species such as *Chrysanthmum* and *Argyranthemum* (Owens and Verhoeven 2017; Verhoeven et al. 2017). PLRV is a phloem-limited virus and does not invade AD (Valkonen 2007), which is also confirmed in the present study, while PSTVd is present in AD of plants (Zhang et al. 2015). PVS is a difficult-to-eliminate virus (Kushnarenko et al. 2017), indicating that its infectious ability of shoot tips is stronger than that of PLVS. Thus, the three pathogens investigated in the present study represent a wide range of viruses and viroid in terms of taxonomy and infectious ability. We previously reported successful preservation of ASGV in cryopreserved shoot tips of apple "Gala" (Wang et al. 2018b). Gene sequencing of the coat protein (CP) and movement protein (MP) of the ASGV genome showed that cryopreserved ASGV shared 99.87% nucleotide identities with shoot tip culture-preserved virus, indicating that cryopreserved virus is genetically stable. In addition, using the same potato cultivar and the cryogenic protocol, Wang et al. (2014b) reported no polymorphic bands were detected by genetic markers in plants recovered from cryopreservation, indicating that the plants recovered from cryopreservation were genetically stable, as already proven in many plant species (Wang et al. 2014a, 2018a; Li et al. 2017).

In this study, shoot tip cryopreservation produced 100% of PVS- and PSTVd-preserved plants, regardless of the shoot tip size. However, cryopreservation of 0.5-mm-shoot tips completely failed to preserve PLRV, and of 1.5-mm shoot tips resulted in 35% of virus-preserved plants and 65% of virus-free plants. In order to understand why frequencies of PLRV preservation varied with the size of shoot tips, cell survival patterns and virus localization were conducted. Results showed PLVR was not present in AD and LPs 1–3, but it was readily found in LP 4 and older tissues. The majority of cells in AD and some cells in LPs 1–3 survived following cryopreservation. A few cells in LP 4 survived in about 30% of cryopreserved shoot tips tested. This figure is almost equal to 35% of the virus-preserved shoots. All the data generated above provided explanations to varying frequencies of PLRV-preserved shoots recovered from cryopreservation when 0.5-mm- and 1.5-mm-shoot tips were used for cryopreservation.

When the viruses and PSTVd are cryopreserved in shoot tips, cryopreservation durations of shoot tips, shoot regeneration of cryopreserved shoot tips, and propagation efficiency of the recovered shoots are important factors determining pathogen preservation efficiency. Once samples are stored in LN, cellular divisions and metabolic processes are arrested, and theoretically, plant materials can be stored for a definitive period of time (Benson 2008). In plant preservation, a sample is considered as successfully cryopreserved if it has a minimum recovery rate of ≥ 30% (Vollmer et al. 2017). Potato is a plant that is more vulnerable to cryopreservation, and shoot regrowth levels were generally high (> 50%) in most of the previous studies (Wang et al. 2009a; Vollmer et al. 2017). Shoot regrowth levels of 58–60% were obtained in the present study, which can be considered high enough for cryopreservation (Vollmer et al. 2017). Previous studies showed that shoot regrowth levels maintained unchanged in potato shoot tips that had been cryopreserved for up to 10 years (Keller et al. 2006). In the present study, although shoot proliferation levels of the pathogen-cryopreserved shoots were lower than that of the control after 12 weeks of subculture, they reached similar levels as the control after 18 weeks of subculture. In the present study, at least seven shoot nodes can be produced in each subculture cycle. In general, single nodal shoots are used, with 3 weeks for each subculture cycle in micropropagation of potato. Therefore, the micropropagation index is n^7, where n represents subculture times. These data demonstrate pathogen-preserved shoots recovered from cryopreservation can be efficiently micropropagated. Thus, once a single virus-infected shoot is obtained, it can be fast micropropagated in a short time. In addition, infectious abilities of the viruses- and viroid-preserved plants recovered from cryopreservation were verified by grafting and mechanical inoculation to the potato host.

In conclusion, PLRV, PVS, and PSTVd were for the first time successfully cryopreserved in shoot tips. These pathogens represent a diverse range of viruses and a viroid in term of the infectious ability and taxonomy. Shoot tip cryopreservation requires liquid nitrogen, but not any extra equipment, and can be conducted in common tissue culture laboratories. Shoot tip cryopreservation opens a new avenue for long-term preservation of viruses and viroids.

Author contribution statements J-W Li: performance of experiments, data collection and analysis, and preparation of manuscript; M-R Wang and Z-H Cui: assistance to the performance of experiments, data collection, and analysis; H-Y Chen: assistance to the performance of experiments; Z Zhang: valuable discussions; D-R Blystad: valuable discussions; L Zhao: financial support, experimental design, and preparation of manuscript; Q-C Wang: chief scientist of the project, financial supports, experimental design, and preparation of manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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